

DISSERTATIONS IN
**FORESTRY AND
NATURAL SCIENCES**

JOUNI HEIKKINEN

*Novel Applications of
Pseudomonas sp. Bacterial
Strains in Rainbow Trout
Aquaculture*

PUBLICATIONS OF THE UNIVERSITY OF EASTERN FINLAND
Dissertations in Forestry and Natural Sciences



UNIVERSITY OF
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AUTHOR: JOUNI HEIKKINEN

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No 123

Academic Dissertation

To be presented by permission of the Faculty of Science and Forestry for public examination in the Auditorium in Tietoteknia Building at the University of Eastern Finland, Kuopio, on October, 4, 2013, at 12 o'clock noon.

Department of Biology

Kopijyvä Oy

Kuopio, 2013

Editors: Profs. Pertti Pasanen

Pekka Kilpeläinen and Matti Vornanen

Distribution:

Eastern Finland University Library / Sales of publications

julkaisumyynti@uef.fi

www.uef.fi/kirjasto

ISBN: 978-952-61-1234-3 (nid.)

ISBN: 978-952-61-1235-0 (PDF)

ISSNL: 1798-5668

ISSN: 1798-5668

ISSN: 1798-5676 (PDF)

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ABSTRACT

Fungal and bacterial diseases cause major losses in rainbow trout aquaculture. Therefore, formalin baths are currently commonly used to prevent infections by pathogens like *Saprolegnia* sp. during egg incubation. However, there are safety and environmental concerns related to the use of formalin in hatcheries.

Rainbow trout fry syndrome (RTFS) and cold water disease, caused by *Flavobacterium psychrophilum*, are major problems during the fry and juvenile stage. There has been intense vaccine development against RTFS, but so far no commercial vaccine is available and hence antibiotics are currently the only method to treat this disease. The use of antibiotics in disease treatment causes an increased risk of the development of antibiotic resistant bacterial strains. Hence, there is an acute need for alternative disease prevention protocols.

Probiotics are widely used in health promoting food products for humans, but also in feed of homoeothermic animals. In aquaculture, utilization of probiotics has been vastly studied during the last decade.

In this thesis, sustainable methods to prevent infections caused by *Saprolegnia* sp. during rainbow trout egg incubation and by *Flavobacterium psychrophilum* during the fry and juvenile stage of rainbow trout aquaculture, were studied.

Protective bacterial strains against *Saprolegnia* sp. infections on rainbow trout eggs were screened and tested under experimental conditions *in vivo*. Furthermore, supportive water treatment methods for bacterial culture utilization in rainbow trout egg incubation were developed and their efficiencies were evaluated.

High dose (400 mWs/cm²) UV-irradiation of hatchery inlet water decreased rainbow trout egg mortality significantly at the eyed egg stage and utilization of protective bacterial cultures, *Pseudomonas* sp. M162, *Pseudomonas* sp. M174 and *Janthinobacterium* sp. M169 enhanced this effect. Supplementation of the protective cultures did not increase the mortality of the eggs.

Feeding the rainbow trout fry with *Pseudomonas* sp. M174 reduced the mortality that occurred during the experimental *Flavobacterium psychrophilum* infection. The mode of probiotic action was to evoke immunostimulatory effects and siderophore production. *Pseudomonas* sp. M162 also decreased *Flavobacterium psychrophilum* related mortality, while the probiotic effect resulted mainly through immunostimulation. Both strains were found to be safe for the fish.

As a conclusion, this thesis has demonstrated the remarkable potential of *Pseudomonas* sp. M162 and M174 strains and novel applications in which they can be utilized as protective cultures and probiotics in rainbow trout aquaculture.

Universal Decimal Classification: 591.2, 591.619, 597.552.51, 639.3.09

CAB Thesaurus: fish culture; hatcheries; fish diseases; disease prevention; pathogens; probiotics; Pseudomonas; Flavobacterium psychrophilum; Saprolegnia; rainbow trout; fish eggs; fry; ultraviolet radiation; siderophores; immunostimulation

Yleinen suomalainen asiasanasto: kalanviljely; kalataudit; taudinaiheuttajat; sienitaudit; bakteeritaudit; probiootit; kirjolohi; mätä; ultraviolettisäteily

Acknowledgements

The work described in this thesis has taken almost a decade to accomplish and have seen the department changing its name from Institute of Applied Biotechnology to Department of Biosciences and finally Department of Biology. I would like to thank the staff at the Department for providing me a pleasant environment to work.

I wish to express my sincere thanks to Professor Atte von Wright and Dr. Jouni Vielma, my supervisors, for their supportive and valuable comments during the preparation of manuscripts and writing the thesis

I want to thank Professor Kenneth Cain and Dr. Jose Luis Balcazar, for kindly agreeing to review this thesis and giving thoughtful comments that significantly improved the thesis.

I also express my warm thanks to my co-authors Dr. Marja Tirola and Dr. Lotta-Riina Sundberg, Mrs. Päivi Eskelinen, Ms. Satu Mustonen, Ms. Dina Navia-Paldanius, Dr. Tiina Korkea-aho, Dr. Kim Thompson, Ms. Anna Papadopoulou, Professor Brian Austin and professor Alexandra Adams.

I am particularly grateful to Dr. Paula Henttonen, for teaching me the secrets of aquaculture and fish biology and for her encouraging and constructive comments on thesis structure.

I wish to express my sincere thanks to Dr. Hannu Mölsä, for hiring me into the ProBio AquaFeed-project and finally to working with *Saprolegnia*.

My sincere thanks goes to the personnel of Fish Research Unit, Marko Kelo, Mikko Ikäheimo and Kauko Strengell. Without

your technical knowledge and help, these experiments would have been, if not impossible, at least much more difficult.

My warm thanks to Ms. Roseanna Avento for assistance in language issues and Dr. Jenny Makkonen for all instructive assistance you gave me. It was much easier to walk the same route a few months later than you.

I am grateful to my colleagues at Food and Nutrition Biotechnology laboratory, Mirja, Elvi, Jenni, Outi, Kristiina, Kati, Riitta, Eeva-Liisa, Toni, and Paula. It was always nice to come to work in the lab.

I want to thank my brothers and sisters in Claybay Skeleguins for giving me possibility to start ice-hockey at the age of 35. The energy and enthusiasm I have got from our practices at 7am helped me a lot during the writing process.

I express my warmest thanks to my friends Heikki and Mikko. The annual hunting trips to Nurmes, Ilomantsi and Lieksa have been a necessary battery recharging for me. I really hope that "Operation Capercaillie" still continues.

I thank my parents Martti and Pirkko and my sister Heidi for their support. Even though you probably did not always understand what I was doing, you always supported me in it.

And last but not least I would like to thank my wife Tanja for her love and support during this challenging writing process and to my little son, Jesse, for being a boundless source of joy and getting my thoughts away from this thesis.

Financial support for this study was provided by Kemira Grow-How Oy, Savon Taimen Oy and Ministry of Agriculture and Forestry, Finland.

Kuopio, September 2013 *Jouni Heikkinen*

LIST OF ABBREVIATIONS

ARISA = Automated ribosomal intergenic spacer analysis

ASA = atypical *Aeromonas salmonicida*

ASS = furunculosis caused by *Aeromonas salmonicida* ssp. *salmonicida*

BKD = Bacterial kidney disease

CFU = colony forming unit

CWD = Cold water disease

ELISA= Enzyme-linked immunosorbent assay

ERM = Enteric red mouth disease

IHN = Infectious hematopoietic necrosis

i.m. = intramuscular

i.p. = intraperitoneal

IPN = Infectious pancreatic necrosis

PCA = Principle component analysis

RTFS = Rainbow trout fry syndrome

SD = Sleeping disease

TBC = Total bacterial counts

TSA =Tryptone soy agar

TSB = Tryptone soy broth

TYES =Tryptone yeast extract salts

VHS = Viral haemorrhagic septicemia

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals I-IV.

- I.** Heikkinen, J, Mustonen, SM, Eskelinen, P, Sundberg, L-R, von Wright, A. Prevention of fungal infestation of rainbow trout (*Oncorhynchus mykiss*) eggs using UV irradiation of the hatching water. *Aquacultural Engineering*, 2013, 55, 9-15.
- II.** Heikkinen, J, Tirola, M, Mustonen SM, Eskelinen P, Navia-Paldanius, D, von Wright, A. Ultraviolet irradiation of hatchery water and protective bacterial cultures as suppressor of *Saprolegnia* infections in rainbow trout (*Oncorhynchus mykiss*) eggs. Submitted manuscript
- III.** Korkea-aho, T, Heikkinen, J, Thompson, K, von Wright, A, Austin, B. *Pseudomonas* sp. M174 inhibits the fish pathogen *Flavobacterium psychrophilum*. *Journal of Applied Microbiology* 2011, 111, 266-277.
- IV.** Korkea-aho, TL, Papadopoulou, A, Heikkinen, J, von Wright, A, Adams, A, Austin, B, Thompson, KD. *Pseudomonas* M162 confers protection against rainbow trout fry syndrome by stimulating immunity. *Journal of Applied Microbiology* 2012, 113, 24–35.

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AUTHOR'S CONTRIBUTION

The author took part in planning and design of the experiments and performed all analyses for cultivable microbes in Studies I and II, incubation trial in study I and had the main responsibility in writing and submitting the articles. In articles III and IV, the author performed isolation, screening and identification of probiotic strains and participated in writing and reviewing processes.

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1 Introduction

Aquaculture is fastest the growing sector of animal food-production in the world. During the last forty years, the average annual growth rate of aquaculture has been 6.6% (FAO, 2010). At the same time, the capture of the wild fish has reached its limits, emphasizing the significance of aquaculture in protein supply to the world increasing global population.

Intensive fish farming is only possible through effective feeding and high fish densities, but this increases the risk of disease outbreaks. The main risks are viral and bacterial diseases, which occasionally are responsible for severe losses to the industry (Stone et al., 2008, Mardones et al., 2011). Preventive measures, like vaccination, have been developed against some diseases, but antibiotic medication to combat acute infections is still a common practice. Probiotics have been suggested as alternatives to reduce the use of antibiotics (Balcazar et al., 2006a) and the risk of selection for antibiotic resistant bacterial strains (Miranda and Zemelman, 2002a).

The highest mortalities in salmonid aquaculture occur during egg incubation and fry period (Bootland and Leong, 2011, Munro and Midtlyng, 2011, Starliper and Schill, 2011, Thoen et al., 2011). Hence, utilization of probiotics and protective cultures to prevent diseases should be focused on these early life stages of salmonid fish with emphasis on those diseases without environmentally safe treatment method.

Rainbow trout fry syndrome (RTFS) and coldwater disease (CWD) are caused by *Flavobacterium psychrophilum* resulting in severe mortalities during the fry and juvenile stage (Starliper and Schill, 2011). Early occurrence of RTFS enforces the farmer to either accept the mortalities or using antibiotics for treatment.

Formalin treatment is the most common method used to prevent salmonid egg mortality due to *Saprolegnia* infections (Gieseke et al., 2006). However, formalin is a hazardous chemical which may cause risks to hatchery personnel and the environment. Keeping this in mind, there is no environmentally safe and effective treatment method against *Saprolegnia* infections on rainbow trout eggs.

Saprolegnia is capable of attaching to dead rainbow trout eggs, but not directly on living ones (Kitancharoen et al., 1997, Thoen et al., 2011). Either maternally transferred immune system components, bacterial epibionts attached on egg surface or a combination of both prevents the attachment of *Saprolegnia* spores. Several bacterial strains possess fungicidal activities, but their effects on prevention of *Saprolegnia* infections in rainbow trout egg incubation *in vivo* have not been studied.

In this thesis, applications of *Pseudomonas* sp. strains M162 and M174 for the control of *Saprolegnia* sp. infections during egg incubation and *Flavobacterium psychrophilum* mortalities in rainbow trout aquaculture has been assessed in different life stages.

2 Review of literature

2.1 RAINBOW TROUT

The rainbow trout (*Oncorhynchus mykiss*) belongs to the order of Salmoniformes and Salmonidae-family; natural habitats of the species are the river tributaries into the Pacific Ocean in Asia and North America (Helfman et al., 2009). Subsequently, the species has been introduced into several water bodies around the world, which has also caused a concern of the rainbow trout's capability to pose a threat to local salmonid species (Fausch, 2007, Seiler et al., 2009, Peeler et al., 2011).

The species was named by German biologist Johann Julius Walbaum in 1792. Species was renamed as *Salmo gairdneri* by Richardson in 1836, but utilization of DNA technology in taxonomy research has revealed closer species relationship with Pacific salmon (*Oncorhynchus* species) than *Salmo* species and hence the name was returned to the original *O. mykiss* (Smith and Stearley, 1989).

Rainbow trout's name derives from the broad purple colored region in operculum and around lateral line, which reflects also other colors (Koli, 1990) (Fig. 1). The dorsal side of the fish is from blueish to greenish and it is full of small black dots which can be found below the lateral line, but usually not from the ventral side, which is usually silvery. Rainbow trout's common appearance is more robust than that of brown trout (*Salmo trutta*) or Atlantic salmon (*Salmo salar*). The coloration tends to vary depending on the location and age of the fish.

Three ecologic forms of rainbow trout can be distinguished: river living forms, cold lake living forms (kamloops) and anadromous forms (steelhead)(Koli, 1990, Groot, 1996). In their

natural habitats, rainbow trout reproduces during spring in brooks and rivers. Males reach sexual maturity in Finland usually after 2-3 and females after 3-4 years (Kause et al., 2003).



Figure 1. Rainbow trout (*Oncorhynchus mykiss*) with typical spawning color during the egg stripping.

The spawning color of the male is darker than outside of the spawning period and a small hook is formed in the lower jaw (Koli, 1990). A female produces on average about 2000 eggs per kilogram of live weight (Purser and Forteach, 2003) and digs a redd with her tail where the eggs are laid (Groot, 1996). The eggs hatch after roughly 300-400 day degrees (Billard and Jensen, 1996). After the energy from the yolk sac is consumed, the larvae start to feed on plankton. As the larvae grow into fingerlings they shift their diet to insect larvae, crustaceans and insects on the water surface (Koli, 1990). When rainbow trout reach a size of 35-40cm, they become predators, and small fish form their main source of energy. The normal weight of the rainbow trout in natural conditions in Finland is 0.5-3 kg, but the average weight of rainbow trout in endemic regions is considerably higher (Groot, 1996). The maximum size of the rainbow trout varies with stock, region and habitat.

2.2 RAINBOW TROUT AQUACULTURE

In 2010, the aquaculture production of salmonid fish amounted to 2.41 million tonnes, with a value of 11.6 billion USD (FAO, 2011). The most important salmonid aquaculture species are Atlantic salmon with a production of 1,43 million tonnes followed by rainbow trout, the production of which reached 0.73 million tonnes in 2010 (FAO, 2011). Rainbow trout aquaculture is widespread around the world; the biggest producers were Chile (30.3 % of world production), Iran (12.6 %), Turkey (11.7 %), and Norway (7.5%). In Finland, rainbow trout is the major cultivated species accounting for 89% of total aquaculture production in 2012 (FGFRI, 2013).

Some of wild salmonids are anadromous fish, which migrate to the sea to grow out and then migrate back to their home rivers for spawning. Similarly in aquaculture, salmonid eggs need freshwater for successful hatching. The larval and juvenile stages are also grown in freshwater. Outgrowing is possible in freshwater, but usually deeper sea or brackish water is preferred due to the better temperature environment, water exchange and hence smaller nutrient loads under the aquaculture cage.

Rainbow trout can tolerate a wide water temperature range, but elongated periods above 20°C commonly increase mortality. Kaya (Kaya, 1978) reported 26°C as the lethal temperature for rainbow trout. The highest feed intake is achieved in 19.5 °C, but since increase in water temperature increases energy needed for metabolism, optimum growth temperature for rainbow trout is 16.5 °C (Wurtsbaugh and Davis, 1977, Jobling, 1981). Salmonids demand a high level of dissolved oxygen in water, preferably close to 90% saturation (at 10 °C). In hypoxic conditions, when oxygen saturation decreases below 53% (at 10 °C), the feed intake decreases significantly (Glencross, 2009). Moderate oxygen supersaturation (<140%) was not found to be harmful for rainbow trout and even enhanced the recovery from transfer (Ritola et al., 1999).

The natural spawning time of rainbow trout is April-May in the northern hemisphere (Groot, 1996), but selective breeding and photoperiod adjustment as can be conducted under aquaculture conditions have elongated egg production season so that it now spans the whole year. Eggs and milt are stripped from broodstock fish and mixed gently, after which the eggs are allowed to swell and are disinfected. Rainbow trout eggs do not tolerate movement within 2-4 days after fertilization until they have reached the eyed-egg stage, when the dead eggs can be removed. The water temperature in a rainbow trout hatchery is commonly 8-12 degrees. Eggs reach the eyed-egg stage after 155.9-179.5 degree days and hatching occurs after 348.4-436 degree days, depending on the temperature of water during the incubation (Billard and Jensen, 1996).

After a yolk sac larva has utilized the nutrients, it begins to start feeding from surface. Special microfeeds have been developed for the first few weeks to fulfill the nutritional demands of the fish. When larva starts feeding, it turns into fry. The fry reaches the fingerling stage when the fish has grown to approximately 2 gram weight and feeding is changed from micro feeds to commercial pellet feed (Halver, 1996). The pellet size is increased according to the size of the fish to minimize the energy needed for feed intake and to reach optimal growth. After 7-8 months from the fingerling state, rainbow trout reaches the portion size of 300-400 grams, which is the marketing size in Central Europe. In Northern Europe, larger fish are preferred and hence fish are slaughtered for market at the size of 1.5-3.0 kg.

2.3 DISEASE RISKS IN RAINBOW TROUT AQUACULTURE ENVIRONMENT

There are many disease risks in rainbow trout aquaculture environment. Although viral and parasite infections cause

severe problems to industry, bacterial and fungal diseases are described here more thoroughly as they are more important with respect to the experimental set up in this thesis.

