TOWARDS THE DEVELOPMENT OF A PROTOCOL FOR THE SELECTION OF PROBIOTICS IN MARINE FISH LARVICULTURE

A thesis submitted in fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

of

RHODES UNIVERSITY

by

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December 2004

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Acknowledgements

After an unsuccessful attempt at a diploma in analytical chemistry in the late 1980s, I thought my tertiary education had come to an abrupt halt. I'll always remember Didee Robinson's words of encouragement when I was feeling sorry for myself – "everything happens for a reason". This is the proof.

Having a poor academic track record, I had some difficulty being accepted into the Faculty of Science at Rhodes University. Professor Mike Brown, Dean at the time, recognised my enthusiasm and desire to pursue a career in ichthyology and granted me admission to this prestigious university. I am forever indebted to him for being able to see potential before even I was aware of it!

In carrying out this research, I spent my time between two academic departments at Rhodes University, namely the Department of Ichthyology & Fisheries Science (DIFS) and the Department of Biochemistry, Microbiology & Biotechnology (DBMB). My HODs at the DIFS, Professors Peter Britz and Tom Hecht and the DBMB, Professors Peter Rose, Rosie Dorrington, Chris Whiteley and Don Hendry are thanked for their help and support.

Financial support as a student is always greatly appreciated. I would like to thank the South African Network for Coastal and Oceanographic Research (SANCOR) and the German Academic Exchange Service (DAAD) for their generous funding of my living expenses. A special thanks to Professors Peter Britz and Tom Hecht for the graduate assistance funding they helped secure for me over the years. John Gillam (Dean of Research office) is sincerely thanked for his help in securing me various bursaries and scholarships during my studies.

Project funding was obtained from SANCOR and the Rhodes University Joint Research Council through Prof Tom Hecht and Dr Horst Kaiser respectively. Your trust in my administration of your funding is deeply appreciated.

The many DIFS fish feeders and air conditioner controllers must be thanked for their assistance over the years – Warren Aken, Peter-John Case, Stephanie Case, Russell Chalmers, Ferdie Endemann, Albert Esterhuizen, Andrew Gordon, Gavin Johnston, Cliff Jones, Larry Oellerman, Ryan Palmer, Guy Paulet, John Radull, Ernst Thompson and the many others who

have provided help when needed. Special mention must go to Ndzaliseko Songongo for his assistance around the marine hatchery.

The DIFS master technician, Terry Longman is sincerely thanked for his help with repairing equipment and manufacturing various bits and pieces to keep the hatchery running. Jack Atherfold and the team of electricians and maintenance staff are thanked for their help with installing and repairing lights, heaters, air-conditioners and other equipment in the hatchery. The "late" Martin Davies from the DIFS is thanked for his help with the marine hatchery and for his entertaining banter, particularly regarding the success of the Welsh rugby team.

To my lab mates at the DBMB, Garth Cambray, Wade Edwards, Sheena Fraser, Christie Roux and James Strong, who offered help and advice to me, the sometimes clueless ichthyologist – Thank you. A special acknowledgement must be made to Justin Jordaan for his assistance with the molecular work. To the many other students of the department who I harassed for information, chemicals etc. - without your help I'd have completely lost the plot!

Sagaran Abboo and his technical staff in the DBMB for always being willing to offer advice and particularly free chemicals… Sagaran will no longer hear the words "Pleeeease Sagaran, I just need about five grams…" Without his help I'd still be trying to order things or waiting for them to arrive!

Professor Santy Daya is thanked for the use of his radioactive lab and Dr Brett Pletschke his help and advice with the enzymology. Cherri-Lynne Mack is acknowledged for her help with the vitamin C analysis and Prof Chris Whiteley for the kind donation of enzyme standards.

Drs Joanne Burgess, Janice Limson and Brett Pletschke allowed me bench space in their labs at the DBMB during the past year. Thank you for providing me with an area to complete the experiments needed to wrap up the thesis. Jeremy Baxter and Professor Sarah Radloff of the Department of Statistics, Rhodes University are thanked for their help with experimental design and the analysis of the results, particularly in Chapters 4. Jeremy Baxter is thanked for his help with the mathematical formulae in Chapter 3. Victor Clarke, the librarian from the South African Institute of Aquatic Biodiversity is thanked for his help during this thesis.

Thank you to Professor Don Hendry from the DBMB for his comments and suggestions on Chapter 3, and Professor Whiteley and Dr Brett Pletschke for their comments on the enzymology in Chapter 5. I'm also indebted to Dr Cliff Jones, friend and colleague, for comments on earlier drafts.

Dr Winston Leukes is thanked for his support and input in the aspects of the experimental design. He is thanked for commenting on the final drafts.

I was privileged to be around the DIFS at a time when Professor Tom Hecht was a member of staff. His passion for things fishy, and relentless quest for knowledge, has been inspirational to my development as a scientist. His insightful and critical ideas toward improving this thesis are valued and greatly appreciated. Enjoy retirement, you deserve every moment of it!

Dr Horst Kaiser, the best supervisor a student could ever ask for and the kind everyone should strive to emulate. He was trusting enough to allow me to do my own thing, yet concerned enough to ensure I was on the right track. Dr K often helped me make sense of sometimes cluttered data and, from our many discussions, has had a huge influence in moulding me into the scientist I am today. Thank you very much for your friendship, advice, support and the unlimited time you so willingly give all your students.

John and Ursula Case are acknowledged for their support during the thesis. Hopefully we will now be able to spend more time at the coast!

My dearest Nana, thanks for your support and interest in my progress over the past few years.

My Mom and Dad, Gail and Dave, who have waited a long time to hear me answer "Yes" to "Have you handed in yet?" and "Will you be wearing the red dress this year?" It's been a long haul and I promise there are currently no immediate study plans and I do plan on getting a "proper job"! Thanks for all your love, encouragement and support during all of my studies. Who would have thought back in 1989 that there would ever be a doctor in the family, let alone that it might be me!

And finally, to my wife, Stephanie Ellen Case, who has hung around Grahamstown while I completed this thesis. Thank you very much for your love, support and encouragement,

especially during the write-up stages when we didn't see much of each other. It is for these reasons that I dedicate this thesis to you. Hopefully I can reciprocate when you tackle your doctorate!

Post-examination acknowledgements

Thank you to my external examiners, Dr Anna Mouton, Professor Einar Ringo and Professor Patrick Sorgeloos for their time and effort in reading this thesis.

Abstract

Manipulation or control of the microbiological aquaculture environment has been identified as an important focus area for future hatchery development. Subsequently, alternatives to obtain control of the microbiological environment are being sought of which the field of probiotics appears highly promising. Probiotics are usually selected based on various *in vitro* characteristics, however, the methods used differ and are sometimes unsuccessful due to poor experimentation. The aim of this work is to contribute towards the development of a protocol for the *in vitro* screening of bacterial candidate probiotics for marine fish larviculture.

To reduce the number of candidate probiotics to be tested *in vivo*, various *in vitro* experiments need to be conducted, each screening for a particular mode of action – antagonism towards pathogen through production of antimicrobial compounds, growth and attachment to fish intestinal mucus, and the production of other beneficial compounds such as vitamins, fatty-acids and digestive enzymes. A total of 108 bacteria species were isolated from the digestive tract of the adult common clownfish, *Amphiprion percula* to screen for potential probiotics to be used in clownfish larval rearing. The antagonistic compounds assay identified twelve isolates which showed antagonism towards two or more aquatic pathogens. This was followed by an *in vitro* test that involved growing the organisms in fish intestinal mucus and modeling their growth parameters. A ranking index (*RI*) was developed using the lag period (λ) and doubling time (t_d) of the organism, where $RI = \frac{1}{\lambda \times t_d} \times 100$. Five candidate probionts (AP1-AP5) with varied growth parameters were used for further *in vitro* experiments. The attachment to mucus assay introduced a novel tool for quantifying competition for attachment sites between candidate probionts and pathogens on mucus. Candidate probiont *Pseudoalteromonas* AP5 reduced the attachment ability of *Vibrio alginolyticus* when added before the pathogen and partially out-competed the pathogen for attachment sites when added

second. *In vitro* screening for the production of beneficial compounds tested the candidate probiotics' ability to produce digestive enzymes - trypsin, lipase and alkaline phosphatase as well as carotenoids and vitamin C. Candidate probiont *Pseudoalteromonas* AP5 produced high levels of the enzymes (98.2, 34.1 and 91.3 mU product liberated.ml^{-1}, respectively) and contained carotenoids while *Kocuria* AP4 contained carotenoids but produced low quantities of enzymes (7.8, 0 and 59 mU product liberated.ml^{-1}, respectively). None of the candidate probiotics produced vitamin C. To eliminate potential pathogenic or toxic candidate probionts, *Artemia* nauplii were exposed to each candidate probiont and the percentage *Artemia* mortality after 24-hours was determined. Candidate probiont AP2 caused high mortality of *Artemia* nauplii (98.4%) and was excluded from further studies. Identification of candidate probionts AP3-AP5 was performed using 16S-rDNA molecular techniques and the bacteria were assigned the names *Bacillus* AP3, *Kocuria* AP4 and *Pseudoalteromonas* AP5, respectively. Two methods of larval probiont delivery were tested – attachment to *Artemia*, and in-water delivery. Attachment to *Artemia* was high for both *Kocuria* AP4 and *Pseudoalteromonas* AP5 (7.2 x10^{3} and 2.7x10^{4} bacteria.nauplius^{-1}, respectively) while the in-water viability experiment showed that *Kocuria* AP4 comprised 23.9% of the total culturable water microflora after 24 hours while *Pseudoalteromonas* AP5 contributed 100%. To validate the findings from the *in vitro* experiments, *in vivo* trials using clownfish larvae were performed. Of the four candidate probiotics tested, only *Kocuria* AP4 showed potential to increase larval survival.

In vitro tests produced a better understanding of the possible mode of action and strategies of competition between bacteria, however, the number of criteria in which a candidate probiont is successful *in vitro* may not be the best predictor for its effectiveness *in vivo*. Commercial studies that reduce between-treatment variation are required to test predictions about the most suitable probiont or combinations thereof.

Chapter 1

Introduction

Introduction

Combined with the problem of antibiotic contamination of aquaculture facilities and livestock, the indiscriminate worldwide use of antibiotics in aquaculture has led to the development of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate (Aoki and Watanabe, 1973; Aoki, 1975; Aoki et al., 1980; Hayashi et al., 1993; DePaola, 1995; Sahul and Balasubramanian, 2000; van der Waaij and Nord, 2000; Bruun et al., 2000; Miranda and Zemelman, 2002). Subsequently, certain antibiotics such as chloramphenicol have been banned in many countries (Robert et al., 1995; FAO website – URL in reference list). As a result of resistant bacterial strains becoming more prevalent and difficult to treat, alternative methods of controlling the microbial environment are being investigated. One of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Ringø and Birkbeck, 1999; Verschuere et al., 2000; Irianto and Austin, 2002a).

In aquaculture, the term "probiotics" is often loosely used to describe a microbial formulation responsible for biocontrol or bioremediation. The word probiotics comes from the Greek "pro bios" meaning "for life". The original definition (Lilly and Stillwell, 1965) of probiotics "substances produced by one protozoan that stimulated the growth of another" was expanded on from an agricultural perspective and defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). If the contribution of a probiotic is in the form of a food additive, e.g. single-cell protein, and not as a live microbial agent it cannot by the above definition be considered a probiotic. The above definition was therefore broadened to include a "mono- or mixed culture of live microorganisms…" (Havenaar and Huis in't Veld, 1992). However, Salminen

et al. (1999) suggested it include "microbial cell preparations or components of microbial cells". Gatesoupe (1999) redefined probiotics for aquaculture as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". The definition provided by Verschuere et al. (2000) includes, 1) the ability of a probiotic to modify the "host-associated or ambient microbial community" and, 2) to improve the quality of its surroundings, both of which can be considered as biocontrol. The definition of Gatesoupe (1999) will be used in this review as it focuses on the oral delivery of the probiont and its ability to improve the health of the host due to its presence in the digestive tract.

While vaccines require the participation of host cells to elicit positive effects, probiotics protect their host against neighboring or invading pathogens by interfering with their cellular functions. Probiotics may protect their host from pathogens by producing metabolites which inhibit the colonisation or growth of other microorganisms or by competing with them for resources such as nutrients or space (Fiorillo et al., 2002; Ouwehand et al., 1999a; Ouwehand et al., 2001; Forestier et al., 2001; Pinchuk et al., 2001; Mukai et al., 2002; Servin and Coconnier, 2003; Vine et al., 2004a; Vine et al., 2004b).

While the addition of potentially probiotic microorganisms to culture water in larval fish systems is a means of biocontrol, it is possible that some may be ingested and have a probiotic effect on the host animal. This is often not investigated (Nogami and Maeda, 1992; Jory, 1998; Moriarty, 1998; Verschuere et al., 1999; Ruiz-Ponte et al., 1999; Douillet, 2000b; Chythanya et al., 2002) as this is considered a method of improving water quality rather than enhancing the intestinal microflora of the animals living in the water.

To optimise the requirements for the species being farmed, intensive aquaculture conditions should be carefully controlled. Microorganisms that can be added to an intensive fish culture system have until recently been poorly available. A range of microorganisms are now available to seed biofilters, improve water quality or provide a food source for livefood organisms. However, the large-scale supply of probiotic bacteria is uncommon as product development has been slow due to lack of expertise, paucity of research and knowledge about their mode of action. Similarly, after more than 20 years of research, the preventative mechanism of probiotics on gut disturbances in humans is still not completely understood (Gismondo et al., 1999). Little is known about the establishment and action of the microflora particularly during the larval stages of aquatic organisms (Olafsen, 2001).

Although many publications about probiotics in aquaculture have been produced during the past decade, the research has generally been of an applied nature with few discussions on the bacterial mode of action. In the fields of medical and agricultural probiotic research, unequivocal evidence of the exact mode of action has been demonstrated (Fuller, 1989). Particularly regarding the use of lactic acid bacteria (Perdigon et al., 1995; Salminen et al., 1998; Kontula, 1999; Tannock, 1999) and knowledge regarding aspects of the organism's biology, culture and suitability has been of benefit to aquaculture (Gildberg and Mikkelsen, 1998; Ringø and Gatesoupe, 1998; Nikoskelainen et al., 2003). As antibiotics become less popular for controlling the aquatic microflora in hatcheries, the concept of treating the early stages of aquatic animals with probiotics has become increasingly popular. Initial research focused on the potential use of probiotics during the adult and juvenile phases of aquatic organisms (Limsuwan and Lovell, 1981; Lovell, 1981; Lovell and Limsuwan, 1982; MacDonald et al., 1986) while research into the use of probiotics in aquatic larviculture began in the late 1980's (Nicolas et al., 1989; Castagna et al., 1989; Gatesoupe, 1990; Munilla-

Moran et al., 1990). With a reduction in the use of antibiotics, the use of probiotics to help control or manipulate the microflora associated with aquatic larval culture has become increasingly popular with a review (mainly on probiotics in aquatic invertebrates) being written by Gomez-Gil et al. (2000). A number of general review articles on the use of probiotics in aquaculture are also available (see Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Skjermo and Vadstein, 1999; Ringø and Birkbeck, 1999; Verschuere et al., 2000; Irianto and Austin, 2002a).

Previous reviews have investigated the use and potential application of probiotics in aquaculture in general while this review focuses on the use of probiotics during the larval stage. The aims of this chapter are (1) to present information on the interaction between probiotics and aquatic larvae in the culture environment, (2) to identify probiotic selection criteria and constraints for the use of probiotics in aquatic larviculture, and (3) to propose a protocol for the screening of candidate probionts in aquatic larviculture.

The larval rearing environment

In aquaculture, the exposure to a diverse bacterial microflora is limited to the resources available. Therefore the gastrointestinal flora usually resembles the microflora initially present in the rearing water, microalgae and livefood (Tanasomwang and Muroga, 1989; Munro et al., 1994; Ringø et al., 1996; Gatesoupe, 1999; Riquelme et al., 2001). Verschuere et al. (1997) showed that the development of the microbial community in *Artemia* populations is influenced by both deterministic and stochastic factors. Deterministic factors include salinity, temperature, feed quality etc., while organisms in the right place at the right time to enter the habitat and proliferate, make up the stochastic factors. The combination of

controllable and chance factors determines the composition of the resulting microflora, making research on probiotics for larviculture both interesting and challenging.

Upon hatching, marine fish larvae possess a sterile (Ringø et al., 1996), immature digestive system (Govoni et al., 1986; Timmermans, 1987; Hansen et al., 1992; Roo et al., 1999) which is colonised by the egg flora at the time of hatching (Hansen and Olafsen, 1990) or through water-borne bacteria as the larvae drink water to osmoregulate (Ringø et al., 1996; Reitan et al., 1998; Olafsen, 2001) or begin feeding (Munro et al., 1993a; Bergh et al., 1994; Huys et al., 2001). Ringø et al. (1996) found that the intestinal microflora of turbot larvae was strongly affected by the addition of *Vibrio pelagius* to the rearing water. Similarly, 70% of the intestinal microflora of cod larvae was comprised of *Lactobacillus plantarum* when exposed to the bacteria in the larval rearing water compared to 1% in unexposed tanks (Strøm and Ringø, 1993).

Both marine and freshwater fishes have each been shown to have a specific indigenous microflora (Horsley, 1977; Sakata, 1990; Ringø and Gatesoupe, 1998). While humans and terrestrial farm animals tend to have an intestinal microflora dominated by Gram-positive obligate or facultative anaerobes (Gatesoupe, 1999), the intestinal microflora of aquatic animals consists mainly of Gram-negative aerobic, obligate anaerobic and facultative anaerobic bacteria, the composition of which may change with environmental stresses (Ringø and Strøm, 1994; Ringø et al., 1997; Kennedy et al., 1998), diet (Munro et al., 1993b; Ringø and Strøm, 1994; Douillet and Langdon, 1994; Gildberg et al., 1995; Ringø et al., 1997) and fish age (Bergh et al., 1994; Prayitno and Latchford, 1995; Olafsen, 2001). The most common members of the microflora of healthy marine fish are *Vibrio* spp., *Pseudomonas* spp. and *Acinetobacter* spp. (Muroga et al., 1987; Tanasomwang and Muroga, 1989; Nicolas et al.,

1989; Mac and Fraile, 1990; Bergh et al., 1994; Munro et al., 1994; Gatesoupe et al., 1997), while *Aeromonas* spp., *Plesiomonas* spp., *Pseudomonas* spp. and members of the family Enterobacteriaceae, dominate the microflora of freshwater species (Ugajin, 1979; Sugita et al., 1988; Sakata, 1990; Sugita et al., 1991a; Ringø et al., 1995). A variety of microorganisms have been used as probiotics to improve the growth or survival of aquatic larval species (Table 1.1).

Table 1.1 - Intestinal probiotics used in fish and shellfish larviculture and the effect on their host.

Microbe	Host species	Effect on host	Reference
	Molluscs		
Roseobacter sp. (strain BS107)	*Pecten maximus*	Short-term improvement in survival	Ruiz-Ponte et al., 1999
Arthrobacter sp. (Strain 77)	*Argopecten purpuratus*	Produces inhibitory compounds. Replaced resident microflora within 24 hours	Riquelme et al., 2000
Strains 11 and C33	*A. purpuratus*	Added with microalgae and colonized digestive tract	Avendano and Riquelme, 1999
Vibrio sp. 33, *Pseudomonas* sp. 11 and *Bacillus* sp. (strain B2)	*A. purpuratus*	Compared to antibiotic treatment, the addition of probiotics increased number of eyed larvae	Riquelme et al., 2001
Strain CA2	*Crassostrea gigas*	Enhanced growth	Douillet and Langdon, 1994
*Aeromonas media (*strain A199*)*	*C. gigas*	Reduced mortality when challenged with *Vibrio tubiashii*	Gibson et al., 1998
	Crustaceans		
Vibrio alginolyticus	*Litopenaeus vannamei*	Survival improved after challenge with *Vibrio parahaemolyticus*	Garriques and Arevalo, 1995
Lactobacillus sporogenes	*Macrobrachium rosenbergii*	Fed as bio-encapsulated probiotic via *Artemia.* Improved growth rate and feed efficiency ration of post-larvae	Venkat et al., 2004
Bacillus S11	*Penaeus monodon* (PL-10)	Post-larvae survival was higher when challenged with *V.harveyi*. Probiont provided cellular and humoral immune defence	Rengpipat et al., 2000

		responses	
VKM-124 *Pseudoalteromonas undina*,	*Penaeus* sp.	Reduced mortality by controlling pathogenic viruses	Maeda et al., 1997
Bacterial strains F3 and PM-4	*Portunus trituberculatus*	Improved crab larval survival. PM-4 repressed growth of *Vibrio* spp. in culture water	Nogami and Maeda, 1992
	Fish		
Bacillus toyoi	*Scophthalmus maximus*	Improved turbot larval growth when added to disinfected rotifers	Gatesoupe, 1989
Bacillus strain IP5832 spores	*S. maximus*	Improved weight gain of larvae and reduced mortality after challenging with *Vibrio*	Gatesoupe, 1991a
S. thermophilus, L. helveticus and *L. plantarum*	*S. maximus*	Added to rotifer cultures which were fed to turbot larvae. Larval survival increased	Gatesoupe, 1991b
Vibrio Pelagius	*S. maximus*	Higher larval survival than controls when added alone or in combination with *Aeromonas caviae*	Ringø and Vadstein, 1998
Vibrio mediterranei Q40 strain	*S. maximus*	Reduced colonization of gut by opportunistic microflora	Huys et al., 2001
V. alginolyticus	*S. maximus*	Presence of probiotic correlated with better survival	Gatesoupe, 1990
L. plantarum and *Carnobacterium* sp.	*S. maximus*	Improved survival after challenge with *Vibrio* sp.	Gatesoupe, 1994
Vibrio (strain E)	*S. maximus*	Improved survival and growth after challenge with *Vibrio splendidus*	Gatesoupe, 1997

Carnobacterium divergens and *Vibrio Pelagius*	*S. maximus*	Beneficial effect of *C. divergens* on survival inconclusive from *in vivo* study	Ringø, 1999
Streptococcus lactis and *Lactobacillus bulgaricus*	*S. maximus*	Six times increase in survival of larvae feed rotifers enriched with probionts	Garcia de la Bande et al., 1992
Roseobacter strain 27-4	*S. maximus*	Improved larval survival over first five days.	Hjelm et al., 2004
L. plantarum	*Gadus morhua*	Opportunistic colonisation reduced by 70%	Strøm and Ringø, 1993
Carnobacterium divergens	*G. morhua*	Improved survival after challenge with *Vibrio anguillarum*	Gildberg et al., 1997
V. salmonicida strain	*Hippoglossus hippoglossus*	Larval survival increased (to 72.8%) compared to control (58.2%) after 32 days post-hatch	Ottesen and Olafsen, 2000
Vibrio strains PB 1-11 and PB 6-1	*H. hippoglossus*	No difference in gut bacterial CFUs or growth between controls and bacteria bioencapsulated in *Artemia franciscana*	Makridis et al., 2001
Mixture of *Pseudomonas* and *Cytophaga/Flavobacterium*	*H. hippoglossus*	Improved larval survival and reproducibility of treatment	Skjermo and Vadstein, 1999
Microbiologically matured water	*H. hippoglossus*	Improved survival of yolk-sac larvae	Vadstein et al., 1993
Bacillus *no. 48*	*Centropomus undecimalis*	Reduced *Vibrio* spp. in the microflora	Kennedy et al., 1998
Pediococcus acidilactici and *Saccharomyces cerevisiae*	*Pollachius pollachius*	Addition of *P. acidilactici* with *Artemia* improved growth of larvae	Gatesoupe, 2002
VKM-124 *Pseudoalteromonas undina*,	*Sparus auratu*	Reduced infection with various pathogenic viruses	Maeda et al., 1997

Arnobacterium sp. (strain K1)	*Oncorhynchus mykiss* fry	Detected in intestine ten days after being administered	Robertson et al., 2000
Carnobacterium sp. Strain 1	*Oncorhynchus mykiss* fry	Fed with pellet diet. No adverse affect on survival	Joborn et al., 1997
Streptococcus faecium M74	*Cyprinus carpio*	Improved growth and food conversion ratio in fry over six weeks	Bogut et al., 1998
Kocuria AP4	*Amphiprion percula*	Improved clownfish larval survival	N.G. Vine, this thesis
Pseudoalteromonas AP5		Reduced *Vibrio* spp. in the culture water microflora	

In fish larvae, the development of the stomach, foregut, midgut and hindgut becomes compartmentalised in terms of pH, enzyme type and activity (Govoni et al., 1986; Gisbert et al., 1998; Ribeiro et al., 1999a; Ribeiro et al., 1999b; Gallagher et al., 2001), thus for the probiotic to be of benefit, it must reach and maintain itself in the part where its effect is to be applied. An inability to thrive in a particular part of the digestive tract is also unlikely to benefit the host. It is therefore important that when screening potential probionts, an understanding of how and where probiotics act within the host is required.

Probiotics used for terrestrial animals or adult fish should be resistant to bile (Nikoskelainen et al., 2001a) if they are to persist in the digestive tract. Selection criteria for probiotics for larvae differ from those used for adult fish in that initially the pH of the larval digestive system is alkaline (Tanaka et al., 1996; Ronnestad et al., 2000). As the larval digestive tract is immature at hatching, the gall bladder has yet to develop and subsequently bile is not secreted until later on during development (Govoni et al., 1986). Therefore, the probiotic is not required to move through an acidic environment en route to the gut and, unlike a probiont designed for adults, does not need to be resistant to acid and bile. Probiotics selected for use during the early stages of larval development do thus not need to be screened for this characteristic.

The composition of the colonising microflora is determined by the interaction between bacteria (Ringø and Birkbeck, 1999) and available nutrients in the form of partially digested compounds, mucus or secretions of the gastrointestinal tract (Fänge and Grove, 1979). Colonisation of the gut by bacteria generally increases at the onset of exogenous feeding, resembling the microflora similar to that of the livefood as opposed to that of the surrounding environment (Muroga et al., 1987; Tanasomwang and Muroga, 1989; Munro et al., 1993b;

Bergh et al., 1994; Munro et al., 1994). For example, Nicolas et al. (1989) showed that non-feeding turbot larvae retained a gut microflora similar to that of the water and not that of the rotifer associated microflora. Colonisation of the turbot gut appears to occur in two steps, firstly, low levels of bacteria (about 5 x 10^2 per larva) were found during the first four days, then between five and 16 days after hatching, the bacteria reached concentrations of 5 x 10^4 per larva (Ringø et al., 1996; Ringø and Vadstein, 1998). Reasons for this are unclear. It could be related to an increase in attachment sites suitable for bacteria as a result of the histological and functional development of the larvae (Ringø et al., 1995) and improved internal environmental conditions for bacterial growth (Ringø and Vadstein, 1998).

To maximise the competitive advantage of probiotics, early delivery seems to be best (Ringø et al., 1996; Ringø and Vadstein, 1998; Gatesoupe, 1999) since bacteria colonising the intestine before first feeding may be able to persist (Hansen and Olafsen, 1999; Olafsen, 2001). Opportunistic pathogens are commonly introduced along with mass-cultured livefood due to concentrated feeding levels combined with the rapid growth rate of the bacterial microflora (Skjermo and Vadstein, 1993) enforcing the concept that probiotic delivery should occur during the early stages of larval development prior to exogenous feeding.

Aquatic candidate probionts for larviculture have been isolated from adults (Olsson et al., 1992; Gildberg et al., 1997; Gram et al., 1999; Riquelme et al., 2000; Rengpipat et al., 2000; Gullian et al., 2004) and healthy larvae (Ringø et al., 1996; Gatesoupe et al., 1997; Gatesoupe, 1997; Ringø and Vadstein, 1998). It has been suggested that the efficacy of probiotics is likely to be highest in the host species from where they were isolated (Verschuere et al., 2000). However, the human probiotic, *Lactobacillus rhamnosus*, enhanced survival of rainbow trout (Nikoskelainen et al., 2003) when challenged with furunculosis (Nikoskelainen

et al., 2001b). Some probiotics used for humans and terrestrial animals have shown promise in aquaculture species (Nikoskelainen et al., 2001a; Nikoskelainen et al., 2001b; Nikoskelainen et al., 2003). Although not a major part of the normal microflora of aquatic animals (Ringø, 2003), lactic acid bacteria have been intentionally introduced into Atlantic salmon (*Salmo salar*) (Gildberg et al., 1995), rainbow trout (*Oncorhynchus mykiss*) (Nikoskelainen et al., 2001b; Nikoskelainen et al., 2003), turbot (*Scopthalmus maximus*) (Gatesoupe, 1994), Atlantic cod (*Gadus morhua*) (Strøm and Ringø, 1993; Gildberg and Mikkelsen, 1998) and pollack (*Pollachius pollachius*) (Gatesoupe, 2002). However, within "a few days" after adding the probionts they were no longer detectable in the gastrointestinal tract of Atlantic salmon or rainbow trout (Joborn et al., 1997). The reasons why lactic acid bacteria are seldom isolated from larvae is probably due to different preferred incubation temperatures and periods, and the absence of glucose from the growth medium (Ringø et al., 1995). Another reason may be due to the rapid gut evacuation rate of larvae which possibly flushes the slow-growing microorganisms before they are able to attach and colonise. The use of probiotics derived from the aquatic environment and used in terrestrial animals has yet to be demonstrated. Due to the infancy of the field, information pertaining to the survival and continued effect of these terrestrial probiotics in the aquatic environment is limited (Gatesoupe, 1999).

At this stage our understanding of the mode of action of probiotics is limited (Klaenhammer and Kullen, 1999; Tannock, 1999). This has been attributed to three factors (Klaenhammer and Kullen, 1999). (1) The complexities of the microbial activity in the gastrointestinal tract and the competition and interaction between the species that influence the environments microecology are poorly understood; (2) Confusion over identification, activity and viability of the probiotics which has contributed to misidentification of cultures; (3) Individual

probiotic strains have been proposed but have yet to be tested in expensive large-scale *in vivo* trials. In the past few years, interest in probiotic formulations for human and animal use has increased and can be attributed to the realisation of concept validation.

Modes of action

An understanding of the mechanisms used by probiotics to compete either with other probiotics or with pathogens is important when designing a protocol for their selection. The selection criteria for human probiotics have focused on methods of processing and production, biosafety considerations and taking into account the part of the body where the organism is active (Huis in't Veld et al., 1994). For aquaculture purposes, suggestions have been the evaluation of the probiotic's ability to out-compete pathogenic strains (Vine et al., 2004a), assessment of the probiotic's pathogenicity, evaluation of the probiotic *in vivo*, and cost considerations (Gomez-Gil et al., 2000). The common modes of action available to human probionts as suggested by Fuller, 1987 include (1) the stimulation of the humoral and/or cellular immune response, (2) modification of the metabolism of bacterial pathogens by changing their enzyme levels, and (3) competitive exclusion through either production of inhibitory compounds which are antagonistic towards pathogens, or by competing for nutrients, attachment sites, or oxygen.

