The fish egg microbiome: diversity and activity against the oomycete pathogen *Saprolegnia*

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Thesis

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Chapter 1

General introduction and thesis outline

Aquaculture is the fastest-growing animal food sector and will further increase to feed the growing human population. Along with this growth, aquaculture is suffering more and more from emerging diseases caused by viruses, bacteria, fungi, oomycetes and parasites. To mitigate these infectious diseases in a sustainable manner, there is an increasing interest in habitat bioaugmentation or introduction of beneficial microbes to protect fish and amphibians against emerging pathogens. To this end, a profound understanding of the diversity and temporal-spatial dynamics of the microbiomes of aquatic organisms and their impact on growth and health of their hosts is required. The research described in this thesis focuses on the characterisation of fungal, oomycete and bacterial communities associated with Atlantic salmon (*Salmo salar*) eggs, the isolation of potential beneficial microbes, and the efficacy of several bacterial and fungal isolates and their metabolites to mitigate oomycete diseases. This introductory chapter will first give an overview of common and emerging infectious diseases in aquatic environments and the measures that are currently used to control these diseases. The current state-of-the-art in fish microbiome research is summarized and the potential functions of the fish microbiome and its role in disease protection are highlighted. Finally, I will present the outline of my thesis.

Aquaculture: opportunities and threats

Aquaculture is defined as ‘the farming of aquatic organisms’ (FAO, 2002). In many parts of the world, fish contribute substantially to the total animal protein supply, in some cases even more than 20% (FAO, 2014). In 2013, the world aquaculture production (70 million tonnes excluding aquatic plants) overtook bovine production (68 million tonnes) and accounted for USD 151 billion (FAO, 2015, 2016). The major aquaculture groups include fish, crustaceans (such as shrimps and lobsters) and molluscs (such as mussels, oysters and scallops) (FAO, 2014). Aquaculture is yet a more environmentally-friendly food producing industry than livestock farming in agriculture due to the natural efficiency of fish in converting feed into animal protein (FAO, 2014). Aquaculture comprised 43% of total world fisheries in 2013 and this proportion is foreseen to exceed 60% by 2030 due to the reduction of capture fisheries and the raise in demand of fish products (FAO, 2014, 2015). Therefore, optimizing the health and growth of aquaculture animals is key to ensure sustainability and profitability of the industry. However, several factors are harmful to the long-term development of aquaculture, such as water pollution by aquaculture wastes, variability of water temperature due to climate change and emerging diseases (Cheung *et al.*, 2010; FAO, 2014).

Besides the common ‘lingering’ aquaculture diseases, such as Vibriosis and Pancreas Disease, other diseases are emerging (FAO, 2014; WHO, 2016). The

major causes proposed for this emergence include changes in environment and climate (e.g. temperature fluctuation), microbial evolution (e.g. resistance to antibiotics), anthropogenic activities (e.g. transmission of pathogens between different regions via transport/trade), changes in aquacultural practices (e.g. hygienic management or restricted regulations of chemical control measures), and social and cultural factors (e.g. consumption of contaminated raw food and subsequent release of manure in the water) (Harvell *et al.*, 1999; Murray and Peeler, 2005; Adlard *et al.*, 2015; WHO, 2016). Pathogenic microorganisms are commonly present in aquatic ecosystems but when the commensal microbial and plankton community is disturbed or the ambient environment (e.g. temperature and season) varies, pathogens can become more prevalent and cause infection (Turner *et al.*, 2009; Romero *et al.*, 2014a). Aquaculture is enormously diverse, including more than 560 species (FAO, 2014), and also has very diverse farming practices. Therefore, it is no surprise that also many infectious diseases are threatening the aquaculture industry, such as diseases caused by viruses, bacteria, fungi, oomycetes, and parasites including protists and metazoans (Table 1).