2.3.1 Bacterial diseases

Salmonid aquaculture with high fish densities creates an environment optimal for high bacterial numbers. Nutrients released from the feed, faeces in the water and drifting scales and mucus provide excellent growth conditions for bacteria. Facultative and opportunistic pathogenic bacteria are ubiquitous in the aquaculture environment, but usually a shift either in the physical barrier or immunological status of fish is needed before there can be a disease outbreak.

The most meaningful disease outbreaks in freshwater environment are caused by *Flavobacterium columnare* and *Flavobacterium psychrophilum*. Columnaris disease, due to *F. columnare*, can be recognized from skin lesions, fin erosion and gill necrosis in fish (Pulkkinen et al., 2010). Columnaris disease is related to elevated water temperatures during the summer months and fish mortality may reach 100% (Suomalainen et al., 2005).

Flavobacterium psychrophilum causes diseases known as rainbow trout fry syndrome (RTFS) and bacterial cold water disease (CWD). The classification of the disease is related to life stage of fish. Some strains have been associated with both diseases (Lorenzen et al., 1997). RTFS is a disease of the early life stages from yolk sac larva to early feeding stage (Nematollahi et al., 2003, Starliper, 2011). The most visible symptom of this disease is anemia which is revealed by pale gills, intestine, kidney and liver (Nematollahi et al., 2003).

CWD outbreaks occur at water temperatures below 16 °C and are most severe and prevalent below 10°C (Starliper, 2011) especially infecting fry and fingerlings (Nematollahi et al., 2003). The initial signs of disease are loss of appetite and eroded fin

tips progressing to severe necrosis in caudal area (Starliper, 2011).

The primary mode of transmission of RTFS and CWD is apparently horizontal and via infectious cells in the water (Starliper and Schill, 2011). Skin injuries and decreased immune status due to stress in the fish facilitates the infection (Madetoja et al., 2000). Furthermore, vertical transmission is an important route for *F. psychrophilum* (Starliper and Schill, 2011). In addition to the recovery of the *F. psychrophilum* from the milt (Madsen et al., 2005), ovarian fluids, egg surfaces (Rangdale et al., 1996) and inside the fertilized and eyed eggs (Brown et al., 1997), the current egg disinfection procedures do not remove the all bacteria from the egg surface (Wagner et al., 2008, Barnes et al., 2009) creating a risk of disease transfer via eggs.

Bacterial kidney disease (BKD) is caused by *Renibacterium salmoninarum* and infections occur in all salmonids. Disease has a chronic nature and mortality occurs often in 6 to 12 month old juveniles and adults prior to spawning. Bacterial kidney disease can spread horizontally from infected fish or vertically via eggs from infected parents (Evelyn et al., 1986). Indeed, *R. salmoninarum* is one of the few bacterial pathogens capable to transmit the disease from parents to progeny, even although the eggs are disinfected after fertilization. Severe infection of *R. salmoninarum* may not show any obvious external signs in fish, which - together with a chronic nature of the disease - makes its detection challenging in some cases (Wiens, 2011).

Enteric red mouth disease (ERM) is caused by *Yersinia ruckeri*, a pathogen from the same genus which causes a severe diarrheal disease in humans and other homoeothermic animals. *Y. ruckeri* causes acute or chronic bacterial septicemias in fish. The most detectable symptoms are anorexia, darkening of the skin and lethargy (Barnes, 2011). A reddening of throat and mouth is commonly present.

In the marine environment, the major bacterial diseases of rainbow trout are furunculosis and vibriosis. *Aeromonas salmonicida* ssp. *salmonicida* (ASS) causes severe septicemia and acute mortality in salmonids; the disease is known as furunculosis (Cipriano and Austin, 2011). In fingerlings, acute mortality may occur without any clinical signs other than darkening of the skin, but in juveniles and adults also hemorrhage at the base of the fins and oral cavity is commonly encountered.

The so-called atypical *Aeromonas salmonicida*-disease (ASA) is caused by a group of *A. salmonicida* strains, different from furunculosis evoking *A. salmonicida* ssp. *salmonicida* (Wiklund and Dalsgaard, 1998, Gudmundsdóttir and Björnsdóttir, 2007). The external signs of the disease often resemble symptoms of acute septicemia, including hemorrhage at the base of the fins and the development of skin ulcers on the side of the body (Gudmundsdóttir, 1998).

Listonella anguillarum (previously known as *Vibrio anguillarum*) is the best known causative agent of the disease called vibriosis, the most common disease of wild and cultured marine fish (Actis et al., 2011). Vibriosis may be caused in salmonids also by *Vibrio ordalii* or *Vibrio salmonicida* (Toranzo et al., 2005). The occurrence of vibriosis is related to water quality, temperature, stress of the fish and pathogenicity of *L. anguillarum* strain (Actis et al., 2011). Typical clinical signs of vibriosis include red spots on the ventral and lateral sides of the fish and dark ulcerating skin lesions. Corneal lesions are also rather common. In acute outbreaks, especially fingerlings may die without any external symptoms.

2.3.2 Fungal diseases

The occurrence of cottonlike mycelia in the skin of the fish or on the surface of eggs in a hatchery is often referred to as water mold infection. Even though the causative agent, *Saprolegnia* sp. was classified as a fungus still in the nineties, the current

taxonomy locates it to the kingdom Chromalveolata (Cavalier-Smith, 1998).

The genus *Saprolegnia* consists of approximately 70 different species but only a minority are connected to fish disease outbreaks. *Saprolegnia* species are secondary pathogens (Van West, 2006), although some pathogenic strains of *Saprolegnia parasitica* have been isolated in high mortality outbreaks, suggesting that the strains could also be primary pathogens (Hussein and Hatai, 2002). Utilization of both sexual and asexual reproduction makes *Saprolegnia* as efficient survivor. Thick walled oospores, produced by sexual lifecycle, enables species to survive harsh conditions and germinate when the conditions have improved (Beakes and Bartnicki-Garcia, 1989). Asexual reproduction instead allows mainly dispersion of the zoospores (van den Berg et al., 2013).

In hatcheries, *Saprolegnia* sp. causes mortality in salmonid eggs by growing its mycelia on dead eggs but it also surrounds quickly adjacent living eggs, finally suffocating them (Smith et al., 1985, Fregeneda-Grandes et al., 2007a, Thoen et al., 2011). Hence, the number of dead eggs may increase exponentially in hatching jars and trays (Smith et al., 1985). A poor fertilization rate increases the possibility of *Saprolegnia* outbreaks in hatcheries. Removal of dead eggs by handpicking decreases the *Saprolegnia* infections on eggs (Barnes et al., 2002), but the procedure is laborious and possible only for vertical incubators in which the eggs are in one layer.

Saprolegnia sp. is capable of infecting also adult fish (Willoughby and Pickering, 1977, Willoughby, 1978). Zoospores attach themselves to the skin of the fish at places in which the mucus layer has been damaged due to parasites (Hatai and Hoshiai 1994, Stueland et al., 2005a), other disease (Bruno et al., 2011), physiological changes prior the spawning period or by handling. The isolates, which cause lesions in salmonids, seems to form a cluster which share a similar secondary cyst coat morphology,

germ tube growth and esterase isozyme patterns (Beakes et al., 1994, Diéguez-Uribeondo et al., 2007, Fregeneda-Grandes et al., 2007b). The mycelium grows usually on side and head of the fish causing electrolyte loss which finally leads to death (Hatai and Hoshiai, 1994). In severe infections, 20-80% of the skin surface area might be covered (van den Berg et al., 2013).

2.3.3 Viral diseases

Marine water may contain up to 10^8 viruses /ml (Suttle, 2005) and a positive correlation has been found between viral abundance and the abundance of bacteria, the chlorophyll concentration and total phosphorus level (Maranger and Bird, 1995). These conditions occur in rainbow trout aquaculture seacages, where economically viable production necessitates high fish densities. Hence also pathogenic viruses are present in farming sites. Viral diseases are responsible for huge economic losses in salmonid aquaculture (Raja-Halli et al., 2006, Saksida, 2006, Stone et al., 2008, Mardones et al., 2011). Most severe outbreaks have been caused by viral hemorrhagic septicemia (VHS) (Stone et al., 2008, Dale et al., 2009), infectious hematopoietic necrosis (IHN) (LaPatra et al., 2001) and infectious pancreatic necrosis (IPN) (Saint-Jean et al., 2003), while mortalities related to sleeping disease are commonly lower (Graham et al., 2003).

2.3.4 Parasites

Fish parasites are a varied group of organisms which include members from protozoans, monogens, cestods, nematodes, trematodes and crustaceans. Most of the representatives are ubiquitous and do not trigger any problems unless the number of parasites per fish increases greatly. High parasite levels in fish are a burden to the immune system and thus they increase the risk of outbreaks of bacterial and viral diseases.

White spot disease is caused by a protozoan, *Ichthyophthirius multifiliis*, in freshwater environment. The organism is probably

the most difficult among unicellular fish parasites with regard to treatment due to its problematic life cycle. The parasitic trophont is able to live on the host epidermis, forming visually detectable white spots, which cause stress and dysfunction of osmoregulation to fish (Shinn et al., 2012). White spot disease leads to significant mortalities in aquaculture, if it is left untreated.

Other significant protozoan species in rainbow trout aquaculture are *Ichthyobodo necator* and *Chilodonella*, which both are also deadly to fish if left untreated (Rintamäki-Kinnunen and Valtonen, 1997).

2.4 HOST-PATHOGEN INTERACTIONS

The host-pathogen interactions have high interspecific functional variation. Hence the mechanisms of interactions are described in this chapter from the point of view of rainbow trout, unless mentioned otherwise.

Fish defense mechanisms consist of two different but synergetic branches: innate (non-specific) and acquired immunity (specific) (Magnadóttir, 2006)(Fig. 2). The fish egg is introduced into an environment which is replete with facultative and opportunistic pathogens and hence innate immunity is needed to enable the survival of fish, before any antigens of pathogens can be introduced to the host and acquired immunity can be developed. The inefficiency and slow response of acquired immunity further emphasizes the importance of innate immunity.

2.4.1 Innate immunity

Innate immunity consists of physical barriers and humoral and cellular immune systems (Magnadóttir, 2006). In addition to its necessity as defense mechanism, it has also role in the development of acquired immune response and homeostasis.

The first obstacles which a pathogen meets while trying to enter the host are physical barriers, consisting of mucous layer, scales, epidermis and gills (Kiron, 2012).

The humoral immune system includes also proteins e.g. lysozyme, complement, lectin, C-reactive protein, interferon, transferrin, haemolysin and anti-proteases.

The cellular immune system consists of macrophages, nonspecific cytotoxic cells and granulocytes, which can be divided into neutrophils, eosinophils or basophils according to their staining properties.

2.4.1.1 Physical barrier

The mucus layer has the capability to trap and slough the pathogens and it also contains humoral immune components (Magnadóttir, 2006). In addition, profuse mucus secretion may detach pathogens from the host. Fast et al. (Fast et al., 2002) found that rainbow trout mucous layer contained predominantly alkaline phosphatase, serine and metalloproteases and lysozyme. Protease activities were higher in rainbow trout adapted to saltwater compared to freshwater reared fish while the opposite was the case for lysozyme activities.

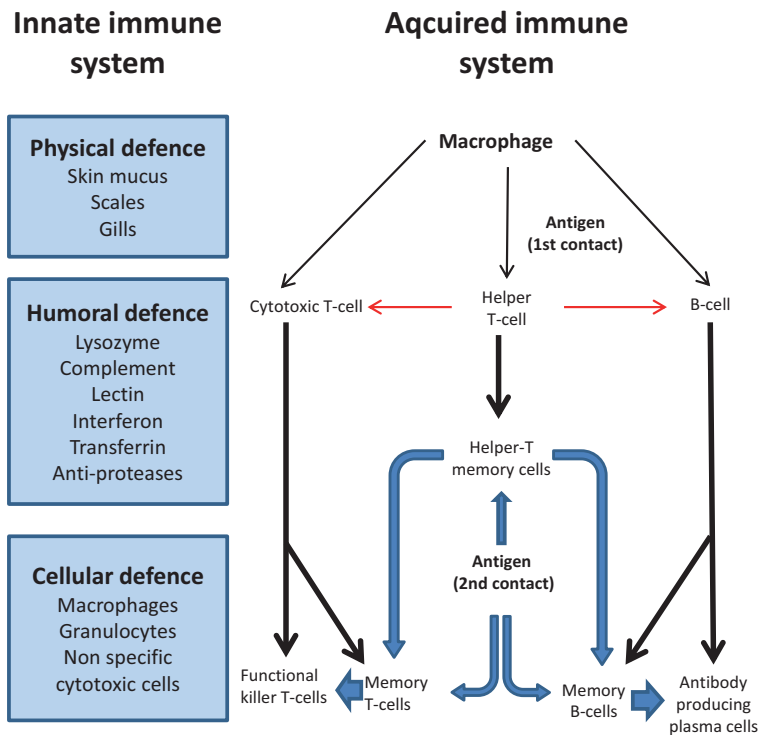


Figure 2. Simplified mechanisms of innate and acquired immunity in fish based on a knowledge of the immunity of mammals, since many of the mechanisms are unclear in fish. The thin black arrows depict presentation of the antigen when the antigen is contacted for the first time. The thin red arrows depict cytokine mediated activation needed for further stimulation of the cell mediated response (left hand side) or antibody mediated response (right hand side). The thick black arrows depict multiplication of the cells or functional modification of cells in response. The blue arrows depict fast and precise response when fish immune system comes into contact with the antigen for the second time. (Modified from Magnadóttir 2006, Reece et al., 2011, Jokinen 2012).

The scaly cover forms a tight physical barrier against parasites. Loss of scales through mechanical injury opens an easy accessible route for pathogens and parasites into the epidermis of fish.

2.4.1.2 Humoral compounds

Non-specific soluble humoral defense substances not only intensify the defensive effect of mucous layer, but also weaken and destroy pathogens in plasma and tissue fluids. The humoral components in the fish innate immune system consist of lytic enzymes (like lysozyme and chitinase), enzyme inhibitors, complement components, agglutinins and precipitins (like lectins), natural antibodies, growth inhibitors, cytokines, chemokines and antibacterial peptides (Alexander and Ingram, 1992, Magnadóttir, 2006).

2.4.1.3 Cellular defense

Cell mediated innate immunity relates to the function of phagocytic cells (granulocytes and monocytes/macrophages) (Neumann et al., 2001) and non-specific cytotoxic cells (Evans et al., 2001). Defensive cells circulate in the bloodstream and they are located in almost every tissue in fish.

2.4.1.4 Complement

The complement system consists of over 20 circulation proteins, which cause a cascade activating the complement response to destroy the pathogen (Jokinen, 2012). Complement may be activated by the classical route through acquired immunity (presented section 2.4.2.), when antibodies become bound to pathogen and complement proteins are then attached to antibodies, activating the complement system (Boshra et al., 2006). In the alternative route, complement proteins attach themselves directly to the surface of the invasive microbe, or to lectin (nonspecific lectin route) complexes that are attached to the cell surface of the microbe.

2.4.1.5 Inflammatory reaction

The inflammation reaction enhances the destruction of pathogens through local and systemic changes in fish (Ingerslev et al., 2010). The blood circulation in the inflammation area increases and hence the permeability of blood vessels increase and there are elevated numbers of granulocytes present (Reite and Evensen, 2006, Jokinen, 2012). Attachment of antigens to

macrophages triggers secretion of cytokines from the cells, which enhances the inflammatory reaction further (Ingerslev et al., 2010). Cytokines act through cytokine receptors and they increase the levels of acute phase proteins, such as amyloid-A and C-reactive protein, which activates complement (Chapter 2.4.1.4.)(Jokinen, 2012).

2.4.2 Acquired immunity

Although first response of acquired immunity is slow, a second contact with pathogen leads to a targeted and fast response (Uribe et al., 2011). Acquired immunity consists of two components: antibody-mediated immunity and cell mediated immunity.

Antibody mediated immunity is based on an immunological memory in which special antigen receptors of lymphocytes recognizes the pattern of foreign material and the lymphocyte starts to multiply (Jokinen, 2012). Daughter cells mature then to antibody secreting B-lymphocytes and killer T-cells. The antibody on the surface of B-lymphocyte recognizes specific antigens directly on the surface of the pathogen (and becomes attached to the antigen receptor). T-lymphocytes instead cannot recognize the antigen directly, but they need to be presented attached to MHC-proteins or in case of extracellular pathogens by monocytes, macrophages, dendritic cells or B-lymphocytes. Phagocytic cells and complement are then capable of becoming attached to the Fc-part of antibody and the pathogen can be destroyed either by phagocytosis or the complement system. Fish antibodies are also capable of neutralizing the pathogen, agglutinating or precipitating the antigens to enhance phagocytosis. Teleost fish mainly have one antibody type, which resembles the human IgM (Jokinen, 2012).

Acquired cell mediated immunity is based on cell mediated destruction of infected cells. It can be viewed as a cascade; cytotoxic T-cells are converted into active killer T-cells with the

contribution of helper T-cells and memory T-cells. A killer T-cell is capable of destroying the infected fish cell through apoptosis.

In aquaculture, acquired immunity is enhanced through vaccination of fish, creating the first contact to the weakened pathogen before the pathogen is actually met. Innate immunity can also be enhanced with immunostimulants, like β -glucan (Djordjevic et al., 2009, Kiron, 2012), or probiotics (see Chapter 2.6.).

2.4.3 Maternal transferred immunity

It is known that components of both innate and acquired immunity are transferred from mother to progeny and they provide protection to the progeny prior to its own immune system is developed (Zapata et al., 2006). Maternal IgM-antibodies (Castillo et al., 1993), complement components (Løvoll et al., 2006), lysozyme (Yousif et al., 1991), and lectins (Tateno et al., 1998) have been found in salmonid fish eggs or larva.

2.4.4 Role of endogenous microbiota

The intestinal microbiota of rainbow trout and its role in health aspects, feed utilization and gene expression have been extensively studied (Heikkinen et al., 2006, Kim et al., 2007, Merrifield et al., 2009a, Merrifield et al., 2009b, Dimitroglou et al., 2009, Mansfield et al., 2010). Autochthonous intestinal microbiota block binding sites from pathogens, support the gut mucosal barrier function (Merrifield et al., 2010a) and research performed with the gnotobiotic zebrafish (*Danio rerio*) suggests that endogenous microbiota have an important role in the development of innate immunity (Rawls et al., 2004).