The enhancement of non-specific immunity by probiotics in humans is well recognised (Gill, 2003) and their effect on stimulating cytokine production, which regulates the production of T-cells, has been documented in some animal species (Maassen et al., 2000; Perdigon et al., 2002). Larval fish have a poorly developed immune system, relying primarily on their non-specific immune response (Vadstein, 1997). In larvae exposed to high bacterial concentrations in the water, a greater amount of mucus-producing saccular cells have been found, suggesting

that non-specific defense mechanisms against invaders were stimulated by the augmented microflora (Ottesen and Olafsen, 2000).

Irianto and Austin (2002b) showed that after feeding trout with probiotics for two weeks, stimulation of cellular immunity was detected with an increase in lysozyme activity and in the number of erythrocytes, macrophages and lymphocytes. Panigrahi et al. (2004) also fed trout a pellet containing the probiotic, *Lactobacillus rhamnosus* JCM1136 resulting in increased levels of non-specific immune response parameters. Stimulation of the immune system by probiotics has also been shown in carp (Stosik and Szenfeld, 1996) and shrimp (*Penaeus monodon*) (Rengpipat et al., 2000). A paucity of research is available regarding the ability of microorganisms to stimulate the immune response system of fish larvae (Olafsen and Hansen, 1992). Therefore, to better understand the possible modes of action used by probiotics, screening for the stimulation of the immune response system should be performed.

The ability of probiotics to modify the metabolism and enzyme levels of the other intestinal bacteria has been conducted in terrestrial animals. For example, probiotic yeast cultures provided to dairy cattle were shown to influence ruminal microbial metabolism by increasing microbial protein production (Miller-Webster et al., 2002). Therefore, research into the use of probiotics to beneficially manipulate the metabolism of the microflora in aquatic organisms, requires urgent attention.

If a probiotic excludes a pathogen by producing antagonistic extra-cellular metabolites, it is possible that the pathogen will eventually develop resistance to the metabolites. It is therefore important that a probiotic has different ways of out-competing pathogens in order to grow. These include (1) production of antagonistic compounds, (2) advantageous growth

characteristics i.e., a short lag-period and a short doubling time, (3) attachment ability to intestinal mucus, and (4) production of other compounds beneficial to the host. These four criteria could be used for the *in vitro* screening of suitable probiotic candidates (Figure 1.1).

Antagonistic compounds

For the purpose of this review, antagonistic compounds are described as chemical substances produced by bacteria which are toxic or inhibitory towards other microorganisms. These substances may be produced as either primary or secondary metabolites and therefore have different modes of inhibitory action. Antibiotics are chemicals usually produced as secondary metabolites which, although created in small quantities, inhibit or kill other microorganisms (Brock and Madigan, 1997). Other inhibitory compounds produced by bacteria include organic acids, hydrogen peroxide (Ringø and Gatesoupe, 1998), carbon dioxide (Gill, 2003) and siderophores (Braun and Braun, 2002; Yoshida et al., 2002). Bacteriocins are proteinacious agents produced by bacteria to inhibit or kill other bacteria. They are ribosomally synthesised unlike antibiotics which are synthesised by other mechanisms (Brock and Madigan, 1997). The genes encoding the proteins involved in processing the bacteriocin are often carried out by plasmids or transposons (Brock and Madigan, 1997) suggesting that the ability to produce bacteriocins can be transferred between bacteria.

Siderophores are iron-complexing chemicals secreted by bacteria and fungi (Braun and Braun, 2002). Bacteria which produce siderophores have highly specific transport proteins in their outer membrane which allow them to utilise Fe^{3+} which is otherwise insoluble in the surrounding medium (Braun and Killmann, 1999). Siderophore-producing bacteria can survive in nutrient-poor environments (Mazoy et al., 1992; Braun and Braun, 2002). Gatesoupe, 1997 showed that the siderophore-producing "Vibrio E" increased the resistance

of turbot larvae against pathogenic *Vibrio splendidus* and improved larval growth. The omission of ferrous salt in the diet of sea bass larvae had no negative effect on larval growth or survival (Gatesoupe et al., 1997) which suggests that the establishment of siderophore-producing bacteria could help control the growth of those opportunistic pathogens that have a low iron uptake. The addition of an efficient iron-chelating probiont to inhibit pathogens must be done with caution as iron limitation may cause a reverse effect and induce proliferation of the pathogen through the expression of the pathogens siderophores (Gram et al., 2001).

The production of antagonistic or inhibitory compounds against any other microflora *in vitro* is no guarantee that the potential probiotic will be effective *in vivo* (Gibson et al., 1998; Ringø and Gatesoupe, 1998; Gram et al., 2001). Additionally, the lack of effectiveness may be a result of selective ingestion by the host (Prieur, 1983; Riquelme et al., 2000) or death of the probiotic microbe in the digestive tract. Ruiz-Ponte et al. (1999) found a strain of *Roseobacter* sp. which only produced an inhibitory compound in the presence of foreign cells. This suggests that *in vitro* antagonism studies using mono-cultured probiotic culture supernatant instead of whole cells may inadvertently result in potential antimicrobial-producing probionts going undetected.

Bacteria with antagonistic activity against other microorganisms were present in low quantities (~2% of the total microflora) in the larval rearing environment of the Chilean scallop, *Argopecten purpuratus* (Riquelme et al., 1997) but may contribute up to 21% in microalgae monocultures (Lodeiros et al., 1991 cited in Avendano and Riquelme, 1999). Once these bacteria enter the gastrointestinal tract, they can be found throughout the digestive tract (MacDonald et al., 1986). The activity of antimicrobial metabolites from the sterile

supernatant of the probiotic *Pseudomonas fluorescens* strain AH2 was still effective *in vitro* after seven days (Gram et al., 2001).

Antagonism may not only be limited to other bacteria. Maeda et al. (1997) isolated a bacterial strain VKM-124 *Pseudoalteromonas undina*, which had vibrio-static activity and inhibited the appearance of the cytopathic effect on prawn epithelioma papillosum cyprini cells. When added to prawn (*Penaeus* sp.) and sea bream (*Sparus aurata*) larval tanks, VKM-124 *P. undina* improved larval survival (compared to a non-treated control), by reducing the infection of the larvae with Sima-aji Neuro Necrosis Virus (SJNNV), Baculo-like viruses, and Irido virus. It is possible that *in vivo*, the probiotic activated the immune system of the exposed organism thereby reducing the viral infection. Further studies should be conducted to confirm whether a reduction in viral count is due to direct antagonism or via the stimulation of the immune system.

Growth in mucus

Bacteria may only produce metabolites during the stationary growth phase (Monaghan et al., 1999), which may not occur in the gut due to constant flushing. Isolating bacteria that produce anti-microbial metabolites is common practice, while little is known regarding what stage of growth the bacteria produce the metabolites and whether the bacteria are able to compete for attachment sites (Vanbelle et al., 1990). Thus, *in vitro* studies may create a false impression of the ability of probiotics to inhibit pathogens *in vivo*. Inability to compete for attachment sites on the mucus of the gut wall suggests that these bacteria may not multiply sufficiently fast to compensate for being flushed from the mucus during gut evacuation.

Screening for organisms with antagonistic abilities towards pathogens may produce a large number of candidate probiotics. A simple method for eliminating candidate probionts with similar growth characteristics is by growing them in standard microbiological media and comparing their growth profiles (Vine et al., 2004a). Bacteria with similar antagonistic abilities and growth profiles can be assumed to be of equal potential, thereby further reducing the pool of candidate probionts to be tested. Vine et al. (2004a) developed a ranking index whereby candidate probionts grown in vitro in fish intestinal mucus were ranked according to the growth profile characteristics, lag period and specific growth rate. This tool enabled the rapid screening of candidate probiotics, however, their results confirm suggestions by other authors (Sugita et al., 1997; Robertson et al., 2000) that growth *in vitro* should not be viewed in isolation since all probiotic bacteria produced antagonistic metabolites. For example, the candidate probiotic with the longest lag period inhibited five of seven pathogens although its ranking index was low while the two highest-ranking probiotics showed combined antagonism to three pathogens (Vine et al., 2004a).

Attachment to mucus

In humans it appears that attachment and the production of anti-microbial compounds by lactic acid bacteria are the critical factors in excluding pathogens (Reid, 1999; Gill, 2003). This appears to be similar in fish where, as Ringø et al. (1998) suggested, attachment of lactic acid bacteria to the mucus layer may serve as the first barrier of defence against invading pathogenic bacteria. Attachment is therefore regarded as a prerequisite for colonisation (Olsson et al., 1996; Joborn et al., 1997; Ringø and Gatesoupe, 1998) and is important in the stimulation of the host's immune system (Schiffrin and Blum, 1999; Collins and Gibson, 1999; Ouwehand et al., 1999b). The superior ability of bacterial pathogens to attach has been related to virulence (Wilson and Horne, 1986; Bruno, 1988) and is considered the first step of

bacterial infection (Bengmark, 1998). Much research has been conducted on the ability of probiotics to attach to the intestinal mucus of fish (Krovacek et al., 1987; Olsson et al., 1992; Vazquez et al., 1997; Andlid et al., 1998; Rinkinen et al., 2003b; Vine et al., 2004b). For these reasons potential probionts should be tested for their ability to adhere to mucus *in vitro* before large-scale trials are attempted as the candidate probiotic may be transient *in vivo* and consequently not contribute to the health of the host organism.

Attachment to mucus may not be the only invasion strategy employed by pathogens, as was the case with *Vibrio anguillarum* O2 which exhibited poor attachment to mucus but was highly virulent (Olafsen, 2001). The human probiotic *Lactobacillus reuteri* did not produce antimicrobial substances, yet, *in vitro* it inhibited the binding of pathogens to receptor sites (Mukai et al., 2002). Competition for attachment may not necessarily be due to competitive exclusion but may also be due to specific inhibitors (Rojas and Conway, 1996) such as those produced by *Lactobacillus fermentum* which inhibited the adhesion of *E. coli* to porcine ileal mucus (Ouwehand and Conway, 1996). An advantage of competitive exclusion based on enhanced attachment is that unlike antibiotics, the factors that inhibit pathogen binding do not necessarily kill the pathogen thereby exerting less selective pressure on the pathogen to evolve resistance (Reid et al., 2001). Therefore, although probiotics may have beneficial effects on host species from which they have not been isolated, attachment ability is not necessarily host-probiont species-specific but rather dependent on the bacterial strain (Rinkinen et al., 2003b).

Production of other beneficial compounds

Beneficial dietary compounds

Bacteria have been isolated from fish intestine that produce various vitamins as secondary metabolites. Vitamin B_{12} is produced by different bacteria isolated from the microflora of a variety of fish species, including carp (*Cyprinus carpio*) (Kashiwada et al., 1970; Sugita et al., 1990; Sugita et al., 1991a), channel catfish (*Ictalurus punctatus*) (Limsuwan and Lovell, 1981), various tilapia species (Lovell and Limsuwan, 1982; Sugita et al., 1991a) and rainbow trout (*O. mykiss*) (Sugita et al., 1991b). Fish species that require dietary vitamin B_{12} do not appear to have these vitamin B_{12}-producing bacteria while those that do, do not require additional dietary B_{12} (Sugita et al., 1991a). Whether the introduction of vitamin B_{12}-producing bacteria into fish species requiring the vitamin would reduce the need for B_{12} inclusion in the diet warrants further investigation.

Unlike most animals fish are unable to synthesise ascorbic acid from glucuronic acid (Merchie et al., 1997) and are therefore dependent on an adequate supply through the feed. Ascorbic acid is involved in the formation of cartilage and fibrous tissue and vitamin C deficiencies may result in broken back syndrome (Merchie et al., 1997). Production of vitamin C by the intestinal microbes of fish has yet to be investigated while the dietary contribution of vitamin K by the intestinal microflora of immature brook trout (*Salvelinus fontinalis*) has been shown (Poston, 1964).

Carotenoids can act as anti-oxidants, scavenging lipid-damaging free radicals. Identification of bacteria producing these pigments is simplified as the colonies are bright yellow or orange when grown on agar media. Brightly pigmented bacteria generally produce more metabolites

than pale strains (Holmstrom et al., 2002). This may help in the initial screening of potential probiotics selected on the basis of their ability to produce these beneficial metabolites.

Marine bacteria have been shown to contain between 36% and 56% crude protein (dry weight) (Brown et al., 1996). The amino acid profile was similar to that of *Saccostrea commercialis* spat suggesting that the bacteria could act as a direct protein source for the developing bivalve (Brown et al., 1996).

The production of short-chain fatty acids by intestinal bacteria has been shown to contribute to the nutrition of the host in humans (Batt et al., 1996) and fish (Clements, 1997), however, the effect on aquatic larvae has not been investigated. Nutritive fortification of rotifers has been achieved with such bacteria at a level higher than that of rotifers fed *Saccharomyces cerevisiae* (Watanabe et al., 1992). In fish, lipids produced by intestinal microbes (Ringø et al., 1992a; Shirasaka et al., 1995) have contributed significantly to the diet of Arctic charr (*Salvelinus alpinus*) (Jostensen et al., 1990; Ringø et al., 1992a), turbot (*S. maximus*) (Ringø et al., 1992b) and in the case of tilapia (*Oreochromis niloticus*), they physically altered the morphology of the digestive system (Kihara and Sakata, 1997).

The bacterial production of highly polyunsaturated fatty acids (HPUFAs) in the larval digestive tract is unknown. A strain of *Shewanella putrefaciens*, isolated from the intestine of Pacific mackerel, produced eicosapentaenoic acid (EPA) as the sole HPUFA. The contribution of EPA amounted to 24-40% of the total fatty acid in the cell (Yazawa, 1996). Rotifers cultured with *S. putrefaciens* and then separated from the bacteria, lost 48% of their EPA content after six hours and 80% after 24 hours (Watanabe et al., 1992). To ensure that high levels of fatty acids are available to the larvae, feeding of EPA and docosahexaenoic acid

(DHA) enriched rotifers to larvae should be immediate or possibly include addition of the HPUFA producing bacteria to the culture water.

Beneficial digestive enzymes

Another contribution that a probiotic may make to its larval host is that of providing it with digestive enzymes. The digestive capability of the larval digestive system develops and changes as the larva grows (Govoni et al., 1986; Cahu and Zambonino-Infante, 1995; Ribeiro et al., 1999b; Gawlicka et al., 2000; Zambonino-Infante and Cahu, 2001). Bacteria isolated from fish digestive systems have been shown to digest chitin (Hamid et al., 1979; MacDonald et al., 1986), starch (Hamid et al., 1979; MacDonald et al., 1986; Gatesoupe et al., 1997), protein (Hamid et al., 1979; Gatesoupe et al., 1997), cellulose (Erasmus et al., 1997; Bairagi et al., 2002) and lipids (Gatesoupe et al., 1997) *in vitro*. Sea bass (*Dicentrarchus labrax*) larvae fed live yeast (*Debaryomyces hansenii* CBS 8339) incorporated diets showed increased activities and concentrations of mRNA trypsin and lipase (Tovar-Ramirez et al., 2004). Contrary to these findings, Pollak and Montgomery (1994) found that the giant bacterium *Epulopiscium fishelsoni* reduced the activity of the digestive enzymes, particularly protease and amylase in the surgeonfish *Acanthurus nigrofuscus*.

The microflora of juvenile Dover sole, *Solea solea*, had a greater proportion of substrate-degrading bacteria than adults (MacDonald et al., 1986) suggesting that these microorganisms possibly contribute to their host by enhancing their limited ability to digest and assimilate nutrients. The degradation of comparatively unattractive complex molecules may release compounds which could be beneficial to the host (MacDonald et al., 1986). The importance of digestive enzymes produced by bacteria remains unclear (MacDonald et al., 1986; Pollak

and Montgomery, 1994) and the contribution of "introduced" or probiotic bacteria has yet to be investigated.

Interaction with livefood

For most cultured marine fish species the most suitable prey at first feeding is rotifers (Sorgeloos et al., 1995; van der Meeren and Naas, 1997). Manipulation of the external microflora of the livefood which is then fed to the larvae has potential application in the delivery of probiotics. The bacterial flora of rotifers was approximately $5x10^3$ bacteria per individual (Skjermo and Vadstein, 1993; Munro et al., 1993a). Attempts to feed rotifers with a considerably higher bacteria load to turbot larvae have proven unsuccessful (Nicolas et al., 1989; Munro et al., 1999). The amount of probiotic cells which adhere to the livefood depends on the probiont, time of exposure and the state (dead or alive) of the livefood organism (Gomez-Gil et al., 1998). As the livefood's bacterial load increases it may reach levels and/or micro-communities may develop which may negatively affect the health of the host larvae. For example, Olsen et al., 1999a found that bacterial overloading of 4-day old *Artemia* fed to halibut larvae resulted in poorer larval growth.

It is well documented that a change in diet selects for a different bacterial community (Shiranee et al., 1993; Ringø and Strøm, 1994; Olsen et al., 2000b). For example, in Arctic charr (*S. alpinus*), alteration of dietary fatty-acids resulted in a major change in contribution of the lactic acid bacterial flora (Ringø et al., 1998). Large numbers of *Vibrio* spp. in the rearing water and larval intestine are usually attributed to the presence of *Artemia* (Verschuere et al., 1999; Olsen et al., 1999a; Olsen et al., 2000b; Eddy and Jones, 2002) which diminish as the fish are weaned onto a formulated diet (Blanch et al., 1997). Through microbial manipulation, livefood can be treated to act as a vector for probiotics. Manipulation includes

the disinfection of the rotifers (Munro et al., 1999; Rombaut et al., 1999) or *Artemia* (Harboe et al., 1994; Abreu-Grobois et al., 1994; Theisen et al., 1998; Liltved and Cripps, 1999) followed by incubation with the probiotic (Gatesoupe, 1991c; Gatesoupe, 1997; Gatesoupe and Lesel, 1998; Makridis et al., 1998; Gatesoupe, 2002). In addition, a positive effect of probiotics on livefood cultures has been documented (Bogaert et al., 1993; Shiri Harzevili et al., 1998; Douillet, 2000a; Douillet, 2000b; Gatesoupe, 2002; Orozco-Medina et al., 2002; Villamil et al., 2003) as has the transfer of these bacteria to larval fish (Gatesoupe, 1997; Gatesoupe and Lesel, 1998; Makridis et al., 1998; Makridis et al., 2001).

Once a probiont has been shown to be effective *in vivo*, the delivery method to the larvae could be tested and refined in a sequence of studies. Some probiotics may be able to attach to live food. Marine fish larvae in particular, require live food and many do not accept artificial diets during the early stages of larval development (Lavens et al., 1995; Hart and Purser, 1996; Yufera et al., 2000). If probiotics can be administered via livefood, their application in marine fish larviculture could be simplified.

The development of a probiotic selection protocol

In vitro screening

A bottleneck in the isolation of suitable probiotics for aquaculture has been the lack of a clearly defined experimental protocol. The proposed research protocol in Figure 1.1 is represented as a hypothetical flow diagram involving a series of experiments that could be conducted for the selection of larval probiotics. By systematically conducting *in vitro* tests on a large number of potential probiotics, less-promising candidates can be excluded, thereby reducing the number of *in vivo* trials required to validate the effectiveness of the probiont. Ranking the four *in vitro* experiments described above as criteria for the selection of larval

probiotics must be performed with caution. For example, the *in vitro* production of antagonistic compounds may not necessarily be more important than attachment to mucus when applied *in vivo*. Experiments investigating the production of antagonistic compounds cannot test the candidate probiont against all potentially competitive bacteria. Therefore, the apparent low production of antagonistic compounds by a particular candidate probiotic may result from the researcher's choice of pathogens it was tested against. It is therefore recommended that researchers, screening for the production of antagonistic compounds, use as many species of competitive bacteria as possible.

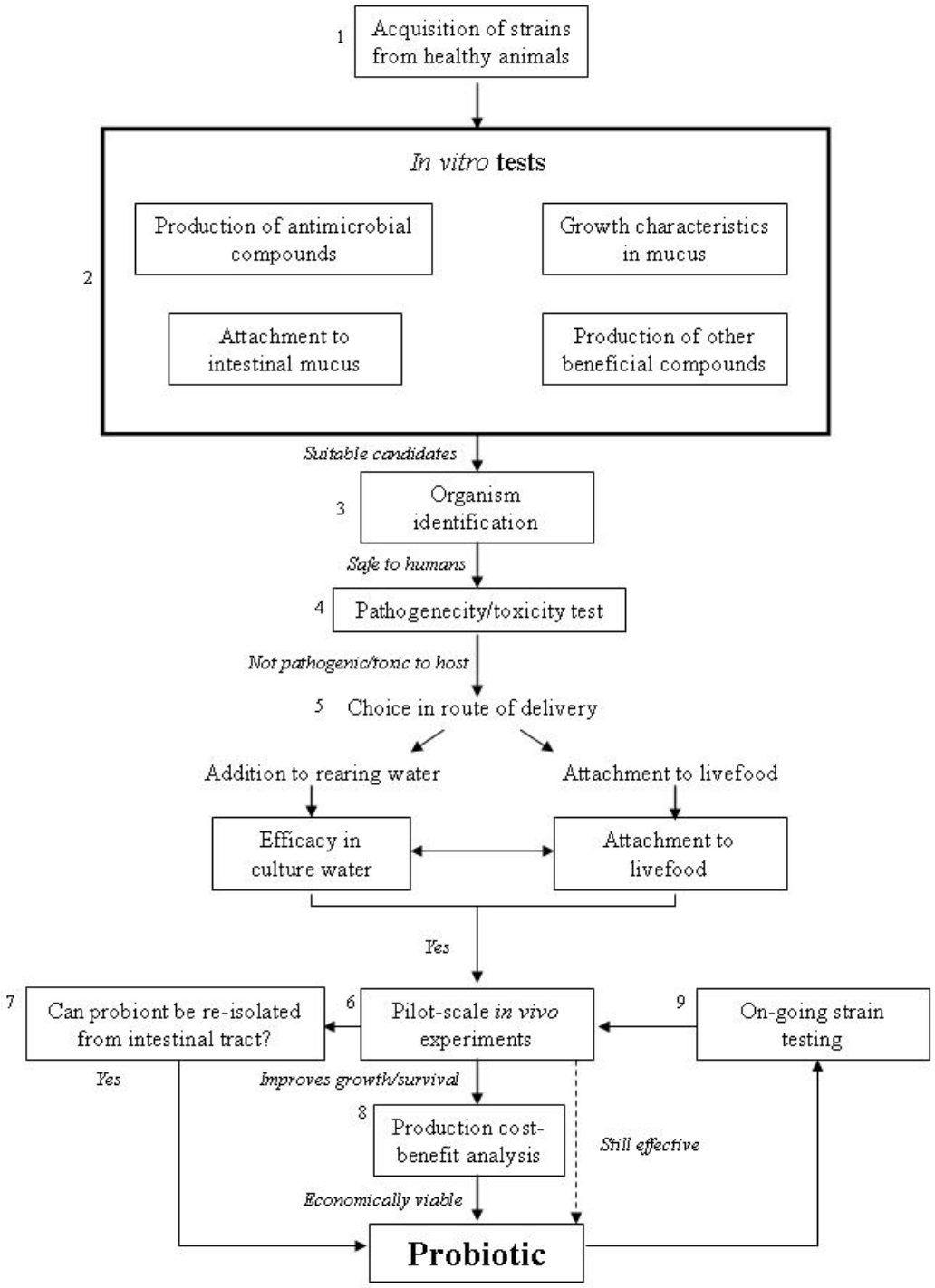

Figure 1.1 - Proposed research protocol for the selection of intestinal probiotics in marine larviculture (with acknowledgements to Verschuere et al., 2000). See explanatory note at end of text.

In vitro experiments should be designed to reduce the number of candidate probionts with each experiment. The first experiment should investigate the production of antimicrobial metabolites as this has been the first method used by other authors in selecting candidate probiotics (Ruiz et al., 1996; Joborn et al., 1997; Gram et al., 1999; Robertson et al., 2000; Spanggaard et al., 2001; Ringø et al., 2004; Hjelm et al., 2004). Testing whether the probiont is capable of growth in intestinal mucus could follow. Modeling the growth profiles helps identify similar strains and some of these can be eliminated (Vine et al., 2004a). The method of ranking the growth characteristics (lag-period and doubling-time) of candidate probiotic bacteria, as proposed by Vine et al. (2004a) would help when dealing with large numbers of candidate isolates. Higher-ranked candidates could be carried over to the third step, that of testing their attachment to mucus. Running this experiment third is due to the use of radioactively labeled the bacteria (Vine et al., 2004b). Therefore any reduction in the amount of radiolabel reduces the cost and associated risks of radioactive exposure. Finally, investigations into the production of other beneficial compounds such as digestive enzymes, fatty acids, vitamins etc. can be performed. These analyses can be expensive and time-consuming and are therefore best tested on a small number of candidate probionts.

The *in vitro* experiments can, and should only be used as an indication of possible successes *in vivo*. Some assumptions regarding the *in vivo* mode of action based upon *in vitro* experiments may not hold. Therefore, the purpose of the *in vitro* experiments is to gain a better understanding of the potential of specific candidate probiotics before subjecting them to costly and time-consuming *in vivo* trials.

Organism identification

As not all initially isolated bacteria are to be used as probionts, identification of candidate probiotics would not need to be performed until after further screening using *in vitro* tests. When the putative probiotic is to be tested *in vivo*, it should ideally be identified down to strain level (SCAN - http://europa.eu.int/comm/food/fs/sc/scan/index_en.html), using either 16S-rDNA or fatty-acid profile techniques (Buyer, 2002). Identification of the candidate probiont can provide potentially useful information regarding its culture requirements, pathogenicity and hence suitability as a candidate probiont. If the particular strain does not exhibit any pathogenesis in the target organism but is a known human or aquatic pathogen, approval by the relevant food and drug administration may be difficult or even impossible thereby making it an unfeasible candidate for further development. Data on the bacteria may also assist in the screening of bacterial transference of antibiotic resistance (SCAN – see above for web address). Although the use of antibiotics in aquaculture has become less popular, they are still used in humans and therefore the possibility of transferring resistance from the microflora of cultured organisms to human bacteria should be prevented.

Since prolonged exposure of obligate anaerobic bacteria to oxygen results in cell death, probiotics should ideally be aerobic or facultative anaerobes. The aerobic method with which probionts are applied to larvae, via the livefood, or in the culture water, suggests that obligate anaerobes are unlikely to thrive. Additionally, sustaining an anaerobic bacteria culture requires more technical expertise and equipment and is more expensive than culturing aerobic bacteria. It is possibly due to the technical considerations required for working with anaerobes that their use as probiotics has been limited, however various potential anaerobic probiotics have been identified for use in aquaculture (Dixon et al., 2001; Ramirez and Dixon,

2003). Delivery of anaerobic probiotics to the larval may be logistically problematic and should be carefully considered before further product development.

Pathogenicity/toxicity of candidate probionts

A probiotic must not be pathogenic or toxic to its host. This can be determined by small-scale challenge tests of the host species using short-term baths in the bacterial suspension or direct addition to the culture water. If a microorganism is to be used as a probiotic for livefood organisms, tests should also be performed on the larvae to which they are to be fed (Verschuere et al., 2000). Irianto and Austin (2002a) working on trout probiotics, selected four out of eleven bacteria which were deemed harmless to the fish following intra-peritoneal or-intra muscular injections.

As Lactic acid bacteria are intrinsically resistant to antibiotics (Salminen et al., 1998), potential pathogens would be difficult to control. Therefore, in humans, candidate probiotics from the genus *Lactobacilli* are rigorously screened for pathogenicity before being passed for human trials (Harty et al., 1994).

Non-pathogenic strains of known pathogenic bacteria do exist and therefore, should be screened *in vitro* before being omitted from further testing. For example, strains of the pathogen *Vibrio alginolyticus* have been isolated from the intestine of apparently healthy larvae (Munro et al., 1993b), and been used as a probiotic in algal production (Gomez-Gil et al., 2002), shrimp (Garriques and Arevalo, 1995; Vandenberghe et al., 1999) and fish culture (Austin et al., 1995). Similarly, a non-pathogenic strain of *Aeromonas hydrophila* has been used as a probiont in rainbow trout (Irianto and Austin, 2002b) while a strain of *Escherichia coli* has been used as a probiotic in humans (Jansen et al., 1998). It is possible that when

pathogenicity is suppressed or lost, other factors such as growth rate or attachment ability which are factors that contribute to their success as pathogens, may influence the microflora to the benefit of its host.

Route of delivery

Exposing the larvae to probiotic concentrations greater than those occurring naturally in the water, increases the chance of probiotics being ingested by the larvae either directly or via the livefood. As a result, the probiotic is able to better establish itself in the mucus and epithelium of the digestive tract (Strøm and Ringø, 1993; Ringø et al., 1996; Ringø and Vadstein, 1998; Huys et al., 2001), and potentially suppress the attachment of pathogenic bacteria. This may be particularly beneficial in larvae with a poorly developed immune system (Vanbelle et al., 1990) as subsequent pathogenic bacterial exposure is suppressed by established probiotics (Gatesoupe, 1994). The health of the host is thereby enhanced by preventing or reducing the proliferation of pathogens or opportunistic bacteria, and the latter rather than primary or specific pathogens contribute most to larval mortality under culture conditions (Vadstein et al., 1993; Munro et al., 1995a; Huys et al., 2001). Another method of manipulating the intestinal microflora is the use of microbiologically matured seawater, which compared to membrane-filtered or disinfected water, has led to an improvement in turbot larval growth (Vadstein et al., 1993; Skjermo et al., 1997). This supports the hypothesis that a mature, established microflora, which is dominated by K-selected species, is able to confer protection on the larvae by reducing the proliferation of *r*-selected species.

Livefood organisms such as rotifers and *Artemia* are filter-feeders and are also known to graze bacteria (Vadstein et al., 1993; Skjermo and Vadstein, 1999; Olsen et al., 2000b). Their ability to act as vectors for the delivery of probiotics to fish larvae (Makridis et al., 1998;

Makridis et al., 2000; Gatesoupe, 2002) allows for the manipulation of the livefood's bacterial community through the addition of probiotics. This in turn reduces the number of opportunistic (*r*-selected species) or pathogenic bacteria from entering the larvae and colonising the intestinal tract.

In vivo validation

Based on the definition of probiotic used in this review, a researcher must be able to re-isolate the probiotic from the intestinal tract of the larvae. If not, it cannot be discounted that larval growth and survival may have been improved due to some other factors such as improved water quality rather than the ability of the probiont to exclude opportunistic or pathogenic bacteria intestinally.