Disease outbreaks can impact a species' economic value by either reduction in yield by mortality or reduction in growth. Also, a bad taste or appearance will reduce the marketability of the products (Lafferty *et al.*, 2015). For example, outbreaks of white spot syndrome virus and yellow head disease virus led to economic losses of around USD 6.5 billion in shrimp aquaculture in Asia from 1991 to 1993 (Lightner, 2011). Sea lice infection causes more than USD 100 million worldwide every year (Johnson *et al.*, 2004). Worldwide, lots of money is invested to minimize the detrimental impact of aquatic diseases, largely by purchasing parasiticides, equipments and feed, but also on research (Costello, 2009).

Table 1 A selection of pathogens of wild and cultivated aquatic hosts.

Pathogen groups	Pathogen types	Hosts	References*
Viruses	Rhabdovirus (infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus, Spring viraemia of carp virus), iridoviruses (red sea bream iridovirus, infectious spleen and kidney necrosis iridovirus), nodaviruses (Viral nervous necrosis virus, Striped jack nervous necrosis virus, betanodavirus)	Fish	(Munday <i>et al.</i> , 2002; Walker and Winton, 2010; Purcell <i>et al.</i> , 2012; Sahoo and Goodwin, 2012)
	White spot syndrome virus, Taura syndrome virus, Yellow head virus, infectious hypodermal and haematopoietic necrosis virus, Infectious myonecrosis virus, nodaviruses (<i>Macrobachium rosenbergii</i> nodavirus, <i>Penaeus vannamei</i> nodavirus)	Shrimp	(Hill, 2002; Walker and Winton, 2010; Thrush <i>et al.</i> , 2012)
	Epizootic haematopoietic necrosis virus, ranaviruses	Fish and amphibians	(Gray <i>et al.</i> , 2009; Walker and Winton, 2010)
	Koi herpesvirus	Common carp and koi carp	(Hedrick <i>et al.</i> , 2000)
	Infectious salmon anaemia virus	Atlantic salmon	(Hastings <i>et al.</i> , 1999; Asche <i>et al.</i> , 2009; Thrush <i>et al.</i> , 2012)
Bacteria	New strain of viral haemorrhagic septicaemia virus (type IV)	Wild freshwater fish	(Lumsden <i>et al.</i> , 2007; Thrush <i>et al.</i> , 2012)
	<i>Vibrio</i> spp., <i>Moritella viscosa</i> , <i>Aeromonas</i> spp., <i>Yersinia ruckeri</i> , <i>Flavobacterium</i> spp., <i>Piscirickettsia salmonis</i> , <i>Edwardsiella</i> spp., <i>Renibacterium salmoninarum</i> , <i>Lactococcus garvieae</i>	Fish	(Ghittino <i>et al.</i> , 2003; Sommeret <i>et al.</i> , 2005)
	<i>Campylobacter</i> spp.	Wild waterfowl	(Abulreesh <i>et al.</i> , 2006)
	<i>Aeromonas hydrophila</i> , a <i>Flavobacterium</i> sp.	Wild common carp	(Monette <i>et al.</i> , 2006)
	<i>Vibrio</i> spp.	Wild biovalves and corals	(Thompson <i>et al.</i> , 2004; Romero <i>et al.</i> , 2014a)
Metazoan parasites	Rickettsia-like organisms (e.g. necrotizing hepatopancreatitis bacterium)	Crustaceans, (e.g. penaeid shrimps)	(Gollas-Galván <i>et al.</i> , 2014)
	<i>Streptococcus</i> spp.	Fish	(Agnew and Barnes, 2007; Geng <i>et al.</i> , 2012)
	the Monogeneans <i>Gyrodactylus</i> spp., the Cestodes <i>Eubothrium</i> spp., the crustaceans <i>Lepeophtheirus salmonis</i> and <i>Caligus</i> spp., the sea lice <i>Lepeophtheirus salmonis</i> , the helminths <i>Anisakis simplex</i> , <i>Opisthorchis felineus</i> and <i>Diphylobothrium</i> spp.	Fish, wild salmonids, freshwater mammals and reptiles	(Dick <i>et al.</i> , 2001; Dupouy-Camet and Peduzzi, 2004; Chai <i>et al.</i> , 2005; Křošek <i>et al.</i> , 2005; Sommeret <i>et al.</i> , 2005; Sripa <i>et al.</i> , 2010; Johnson and Pauli, 2011; Sripa <i>et al.</i> , 2011; Praveitoni <i>et al.</i> , 2012; Pozio <i>et al.</i> , 2013; Hjeltnes, 2014; Powell <i>et al.</i> , 2015)
	the myxozoan <i>Myxobolus cerebralis</i>	Wild rainbow trout	(Hedrick <i>et al.</i> , 1998)
	Platyhelminths	Wild anurans	(Brooks <i>et al.</i> , 2006)
Protozoan parasites	Macroparasites (including Acanthocephala, Annelida, Arthropoda, Cestoda, Monogenea, Nematoda and Trematoda)	Amphibians	(Koprivnikar <i>et al.</i> , 2012)
	<i>Parabrucephalopsis parasituri</i> and <i>Prosorhynchoides ozakii</i>	Fish	(Ogawa <i>et al.</i> , 2004; Urabe <i>et al.</i> , 2007; Ogawa, 2011)
	<i>Ameson</i> , <i>Pleistophora</i> , <i>Psorospermium</i> , <i>Thelohania</i> , <i>Vavria</i> , <i>Mikrocytos</i> , the Amoebae <i>Paramoeba</i> spp., the flagellates <i>Ichthyobodo</i> spp., the dinoflagellate <i>Hematodinium</i> spp., the ciliates <i>Ichthyophthirius multifiliis</i> and <i>Trichodina</i> spp., the Microsporidia <i>Tetramicra brevivulum</i> , the Myxosporeans <i>Tetracapsula byosalmonae</i> , <i>Mikrocytids</i>	Fish, freshwater crayfish, aquatic invertebrates including crabs, lobsters and oysters	(Edgerton <i>et al.</i> , 2002; Camegie and Cochenne-Laureau, 2004; Sommeret <i>et al.</i> , 2005; Small, 2012; Hartikainen <i>et al.</i> , 2014; Powell <i>et al.</i> , 2015)
	<i>Bonania ostreae</i>	Wild and cultivated oysters	(Adlard <i>et al.</i> , 2015)