2.4.5 Routes of infection

Pathogens have three routes by which they can enter the fish: through skin, via gills or gastrointestinal tract. Some of the diseases, like IHN (Bootland and Leong, 2011) and VHS (Smail

and Snow, 2011, Schönherz et al., 2012), are capable to utilize all of these routes. Fungal and parasite infections are typical examples of infections occurring through skin.

The oral infection route is a direct way to the intestinal tract of fish, if the pathogen is tolerant to bile salts and the acidic conditions present in the fish stomach. Oral transmission has been found to be one of the most common infection routes of ASS (Cipriano and Austin, 2011).

In addition to the three major routes, a pathogen can pass vertically from broodstock to progeny in the case of *Renibacterium salmoninarum* (Evelyn et al., 1986). Vertical transmission may occur also in other pathogens, but clear evidence is still missing in most cases.

2.4.6 Immune system avoidance mechanisms of fish pathogens

A mechanism which allows a pathogen to evade, resist or subvert immune system of fish makes it easier to establish an infection. Most bacterial pathogens are destroyed by the fish immune system through phagocytosis, in the first place through reactive oxygen species (ROS) produced by macrophages. Enzymes, like catalase and superoxide dismutase (SOD), produced by *Yersinia ruckeri* may detoxify ROS and hence allow the pathogen to survive and even reproduce inside the macrophage (Ryckaert et al., 2010, Barnes, 2011).

Virulent isolates of *Y. ruckeri* isotype O1 have been found to be resistant to rainbow trout normal serum, which kills avirulent isolates of O1 and other isotypes O2, O5, O6 and O7, suggesting that *Y. ruckeri* may inhibit effects of lysozyme and lectins found in normal serum (Davies, 1991).

A correlation has been reported between *Vibrio ordalii* in host fish blood and the decrease of white blood cells in moribund fish, suggesting that there is the production of a leukocytolytic factor (Actis et al., 2011).

2.4.7 Infection conducive factors like stress

The immune system of fish and hence the susceptibility to diseases is affected by the stress experienced by the fish (Maule et al., 1989, Pickering, 1998). Stress may be related to poor water quality, temperature fluctuation, handling procedures, reproduction or social dominance in the tank (Pickering, 1998). The stress of the fish can be categorized as acute (short termed periods) or chronic stress (elongated periods) (Davis, 2006). The acute stress response activates the immune system through leukocyte mobilization, innate and Th1 responses (Tort, 2011). With chronic stress, energy metabolism is eventually changed since the continuous production of antibodies, complement proteins and production and differentiation of different types of leucocytes leads to a lack of resources and finally to immune suppression.

2.5 CURRENT PATHOGEN AND PARASITE MANAGEMENT

2.5.1 Vaccination

Vaccination provides long lasting prophylactic protection against certain pathogens. Vaccination is performed by either dipping the fish in a vaccine solution, administering the vaccine orally along with the feed or, most commonly, by injecting the vaccine into the intraperitoneal cavity. Vaccine injection is currently the most efficient method, but it is also most expensive due to the high labor costs. Combining two or more vaccines to be injected simultaneously with one needle reduces labor costs and stress on the fish.

Effective vaccinations have been developed against furunculosis, vibriosis, yersiniosis, and IHN-(Bootland and Leong, 2011) and VHS-viruses (Smail and Snow, 2011). Lately, immersion vaccination against cold water disease has been developed, but more experiences from fish farms testing the vaccine are still needed. Injection vaccination against cold water disease has

been used successfully (Madetoja et al., 2006), but injections cannot be given into tiny rainbow trout fry.

Vaccination against IPN, although commonly used, does not give total protection, and outbreaks occur from time to time (Munro and Midtlyng, 2011).

2.5.2 Bath treatments

Parasite outbreaks are treated commonly with treatment baths. When the causative parasite is recognized, the treatment is planned according to the culture environment. Fish cultured in fiberglass tanks allows cleaning of the tank and treatment with a disinfectant chemical substance, such as formalin for a certain period of time and the infection may be eradicated in few days. However, if parasite infected fish are located in earth ponds or earth floor raceways, the lifecycle of parasite has to be taken into account while planning the treatment. The sediments in the pond or raceway may contain intermediate hosts of the parasite which release new parasites after a certain period of time and hence a mere treatment bath will not stop the infection. Currently formalin (*Ichthyobodo necator*, *Ichthyophthirius multifiliis*) (Rintamäki-Kinnunen and Valtonen, 1997, Rintamäki-Kinnunen et al., 2005) and salt (*Chilodonella*) (Rintamäki-Kinnunen and Valtonen, 1997) are used to treat parasite infections in rainbow trout farms.

Formalin baths are the most common method to decrease mortalities by *Saprolegnia* infections during the egg incubation period (Schreier et al., 1996, Barnes et al., 2000a, Barnes and Soupir, 2007). Formalin is a hazardous chemical, which releases fumes which can irritate the eye and respiratory organs. However, the formalin levels used in aquaculture have not caused occupational hazards during the daily operations inside hatcheries (Lee and Radtke, 1998, Wooster et al., 2005). Formalin necessitates precautionary measures, such as sufficient ventilation and an awareness of the risks when handling the

product. However, environmental concerns related to use of formalin have been expressed (Marking et al., 1994).

Rainbow trout eggs are disinfected after the fertilization to prevent transmission of fish diseases. Iodophor treatment (100mg/l free iodine) has been commonly used (Wagner et al., 2008), but lately it was observed that treatment does not provide 100% protection and alternative methods have been investigated (Wagner et al., 2010, Wagner et al., 2012).

2.5.3 Antibiotics

Antibiotics are used in aquaculture for therapeutic purposes against bacterial pathogens, when outbreaks cannot be prevented by vaccination or outbreaks occur before the fish have reached the size suitable for vaccination. Previously, antibiotics were used as growth promoters and prophylactic prevention of disease, but prohibition of the growth promoter use of antibiotics in European Union (Regulation, EC No, 1831) has led to a decrease in amount of antibiotics used in aquaculture, especially in the European Union (Alderman and Hastings, 1998). However, in 2010 Atlantic salmon production in Chile used an enormous amount of antibiotics compared to Europe (BurrIDGE et al., 2010).

There is a clear evidence that excessive use of antibiotics leads to the development of antibiotic resistant bacterial strains in the fish farm environment (Miranda and Zemelman, 2002a, Miranda and Zemelman, 2002b, Miranda and Rojas, 2007, Hesami et al., 2010, Naviner et al., 2011). Antibiotic resistant pathogen strains reduce the response to antibiotic treatment and may lead to high economic losses in fish farm. Furthermore, increased antibiotic resistance in environmental bacterial microbiota may lead to the transfer of antimicrobial resistance to human pathogens. Hence it is essential that antibiotics are used only when necessary and that diseases should be diagnosed properly so that treatment can be targeted accordingly.

The spectrum of antibiotics used in aquaculture is narrower than in human medicine. Amoxicillin, florfenicol, tribrissen (sulfadiazine: trimethoprin (5:1)), oxolinic acid, flumequin, oxytetracycline and erythromycin are used in salmonid aquaculture (Burrige et al., 2010). However, the use of quinolone type antibiotics (oxolinic acid and flumequin) is prohibited in salmon aquaculture in Scotland, Canada and United States due to the importance of the quinolones in human medicine. In addition, the use of erythromycin is not allowed in salmon aquaculture in Norway, Scotland and Canada, but it is still allowed in Chile. In Finnish aquaculture, only oxytetracycline, tribrissen and florfenicol, are allowed to be used with certain limitations.

2.5.4 Selective breeding

Selective breeding programs are used in aquaculture to enhance some desired trait in the fish by parental selection and the formation of numerous combinations of families. Visceral lipid weight (Kause et al., 2007) fillet percentage, fillet weight and late maturity have been used as selective criteria for rainbow trout (Kause et al., 2003, Kause et al., 2007). The enhanced ability to resist certain diseases is one of the most profitable traits in selective breeding programs. Advances have been made in resistance against CWD (Leeds et al., 2010), ERM, RTFS and VHS (Henryon et al., 2005).

2.5.5 Treatment of inlet water

Ozonation and UV irradiation have been used in rainbow trout aquaculture to decrease the pathogen levels in the inlet water, although due to economical profitability these treatments tend only to be performed mainly in hatcheries and in freshwater recirculation aquaculture farms (Liltved et al., 1995, Summerfelt, 2003, Sharrer et al., 2005, Sharrer and Summerfelt, 2007). In marine water, high doses of ozone would be needed to achieve disinfection and it would cause formation of byproducts toxic to fish (Attramadal et al., 2012). Both treatments can decompose

the organic material present in water (Sharrer and Summerfelt, 2007) and the disinfection effect is strongly influenced by the turbidity of the water (Attramadal et al., 2012, Gullian et al., 2012). Even rather low levels of dissolved ozone have been found to be toxic to fish eggs (Forneris et al., 2003). UV irradiation can be utilized to destroy any dissolved ozone in previously ozone treated water (Sharrer and Summerfelt, 2007).

2.6 PROBIOTICS AND PROTECTIVE CULTURES IN AQUACULTURE

Most of the fish diseases occur when an opportunistic pathogen finds a fish with a decreased immunological status due to malnutrition, stress or parasites. Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO, 2001). Although they are used all around the world in foodstuffs and they are extensively used in homoeothermic animal feeds, the use of probiotics in aquaculture is only now reaching the stage when the first commercial probiotics have entered the markets.

2.6.1 Selective criteria of probiotics

Probiotic bacteria for aquaculture applications have been a topic of intensive research during the last two decades (Kesarcodi-Watson et al., 2008, Wang et al., 2008, Merrifield et al., 2010a, Nayak, 2010). Compared to normal homoeothermic animals, the aquaculture environment poses special demands for probiotic candidates. The water temperature for salmonid species is well below the growth optima of conventional probiotic strains like *Bifidobacterium* and *Lactobacillus*. The gastrointestinal tract of salmonid species is relatively short and there are no anaerobic niches.

The primary selective criteria for probiotics are the lack of any pathogenicity to the host or humans, the lack of acquired

antibiotic resistance and tolerance to bile salts and low pH (Kesarcodi-Watson et al., 2008, Merrifield et al., 2010a). The ability to colonize intestinal epithelium surface, adherence to and growth in intestinal mucus, advantageous growth characteristics, antagonistic properties against fish pathogens, production of extracellular digestive enzymes, production of vitamins, indigenous to host species and environment and good processing capabilities have been mentioned as favorable properties of fish probiotics candidates (Merrifield et al., 2010a). Major benefits through probiotic are expected in larval survival, improved immunological status, increased disease resistance, growth stimulation, enhanced utilization of certain feed components, improved gastric morphology, effects on gut enzyme activities, reduced malformations and better stress tolerance of fish (Sealey et al., 2009, Merrifield et al., 2010a, Nayak, 2010, Lauzon and Ringø 2012).

Utilization of probiotic strains, which have shown an effect on humans, has been used also in aquaculture (Nikoskelainen et al., 2001, Panigrahi et al., 2004, Balcázar et al., 2007a). This approach benefits the fact that the strains have proven safety for end user of fish products (Lauzon and Ringø, 2012). However, their optimal environment is different compared to aquaculture environment.

2.6.2 Protective bacterial cultures in aquaculture

Probiotics are commonly considered as feed additives which are delivered orally. However, in fish farming there is also another possible route to administer beneficial bacteria. Addition of beneficial bacteria to tank water may allow the bacteria to colonize skin mucus, fins and gills of the fish and also to enter the gastrointestinal tract and in hatcheries onto the surface of eggs. Although Merrifield (Merrifield et al., 2010a) suggested that the term probiotics could be used also for microbial cells introduced through water, in this thesis, these terms are distinguished to emphasize the possibilities to use protective bacterial cultures in aquaculture. The utilization of protective

bacterial cultures by bathing the eggs or fish in bacteria supplemented water has rarely been studied (Table 1) unlike the situation for oral supplemented probiotics (Table 2).

Table 1. Potential protective bacterial cultures studied for aquaculture purposes

Protective cultures (species)	Pathogen	References	Targeted life stage
<i>Oncorhynchus mykiss</i>			
<i>Pseudomonas</i> sp M174	<i>Flavobacterium psychrophilum</i>	(Korkea-aho et al., 2011)	fingerling
<i>Pseudomonas</i> sp. M162 and M174, <i>Janthinobacterium</i> sp. M169	<i>Saprolegnia</i> sp.	(Heikkinen et al., 2013, submitted manuscript)	eggs
<i>Pseudomonas</i> sp. MT5	<i>Flavobacterium columnare</i>	(Suomalainen et al., 2005)	fingerling
<i>Pseudomonas</i> spp.	<i>Vibrio anguillarum</i>	(Spanggaard et al. 2001)	fingerling
<i>Pseudomonas fluorescens</i>	<i>Vibrio anguillarum</i>	(Gram et al., 1999)	fingerling
<i>Anguilla australis</i>			
Richardson			
<i>Aeromonas media</i> A199	<i>Saprolegnia</i> sp.	(Lategan et al., 2003)	juvenile
<i>Aeromonas media</i> A199	<i>Saprolegnia</i> sp.	(Lategan et al., 2004a)	adult
<i>Bidyanus bidyanus</i>			
(Mitchell)			
<i>Aeromonas media</i> A199	<i>Saprolegnia</i> sp.	(Lategan et al., 2004b)	adult

Protective bacterial cultures used with salmonid fingerlings have resulted in controversial effects. Smith and Davey (Smith and Davey, 1993) found that pseudomonads which were added to tank water reduced stress-induced furunculosis in salmon. On the other hand, Gram et al. (Gram et al., 2001) found no significant reduction in mortality in cohabitant trial with *A. salmonicida*, despite of the promising, *A. salmonicida*-antagonistic results of *Pseudomonas fluorescens* strain AH2 *in vitro*. However, the same strain showed significant reduction of juvenile rainbow trout mortality by addition of the bacteria to the tank water in an immersion challenge test with *V. anguillarum* (Spanggaard et al., 2001). Gram et al. (Gram et al., 1999) emphasized the importance of the sufficient antagonist bacterial

density in disease treatment. To achieve the inhibitory effect *in vivo*, protective bacteria should be added on a regular basis or it should be able to colonize and multiply on or in the host.

2.6.3 Targeted life stages of fish

Olafsen (Olafsen, 2001) postulated that microbiota in the surrounding water would colonize the surface of the eggs and indigenous microbiota of hatched larva would originate from the egg surface microbiota and surrounding water. The effects of probiotics have been tested on larva (Waché et al., 2006, Sealey et al., 2009), fingerling (Kim and Austin, 2006a, Burbank et al., 2011, Korkea-aho et al., 2011, Merrifield et al., 2011, Korkea-aho et al., 2012) and on-growing rainbow trout (Kim and Austin, 2006b, Capkin and Altinok, 2009). The majority of research has been performed with 5-25 gram fish (initial weight).

2.6.4 Supplementation of probiotics

Probiotic bacteria can be added to fish feed most commonly as live supplements (Irianto and Austin, 2002, Burbank et al., 2011, Panigrahi et al., 2011), but freeze-dried (Panigrahi et al., 2011), dead cells (Irianto and Austin, 2003, Panigrahi et al., 2011), disrupted cells (Brunt and Austin, 2005, Newaj-Fyzul et al., 2007), cell-free supernatants (Brunt and Austin, 2005, Newaj-Fuzyl et al., 2007) or spores (Raida et al., 2003) have also been investigated. Live supplements are commonly sprayed on the diet and coated with oil to minimize any bacterial loss to the surrounding water (Irianto and Austin, 2002). Heat (Panigrahi et al., 2011) and formalin (Irianto and Austin, 2003) treatments have been used to inactivate the probiotics. For species utilizing live feed, rotifers can be used as probiotic delivery vector during the larval stage (Vine et al., 2006).

Table 2. Potential probiotic strains which have been investigated for rainbow trout (*Oncorhynchus mykiss*) aquaculture purposes *in vivo* (Modified Merrifield et al., 2010a).

Potential probiotic	Parameters investigated	References
<i>Pseudomonas</i> sp. M174	DR, IR	(Korkea-aho et al., 2011)
<i>Pseudomonas</i> sp. M162	DR, IR	(Korkea-aho et al., 2012)
<i>Lactobacillus plantarum</i> , <i>Lactococcus lactis</i> ssp. <i>cremoris</i> , <i>Leuconostoc mesenteroides</i>	DR, GE	(Pérez-Sánchez et al., 2011)
<i>Lactobacillus sakei</i> , <i>Lactococcus lactis</i> , <i>Leuconostoc mesenteroides</i>	DR, IR, GM	(Balcázar et al., 2007b)
<i>Lactobacillus rhamnosus</i>	DR, GM, GP	(Vendrell et al., 2008)
<i>Lactobacillus rhamnosus</i>	DR, GP	(Nikoskelainen et al., 2001)
<i>Lactobacillus rhamnosus</i>	GM, IR	(Nikoskelainen et al., 2003)
<i>Lactobacillus rhamnosus</i>	GM, IR	(Panigrahi et al., 2004)
<i>Lactobacillus rhamnosus</i>	GM, IR	(Panigrahi et al., 2005)
<i>Lactobacillus rhamnosus</i> , <i>Bacillus subtilis</i> , <i>Enterococcus faecium</i>	IR, GE	(Panigrahi et al., 2007)
<i>Lactobacillus rhamnosus</i>	IR	(Panigrahi et al., 2010)
<i>Lactobacillus rhamnosus</i>	GE	(Panigrahi et al., 2011)
<i>Enterococcus faecalis</i>	BC, GP, IR, DR	(Rodríguez-Estrada et al., 2009)
<i>Enterobacter amnigenus</i> , <i>Enterobacter</i> sp	DR	(Burbank et al., 2011)
<i>Bacillus subtilis</i>	DR, GM, IR	(Newaj-Fyzul et al., 2007)
<i>Bacillus subtilis</i> , <i>B. licheniformis</i>	DR, IR	(Raida et al., 2003)
<i>Bacillus subtilis</i> , <i>B. licheniformis</i>	BC, FU, GM, GP	(Bagheri et al., 2008)
<i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>Enterococcus faecium</i>	BC, FU, GM, GP, IR	(Merrifield et al., 2010b, Merrifield et al., 2010c)
<i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>Enterococcus faecium</i> , <i>Pediococcus acidilactici</i>	GH, GM	(Merrifield et al., 2010d)
<i>Bacillus</i> spp, <i>Aeromonas sobria</i>	DR, IR	(Brunt et al., 2007)
<i>Bacillus</i> spp, <i>Aeromonas sobria</i>	IR	(Brunt et al., 2008)
<i>Bacillus</i> spp, <i>Aeromonas sobria</i>	IR	(Abbass et al., 2010)

Parameters investigated: BC=Body composition, BE=Brush border enzymes, DR=disease resistance, FU=feed utilization, GH=gut histology, GM=gut microbiota (inclusive probiont colonization), GP=growth performance, IR=immunological/haematological response, SM=skeletal malformation, GE=gene expression.