It has been suggested that candidate probiotics should be tested by challenging them *in vivo* through the addition of a representative pathogen (Verschuere et al., 2000). However, larval mortality related to the composition of the microflora can usually be attributed to opportunistic rather than pathogenic bacteria (Olafsen, 2001). Additionally, as with the choice of pathogens for the *in vitro* antagonism experiment, *in vivo* experiments cannot test all possible pathogens (usually testing fewer than in the *in vitro* tests) making it difficult to draw conclusions on the tested pathogens when they may not occur as part of the normal or natural larval system microflora. Therefore, for the selection of probiotics for larviculture, the *in vivo* challenge experiment is not necessary.

Production cost-benefit analysis

If the probiotic is to be commercialised, an economic analysis on the potential commercial production is required upon the successful completion of the *in vivo* trials. Investigations into

the economic viability of different product formulations, packaging options and dosing recommendations would need to be completed.

Upon successfully completing the screening stages discussed above, the microorganism can be called a probiotic. To ensure that the probiotic remains viable, on-going strain testing is required, including aspects of storage and efficacy.

Probiotic selection constraints

Dosage and dosing-frequency

If probiotics are to persist and make any contribution to the indigenous microflora, initially, their introduction would need to be done on a regular basis and/or at a concentration higher than that of the already established microbial community (Verschuere et al., 2000).

The dose-effect relationship must be carefully determined to avoid overdosing which may result in a lower probiont efficacy while increasing costs or conversely under-dosing which reduces the efficacy of the probiont. For the purposes of administering probiotics, a dose can be defined as the concentration (in number of probiotic cells.ml^{-1}) that is available to the aquatic host. This can be added directly to the water, attached to the livefood, or, depending on the manufacturing process, be incorporated into the artificial diet (i.e., an extruded pellet would probably kill the microorganism during the extruding process). Additional doses may be required to maintain the desired probiotic concentration in the culture water, the frequency of which may depend on the probiotic species, stage of fish development, diet, culture conditions and desired intestinal probiotic concentration.

It has been suggested that aquaculture systems cannot support bacterial concentrations greater than 10^6 cells ml^{-1} (Maeda, 1994). *L. rhamnosus* at a dose of 10^9 CFU.g^{-1} of feed protected rainbow trout from experimentally induced furunculosis (Nikoskelainen et al., 2001b) and Gomez-Gil et al., 2000 found that the concentration of added bacteria rarely exceeded the dosage (10^7 cells.ml^{-1} culture water) while it decreased over 72 hours at a rate depending on the bacterial species. Doses between 10^4 and 10^6 cells.ml^{-1} in the culture water of strain 77 (*Arthrobacter* sp.) were capable of displacing the resident microflora of larval Chilean scallop, *A. purpuratus* after 24 hours of incubation (Riquelme et al., 2000). In *Crassostrea gigas*, additions of CA2 bacteria at a final concentration of 10^5 cells.ml^{-1} increased larval growth (Douillet and Langdon, 1994). *Roseobacter* 27-4 inoculated at 10^7 cells.ml^{-1} improved turbot (*S. maximus*) larval survival better than concentrations of 10^3 to 10^5 cells.ml^{-1} (Hjelm et al., 2004). Challenge experiments in which juvenile rainbow trout were exposed to the probiotic *P. fluorescens* AH2 at a concentration of 10^5 cells.ml^{-1} followed by exposure to *V. anguillarum* at 10^4 to 10^5 cells.ml^{-1} in the system water, reduced accumulated mortality by 46% (Gram et al., 1999). Nicolas et al., 1989 found that turbot larvae refused to eat rotifers with high bacterial loads of 10^5 bacteria per rotifer suggesting that overloading the livefood organisms with bacteria may be not always be beneficial to the larvae. Although culture conditions, probiotic and fish species may have an influence, a dose maintaining between 10^4 and 10^6 cells.ml^{-1} in the culture water appears to be sufficient in introducing a probiotic capable of dominating the intestinal microflora.

A single dose may be used for probiotics capable of surviving within the gut and attaching to the mucus and/or intestine. However, since doses higher than 10^6 cells ml^{-1} may be unnecessarily high (Maeda, 1994), regular dosing at lower concentrations may be required to ensure that the bacteria persist in the water and subsequently the intestinal tract. By their

sustained presence, periodical additions may also assist in maintaining the desired microflora at levels which inhibit the development of pathogenic or opportunistic bacteria. After a 24-hour exposure to the probiotic "strain 77" at concentrations of 10^4 and 10^6 cells.ml^{-1}, Riquelme et al. (2000) found that almost 100% of the gut of Chilean scallop, *A. purpuratus* larvae was dominated by the probiotic. Probiotic levels in the gut were maintained by providing a concentration of 5 x 10^3 cells.ml^{-1} "every few days" (Riquelme et al., 2001). Two doses of probiotic bioencapuslated *Artemia* was insufficient to predictably influence the intestinal species composition of halibut larvae over a ten day period (Makridis et al., 2001).

Shelf-life and storage

When bacteria are cultured under artificial conditions, i.e. with an excess of nutrients and without competition from other organisms, they may loose the ability to produce compounds which would otherwise have been produced under stressful conditions. For example, sub-cultures of the industrially cultured Gram-positive, spore-forming, *Clostridium acetobutylicum* are known to lose their ability to produce the acids for which they are cultured (Kashket and Cao, 1995). This problem of strain degeneration is important when considering Gram-negative candidate probionts as unlike some Gram-positive bacteria, they are not endospore-forming. Therefore to maintain viable cultures, Gram-negative candidate probionts require more frequent sub-culturing on solid or liquid media.

Since the production of inhibitory metabolites depends on the media upon which the bacteria are cultured (Olsson et al., 1992), culture methods and media which eliminate or reduce the loss of these selective criteria need to be used. Nikoskelainen et al. (2001a) found that three lactic acid probionts lost the ability to produce antimicrobial compounds, antagonistic towards fish pathogens. Although it is possible that the lack of antimicrobial production was due to

sub-culturing, the authors attributed it in part to different screening methods. Ringø et al. (2004) suggest that the ability of probiotics to produce metabolites which inhibit the growth of other microorganisms, can be lost due to storage and sub-culturing. Therefore, it is recommended that small-scale *in vivo* testing remains ongoing to ensure the efficacy of the probiotic remains at a level beneficial to the larvae (Figure 1.1).

Some Gram-positive organisms have the advantage of forming endospores thereby making culturing and long-term storage easier. A simple and rapid means of culturing, storing and administering probiotics to larvae would be preferred by aquaculturists, however, with many candidate probiotics being Gram-negative, sterile laboratory techniques, equipment and skills are required to ensure product quality, all at extra expense. Therefore, Gram-negative probiotics which require inexpensive equipment and techniques would be of great benefit to the industry.

Non-bacterial sources of probiotics

Yeasts have been identified which exhibit probiotic characteristics. With our vast knowledge of yeasts, particularly their biology, genetics and safety issues, their application as potential probiotics is good.

Yeasts are not affected by antibiotics. This is advantageous in probiotic preparations used for preventing disturbances in the normal microflora in the presence of antibacterial metabolites. Strains of *S. cerevisiae* and *D. hansenii* isolated from salmonids (Andlid et al., 1999) have been shown to attach and grow in fish intestinal mucus (Vazquez et al., 1997; Andlid et al., 1998) while in rats, a protease produced by *Saccharomyces boulardii* was shown to interfere with toxin A from *Clostridium difficile* (Castagliuolo et al., 1996). A strain of *S. boulardii*

has been successfully used as a probiotic for *Artemia* nauplii, conferring resistance against a pathogenic Vibrio (Patra and Mohamed, 2003) however, the ability to transfer resistance to fish or shrimp larvae was not tested. In sea bass (*D. labrax*) larvae, *D. hansenii* CBS 8339 exhibited a probiotic effect, improving larval development by promoting intestinal maturation and increasing the ability to absorb nutrients (Tovar-Ramirez et al., 2004).

The use of greenwater is common in the larval rearing of many marine fish species (Reitan et al., 1997; Dhert et al., 1998). As bacteria may extend the stationary phase of the algae (Avendano and Riquelme, 1999) which is ideal for larval rearing as the culture of many species utilise the stagnant water technique. The presence of microalgae in the culture water has been shown to influence the composition of the surrounding microflora, often reducing the number of potentially pathogenic or opportunistic bacteria (Douillet and Langdon, 1994; Avendano and Riquelme, 1999; Gomez-Gil et al., 2002; Eddy and Jones, 2002). The presence of algae (and possibly its associated microflora?) in seabass larval-rearing water has been shown *in vivo* to increase the production of larval digestive enzymes at both pancreatic and intestinal levels (Cahu et al., 1998). Apart from the know beneficial effects of algae on larval survival (i.e. provides contrast to aid feeding, food for livefood) the use of greenwater during the early stages of larval rearing may also benefit the larvae in extending the stationary growth phase of the algae, controlling the culture water microflora and improving digestive enzyme production.

Concluding remarks

Although there are many choices of biocontrol microorganisms, typically used in shrimp or pond culture, there are currently only a few commercial "intestinal" probiotics used specifically during the larval stages of aquatic organisms. Many of the probiotics discussed in

this review have been tested in small or pilot scale facilities, however, it would appear that none have been tested repeatedly on a commercial scale. The general lack of empirical data on the large-scale testing of intestinal probiotics therefore warrants urgent investigation.

Due to space limitation in many hatcheries better larval survival does not necessarily equate to higher production. Probiotics are of benefit to hatcheries as they improve larval quality in terms of health and growth potential, and by improving larval survival, the number of broodstock needed to produce the required number of larvae can be reduced. Broodstock is expensive to obtain and maintain, as for example in the case of shrimp, and therefore an increase in larval survival means that fewer broodstock animals are required for production. The probiont *Vibrio salmonicida* increased halibut (*Hippoglossus hippoglossus*) larval survival by 14.6% (Ottesen and Olafsen, 2000) which equates to either an increase in production or an equivalent reduction in the number of broodstock required to produce the same number of larvae.

Reasons for the low numbers of commercial intestinal probionts for aquatic larviculture can (in no particular order) be attributed to (1) the infancy of the field of research which only gained momentum in the late 1990s, (2) the inconsistent nature of *in vivo* experiment results, particularly on a large scale, (3) the type of organisms used tend to be Gram-negative, nonspore-forming, making upscale processing difficult, (4) methods of delivering the probiotics to the larvae have not been optimised, (5) the potential effects of the probiont on fish health during grow-out has not been examined, (6) possible hesitancy from industry who until recently have stood to lose more during the initial R&D stages. As these points are addressed over the next few years, it seems reasonable to assume that commercially available larval probiotics will soon enter the aquaculture market.

Explanatory note for Figure 1.1 (numbers in brackets shown in figure)

Suitable probiotics should ideally be derived from healthy individuals (1), preferably of the species into which they are to be introduced. As a result, problems regarding pathogenicity towards the host organism are reduced. After initial isolation there are likely to be a large number of bacteria available for screening. The aim of the *in vitro* tests (2) is to rapidly reduce the initially available large pool of candidate probionts to a more acceptable number for further testing (see text for further information regarding the order in which the tests should be conducted). Probiotic candidates can then be identified (3) using microbiological/phenotypic tests or, more accurately, using 16S-rDNA techniques. Each identified organism needs to be carefully researched and assessed regarding its potential pathogenicity/toxicity towards humans. Small-scale pathogenicity/toxicity tests on the host organism can then be conducted to further eliminate some probionts (4). Once suitable candidates have been selected based on the *in vitro* results, the route of delivery should be tested. The choice of probiotic delivery (5) may be influenced by certain factors related to the biology of the fish species, for example the time required from hatching to the start of exogenous feeding. A long time may favour adding the probiotic to system water with the aim of increasing the exposure of the larvae to the probiotic. In cases where the water in the larval rearing system is frequently being replaced, attachment of probionts to livefood may be preferable. The prolonged efficacy of the candidate probiont in the larval rearing system needs to be confirmed before adding it to the water can be recommended. Similarly, experiments testing the attachment of the candidate probiotics to livefood organisms should be conducted to ensure that sufficient candidate probionts attach and are therefore available to the larvae when feeding on the livefood. Final validation can only be performed *in vivo* (6). If a suitable candidate shows good potential *in vivo* and further upscaling and production is anticipated, a cost-benefit analysis needs to be performed (7). This should include aspects

such as product formulation, packaging and the dosing recommendations. To be considered a true "intestinal" probiotic, the organism must have been isolated from the intestinal tract of the larvae (8). If not, it cannot be discounted that larval growth and survival may been improved due to some other factors such as improved water quality rather than the probiont's ability to exclude opportunistic or pathogenic bacteria. Finally, a probiont that is economically viable to produce can be considered a marketable probiotic. Due to the problem of strain degeneration, on-going pilot-scale *in vivo* experiments should be conducted to test the reliability of probiont efficacy (9).

Thesis research objectives

Manipulation or control of the microbiological aquaculture environment has been identified as an important focus area for future hatchery development (Sorgeloos et al., 1995). With the indiscriminant use of antibiotics in hatcheries, widespread resistance of various bacterial species has occurred, resulting in an increased incidence of disease. Subsequently, alternatives to obtain control of the microbiological environment are being sought of which the field of probiotics appears highly promising.

Currently, researchers select probiotics based upon various *in vitro* characteristics, however, the methods used to make their final selections differ and are sometimes unsuccessful due to poor experimentation. The aim of this work is to contribute towards the development of a protocol for the *in vitro* screening and selection of bacterial candidate probiotics which can be used in marine fish larviculture.

The objectives were :

1. To investigate the modes of action used by probiotics in marine fish larvae and from these, design *in vitro* tests to identify candidate probiotics.
2. To develop a protocol for the selection of marine fish larvae probiotics using the *in vitro* tests.
3. To assess whether the candidate probiotics identified *in vitro*, were effective *in vivo* using clownfish (*A. percula*) larvae.

Thesis outline

This thesis contains the results of both experimental and bibliographical work related to the research objectives.

In **Chapter 1** (Introduction) an overview is given on the interaction between probiotics and aquatic larvae in the culture environment as well as current probiotic selection criteria and the constraints for the use of probiotics in larviculture.

To reduce repetition, **Chapter 2** (General materials and methods) describes in detail, the various techniques and methods used repetitively in the experimental chapters throughout the thesis.

Chapter 3 (Growth in mucus) proposes a method for screening candidate probionts using their growth characteristics *in vitro*. The growth characteristics are modeled and from the resulting growth model coefficients, a ranking index is determined to assist in selecting candidate probionts.

In **Chapter 4** (Attachment to mucus) the candidate probionts are assessed for their ability to attach to intestinal mucus as well as their ability to compete with two pathogens for attachment sites. This chapter introduces a novel technique using radio-labeled isotopes to label the bacteria which allows for quantification of the competition between candidate probiont and pathogen.

Chapter 5 (Production of beneficial compounds) quantifies the production of various compounds produced by the candidate probionts which are considered beneficial to the host.

In **Chapter 6** (Organism identification and pathogenicity/toxicity) three of the candidate probionts are identified to genus level using 16S-rDNA techniques. Based on the identifications, a brief literature review on the genus of each of the candidate probionts is provided. To help eliminate any potential pathogens or toxin producers amongst the candidate probionts, the probionts are incubated with newly-hatched *Artemia* for 24 hours and *Artemia* mortality recorded.

Chapter 7 (Attachment to *Artemia* and in-water viability) investigates the two methods of probiotic delivery – attachment to *Artemia* and the addition of the probiont to the larval culture water.

Chapter 8 (*In vivo* validation) is the final experimental chapter and tests the candidate probionts *in vivo* using clownfish (*Amphiprion percula*) larvae.

In **Chapter 9** (General discussion) the results are discussed in the framework of the research objectives. Conclusions are drawn and research objectives for further work on probiotics in larval rearing are suggested.

Chapter 2

General materials and methods

During this study a number of techniques were common to different experiments. To reduce repetition, these techniques are described below.

Probiotic culture maintenance

After isolation and initial screening for antibiotic activity (Chapter 3), the candidate probionts were maintained at -80°C as well as on agar slants. The frozen stock cultures were prepared by growing the probionts in marine broth 2216e for 48 hours at 25°C. A volume of 320 µl of 1:1 stock of marine broth and sterile glycerol was added to 800 µl of the probiotic culture in a sterile microcentrifuge tube. The tubes were snap-frozen in liquid nitrogen and transferred to a -80°C deepfreeze.

Working cultures were maintained on 10ml marine agar 2216e slants. After incubation at 25°C until bacterial growth was evident, the slants were maintained at room temperature in a cupboard. From these, new slants were restreaked every four to six months unless contamination occurred. When contamination of the slants occured, new slants were prepared from the -80°C stock cultures.

Preparation of probiotic bacteria

A pure colony from the marine agar 2216e (Difco) slant stock culture was aseptically inoculated into an Erlenmeyer flask containing 200 ml Marine Broth 2216e (Difco) and incubated on an orbital shaker at 200 rpm for 48 hours at 25°C. The broth was then transferred to a sterile 250 ml centrifuge tube and centrifuged at 2000 *g* for ten minutes. The supernatant was poured into a sterile flask and kept at 4°C, while the bacterial pellet was resuspended in sterile seawater.

Cell counts of each probiont were performed using a haemocytometer and cell density was related to absorbance assuming the Beer-Lambert law. This enabled rapid adjustment of the cell density/concentration in the water depending on the application or experiment the probionts were required for.

Mucus preparation

Due to the large volume of mucus required, mucus from the spotted grunter, *Pomadasys commersonnii* (Haemulidae), was used. This species was chosen as it was easily available and, like *Amphiprion percula*, it is omnivorous. Mucus was collected from the intestinal tract of three adult fish, suspended in sterile NSS (nine-salt solution: NaCl, 17.6g; Na_2SO_4, 1.47g; $NaHCO_3$, 0.08g; KCl, 0.25g; KBr, 0.04; $MgCl.6H_2O$, 1.87g; $CaCl_2.H_2O$, 0.41g; $SrCl_2.6H_2O$, 0.008g; H_3BO_3, 0.008g in 1000 ml double-distilled H_2O (Olsson et al., 1992)) and centrifuged twice at 8000 *g* for 15 minutes to remove particulate matter. The remaining supernatant was filter-sterilised through a 0.22 μm filter, diluted to 0.25 $mg.ml^{-1}$ using Bradford's protein assay (Bradford, 1976), and kept at 4°C.

Clownfish broodstock maintenance

Adult breeding pairs of clownfish (*A. percula*) were individually housed in 100 l aquaria, each with its own filtration system. Each tank contained a PVC pipe upon which the breeding pair spawned. Water quality variables were maintained within limits acceptable to this species. The fish were fed twice daily on a diet of minced squid, fish, mussels and seaweed.

Clownfish larval rearing

On the afternoon on which hatching was to take place, the PVC pipe to which the eggs were attached was removed from the broodstock tank. The PVC pipe was placed in a 5 l bucket

containing water from the broodstock tank. A light stream of bubbles was directed over the eggs to ensure agitation and oxygenation, simulating that provided by the broodstock fish. The bucket was floated in one of the buckets in the larval rearing system described below.

The larval rearing system consisted of a 600 l water bath heated to 25°C. Suspended in it were six 25 l buckets which held the larvae. Each bucket was aerated with an airstone. A stagnant larval rearing system was used for the four-day period. Only one probiotic was tested at a time with the number of eggs determining the number of replicates. Unless a minimum of two replicates of 50 fish each could be used for the control and probiotic-treated larvae, the experiment was abandoned.

The larval rearing tanks contained 17 l of 0.1 μm filtered seawater and were maintained at 25°C. On the afternoon that hatching was to occur, three litres of microalgae, *Nannochloropsis oculata* were added per tank. To provide food for the rotifers, two litres of algae were added for the first three days. Screens were placed over the outlets to prevent the larvae from being flushed out.

Rotifers (*Brachionus plicatilis*) were harvested from rotifer culture tanks using a 100 μm screen. After rinsing under freshwater for 30 seconds, the rotifers were suspended in a cone containing 1 l of seawater and aerated. The rotifers were enriched before feeding to the larvae using 10 ml Super Selco (INVE) per cone. Rotifers were added to the larval tanks at a density of 5-8 individuals.ml^{-1}.

Lights were turned off at sunset and remained off for two hours before a subdued night light came on for the duration of the night. Lights came on the following morning and the photoperiod for the remainder of the experiment was 12 hours light : 12 hours dark with a subdued night light being used during the dark period.

Artemia hatching and preparation

Brineshrimp cysts (EG *Artemia* cysts, INVE) were rehydrated for one hour before being decapsulated in 3.5% sodium hypochlorite according to the method suggested by Lavens and Sorgeloos, 1996. After 24 hours incubation at 25°C under constant light, the nauplii were siphoned onto a 100 μm screen and washed with seawater. The nauplii were counted and suspended in seawater at the concentration required for the relevant experiment.

Statistical analysis

All data analysis was performed using the statistical software package *STATISTICA* v6 (Statsoft Inc., Tulsa, OK, USA).

Chapter 3

In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus

Published in *FEMS Microbiology Letters* 231(1): 145-152 (2004)

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Introduction

The use of probiotics in aquaculture is becoming increasingly popular, having recently been defined as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health" (Gatesoupe, 1999). To date, the screening of probiotics has been pragmatic, lacking meaningful selection criteria, and they are often assessed only on their ability to produce anti-microbial metabolites (Gatesoupe and Lesel, 1998; Rengpipat et al., 1998; Rico-Mora et al., 1998; Gibson et al., 1998; Cai et al., 1998; Gibson, 1999).

Isolating bacteria that produce anti-microbial metabolites is common practice, while experiments to determine at what stage of growth the bacteria produce the metabolites and whether the bacteria are able to compete for attachment sites (Vanbelle et al., 1990) are seldom performed. Such experiments are necessary as bacteria may only produce metabolites during the stationary growth phase (Monaghan et al., 1999), and this phase may not occur in the gut due to constant flushing. Thus, *in vitro* studies may create a false impression of the ability of probiotics to inhibit pathogens *in vivo*. Any inability to compete for attachment sites on the mucus of the gut wall suggests that these bacteria may not multiply sufficiently fast to compensate for being flushed from the mucus during gut evacuation.

Therefore, screening of candidate probiotics preferably requires that the various selective criteria such as antagonism, production of beneficial compounds, attachment and growth are all considered. This paper suggests a protocol for the isolation and selection of potential probiotic bacteria based on their *in vitro* growth characteristics. Bacterial growth characteristics such as lag period and doubling time are influenced by more factors than were tested in this study. Potential variables are pre-incubation, composition and concentration of

ingredients in culture media and temperature. This experiment used the same parameters for all organisms tested thereby allowing for direct comparisons between organisms. Future studies could test and modify the proposed ranking concept depending on different experimental conditions or additional variables. Here, the concept of a ranking index (RI) is proposed to screen potential aquaculture probionts before being tested, thereby reducing the time and expense of *in vivo* studies.

Materials and methods

Isolation and basic screening

Five adult common clownfish, *Amphiprion percula*, were killed by severing the spinal column. Each fish was surface washed for one minute with 0.1% benzylkonium chloride to remove external bacteria. Under sterile conditions, the gut region was dissected and separated into stomach and intestine, and then sliced open. Samples from the two regions were individually masticated in one ml of sterile seawater. Serial dilutions were made of each sample to dilutions of 10^{-8} from which aliquots of 100 µl were placed on Petri dishes containing media chosen for their general and selective abilities; marine agar is a general non-selective, heterotrophic bacterial media, MacConkey agar is selective for enterobacteriaceae, Thiosulphate-Citrate-Bile salt Agar (TCBS) selects for the genus *Vibrio*, and Pseudomonas-isolating agar selects for the genus *Pseudomonas*. The media were prepared according to the manufacturer's directions (DIFCO). The Petri dishes were incubated for 48 hours at 25°C after which colony-forming units (CFU) were counted. Counts between 30 and 300 CFUs were used for analysis.

Antagonism to pathogens

A bacterial growth inhibition study to test for the production of anti-microbial metabolites by the isolates was performed using seven fish pathogens: *Aeromonas hydrophila, A. salmonicida, Vibrio harveyi, V. anguillarium, V. damsela, V. alginolyticus* and *Carnobacterium piscicola*. Pathogens were incubated in marine broth for 24 hours at 25°C and incorporated into pour plates. Wells were cut into the agar and filled with 100 µl of the marine broth isolates. The presence of anti-microbial metabolites produced by the isolates inhibited the growth of the pathogen producing a zone of inhibition around the well. To determine at what stage of growth the anti-microbial metabolites were produced, a 50 ml volume of marine broth was inoculated with 4 ml from a 36-hour culture and incubated on a shaker at 25°C. Five-ml samples were taken every six hours for 48 hours, centrifuged (5000 rpm for 10 minutes) and the supernatant was filtered through a 0.22 µm filter. Pathogen pour plates, made as described above, were then inoculated with 100 µl of the supernatant. After 48 hours, zones of inhibition were recorded as being present or absent.

Growth

To further reduce the number of candidate probionts for *in vivo* studies, those that inhibited only one pathogen were excluded leaving twelve candidate probionts (from an original total of 106 isolates). Based on antagonism to the same pathogens, similar colony morphology characteristics (Gram stain, shape, colour, motility, oxidase production, catalase production and motility) and similar growth profiles in marine broth, duplicate candidate probionts were eliminated. The growth profiles of the bacteria were determined by inoculating the wells of a sterile 96-well microtitre plate with 250 µl of marine broth to which a 20 µl volume of 24-hour old marine broth bacterial culture was added. The plate was incubated at 25°C and the OD recorded at 640 nm (BioTek Powerwave ELx808 Microplate Reader/ KC Junior

software) every 15 minutes for 36 hours. Each culture was inoculated in triplicate and the readings of the profiles were averaged.

Growth profiles of five candidate probionts and two pathogens (*A. hydrophila* and *V. alginolyticus*) were used to compare characteristics such as length of lag phase (λ) and maximum specific growth rate (μ). These two pathogens were selected as they are both known to grow on intestinal mucus (Takahashi and Fujino, 1984; Sugita et al., 1987).

To assess their potential ability to grow in the gut, all bacteria were grown in marine broth or in intestinal fish mucus that was filter-sterilized (0.22 μm) and diluted to one mg/ml using Bradford's protein assay (Bradford, 1976). The bacteria were inoculated in triplicate into wells of a sterile microtitre plate with 250 μl of sterile mucus or marine broth to which a 20 μl volume of a 36-hour old marine broth bacterial culture was added. The plate was incubated for 36 hours at 25°C during which time the OD was read every 15 minutes at 640 nm.

The OD_{640} reference values were fitted to the logistic (1) and Gompertz functions (2) (Zar, 1984). These functions are non-linear models in which the exponential increase or decrease is corrected by an exponential term.

(1) $$y(t) = \frac{a}{1 + be^{(-ct)}}$$ (logistic growth model)

(2) $$y(t) = ae^{[-e^{(b-ct)}]}$$ (Gompertz growth model)

where e = natural log (2.718), y = population size, t = the incubation time and a, b and c are estimated coefficients.

The estimates within most sigmoidal growth curves usually have no biological meaning and therefore both models were rewritten according to the method proposed by Zwietering et al., 1990 who substituted the mathematical parameters with A (maximum biomass yield), μ (maximum specific growth rate) and λ (lag period) (Figure 3.1). This is achieved by deriving the biological parameters as a function of the parameters of the basic function and then substituting them into the equations (1) and (2), respectively. An example using the logistic growth equation (1) follows:

To determine the inflection point of the curve, the second derivative of the function with respect to t was calculated (3).

(3) $$\frac{d^2y}{dt^2} = abc^2e^{-ct}\frac{be^{-ct}-1}{(1+be^{-ct})^3}$$

At the inflection point, where $t = t_i$, the second derivative is equal to zero (4).

(4) $$\frac{d^2y}{dt^2} = 0 \rightarrow t_i = \frac{Ln(b)}{c}$$

The maximum specific growth rate (μ) was derived by calculating the first derivative at the inflection point (t_i).

(5) $$\mu = \left(\frac{dy}{dt}\right)_{t_i} = \frac{ac}{4}$$

Here, c can be substituted by $c = 4\mu/a$.

The lag period (λ) is defined as the t-axis intercept of the tangent line through the inflection point and can be described as:

(6) $$y = \frac{a}{2}\left(\frac{1}{2}c.\lambda + 1 - \frac{Lnb}{2}\right)$$

Using equations 4, 5 and 6 yields:

(7) $$\lambda = \frac{2}{c}\left(\frac{Lnb}{2} - 1\right)$$

The parameter b in the Logistic equation (1) was substituted by:

(8) $$b = e^{\left(\frac{4\mu m\lambda}{a}+2\right)}$$

The asymptotic value is reached for t approaching infinity:

(9) $$t \to \infty : y \to a \Rightarrow A = a$$

The parameter a in the Logistic equation (1) can be substituted by A, yielding the modified logistic equation:

(10) $$y = \frac{A}{\left\{1 + e^{\left[\frac{4\mu_m}{A}(\lambda - t) + 2\right]}\right\}}$$

Similarly, the Gompertz equation can be solved using the parameters μ, λ and A to produce:

(11) $$y = Ae^{\left\{-e^{\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]}\right\}}$$

The growth models were fitted to the data for the period from the start of incubation (t=0) to the start of the stationary phase since the osmotic swelling of bacteria in a hypotonic medium can considerably decrease the opacity, probably through lowering the refractive index of the cells (Pirt, 1985). Curve fitting and estimation of the kinetic parameters was performed using the Lavenberg-Marquardt method (Marquardt, 1963), which minimizes the sum of squares differences between measured and predicted values. Starting values are estimated by searching for the steepest ascent of the curve between four data points (estimation of μ) and where this intersects the x-axis (estimation of λ), the final data point is taken as an estimation for the asymptote (A).

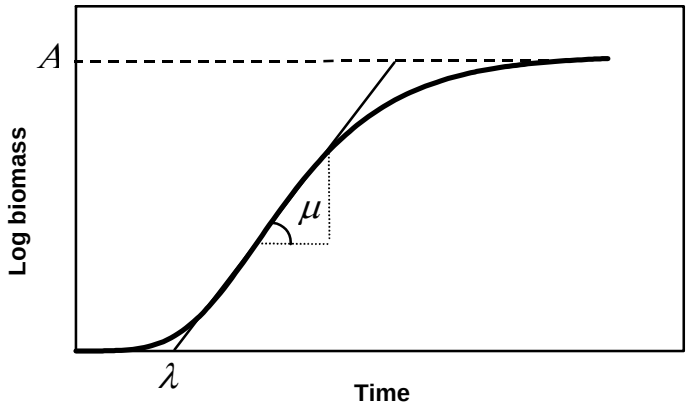

Figure 3.1 - A growth curve and its phases (*A* = maximum biomass yield, μ = maximum specific growth rate, and λ = lag period (From Zwietering et al., 1990).