<i>Dermocystidium</i> spp.	Fish	(Rowley <i>et al.</i> , 2013)
<i>Amphibiocystidium</i> spp.	Amphibians	(Rowley <i>et al.</i> , 2013)
<i>Sphaerothecum destruens</i>	Cyprinid	(Gozlan <i>et al.</i> , 2009; Gozlan <i>et al.</i> , 2014; Adlard <i>et al.</i> , 2015)
<i>Ichthyophonus hoferi</i>	Wild clupeids	(Rahimian and Thulin, 1996; Gozlan <i>et al.</i> , 2014)
<i>Paecilomyces</i> , <i>Ochroconis</i> , <i>Mucor</i> , <i>Phoma</i> spp.	Various hosts	(Gozlan <i>et al.</i> , 2014)
<i>Fusarium falciforme</i> and <i>Fusarium keratoplasticum</i>	Sea turtles	(Sarmiento-Ramírez <i>et al.</i> , 2014)
<i>Batrachochytrium dendrobatidis</i>	Amphibians	(Daszak <i>et al.</i> , 1999; Daszak <i>et al.</i> , 2003; Garner <i>et al.</i> , 2005; Alan Pounds <i>et al.</i> , 2006; Peeler <i>et al.</i> , 2011)
<i>Veronaea botryosa</i>	Sturgeon	(Steckler <i>et al.</i> , 2014)
<i>Exophiala</i> spp.	Amphibian, crabs, fish and turtle	(Carmichael, 1967; McGinnis and Ajello, 1974; de Hoog <i>et al.</i> , 2011)
<i>Enterospora nucleophile</i>	sea bream	(Palenzuela <i>et al.</i> , 2014)
<i>Lacazia loboi</i>	Delphinidae	(Paniz-Mondolfi <i>et al.</i> , 2012)
<i>Oomycetes</i>	Freshwater fish and amphibians, especially salmonids in aquaculture	(van West, 2006; Fernández-Benítez <i>et al.</i> , 2008; Phillips <i>et al.</i> , 2008; van den Berg <i>et al.</i> , 2013)
<i>Aphanomyces invadans</i>	Freshwater and brackish water fish	(Oldtmann, 2012)
<i>Achlya debaryana</i>	Channel catfish	(Phillips <i>et al.</i> , 2008)
Chlamydia	Various aquaculture animals	(Sirde <i>et al.</i> , 2014)