Table 2 continues	Parameters investigated	References
Potential probiotic		
<i>Pediococcus acidilactici</i>	BC,FU,GM,GP,IR	(Merrifield et al., 2011)
<i>Pediococcus acidilactici</i> , <i>Saccharomyces cerevisiae</i>	BC,FU,GM,GP,SM	(Aubin et al., 2005)
<i>Carnobacterium divergens</i> , <i>Carnobacterium maltaromaticum</i>	IR	(Kim and Austin, 2006a, Kim and Austin, 2006b)
<i>Carnobacterium inhibens</i>	DR, GM	(Robertson et al., 2000)
<i>Aeromonas sobria</i>	DR, IR	(Brunt and Austin, 2005)
<i>Aeromonas sobria</i> , <i>Brochothrix thermosphacta</i>	DR, IR	(Pieters et al., 2008)
<i>Aeromonas sobria</i>	DR, IR	(Arijo et al., 2008)
<i>Aeromonas hydrophila</i> , <i>Vibrio fluvialis</i> , <i>Carnobacterium spp.</i> , <i>Carnobacterium inhibens</i> , <i>V. alginolyticus</i> and an unidentified Gram-positive coccus	DR, IR, GM	(Irianto and Austin, 2002b)
<i>Aeromonas hydrophila</i> , <i>Vibrio fluvialis</i> , <i>Carnobacterium spp.</i> , and an unidentified Gram-positive coccus	DR, IR	(Irianto and Austin, 2003)
<i>Saccharomyces cerevisiae</i> , <i>Debaromyces hansenii</i> , <i>Rhodotorula glutinis</i>	GM	(Andlid et al., 1995)
<i>Saccharomyces cerevisiae</i> , <i>Saccharomyces boulvardii</i>	BE, GM	(Waché et al., 2006)
<i>Saccharomyces cerevisiae</i>	GP	(Barnes and Durben, 2010)
<i>Saccharomyces cerevisiae</i>	GP, DR, IR, GM	(Tukmechi et al., 2011)
<i>Saccharomyces cerevisiae</i> , <i>Enterococcus faecium</i> , <i>Lactobacillus acidophilus</i> , <i>Lb. casei</i> , <i>Lb. plantarum</i> and <i>Lb. brevis</i>	DR, GP, GH, GE	(Sealey et al., 2009)
<i>Kocuria Sm1</i>	DR, IR	(Sharifuzzaman and Austin, 2009)
<i>Kocuria Sm1</i>	DR, IR	(Sharifuzzaman and Austin, 2010a, Sharifuzzaman and Austin, 2010b)
<i>Kocuria Sm1</i> , <i>Rhodococcus SM2</i>	DR, IR	(Sharifuzzaman et al., 2011)
<i>Enterobacter cloacae</i> , <i>Bacillus mojavensis</i>	DR, IR, GP	(Capkin and Altinok, 2009)
Parameters investigated: BC=Body composition, BE=Brush border enzymes, DR=disease resistance, FU=feed utilization, GH=gut histology, GM=gut microbiota (inclusive probiotic colonization), GP=growth performance, IR=immunological/haematological response, SM=skeletal malformation, GE=gene expression.		

Various doses of probiotic supplementation have been tested in several *in vivo* trials (Gram et al., 1999, Sharifuzzaman and Austin, 2010a, Panigrahi et al., 2011). The dose dependency of probiotics to physiological effects in fish is still poorly studied.

Sharifuzzaman and Austin (Sharifuzzaman and Austin, 2009) investigated the effect of duration of probiotic supplementation on immunological parameters of rainbow trout. The highest protection against i.p. challenge with *Vibrio anguillarum* was achieved after a two week period of probiotic feeding and the beneficial effects slightly decreased with time despite of the fact that probiotic feeding was continued.

The first commercial probiotic fish feed, Biomar Inicio Plus, arrived on the market in 2009, utilizing *Pediococcus acidilactici* strain. In 2012 Biomar released another probiotic feed series, LARVIVA Pro. So far, other major fish feed producers, EWOS and Skretting, have concentrated prebiotic applications instead of probiotic supplemented feed.

Although the use of probiotics in aquaculture have shown promising results on several occasions, one should remember that numerous variables, like rearing environment, indigenous microbiota and physiological status of the fish affects to the outcome of probiotic feeding (Merrifield et al., 2010c).

Furthermore, probiotic strains must be administered continuously to fish to maintain the balance between the probiotic strain and indigenous microbiota of the fish and achieve beneficial health effects (Balcázar et al., 2007a).

2.7 THE POTENTIAL OF GENUS *PSEUDOMONAS* AS PROBIOTICS AND PROTECTIVE CULTURES IN AQUACULTURE

The Genus *Pseudomonas* belong to gammaproteobacteria and the 128 *Pseudomonas* species constitutes one of the most diverse genera in the world. Due to the diversity of the genus, those micro-organisms may be found in almost every place on earth from the polar glaciers to deserts, mostly in water, soil and plants (Peix et al., 2009). Pseudomonads are gram negative, catalase positive, aerobic or microaerophilic straight or slightly curved rods, which are usually motile. Most of the *Pseudomonas* species cannot grow below pH 4.5 (Holt et al., 2000), which limits their possibility to enter the gastrointestinal tract. However, pseudomonads have been isolated several times from the intestine of healthy salmonid fish and fish eggs (Spanggaard et al., 2000, Huber et al., Merrifield et al., 2009, Mansfield et al., 2010, Bergmann et al., 2013).

Several *Pseudomonas* species are opportunistic pathogens of plants, animals and also humans. *P. aeruginosa* is a major cause of nosocomial infections all over the world (de Bentzmann and Plésiat, 2011). The extensive use of antibiotics has led to the appearance of antibiotic resistant strains of *P. aeruginosa*, which is also sign of the great adaptability of *Pseudomonas*.

Some *Pseudomonas* species respond to the growth limiting availability of iron by producing low molecular weight iron chelators, siderophores (Cornelis and Matthijs, 2002), which increase the competitiveness of the species in iron deficient environments. This ability has been found effective for inhibiting the growth of bacterial fish pathogens *in vivo*, like *Listonella anguillarum* (Spanggaard et al., 2001).

Some of the *Pseudomonas* strains have been associated with fish diseases. *Pseudomonas putida* (Altinok et al., 2006) has been reported to cause a disease outbreak in rainbow trout fry and *P.*

luteola (Altinok et al., 2007) in juvenile rainbow trout. Strains of *P. anguilliseptica*, *P. chlororaphis*, *P. pseudoalcaligenes* and *P. plecoglossicida* have also been isolated from fish (Hatai et al., 1975, Austin and Stobie, 1992, Nishimori et al., 2000, Ferguson et al., 2004). It is believed that some *P. fluorescens* strains are opportunistic fish pathogens (Roberts and Horne, 1978, von Siebenthal et al., 2009).

3 Objectives

The objective of this study was to screen and identify potential probiotics or protective culture strains to be utilized in the aquaculture environment. Probiotics and protective cultures may provide a method to inhibit losses against the kinds of fish diseases for which there is no vaccine available or protection is otherwise difficult.

Specific aims of the study were:

I) To find an effective hatchery inlet water treatment method against *Saprolegnia* infections on rainbow trout (*Oncorhynchus mykiss*) eggs, which would also support the utilization of protective bacterial strains in the hatchery

II) To find antagonistic *Saprolegnia* sp. bacterial strains, which would be suitable for utilization in the aquaculture environment

III) To clarify the mechanism of action of *Pseudomonas* sp. strains M162 and M174 as probiotic feed supplement

IV) To test the effect of *Pseudomonas* strains as probiotics for juvenile rainbow trout (*O. mykiss*)

4 Materials and methods

4.1 EXPERIMENTAL DESIGN OF THE *IN VIVO* TRIALS

The list and names of the *in vivo* experiments performed in this thesis are presented in Table 3.

Table 3. *In vivo* experiments performed in this thesis

Experiment	Study	Life stage
Incubation trial I	I	egg
Adhesion trial	II	egg
Incubation trial II	II	egg
Challenge trial I	III	juvenile
Challenge trial II	III	juvenile
Challenge trial III	IV	fry
Safety trial I	III	juvenile
Safety trial II	IV	juvenile
Colonization trial I	III	juvenile
Colonization trial II	IV	juvenile
Immunology trial I	III	juvenile

4.2 BIOLOGICAL SAMPLES

4.2.1 Hatcheries and fish farm

Study I *in vivo* trials (Incubation trial I) were performed in the fish research unit hatchery, University of Kuopio, utilizing the Lake Kallavesi water. The adhesion trial in Study II was conducted in the University of Kuopio laboratory and incubation trial II in the Finnish Game and Fisheries Research Institute, Laukaa station hatchery, utilizing Lake Peurunkajärvi basin water.

Studies III and IV were performed in the University of Stirling, Institute of Aquaculture, Aquaculture Research Facility, Scotland.

4.2.2 Fish

Rainbow trout eggs were purchased for studies I and II from Savon Taimen Oy, Rautalampi, Finland. Juvenile rainbow trout used in studies III and IV were purchased from Frandy fish farm, Clackmannanshire, Scotland.

4.2.3 Bacterial strains

Bacterial strains *Pseudomonas* sp. M162, *Pseudomonas* sp. M174 and *Janthinobacterium* sp. M169 were isolated from the surface of the healthy rainbow trout eggs, identified by partial sequencing of 16S rRNA gene as described in study III. Sequences of *Pseudomonas* sp. M162 and M174 and *Janthinobacterium* sp. M169 were positioned to EMBL Nucleotide Sequence Database under accession numbers FN548143, FN548144 and FN548145, respectively.

Flavobacterium psychrophilum strains used in Study IV were ST2/00 (Madetoja et al., 2002), 413 (Dr. Lotta-Riina Sundberg, University of Jyväskylä, Finland) and JIP02/86 (Duchaud et al., 2007).

4.3 ANALYTICAL METHODS

4.3.1 Water analyses (Studies I and II)

The quality of inlet water was monitored from samples taken before the incubation tanks. Water temperature, dissolved oxygen content, pH, conductivity, chemical oxygen demand (COD), formate and acetate concentrations were analysed as described in Studies I and II.

4.3.2 Rainbow trout egg total aerobic bacterial counts (Studies I and II)

Total aerobic bacterial counts of rainbow trout egg surface microbiota were determined with R2A-agar (LAB M, Bury, England) with plates being incubated at 15°C for 7 days.

4.3.3 Microbial diversity analyses of rainbow trout eggs (Studies I and II)

Microbial community structure on rainbow trout eggs was studied from each treatment group by using length heterogeneity analysis of PCR amplified 16S rRNA-genes (Suzuki et al., 1998) as described in study I.

The microbial biodiversity present on rainbow trout eggs was analyzed using automated ribosomal intergenic spacer analysis (ARISA, (Fisher and Triplett, 1999) as described in Study II.

4.3.4 Screening of *Saprolegnia* inhibiting strains (Study II)

A total of 360 bacterial strains isolated from healthy rainbow trout eggs, intestine of healthy rainbow trout fingerlings and rearing tank water were screened for their ability to prevent growth of *Saprolegnia* hyphae by the modified method of Stueland et al. (Stueland et al., 2005b) as described in Study II.

4.3.5 Siderophore production (Studies III and IV)

Siderophore production of *Pseudomonas* sp. strains M162 and M174 was tested with three different methods as described in study III. Siderophores were detected with CAS (Chrome Azurol S) agar (Schwyn and Neilands, 1987) from bacterial suspension and filtered bacterial supernatant. CAS assay solution (Schwyn and Neilands, 1987) was utilized to determine siderophores from sterile filtered supernatants. The presence of siderophores in filter-sterilized supernatants was detected spectroscopically measuring absorbance between 300 and 500 nm.

4.3.6 Antagonism assays *in vitro* (Studies III and IV)

The antagonism activity of *Pseudomonas* sp. strains M162 and M174 was tested against *Flavobacterium psychrophilum* strains

ST2/00 and 413 using automated growth chamber and turbidity reader Bioscreen C (Thermo Labsystems). In the first trial, growth inhibition properties of cell-free supernatant filtrates were tested from probiotics bacteria grown in brain-heart infusion broth medium (BHI). In the second trial, the antagonistic activity of cell-free supernatant filtrates was tested in Fe-deficient and Fe-sufficient M9 medium fortified with casamino acids.

4.3.7 Haematological and immunological analyses (Studies III and IV)

Erythrocyte and leukocyte counts were determined from whole blood using a haemocytometer. Lysozyme activity was analyzed from homogenized intestine sample supernatants (Study III) and serum (Studies III and IV). The respiratory burst activity assay was carried out according to Secombes (Secombes, 1990).

Phagocytosis activity of head kidney macrophages were determined by percentage of yeast engulfed by macrophages as described in Study III.

The superoxide anion (O_2^-) production by head kidney macrophage suspensions was measured via the conversion of nitroblue tetrazolium to formazan, following the method of Secombes (Secombes, 1990).

The level of total IgM in the serum of experimental fish were determined with enzyme-linked immunosorbent assay (ELISA) as described in Study IV. The levels of natural antibodies in the serum were determined using indirect ELISA as described by Kachamakova et al. (Kachamakova et al., 2006). The levels of specific antibodies against *Pseudomonas* M162 were measured using a slight modification of the indirect ELISA method of Kachamakova et al. (Kachamakova et al., 2006), which is described in Study IV.

The serum bacterial killing activity was determined according to Sharifuzzaman and Austin (Sharifuzzaman and Austin, 2010b), by comparing the growth of *F. psychrophilum* strain JIP02/86 in the presence and absence of serum from the experimental fish.

4.3.8 Microbial analysis from gill arch and intestinal contents (Studies III and IV)

The first gill arch on the right side and intestinal contents of M174 fed fish were removed, weighed and transferred into 900µl of 0.02M phosphate buffered saline (PBS, pH 7.2) and placed on ice prior to homogenization. Bacterial counts were determined from TYES and *Pseudomonas* agar incubated for up to 8 days at 15 °C. Representative colonies were selected for Gram-staining and the oxidase test (Oxidase strips, Fluka) and were further analyzed by PCR.

The microbial analysis of M162 fed fish was slightly modified and is described in Study IV.

4.4 RAINBOW TROUT EGG INCUBATION TRIALS

4.4.1 Rainbow trout egg incubation trial I (Study I)

In incubation trial I, inlet hatchery water was treated with 1) ozone + H₂O₂ (theoretical feed 25 mmol/h and 2.5 mmol/h, respectively, in the treated water, theoretical ozone concentration was 8 mg/l and the contact time was 6.8 min.) 2) UV-irradiation (400 mWs/cm²) or 3) UV-irradiation (400 mWs/cm²) + H₂O₂ (5mg/l). Two groups (no treatment and formalin bath 1000ppm, 20 min, three times a week after the visual appearance of *Saprolegnia* hyphae) were used as controls.

Incubation was performed in triplicate in incubation tanks (total volume 9l) containing the incubation tray (Fig. 3). Waterflow was forced to go through tray area only. The eggs (0.1 liter per

tray) were carefully poured onto the incubation tray. Each tank was photographed daily to document *Saprolegnia* infections on the eggs during the incubation. The water temperature was 10 °C during the experiment. Fifteen eggs were taken from each tray with a sterile spoon for microbial analyses at 1, 10 and 20 days after fertilization. Five eggs for microbial community analyses were taken after ten days of incubation. The incubation trial was ended 28 days after fertilization.

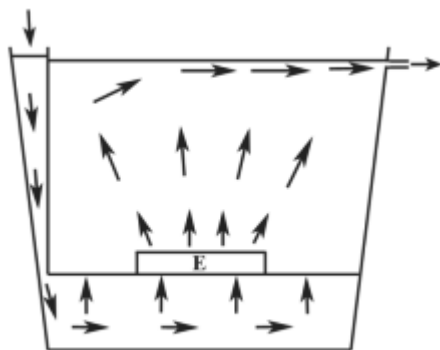


Figure 3. Incubation tank used in Studies I and II. Direction of waterflow is shown with arrows and incubation tray in which eggs were placed is denoted with E.

4.4.2 Adhesion trial and safety of the bacterial strains towards rainbow trout eggs (Study II)

The safety and adhesion properties of ten bacterial strains screened according to *Saprolegnia* inhibition (see Chapter 4.2.4.) were tested *in vivo* with microbial analysis (4.2.2.) and microbial diversity analysis (4.2.3.). Ten selected bacterial strains were tested, and the controls were incubated without any addition of bacteria.