The doubling time (t_d), which is the time taken to double the biomass during the exponential growth period is described as:

$$t_d = \frac{Ln(2)}{\mu} \quad (12)$$

In two cases, one of the three growth profiles was distinctly different from the other two (probably due to contamination) and was discarded in the analysis. To provide an equal starting point for growth of each bacteria, the data were blanked using the average absorbance at t=0.

The model for each bacteria was chosen based on the results of the following four steps: (i) the calculation of r^2, the coefficient of determination, (ii) a residual pattern analysis, (iii) the

biological interpretation of parameters μ, λ and A, and (iv) an estimation of the models' F-value.

Since this experiment was designed to aid the screening of large numbers of potential fish probiotics, a ranking index (RI) was developed using the parameters' doubling time (t_d) and lag period (λ) converted into integer hours and minutes (i.e., 3h40 = 3.67).

$$RI = \frac{1}{\lambda \times t_d} \times 100 \quad (12)$$

The RI hypothesises that a bacterium with a short lag period and short doubling time has a better chance of out-competing other bacteria based on their growth characteristics.

Results

Isolation and screening

Bacterial growth on the various media is summarized in Table 3.1.

Table 3.1 – Bacterial cell concentrations based on colony forming units (CFUs) isolated from the gut and stomach of the common clownfish *Amphiprion percula* on various standard microbiological media. N = number of plates on which CFUs ranged between 30 and 300.

Medium	Gut (CFU.mL^{-1})	N	Stomach (CFU.mL^{-1})	N
Marine Agar 2216e	6.78×10^{6}	11	2.72×10^{7}	12
MacConkey Agar	8.75×10^{4}	6	1.75×10^{5}	1
TCBS Agar	4.01×10^{6}	4	3.08×10^{3}	2
Pseudomonas Isolating Agar	6.75×10^{5}	2	1.10×10^{6}	1

TCBS - Thiosulphate-Citrate-Bile Salt

Antagonism to pathogens

Pathogenic bacterial growth was greatest on marine agar. The number of vibrios in the intestine was high and contributed 59% as compared to pseudomonads, which contributed less than 10%. The stomach microflora was composed of 4% pseudomonads and less than 0.001% vibrios. Antimicrobial metabolites were produced by all candidate probionts during the stationary phase and no zones of inhibition were observed during the lag or exponential phase.

The five candidate probiotics collectively showed antagonistic activity against the seven pathogens. Candidate probiotic AP3 showed the greatest antagonistic activity, inhibiting five of the pathogens. The pathogens *V. damsela* and *C. piscicola* had the highest number of antagonists with four each (Table 3.2).

Table 3.2 - Antagonism (X) between candidate probiotics (AP1–AP5) and fish pathogens grown on marine agar. The plates were pathogen pour plates within which wells had been punched and the candidate probiotic was added in marine broth. *A = Aeromonas, V = Vibrio, C = Carnobacterium.*

Probionts	**Pathogens**						
	A. salmonicida	***A. hydrophila***	***V. alginolyticus***	***V. harveyi***	***V. anguillarium***	***V. damsela***	***C. piscicola***
AP1			X				X
AP2						X	X
AP3	X	X		X	X	X	
AP4						X	X
AP5			X			X	X

Growth

All models gave a visually good fit to the data ($p<0.01$). Only models with r^2 values greater than 0.90 were considered for analysis. The residuals were plotted for each model and checked by eye to ensure they conformed to a normal distribution. *F*-values were determined for each model with the best model being chosen based on the highest r^2 value and highest *F*-value. The Gompertz model was the best-fitting model for eight of the 15 growth profiles.

All bacteria grew in both the marine broth and gut mucus. The growth profiles in marine broth were typical, single-phase profiles. Growth in mucus, however produced a diauxic profile for *V. alginolyticus* (Fig. 3.2). The lag period was greater for all bacteria in mucus when compared to growth in marine broth (Table 3.3): however, μ was greater for AP1, AP4

and *V. alginolyticus* when grown in mucus. The growth parameters of candidate probiotic and pathogenic bacteria grown in marine broth and intestinal fish mucus are shown in Table 3.3. Figure 3.2 depicts the growth of candidate probionts AP2 and AP5, and pathogens *V. alginolyticus* and *A. hydrophila* grown in fish gut mucus. No model for *V. alginolyticus* was plotted because the profile is diauxic and rather two separate models were used, one for each λ, μ, and A of the two stages. The growth curves in Figure 3.2 are representative of others obtained in this study.

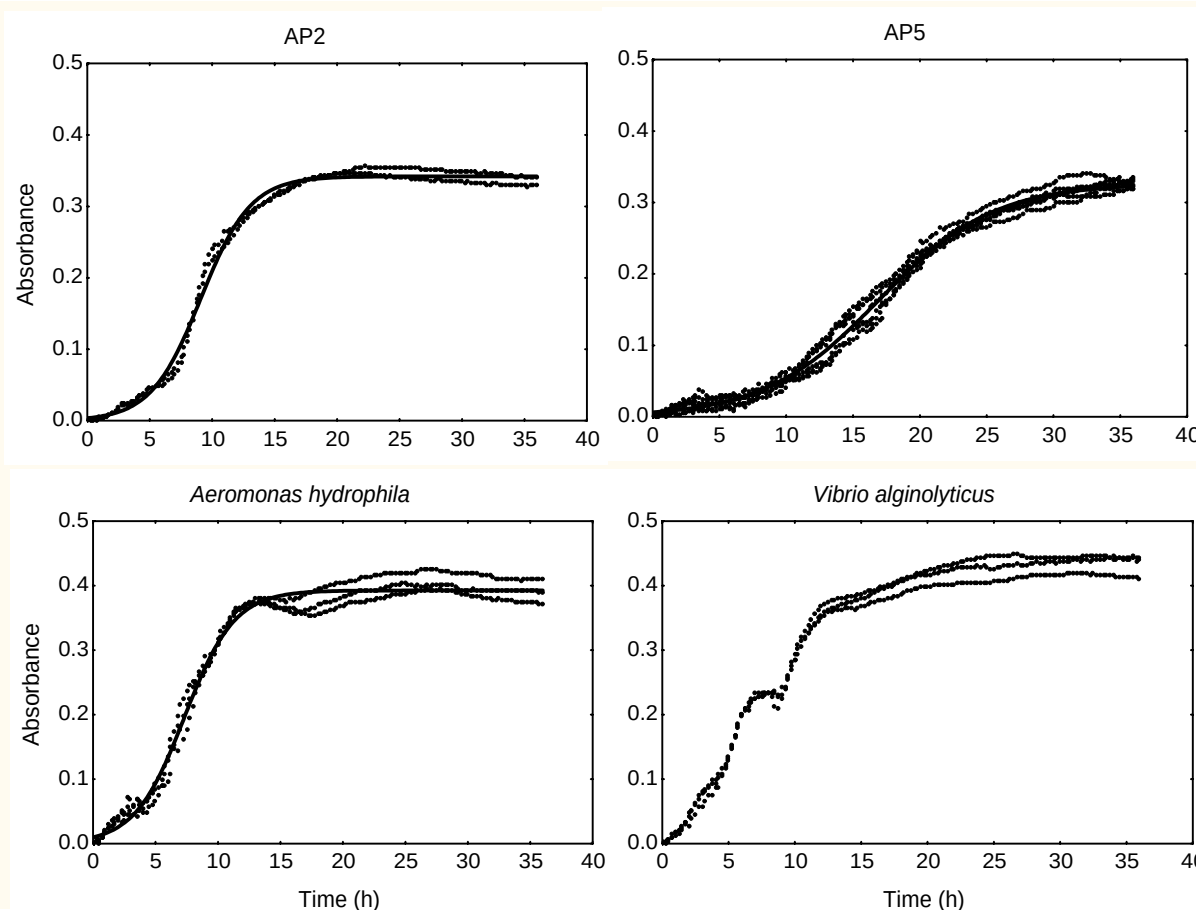

Figure 3.2 - Growth profiles and models of candidate probiotics AP2 and AP5, and the pathogen *Aeromonas hydrophila* grown in intestinal fish mucus. Due to the diauxic growth profile of *Vibrio alginolyticus* only the data points are shown.

The ranking indices of AP1, AP2 and AP5 was highest for that among all candidate probionts when grown in fish intestinal mucus (Table 3.3). The average RI for the pathogens was 2.65 for marine broth and 2.89 for mucus (only the first model of *V. alginolyticus* growth is included).

Table 3.3 - Growth parameters of five candidate probionts and two pathogens grown in marine broth and fish intestinal mucus. Parameters *A* (asymptote), μ (specific growth rate) and λ (lag period) were estimated using either the logistic or Gompertz equations, which were selected based on criteria discussed in the text. Ranking Index = 1/(doubling time (t_d) x λ) x 100.

*** negative values not given.**

Treatment	Model	Model parameters			t_d	r^2	F-Value	Ranking Index
		A	μ	λ				
Marine Broth								
AP1	Gompertz	0.376	0.044	2:23	15:43	0.943	17138	2.67
AP2	Logistic	0.397	0.062	3:31	11:14	0.996	38315	2.54
AP3	Logistic	0.447	0.047	13:18	14:45	0.998	103220	0.51
AP4	Gompertz	0.208	0.006	*	114:11	0.983	40534	*
AP5	Gompertz	0.233	0.021	0:44	32:33	0.972	37260	4.14
A. hydrophila	Gompertz	0.342	0.035	2:08	19:58	0.991	112181	2.35
V. alginolyticus	Gompertz	0.333	0.036	1:45	19:28	0.994	189448	2.94
Mucus								
AP1	Logistic	0.376	0.050	3:49	13:54	0.981	47830	1.89
AP2	Logistic	0.342	0.043	5:05	16:13	0.994	81669	1.21
AP3	Gompertz	0.822	0.017	14:47	39:41	0.975	7903	0.17
AP4	Gompertz	0.189	0.007	22:52	95:58	0.986	10055	0.05
AP5	Logistic	0.327	0.019	8:19	36:33	0.993	118839	0.33
A. hydrophila	Logistic	0.393	0.049	3:20	14:06	0.985	63874	2.13
V. alginolyticus	Logistic	0.250	0.047	1:51	14:50	0.986	8179	3.65
1st model								
2nd model	Gompertz	0.203	0.025	0:37	27:13	0.94	15910	6.04

The candidate probiont AP4 showed slow growth in marine broth and failed to reach an asymptote after 36 hours. As a result, the line depicting growth was almost linear and the $y \Rightarrow 0$ portion of the curve intercepts the t-axis on the negative quadrant to yield a negative λ.

Discussion

The aim of this study was to provide a method for the *in vitro* screening of potential probiotics that could be applied to combat fish pathogens under aquaculture conditions. To accomplish this, the growth of five probiotics *in vitro* was compared to that of two pathogens. Using estimates of coefficients from two best-fit non-linear growth models, a ranking index was produced by combining estimates for doubling time t_d and lag period λ. The assumption was that a probiotic will be most competitive *in vivo* if it has a short lag phase and a fast growth rate. However, as growth is not the only criterion useful in screening potential probiotics, other modes of competition such as fast growth and ability to attach to the intestinal tract can be considered together with the probiotic's ability to produce substances antagonistic to pathogens.

In marine broth, candidate probiotics with a shorter lag period and a greater doubling time than the pathogens were AP5 and AP1, respectively. In mucus, the μ value of AP1 (0.050) was slightly higher than that of the pathogens (0.049 and 0.047) and the lag period was longer. Thus, it is hypothesised that these bacteria cultured in fish mucus *in vitro* before inoculation into the larval fish culture system during the exponential phase may be able to compete in the gut with pathogens that require time λ to reach the exponential phase. AP5 grown in marine broth had a shorter lag period than any of the pathogens. However, it has a much lower μ value which suggests that the time gained due to the shorter lag period may be offset by the pathogen's faster specific growth rate (μ).

I suggest that lag period and doubling time are the most appropriate criteria for the comparison of growth patterns while the value representing the maximum biomass yield (*A*) of the bacteria should not be used as a means of comparison. This is due to differences in absorbance values of the probiotics and pathogens caused by the changing size and shape of the microorganisms since these features alter the degree and direction of light scatter (Pirt, 1985). Such species-specific absorbance values limit direct comparison of growth profiles.

These results confirm suggestions by other authors that growth should not be viewed in isolation since all probiotic bacteria produced antagonistic metabolites (Sugita et al., 1997; Robertson et al., 2000). For example, the probiotic with the longest lag period (AP3) inhibited five of the seven pathogens although its ranking index was low. The two highest-ranking probiotics showed combined antagonism to three pathogens. Thus, the ability to inhibit the growth of competitors through the production of anti-microbial metabolites is one way a bacterial species is able to establish itself over competitors. In marine fish, the gut's bacterial microflora varies from species to species with intraspecific interactions (Ringø and Birkbeck, 1999). The major taxonomic groups contributing to the healthy intestinal flora of marine fish species include *Vibrio, Pseudomonas, Acinetobacter* and *Achromobacter*, followed by *Micrococcus*, *Bacillus* and representatives from the family Enterobacteriaceae making up the majority of the remainder (Liston, 1957; Colwell, 1962; Newman et al., 1972; Ringø and Strøm, 1994). In aquaculture, non-pathogenic strains of known pathogens have been successfully used as probiotics (Austin et al., 1995; Gomez-Gil et al., 2002; Chythanya et al., 2002), however, testing whether strains are possibly pathogenic before large-scale use, is strongly advised. Due to the presence of pathogenic and opportunistic bacteria in the environment (Skjermo et al., 1997), these organisms need to be suppressed to reduce their

proliferation and consequently the incidence of disease. This study provided for the first time an estimation of the growth pattern of two selected pathogens, *A. hydrophila* and *V. alginolyticus*, in fish intestinal mucus. The pathogen *A. hydrophila* exhibited a diauxic growth profile when grown in mucus and a single growth profile when grown in marine broth. This type of growth has been noted in *Escherichia coli* as it sequentially utilizes glucose and then lactose (Pirt, 1985). Fast-growing pathogenic bacteria like *V. alginolyticus* and *A. hydrophila* are considered *r*-selected species (Andrews, 1984). The rapid growth of these pathogens in intestinal mucus confirms that the intestinal tract serves as a site of growth (Balebona et al., 1998). If these bacteria are present in the culture environment in low numbers, and given that their lag period appears to be shorter and their maximum specific growth rate higher than that of other bacteria, these species could reach potentially harmful concentrations under *in vivo* conditions.

The results have practical applications. For example, based on the ranking indices of the candidate probionts grown in mucus, AP1 has one growth parameter (μ) greater than that of the pathogens tested. However, the manner in which the potential probiont grows in a bacterial community may be different. Strategies to increase probiotic concentration include the inoculation of probiotics and pre-conditioning of these bacteria to reduce the lag period. It should be tested whether or not such strategies will allow the probiotic bacteria to dominate initially and thereby gain a competitive advantage. This could become an important aspect under *in vivo* conditions where both attachment and nutrient supply differs from that found in *in vitro* studies. Here, the growth of the bacteria may be less than the rate of flushing from the intestine, and the bacteria may become unable to attach to the intestinal mucus or the intestinal wall where they will be flushed before reaching a viable population level (Cahill, 1990). A probiotic may colonise the fish intestinal tract and prevent the proliferation of

pathogenic or opportunistic bacteria if certain of its growth characteristics are superior to that of the pathogen. This experiment identified no bacteria grown in mucus with a ranking index greater than that of the two pathogens. Pathogens are usually fast-growing (Andrews and Harris, 1986), *r*-selected strategists. Thus, a high starting concentration of probiotics may make them more competitive. This would favour the use of AP3, which showed relatively high inhibition potential. Knowing its growth pattern, the bacteria could be cultured in large numbers before addition to the aquaculture system. For example, as the probiont reaches its maximum specific growth rate μ, the pathogen, having a smaller population size, will only be reaching its own maximum μ at an overall smaller population size. Although the doubling time of some the candidate probiotics may be longer than those of the pathogens, it must be noted that the concentration of probionts introduced into the water is likely to be much higher (from 10^5 to 10^7 cells.ml^{-1}) than that of the pathogens. A slow rate of bacterial colonization has been shown to be beneficial to turbot larvae during the critical period of gut development (Munro et al., 1993b), suggesting that an optimal dose exists that allows sufficient numbers of the probionts to compete with the pathogens.

Substrate availability is another factor to be consider when predicting the outcome of *in vivo* studies based on results obtained from *in vitro* experiments. The Monod relation (Monod, 1949) predicts bacterial growth rate (μ) as a function of substrate concentration. Mucus in the intestine is likely to be a replenishable growth substrate occurring at concentrations greater than that used in this experiment. Likewise, *in vivo* bacterial growth may be faster than that found in this experiment. Consequently, the ranking indices of some bacteria may increase as λ would remain constant while μ increases.

Furthermore, the effect of probiotics on previously established microflora and on the proliferation of opportunistic bacteria both *in vitro* and *in vivo* warrants further investigation using microbiologically matured seawater with its diverse microflora. Matured seawater is dominated by non-opportunistic (K-strategist) bacteria with a high substrate affinity and better competitive ability at low substrate supply as compared to opportunist (*r*-strategist) bacteria (Andrews and Harris, 1986; Skjermo and Vadstein, 1999). For example, microbial maturation of tank water has been shown to enhance larval survival and growth (Skjermo and Vadstein, 1999). The bacterial concentration of seawater has been shown to range from 7 x 10^3 cells.ml^{-1} (Eddy and Jones, 2002) to 3.7 x 10^4 cells.ml^{-1} (Riquelme et al., 2001) while microbiologically matured seawater contains approximately 7.5 x 10^4 bacteria.ml^{-1} (Salvesen et al., 1999) and relatively more K-selective species (Skjermo et al., 1997).

In conclusion, probiotics *in vivo* may become more competitive than predicted from experimental observations of their growth patterns or their starting population density as the production of anti-microbial metabolites or better attachment ability lead to inhibition *in vitro.* The production or effect of inhibitory metabolites depends on the media on which the probiotic is cultured (Olsson et al., 1992). Further tests examining the production of anti-microbial metabolites in fish mucus need to be carried out to verify preliminary conclusions from this study and account for the factors that affect the ranking of candidate probiotics *in vivo.* Furthermore, the methods developed in this study can help in the screening process of probiotics in human and terrestrial animal nutrition.

Chapter 4

Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus

Published in *Journal of Fish Diseases* 27: 319-326 (2004)

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Introduction

The worldwide increase in bacterial resistance to antibiotics (van der Waaij and Nord, 2000) has stimulated investigations into the use of probiotics. In aquaculture, antibiotics are discharged into the environment causing the occurrence of resistant bacteria on fish farms (Aoki et al., 1980; DePaola, 1995; Kerry et al., 1997; Miranda and Zemelman, 2002) or in the sediment below net cages (Bjorklund et al., 1990). With tighter regulations on the use of antibiotics in aquaculture, the use of probiotics has increasing potential (Austin et al., 1995; Ruiz et al., 1996; Gatesoupe and Lesel, 1998; Rengpipat et al., 1998; Gibson et al., 1998; Barker, 1998; Shiri Harzevili et al., 1998; Gatesoupe, 1999; Skjermo and Vadstein, 1999; Gram et al., 1999; Ringø and Birkbeck, 1999; Gomez-Gil et al., 2000). Probiotics make up part of the resident microflora and contribute to the health or well-being of their host (Gatesoupe, 1999). To remain within their host, they must either attach to the intestinal tract or grow fast enough to prevent them from being flushed out by the movement of food through the digestive tract. By attaching to the intestinal mucosa, probiotics can extend their time within the gut thereby influencing the gastrointestinal microflora of their host (Andlid et al., 1998; Kirjavainen et al., 1998; Mack et al., 1999; Ouwehand et al., 2001; Forestier et al., 2001).

Several candidate probiotic bacteria that produce anti-microbial metabolites have been isolated (Austin et al., 1995; Ruiz et al., 1996; Canganella et al., 1996; Robertson et al., 2000; Gatesoupe, 2002; Vine et al., 2004a); however, it has not been established at what stage bacteria produce the metabolites and whether or how well they compete for attachment (Gatesoupe, 1989). There is a need for such experiments as candidate probiotic bacteria may only produce anti-microbial metabolites during the stationary growth phase (Monaghan et al., 1999; Vine et al., 2004a). Therefore, if the probiotic bacteria are unable to attach to the

intestinal mucus, the stationary growth phase may not be achieved in the gut due to them being flushed out. Where attachment has been observed experimentally *in vitro*, expectations that probiotics will inhibit pathogens *in vivo* may be unfounded. Therefore, hypotheses derived from *in vitro* studies about interactions between bacteria need to be tested *in vivo*. However, before testing probiotics *in vivo* an understanding of the interaction between pathogens and probionts and factors governing competition between them is necessary. In particular, the effect of pathogens on the attachment ability of probiotics has not been tested. Characklis and Marshall (1990) defined attachment as the capture and/or entrapment of cells in a biofilm. This refers to the interaction of the bulk liquid (or in this case, mucus) compartment components with the biofilm components, which in contrast to adsorption, occurs at the liquid-substratum interface (Characklis and Marshall, 1990).

Adhesion of probiotic bacteria to the intestinal mucosa has been shown to enhance their antagonistic activity against pathogens (Coconnier et al., 2003). In humans, the antibody titres in serum treated with probiotic bacteria were directly correlated with the adherence ability of that strain (Juntunen et al., 2001) suggesting that probiotic attachment enhanced the health of its host. In a healthy gut, attachment may allow the probiotic to exert its beneficial effects whilst in a diseased gut it may reduce the possibility of pathogen translocation when the host's defence mechanisms are impaired (Apostolou et al., 2001).

Candidate probionts were isolated from adult clownfish (*Amphiprion percula*), a popular marine aquarium species. Five previously unstudied candidate probionts isolated from this clownfish species, and two fish pathogens were tested for their ability to attach to mucus and to compete for attachment sites. This paper introduces a novel tool for the *in vitro* assessment

of competition for adhesion between micro-organisms using two different labelled isotopes.

Materials & methods

Two radioactive isotopes were used to monitor attachment and quantify competition between five probiotics and two pathogens. The candidate probionts had been isolated from the intestinal tract of the common clownfish (*A. percula*) (Chapter 3). Five candidate probionts (AP1-AP5) were cultured in 10 ml of marine broth containing 100 µl [^{3}H] thymidine ([*methyl*-1,2-^{3}H]thymidine, 185 MBq/5µl; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The pathogens, *Vibrio alginolyticus* and *Aeromonas hydrophila*, were cultured in the same medium except that it was enriched with 100 µl [^{14}C] thymidine (185 MBq/5µl; Amersham Pharmacia Biotech Inc.) as the radioactive label. All cultures were incubated at 25°C for 36 hours and then centrifuged at 2000 *g* for 10 minutes. The centrifuged pellet was resuspended in sterile Nine Salt Solution (NSS) (Olsson et al., 1996) and cell density was adjusted to approximately 10^{8} cells.ml^{-1}. Triplicate samples from the original culture of 250 µl were taken for radiolabel incorporation analysis.

Due to the large volume of mucus required, mucus from the spotted grunter, *Pomadasys commersonnii* (Haemulidae), was used. This species was chosen as it was easily available and, like *A. percula*, is omnivorous. Mucus was collected from the intestinal tract of three adult fish, suspended in NSS, and centrifuged twice at 8000 *g* for 15 minutes to remove particulate matter. The remaining supernatant was filter-sterilised through a 0.22 µm filter, diluted to 0.25 mg.ml^{-1} using Bradford's protein assay (Bradford, 1976), and kept at 4°C.

The choice of the pathogen *V. alginolyticus* was based on its ability to colonise the gastrointestinal tract (Sugita et al., 1987). The pathogen *A. hydrophila* has been isolated from

the intestine of infected fish and proliferates in the gut (Takahashi and Fujino, 1984; Bricknell et al., 1999) and the presence of adhesive factors such as adhesin which mediates bacterial adhesion to cells has been suggested (Krovacek et al., 1987). Pathogen cultures were obtained from Onderstepoort Veterinary Faculty (University of Pretoria, South Africa), having been previously isolated from diseased fish.

The method used in the in vitro assay was based in part on past studies (Olsson et al., 1992; Rinkinen et al., 2000). A volume of 250 µl mucus was immobilised in sterile microtitre plates (Corning Costar, Cambridge, MA, USA) and incubated overnight at 4°C. Any unbound mucus was removed by washing the wells twice with 250 µl NSS after which 200 µl aliquots of the washed and labelled probionts were added to the well for two hours. The wells were washed twice with 250 µl sterile NSS to remove any non-adhering bacteria. Each treatment was performed in triplicate and wells containing only NSS served as controls.

This assay differed from previous studies in that bacteria from the mucus and NSS control wells were then removed as described below, while labelled candidate probiotic cultures were added to the wells containing pathogens and *vice versa* for four hours to allow for competition for attachment on the mucus. After this, the wells were washed twice with 250 µl sterile NSS to remove non-adhering bacteria. The adhering bacteria were then removed using 250 µl 10% sodium dodecyl sulfate (SDS) added to each well and incubated at 60°C for one hour.

The 250 µl contents of each well was added to scintillation vials containing 4 ml scintillation fluid and left for twelve hours. Radioactivity was determined by liquid scintillation counting ^{3}H in the 0-400 channel and ^{14}C in the 400-670 channel. The number of adhering bacteria

(candidate probionts and pathogens) was assessed as the radioactivity recovered from the wells compared to the radioactivity of the original bacterial suspension.

Statistics

Differences in adherence amongst and between the candidate probiotics and pathogens as well as the sequence in which they were added were investigated. To achieve this, the average number of attached cells were tested for significant differences between treatments using ANOVA and a Student's *t*-test. In two cases one of the triplicate's missing values was estimated using the average of the two known observations. A MANOVA was deemed inappropriate due to second order interactions as well as the small sample size. Also, as interaction effects existed, the factors' effects did not have a clear interpretation. Therefore, it was not deemed advisable to proceed with multivariate tests, and two univariate three-way analyses of variance (one for each variable) were conducted, as recommended by Johnson and Wichern, 1998. The three factors tested in the ANOVA were (i) the sequence of addition (probiotic then pathogen, pathogen then probiotic), (ii) the probiotic (AP1-AP5) and (iii) the pathogen (*A. hydrophila* or *V. alginolyticus*). Where main effects were significant, Scheffe's test was used to determine which levels were significantly different at the 5% level of significance. In addition, the competition data (i.e. differences in adherence between probionts and pathogens) were compared with their respective control using an ANOVA. The controls were the five probionts and two pathogens on their own.

Results

Incorporation of radioactive label was in the range of 7.7 $x10^{-4}$ to 7.4 $x10^{-2}$ nCi per cell. Incorporation of ^{3}H ranged from 1 858 cpm per 10^{8} cells (AP3) to 6 246 cpm per 10^{8} cells (AP4) and ^{14}C incorporation ranged from 1 752 cpm per 10^{8} cells (*A. hydrophila*) to 1 985

cpm per 10^8 cells (*V. alginolyticus*). Attachment of the control candidate probiotic cells to the mucus ranged from 6.7 x 10^5 cells per well (AP1) to 2.3 x 10^6 cells per well (AP3). The attachment of the pathogens was 1.6 x 10^6 cells per well for *V. alginolyticus* and 2.4 x 10^6 cells per well for *A. hydrophila*.

With the exception of AP1, all candidate probionts showed greater attachment to intestinal mucus than to the surface of the uncoated well. Attachment of AP1 to mucus was less than half while AP5 was more than thrice the attachment on the uncoated well. Between the controls, the average attachment was significantly different for the candidate probiotics ($p<0.01$) (Figure 4.1), but not the pathogens (Figure 4.2). The three-way factorial ANOVA showed that the effects of sequence of addition, candidate probiotic attachment and pathogen attachment were all significant ($p<0.01$) as were the interactions between sequence of addition and probiotic attachment and pathogen attachment ($p<0.05$).

The attachment success of AP3 was greater when the bacteria were added after *A. hydrophila* and, except when added either before or after *V. alginolyticus*, where it was significantly different ($p<0.05$) to all the other combinations (Figure 4.1). When added after the pathogens, both AP5 and AP4 with *V. alginolyticus* showed less than 100% attachment relative to their controls (Figure 4.1). In all other combinations attachment was improved in those bacteria added second.

A. hydrophila showed the greatest attachment of the pathogens when added after AP2. Attachment was different ($p<0.05$) from all others (including *V. alginolyticus*) except when it was added after AP1, AP3 and AP4 (Figure 4.2).

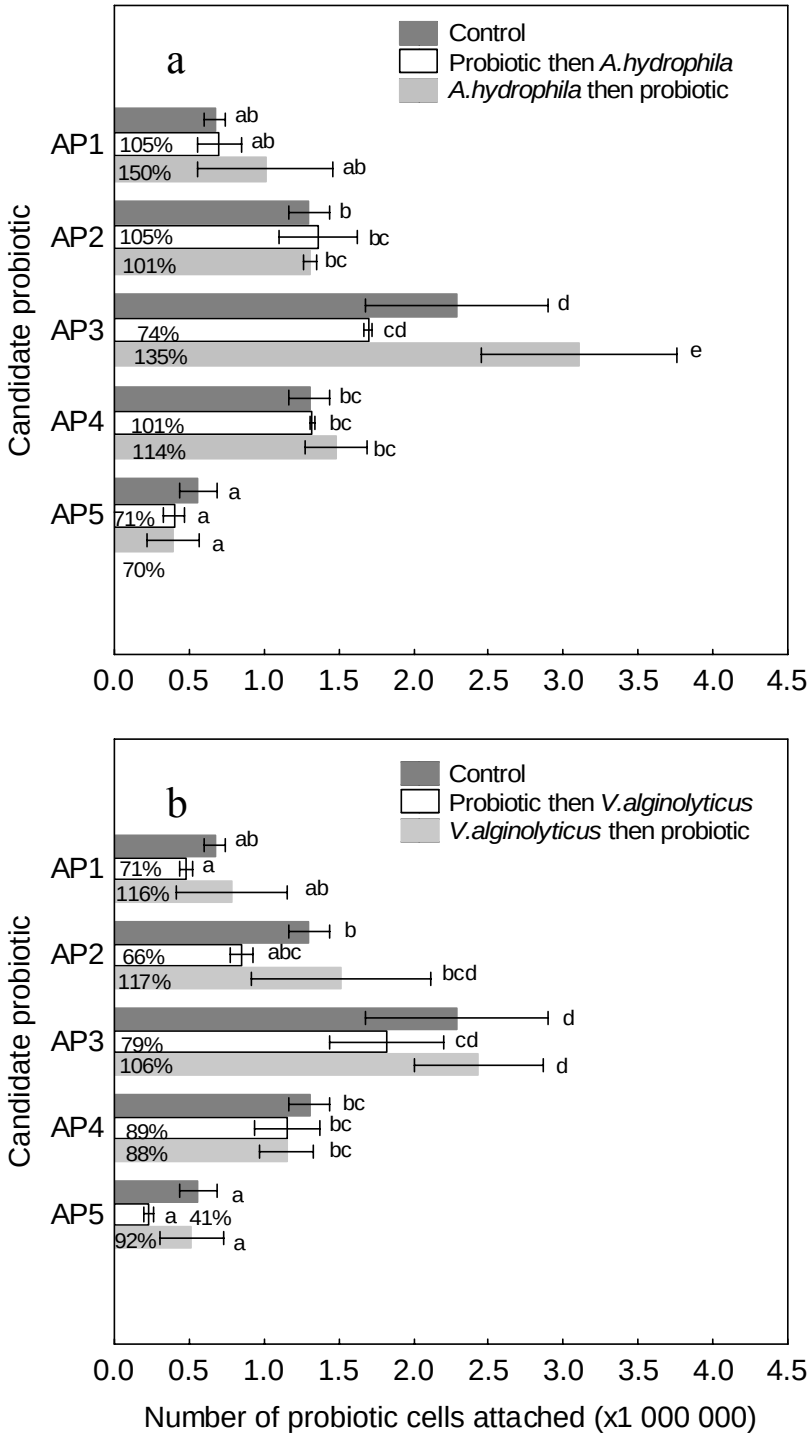

Figure 4.1 - Mean number ± standard deviations (columns) and percentage average change (text in columns) of candidate probiotic bacterial cells (AP1-AP5) attached to intestinal fish mucus before and after addition of pathogens (a) *Aeromonas hydrophila* and (b) *Vibrio alginolyticus*. Control candidate probionts were added alone. Different letters ($^{a-e}$) indicate statistically significant differences ($p < 0.05$).