* The prime sources of the contents and references of the table are Walker and Winton (2010), Sommeret *et al.* (2005) and Gozlan *et al.* (2014).

Control measures of aquatic diseases

Several measures are taken to prevent or cure aquatic diseases, including vaccinations, breeding for resistance, control by chemicals and antibiotics, and biological control.

Vaccination

Vaccines are globally applied to trigger immunity of fish to infectious diseases, mainly because they have high efficacy and low cost (Komar *et al.*, 2004). Traditional antigen vaccines are prepared by attenuating or killing microbes. They are applied by immersion, injection or oral uptake and mainly effective against bacterial and viral diseases (Heppell and Davis, 2000; Plant and LaPatra, 2011). Vaccines are used for preventing instead of curing diseases (Yanong, 2009) and therefore they are not applicable for controlling sudden disease outbreaks. Other disadvantages of vaccination are the labour intensiveness of applying vaccines by injection (Yanong, 2009), the need for re-application in the case of immersion vaccination and the instability of oral vaccines (Komar *et al.*, 2004). Moreover, injection can cause stress on fish and make them more susceptible to other pathogens (Yanong, 2009). DNA vaccines, which consist of encoding sequences expressing the antigens directly in the recipients, have been developed because of easier production, low production cost, stability, easy modification and lower risk of accidental infection due to incomplete attenuation (Davis and McCluskie, 1999; Heppell and Davis, 2000; Evensen and Leong, 2013). DNA vaccines can effectively protect rainbow trout (*Oncorhynchus mykiss*) from infectious hematopoietic necrosis and viral haemorrhagic septicaemia (Anderson *et al.*, 1996; Lorenzen *et al.*, 1998), and exerted a high effect against fish rhabdoviruses (Kurath, 2008).

Breeding for disease resistance

In the past decades, many fish breeding programs have been launched to improve disease resistance in aquaculture. To date, however, only a small percentage of aquaculture production is based on genetically improved stocks because of a low success rate of selective breeding (Gjedrem, 2012). Nevertheless, selective breeding has recently achieved resistance against the grass carp reovirus in grass carp (*Ctenopharyngodon idellus*) (Huang *et al.*, 2015) and against sea lice in Atlantic salmon (Gharbi *et al.*, 2015). In addition, crossing a brown trout (*Salmo trutta*) female with an Atlantic salmon male has resulted in resistance against

amoebic gill disease (Maynard *et al.*, 2016). However, to our knowledge, breeding has not yet resulted in fish species, with genetic resistance against fungal or oomycete diseases.

Control by chemicals and antibiotics

Chemicals like formalin, hydrogen peroxide and NaCl are widely used to control aquaculture diseases (Plumb, 1991; Harikrishnan *et al.*, 2010; Bruno *et al.*, 2011; Harikrishnan *et al.*, 2011). However, for many chemicals there are concerns on safety for the farmers and their adverse environmental effects. Therefore, formalin is foreseen to be banned soon (Bruno *et al.*, 2011). Antibiotics, such as ampicillin, tetracycline and streptomycin, are commonly used to control aquaculture diseases (Defoirdt *et al.*, 2011; Romero *et al.*, 2012). Concerns on the application of antibiotics have risen due to the development of antibiotic resistance in several aquatic pathogens, the putative risk of transfer of antibiotic resistance to human or animal pathogens, and the detrimental effects of residues of antibiotics in food products on human health (Cabello, 2006; Heuer *et al.*, 2009; Cabello *et al.*, 2013). To evaluate the efficacy of a new chemical therapy, a profound knowledge on the ecology and pathogenicity of the pathogen is required, as well as extensive toxicity testing and environmental risk assessment before application of the treatment in the aquaculture industry.