Trial was performed in 60 ml sterile plastic containers at +8 °C with UV-treated Lake Peurunkajärvi basin water with five replicates. Fifty ml of water was measured with a syringe into

containers and 70 fertilized rainbow trout (*O. mykiss*) eggs were carefully placed into container. Then 50 µl bacterial suspension was added into the container to achieve a bacterial level of 10^5 CFU/ml in containers. After 4 days' incubation, 40 ml of water was removed with a syringe and carefully replaced with fresh water. For each treatment, a sterile syringe was used to avoid cross contamination of strains. Eggs were incubated for an additional 2 days after which a total of 35 eggs per container were removed using a sterile spoon for microbial (20 eggs) and automated ribosomal intergenic spacer (ARISA, 15 eggs) analyses. Water was replaced with fresh water as described earlier, and the replacement was repeated after 2 days' incubation. After a total of 11 day's incubation the rest of the eggs in the containers were removed to be subjected to microbial and ARISA analyses. The mortality of the eggs was monitored during the trial to evaluate safety of the bacterial strains on the eggs.

4.4.3 Rainbow trout egg incubation trial II (Study II)

In incubation trial II, the inlet water of five treatment groups (UV400 control, UV400-M162, UV400-M169, UV400-M174 and UV400-IM) was treated with UV-irradiation (400 mWs/cm²) while six treatment groups (UV0 control, UV0-M162, UV0-M169, UV0-M174, UV0-IM and UV0-formalin bath) were incubated with non-treated water.

Incubation was performed with 5 replicates for each treatment in incubation tanks (9 l or 10 l, location of different sized tanks to treatment groups were randomized) in which the incubation tray was placed (Fig 3.). The eggs (0.05 liter per tray) were carefully poured onto the incubation tray. Then water flow was stopped and 500 µl of bacterial suspension were added onto the eggs according to Table 4 to achieve a final protective bacteria density 1×10^5 CFU/ml. After 24 hours, waterflow was turned on. The bacterial addition was repeated similarly after 7 days.

Table 4. Treatment groups (n=5) in rainbow trout incubation trial II

Treatment group	UV treatment of inlet water	Addition of protective bacteria	Added bacteria strain
UV400-control	+	-	-
UV400-M162	+	+	<i>Pseudomonas</i> sp. M162
UV400-M169	+	+	<i>Janthinobacterium</i> sp. M169
UV400-M174	+	+	<i>Pseudomonas</i> sp. M174
UV400-Mix	+	+	Bacterial mixture
UV0-control	-	-	-
UV0-M162	-	+	<i>Pseudomonas</i> sp. M162
UV0-M169	-	+	<i>Janthinobacterium</i> sp. M169
UV0-M174	-	+	<i>Pseudomonas</i> sp. M174
UV0-Mix	-	+	Bacterial mixture
UV0-Formalin bath	-	-	-

The formalin bath (1000 ppm, 20 min) was performed three times a week for UV0-formalin bath group after the visual appearance of *Saprolegnia* hyphae on the eggs on day 12. The incubation trial was ended after 28 day's post fertilization.

4.5 SAFETY OF PROBIOTICS STRAINS FOR FISH (STUDIES III AND IV)

The safety of the probiotic strains *Pseudomonas* M162 and M174 was evaluated by giving a i.p. injection of bacterial suspension (10^7 CFU in 0.1 ml of sterile saline per fish) and then a challenging trial was conducted as described in Study III.

4.6 PROBIOTIC FEED PREPARATION (STUDIES III AND IV)

Pseudomonas M162 and M174 were grown for 2-3 days in tryptone soya broth (TSB; Oxoid) at 22 °C and a suspension in sterile saline was made as described in Study III. The suspension was mixed with fish feed (Skretting, Invergordon, UK), dried and stored at 4 °C. Fresh batches were prepared weekly and

bacterial numbers were detected by plate counting on tryptone soya agar (TSA; Oxoid) incubating 2 days at 22 °C.

4.7 CHALLENGE TRIALS I, II AND III (STUDIES III AND IV)

Two fish trials were conducted to determine the potential activity of M174 against *F. psychrophilum* JIP02/86.

In challenge trial I, 13 rainbow trout per tank were placed in plastic fiber tanks in duplicate. The fish were fed either with commercial diet or M174 supplemented diet ($4 \times 10^5 - 10^6$ CFU g⁻¹, described at chapter 4.7.) for 14 days at 2% fish biomass. Water temperature during the trial was 11-13 °C and fish were acclimated in the tanks for 7 days prior to the supplement feeding.

After two weeks of feeding, all fish were anaesthetized and challenged intramuscularly (i.m.) with *F. psychrophilum* JIP02/86 at 8×10^4 per fish (previously determined as LD₅₀ for this isolate). The challenge experiment was ended 18 days' postinjection.

The relative percentage survival (RPS) was determined as: $RPS = 1 - (M174 \text{ fed mortality } \% / \text{ control mortality } \%) \times 100$ (Amend, 1981).

In challenge trial II, four groups of tanks, 30 fish each, were used in duplicate. Fish were acclimated for 7 days and fed daily with a commercial diet at a rate of 2% of biomass daily. Then fish were fed with M174 supplemented diet ($1-2 \times 10^9$ CFU g⁻¹) or saline supplemented diet (control) for two weeks and challenged with *F. psychrophilum* JIP02/86 (2×10^4 per fish, i.m.). Challenging was continued for another 21 days' postinjection. Water temperature during the experiment was 12-16 °C.

The potential activity of M162 against *F. psychrophilum* JIP02/86 was determined in challenge trial III. The M162 supplemented feed was prepared as described in chapter 4.7.

The rainbow trout fry were divided into duplicate tanks (30 each). The fish were fed with commercial diet (initial weight 2.1g) or M162 (initial weight 1.9g) supplemented feed ($5 \times 10^7 - 2 \times 10^9$ CFU g⁻¹) for two weeks and challenged with *F. psychrophilum* JIP02/86 as described above. The challenge was continued for 21 days postinjection. This challenge trial was repeated twice to verify the results.

In order to estimate the mortalities, kidney and spleen samples were collected from challenged fish and cultured on TYES agar. Representative yellow colonies were collected from plates and confirmed using *F. psychrophilum* specific PCR as described in Study III.

4.8 IMMUNOLOGICAL EFFECTS OF M174 SUPPLEMENTED DIET (STUDY III)

In order to identify possible effect of M174 on the immune system of fish, rainbow trout fingerlings (average weight 12g) were placed in four tanks, 23 fish per tank each. Fish were acclimatized in tanks for 10 days after which the fish in two tanks were fed with M174 supplemented feed ($7 \times 10^7 - 1 \times 10^9$) and in two tanks with saline supplemented feed as controls for two weeks. Blood and head kidney samples for immunological analyses were taken from 10 fish per tank as described in Study III.

The rest of the fish were fasted for 72 hours and 5 fish per tank were analyzed to confirm colonization of intestine with M174.

Samples for evaluation of effects of M162 supplemented feed on the fish immune system were taken from a colonization trial, described below.

4.9 ADHESION OF THE PROBIOTICS TO FISH (STUDIES III AND IV)

Adhesion properties of M174 on rainbow trout intestine and gills were determined with feeding and bathing trials. The feed and bathing suspension was prepared as described in Chapter 4.5.

Rainbow trout (average 8.9g) were divided into 9 tanks each with five fish per tank and acclimatized for 7 days prior to bathing or probiotic feeding. Water temperature during the trial was 8.5-12 °C.

Three tanks were fed with M174 supplemented feed (Table 5), three tanks were fed with control feed and bathed with M174 suspension three times a week and three tanks were fed with control feed and bathed three times a week with saline as the negative control. Bathing was carried out by stopping the waterflow, adding the bacterial suspension into the tank and continuing waterflow after a one hour cessation.

After a feeding and bathing period lasting two weeks, fish were fasted for 24 hours and intestinal and gill samples were taken from 2 fish per tank as described in Chapter 4.2.8. The rest of the fish were fed daily with control feed for another two weeks after which a second set of samples were collected for gill and intestinal microbial analyses.

Table 5. Colonization trial groups used for evaluation of adhesion properties of *Pseudomonas* M174 and M162 and total bacterial counts (TBC) of probiotic feed and bath suspension.

Group	Feed	Feed TBC	Bath	Bath TBC	n	Sampling	Study
Feed	M174	2×10^6 - 3×10^7	-	-	3	Wk 2 & 4	III
Bath	Control	-	M174	8×10^5 - 3×10^6	3	Wk 2 & 4	III
Control	Control	-	Saline	-	3	Wk 2 & 4	III
Feed	M162	5×10^7 - 2×10^9	-	-	3	Wk 2 & 3	IV
Control	Control	-	-	-	3	Wk 2 & 3	IV

The colonization abilities of M162 were evaluated by dividing rainbow trout fingerlings (initial average weight 10 ± 0.21 g) into six tanks containing 18 fish per tank. Three tanks of fish were fed with M162 supplemented diet and other three tanks with control diet for two weeks (Table 5). After two weeks of feeding the probiotic diet, samples were taken of 12 fish per treatment for immunology analyses and 9 fish per treatment for microbial analyses (week 2 sampling). The remaining fish were fed with control diet for one week after which a second set of samples was taken (week 3 sampling).

4.10 STATISTICAL ANALYSES

I

One-way ANOVA test was used to test differences between the treatments and two-sided Dunnett t-test to test differences between the treatment groups against the control group. Differences in the OTU profiles between treatments were analyzed using ANOVA and hierarchical cluster analysis, which was performed to calculate the similarity and squared Euclidian distance for each sample. All statistical analyses were performed with SPSS for Windows, (versions 13.0 and 14.01).

II

The effects of supplementation of protective bacterial culture strains and their combination on mortality of rainbow trout eggs

incubated in UV irradiated water and non-treated water were analyzed with ANOVA and two sided Dunnett t (2-sided) post hoc test to compare treatments to control. Prior to the test, the mortality data of UV0 group was reciprocally transformed to meet the assumptions of ANOVA. The effect of UV irradiation was analyzed with the Mann-Whitney U nonparametric test. The effects of UV irradiation in total aerobic bacterial levels on egg surfaces were analysed with ANOVA and Dunnett t (2-sided) post hoc test to compare treatments to control. The effects of the UV irradiation of inlet water on the number of fragment lengths on egg surface microbiota compared to non-treated water and formalin control were analysed with ANOVA and LSD post-hoc test. The effects of supplementation of protective single strain bacterial cultures on the number of fragment lengths on egg surface microbiota compared to non-supplemented control, formalin control and supplementation of mixture of these three single strains were analysed also with ANOVA and LSD post-hoc test. The beta diversity of microbial communities in the incubation trial was analysed using principal component analysis. Statistical analyses were conducted with IBM SPSS Statistics version 19 and PC-Ord (MJM software).

III

Differences in the bacterial growth curve in the *in vitro* experiment were analyzed using the nonparametric Mann-Whitney test. The growth curve differences of *Pseudomonas* M174 supernatant, grown with or without added iron, were tested with a one-way analysis of variances (ANOVA) with least significant difference (LSD) multiple comparison. Differences between tanks in same treatment in the *in vivo* experiments were analyzed with the χ^2 test, before tanks were combined and treatments compared. Cumulative mortality, bacterial counts in fish gills and intestine between different treatment groups were compared with the nonparametric Mann-Whitney test. Lesion appearance and *Flavobacterium* isolation percentage between different treatment groups were analyzed with χ^2 test.

Differences in haematological and immunological parameters were analyzed with independent samples *t* test. Serum lysozyme and respiratory burst data were Lg transformed to meet the assumptions of the *t* test. All the statistical analyses were performed with SPSS 16.0 for Windows software.

IV

The growth curve differences of *E. psychrophilum* with and without M162 supernatant, siderophore percentage production with and without iron and cumulative mortality comparison were analyzed using the nonparametric Mann – Whitney test. The relative percent survival (RPS) was determined according to Amend (Amend, 1981). The difference in the growth curves with or without added iron were tested with one-way analysis of variance (ANOVA) with a least significant difference (LSD) multiple comparison. The comparisons between M162 fed fish and control fish in immunological and microbiological analyses, as well as serum killing activity, were tested with Student's *t*-test. All the statistical analyses were performed using SPSS 19.0 for Windows software (SPSS Inc., IBM Statistics 19, USA).

5. Results

5.1 WATER ANALYSES (STUDIES I AND II)

The quality of the inlet water in all treatment groups in Study I was uniform during the whole trial with respect of pH, conductivity, temperature, oxygen content and hydrogen peroxide concentration (treatment groups $O_3 + H_2O_2$ and UV + H_2O_2 , Table 6). The ozonized water was supersaturated with oxygen (saturation grade about 240 %). Table 6 shows the values of parameters that represent organic matter (COD_{Mn} , UV-absorbance, acetate, formate) only from the first and the last day of the trial. Actually, these parameters were analyzed two or three times per week, but the means were not calculated because the values were increasing during the whole trial. The increase of the organic matter in the water resulted from the spring turnover of in Lake Kallavesi. COD_{Mn} and UV-absorbance increased about 30 % during the trial. COD_{Mn} could not be analysed from treatments 3 and 5 since H_2O_2 interfered with the analysis. The concentrations of acetate and formate increased also during the trial. $O_3 + H_2O_2$ -treatment decreased the UV-absorbance by 40 %, but UV- and UV + H_2O_2 -treatments did not have any influence on that parameter. $O_3 + H_2O_2$ -treatment produced an almost tenfold increase in acetate and formate concentrations. When water was treated with UV + H_2O_2 , up to fivefold increase in the formate and there was a 30-120% increase in the acetate concentration. UV-treatment did not have any significant effect on the concentrations of acetate or formate in Studies I or II (Table 6 and 7).

Table 6. Physical and chemical characteristics of the inlet water after different treatments. The values of the parameters that represent organic matter (COD_{Min}, UV-absorbance, acetate, formate) are presented from the first and the last day of the trial. The value of untreated water comprises water for control and formalin treated hatching trays (treatments 1 and 2).

Parameter	average ±SD (n)	Untreated water	O₃ + H₂O₂	UV	UV + H₂O₂
pH		6.9 ± 0.1 (12)	6.7 ± 0.1 (12)	6.9 ± 0.1 (12)	6.9 ± 0.1 (12)
Conductivity [µS/cm]		57 ± 1 (12)	59 ± 1 (11)	57 ± 1 (12)	57 ± 1 (12)
Temperature [°C]		10 ± 0.3 (12)	10 ± 0.5 (12)	10 ± 0.4 (12)	10 ± 0.6 (12)
Oxygen content [mg/l]		11 ± 0.4 (4)	27 ± 2.7 (4)	10 ± 0.2 (4)	11 ± 0.6 (4)
H ₂ O ₂ concentration [mg/l]		na.	0.7 ± 0.1 (12)	na.	4.4 ± 0.2 (11)
COD _{Min} (A) [mg/l]		12.7 (1)	na.	11.8 (1)	na.
COD _{Min} (B) [mg/l]		16.4 (1)	na.	15.8 (1)	na.
UV-absorbance (A) [1/cm]		0.44 (1)	0.26 (1)	0.43 (1)	0.43 (1)
UV-absorbance (B) [1/cm]		0.55 (1)	0.33 (1)	0.53 (1)	0.53 (1)
Acetate (A) [µg/l]		26 (1)	240 (1)	32 (1)	57 (1)
Acetate (B) [µg/l]		122 (1)	372 (1)	133 (1)	159 (1)
Formate (A) [µg/l]		36 (1)	366 (1)	39 (1)	111 (1)
Formate (B) [µg/l]		52 (1)	459 (1)	70 (1)	304 (1)

Symbols: COD_{Min} = chemical oxygen demand, na. = not analysed, (A) = measured at the beginning of the trial, (B) = measured at the end of the trial

Table 7. Physical and chemical characteristics of the untreated (UV0) and UV irradiated (UV400) inlet water.

Parameter mean \pm SD (n)	UV0	UV400
pH	7.1 \pm 0.07 (5)	7.1 \pm 0.03 (5)
Conductivity (μ S/cm)	51 \pm 0.3 (5)	51 \pm 0.3 (5)
Temperature ($^{\circ}$ C)	8 \pm 0.5 (5)	8 \pm 0.4 (5)
COD _{Mn} (mg/l)	6.8 \pm 0.1 (5)	6.8 \pm 0.1 (5)
Color (mg PtCo/l)	26 \pm 0.8 (5)	25 \pm 0.5 (5)
UV-absorbance (1/cm)	0.21 \pm 0.001 (5)	0.21 \pm 0.001 (5)
Turbidity (NTU)	0.17 \pm 0.02 (5)	0.16 \pm 0.02 (5)
Acetate (μ g/l)	18 \pm 1 (5)	19 \pm 2 (5)
Formate (μ g/l)	29 \pm 4 (4)	34 \pm 7 (5)
Oxalate (μ g/l)	29 \pm 5 (5)	34 \pm 5 (5)

Symbols: COD_{Mn} = chemical oxygen demand

5.2 EGG SURFACE MICROBIOTA (STUDIES I AND II)

The total aerobic bacteria counts on rainbow trout egg surface increased during the incubation trial (Fig. 4) After 20 days' incubation, the highest counts were found in control eggs and eggs incubated with O₃ + H₂O₂ treated water. As expected, formalin baths decreased bacterial counts on the egg surfaces. However, formalin baths slightly increased the number of OTU's, compared to control (Fig. 5). All three inlet water treatments instead decreased the number of OTU's.

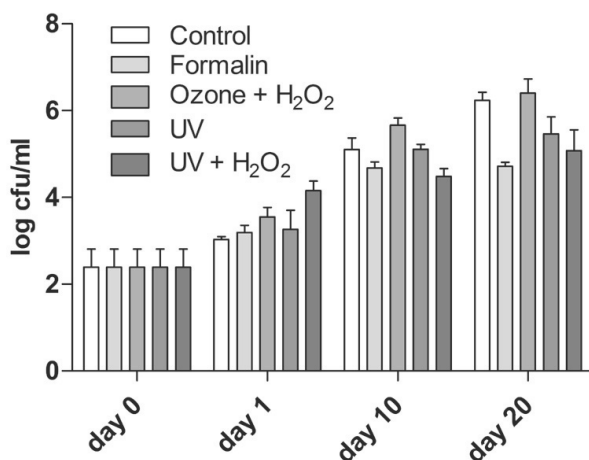


Figure 4. Total aerobic bacterial counts of surface of rainbow trout eggs during the incubation trial (mean+ SD, n=3).