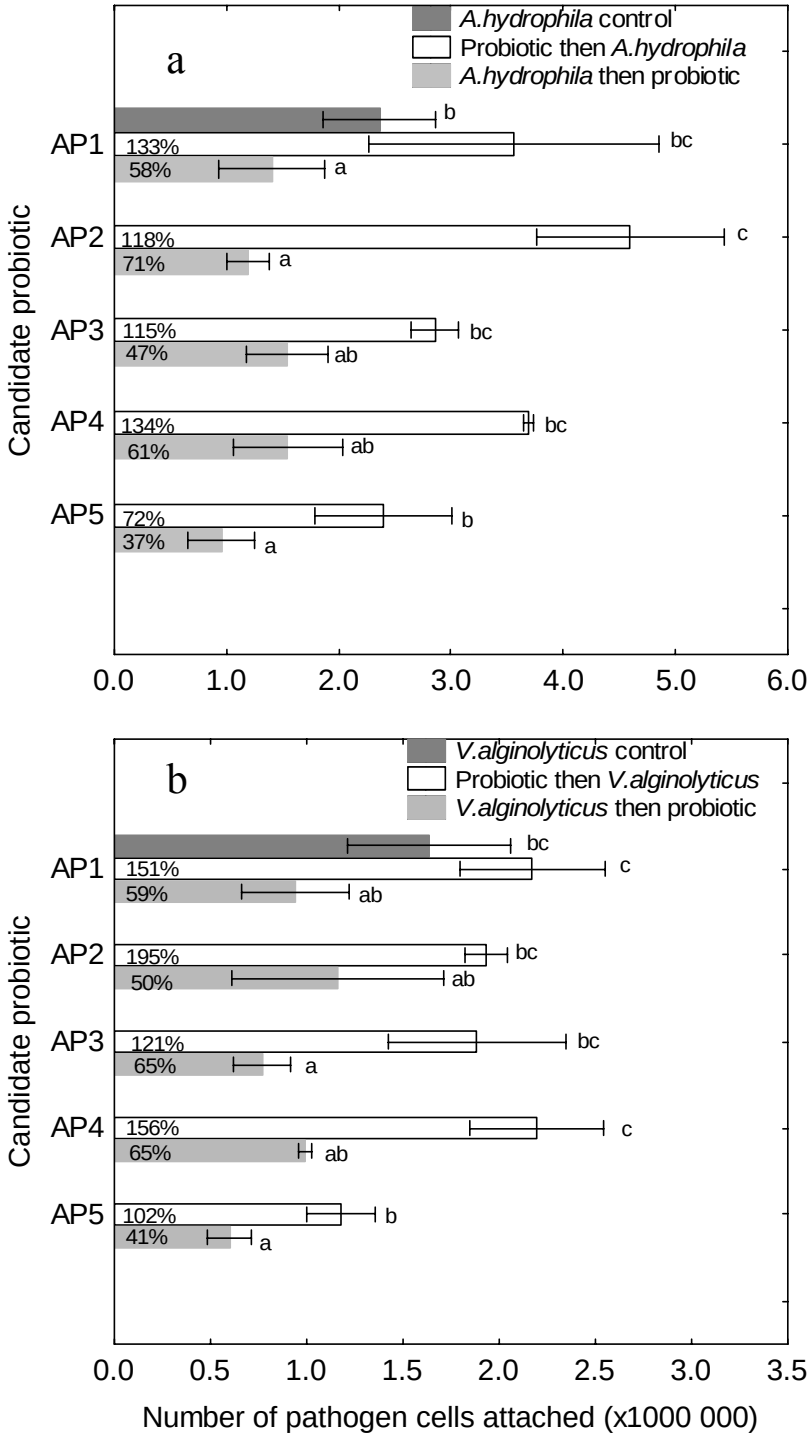

Figure 4.2 – Mean number ± standard deviations (columns) and percentage average change (text in columns) of (a) *Aeromonas hydrophila* and (b) *Vibrio alginolyticus* cells attached to intestinal fish mucus before and after addition of candidate probionts AP1-AP5. Control pathogens were added alone. Different superscripts ($^{a-e}$) indicate statistically significant differences ($p < 0.05$).

Discussion

The use of two isotopes as a novel tool to show competition between bacteria

The isotope method used in this study made it possible to identify competition between bacteria for attachment on mucus. Overall, probiotic attachment was greater when the candidate probiotics were added after the pathogens. Similarly, pathogen attachment was improved when they were added after the candidate probiotics. This can be interpreted in a few ways: – for example, the attached bacteria may have modified the mucus structure, creating more or better attachment sites for the bacteria added. Alternatively, the bacteria added second may have competed with the attached bacteria for attachment sites. Since different radiolabels were used to identify the candidate probiotics and pathogens it was possible to determine the competitiveness of each, thus using a technique that other studies have not applied. Using this tool, it could be demonstrated that pathogens could displace candidate probiotics and vice versa.

Data reporting

This study has also highlighted the importance of data reporting. For example, attachment success has been described as a function of the number of cells attached and the total number of cells available (Ouwehand et al., 2000; Juntunen et al., 2001; Rinkinen et al., 2003a). Using percentage change makes comparisons with other studies difficult where organism concentrations differ. An example of this is AP1 which showed 105% attachment when added after *A. hydrophila* but was significantly different (when analysed as cells attached per well) from the 106% of AP3 when added after *V. alginolyticus* (Figure 4.1). Although the percent change is an easily interpreted concept, to prevent confusion and provide comparisons, future studies should report the number of cells attached per well together with the percent change data.

Growth profile and attachment success

Adhesion appears to be greater during the stationary phase than during the logarithmic growth phase (Blum et al., 1999). All candidate probiotics and pathogens attached to and were capable of growing in the mucus used for this study (Vine et al., 2004a). Cells for this experiment were taken from 36-hour marine broth cultures during the stationary phase of probionts AP1, AP2, AP3 and AP5 as well as the pathogens *A. hydrophila* and *V. alginolyticus*. This was possible since the growth curves for the respective bacteria had been described (Vine et al., 2004a). Therefore, the ability of either the pathogens or the probionts to attach to mucus cannot be attributed to any differences in the growth phase of these bacteria. Only the probiont AP4 was an exception as it had a long lag-period when grown in mucus (Vine et al., 2004a); it was in the exponential growth phase when used in this experiment.

Sequence of attachment by bacteria

The results indicate that attachment of the pathogens is generally enhanced by the presence of the probiotics. However, the addition of probiotics after the pathogens results in reduced pathogen attachment relative to the controls (Figure 4.2). Only AP5 caused a lower attachment success of *V. alginolyticus* when added before the pathogen (Figure 4.2). When AP5 was added first, the average attachment change was 41% compared to 72% when added after *V. alginolyticus* (Figure 4.2) suggesting that the probiotic was displaced but enhanced attachment of the pathogen did not occur. Conversely, when *V. alginolyticus* was added first followed by AP5, attachment change was 37% (Figure 4.2) while AP5 had 92% attachment change when added second (Figure 4.1). This implies that the pathogen was displaced for attachment sites by the probiotic. From these findings it would appear that of the candidate

probiotics tested, AP5 has the greatest potential to beneficially influence the intestinal microflora through manipulating the attachment of other microorganisms. This does not mean that candidate probionts AP1-AP4 are not potentially suitable candidate probiotics as other criteria such as the production of antimicrobials, enzymes or fatty acids are also important considerations when selecting probiotics, which may be better than AP5.

Since the mucus layer covering the intestinal cells is the initial contact surface for ingested micro-organisms it is considered an important site for bacterial adhesion and colonisation (Olsson et al., 1992; Mikelsaar et al., 1998; Ouwehand et al., 2001). A possible explanation for this competition for attachment comes from a previous study on these probionts which showed that the candidate probiotics used in this experiment produced antimicrobial metabolites antagonistic to a range of aquatic pathogens (Vine et al., 2004a). However, explaining how these candidate probiotics affected the attachment of the pathogens was not part of the study objectives and is worth investigating in future studies. Contributing to this, Forestier et al., 2001 showed that the addition of the human probiotic *Lactobacillus casei* subsp. *rhamnosus* before, during or after the incubation with three human pathogens to an intestinal cell line was shown to reduce the adhesion success of the pathogens. It has been hypothesised that the presence of the probiont may restrict the access of pathogens to tissue receptors by steric hindrance (Forestier et al., 2001) or by blocking the receptor with specific adhesin analogues (Tuomola et al., 1999). The adherence of strains of the human probiotics, *Lactobacillus acidophilus* (Bernet et al., 1994) and *Lactobacillus johnsonii* (Blum et al., 1999) were influenced by the presence of a proteinaceous promoting factor in the bacterial culture supernatant.

Similarly, some bacteria are capable of preventing the adhesion of other micro-organisms due to factors other than the production of antimicrobial substances such as antibiotics or siderophores (Mukai et al., 2002). Although all the candidate probiotics in this study were shown to attach to mucus, it seems the ability of candidate probiont AP5 to attach to mucus and inhibit the attachment of pathogens, by its presence, might suppress the growth of the pathogen in the digestive tract (Figure 4.1). However, should conditions arise resulting in stress of the host, this may give rise to a change in the intestinal microflora (Ringø et al., 1997), giving pathogens an opportunity to compete. Thus, the most favourable timing of probiotic addition to optimise attachment while reducing proliferation of pathogens or opportunistic bacteria should be determined.

In conclusion, bacteria isolated from adult common clownfish have the potential of colonising intestinal mucus and consequently may serve as prophylactic and therapeutic agents. These findings will be used to further refine a protocol for the selection of probiotics in marine fish larviculture.

Chapter 5

Production of beneficial compounds by candidate probiotic bacteria

Introduction

In many studies the selection of probiotic bacteria has primarily been based on their ability to produce anti-microbial metabolites (Gatesoupe and Lesel, 1998; Rengpipat et al., 1998; Rico-Mora et al., 1998; Gibson et al., 1998; Cai et al., 1998; Gibson, 1999). The production of beneficial products should also be taken into account when selecting probiotics. Microorganisms are capable of producing complex molecules, either directly, as part of their metabolic activities, or indirectly, when they die. The compounds which may be of benefit to the host include pigments (Holmstrom et al., 2002), proteins (Klein et al., 1998), fatty acids (Jostensen et al., 1990; Shirasaka et al., 1995; Yazawa, 1996), vitamins (Sugita et al., 1991a; Sugita et al., 1991b) and digestive enzymes (Cahill, 1990; Pel et al., 1990; Pollak and Montgomery, 1994; Chandramohan, 1997; Chandrasekaran, 1997; Singh et al., 1998; Hansen and Olafsen, 1999; Ramirez and Dixon, 2003).

Screening for these compounds can be performed *in vitro* using standard microbiological and biochemical techniques. The presence or production of beneficial compounds is however, no guarantee that benefit will be transferred to the host organism. Validation of the probiont's beneficial mode of action can only be confirmed when tested and assayed *in vivo*.

This chapter aims to contribute to the development of a protocol for the selection of probiotic bacteria using *in vitro* experiments to screen for the production of beneficial pigments, vitamins and digestive enzymes.

Materials and methods

Clownfish larvae (*A. percula*) were hatched and reared according to the methods described in Chapter 2. To assay for enzymes present in the larval digestive system at the time of

hatching, three samples of ten larvae each were collected from the larval rearing tank one hour after hatching. The larvae were frozen at -18°C. Each sample was homogenised in 300 µl 0.2% sterile saline, pH 7.5 and then centrifuged at 8000 *g* for 10 minutes. The supernatant was removed and stored at 4°C for analysis.

Following the methods described in chapter 2, all five candidate probionts (AP1 to AP5) were prepared to a concentration of approximately 10^8 cells.ml^{-1}. Quantification of the pigments in the candidate probionts was not performed but the colour of the cultures on marine agar was noted. Vitamin C content of whole cells and culture supernatant was determined using the method described by Plummer (1978).

To provide an idea as to what enzymes the candidate probionts produce, the bacterial isolates were inoculated into an APIZYM (bioMerieux Vitek) test system. The system provides rapid identification of constitutive enzymes based on colour development due to enzyme activity.

The enzymes chosen to quantitatively analyse were alkaline phosphatase, lipase and trypsin. The enzymes were assayed by testing the marine broth culture supernatant in which the probionts had grown. The supernatant was prepared according to the methods described in chapter 2. Sterile marine broth was used as a control for each assay. All assays were carried out in triplicate with a BioTek Powerwave ELx808 Microplate Reader with KC Junior software using commercial enzymes as standards.

Alkaline phosphatase was assayed using the Alkaline Phosphatase substrate system (SIGMA) which uses *p*-nitrophenyl phosphate as a substrate. A 400 µl volume of the substrate was added to 100 µl of the sample and made up to 1000 µl with 10 mM ammonium bicarbonate

buffer (pH 7.8). The tubes were incubated in the dark for 30 minutes at 25°C then centrifuged at 8000 *g* for 10 minutes. In triplicate, 250 µl of the supernatant was pipetted into a well of a microtiter plate and the wavelength of the contents measured at 405 nm.

Lipase was assayed using the method proposed by Albro et al. (1985). A 300 µl volume yielding a final concentration of 1.4mM *p*-nitrophenyl myristate substrate (FLUKA) was added to 200 µl of the sample and made up to 1000 µl with 25 mM ammonium bicarbonate buffer with 0.5% (v/v) Triton X-100 (pH 7.8). The tubes were incubated in the dark for 30 minutes at 25°C then centrifuged at 8000 *g* for 10 minutes. In triplicate, 250 µl of the supernatant was pipetted into a well of a microtiter plate and the wavelength of the contents measured at 405 nm.

Trypsin was assayed following the method described by Erlanger et al. (1961) where 1mM Nα-Benzoyl-DL-Arginine *p*-nitroanilide (BAPNA) hydrochloride (SIGMA) was used as the substrate. A 300 µl volume of the substrate was added to 200 µl of the sample and made up to 1000 µl with Tris-HCl buffer (0.1 M and 1 mM respectively). The tubes were incubated in the dark for 30 minutes at 25°C then centrifuged at 8000 *g* for 10 minutes. In triplicate, 250 µl of the supernatant was pipetted into a well of a microtiter plate and the wavelength of the contents measured at 405 nm. The protein content of the enzyme extract was determined spectrophotometrically at 595 nm according to Bradford (1976).

Based on a standard curve of absorbance plotted against product concentration, the concentration of each of the assay products was estimated using the coefficients from a least-square regression model. Enzyme activity was expressed as total activity per larvae ($mU.larva^{-1}$) or bacteria ($mU.ml^{-1}$ supernatant) where 1 mmole of product is liberated during 1

minute of hydrolysis per larva or per ml of cell supernatant at 25°C. Preliminary experimental assays were used to determine the optimal dilutions of the various samples to ensure the enzyme activities of the samples were within the optimal range of the respective assays.

Enzymatic activity data from the individual assays were compared using a Kruskal-Wallace test followed by a multiple range test to identify differences in enzyme concentration between the candidate probionts for each enzyme.

Results

The identification and characteristics of candidate probionts AP3, AP4 and AP5 are provided in chapter 6. Candidate probiont AP3 was identified as *Bacillus* AP3, AP4 as *Kocuria* AP4, and AP5 as *Pseudoalteromonas* AP5.

Only candidate probionts *Kocuria* AP4 and *Pseudoalteromonas* AP5 had pigmented colonies. The colonies of *Kocuria* AP4 were bright yellow while *Pseudoalteromonas* AP5 colonies were bright orange when grown on marine agar 2216e. No vitamin C was found in the culture broth or lysed cells of any of the candidate probionts.

Alkaline phosphatase was produced by all candidate probionts (Table 5.1) with AP3, *Kocuria* AP4, and *Pseudoalteromonas* AP5 equally producing the most (>40 nmoles).

Table 5.1 – APIZYM enzyme profiles of five candidate probiotics isolated from common clownfish, *Amphiprion percula*.

Enzyme	Candidate probionts				
	AP1	**AP2**	**AP3**	**AP4**	**AP5**
Alkaline phosphatase	+	++	+++	+++	+++
Esterase lipase (C8)	+	-	+	-	+
Trypsin	-	-	-	-	+

- = no activity, + = presence of enzyme from 10-20 nmoles, ++ = 20-30 nmoles and +++ = >40 nmoles

Using the biochemical assays, sterile marine broth contained none of the enzymes assayed and therefore all enzymes detected in the supernatant originated from the candidate probionts. Table 5.2 shows the enzyme activity of the culture supernatant of the five candidate probionts and newly hatched clownfish larvae. All of the candidate probionts produced trypsin and alkaline phosphatase while the larvae produced all three enzymes. *Kocuria* AP4 was the only candidate probiont that did not produce lipase.

Table 5.2 – Average enzyme activities (± Std Deviation) of three enzymes produced by candidate probionts and clownfish (*A. percula*) larvae. Enzyme activity determined as mU product liberated.ml^{-1} supernatant or mU.larva^{-1}.

Candidate probiont	Enzyme activity		
	Trypsin	**Alkaline phosphatase**	**Lipase**
AP1	$7.65^{a} \pm 0.55$	$101.44^{ab} \pm 6.15$	$9.24^{ab} \pm 0.35$
AP2	$14.94^{ab} \pm 0.83$	$75.21^{ab} \pm 2.02$	$20.46^{ab} \pm 1.98$
AP3	$10.93^{ab} \pm 1.45$	$172.66^{b} \pm 2.05$	$5.27^{ab} \pm 0.5$
Kocuria AP4	$7.83^{a} \pm 0.83$	$59.0^{a} \pm 1.07$	0^{a}
Pseudoalteromonas AP5	$98.18^{b} \pm 3.01$	$91.28^{ab} \pm 2.23$	$34.05^{b} \pm 1.49$
A. percula larvae	0.65 ± 0.02	0.46 ± 0.03	0.13 ± 0.02

[a-b] Different letters indicate significant differences between candidate probionts ($p < 0.05$) in that column.

Discussion

The introduction of exogenous compounds to the developing larvae as a means of assisting or improving growth and/or survival raises two questions: 1) what makes them beneficial? and, 2) do larvae require more than their natural levels?

As mentioned in Chapter 1, the aim of the *in vitro* studies is to provide researchers with simple and rapid methods for screening large numbers of candidate probionts. The criteria considered for screening in Chapters 3 (antagonism) and 4 (attachment to mucus) were based on work previous performed as described in the literature. It is apparent that the number of potentially beneficial compounds that could be assayed for is extremely large. Besides being time consuming and expense, there is no reason why each candidate probiont could not be

screened for potentially beneficial compounds using Nuclear Magnetic Resonance (NMR) etc. These methods compromise the aims of the original screening protocol of being simple and rapid. There is, of course, no reason why a smaller sample of candidate probionts which have successfully been screened to a later stage of the protocol shown in Figure 1.1 couldn't be assayed using the above methods. The results may assist the researcher in identifying the possible mode of action of the candidate probiont *in vivo*.

From an evolutionary perspective it seems reasonable to assume that larvae hatch with the ability to create the compounds necessary for survival and development. If these compounds have evolved to these levels, why would it be beneficial to increase them further from an exogenous source? In aquaculture, larvae are reared in an artificial environment which has many disadvantages such as livefood quality and quantity. An aim in aquaculture is to improve growth rates and therefore increasing the enzyme levels may lead to improved growth rates. The effect of additional, exogenous enzymes has shown to increase growth in gilthead sea bream (*S. aurata*) larvae (Kolkovski et al., 1993) and Atlantic salmon (*S. salar*) (Carter et al., 1994), but was ineffective in sea bass larvae (*Dicentrarchus labrax*) (Kolkovski et al., 1997b). A problem with many cultured species is the assimilation of artificial diets during the early stages of larval development. It has been suggested that this is due to the low enzyme levels capable of digesting the food (Govoni et al., 1986; Kolkovski et al., 1993; Kolkovski et al., 1997a). Therefore, the addition of probiotic bacteria capable of producing beneficial enzymes may assist in the digestion of artificial foods, thereby reducing the livefood feeding period and subsequent associated costs.

Pigments

The production of inhibitory metabolites by bacteria appears to correlate with the expression of pigments (Holmstrom et al., 2002; Egan et al., 2002) and additionally, pigments in the form of carotenoids are important in the production of various vitamins (Ronnestad et al., 1998a). For example, the vitamin A in the eyes of copepod fed halibut (*H. hippoglossus*) is mainly derived from dietary carotenoids (Ronnestad et al., 1998a) compared to larvae fed *Artemia* which have lower levels of vitamin A (Ronnestad et al., 1998b). It is therefore possible that the source of the pigment influences the levels of vitamin A (Shields, 2001). Astaxanthin has been isolated from *Kocuria rosea* (Coello et al., 2003) and *Pseudoalteromonas* NCIMB 41083 (Spragg, 2003). If the primary carotenoids of marine copepods are lutein and astaxanthin, which are considered beneficial to halibut larvae (Ronnestad et al., 1998b), both candidate probionts could be of benefit to the larvae. An orange strain of *Kocuria polaris* sp. nov. contained six water-insoluble pigments, the production of which were independent of growth conditions or culture media (Reddy et al., 2003). Based on the findings by Reddy et al. (2003), pigment production of *K. polaris* sp. nov. is independent of growth substrate and it therefore seems worth testing if pigments may be produced in the digestive system of the larvae and if the larvae are capable of utilising them.

Vitamins

Although none of the candidate probionts used in this study produced vitamin C, bacteria are known to produce this vitamin (Hancock and Viola, 2001). Fish are unable to synthesise vitamin C and are dependent on a constant supply through their food (Chatterjee, 1973). Increasing the levels of vitamin C available to the larvae can be achieved through enrichment of the livefood (Merchie et al., 1995a; Merchie et al., 1995b; Merchie et al., 1997; Olsen et al., 2000a). The production of water-soluble vitamins by intestinal bacteria would be

beneficial as administering these vitamins to larvae is difficult as they leach into the culture water and are therefore unavailable to the larvae. According to Coulson (1983), the rate of protein synthesis in larvae is an order of magnitude greater than that of adult fish. This was hypothesised to be due to the "scaling down" effect between the rate of blood flow and the ratio between food particle size, accessibility of enzymes and the intestinal surface (Coulson, 1983). Based on this hypothesis, if the vitamins are produced inside the larvae, the ingestion is likely to be higher due to the higher localised concentration of the vitamin.

Enzymes

Assays were conducted for alkaline phosphatase, trypsin and lipase. Alkaline phosphatase and trypsin are predominant and considered important during the early stages of larval development (Cousin et al., 1987; Ribeiro et al., 1999a; Gawlicka et al., 2000) while lipase is considered important yet is not commonly found in developing larvae (Govoni et al., 1986; Baglole et al., 1998; Martinez et al., 1999).

It would be possible to assay for a large number of digestive enzymes however, to be efficient, it is recommended that initial qualitative, *in vitro* tests such as the APIZYM system used in this study and by Ramirez and Dixon (2003) are used to get an idea of what enzymes are present. Another rapid method for enzyme screening involves plating the candidate probiotics on selective media such as those used by Bairagi et al. (2002). The three enzymes were chosen for this experiment based on results obtained from the APIZYM test system and their importance during the early stages of the larval digestive process.

During the early larval stages, before the development of a functional stomach, the pH of the larval digestive tract is alkaline (Walford and Lam, 1993; Tanaka et al., 1996; Ronnestad et

al., 2000). Subsequently, alkaline phosphatase dominates (over acid phosphatase) during these stages (Martinez et al., 1999). Since probiotics are applied during the initial stages of larval development, assaying for acid phosphatase was not performed.

If the generation time of the bacteria is less than the rate of intestinal tract flushing, and the bacteria are unable to attach to the intestinal mucus or intestine, they will be flushed out before reaching a viable population level in the digestive tract (Cahill, 1990). In Chapter 4 it was shown that all five candidate probionts were capable of attaching to intestinal mucus. The continuous production of mucus in the digestive tract combined with the continual intake of livefood by the larvae suggests that a constant source of nutrients are available to the intestinal microflora. Therefore, based on the assumption that there is an unlimited supply of nutrients and the bacteria can reach the stationary phase in the digestive system, the only limit to biomass concentration is the maximum packing density of the cells in the biomass (Pirt, 1985).

The enzymes produced by the candidate probionts were assayed in marine broth in which the bacteria reached a concentration of 10^8 cells.ml^{-1}. Although this bacterial concentration is unlikely to be reached in the larval digestive system (Chapter 8), a level of 1.7×10^6 cells.ml^{-1} (Chapter 7) may occur in the culture water. Since the carrying capacity of a bacterial culture is limited by nutrient concentration (Monod, 1949), it is, therefore, unlikely that the nutrients available to the candidate probionts in the larval culture water are greater than that provided by marine broth. In a stagnant water larval culture environment the bacterial flora eventually reaches the stationary phase (Salvesen et al., 1999) and may therefore produce enzymes at concentrations lower than those produced by the candidate probionts when grown in marine broth. These enzymes may be ingested along with water or livefood, ending up in the

digestive tract where they may contribute to the larval digestive process. This is speculative and requires analysis of the extracellular enzymes in the rearing water as well as their possible contribution to larval digestion.

The influence of exogenous enzymes on larval development has been widely debated. Some authors suggest that exogenous enzymes provided by livefood enhance development (Lauff and Hofer, 1984; Moyano et al., 1996; Lazo et al., 2000; Garcia-Ortega et al., 2000), while others have concluded that they do not lead to any improvement (Kolkovski et al., 1997a; Kurokawa and Suzuki, 1998; Gawlicka et al., 2000; Koven et al., 2001; Cahu and Zambonino-Infante, 2001). The contribution of the intestinal microflora to enzyme production was not investigated. The contribution of digestive enzymes to fish from bacterial sources has received some attention (Pollak and Montgomery, 1994; Singh et al., 1998; Bairagi et al., 2002; Ramirez and Dixon, 2003) and may help explain differences in results where there have been discrepancies regarding the enzyme contribution of livefood. For example, the presence of algae in the larval rearing water enhances the rate of digestive enzyme development in sea bass (*D. labrax*) larvae (Cahu et al., 1998). However, it is possible, that the algal-associated microflora may be responsible for contributing to the digestive enzymes.

The digestive system of *A. percula* larvae is differentiated before the embryo hatches and appears functional before the yolk-sac is completely absorbed at three days after hatch (Gordon and Hecht, 2002). Upon hatching, the pH of the larval gut is alkaline becoming acidic after the development of the stomach (Walford and Lam, 1993; Tanaka et al., 1996; Ronnestad et al., 2000). Alkaline phosphatase and trypsin have optimal activity at high pH (Hidalgo et al., 1999) and therefore dominate during the early larval stages. In *A. percula*

larvae, trypsin activity was 0.65 mU.larva^{-1} which appears higher than the 0.17 mU.larva^{-1} found in seven-day-old halibut (*H. hippoglossus*) larvae (Rojas-Garcia and Ronnestad, 2002) and ≈ 0.5 mU.larva^{-1} in two-day-old sole (*Solea senegalensis*) (Ribeiro et al., 1999a). First-feeding in marine fish larvae which have short yolk-sac periods have increased trypsinogen/trypsin content and increased trypsin activity (Lauff and Hofer, 1984; Gawlicka et al., 2000), while larvae (such as halibut) which have a longer yolk-sac period, have lower levels of enzyme activity (Shields, 2001). Clownfish larvae are generally larger and relatively more developed than other marine species (Hoff, 1996), which may explain the higher enzyme activities.

During larval development, trypsin levels increase for a few days followed by a decrease to a constant level (Cahu and Zambonino-Infante, 1994; Ribeiro et al., 1999a). Thus during the initial phase of larval development additional enzymes may benefit the larvae. The supernatant of candidate probiont *Pseudoalteromonas* AP5 had the highest trypsin enzyme activity of the candidate probionts at 98.1 mU.ml^{-1} supernatant while the other candidate probionts ranged from 7.65 to 14.94 mU.ml^{-1} supernatant. The larval trypsin activity was 0.65 mU.larva^{-1}, which suggests that the additional trypsin supplied by the bacteria could benefit the larvae. Exogenous trypsin, activated by the high pH 9.0 of the hindgut of whitefish (*Coregonus* sp.) larvae greatly contributed to total trypsin activity (Lauff and Hofer, 1984) and therefore trypsin introduced by bacteria may play a role in increasing larval trypsin enzyme levels.

The activities of the different larval digestive enzymes change during the course of development (Ribeiro et al., 1999a; Gawlicka et al., 2000; Rojas-Garcia and Ronnestad, 2002). Phosphatases are involved in nutrition processes, mineralization and the internal

transport and hydrolysis of phosphorylated proteins (Letelier et al., 1985), while alkaline phosphatase is associated with the absorption of glucose, lipid, calcium and inorganic phosphate (Moss, 1992; Eguchi, 1995). Alkaline phosphatase in clownfish larvae was 0.46 mU per individual larva compared to ≈0.45 mU per individual larva for *S. senegalensis* (Ribeiro et al., 1999a). The enzyme's activity decreases during larval development while acid phosphatase levels increase due to the development of the stomach (Cousin et al., 1987; Martinez et al., 1999). Candidate probiont *Bacillus* AP3 had the highest alkaline phosphatase activity of the probiont candidates at a level of 172.7 $mU.ml^{-1}$ supernatant. The production of alkaline phosphatase by bacteria during the larval developmental period when the gut pH is high, may contribute to the larval pool of digestive enzymes although experiments are required to confirm this.