Biological control

Biological control, defined as ‘the use of living organisms as control agents for pests, weeds and diseases’ (Waage, 2007), has been proposed as a promising measure to tackle aquaculture diseases in a more sustainable manner. The majority of the biological control in aquaculture includes potential probiotic bacteria but also parameciidae, bacteriophages, fungi, yeasts and microalgae are considered (Neospark; Scholz *et al.*, 1999; Marques *et al.*, 2006; Khairnar *et al.*, 2013; Schmeller *et al.*, 2014). The potential probiotic bacteria to date mostly include species in the genera *Vibrio*, *Bacillus*, *Pseudomonas* and *Lactobacillus* (Table 2), which were randomly selected and explored for their effects on fish growth and disease (Verschuere *et al.*, 2000; Newaj-Fyzul *et al.*, 2014; Hai, 2015; Newaj-Fyzul and Austin, 2015). Additionally, natural products like short-chain fatty acids, polyhydroxy-alkanoates, bacterial-derived cyclic lipopeptide biosurfactants, and plant and fungal extracts are being investigated as potential measures to suppress aquaculture diseases (Immanuel *et al.*, 2004; Harikrishnan *et al.*, 2012; Reverter *et al.*, 2014; Liu *et al.*, 2015). Several compounds (i.e. quorum sensing

antagonists) can inhibit the expression of the virulence genes of bacterial pathogens and are applied as antivirulence therapy (Defoirdt *et al.*, 2004; Clatworthy *et al.*, 2007; Defoirdt *et al.*, 2011).

The underlying modes of action of probiotics include boosting the immune response, improving nutrient uptake, enhancing growth and survival, disease suppression by producing pathogen-inhibitory compounds, prohibiting pathogen colonization by competitive exclusion, and optimizing water quality by eliminating harmful elements like ammonium and nitrite (Verschuere *et al.*, 2000; Martínez Cruz *et al.*, 2012; Newaj-Fyzul *et al.*, 2014; Hai, 2015; Newaj-Fyzul and Austin, 2015). Microbes can be applied directly in culture water or conveyed by (live) feed. For many promising candidates, however, only a few are commercialized or licensed. These include *Bacillus*, *Lactobacillus*, *Enterococcus*, *Paracoccus*, *Pediococcus* and *Thiobacillus* species marketed as a.o. BioMar, Biomin® or SporeGen®. Constraints for commercial application of probiotics are concerns with long-term safety for the environment, limited shelf-life, high production costs and stringent storage and transport conditions of the microbial inoculants. Also consistency of performance under different environmental conditions, their relatively narrow activity spectrum and compatibility with other applications are points of concern to the industry. Even though probiotics are considered as promising candidates in sustainable control of diseases in aquaculture, patenting, registration and commercialization of probiotics are complicated due to the strict rules and regulations involved.

Microbiomes of aquatic animals

In recent years, many microbiome studies have described the composition of microbial communities in humans, insects, plants, soils and water. These studies clearly point to the importance of microbiomes for pathogen protection of their eukaryotic hosts (Round *et al.*, 2010; Mendes *et al.*, 2011; Weiss and Aksoy, 2011; Everard *et al.*, 2013; Stecher *et al.*, 2013; Turner *et al.*, 2013; Liu *et al.*, 2014). As a result, habitat bioaugmentation has been proposed as a promising approach to mitigate emerging diseases of fish and amphibians in natural ecosystems and commercial aquaculture. This requires profound understanding of the diversity and activity of the microbiomes associated with their aquatic hosts. In the next paragraphs the current status of fish microbiome research, including investigations into the diversity, dynamics and functions of microbiomes for their host (Figure 1) will be described.