Hierarchical cluster analysis revealed that egg-associated bacteria at UV-irradiation and H₂O₂ treated group significantly differed from other treatments (Fig. 6) Furthermore, all replicates from the O₃ + H₂O₂ treatment were located in same branch.

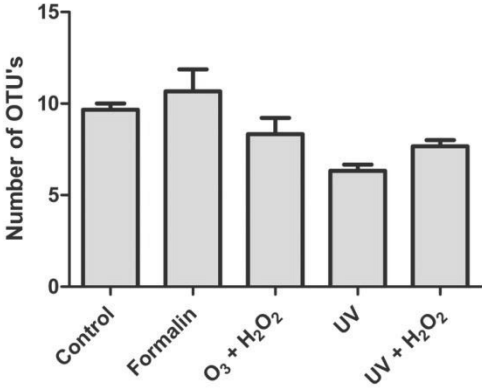


Figure 5. Average number (mean± S.E., n=3) of operational taxonomical units (OTU's) in surface microbiota of fish eggs detected by LH-PCR.

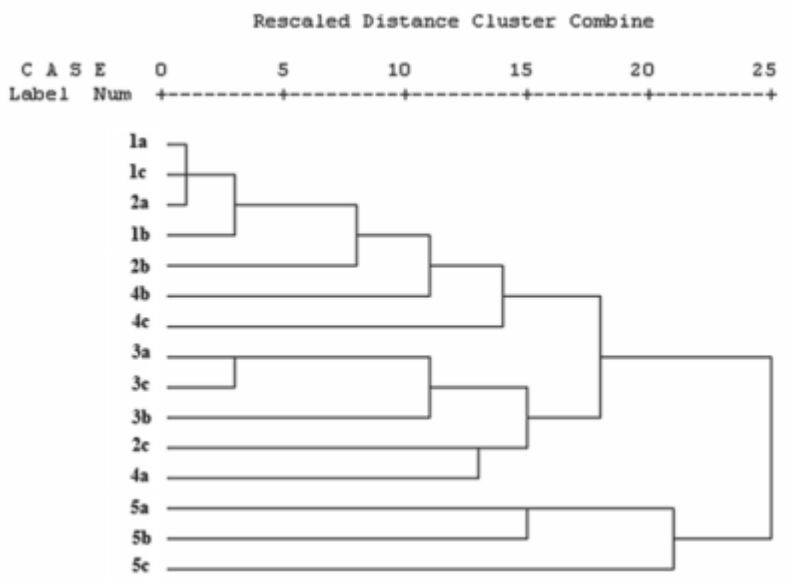


Figure 6. Hierarchical cluster analysis of the surface microbiota on fish eggs as detected by LH-PCR. Treatments 1-5 are 1 = control, 2 = formalin bath, 3 = O₃ + H₂O₂, 4 = UV irradiation, 5 = UV irradiation + H₂O₂, a, b and c indicate replicates from each treatments.

In Study II, total aerobic bacteria levels on the eggs after placing the eggs on trays were 2.54 ± 0.23 log CFU/ml. After two weeks' incubation, significantly higher total bacterial counts were found in UV400 groups than in the UV0 groups (7.27 ± 0.15 log CFU/ml vs 6.7 ± 0.26 log CFU/ml, ANOVA, $p = 0.000$). After four weeks' incubation, *Saprolegnia* infections were so advanced in UV0 groups that microbial samples could not be taken. In UV400 groups slight decrease in total bacteria counts was found as compared to results after two weeks' incubation. In the M169 (6.66 ± 0.2 log CFU/ml) and inoculation mixture (6.61 ± 0.2) log CFU/ml) supplemented groups, the total bacterial counts on eggs were lower than in the control group (7.13 ± 0.25 log CFU/ml, (ANOVA, Dunnett t (2-sided), $p = 0.016$; $p = 0.007$, respectively).

In the ARISA analysis (Fig. 7), PCR products were classified as length categories and effects of inlet water UV irradiation and protective culture supplementation on number of fragment lengths were analysed. The number of observed peak sizes varied between 12 and 50 per sample. After two week's incubation, the UV400 groups had significantly fewer detectable fragment lengths than eggs in the UV0 groups (21.7 ± 7.1 vs 26.6 ± 8.2 , $p=0.034$). Inoculation with protective bacterial cultures significantly decreased the number of detected fragment lengths ($p=0.04$), but instead formalin treatment increased the number of fragment sizes.

After two weeks' incubation, biomarkers of strains M162 and M169 were below the detection limit (0.5% proportion of total peak area) and strain M174 could not be confirmed since there was a peak of the same size also in the control units which had received no bacterial supplementation. Principle component analysis (data not shown) revealed that the bacterial diversity in the UV400 group was more scattered than in UV0 groups including formalin treated units. Two PCA components, UV-

irradiation of inlet water and supplementation of protective bacterial cultures, explained 27% of the total variance.

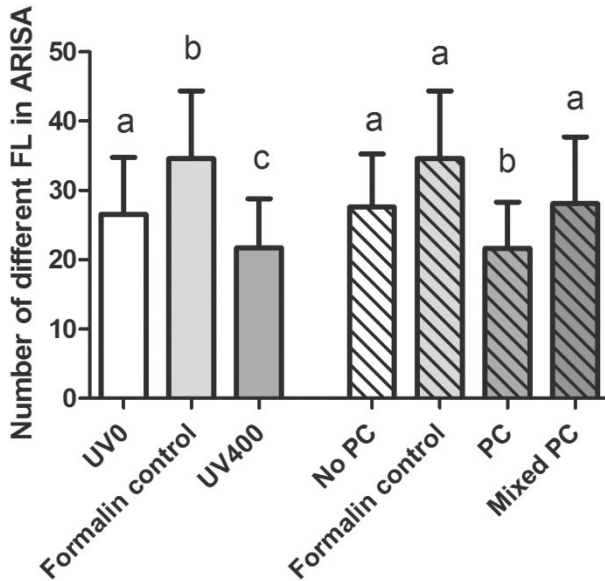


Figure 7. Number of different fragment lengths (FL) in ARISA analysis on rainbow trout egg surface hatched with non-UV treated (UV0) or UV-treated (UV400) water or formalin bathed controls, without supplemented protective cultures (No PC), single protective culture strain (PC: *Pseudomonas* sp. M162, *Pseudomonas* sp. M174 or *Janthinobacterium* sp. M169) or combination of these three strains (Mixed PC). Different letters on top of the bars denote a significant difference ($p < 0.05$) between the treatments.

5.3 EGG MORTALITIES (STUDIES I AND II)

Treatment of inlet water with high dose (400 mWs/cm²) of UV irradiation (mean mortality 14.3%) significantly reduced mortality of rainbow trout eggs during the incubation compared to control (mean mortality 77,3%) in Study I (ANOVA, $p = 0.011$, Fig. 8). Formalin baths and inlet water treatment with either O₃ + H₂O₂ or UV + H₂O₂, decreased mortality compared to control, although the result was not statistically significant. UV-

irradiation of inlet water was capable of delaying the appearance of *Saprolegnia* infected rainbow trout eggs (Fig. 9).

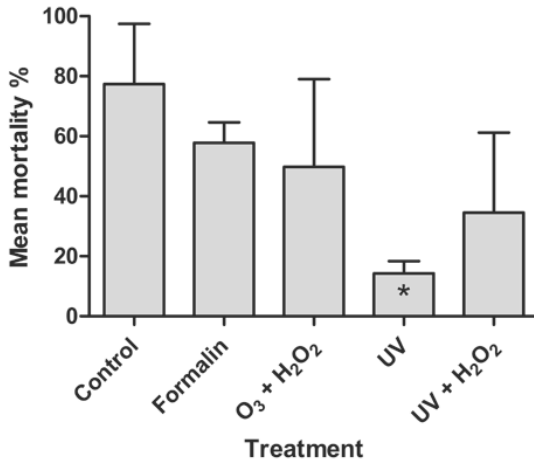


Figure 8. Mortality percentage of rainbow trout eggs in incubation trial represented as (mean+ SD, n=3). Significant difference is presented with * (p<0.05).

Addition of protective cultures on eggs did not increase the mortality of eggs in the adhesion trial. The total mortality in adhesion trial was 0-1.4%.

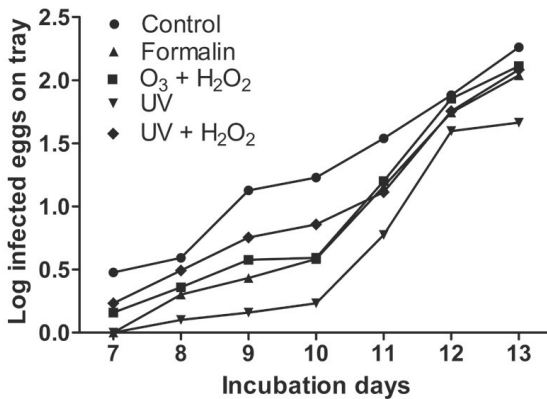


Figure 9. Logarithmic cumulative appearance of infected eggs on hatching trays with the different treatments (n=3) during the incubation days 7-13

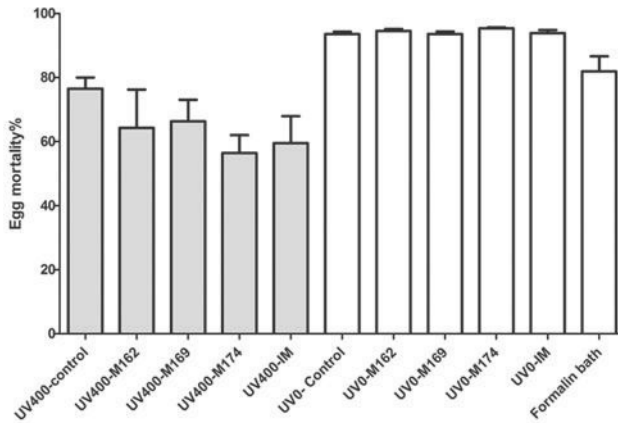


Figure 10. Mortality percentage of rainbow trout eggs in incubation trial. Values are presented as means (n=5) \pm SD. Groups denoted as UV400 were incubated with UV-treated (400 mWs/cm²) inlet water and groups denoted as UV0 with non-treated incubation water. Groups marked with M162, M169 or M174 were supplemented with bacterial suspension, groups referred to as IM were supplemented with an equal mixture of M162, M169 and M174. Formalin bath group was treated with formalin bath 1000 ppm, 20 minutes, three times a week, after the first *Saprolegnia* hyphae became visually detected.

UV-irradiation (400 mWs/cm²) of inlet water significantly decreased mortality (t-test, $p = 0.000$) of rainbow trout eggs during the incubation also in Study II (Fig. 10) Addition of bacterial cultures *Pseudomonas* sp. M162, *Janthinobacterium* sp. M169, *Pseudomonas* sp. M174 and their mixture on egg jars increased egg survival in the UV400 group, but the addition did not have any effect in the UV0 group. The lowest egg mortality was found in M174 supplemented UV400 group (mean egg mortality 56.4%). Formalin baths increased significantly survival in the UV0 group (ANOVA, $p = 0.001$).

Eggs in all experiment trays were affected by *Saprolegnia* infection during the trial. *Saprolegnia* hyphae appeared

approximately 2 days earlier in the UV0-groups compared to the UV400 groups.

5.4 IMMUNOLOGICAL EFFECTS AND COLONIZATION (STUDIES III AND IV)

5.4.1 Siderophore production of *Pseudomonas* sp. M162 and M174

While cultured in CAS agar plates, M162 produced strong orange halos regardless of the iron concentration used in the medium or on the days grown.

Strain M174 produced strong colour change during the first 2 days of incubation and orange halos were produced on the CAS agar with 0, 10 and 100 μmol added iron concentrations. Orange halos around the wells were found from cell-free supernatant samples, when M174 had been grown for 5 or 7 days under iron-deficient conditions

Siderophore production of M174 was clearly detected from cell-free supernatant filtrates made from 3, 5 and 7 days grown cultures in iron-limited M9C-medium (Fig. 11). When M174 was cultured in M9C medium, supplemented with 10 μmol iron, no siderophores were detected.

Siderophore production of M162 was detected in CAS liquid assay at levels of 82.6% on days three, five and seven and the values differ from cultures supplemented with 10 μmol iron (Mann-Whitney $p \leq 0.05$, Fig. 12.).

A clear absorbance peaks was detected around 380 nm for M174 (Fig. 13) and at 384nm for M162.

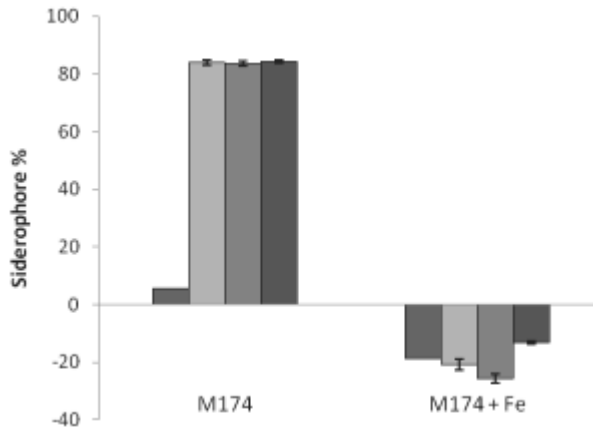


Figure 11. Siderophore percentage (average \pm SD) in Chrome azurol S liquid assay when *Pseudomonas* M174 was grown in the presence of 10 μ mol Fe or without added iron for one (■), three (▒), five (■) and seven (■) days using cell-free supernatants.

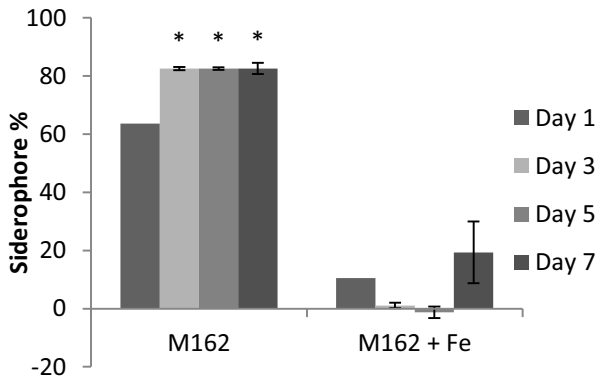


Figure 12. Siderophore percentage (average \pm SD) of *Pseudomonas* M162 sterile filtered supernatants, when grown in iron sufficient (M162 + Fe) or iron deficient (M162) media. Supernatants collected from cultures grown 1, 3, 5 and 7 days. *Statistically significantly different siderophore percentage from the same day iron sufficient grown supernatant (Mann-Whitney, $P \leq 0.05$).

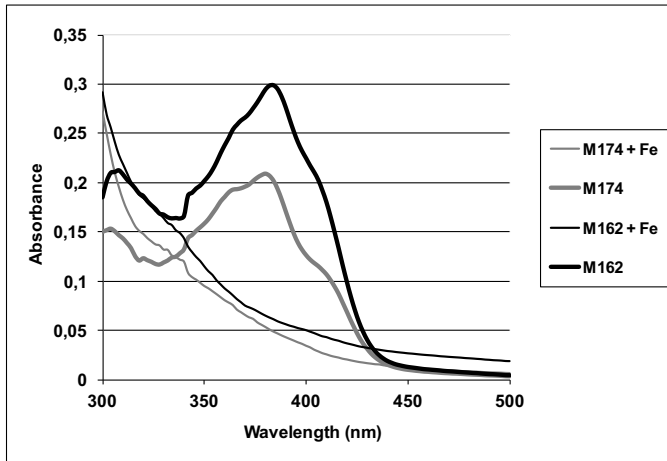


Figure 13. The absorbance spectra of the *Pseudomonas* M174 and M162 supernatants from seven day old cultures grown with 10 μmol Fe or without added iron.

5.4.2 Antagonistic activity against *Flavobacterium psychrophilum* *in vitro*

When *Pseudomonas* M174 was grown in BHI medium, the supernatant was inhibitory to *F. psychrophilum* strain 413 (Mann-Whitney, $p = 0.024$, Fig. 14a). The growth of *F. psychrophilum* strain ST2/00 was also reduced, but the inhibitory effect was not statistically different as compared to the growth of pathogen strains without M174 supernatant (Mann-Whitney, $p = 0.233$, Fig. 14b). The supernatant of M174, when grown in iron depleted medium, inhibited growth of both *F. psychrophilum* 413 (ANOVA $p = 0.022$, Fig. 14c) and ST2/00 (ANOVA, $p = 0.02$, Fig. 14d). This inhibition was not detectable in the presence of 10 μmol Fe (Fig. 14 c-d).

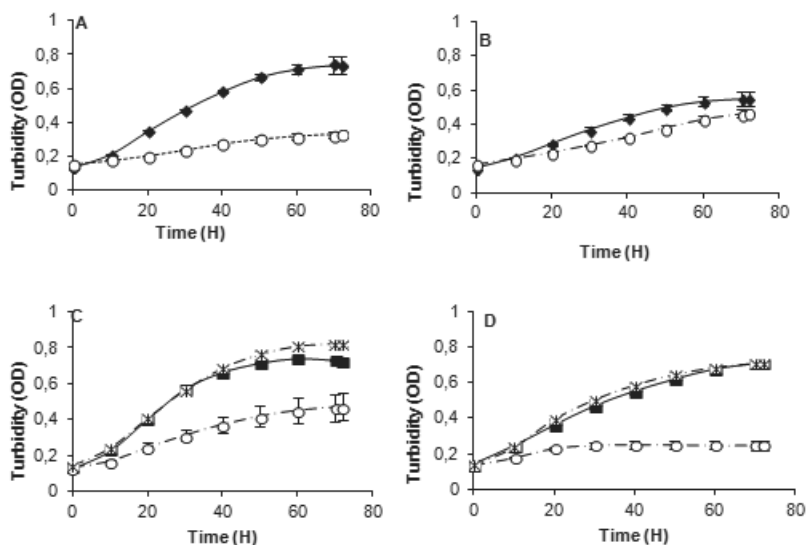


Figure 14. Growth (\pm SD) of (A) *Flavobacterium psychrophilum* 413 (—●—) and (B) *F. psychrophilum* ST2/00 (—●—) with the *Pseudomonas* M174 (-○-) supernatant. Supernatants were from the protective bacteria cultures grown in BHI broth. Growth (\pm SD) of (C) *F. psychrophilum* 413 (—■—), with M174 supernatant grown in Fe deficient M9C media (-○-) or with M174 supernatant grown in Fe sufficient M9C media (-*-) and (D) *F. psychrophilum* ST2/00 (—■—), with M174 supernatant grown in Fe deficient M9C media (-○-) or with M174 supernatant grown in Fe sufficient M9C media (-*-).