Lipase activity in clownfish larvae was 0.13 $mU.larva^{-1}$ while the candidate probionts produced up to 34.05 $mU.ml^{-1}$ supernatant (*Pseudoalteromonas* AP5). In fish, the major lipase appears to be non-specific and bile-salt dependent (Gjellesvik et al., 1992) and the secretion of this lipase appears to be induced by food (Hoehne-Reitan et al., 2001). This may explain the relatively low levels of lipase activity found in newly hatched clownfish larvae as they had not yet started feeding. The activity of lipase tends to increase during the early stages of larval development before leveling out prior to metamorphosis (Martinez et al., 1999; Gawlicka et al., 2000).

The candidate probiont with the highest overall enzyme activity was *Pseudoalteromonas* AP5. The genus has been shown to produce a variety of compounds (Holmstrom and Kjelleberg, 1999; Holmstrom et al., 2002). *Pseudoalteromonas haloplanktis* strain S5B produces trypsin-like proteases thought to cause fish spoilage (Odagami et al., 1993), while

Pseudoalteromonas espejiana has been used to digest *Ulva*, the macroalgae, producing single-cell algal detritus which can be consumed by *Artemia* (Uchida et al., 1997). Based on the production of beneficial compounds, the ability of *Pseudoalteromonas* AP5 to produce the highest enzyme activities for trypsin and lipase out of the five candidate probionts, combined with the orange pigmentation, makes it the most suitable candidate for *in vivo* screening.

The ability of the candidate probionts to produce the various enzymes would appear a viable option for increasing the digestive capabilities of the larvae. This may provide aquaculturists with the option of feeding the larvae with artificial diets from an earlier stage, thereby reducing the costs associated with feeding livefood. Since the source of food may have an influence on enzyme activity (Cahu and Zambonino-Infante, 2001), it is recommended to compare the enzyme activities of wild and captive bred larvae. This may assist aquaculturists in optimising the larval enzyme levels thereby reducing the unnecessary addition of "suspected" beneficial enzymes.

Investigating what happens when beneficial enzymes are introduced (via probiotics) to the digestive process needs to be considered. In order to test whether the candidate probionts contribute to the larval digestive enzymes, *in vivo* experiments should be performed whereby the larvae are exposed to the probionts for varying periods and then assayed for enzyme activity. The enzymes can only be considered beneficial if growth and/or survival improves as a function of enzyme activity. However, to eliminate the possibility that the elevated enzymes activities are solely responsible for improved growth/survival, an experiment whereby only the enzymes are introduced should be performed comparing larval growth and/or survival.

Chapter 6

Identification of candidate probionts and their toxicity towards *Artemia*

Introduction

One of the steps in the assessment of suitable candidate probionts is their identification and knowledge about their toxicological characteristics. Depending on the species, once an organism has been identified, a large amount of biological information may be sourced from the literature. This may include details such as information on optimal culture conditions and possible toxic or pathogenic traits. Although standard phenotypic identification methods are available, the use of 16S-rDNA identification has become more acceptable due to its greater degree of accuracy.

Although the identified bacteria may not be listed as being toxic or pathogenic, it is advisable that toxicity tests are carried out. For example, *V. alginolyticus* is a known pathogen to gilt-head sea bream (*S. aurata*) (Balebona et al., 1998), shrimp (*Litopenaeus vannamei*) (Vandenberghe et al., 1999) and diatom cultures (Rico-Mora et al., 1998). However, it has also been used successfully as a probiotic for Atlantic salmon (*S. salar*) (Austin et al., 1995), shrimp (*Penaeus* spp.) (Jory, 1998) and algae (Gomez-Gil et al., 2002). This example emphasises the possibility that a previously identified, known pathogen can have non-pathogenic/non-toxic strains which may be of benefit to the host. However, a possible scenario is that a typical non-pathogenic/non-toxic bacterial species may have pathogenic/toxic strains which would need to be eliminated using a screening test.

Many bacteria produce exotoxins (Brock and Madigan, 1997). These toxins may be in the form of antibiotics, siderophores or peroxidases (see Chapter 1), which inhibit the growth of other bacteria and may be toxic to eukaryotes (Holmstrom et al., 2002). Therefore, it is important that the pathogenicity/toxicity test is also performed on the probiotic culture supernatant to detect whether these toxins are present. Since these experiments are usually

run over a short time period, it is often not long enough for the bacteria to produce any substantial amount of the toxins in the presence of the *Artemia.* The use of the culture supernatant provides a convenient alternative.

This chapter describes the 16S-rDNA identification of candidate probionts AP3, AP4 and AP5 and the information from the literature regarding their suitability as candidate probiotics. The pathogenic or toxic ability of all five probionts as well as their culture supernatants were tested using the brineshrimp *Artemia* as a study organism. Candidate probionts AP1 and AP2 were not identified using 16S-rDNA techniques as they were not deemed suitable candidate probionts based on the pathogenecity/toxicity results and *in vitro* experiments (Chapters 3, 4 and 5).

Materials and methods

Identification of candidate probionts

Candidate probionts were Gram-stained and examined using light microscopy (magnification x1000). The production of catalase, oxidase and indole was tested using standard techniques.

Extraction and purification of bacterial DNA was performed on individual colonies using a QIA tissue extraction kit (QIAGEN Inc. Chatsworth, CA). The template DNA concentration was quantified using a GeneQuant spectrophotometer and diluted to approximately 250 ng/ml. From the template stock solution, 5µl of the purified DNA was PCR amplified in duplicate using 5µl for each of two pairs of primers – 907F (5`-AAA CTC AAA GGA ATT GAC GG-3`), 1401R (5`-CGG TGT GTA CAA GAC CC-3`), GM5F (5` - CCT ACG GGA GGC AGC AG – 3`) and 901R (5` -CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC GTC AAT TCC TTT GAG TTT – 3`) at a concentration of 50ρmoles

per reaction. PCR Mastermix (Promega) was added to PCR tubes and the volume made up to a final of 100µl with triple distilled sterile water. The thermal cycling program was as follows: initial denaturation at 94°C for 10 minutes, followed by 36 cycles of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 90 seconds, while the 36th cycle, the final extension step, ended with a 10-minute period at 72°C. The tubes were then cooled and maintained at 4°C until gel analysis.

The amplified PCR product was analysed by gel electrophoresis using a 2% agarose gel containing ethidium bromide. The gel was immersed in TBE buffer and run at 80V for 3 hours. A reference ladder marker (λ phage digested with *pst*), was used to provide an indication of fragment size and once completed, the gel was examined with a UV transilluminator.

The PCR product was cleaned using a QIAgen PCR cleaning kit and the product was eluted and stored in triple distilled water. Sequencing was performed in forward and reverse on the ABI Prysm Genetic Analyser (Applied Biosystems, USA) using 20 nmoles of the PCR product, 4µl Big Dye 4 Terminator dye, 4µl dilution buffer and 50nmoles of one of the primers.

Resulting chromatographs were viewed using the Chromas 2.2 software (Technelysium Pty Ltd). Sequences from the same organism were merged using the software CLUSTAL X and submitted to the Advanced BLAST search program of the NCBI for identification. Related sequences were aligned with the default settings of CLUSTAL X (Thompson et al., 1997) which allowed for taxonomic assignment to genus level. For each sequenced probiont, the

two species from the NCBI database with the closest matched sequences were researched in the literature for information pertaining to their potential as probionts.

Pathogenicity/toxicity of candidate probionts towards Artemia

Candidate probionts and culture supernatant were prepared according to the methods discussed in chapter 2. Brine shrimp, *Artemia* sp. nauplii were hatched and prepared to a density of 200 nauplii.ml^{-1} according to the method described in Chapter 2.

To indicate the possible pathogenic/toxic effect of an organism or compound, a brine shrimp bioassay is often used (Harwig and Scott, 1971; Feuillade et al., 1996; Jann-Para et al., 2004). The assay involves incubating *Artemia* nauplii with extracts from or with the actual test organism. Survival of the nauplii is then compared to a control to determine the pathogenecity/toxicity of the test organism. A 100 µl volume of SSW containing approximately 20 newly hatched nauplii was added to each well of a 96-well microtitre plate. For six replicates for each treatment, 100µl of either probiotic bacterial suspension or the supernatant from each probiont was added to the *Artemia*. The control for the bacterial suspension was SSW, while sterile marine broth was used as the control for the supernatant. After incubating the plate at 25°C for 24 hours, the number of dead *Artemia* were counted in each well. A drop of concentrated formalin was then added to each well to kill the live nauplii and the total number of *Artemia* per well was counted. Percentage survival was determined for each well and a Kruskal-Wallis test was used to determine differences in percent *Artemia* survival between candidate probionts at the $p<0.05$ level. A post-hoc multiple means comparison test was used to show differences between treatments. Analysis was performed separately on the probiont cell suspension and culture supernatant.

Results

Identification of candidate probionts

The genus obtained from the NCBI database and the allocated names for the three probionts are listed in Table 6.1. The 16S-rDNA genetic base sequences of the three candidate probionts are listed in Appendix 1.

Table 6.1 – Identification of candidate probionts by 16S-rDNA analysis.

Original name	Gram stain	Production of			16S-rDNA closest related species from NCBI BLAST	Allocated name
		Cat	Ox	Ind		
AP3	+	+	+	+	*Bacillus subtilus* *Bacillus pumilus*	*Bacillus* AP3
AP4	+	+	-	-	*Kocuria varians* *Kocuria rhizophila*	*Kocuria* AP4
AP5	-	+	+	-	*Pseudoalteromonas piscicida* *Pseudoalteromonas flavipulchra*	*Pseudoalteromonas* AP5

Cat= Catalase, Ox = Oxidase, Ind = Indole

Pathogenicity/toxicity of candidate probionts on Artemia

Survival of *Artemia* exposed to the candidate probiont bacterial suspensions and marine broth supernatant is shown in Table 6.2

Table 6.2 – Percent survival of *Artemia* nauplii after 24 hours exposure to candidate probiont cells and their marine broth culture supernatant. Controls were incubated in sterile seawater and sterile marine broth.

Candidate probiont	Probiotic cells	Culture supernatant
	% survival ± Std Deviation	% survival ± Std Deviation
Control	7.3 ± 8.3 ^{ab}	12.3 ± 8.8 ^{m}
AP1	11.1 ± 7.5 ^{ab}	54.2 ± 32.3 ^{no}
AP2	1.6 ± 2.4 ^{c}	24.6 ± 5.2 ^{mn}
AP3	13.1 ± 7.1 ^{ab}	19.8 ± 8.4 ^{mn}
AP4	28.3 ± 21.4 ^{b}	64.4 ± 27.4 ^{n}
AP5	12.5 ± 8.8 ^{ab}	14.6 ± 9.0 $^{m\ o}$

Different superscript letters ($^{a\text{-}c;\ m\text{-}o}$) indicate significant differences within that column ($p < 0.05$).

Discussion

The discussion will address the relevant literature on each of the three 16S–rDNA identified candidate probionts in terms of their biology and their use for biotechnological applications. The second part of the discussion discusses the results of the pathogenecity test and how it may influence the selection of the candidate probionts.

Identification of candidate probionts

Bacillus spp.

This genus is recognised as rod shaped, Gram-positive, endospore-forming, microorganisms. The BLAST NCBI database classification of the genus is as follows -

Phylum Firmicutes
Class Bacilli
Order Bacillales
Family Bacillaceae
Genus Bacillus

Eleven of 27 strains of aerobic-endospore forming bacilli isolated from marine invertebrates and seawater were *Bacillus subtilus* while one was *B. pumilus* (Ivanova et al., 1999). All of the isolated marine bacilli were able to utilise a wide range of organic compounds and were halo- and alkalitolerant, reflecting their metabolic flexibility (Ivanova et al., 1999).

Antibiotics may be produced by bacteria during periods of stress such as during spore formation (Brock and Madigan, 1997). *B. subtilis* is widely distributed in the environment and forms endospores, the probiotic activity of which is still unclear (Sullivan and Nord, 2002). Marine strains of *B. subtilus* have been found which produce novel metabolites (El Helow, 2001).

Gatesoupe (1999) suggested that it was improbable that *Bacillus* would multiply in the digestive tract of marine organisms. However, Gullian et al. (2004) re-isolated an inoculated probiotic (*Bacillus* P64) from the hepatopancreas of shrimp but the authors were careful to

recommend the importance of repeated inoculations, suggesting that the bacteria may be transient rather than capable of establishing themselves.

As a potential probiotic, *B. subtilis* has been isolated from the rearing water of the common snook (*Centropomus undecimalis*) where it suppressed the growth of *Vibrio* spp. in the rearing water (Kennedy et al., 1998). This suggests that the bacteria may be suitable for biocontrol of the culture microflora.

Being a baccilid, the candidate probiont, *Bacillus* AP3, is capable of forming endospores. This is a desirable trait for a probiotic as it means that storage and packaging is simpler, subsequently requiring less technical expertise for delivery to the larvae. However, preliminary *in vivo* trials using clownfish larvae showed that this candidate probiont increased mortality compared to a control (results not shown), and it was therefore excluded from further tests.

Kocuria spp.

This genus has bacteria recognised as coccoid, Gram-positive, non-endospore-forming, aerobic, non-halophilic microorganisms. The BLAST NCBI database classification of the genus is as follows -

Phylum Actinobacteria
Class Actinobacteria
Order Actinomycetales
Suborder Micrococcineae
Family Micrococcaceae

Genus Kocuria

The genus *Kocuria* was first proposed by Stackebrandt et al. (1995) to accommodate pylogenetically distinct members formally classified in the genus *Micrococcus*.

Members of the genus *Kocuria* have been isolated from soil, mammalian skin, rhizoplane, freshwater and marine habitats (Reddy et al., 2003; Kim et al., 2004). Not much is known of their pathogenecity to mammals or humans, but under the previously named genus *Micrococcus*, they were not considered a primary pathogen (Kocur, 1986).

A species isolated from marine sediment, *Kocuria marina* sp. nov. is capable of growth at high salinities (15% NaCl in growth media) (Kim et al., 2004). From a biotechnological perspective, *K. rosea* has been used to produce feather meal where it improved the essential amino acid content of the meal and produced the pigment astaxanthin (Coello et al., 2003).

Pseudoalteromonas spp.

This genus has bacteria recognised as straight or curved rods, Gram-negative, non-endospore-forming, obligatively aerobic, generally motile microorganisms. All species require a seawater base for growth (Gauthier et al., 1995). The BLAST NCBI database classification of the genus is as follows -

Phylum Proteobacteria

Class Gammaproteobacteria

Order Alteromonadales

Family Alteromonadaceae

Genus Pseudoalteromonas

Members of the genus *Pseudoalteromonas* have to-date, only been isolated from the marine environment (Holmstrom and Kjelleberg, 1999). *P. espejiana* has been used to digest *Ulva*, the macroalgae, producing single-cell algal detritus which can be consumed by *Artemia* (Uchida et al., 1997). The bacteria were attached to the *Ulva* suggesting that the digestion of the *Ulva* was by attached bacteria rather than those which were free-living (Uchida et al., 1997). This suggests that the species is of benefit when it is attached, much like *Pseudoalteromonas* AP5 which attached to intestinal mucus and out-competed pathogens for attachment sites (Chapter 4). *Pseudoalteromonas piscicida* is generally isolated from red tide seawater (Bein, 1954; Venkateswaran et al., 2003) and releases a toxin thought to kill fish (Bein, 1954). It has however also been found to reduce *Vibrio* spp. in shrimp culture water (Saulnier et al., 2000). Similarly, Maeda et al. (1997) identified a strain of *Pseudoalteromonas undina* which repressed the growth of harmful bacteria and viruses, improving the growth of striped jack (*Caranx delicatissimus*) and *Penaeus* sp. larvae.

Holmstrom and Kjelleberg (1999) suggested that *Pseudoalteromonas* is capable of switching the expression of antimicrobial activity on or off depending on fluctuations and stimuli present in cells immediate environment. Auto-inhibition has been demonstrated to occur within the genus (Holmstrom and Kjelleberg, 1999) and is probably to maintain microbial diversity within a microhabitat (DeFreitas and Fredrickson, 1978).

Pigmented bacteria of the genus *Pseudoalteromonas* produce metabolites which may be algicidal (Lovejoy et al., 1998), inhibit the growth of bacteria and fungi (Holmstrom et al., 2002), agarolytic (Uchida et al., 1997), or discourage settlement of invertebrate larvae (Lau et al., 2002). Conversely, extracellular metabolites produced by *Pseudoalteromonas* have also

been found to promote the survival of other marine organisms as well as promoting the settlement of oyster and tunicate larvae (Holmstrom and Kjelleberg, 1999). Pigmented bacteria have a greater ecological advantage and are more likely to survive the effects of desiccation (Grinsted and Lacey, 1973) which could be of use when formulating packaging. The colonies of *P. piscicida* isolated by Lee (2003) were yellow, unlike the bright orange colour of the *Pseudoalteromonas* AP5 colonies.

Hentschel et al. (2001) isolated nine bioactive strains of *Pseudoalteromonas* from marine sponges. The *Pseudoalteromonas* spp. produced a variety of antagonistic compounds which inhibited the growth of other bacteria but not the eukaryotic fungus, *Candida albicans*. The authors hypothesised that since the bacteria resided on or within the surfaces of the sponges, production of cytotoxic compounds targeting eukaryote cells would possibly harm the host animal (Hentschel et al., 2001).

Regarding their interaction with larvae and their potential as a probiont, *P. haloplanktis* was one of the predominant bacteria in the intestinal tract of larval turbot (Huys et al., 2001), while *Pseudoalteromonas* spp. dominated the intestinal flora of cultured halibut (*H. hippoglossus*) larvae (Verner-Jeffreys et al., 2004).

With the information gained from this study and elsewhere, the candidate probiont *Pseudoalteromonas* AP5 was selected as one of the most likely candidates and was therefore used in the remaining experiments.

Pathogenicity/toxicity of candidate probionts

After hatching, instar 1 *Artemia* nauplii utilise their endogenous reserves for the first 6-8 hours (Han et al., 2000). The *Artemia* nauplii were used immediately after hatching and were therefore not utilising the bacteria as a food source, however, upon switching to an exogenous food source the nauplii may consume the candidate probionts.

Incubation with probiotic cells (Table 6.2) suggests that AP2 should not be pursued as a potential probiotic as *Artemia* survival was reduced. Although *Artemia* survival in the supernatant of AP2 was not different from the control, incubation with the bacterial suspension treatment resulted in greater mortality and was more pathogenic than the control and AP4.

Survival of *Artemia* exposed to candidate probiont AP1 was only improved in the supernatant treatment. *Artemia* exposed to *Kocuria* AP4 survived better than the control in the supernatant and bacterial cell suspension (Table 6.2). This suggests that if *Kocuria* AP4 does not serve as a suitable probiotic for fish larvae, it has potential as a probiont for *Artemia* and possibly other crustacean larvae. Various authors have demonstrated the ability of monoxenic bacterial cultures to improve *Artemia* growth and survival (Verschuere et al., 1999; Orozco-Medina et al., 2002; Villamil et al., 2003). Preemptive colonisation of the culture water with the bacteria had an influence on the microflora communities that developed in the culture water or that were associated with the *Artemia* (Verschuere et al., 1999). The growth of *Kocuria marina* sp. nov. in high salinities (Kim et al., 2004) further supports the potential of the genus to act as a probiont for *Artemia*.

During the 24-hour incubation period, changes occur within the *Artemia* culture environment. Firstly, an increase in bacterial growth may result in a decrease in oxygen available to the *Artemia,* and secondly, the waste-products produced by the bacteria and *Artemia* all concentrate in the culture environment. It is therefore recommended that similarly designed experiments aiming to test probiotic ability but running longer than 24 hours should account for these changes.

This study allows developers of probiotics to test the pathogenic/toxic ability of candidate probionts by checking both their cell suspensions and culture supernatants for their respective pathogenic and toxic capabilities. The experiment provided useful information regarding the pathogenicity/toxicity of the five candidate probionts, further focusing the probiotic selection protocol with the elimination of candidate probiont AP2.

Chapter 7

Attachment to *Artemia* nauplii and in-water viability of candidate probionts

Introduction

Upon hatching, the digestive system of marine larvae is sterile (Timmermans, 1987) and the subsequent establishment of the larval gut microflora is non-selective (Cahill, 1990; Hansen and Olafsen, 1999). The bacterial colonisers are important as once they establish themselves they may persist, thereby, dominating the bacterial composition of the developing larvae. Under culture conditions it is possible to manipulate the associated microflora in the larval rearing environment with the aim of improving larval survival (Riquelme et al., 1997; Salvesen et al., 1999; Maeda, 1999).

Although the ability of the probiont to attach to livefood organisms is not always tested (e.g., Gatesoupe, 1997; Makridis et al., 1998; Skjermo and Vadstein, 1999; Verschuere et al., 1999), the attachment ability of suitable candidate probionts for larviculture should be investigated. Alternatively, the probiotic can be added directly to the rearing water (Strøm and Ringø, 1993; Ringø et al., 1996; Ottesen and Olafsen, 2000), in which case the persistence of the probiont in the water should also be tested.

The choice of a probiotic delivery method may be influenced by certain factors related to the biology of the fish species, for example the time required from hatching to the start of exogenous feeding. A period of a week or more would probably favour adding the probiotic to the system water with the aim of increasing the exposure of the larvae to the probiotic. In cases where the water in the larval rearing system is frequently being replaced, attachment of probionts to livefood may be preferable.

The prolonged viability of the candidate probiont in the larval rearing system needs to be confirmed before adding it to the water can be recommended. Similarly, experiments testing

the attachment of the candidate probiotics to livefood organisms should be conducted to ensure that sufficient candidate probionts attach and are therefore available to the larvae when feeding on the livefood.

This aim of this chapter was to assess the ability of candidate probionts to a) attach to livefood organisms (*Artemia*), and b) to remain viable in the larval rearing culture water, respectively. The candidate probionts *Kocuria* AP4 and *Pseudoalteromonas* AP5 were selected in this experiment as their distinctive colour and colony characteristics make identification and counting on agar media easier.

Materials and methods

Probiont attachment to Artemia

Brine shrimp nauplii were hatched according to the method described in Chapter 2. A sample of nauplii was counted and suspended in SSW at a density of 100 nauplii.ml^{-1} as a non-sterilised treatment. The remaining nauplii were immersed in a sodium hypochlorite (5 mg.l^{-1} active chlorine) bath for 20 minutes (Gomez-Gil, 1994), and rinsed with 2 l of SSW until the smell of chlorine was no longer evident. The nauplii were then resuspended in SSW at a density of 100 nauplii.ml^{-1} for the probiotic attachment treatments.

Candidate probionts AP4 and AP5 were prepared according to the methods described in Chapter 2. The probiotics were added at a concentration of 10^8 cells.ml^{-1} to flasks containing *Artemia* and incubated for one hour to allow for attachment, an exposure duration suggested by King (2002). A control (*Artemia* to which no bacteria were added), was also incubated in SSW. Each treatment was duplicated. After the incubation period each flask of nauplii was drained through a 100 μm screen and washed with 100 ml SSW to remove any non-adhering

probiotics and the nauplii were resuspended in 10 ml SSW. To separate the attached probiotics from the *Artemia* the nauplii were sonicated at 50 Hz for ten minutes in a bath sonicator. The duration of sonication was deemed most suitable by King, 2002 to remove the maximum amount of bacteria while not killing the *Artemia.* Damaging the *Artemia* may result in the release of ingested bacteria which would increase bacterial CFU counts, thereby creating a false, elevated level of assumed attachment.

Serial dilutions were made of each sonicated sample and 0.1 ml volumes were plated onto marine agar and incubated at 28°C for 48 hours. Only counts from plates containing between 30 and 300 CFU's were used for quantification.

Probiont viability in culture water

Probionts *Kocuria* AP4 and *Pseudoalteromonas* AP5 were prepared as described in Chapter 2. Six 320 l tanks were prepared, each containing algae and rotifers at densities described in Chapter 2 for clownfish larval rearing. A sample of water was taken from each tank to describe the bacterial microflora prior to inoculation with the probionts. Serial dilutions were made to 10^{-4} and, in duplicate, spread on Marine agar 2216e (Difco), TCBS Agar (Biolab) and *Pseudomonas* Isolation Agar. The plates were incubated at 25°C for 60 hours before reading individual CFU counts. Only counts from plates containing between 30 and 300 CFU's were used for quantification.

Two tanks served as controls (no probiotic added) while the probionts were each added to two tanks to yield a final concentration of approximately 10^5 cells.ml^{-1}. Bacterial samples were plated (as described above) prior to the addition of the probionts, and after 4 and 24 hours to determine the viability of the probiont in the system water.

Data analysis

Due to the small sample sizes, and the associated difficulties to test for equality of variance and normal distribution of residuals, a Kruskal-Wallace test was used to check for differences ($p<0.05$) between medians in bacterial counts. To identify differences between treatments, post-hoc comparisons were performed using a median test.

Results

Probiont attachment to Artemia

Bacterial counts on *Artemia* not exposed to probiotics were significantly different from those exposed to the candidate probionts (Table 7.1).

Table 7.1 - Mean (minimum - maximum) counts of bacteria attached to *Artemia* nauplii before and after exposure to candidate probionts. N = No. of plates with 30-300 CFUs

Artemia treatment	Attached bacteria per nauplius	N
Sterilised	340 ^{a} (300 – 380)	2
Non-sterilised	970 ^{b} (900 – 1000)	3
Control (no bacteria added)	600 ^{ab} (190 – 1000)	3
Kocuria AP4 attached	7.2×10^3 ^{c} (5×10^3 - 1.0×10^4)	6
Pseudoalteromonas AP5 attached	2.2×10^4 ^{c} (8.2×10^3 – 3.6×10^4)	4

Different superscript letters ($^{a-c}$) depict significant differences within the column ($p<0.05$).

Probiont viability in culture water

In the control tanks the total number of bacteria rose from approximately 4.9×10^4 to 4.6×10^5 bacteria.ml^{-1} over the 24-hour period, whereas the probiont *Kocuria* AP4 counts rose from

an average of 3.8 x10^4 (pre-inoculation) to 1.1 x 10^5 bacteria.ml^{-1}, and *Pseudoalteromonas* AP5 increased from an average of 1.9 x 10^4 (pre-inoculation) to 1.7 x 10^6 bacteria.ml^{-1} within 24 hours.

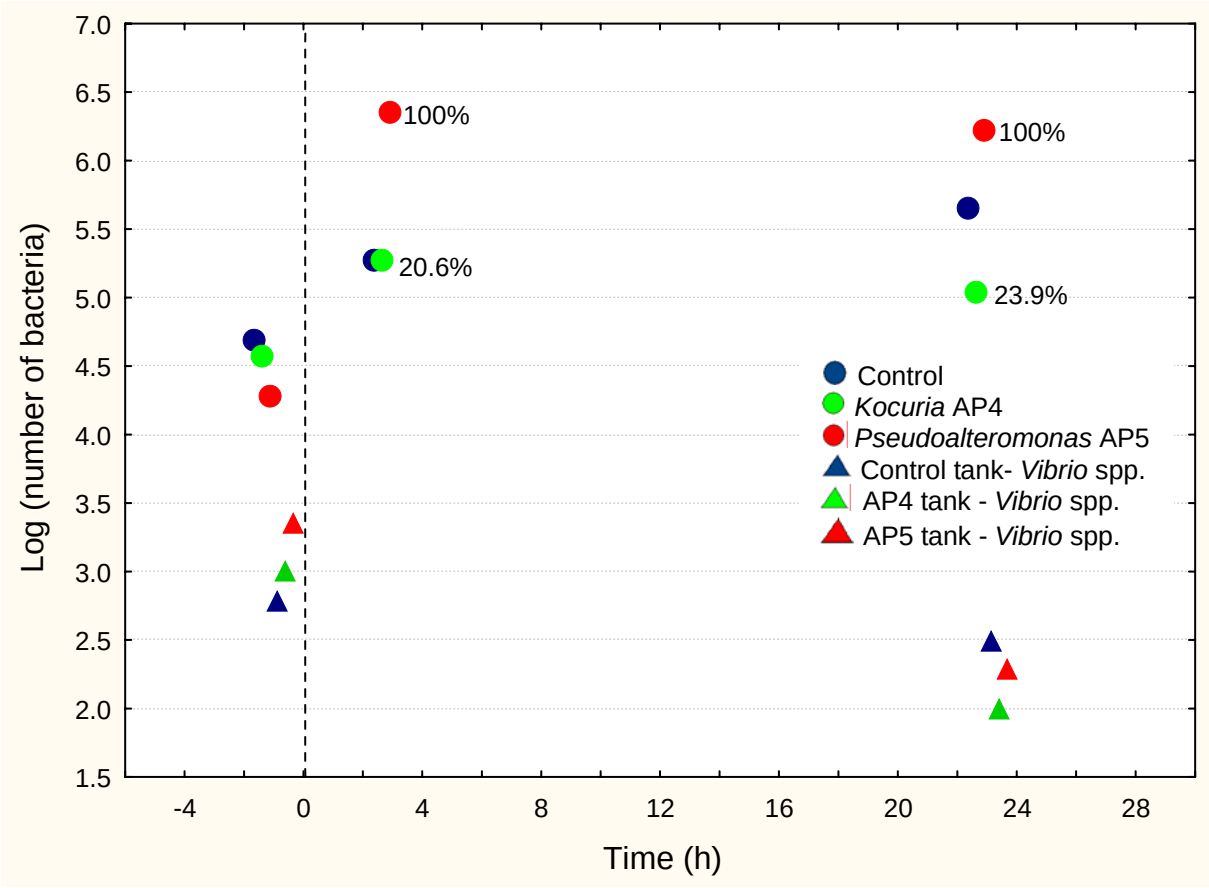

Figure 7.1 - Mean total counts of total heterotrophic bacteria and *Vibrio* spp. in the water from clownfish rearing tanks. Tanks contained probiotics *Kocuria* AP4 and *Pseudoalteromonas* AP5 and a control without probiotics. Water samples were collected prior to inoculation (0), and 4 and 24 hours after addition of the probionts. Percent values indicate contribution of the probiont *Kocuria* AP4 or *Pseudoalteromonas* AP5 to total number of heterotrophic bacteria sampled.

The contribution of the probionts to the total number of bacteria counted was high. *Pseudoalteromonas* AP5 contributed 100% to both the four hour and 24-hour samples. Probiont *Kocuria* AP4 made up 20.6% of the four hour sample and 23.9% of the 24-hour sample.

Tanks inoculated with *Pseudoalteromonas* AP5 reduced the original, pre-inoculation levels of *Vibrio* spp. by approximately one order of magnitude from 2300 to 196 cells.ml^{-1}, and *Kocuria* AP4 reduced the *Vibrio* levels from 1000 to 100 cells.ml^{-1}. The *Vibrio* spp. levels in the control tanks changed from 600 to 307 cells.ml^{-1} over the 24-hour period.