Figure 1 Schematic overview of different life-support functions of the fish microbiome and the impact of the host and ambient environment on microbiome diversity, assembly and functions.

The fish microbiome – who is there?

The composition of microbiomes of aquatic animals is highly dependent on the ambient aquatic habitat. To study microbiomes associated with aquatic animals and their environment, culture-dependent methods such as plating on general and selective culture media have been widely used. Due to the awareness that presumably less than 1% of the viable microbes in various aquatic environments are culturable (Amann *et al.*, 1995), culture-independent methods have been developed. Besides quantitative PCR, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), clone library sequencing, and 16S rRNA gene sequencing, more and more advanced ‘omics techniques are being used to study the composition and functions of microbiomes of a wide variety of eukaryotic hosts including aquatic hosts (Wang *et al.*, 2010; Leonard *et al.*, 2014; Liu *et al.*, 2014; Merrifield and Rodiles, 2015).

The skin, gill and gastrointestinal (GI) tract are considered as the major portals for microbes from external to internal tissues (Merrifield and Rodiles, 2015). These mucosal tissues are important physical barriers against pathogen invasion and provide a unique environment for commensal microbes. The majority of the mucosal microbes is coated with host immunoglobulins (IGs). The immunoglobulins are the antibodies that recognize and bind to the microbes and activating the immune response. Therefore IGs are suggested to be important for the adaptive immune response differentiating between commensal bacterial microbiota and pathogens (Zhang *et al.*, 2010; Xu *et al.*, 2013). In fish, each

mucosal tissue has its own specific adaptive immune system (Salinas, 2015) and therefore it is likely that the microbiota are coated with different Ig isotypes depending on the tissue type (Tacchi *et al.*, 2014). Moreover, as described below, also the microbiomes of the different mucosal tissues vary.

Skin

The slimy and viscous epidermal mucus of the skin of aquatic animals is the natural primary guardian against the external environmental influences and also provides a home for the commensal bacteria to live and to form a shield against invasive pathogens (Hansen and Olafsen, 1999; Merrifield and Rodiles, 2015). The mucus and the protective scales of the fish skin contain more aerobic than anaerobic microbes (Merrifield and Rodiles, 2015). In general, the fish skin houses 10^2 - 10^4 bacteria per cm^2 (Austin, 2006). Fish from warm waters tend to harbour more Gram-positive bacteria in their skin such as *Bacillus* and *Micrococcus* spp., while in the skin of fish from temperate waters, Gram-negative bacteria, including *Acinetobacter*, *Flavobacterium*, *Moraxella*, *Photobacterium*, *Pseudomonas* and *Vibrio* spp. are more abundant. Skins of fish from tropical waters have higher numbers of Enterobacteriaceae (Hansen and Olafsen, 1999; Gram and Ringø, 2005). The skin microbiome of aquatic animals is influenced by the ambient environment, including water quality, load of potential pathogens, oxygen level, pH and temperature (Horsley, 1973, 1977), but also host genotype appeared to be important for the composition of skin specific microbiome (Boutin *et al.*, 2014). It was argued that the skin microbiomes are simply present on the cuticle surface without substantial interference with the skin (Crouse-Eisnor *et al.*, 1985). However, more studies are showing that skin microbes are playing a role in defence and immune responses (Xu *et al.*, 2013; Lowrey *et al.*, 2015). In rainbow trout, the highest diversity in bacterial community was found on the skin when compared to the gills and gut, and several skin isolates inhibited the growth of the aquatic pathogens *Saprolegnia australis* and *Mucor hiemalis* *in vitro* (Lowrey *et al.*, 2015; Salinas, 2015). The skin microbiome can produce polymers to reduce friction generated between the skin and water during swimming and to facilitate locomotion of fish (Sar and Rosenberg, 1989). Compared to the bacterial microbiome, the fungal microbiome of skin is less studied. It was reported that the fungal and yeast population represented less than 2.3% whereas bacteria represented more than 97.7% of the total culturable microbial community of Atlantic salmon (Landeira-Dabarca *et al.*, 2013). *Alternaria*, *Aspergillus*, *Cladosporium* and *Fusarium* represented the most dominant fungal genera in the exoskeleton of crayfish (*Procambarus clarkii*) (Dörr *et al.*, 2012).