5.4.3 Antibodies in serum

The total IgM content of serum of M162 fed fish (OD450 1.34 ± 0.195) was higher after three weeks' feeding compared to control group (OD450 1.16 ± 0.23 , t-test, $p = 0.045$, Fig. 15a). No differences were found in the levels of specific antibodies against M162 between the groups (Fig. 15b) either at week 2 (t-test, $p = 0.093$) or week 3 (t-test, $p = 0.251$) or in the natural antibodies on week 2 or 3 (t-test, $p = 0.338$ and $p = 0.227$, respectively, Fig. 15c).

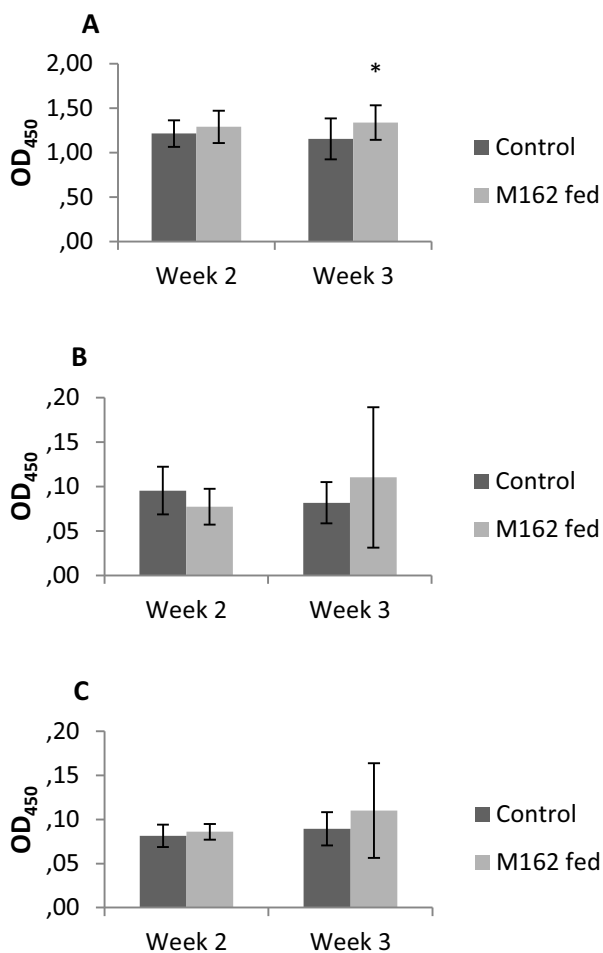


Figure 15. Total antibody IgM (A), M162 specific antibody (B) and natural anti-BSA antibody (C) activity of fish fed with M162 supplemented feed and control feed. (average \pm SD). Samples taken on week 2 and week 3. *Statistically significantly different with respect to the control.

Table 8. Haematological and innate immunity parameters (average \pm SD) in control and M174 probiotic treatment groups (n = number of fish used) (Study III) and in control and M162 probiotic treatment groups (Study IV).

Treatment	Erythrocytes ($1 \times 10^8 \text{ ml}^{-1}$)	Leucocytes ($1 \times 10^7 \text{ ml}^{-1}$)	Respiratory Burst Activity [†]	Phagocytic Activity (%)	Lysozyme Activity (U-1 min-1 ml-1)	
					Plasma	Intestine
M174	10.6 \pm 3.3 (n = 17)	9.99 \pm 3.9 (n = 20)	0.21 \pm 0.09* (n = 18)	23.2 \pm 11.3 (n = 13)	1703 \pm 775 (n = 18)	2481 \pm 871 (n = 19)
Control	12.2 \pm 3.7 (n = 17)	9.8 \pm 2.7 (n = 19)	0.15 \pm 0.08 (n = 17)	29.4 \pm 14.3 (n = 10)	1476 \pm 528 (n = 16)	2518 \pm 1055 (n = 20)
Week 2						
M162	10.6 \pm 2.4 (n=12)	4.7 \pm 0.97* (n=12)	0.7 \pm 0.49 (n=5)	22.4 \pm 8.9 (n=10)	119.5 \pm 61.2 (n=12)	-
Control	9.2 \pm 1.99 (n=10)	5.7 \pm 0.97 (n=10)	0.4 \pm 0.3 (n=10)	20.8 \pm 10.9 (n=11)	172.2 \pm 171.7 (n=9)	-
Week 3						
M162	9.5 \pm 1.3 (n=12)	7.6 \pm 2.6* (n=12)	0.03 \pm 0.001 (n=12)	12.6 \pm 3.7 (n=11)	893.3 \pm 622.7* (n=12)	-
Control	10.3 \pm 1.2 (n=12)	5.9 \pm 0.9 (n=12)	0.03 \pm 0.006 (n=12)	17.7 \pm 9.4 (n=11)	395.8 \pm 332.9 (n=12)	-

*statistically different from control (ANOVA, p < 0.05) [†]Respiratory Burst Activity (OD₆₃₀ adjusted to 10⁵ cells)

5.4.4 Haematological analyses

Respiratory burst activity was significantly higher with fish fed with M174 for two weeks (0.21 ± 0.09) as compared to control group (0.15 ± 0.08 , t-test, $p = 0.049$, Table 8). No differences were found in erythrocyte and leukocyte numbers, phagocytic or lysozyme activity between the M174 fed fish and the control group after two weeks' feeding.

Significant difference in leukocyte numbers was detected after two weeks' ($p = 0.032$, t-test) feeding with M162 (Table 8). One week after probiotic feed withdrawal, leukocyte counts were still higher in the probiotic group (t-test, $p = 0.05$) when compared to the control group (Table 8). No differences were found in erythrocyte counts between M162 fed fish and control group either after two (t-test, $p = 0.173$) or three weeks from the beginning of the trial (t-test, $p = 0.111$).

After two weeks' feeding the fish with M162, no significant difference in lysozyme activity could be detected between probiotic and control groups (t-test, $p = 0.4$). However, one week after withdrawing the probiotic feed, serum lysozyme activity in probiotic group was significantly higher than in control group (t-test, $p = 0.026$).

No statistical difference was found in phagocytic activity or respiratory burst activity of head kidney leukocytes between M162 fed fish and control group (Table 8).

There was no significant difference in the per cent survival of *F. psychrophilum* in the serum killing assay between the probiotic group and the control group on week 2 (t-test, $p = 0.441$) or week 3 (t-test, $p = 0.437$) (Fig. 16).

5.4.5 Safety of the probiotic bacteria

No mortalities or signs of the disease were detected in the control group or in fish injected i.p. with *Pseudomonas* M162 or M174.

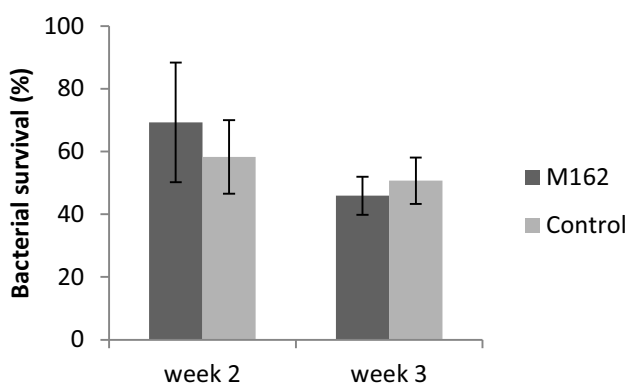


Figure 16. Percentage survival of *F. psychrophilum* JIP02/86 after incubation with serum from fish fed with M162 supplemented feed (■) and control feed (□). Serum was taken on week 2 and week 3 of the experiment. Data are expressed as mean \pm SD.

5.4.6 Colonization of *Pseudomonas* M162 and M174

After two weeks of feeding or bathing with *Pseudomonas* M174, viable M174 identified bacteria were found in the gills of M174 supplemented diet fed fish (Fig. 17a). This significantly differed from the control (Mann-Whitney, $p = 0.022$). From intestinal samples, viable M174 cells were found in all treatment groups. The average *Pseudomonas* counts were highest in the intestine of M174 fed fish (1.9×10^4 CFU per 0.1g of intestinal content) but only negligible in the control group (15.2 CFU per 0.1g), with the difference between the groups being significant (Mann-Whitney, $p = 0.02$, Fig. 17b). Viable *Pseudomonas* cells were found also from M174 bathed group (average 4.2×10^3 CFU per 0.1g), but the difference compared to the control group was not statistically significant (Mann-Whitney, $p = 0.674$).

Two weeks after the cessation of M174 supplementation and bathing, only one viable M174 colony was found in the intestine of control groups and none in the gills or intestine of treated groups.

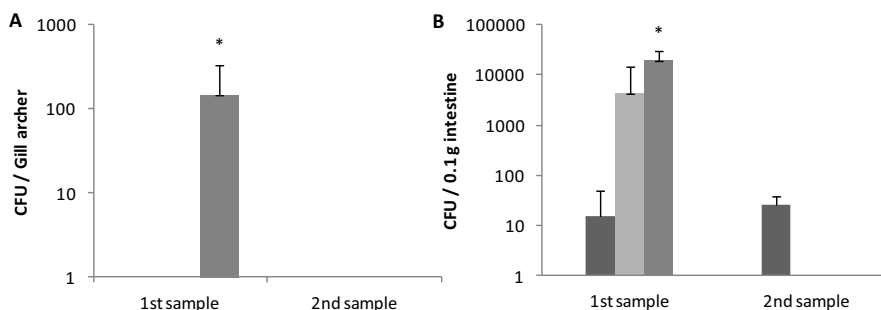


Figure 17. Number of *Pseudomonas* M174 colonies (\pm SD) in fish gill arch (A) and intestine content (B) in samples taken at two different time points, after (■) control feeding and bathing, (■) M174 bathing and (■) M174 feeding. (*group is statistically different from control group, Mann-Whitney test, $p < 0.05$).

After two weeks of feeding M162 supplemented diet, the average numbers of M162 in the intestine of the probiotic-fed group were 1.2×10^4 CFU g^{-1} of intestine content which was equivalent of 23.7% of the total colony count (Table 9). The replacement of M162 supplemented diet with control diet for 1 week decreased the proportion of M162 in the total intestinal bacteria to 0.8% in probiotic-fed group. While the levels of *Pseudomonas* M162 were significantly higher in the M162 fed group compared with the control group (t-test, $p = 0.039$, Table 9) after two weeks, there was no significant difference in the amount of M162 present in the intestine content between M162 fed fish and the control group at week 3 (t-test, $p = 0.349$, Table 9).

5.4.7 Challenge experiments

Feeding the rainbow trout fry with *Pseudomonas* M174 supplemented feed decreased the cumulative mortality in the challenge experiments (Fig. 18 a-b). In challenge trial I, data

were analyzed from only two single tanks and data from replicate tanks were excluded as the challenge was not successful in these tanks. Cumulative mortality in control group was 45% which was statistically different from the M174 fed fish where mortality was 23 % (Mann-Whitney, $p = 0.000$, Fig. 18a). The RPS value in first challenge experiment was 49.1% (Table 10).

Table 9. Number of fish, total colony count and M162 colony count (\pm SD) on *Pseudomonas* specific agar of intestine samples taken on weeks 2 and 3
*Statistically significantly different from control.

Treatment	No. of fish isolated with M162 / Total fish	Total colonies CFU/g	M162 colony count	Ratio (M162 colonies / total colonies)
<u>Week 2</u>		<u>CFU/0.04g</u>		
Probiotic	5/9	$4.9 \pm 10 \times 10^4$	$1.2 \pm 1.5 \times 10^4$ *	23.7%
Control	1/9	$2.6 \pm 7.7 \times 10^6$	222 \pm 667	0 %
<u>Week 3</u>		<u>CFU/0.08g</u>		
Probiotic	1/9	$2.6 \pm 6.7 \times 10^6$	$2 \pm 6 \times 10^4$	0.8%
Control	1/9	$1.3 \pm 2.6 \times 10^7$	111 \pm 333	0 %

A similar end result was achieved in the second challenge experiment, in which data from replicated tanks were combined in the mortality analysis (chi-square test between M174 fed fish tanks $p=0.445$ and control $p= 0.713$). Cumulative mortality in control groups was 57% compared to 41% in the M174 fed fish (Mann-Whitney, $p=0.047$, Fig. 18b). The RPS value was 28.1% in second challenge experiment (Table 10).

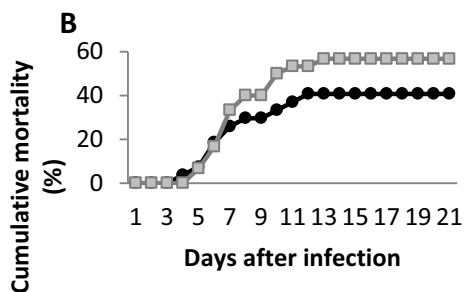
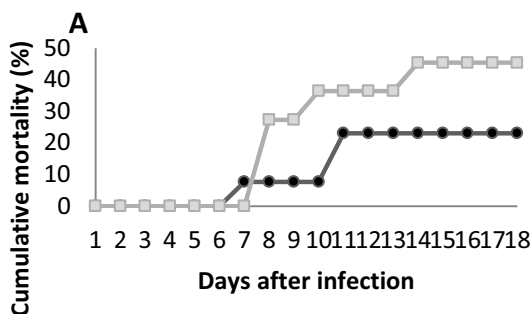


Figure 18. Cumulative mortality percentage of control (■) and M174 fed (●) rainbow trout fry after intramuscular challenge with *Flavobacterium psychrophilum* in experiment 1 (A) and experiment 2 (B).

Table 10. Relative survival (RPS), number of fish used, average initial and after 14 days of feeding weight, lesion percent and *Flavobacterium psychrophilum* isolation percent between M174 fed and control fish after being challenged with *F. psychrophilum* in two different experiments.

Experiment	Dietary group	% RPS	Number of fish	Average initial weight (g)	Average weight after 14 days (g)	% fish with lesions	% fish with <i>F. psychrophilum</i> isolated
1	M174	49,1	13	3,1	4,1	38,5	23,1*
	Control		11	3,5	5,1	45,5	63,6
2	M174	28,1	27	2	3,4	44,4	48,1
	Control		30	2,1	3,5	60	56,7

*Statistically different from control (χ^2 , $p < 0.05$)

Cumulative mortality at the end of the challenge experiment in fish fed with *Pseudomonas* M162 was 35%, which significantly differed from the control group, where mortality was 57% (Mann-Whitney, $p = 0.005$, Fig. 19). The RPS value of experimental infection was 39.2%.

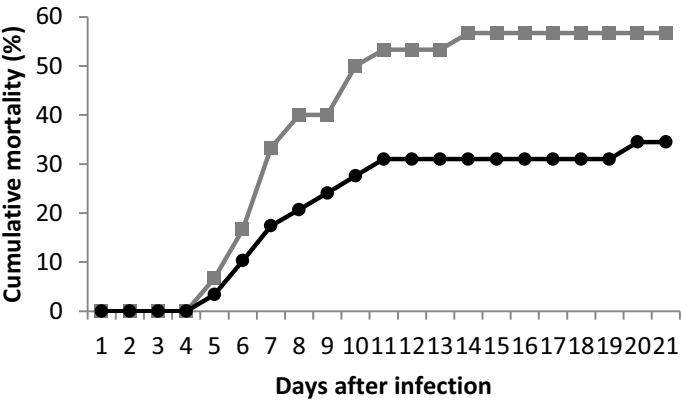


Figure 19. Cumulative mortality of fish fed two weeks with *Pseudomonas* M162 supplemented feed (●) or control feed (■) then infected with an intramuscular injection of *F. psychrophilum* JIP02/86. Statistically significantly different mortality between groups (Mann–Whitney, $P = 0.005$)

6 Discussion

6.1 EGG INCUBATION

Members of the Oomycete species *Saprolegnia* are ubiquitous around the world in freshwater lakes and rivers (Van West, 2006), and are responsible for significant economic losses around the world in aquaculture (Hussein and Hatai, 2002). Utilization of inlet water disinfection methods have been postulated as a possible solution to saprolegniosis in fish eggs (Rahkonen and Koski, 2002).

Forneris and his co-workers (Forneris et al., 2003) found that 0.1 ppm doses of daily ozone treatment increased the hatching rate in brown trout (*Salmo trutta*), but 0.3 ppm every second day seemed to be above the toxicological threshold. This suggests that there is a very narrow range of doses which are simultaneously effective against *Saprolegnia* and safe for salmonid eggs. In Study I, treatment of inlet water with $O^3 + H_2O_2$ was engineered so that no soluble ozone would reach the eggs during the incubation. Mortality in this treatment group was 49.8%, which is considerably higher than that described by Forneris (Forneris et al., 2003).

In advanced oxidative processes (AOP), strong oxidative hydroxyl radicals ($OH\cdot$) are formed, which readily react with any organic matter in water. Production of hydroxyl radicals in water is commonly produced by the combination from two of the following processes, ozonation, UV-irradiation or hydrogen peroxide (Lamsal et al., 2011, Jin et al., 2011). AOP methods have not been used previously in *Saprolegnia* prevention, but they have been extensively studied in drinking water technology (Shiina et al., 2006, Lamsal et al., 2011), wastewater management

and their potential in recirculation aquaculture has been evaluated recently (Sharrer and Summerfelt, 2007).