Pseudomonas spp. were present in culture water in all tanks, however, the numbers were too low (<30) to quantify their contribution to the total microflora.

Discussion

The two applications, 1) attachment of the candidate probionts to *Artemia*, and 2) addition of the candidate probionts to the larval rearing water, were both successful in making the candidate probionts available to the larvae. However, it is not known whether the probionts were consumed by the larvae and if they colonise the digestive tract.

The timing of livefood availability can determine larval rearing success. *Artemia* nauplii utilise their endogenous energy reserves within the first six to eight hours when hatched under optimal environmental conditions, and therefore do not require an exogenous food source (Lavens and Sorgeloos, 1996; Sorgeloos et al., 1998). During this period the nauplii are smallest as well as the most nutritious to the larvae due to their high energy reserves (Sorgeloos et al., 2001). Therefore, it would be beneficial if probionts would attach to

Artemia within six hours of incubation. If the attachment period extends into the instar 2 stage of the nauplii, the probiotic is likely to be consumed by the *Artemia* which are known to feed on bacteria (Gorospe et al., 1996; Olsen et al., 2000b; Orozco-Medina et al., 2002). This may be nutritionally beneficial to the *Artemia* and may also become a source of probiotics for the larvae when the *Artemia* are added to the rearing tank. This experiment showed that probiont attachment was possible after one hour of incubation, at a time when *Artemia* have their highest nutritional value.

Bacterial attachment to livefood organisms is influenced by 1) the already present microflora the livefood (Munro et al., 1993b), 2) preparation method and metabolic stage of the probionts (Fletcher, 1990; Blum et al., 1999), 3) the probiotic/livefood incubation environment (Gomez-Gil et al., 1998; Gatesoupe, 2002), and 4) the surface structure of the bacteria (Santos et al., 1991).

Attachment to the external surfaces of livefood organisms

The external surface of *Artemia* is known to harbour a high bacterial load (Sahul and Balasubramanian, 2000; Gatesoupe, 2002). Bacterial counts range from 3.8 x 10^3 CFU.nauplius^{-1} (Sahul and Balasubramanian, 2000) to 1.2 x 10^7 CFU.ml^{-1} (Austin and Allen, 1981). In this study, newly hatched, non-sterile nauplii contained 9.7 x 10^2 bacteria.nauplius^{-1}, which was reduced to 3.4 x 10^2 bacteria.nauplius^{-1} after sterilisation in sodium hypochlorite. Although there was a significant difference between the attached microflora of sterilised and non-sterilised *Artemia*, the difference was small when compared to *Artemia* exposed to *Pseudoalteromonas* AP5. Therefore, although sterilisation does reduce the level of attached bacteria, the difference between the two suggests from a practical and cost perspective, that it may be unnecessary. To confirm whether sterilisation is necessary, studies should be

performed whereby sterilised and non-sterilised *Artemia* are exposed to individual probiotics, thereby providing directly comparative data on the success of probiont attachment.

Cell preparation and metabolic state

The candidate probiont *Pseudoalteromonas* AP5 made up 100% of the culturable microflora of the water (Figure 7.1). *Kocuria* AP4 only contributed 23.9% after 24 hours, and this may be attributed to its slow growth (Chapter 3). It should be tested if the competitiveness would improve if exposure were extended into the stationary phase of the probiont. Bacterial attachment, for example, may be influenced by the metabolic stage of the bacteria where attachment is greater during the stationary phase compared to the logarithmic growth phase (Blum et al., 1999). Only *Kocuria* AP4, was harvested during the logarithmic phase, as determined in Chapter 3.

Furthermore, the attachment ability of the bacteria to *Artemia* is affected by the method of cell preparation as the bacteria may have mucus capsules which are removed during centrifugation and washing (Fletcher, 1990). These capsules consist of polysaccharides or proteins and need time to regenerate (Brock and Madigan, 1997) suggesting that during the period of capsule regeneration, attachment may be impaired. Further studies are required to 1) better understand the morphology of the bacteria used in this study, and 2) test whether the loss of mucus capsules plays a role in the attachment success in these probionts.

An example of a successful application using *Artemia* was given by Rengpipat et al., 1998 who enriched *Artemia* with *Bacillus* S11 and then fed them to larval tiger shrimp, *P. monodon*. Larvae fed the probiont had a shorter development time and better survival after being challenged with *V. harveyi*. Venkat et al. (2004) fed *Lactobacillus sporogenes* to

shrimp, *Macrobrachium rosenbergii* using *Artemia* as a vector. The probiotic-fed shrimp had improved growth and a better food conversion ratio. Similarly, Gatesoupe (2002) fed two commercial probiotics, Bactocell (*Pediococcus acidilactici*) and Levucell (*Saccharomyces cerevisiae*) to pollack larvae (*P. pollachius*) via *Artemia* and reported improved larval growth.

The spores of the bacterium *Bacillus* IP5832 attached to rotifers within one hour and were able to modify the microflora of larval turbot (*S. maximus*) (Gatesoupe, 1993). Larval survival was higher in rotifer/probiotic fed larvae which were challenged with a pathogenic *Vibrio* species (Gatesoupe, 1993). This is similar to the incubation period of the probionts used in this experiment. The probiotic concentration used in this study was similar to that used by Gomez-Gil et al. (1998) who incubated nauplii with probiotic bacteria (HL57) at 10^8 cells.ml^{-1} for two hours and found a maximum attachment of 4.55 x 10^3 CFU.nauplii^{-1}. Attachment of *Kocuria* AP4 (7.2 x 10^3 CFU.nauplii^{-1} - Table 7.1) was similar to that found by Gomez-Gil et al. (1998) but *Pseudoalteromonas* AP5 had better attachment to *Artemia* when exposed for the same incubation period and at the same concentration (Table 7.1). The range of exposure times and probiont concentrations used in different studies shows the need to conduct experiments with each candidate probiont to determine the conditions required for optimum attachment.

King (2002) found that mixed probiotic cultures adhered to *Artemia* better than one species only. In the culture environment the microflora changes over time (Olafsen, 2001). It is thus possible that the use of different bacteria could be more effective and more stable over time than exposure to only one species.

Makridis et al. (2001) found that two doses of probiotic-bioencapuslated *Artemia* were insufficient to predictably influence the intestinal microflora of halibut larvae over a ten-day period. Two possible reasons are, 1) the attached bacteria were incapable of surviving in the intestinal tract, and 2) that too few probionts were attached to the nauplii and were therefore unable to establish themselves and colonise the digestive tract.

In manipulating the bacterial load of livefood organisms, care must be taken not to alter it to a stage where the levels and/or microorganism communities negatively affect the health of the host larvae. This was demonstrated by Olsen et al. (1999b) who found that when halibut larvae were fed 4-day old *Artemia* with large numbers of bacteria attached to them, poor larval growth was observed. Survival of early-stage halibut larvae was higher when a slow rate of gut microflora colonisation occurred (Munro et al., 1993b). Similarly, attempts to feed rotifers with a high bacterial load to turbot larvae have proven unsuccessful and resulted in increased mortality (Nicolas et al., 1989).

In-water viability of candidate probionts

During the early stages of turbot (*S. maximus*) larval rearing, stagnant water systems have been used for up to eleven days (Huys et al., 2001). Similarly, clownfish larval survival was improved in stagnant water culture systems for the first few days after hatching (Hoff, 1996).

Various factors influence the viability of probiotics in larval rearing water. These factors, (some of which have been discussed above, the others will be discussed below) include, 1) initial probiotic concentration in the culture water, 2) environmental conditions (i.e., temperature, salinity, dissolved oxygen), 3) nutrient availability for probiont growth, 4)

interaction between probionts and algae (Austin et al., 1992; Skjermo and Vadstein, 1993) and 5) the growth characteristics of the probiont (Chapter 3).

Maeda (1994) suggested that aquaculture systems cannot support bacterial concentrations greater than 10^6 cells.ml^{-1} although levels of 10^7 cells.ml^{-1} may be reached upon addition of algae and livefood (Salvesen et al., 1999). Additions of CA2 bacteria to the oyster (*C. gigas*) rearing water at a concentration of 10^5 cells.ml^{-1} increased larval growth Douillet and Langdon (1994) while turbot (*S. maximus*) larval survival was improved when *Roseobacter* 27-4 was inoculated at 10^7 cells.ml^{-1} compared to concentrations of 10^3 to 10^5 cells.ml^{-1} Hjelm et al., 2004.

Munro et al. (1995b) fed rotifers enriched with bacteria to turbot larvae (*S. maximus*), which increased the bacterial concentration in the rearing water over a four-day period. Within 3 to 4 days of first feeding the number of bacteria in the digestive tract of turbot larvae reached 1 x 10^4 to 5 x 10^5 CFU.larvae^{-1}. The studies by Munro et al. (1995b) and others (Nicolas et al., 1989; Munro et al., 1993b; Munro et al., 1994; Huys et al., 2001) have shown that the larval intestinal microflora is influenced by the larval food source and rearing conditions. This suggests that the presence of candidate probionts attached to livefood may seed the culture water, which in return would provide additional bacteria to attach to untreated livefood which is added later.

Skjermo and Vadstein (1993) found that the bacterial microflora of halibut larval tanks with algae was more diverse than in tanks with no algae. Larvae kept in clear water had three times more bacteria in their gut than those kept in green-water systems (Skjermo and Vadstein, 1993). This is thought to be due to the "mature" type of bacterial environment

where opportunistic (*r*-selected) bacteria are uncommon and therefore have less chance of colonising the larval gut (Vadstein et al., 1993). Salvesen et al. (1999) showed that the growth of larval turbot (*S. maximus*) improved after the addition of algae. Similarly, the green-water rearing technique had higher survival in clownfish larval rearing than when using a clear water system (Hoff, 1996).

Because the growth characteristics of each probiont are different under controlled conditions (Chapter 3), experiments are required to determine whether probiont growth or at least survival, exists in the larval culture environment. Although *Kocuria* AP4 had a very long lag period (Chapter 3) and its growth phase had yet to reach an asymptote after 36 hours, it was capable of surviving in the larval culture water and contributed 23.9% to the total microflora after 24 hours (Figure 7.1). Based upon discussions in Chapter 3, *Pseudoalteromonas* AP5 has growth characteristics ideally suited for a probiont as it has a shorter lag period and faster doubling time than *Kocuria* AP4.

Strøm and Ringø (1993) exposed larval cod (*G. morhua*) to the probiotic *L. plantarum* and found that after one hour, approximately 90% of 10^4 bacteria.ml^{-1} in the culture water was made up of the probiotic. The growth and establishment of *Pseudoalteromonas* AP5 in the rearing water after 24 hours suggests that this candidate probiotic was capable of out-competing the other heterotrophs to a degree where 100% of the cultivated bacteria were *Pseudoalteromonas* AP5. However, only 0.01 to 10% of marine bacteria are capable of growth on general culture media ((OLAFSEN2001}) and therefore, it is likely that the probiont did not exclude all other bacteria (as *Vibrio* spp. were also detected) but rather only those capable of growth on agar media. Villamil et al. (2003), using *Lactobacillus brevis*, was able to almost completely eliminate the *V. alginolyticus* load attached to *Artemia* while

only high doses of the probiont managed to reduce *Vibrio* spp. occurring in the culture water. The addition of the yeast *Saccharomyces boulardii* to *Artemia* nauplii did not significantly change the composition of both Vibrio and other aerobic flora (Patra and Mohamed, 2003). This experiment, however, showed that both probionts were capable of reducing the total Vibrio counts in the larval rearing water after 24 hours. The ability to reduce the water pathogen levels suggests that the candidate probionts may have an application as biocontrol agents in the culture environment should they prove unsuccessful as probiotics.

Although this experiment has shown that the probionts *Kocuria* AP4 and *Pseudoalteromonas* AP5 are capable of attaching to *Artemia* as well as remaining viable in the culture water, it is no guarantee that the larvae will be able to utilise them. It remains to be tested whether the probionts are able to enter the larval digestive tract and establish themselves. This will be addressed in Chapter 8. In addition, future experiments should investigate the period probionts remain attached to the nauplii once exposed to the larval rearing system and whether the probionts seed the culture water. It is hypothesised that if the probiont remains effective in the larval rearing tank, it will attach to the livefood thereby contributing to the organisms external microflora.

Future studies should focus on determining the optimal dosage and dosing frequency of the probionts in the culture water. This information would assist aquaculturists whether the livefood should be enriched once or repeatedly, or, if the culture water would provide a viable source of probionts which attach naturally to the livefood. Experiments should also be undertaken to examine which mode of delivery is the best in providing the larvae with the probiotic. These studies would involve examining the succession of the larval gut microflora after the addition of the probionts.

Chapter 8

In vivo validation of candidate probionts

Introduction

The selection of probiotics for larviculture generally involves *in vitro* experiments whereby the candidate microorganisms are screened for production of anti-microbial metabolites (Ruiz et al., 1996; Joborn et al., 1997; Gram et al., 1999; Robertson et al., 2000; Spanggaard et al., 2001; Ringø et al., 2004; Hjelm et al., 2004; Vine et al., 2004a) and/or their ability to attach to, or grow in intestinal mucus (Olsson et al., 1992; Joborn et al., 1997; Vazquez et al., 1997; Andlid et al., 1998; Rinkinen et al., 2003b; Vine et al., 2004a; Vine et al., 2004b). However, no matter how well the candidate microorganisms perform in laboratory tests, *in vivo* tests must be conducted in order to validate the probiotic effect.

To reduce the number of candidate probionts, *in vitro* experiments should be designed to provide researchers with a smaller pool of probionts that require fewer *in vivo* tests which are expensive and time-consuming. Discrepancies between the results of *in vitro* and *in vivo* probiont suitability experiments have occurred, whereby, based on *in vitro* results, the most "likely" candidate probionts were unable to improve larval survival or growth *in vivo* (Riquelme et al., 1997; Rico-Mora et al., 1998; Spanggaard et al., 2001). For example, only two out of ten microflora strains selected on their *in vitro* ability to inhibit the growth of *Vibrio anguillarum* were able to confer any protection to rainbow trout *in vivo* (Spanggaard et al., 2001).

Under Gatesoupe's (1999) definition of probiotics as used in this thesis, probiotics are "microbial cells that are administered in such a way as to enter the gastrointestinal tract *to be kept alive*, with the aim of improving health". Transient microflora are therefore not included in this definition and therefore the ability of the probiont to remain within the gastrointestinal tract of the host should be tested.

This chapter investigated the *in vivo* effect of four candidate probionts on clownfish *A. percula*, larval survival. In addition, due to its fast growth, orange colour and distinctive colony morphology on marine agar, candidate probiont AP5 was tested for its ability to enter and remain within the larval intestinal tract.

Materials and methods

Larval food retention period

To determine the rate at which food moves through the larval digestive system, newly hatched larvae were fed and then sampled over a period to assess the stage of gut fullness. Clownfish larvae were hatched according to the methods discussed in Chapter 2. Before the addition of algae and rotifers, five larvae were removed as a control (no food - empty gut) sample. Five larvae were removed every hour and examined under a dissecting microscope to assess the fullness of their gut. The gut was considered full when the entire tract was green, coloured by the presence of algae.

Contribution of candidate probiont Pseudoalteromonas *AP5 to intestinal microflora*

For candidate probiont *Pseudoalteromonas* AP5, experiments were performed to confirm its presence in the larval digestive tract. For this study clownfish larvae were hatched according to methods described in Chapter 2 with the exception that 60 l tanks were used instead of the 20 l buckets. *Pseudoalteromonas* AP5 was prepared to a concentration of 10^8 cells.ml^{-1} according to the methods described in Chapter 2.

Two tanks served as controls while AP5 was added to two experimental tanks to yield a final concentration of approximately 10^5 cells.ml^{-1}. A sample of five larvae was taken from each of

the tanks prior to addition of the probiont. The larvae were rinsed in 0.1% benzalkonium chloride for one minute followed by two washes in sterile seawater. The larvae were transferred to a sterile microcentrifuge tube and homogenized in the tube using a sterile micropestle. Serial dilutions were made to 10^{-4} and, in duplicate, spread on Marine agar 2216e (Difco), TCBS Agar (Biolab) and *Pseudomonas* Isolation Agar (Difco). The plates were incubated at 25°C for 60 hours before reading individual CFU counts. Counts from plates containing between 30 and 300 CFUs were used for analysis.

Samples of the larval intestinal microflora were plated prior to addition of the probiont, and after 14 and 24 hours to determine the percentage contribution of the probiont to the microflora of the larval digestive system.

In vivo *experiments*

Clownfish larvae (*A. percula*) were reared according to the methods described in Chapter 2. Nine rearing experiments had survival data which could be analysed (>10% survival). Percentage survival was compared between controls and probiotic treatments for the four candidate probionts AP1, *Bacillus* AP3, *Kocuria* AP4 and *Pseudoalteromonas* AP5 (AP2 had been excluded based on pathogenicity/toxicity to *Artemia* in Chapter 6). Due to the small and therefore limited dataset, differences in percentage survival between controls and probiont-treated larvae were analysed using a Mann-Whitney U test at the $p<0.05$ type 1 error level. Post-hoc comparisons were performed using a median test to show differences between treatments.

The number of larvae added to each tank for the *in vivo* rearing experiments ranged from 54 to 150. Depending on the number of larvae available at the time of hatching, the number of

replicates ranged from two to four each for both the probiotic and control. Since each candidate probiont was tested separately with its own controls, comparisons *between* probionts could not be performed.

Results

Larval food retention period

After two hours the algae was distributed completely along the digestive tract from the mouth to the anus. All larvae sampled after one hour had algae in approximately two thirds of their digestive tract.

Contribution of candidate probiont Pseudoalteromonas *AP5 to intestinal microflora*

No *Vibrio* spp. were detected in the intestinal tract of the larvae in the control or probiont treatments. *Pseudomonas* spp. were present in the larval gut, however the numbers were too low (<30 CFUs) to provide an accurate count of their percent contribution to the intestinal microflora.

The contribution of *Pseudoalteromonas* AP5 to the larval intestinal microflora sampled 14 and 24 hours after addition is shown in Table 8.1.

Table 8.1 - Total number of bacteria and contribution of probiont *Pseudoalteromonas* AP5 in the digestive system of clownfish (cells.larva^{-1}). The probiont was added to the rearing water after the zero-hour sample was taken.

Time (h)	Treatment	Total bacteria ± Std Deviation	Probiont	% probiont
0	Control	$1.10 \times 10^2 \pm 1.18 \times 10^2$		
14	Control	$3.52 \times 10^4 \pm 3.19 \times 10^4$		
14	Probiotic	$1.00 \times 10^4 \pm 1.56 \times 10^4$	2.00×10^3	20.0
24	Control	2.38×10^4		
24	Probiotic	9.50×10^3	1.15×10^3	12.1

In vivo *experiments*

Kocuria AP4 was the only candidate probiont which improved larval survival compared to the control (Table 8.2).

Table 8.2 – Percent survival (± Standard Deviation) of clownfish larvae not treated (control), or exposed to candidate probiotics. n = number of replicates.

Candidate probiotic	Control % survival ± SD	n	Probiont % survival ± SD	n
AP1	24.1±6.1	2	32.9±6.2	3
Bacillus AP3	27.6±8.1	7	17.4±15.7	9
Kocuria AP4	23.9±11.0*	5	42.0±17.0*	6
Pseudoalteromonas AP5	15.9±8.2	7	29.6±23.0	9

* denotes significant differences between control and probiotic treatment ($p<0.05$).

Discussion

Larval food retention period

Knowledge of the food retention time provides an idea of how fast probiotic bacteria could move through the intestinal tract of the larvae. In newly hatched *A. percula* larvae, complete movement of algae occurs within two hours. Therefore, in a circulating larval culture environment which gradually flushes the probiotic from the system, the probiotic would have to attach to the larval intestinal tract within two hours. However, as the rate of food movement through the tract decreases as the larvae develop (Govoni et al., 1986), a longer attachment exposure period would occur, thereby allowing slow or poorly attaching probiotics to colonise the digestive tract.

After hatching, larvae drink water to osmoregulate, resulting in the uptake of surrounding bacteria (Ringø et al., 1996; Reitan et al., 1998; Olafsen, 2001). The gut of larvae is initially colonized by bacteria from the water but changes after feeding (RINGO1998B}) to resemble the microflora associated with the livefood (Munro et al., 1995a). This may explain the presence of bacteria in the gut of the larvae taken prior to the addition of *Pseudoalteromonas* AP5. Investigations into optimising the period between exposure to the probiotic and addition of the livefood should be performed. By initially maintaining a constant supply of the probiotic at high concentrations, the larvae would continually be exposed to the probionts, increasing the likelihood that the probionts will be consumed and delivered to the larval digestive system.

Contribution of candidate probiont Pseudoalteromonas *AP5 to intestinal microflora*

In this experiment, approximately 10^5 cells.ml^{-1} of candidate probiont *Pseudoalteromonas* AP5 were added to each tank which is similar to that used by Riquelme et al., 2000 who

dosed between 10^4 and 10^6 cells.ml^{-1} of strain 77 (*Arthrobacter* sp.) in the culture water of larval Chilean scallop, *A. purpuratus*. The probiont was capable of displacing the resident microflora after 24 hours of incubation Riquelme et al. (2000) while the addition of probiotics at a concentration of 5 x 10^3 cells.ml^{-1} "every few days" ensured their residency in the larval scallop digestive tract (Riquelme et al., 2001). Doses higher than 10^7 cells.ml^{-1} would appear to be wasteful as Gomez-Gil et al., 2000 found that the concentration of added bacteria rarely exceeded the dosage (10^7 cells.ml^{-1} culture water) and that it decreased over 72 hours at a rate depending on the probiont.

The average number of bacteria isolated after 24 hours from the gut of control *A. percula* larvae was 2.38 x 10^4 cells.larva^{-1} compared to 9.5 x 10^3 cells.larva^{-1} for larvae exposed to candidate probiont *Pseudoalteromonas* AP5 (Table 8.2). This suggests that the presence of the probiont reduces the proliferation of other bacteria. Similarly, nine-day old cod (*G. morhua*) larvae exposed to probiotic *L. plantarum* on day five, contained approximately 10^2 bacteria.larva^{-1} compared to untreated larvae which possessed approximately 10^4 bacteria.larva^{-1} (Strøm and Ringø, 1993). However, it has been demonstrated (Munro et al., 1994; Huys et al., 2001) that there is no correlation between larval survival and the total number of intestinal bacteria. Therefore, the microflora composition of the larval intestinal tract probably plays a greater role in larval survival than the total number of bacteria present in the larval gut.

Pseudoalteromonas haloplanktis and *Vibrio campbellii* were the predominant bacteria isolated from the intestinal tract of larval turbot (Huys et al., 2001). Similarly, members of the genus *Pseudoalteromonas* were the dominant flora in the gut of cultured halibut (*H. hippoglossus*) larvae (Verner-Jeffreys et al., 2004). Although one of the isolates (strain

TG15-07) exhibited antagonism towards other microorganisms *in vitro* it was not capable of completely out-competing the other intestinal microbes *in vivo* (Verner-Jeffreys et al., 2004). Conversely, *Pseudoalteromonas* HQ was isolated from turbot (*S. maximus*) larvae undergoing mass mortality although *in vivo* larval experiments were not able to reproduce mortality curves (Hjelm et al., 2004). Therefore, since members of the genus *Pseudoalteromonas* have been isolated from the digestive tract of larvae, both in this study and others (Huys et al., 2001; Verner-Jeffreys et al., 2004; Hjelm et al., 2004), it is possible that it has the ability to colonise the larval digestive system.

In this study, the candidate probiont *Pseudoalteromonas* AP5 made up 20% and 12.1% of the intestinal microflora after 14 and 24 hours, respectively. Munro et al. (1999) were only able to re-isolate 0.006% of a mutant *Aeromonas* strain from the gut of turbot larvae after days three and four post-hatch. Further similar experiments were unsuccessful in isolating the bacteria from the larvae. The authors concluded that the bacteria were out-competed for growth substrates in the gut and suggested using a range of probiotics to increase the chance that one (or more) of the probionts becomes established. One of the reasons for the poor re-isolation of the *Aeromonas* strain may be that the rotifers (with bacteria attached) were added too late after hatching and therefore the bacteria had to compete with already established bacteria in the gut. It should be tested if adding the bacteria to the rearing water early on, during the larval endogenous feeding stage would be more successful.

It was found that candidate probiont *Pseudoalteromonas* AP5 dominated the microflora of the larval rearing water, contributing up to 100% after 24 hours (Chapter 7). The lower contribution of *Pseudoalteromonas* AP5 to the total microflora in the intestinal tract after 24 hours may be as a result of the gut evacuating at a rate faster than the bacteria were capable of

attaching and/or growing. Although *Pseudoalteromonas* AP5 was the most effective of the five candidate probionts in competing for attachment sites with *V. alginolyticus* (Chapter 4), its overall attachment to intestinal mucus was the lowest (Chapter 4). When comparing the growth in mucus (based on the ranking index) of the five candidate probionts from Chapter 3, *Pseudoalteromonas* AP5 was third and had a lag period of 8 hours 19 minutes and a doubling-time of 36 hours 33 minutes (Chapter 3). With a gut evacuation rate of less than two hours in newly hatched *A. percula* larvae, and, without the ability to attach to the intestinal mucus, candidate probionts are unlikely to be able to reproduce to levels where they contribute significantly to the intestinal microflora.

In vivo *experiments*

To ensure that results are conclusive statistically, lab-scale *in vivo* testing requires replication. When increasing the number of replicates, the number of available larvae per tank reduces resulting in smaller quantities of larvae per tank or smaller tank volumes being required. As a result of having high replication but lower volumes, a paradoxical relationship seems to occur whereby poor reproducibility of probiotic-treated larval rearing experiments is not uncommon (Ringø and Vadstein, 1998; Huys et al., 2001, this study).

Huys et al. (2001) ranked larval rearing experiments of turbot as successful if survival was >35%, average (10%-35%) and poor (<10%). Using the green-water larval rearing technique, turbot larval survival is approximately 12% from egg to weaning (Shields, 2001) however, during this rearing period, success under commercial conditions is considered to be in the range of 80 to 95% (Dhert et al., 1998). Hjelm et al. (2004) used turbot (*S. maximus*) larvae which were individually placed in 2 ml sterile seawater to which either candidate probiotics or no bacteria were added. Larvae were checked daily for five days and cumulative mortality

recorded. Although some experiments showed differences between treatments, reproduction of these results were inconsistent (Hjelm et al., 2004).

In comparison, the overall poor reproducibility and survival 22.5%±15.8 (range 0 to 54.6%) of clownfish in this study would appear to be lower than the 47.9% (range : 29 to 62%) obtained in commercial clownfish production after 20 to 30 days larval rearing (Hoff, 1996). High variability between treatments was also evident in the study on the survival of turbot larvae (*S. maximus*) by Salvesen et al. (1999). Furthermore, the composition of the gut microflora may differ within treatments even when the same rearing conditions are used (Huys et al., 2001), suggesting that reproducibility of results in small-scale larval rearing experiments may be low requiring cautious interpretation. The poor survival of larvae in all experiments may be at least partially attributed to handling stress at the time of hatching. The stress of individually pipetting the larvae into the tanks may have increased mortality as fish larvae are known to be intolerant of handling stress during the early stages of development (Kaji et al., 1999; Hjelm et al., 2004).

Although *A. percula* broodstock provide a batch of eggs every two to three weeks, a hindrance of this study was the small number of eggs and viable larvae produced (circa 500 per spawning). Most studies on turbot or halibut have greater flexibility in larval stocking densities and numbers of replicates as these species produce thousands to millions of eggs per spawning. To increase the statistical confidence of the results, this study used as many replicates as was possible (determined by the number of larvae that hatched) with as many larvae as possible in each replicate. Another experiment to test the success of a probiont on survival is that of Strøm and Ringø (1993) who used "approximately" 300 cod larvae (*Gadus morhua*) per tank with duplicate replicates for the probiont and the control. No statistics were

used in the analysis of the results. Munro et al. (1995b), working on turbot larvae, used one replicate for the control and two for each probiotic treatment, while the total number of larvae used for each of the 20 replicates varied from seven to 48. Analysis was simple, involving a χ^2-test, comparing the survival of the single control against the two probiont replicates.

Due to possible variability in the environmental parameters during the larval rearing experiments, the different probionts were not compared against each other in terms of their success on improving larval survival. The only way in which the effect of experimental bias can be reduced is to run a larger experiment, whereby two probionts are tested against each other as well as a control with multiple replicates for each. Unfortunately time did not permit this during this study, although the small number of larvae produced by clownfish would also have made it impractical.

The only candidate probiont which improved the survival of clownfish larvae was *Kocuria* AP4. The probiotic was tested twice with a total of five and six control and probiotic replicates, respectively (Table 8.2). Percentage survival of the larvae in the probiotic treatment was 29.6% compared to 15.9% survival of the control and therefore survival of clownfish larvae was increased two-fold compared to the control. In a commercial setting this would equate to a considerable saving as only half of the broodstock would be required to produce the original quantity of larvae or alternatively, production could be doubled. *Kocuria* AP4 also improved the survival of *Artemia* (Chapter 6) and therefore has potential as a probiotic for both *Artemia* and clownfish larvae. Similarly, Hjelm et al. (2004) found only one potential probiotic for turbot larvae out of 34 bacteria that exhibited anti-bacterial activity (from an original 400 isolates).

This study has shown that of the five candidate probiotics which have various strengths and weaknesses in their ability to colonise the gut and be of benefit to their host, only one exhibited any beneficial effects. As it would appear that *Kocuria* AP4 is beneficial to species across different taxonomic phyla (Chapter 6), future experiments should be conducted on the larvae of other cultured marine species.

Chapter 9

General discussion

The aim of this thesis was to contribute towards the development of a protocol for the isolation and selection of probiotics for marine fish larviculture. Selection of the probionts was based on their ability to meet certain criteria shown in Figure 1.1. In the aims and context of this thesis, the acquisition of probiotic *Kocuria* AP4 was particularly beneficial as results regarding this bacteria contributed to the understanding of the selection process.

This discussion will draw on information from the experimental chapters (Chapters 3 to 8). To set about answering this question, a number of aspects need to be considered, firstly, what is the **contribution** of the various experiments in terms of how probiotics could be selected for larviculture? Drawing on this information and that of the literature, the second question can be posed regarding whether the ***in vitro* selection criteria** are good predictors for successful *in vivo* application. Finally, this leads to a critical evaluation of the proposed **selection protocol** presented in Chapter 1. The chapter concludes with suggestions for future research in larval probiotics.