Gill

Fish gills contain a high diversity and richness of microbiota, the composition of which is mainly affected by water quality, seasonal environmental changes and host genotype (Merrifield and Rodiles, 2015). The gill microbiome functions as primary barrier against external pathogens (Larsen and Arias, 2014). Due to the consistent water exchange in the gills, many gill-associated bacteria and fungi have attachment structures and they tend to colonize in protected niches of the gill rather than the surface exposed areas (Mudarris and Austin, 1988; Hansen and Olafsen, 1999; Wang *et al.*, 2010). Typically, the aerobic bacterial population ranges from 10^3 to 10^6 CFU per gram of gill tissue in various fish species (Merrifield and Rodiles, 2015). Anaerobic bacteria are rarely present in fish gill or skin. Facultative anaerobic bacteria, mainly *Vibrio* species, are found in pelagic fish (Lougovois and Kyrana, 2005). The core bacterial microbiome of the gills of at least 19 fish species is composed of *Achromobacter*, *Aeromonas*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Vibrio* species (Cahill, 1990). For Atlantic salmon, the gill microbiome was similar to the skin microbiome and also reflected the microbiome of the ambient water (Horsley, 1973). In contrast, the gill microbiome of healthy turbot (*Scophthalmus maximus*) was distinct from the ambient water (Mudarris and Austin, 1988). So far, studies on the fungal microbiome of fish gills are limited. Wang *et al.* (2010) reported that the Ascomycota as the most dominant fungal phyla of gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalobrama amblycephala* Yih) (Wang *et al.*, 2010).

Gastrointestinal tract

Of the mucosal microbiomes of fish, the gut bacterial microbiome is studied most intensely. In general, the fish GI tract houses up to 10^8 aerobic heterotrophic bacteria and up to 10^5 anaerobic bacteria per gram of tissue wet weight (Austin, 2006). It has been commonly agreed that the GI tract of newly hatched fish larvae is nearly sterile. The microbial population is acquired from the water, live and artificial feed, and establishes shortly after hatching (Ringø and Birkbeck, 1999).

Besides the bacterial microbiome, the yeast microbiome of fish gut has attracted wide attention, mainly due to the fact that several yeasts can stimulate the host immune response, growth, development and metabolism of the hosts (Gatesoupe, 2007). The population density of yeast in fish gut ranges from undetectable quantities to 10^7 CFU per gram of gut matter and the two most reported fungal phyla are the Ascomycota and Basidiomycota (Gatesoupe, 2007; Romero *et al.*, 2014b). Concerning filamentous fungi, only little is known: a

Trichoderma sp. was found in catfish *Hypostomus strigaticeps* (Cardone *et al.*, 2006) and *Cladosporium*, *Dothideomycetes* and *Pseudocercospora* species were found in rainbow trout (Geurden *et al.*, 2014).

It should be noted that the gut is also a habitat for potential pathogenic microbes. Among these are bacteria such as *Aeromonas*, *Edwardsiella*, *Mycobacterium*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas*, *Vibrio* and *Yersinia* species, and *Alteromonas*, *Flavobacterium*, *Moraxella* that are associated with diseases in early developmental stage of fish (eggs, larvae or juveniles) (Hansen and Olafsen, 1999; Bøgvold and Dalmo, 2014). Besides, yeasts, such as *Candida*, *Cryptococcus* and *Trichosporon* species, are known to cause diseases on e.g. *Oncorhynchus rhodurus*, *Oncorhynchus tshawytscha*, *Sparus aurata* and *Tinca tinca* (Gatesoupe, 2007).