Treatment of inlet water with $O^3 + H_2O_2$ caused a tenfold increase in acetate levels and a fivefold increase in formate levels. The rapid increase of these low molecular weight organic acid levels by ozonation of lake water has been described by Hammes and co-workers (Hammes et al., 2006, Hammes et al., 2007). In that study, the formate levels produced due to ozonation of lake water were also higher than the acetate levels.

Increased acetate and formate levels in inlet water may serve as a growth substrate (Gerstmeir et al., 2003) for certain microbes during incubation of eggs. Indeed, the highest total bacteria counts were found on eggs incubating in the $O^3 + H_2O_2$ treated water. High bacterial counts on the egg surfaces may have a role in egg mortality through oxygen deprivation (Barker et al., 1989), but no correlation has been found between cultivable bacterial numbers on eggs and egg mortality (Barker et al., 1990, Barker et al., 1991). However, while only a proportion of bacteria on salmonid eggs are cultivable (Romero and Navarrete, 2006), a significant correlation between bacterial density on eggs as determined by scanning electron microscopy and egg mortality has been found (Stephenson et al., 2003, Barnes et al., 2005). An increase in the bacterial counts is related to the decrease in the numbers of species present on eggs (Barker et al., 1989), which is in agreement with the present results in the group incubated with $O^3 + H_2O_2$ treated water. However, in both UV and UV+ H_2O_2 treated groups total aerobic bacterial counts and the number of OTU's, which describe relative numbers of species biodiversity, were smaller than in control.

Although the results of the ARISA analysis, which was used in study II to evaluate bacterial biodiversity on egg surfaces, cannot be directly compared to those obtained with the LH-PCR method results used in study I, some similar trends can be seen.

Formalin treatment of eggs increased the number of fragment lengths in the ARISA analysis in study II and a similar effect on number of OTU's could be seen in Study I. UV-irradiation of inlet water instead decreased the number of fragment lengths in Study II and reduced the number of OTU's in study I. ARISA and LH-PCR analyses have been used for fingerprinting molecular diversity of fish pathogenic strains (Suomalainen et al., 2006) and to resolve the bacterial community in aquaculture environment (Arias et al., 2006), but not in salmonid eggs.

In studies I and II, the formalin treatments were started after the first signs of fungal infestation. No prophylactic formalin treatment was applied, because in 2005-2006 (the time of studies I and II) this practice was not encouraged by the National Veterinary and Food Research Institute (EELA, from May 2006 onwards Finnish Food Safety Authority EVIRA) and the FGFRI (Finnish Game and Fisheries Research Institute) complied with this recommendation.

UV-irradiation did not affect the physical or chemical parameters of inlet water as compared to control in either incubation trials I or II, although the turbidities of the water used in the studies were different. Compared to ozonation, it seems that UV-irradiation does not produce growth substrates for bacteria, at least to the same extent. The low-pressure vapour lamps, used in the study to obtain UV irradiation, produce almost no by-products and do not affect the biological stability of the water (Hijnen et al., 2006).

Hierarchical cluster analysis of the surface microbiota of rainbow trout eggs showed that inlet water treatment with UV + H₂O₂ seemed to modify egg surface microbiota so that it was divergent from the other groups. Furthermore, O₃ + H₂O₂ created its own branch in the hierarchical cluster analysis, which may suggest that hydrogen peroxide or AOP have modifying role on egg surface microbiota, most probably due to the increase in the formate and acetate levels.

A progressive increase in aerobic bacterial counts was seen both in controls and all treatment groups in Study I, even though treatments of inlet water were effective enough to significantly reduce the aquatic bacterial load. The finding was in accordance with Barker et al. (Barker et al., 1989), and Barnes (Barnes et al., 1999, Barnes et al., 2000b) who observed a similar progressive increase in the bacterial counts on salmonid eggs during the incubation. This may be explained by the increase in the numbers of dead eggs on the trays, which release nutrients to further increase the bacterial growth (Barnes et al., 1999).

UV-irradiation of inlet water decreased the mortality of rainbow trout eggs significantly as compared to control. This seems to be the very first study to report a decline in mortality due to *Saprolegnia* infections in salmonid eggs after UV- irradiation of inlet water. UV-irradiation has been used and extensively studied in aquaculture (Summerfelt et al., 2004, Sharrer et al., 2005, Sharrer and Summerfelt, 2007, Summerfelt et al., 2009, Mamane et al., 2010, Attramadal et al., 2012, Gullian et al., 2012, Cobcroft and Battaglene, 2013), but its effects in the prevention of *Saprolegnia* infections have been poorly studied. Furthermore, the UV-irradiation doses used in aquaculture are much smaller than the 400 mWs/cm² used in Studies I and II, since many fish pathogens are destroyed by UV doses as low as 30 mWs/cm² (Liltved et al., 1995).

Decreased mortality was found also in the groups incubated with O₃ + H₂O₂ and UV + H₂O₂ treated water and formalin bath control, but these did not differ significantly from the no treatment control. The formalin bath in the trial was started when *Saprolegnia* hyphae were visually detected. Starting the formalin baths at the very beginning of the incubation would most probably have led to better incubation result.

In Study II, UV-irradiation of inlet water significantly decreased the mortality of the eggs, even though the dose of UV-

irradiation decreased during the incubation trial due to an accumulation of organic material on the surface of the UV-lamp. The result confirms the previous finding, even taking into account that the water characteristics differed between studies I and II. The mortality amongst all experimental groups was high. The fertilization rate of the eggs was lower than normally expected for rainbow trout in Finland in February. Hence the high numbers of dead eggs on trays at the beginning of the trial probably enhanced *Saprolegnia* growth in the incubation trays (Piper et al., 1982, Kitancharoen and Hatai, 1996). It is known that the quality of the eggs (Azuma et al., 2003) and maternally transferred immune parameters (Løvoll et al., 2006, Mulero et al., 2007) affects mortality of the eggs.

Supplementation of incubation trays with suspension of *Pseudomonas* sp. M162, *Janthinobacterium* sp. M169 or *Pseudomonas* sp. M174 or their mixture decreased mortality as compared to control, when inlet water was treated with UV irradiation. Although, the difference was not significant in any group, 20% lower mortality in group supplemented with *Pseudomonas* M174 could have economic significance to fish farmers. However, the incubation trial was performed with 2-3 egg layers on the incubation trays and effect of protective cultures in production scale egg incubation should be investigated. Hansen and Olafsen (Hansen and Olafsen, 1989) proposed the concept of manipulation of egg surface microbiota of cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) during egg incubation. This seems to be the first time, when protective bacterial cultures have been used in the prevention of *Saprolegnia* infections in salmonid egg incubation.

Supplementation of bacterial cultures on eggs incubated with non-treated inlet water did not have any effect on mortality. It is obvious that the protective cultures used in this incubation trial need to possess a competition advantage against other bacterial species, and simply stopping the waterflow for 24 hours after supplementation was sufficient. The protective strains were

isolated from the eggs during incubation trial I and were hence introduced to the new environment in incubation trial II.

Molecular biology studies revealed that strains M162 and M169 were below the detection limit in the samples one week after the supplementation. This suggests that these strains were not capable to compete for attachment sites on the egg surface in incubation trial II. The presence of strain M174 on the egg surfaces one week after supplementation could not be confirmed, since there was an overlapping peak with a similar sized fragment also in the control group. Strains M162 and M174 produced siderophores (Study III and IV) while no siderophore production was found with *Janthinobacterium* sp. M169 strain (unpublished data). The iron content of the incubation water was not measured during the trials. Hence one can only speculate on the role of siderophore production of protective strains in colonization of egg surfaces. Iron concentrations of Lake Kallavesi (4.65-5.55 μM) and Lake Peurunkajärvi (0.54-0.73 μM) in common water quality analyses were lower than the level used in the siderophore production assay (10 μM), which suggests that siderophore production by the protective strains may have had a role in colonization of the egg surfaces during the incubation in Studies I and II. While the iron concentration in Lake Peurunkajärvi was almost ten times lower than in Lake Kallavesi, siderophore production did not have any major influence in Study II; or perhaps the iron content of the Lake Peurunkajärvi water was so low, that no real competition advantage could be achieved.

Suitable water treatment may provide a competition advantage for protective bacterial culture used in rainbow trout egg incubation. Although UV-irradiation enhanced the effect of protective cultures, it should be noted that further assistance to protective culture survival on eggs may be needed. The protective bacterial levels supplemented on eggs in study II were relatively low as compared to the levels used for probiotic purposes (Nikoskelainen et al., 2001, Brunt et al., 2007), but

because high bacterial counts on eggs may have a role in egg mortality (Sauter et al., 1987), a high supplementation level was avoided.

The recent study of Ali et al. (Ali et al., 2013) revealed that *Saprolegnia* sp. are capable of forming biofilms. In incubation trial II, pipelines were not disinfected prior to the trial. Hence there is the possibility that pipeline biofilms, which could have been formed prior to the trial, may have been an additional source of *Saprolegnia* zoospores and cysts on experimental trays.

6.2 PROBIOTIC EFFECTS OF M162 AND M174

The antagonistic effects of *Pseudomonas* sp. strains M162 and M174 on the fish pathogen, *Flavobacterium psychrophilum*, and their modes of action were evaluated in Studies III and IV. The probiotic effects of the strain M162 resulted mainly through an immunostimulatory action, while that of M174 was attributed to both siderophore production and immunostimulatory effects. Probiotic stimulation of serum lysosome activity (Panigrahi et al., 2004, Newaj-Fyzul et al., 2007, Sharifuzzaman and Austin, 2010b), leukocyte counts (Capkin and Altinok, 2009), total IgM-antibody activity and respiratory burst activity (Nikoskelainen et al., 2003, Sharifuzzaman and Austin, 2010a, Sharifuzzaman and Austin, 2010b) have been reported for several other bacterial species.

The successful experimental infestation of rainbow trout with *F. psychrophilum* has proved challenging (Michel et al., 1999, Decostere et al., 2000), which may have limited probiotic applications against this significant pathogen. It does seem that utilization of *Pseudomonas* sp. M174 (Study III) was the first reported probiotic strain to be antagonistic to *F. psychrophilum in vivo*. Since then, also Burbank and his coworkers (Burbank et al., 2011) found that *Enterobacter* sp. strains were able to decrease

mortalities after a *F. psychrophilum* challenge in juvenile rainbow trout.

Intramuscular injection was used in Studies III and IV to challenge the rainbow trout fry with *F. psychrophilum*. This kind of challenge bypasses the physical defence and protective immune mechanisms of skin (Lorenzen et al., 2010) and cannot be directly compared to natural cohabitation challenge. However, it is the most commonly used method together with intraperitoneal challenge (Obach and Laurencin, 1991, Högfors et al., 2008, LaFrentz et al., 2008, Castillo et al., 2012).

The probiotic strain should not compromise the health of the target species. Although some of the *Pseudomonas* species have been reported as being opportunistic fish pathogen (see Chapter 2.7.), an intraperitoneal injection of *Pseudomonas* sp. strains M162 and M174 did not cause mortality or signs of the disease in the tested fish. The potential of *Pseudomonas* species as probiotics has been found also by other investigators (Bly et al., 1997, Gram et al., 1999, Gram et al., 2001, Spanggaard et al., 2001, Suomalainen et al., 2005, Strom-Bestor and Wiklund, 2011).

Utilization of probiotics to try to prevent bacterial disease outbreaks may decrease the need of antibiotic treatments in rainbow trout aquaculture (Nayak, 2010). Since the aquaculture environment provides favorable conditions for the development of antibiotic resistant strains through periodic introduction of antibiotics to rearing water during the disease outbreak (Schmidt et al., 2001, Miranda and Zemelman, 2002a), probiotics may be viewed as contributing to the sustainable control of pathogens.

Cold water disease and rainbow trout fry syndrome have been treated traditionally with antibiotics. The loss of appetite related to the diseases impedes administration via antibiotic supplemented feed. While problems caused by RTFS appear already when the fry are only 0.2-0.5g, the fry should be

vaccinated as early as possible (Lorenzen et al., 2010). During recent years, advances in CWD vaccine development have led to the first commercial vaccines. Although promising results with RTFS dip vaccines have been achieved in test trials in the field, no commercial products are available at the moment.

Some probiotics are beneficial for the control of multiple fish diseases (Brunt et al., 2007), which is not surprising if the mode of action of the probiotic is either through stimulation of the innate immune system or via the production of antibacterial substances. Immune enhancement is especially relevant in the case of fry-stage diseases like RTFS.

The probiotic effects of M162 apparently resulted mainly from immunostimulation, while M174 exerted its effects through siderophore production and immunostimulation. Immunostimulatory effects have been observed in several probiotic species (Panigrahi et al., 2005, Balcázar et al., 2007b, Newaj-Fyzul et al., 2007), but the siderophore production of the probiotic strains tested in the aquaculture environment is limited to some of the *Pseudomonas* (Spanggaard et al., 2000, Strom-Bestor and Wiklund, 2011), *Aeromonas* (Brunt et al., 2007) and *Bacillus* (Laloo et al., 2010) strains.

Some of the *Flavobacterium psychrophilum* strains are capable of enhancing their iron uptake via siderophore production (Moller et al., 2005). In study III, M174 showed antagonistic activity against *F. psychrophilum* growth *in vitro* in iron deficient growth media. The siderophore production of *F. psychrophilum* strains 413 and ST/00 were not studied. Hence, it cannot be stated whether the antagonistic activity of M174 strains was related to siderophore production or by outcompeting the *F. psychrophilum* strains in siderophore production. Siderophores can be categorized into catecholates, phenolates, hydroxamates and carboxylates, although increased knowledge of new siderophores has resulted in the identification of mixed type siderophores (Miethke and Marahiel, 2007). It is not known if there are

differences in iron uptake efficiency between different types of siderophores in the aquaculture environment, but this may have role in the competition between the probiotic and the pathogen for iron resources.

Several probiotic strains have shown significant effects on serum lysozyme activity (Irianto and Austin, 2002, Kim and Austin, 2006, Brunt et al., 2007, Sharifuzzaman and Austin, 2009, Merrifield et al., 2010b). M162 caused a significant increase in lysozyme activity after 3 weeks, but there was no difference compared to control after 2 weeks. In study III, the effects of M174 on immunological parameters were measured only after two weeks. Hence, it is possible that some of the immunostimulatory effects of the probiotic feeding would not have been apparent at that time. Balcazar et al. (Balcázar et al., 2007a) described a similar significant increase in serum lysozyme activity in fish fed with either *Lactococcus lactis* or *Leuconostoc mesentroides* for 3 weeks, but not for 2 weeks. Similarly in that trial, the probiotic supplemented feed was changed to control feed after two weeks. On the other hand, Sharifuzzaman and Austin (Sharifuzzaman and Austin, 2009) detected the greatest impact on humoral immune parameters after 2 weeks, after which the stimulating effect decreased, although in that trial probiotic feeding was continued until the end of the trial.

Respiratory burst activation is also one of the most typical probiotic modes of action (Nikoskelainen et al., 2003, Brunt and Austin, 2005, Balcázar et al., 2006, Balcázar et al., 2007b, Brunt et al., 2007, Pieters et al., 2008). M174 evoked a significant increase in respiratory burst activity and M162 increased the activity after two weeks of probiotic feeding, although not statistically significantly.

The effect of probiotic feeding on the acquired immune system of fish seems to be related to the increased level of total immunoglobulin in plasma. Oral administration of M162

increased significantly total IgM-levels on week 3, but not on week 2. This finding is in agreement with previous studies (Nikoskelainen et al., 2003, Panigrahi et al., 2005), suggesting that increased antibody production may not be apparent in a two week feeding trials.

M162 and M174 strains adhered to and survived in rainbow trout intestine, but the levels of the probiotics dropped at the end of the probiotic supplementation. This finding is in accordance with previous reports (Nikoskelainen et al., 2003, Panigrahi et al., 2005, Balcázar et al., 2007b). This suggests that the probiotic bacteria were not able to permanently colonize and multiply in the intestine of the fish, which is a common finding when the probiotic strain has not been isolated from the intestine of the fish (Nikoskelainen et al., 2003, Panigrahi et al., 2005) or its optimal growth temperature is significantly higher than the rearing water temperature (Balcázar et al., 2007b).

7 Conclusions

Treatment of inlet water with a high dose (400 mWs/cm²) of UV-irradiation decreased significantly egg mortality in incubation trials; this represents a novel method to decrease the losses encountered during rainbow trout egg incubation.

Treatment of inlet water was able to modify the microbial population on the surface of rainbow trout egg.

The simultaneous presence of protective bacteria, *Pseudomonas* sp. M162, *Janthinobacterium* sp. M169 and *Pseudomonas* sp. M174, enhanced the effect of UV irradiation in controlling the *Saprolegnia* infections of the rainbow trout eggs during incubation.

Addition of protective bacteria *Pseudomonas* sp. M162, *Janthinobacterium* sp. M169 and *Pseudomonas* sp. M174 alone did not decrease the mortality of rainbow trout eggs.

Pseudomonas sp. strains M162 and M174 inhibited the growth of *Flavobacterium psychrophilum* *in vitro* and decreased the mortality of the rainbow trout fingerlings in challenge experiments *in vivo*.

The mode of action of the probiotic strains M162 and M174 were elucidated and both strains exhibited immunostimulatory effects while the strain M174 affected also exerted effects through siderophore production.

Both strains were found to be safe for the fish and capable of colonizing fish intestine transiently.

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JOUNI HEIKKINEN

*Novel Applications of
Pseudomonas sp. Bacterial
Strains in Rainbow Trout
Aquaculture*

Fungal and bacterial diseases cause remarkable losses in rainbow trout aquaculture. In this work, applications of *Pseudomonas* sp. strains M162 and M174 for the control of *Saprolegnia* sp. infections during egg incubation and *Flavobacterium psychrophilum* mortalities in rainbow trout aquaculture has been assessed. The thesis provides new procedures to prevent *Saprolegnia* infections on eggs and indicates the probiotic effect and mode of action of M162 and M174 strains against *F. psychrophilum* *in vivo*.



UNIVERSITY OF
EASTERN FINLAND

PUBLICATIONS OF THE UNIVERSITY OF EASTERN FINLAND
Dissertations in Forestry and Natural Sciences

ISBN 978-952-61-1234-3