In vitro tests

Antagonistic compounds

Initial selection criteria of probionts include screening for the production of antimicrobial metabolites, particularly those which inhibit the growth of pathogens. For practical reasons, only seven pathogens were used in this study. They were obtained from the Onderstepoort Veterinary Faculty, University of Pretoria, South Africa. It is recommended that the screening for antagonism be performed using a large number of pathogens, ideally from different genera. Some bacteria may exhibit antagonism towards pathogens tested *in vitro*, thereby leading to potentially false assumptions regarding their action *in vivo* (Robertson et al., 2000; Gram et al., 2001; Spanggaard et al., 2001; Alavandi et al., 2004).

Growth in mucus

Chapter 3 tested the ability of the candidate organisms to produce antagonistic compounds against seven fish pathogens. Candidate probiont *Bacillus* AP3 exhibited antagonism towards five of the pathogens, *Pseudoalteromonas* AP5 against three and the remaining three candidates were effective against two pathogens. *Kocuria* AP4 showed antagonism towards two pathogens, and improved larval survival. Other researchers (Gibson et al., 1998; Ringø and Gatesoupe, 1998; Gram et al., 2001), have also detected antagonism of candidate probionts *in vitro* yet were unable to improve larval survival or growth *in vivo*.

The production of antagonistic compounds depends on the culture media (Olsson et al., 1992; Fang et al., 1996). Therefore, the media used for screening for antagonism should be similar to that of the environment in which the probionts will settle. In this study, DIFCO marine agar 2216e was used to identify antagonism of the candidate probionts as the probionts were exposed to seawater in the culture environment. If larger volumes of mucus had been available, screening for antagonism could have been done using a mucus-based agar as this may be more similar to the nutrient source available to the probionts in the digestive tract than other commonly used media.

The ranking index suggested in Chapter 3 allows for the comparison of the growth characteristics between candidate probionts grown under similar conditions. The method described in Chapter 3 can be modified to the conditions of the species for which the probiotic is being screened. For example, experiments should be run at the temperature which the host and consequently the bacteria are to be exposed to. As a result, direct comparisons between different experiments may not always be reasonable. If a multiplate reader is unavailable,

growth curves can be determined using standard microbiological methods whereby candidate probionts are grown in flasks, sampled at regular intervals and the cell density measured spectrophotometrically.

Jacobsen et al., 1999 screened 47 strains of *Lactobacillus* spp. as potential human probiotics using *in vitro* techniques. The candidate probiotics were selected based on their production of antimicrobial metabolites, attachment ability to Caco-2 cells and tolerance to pH 2.5. When five of these were tested *in vivo*, survival of the probionts in the human gut was chiefly attributed to their ability to attach to the intestinal cells as well as tolerate a low pH. However, strong-adhering probionts, tolerant of a low pH were not capable of prolonged colonisation after administration (Jacobsen et al., 1999). In this study, *Bacillus* AP3 showed the highest level of attachment to mucus both individually and when challenged with two pathogens (Chapter 4) and it displayed antagonism towards five of the seven pathogens (Chapter 3). However, it was toxic to *Artemia* (Chapter 6) and did not improve clownfish survival (Chapter 8). *Pseudoalteromonas* AP5 showed the greatest ability to exclude pathogens by competing for attachment to mucus (Chapter 3), and had the shortest lag period when grown in mucus (Chapter 3). It also produced pigments and potentially beneficial compounds (Chapter 5) and reduced the total *Vibrio* count in the rearing water (Chapter 8). Despite these characteristics, clownfish larval survival did not appear to be improved under the conditions in which *Pseudoalteromonas* AP5 was tested *in vivo.* The only candidate probiont which showed potential to improve larval survival was *Kocuria* AP4. Although able to attach to mucus (Chapter 4), it failed to reach the stationary phase after 36 hours (Chapter 3). Although it produced pigments and comparably low levels of potentially beneficial enzymes (Chapter 5) and had below-average antagonism towards pathogens (Chapter 3), it improved survival of both *Artemia* (Chapter 6) and clownfish larvae (Chapter 8).

Attachment to mucus

In Chapter 4 the attachment to mucus assay was developed to determine the competitive exclusion of individual pathogens by individual probionts and *vice versa*. Although not performed in this study, the probionts and pathogens could each be separately labelled with both radiolabels. If a probiotic regime was to involve the addition of a number of different probionts which collectively improve the health of the larvae, it could be of interest to determine how each affects the attachment ability of the others. A multi-factorial design could be used to test the different probionts against each other as well as against pathogens. Such a study could be designed to determine competition between two or more probionts. After *in vivo* experiments have identified probiotics, the probionts could then be re-tested using this protocol which would provide information to help determine at what initial concentrations, relative proportions and in what order each probiont should be given to the larvae.

Production of beneficial compounds

The contribution of exogenous enzymes to larval growth and/or survival is a contentious issue (see Chapter 5). For example, the problem with poor larval growth when feeding inert diets has been attributed to the absence of pepsin activity (Govoni et al., 1986; Dabrowski and Culver, 1991). However, larval sea bass (*D. labrax*) fed inert diets had three times higher pepsin activity compared to those fed livefood, although growth remained poor (Zambonino-Infante and Cahu, 1994). This study did not test the influence of enzymes produced by bacteria on larval fish digestive processes. If enzyme levels of larvae containing probiotics were compared to non-exposed larvae, the influence of the other intestinal microflora which may contribute to the larval enzymes cannot be excluded. The contribution of the bacterial enzymes may lead to overestimations of the larval digestive capabilities. A possible method

for testing the contribution of bacterial enzymes would involve comparing the enzyme activity between gnotobiotic larvae and gnotobiotic larvae supplied with the different candidate probionts.

Pathogenicity/toxicity of candidate probionts

Non-pathogenic strains of bacteria may prove useful as probiotics, such as *V. alginolyticus* (Austin et al., 1995; Rico-Mora et al., 1998; Gomez-Gil et al., 2002; Villamil et al., 2003), but these bacteria may develop pathogenic traits. Although they may not kill the host, they may be of concern to human safety. Thus, the genotypic characterisation of all candidate probiotic strains is essential (Vandenberghe et al., 1999). The quality of finfish or species cultured for food must meet government and industry standards regarding exposure to disease-causing organisms. Therefore, the potential transfer of zoonotic bacteria must be carefully assessed in aquaculture. In addition, long-term testing of these strains is recommended. The probiotic must be tested and passed as safe for human consumption. With regard to the ornamental fish trade the possibility of an indirect transfer of probionts/pathogens from fish to humans (i.e., via the culture water) may need to be investigated.

Route of delivery

This project was designed to use *Artemia* as the first livefood organism for clownfish as the larvae are capable of feeding on the instar 1 nauplii. However, during the initial *in vivo* experiments (Chapter 8) it became apparent that the removal of the larger nauplii from the culture water was difficult, as a stagnant culture system had to be used for these studies. Therefore, delivering the probionts via *Artemia* was abandoned, leaving in-water dosing as an alternative method of delivery while feeding rotifers as livefood. It is, however, recommended that studies testing the attachment of probiotics to rotifers be conducted. To

provide the best mode of probiotic delivery to the larvae, a study comparing the effectiveness of in-water addition with attachment to livefood is suggested.

Many of the probiotic lactic acid bacteria used in aquaculture are of terrestrial origin. Depending on the species, lactic acid bacteria levels are stable in seawater for the first 2-4 days before dropping exponentially (Planas et al., 2004). Therefore, prolonged survival of the organism in the marine environment is essential to ensure that a sufficiently high level of bacteria is available to the larvae.

The gut evacuation period of the intestinal tract of clownfish larvae is approximately 2 hours (Chapter 8) which is shorter than the doubling time of *Kocuria* AP4 (96 h – Chapter 3) suggesting that the probiont may not be able to establish itself in the larval gut. A single dose of *Kocuria* AP4 was added to larval clownfish rearing water at a concentration of $\approx 10^5$ cells.ml^{-1} (Chapter 7). After 24-hours, the level of *Kocuria* AP4 was $\approx 2.9 \times 10^5$ cells.ml^{-1}, suggesting that the probiont is capable of surviving and possibly growing in the culture water. Therefore, the persistence of *Kocuria* AP4 in the culture water may have provided the larvae with a constant supply of the probiotic. A stagnant larval rearing system used in this study (Chapter 8) probably contributed to the probiotic persisting in the water. However, if the water in the larval culture environment is constantly being replaced, such as in a flow-through system, the development of a dosing schedule should be considered. The minimum dose should provide sufficient quantities of the probiont to the larvae to ensure it is able to enter and colonise the digestive tract. Depending on the mode of probiont delivery, the schedule should aim to ensure the development of a viable population in the larval gut and should therefore consider 1) the concentration of the probiotic that is required (i.e., cells.ml^{-1} in the culture water or number of probionts adhering to *Artemia*), and 2) the frequency of addition.

To what extent are *in vitro* selection criteria valid for selecting probiotics?

Probiotic validation

Bacteria that contribute to the adult fish's normal microflora may provide the larvae with protection against opportunistic or pathogenic bacteria (Olsson et al., 1992; Gatesoupe, 1994; Ottesen and Olafsen, 2000). It therefore seems reasonable to screen for candidate probiotics isolated from adults of the same species into which they are to be introduced. This raises the question about Gatesoupe's (1999) definition of a probiotic, particularly regarding the "…aim of improving health". In fish, the term "health" has been described as the state of an individual living in the absence of disease and "is reflected in the capacity of an individual fish, …to adapt to, respond to, and meet life's challenges…" (Stephen and Thorburn, 2004). If the probiont is isolated from unhealthy adult fish, does this, according to the definition of Gatesoupe (1999), exclude it as a probiotic? The mode of action of the probiotic may have no effect against certain pathogens which may, for example, have a different point of entry (i.e., gills, wounds) from where the probiotic is naturally located in the digestive tract. It therefore seems possible that fish are capable of supporting the probiotic within the microflora even though they may exhibit symptoms of disease. As health is difficult to quantify in larvae, it is suggested to use larval survival and growth as response variables.

Based on the findings from this thesis, the review of the literature, and hypothesis made during this work, it is proposed that for a microorganism to be considered a probiotic it should meet the following criteria 1) the microbe can be isolated from healthy subjects and grown in pure culture; 2) upon inducing "naive" subjects the probiotic improves their survival and/or growth, and 3) the probiont can be re-isolated from the healthy, induced individuals. As

mentioned above, it should be noted that the microbe may also exist in a diseased fish as the pathogen may not be affected by the mode of action of the probiont.

This study was able to demonstrate criteria 1 and 3 for *Pseudoalteromonas* AP5. Criterion 2 was tested but was not confirmed. *Kocuria* AP4 confirmed criteria 1 and 2 but was not tested for criterion 3. Future studies should be conducted to ensure that the probiont *Kocuria* AP4 can be re-isolated from larvae, thereby fulfilling all suggested criteria.

According to Klaenhammer and Kullen (1999), the selection criteria of human probiotics fall into four basic categories – Appropriateness, Technological suitability, Competitiveness, and Performance and functionality. Of these categories, *Kocuria* AP4 met all criteria for "appropriateness" and fulfilled most of the criteria for the other three. The criteria are designed for human probionts and therefore include aspects such as resistance to bile and acid, and being antimutagenic and anticarcinogenic, traits which were not considered necessary in the selection of probionts for fish larviculture. Criteria which were not tested for in *Kocuria* AP4 but which may be of use for the screening of probiotics were their immunostimulatory ability, genetic stability and genetic amenability. Therefore, it would appear that the selection process proposed in this thesis covers the majority of criteria discussed by Klaenhammer and Kullen (1999) and adds others which are relevant to the target host organism such as the probiont's ability to attach to livefood and its in-water viability.

Based on the fact that different rearing tanks with similar conditions may have distinctly different microflora communities (Grisez et al., 1997; Huys et al., 2001), selecting candidate probionts from tanks where larvae had the best survival, such as in turbot (*S. maximus*) by Huys et al. (2001) is a novel approach. The method used by Huys et al. (2001) does provide a

starting point in selecting candidate probionts, however, the assumption upon which the selection is based is possibly flawed. The assumption is that larvae will do better in tanks which by chance develop a microflora which *does not kill* them. Therefore, since no tank can be considered a "control treatment" the level of larval survival has no reference. Developers of probiotics should therefore be careful when using the method of Huys et al. (2001) for selection, and through repeated studies, should test if the bacteria isolated from tanks with high survival, are in fact from tanks which *repeatedly* perform better than average. Additionally, the poor ability of many marine organisms to be cultured on artificial media (Olafsen, 2001) combined with possible interactions between bacteria of which some may not be cultivable, makes the method described by Huys et al. (2001) difficult to apply.

Identifying probiotic modes of action

Identifying the various modes of action could be performed using different techniques, each aimed at helping identify a particular mechanism. A probiont may use various mechanisms to remain in the digestive tract and therefore modes of action are not mutually exclusive. To test whether antagonism, for example through the production of antimicrobial metabolites, contributes to the success of the probiotic, requires extensive screening for antimicrobial compounds and an understanding of the characteristics. This could be achieved using NMR or other similar techniques. Once the bacteria have colonised the larval digestive tract, the larvae could be sampled and the composition of the intestinal microflora determined. Unexposed larvae would serve as controls from which the total microflora could be determined in the absence of the probiotic. In the larvae exposed to the probiont, the survival of the predominant microflora (other than the probiotic) could be tested against the antagonistic compounds (identified and purified from the probiont). The difference between

the antagonism towards the microflora of exposed and control larvae would partly provide an indication of the success of each antagonistic compound in reducing the microflora.

To test whether growth characteristics of the probiont contribute to its success in the gut, larvae could be exposed to the probiotic using either in-water treatment or exposure via livefood as discussed in Chapter 7. If the probiotic was then removed from the larval rearing system either by completely exchanging the water, removing the livefood or transferring the larvae, only those probiotics that have entered the digestive system would be capable of colonising the gut. Depending on the species and culture conditions (i.e., temperature), frequent larval samples could be taken and the intestinal microflora could be assayed using standard microbiological plating techniques. If the probiotic mode of action is exclusion due to improved growth, it should dominate over opportunistic or pathogenic bacteria in the digestive tract.

Competitive exclusion by the probiont through competition for attachment sites could be assessed *in vivo*. To provide a means of quantification, the probiotic would need to be labelled using immunocytochemical techniques and then added to the larval culture system. Once incorporated in the larval gut, repeated larval gut samples could be prepared for TEM analysis and the contribution of labelled bacteria (probiotics) attached to the intestinal mucus could be determined. This method would also provide information regarding the region of probiont attachment, which may provide further clues as to the activity of potentially beneficial compounds in that region. For example, attachment confined to the stomach area or where the pH is low, suggests that enzymes with high activity at low pH may dominate.

Host specificity

In some fish species no host specificity could be established regarding colonisation by probiotic *C. divergens* (Gildberg and Mikkelsen, 1998; Ringø, 1999). Other authors (Austin et al., 1995; Gram et al., 1999; Irianto and Austin, 2002) have isolated a probiotic from one species and successfully used it in another. The ability of *Kocuria* AP4 to improve survival of *Artemia* nauplii (Chapter 6) and clownfish larvae (Chapter 8) suggests that it should be tested on the larvae of other marine fish and crustacean species.

Strain degeneration

During this study it was noted that the ability of probionts to inhibit the originally tested pathogens had diminished after storage. Strain degeneration should be tested to prevent the introduction of non-viable probionts. Other studies (Nikoskelainen et al., 2001; Ringø et al., 2004) have shown that the ability of probiotics to produce antimicrobial metabolites, which inhibit the growth of other microorganisms, can be lost due to storage and sub-culturing.

Many microbes only produce extracellular and potentially beneficial metabolites if they are essential for their survival (Pirt, 1985). In most cases, culturing bacteria on general nutrient media provides the organism with all the essential nutrients to grow and reproduce. However, the ability of bacteria to produce these metabolites can be lost with successive generations (Kashket and Cao, 1995). Further research is required regarding the problem of strain degeneration, particularly regarding its prevention or reduction and more importantly, its reversal, which would allow for the testing of degenerated candidate probionts.

Can the proposed probiotic selection protocol be justified?

For the description of microbial ecosystems the *r*/K-concept by Andrews and Harris (1986) could be used. *r*-Selected species are generally considered fast-growing opportunists with a low substrate affinity. Conversely, K-selected species are regarded as slow-growing specialists with high substrate affinity. In substrate-limiting environments, K-selected species eventually dominate due to their good competitive ability at low substrate supply (Andrews and Harris, 1986). As *Kocuria* AP4 is relatively slow growing as compared to the other candidate probionts (Chapter 3), it fits this criterion known for K-selected species. With its fast growth in marine broth (Chapter 3), candidate probiont *Pseudoalteromonas* AP5 shows an *r*-selected growth characteristic.

Gram-negative bacteria are considered to cause the majority of bacterial problems associated with fish diseases (Shotts and Teska, 1989). With the exception of terrestrially derived Gram-positives, i.e., lactic acid bacteria, the selection of probiotics in marine aquaculture is dominated by Gram-negative species (Austin et al., 1995; Ruiz et al., 1996; Gibson et al., 1998; Gram et al., 1999; Spanggaard et al., 2001; Chythanya et al., 2002). The selection of Gram-negatives is probably due to the suitability of their *r*-selected strategy, which provides initial, rapid, direct competition, i.e., for nutrients, attachment, etc., with the potentially harmful, opportunistic or pathogenic bacteria which are also fast-growing.

As the aquatic culture environment stabilises and matures, the microflora may become a community dominated by K-selected species (Salvesen et al., 1999). Although not part of the dominant microflora of aquatic organisms (Ringø, 2003), slow growing lactic acid bacteria have received attention regarding their use as probiotics in aquaculture (Gildberg et al., 1997; Byun et al., 1997; Ringø and Gatesoupe, 1998; Nikoskelainen et al., 2003; Ringø, 2003;

Ringø et al., 2004; Venkat et al., 2004). The contribution of Gram-positive bacteria to the intestinal microflora of adult fish is greater than in larvae (Ringø et al., 1995; Ringø and Gatesoupe, 1998). Therefore, although *r*-selected probionts may appear suitable during the early stages of larval rearing, the potential benefits of K-selected species should be considered if a long-term probiotic treatment is required.

By comparing the growth characteristics of the candidate probionts (Chapter 3) it would be possible to group them as either *r*- or K-selected species. However, the *r*/K concept describes a continuum and is built on a variety of criteria. Thus, using the growth curves developed in Chapter 3, bacteria could also be classified according to the lag-period and doubling-time for each particular species. This, in combination with the use of the *r*/K concept would become a tool for their application as probionts.

The *in vitro* tests for antagonism, attachment to mucus and production of beneficial compounds could be performed on both *r*- and K-selected species. Identification and toxicity tests would provide additional information and reasons for excluding unsuitable candidate probiotics. Based on growth data (Chapter 3), when the in-water validation and *in vivo* experiments are performed, the researcher could estimate when to sample for bacteria to confirm in-water viability and persistence in the larval gut.

Mixed probiotic treatments

The probiotic selection protocol proposed in Chapter 1 was designed to select *r*-selected species, however, the protocol can be modified when choosing K-selected probiotics. The major difference for the selection of *r*- and K-selected species would be in determining their *in vitro* growth characteristics. It is hypothesised that faster growing (*r*-selected) species

would initially be able to compete in the larval gut with opportunistic or pathogenic bacteria. As the fish matures and the contribution of Gram-positive, K-selected species increases (Campbell and Buswell, 1983; Ringø and Gatesoupe, 1998), the proportion of *r*-selected species may be reduced. Therefore, to ensure that probiotics remain within the digestive system for as long as possible, it should be tested if simultaneously introducing *r*- and K-selected species will contribute more to the intestinal microflora throughout larval development than the addition of one group. Since the K-selected probionts would initially be competing with *r*-selected species, knowledge of the K-selected organism's growth and attachment ability would be required so that initial doses can be determined. Depending on the growth and attachment characteristics, doses may differ in the proportion of *r*- and K-selected species, the timing of dosages (i.e., K-selected before *r*-selected) and the dose frequency (i.e., 2 x K-selected probionts and 1 x *r*-selected probiont doses per day). The suggestions made here are hypotheses that must be falsified in future studies. Such studies would lead to both a fundamental understanding of the mechanism applied by microorganisms during competition and their application.

The introduction of a suite of probiotics which colonise all areas of the gastrointestinal tract could be considered for juvenile fish or larvae with differentiated digestive systems. These probionts may have different selection criteria based upon the region of the digestive system where they settle. For example, probionts which are to *remain* and act in the stomach should be tolerant of low pH, capable of rapid growth (as the cells lining the stomach are constantly being sloughed), while probionts selected for use in the intestine should be able to attach to mucus and be capable of rapid growth. By selecting a number of both *r*- and K-selected species (based on their growth characteristics) the researcher would have a number of options available for the development of a suite of probiotic bacteria.

Would a mixture of probiotics that each produce antagonistic compounds reduce each others ability to grow? Investigations would be required to test individual probionts against each other *in vitro* for competition for attachment sites and competitive growth. Should synergy occur between probionts or the competition between them be insignificant, *in vivo* tests would be needed for validation.

It may be experimentally difficult to 1) validate whether providing different probiotics simultaneously to occupy separate niches of the digestive system collectively improve larval growth or survival and, 2) to optimise the process. Multi-factorial *in vitro* experiments would need to screen for possible exclusion of one probiont by another either through production of antimicrobials, growth or attachment to mucus. Similarly, multi-factorial *in vivo* experiments would need to test probiont combinations, probiotic concentrations and the best timing of sequential additions on larval growth and/or survival. Due to the uniqueness of the individual larval tank environment combined with the difficulties experienced during *in vivo* studies, it is recommended that a large number of replicates are used.

Future considerations

Where do probiotics work in the larval digestive tract?

Due to poor differentiation of the digestive tract in larval fish, probiotics used in larviculture should be capable of colonising the entire intestinal tract. As the larvae develop, the digestive tract differentiates into the stomach and the intestinal sections. This raises the question - are the chosen probiotics settling in the same area as the pathogen/opportunistic bacteria? Although bacteria with the ability to degrade complex molecules are located throughout the

digestive tract (MacDonald et al., 1986), a probiotic may be ineffective if it does not occur within the same region of the digestive system as the pathogen (Ringø et al., 2003).

It should be tested if probiotics isolated from the intestine are actually functioning in the intestine or if they are capable of adapting to function in other areas such as the stomach. For example, a probiotic selected for its ability to produce beneficial compounds should be able to occupy the larval hindgut region where nutrient uptake occurs (Olafsen and Hansen, 1992). Thus, identifying where the probionts settle would require further testing, possibly by providing gnotobiotic larvae with individual probiotics and then checking whether they are capable of colonising the entire digestive tract from mouth to the posterior end of the hind gut.

Translocation or the relocation of the indigenous intestinal microflora from the lumen is a complex process in the pathogenesis of opportunistic infections in fish (Ringø et al., 2003). Using Transmission Electron Microscopy (TEM) techniques, the region of the intestinal tract where translocation of pathogens occurs has been identified (Ringø et al., 2003). Probiotics could then be supplied to larvae which are known to attach and colonise in the same region as pathogens, thereby competing with the pathogens, possibly reducing their numbers and ability to translocate from the lumen (Ringø et al., 2003).

Although usually selected for their ability to exclude particular pathogens, the long-term effect of probiotics on the subsequent microflora has not been investigated. It is possible that certain probiotics may initially change the microflora to the host's advantage but, in doing so, they may prevent other autochthonous microorganisms from becoming established, thereby ultimately harming the host.

Genetically engineered probiotics

Now a common tool, genetic engineering will allow us to create probiotics which are capable of faster growth, better attachment and the production of a variety of beneficial compounds. Since the health of the host improves when its gut contains a diverse microflora, using a single-species probiotic may not be as efficient as using a group of representative bacteria which have been altered to enhance the health or better still fight infections in the gut. The genes coding for beneficial enzymes, vitamins or fatty acid production could be isolated and introduced into other bacteria. The target bacteria could be a known probiont with a mode of action (i.e., good attachment ability) that is different to that of the introduced gene, potentially improving the ability of the probiont to elicit several beneficial effects.

Pseudoalteromonas *AP5 as a biocontrol agent*

Compared to stagnant systems, recirculating systems for rearing halibut larvae have a relatively stable water quality which improves larval survival possibly by beneficially altering the larval gut microflora (Verner-Jeffreys et al., 2004). The dominant bacteria in the gut of healthy halibut larvae were *Pseudoalteromonas* spp. which also showed antagonism towards other bacteria (Verner-Jeffreys et al., 2004). In another study, *P. undina* strain VKM-124 was strongly antagonistic towards pathogens, and protected prawn larvae from various viral infections (Maeda, 1999). When mixed with an artificial diet and fed to prawns (*Penaeus* sp.), the microorganism reduced the sulfide and organic matter levels of the pond sediment, resulting in suppression of pathogens and improved growth (Maeda, 1999). The experiments used in this thesis have shown that *Pseudoalteromonas* AP5 is antagonistic towards a number of pathogens (Chapter 3), is non-toxic to *Artemia* nauplii – (Chapter 6) and is capable of remaining in the culture water for at least 24 hours (Chapter 7). Further studies using

candidate probiont *Pseudoalteromonas* AP5 as a potential biocontrol agent could therefore be performed.

Conclusion

The aim of this thesis was to contribute towards the development of a protocol for the selection of probiotics for marine larviculture. Using a suggested protocol, the probiont, *Kocuria* AP4 was identified. *Kocuria* AP4 was average or featured poorly in the *in vitro* tests and therefore the hypothesis that probiotics can be selected exclusively based on *in vitro* criteria was not supported. It appears that the *in vitro* success of a candidate probiont does not guarantee that it will be of any benefit *in vivo*. Compared to the typically high number of potentially suitable probiotics selected during *in vitro* tests (Riquelme et al., 1997; Jacobsen et al., 1999; Spanggaard et al., 2001; Hjelm et al., 2004), the small number of successful probionts suggests that until a probiont has been proven effective *in vivo*, the conclusions drawn from its *in vivo* ability must be viewed with caution. Although the final number of candidate probionts which are effective *in vivo* may be low, *in vitro* screening may still be necessary due to the expense and logistical aspects of conducting *in vivo* trials. This study has shown that the selection of probionts through the use of *in vitro* studies was possible, thus emphasising the potential usefulness of this approach. Additionally, the poor reproducibility of the *in vivo* trials in this study suggests that further *in vivo* studies need to be completed to increase the data set, and thereby allow for more confident recommendations regarding potential probiotics. This study has also shown the difficulties involved with a selection protocol based on *in vitro* studies.

Although the *in vitro* tests may incorrectly suggest certain candidate probionts that could be of benefit *in vivo*, the *in vitro* tests do provide a method for reducing the number of candidate

probionts. It is possible, however, that a probiotic may exert its beneficial effects based on a mode of action not assayed for *in vitro*. In this case, the bacteria would be discarded along with the others that did not perform *in vitro*. The *in vitro* tests should therefore be designed to cover as many potential modes of action of the candidate probiont as possible.

As the field of probiotics in marine larviculture develops, a better understanding of how and why the mode of action differs amongst probiotics will be needed. Using known probionts, their individual modes of action could be determined. *In vitro* experiments could then focus on producing results which can be confirmed by the success of the probiont *in vivo*, thereby improving the reliability of *in vitro* tests for identifying probiotics. However, probiotic ability may not necessarily be attributable to one particular *in vitro* test criterion, but may be a function of several criteria acting synergistically. Establishing which criteria are more important than others and how they interact with each other to improve health and survival should be the aim of future research in marine probiotics.

This study has shown advantages and disadvantages of using a combination of *in vitro* and *in vivo* tests to screen for probionts. *In vitro* tests produced a better understanding of the possible mode of action and strategies of competition between bacteria. These insights can hardly be gained from *in vivo* studies and are of fundamental importance. However, the number of criteria in which a candidate probiont is successful *in vitro* may not be the best predictor for its application of *in vivo* tests. Large-scale commercial studies that reduce between-treatment variation are required to test predictions about the most suitable probiont or combinations thereof. Thus, through the collaboration between laboratory studies and commercial testing, basic research can make a contribution to the development of probiotics.

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Appendix

Appendix 1

16S-rDNA sequences for candidate probionts

Bacillus AP3

```
1   gcacaagcgg  cggagcatgc  ggattaattc  gatgcaacgc  gaagaacctt
51  accaaggctt  gacatacacc  gggcacaagc  ggcggagcat  gcggattaat
101 tcgatgcaac  gcgaagaacc  ttaccaaggc  ttgacataca  ccggaccggg
151 ccagagatgg  tctttccccc  ttgtggggct  ggtgtacagg  tggtgcatgg
201 ttgtcgtcag  ctcgtgtcgt  gagatgttgg  gttaagtccc  gcaacgagcg
251 caaccctcgt  tctatgttgc  cagcacgtga  tggtggggac  tcataggaga
301 ctgccggggt  caactcggag  gaaggtgggg  atgacgtcaa  atcatcatgc
351 cccttatgtc  ttgggcttca  cgcatgctac  aatggccagt  acaatgggtt
401 gcgatgccgc  gaggtggagc  taatcccaaa  aagctggtct  cagttcggat
451 cgtggtctgc  aactcgacca  cgtgaagtcg  gagtcgctag  taatcgcaga
501 tcagcaacgc  tgcggt
```

Kocuria AP4

```
1   ttaccaggtc  ttgacatcct  ctgacaaccc  tagagatagg  gctttccctt
51  cggggacaga  gtgacaggtg  gtgcatggtt  gtcgtcagct  cgtgtcgtga
101 gatgttgggt  taagtcccgc  aacgagcgca  acccttgatc  ttagttgcca
151 gcatttagtt  gggcactcta  aggtgactgc  cggtgacaaa  ccggaggaag
201 gtggggatga  cgtcaaatca  tcatgcccct  tatgacctgg  gctacacacg
251 tgctacaatg  gacagaacaa  agggctgcga  gaccgcaagg  tttagccaat
301 cccat
```

Pseudoalteromonas AP5

```
1   gcacaagcgg  tggagcatgt  ggtttaattc  gatgcaacgc  gaagaacctt
51  acctacactt  gacatacaga  gaacttacca  gagatggttt  ggtgccttcg
101 ggagctctga  tacaggtgct  gcatggctgt  cgtcagctcg  tgttgtgaga
151 tgttgggtta  agtcccgcaa  cgagcgcaac  ccctatcctt  agttgccagc
201 gattcggtcg  ggaactctaa  ggagactgcc  ggtgataaac  cggaggaagg
251 tggggacgac  gtcaagtcat  catggccctt  acgtgtaggg  ctacacacgt
301 gctacaatgg  caggtacaga  gagcagcgag  ctagcgatag  tgagcgaatc
351 ccttaaagcc  tgtcgtagtc  cggattggag  tctgcaactc  gactccatga
401 agtcggaatc  gctagtaatc  gcanatcaga  atgttgcggt  gaatac
```