The commensal GI tract microbes play a role in nutrition acquisition (Nayak, 2010; Montalbán-Arques *et al.*, 2015), as was shown for gnotobiotic zebrafish (*Danio rerio*) that were defect in the uptake of protein macromolecules (Bates *et al.*, 2006). The microbes can produce exogenous enzymes to facilitate the digestibility of food and degradation of large and complex molecules, such as chitin, protein and starch (Austin, 2006; Nayak, 2010; Ray *et al.*, 2012; Merrifield and Rodiles, 2015; Montalbán-Arques *et al.*, 2015). The gut microbes can also produce vitamins and eicosapentaenoic acid to enhance the health of the host (Yazawa *et al.*, 1988; Austin, 2006). Moreover, the microbiome of the GI tract can also be the main food source for its hosts, as was shown for Asian silver carp (*Hypophthalmichthys molitrix*) and gizzard shad (*Dorosoma cepedianum*) where the Cyanobacteria were most abundant in the GI tract (Ye *et al.*, 2014). It is well known from studies in humans and animals that nutrients can alter the microbiome composition of the GI tract (Kau *et al.*, 2011; Kostic *et al.*, 2013) and this is also the case in fish as was shown in Arctic charr (*Salvelinus alpinus*) when fed with low or high level of carbohydrate (Ringø and Olsen, 1999). Besides nutritional uptake, the commensal GI tract microbiomes play an important role in defence against pathogens by enhancing and maintaining immune functions (Nayak, 2010; Maynard *et al.*, 2012; Montalbán-Arques *et al.*, 2015). For example, commensal microbiomes of zebrafish larvae were shown to exhibit immunoregulatory activity, via for example stimulating genes encoding antiviral components, against viral infection before the adaptive immunity became mature (Galindo-Villegas *et al.*, 2012). Moreover, starved seabass showed the enrichment of genes encoding antimicrobial compounds in the GI tract microbiome indicating that the microbiome can protect the host from invasion of pathogens under stressed conditions (Xia *et al.*, 2014). Various studies have shown that several members of the GI tract microbiome are capable of producing inhibitory compounds against pathogens, including *Carnobacterium* against *Aeromonas salmonicida*, *Acinetobacter* against *Vibrio*

campbellii, *Vibrio* sp. against *Pasteurella piscicida*, and yeasts against *Vibrio anguillarum* (Sugita *et al.*, 1997; Ringø *et al.*, 2000; Liu *et al.*, 2010; Caruffo *et al.*, 2015).

Compared to research on bacterial microbiomes, much less studies have been conducted on archaeal and protozoan microbiomes of the GI tract of fish. Archaeal species were found in several aquatic organisms, including the GI tract of flounder (*Platichthys flesus*) and grey mullet (*Mugil cephalus*) (van der Maarel *et al.*, 1998). Also protozoa belonging to the genera *Balantidium* and *Paracichlidotherus* were found in the GI tract of surgeonfishes (family Acanthuridae) (Grim *et al.*, 2002). Protozoa are hypothesized as a potential ecological contributor to the fish gut, but clearly more research is needed to prove their exact functions for their hosts (Romero *et al.*, 2014b).

The fish microbiome: for better or for worse?

Besides the commensal microbes, the fish microbiome can also be a source of pathogens as they can utilize the aquatic environment not only to disperse, but also to propagate (Hansen and Olafsen, 1999). Climate change, anthropogenic activity, disturbance of ecosystems, introduction of pathogens, and evolution of pathogens are some of the factors that can enhance the frequencies of existing and (re-) emerging pathogens (De Silva and Soto, 2009; Pulkkinen *et al.*, 2010; Engering *et al.*, 2013; Liu *et al.*, 2014; Tomova *et al.*, 2015). For example, the spread of parasitic protozoans *Haplosporidium nelsoni* and *Perkinsus marinus* in Eastern oyster (*Crassostrea virginica*), and the bacterial pathogen *Vibrio parahaemolyticus* were triggered by increased temperatures in seafood products in the south of Chile and in oysters in Alaska (Hofmann *et al.*, 2001; De Silva and Soto, 2009).

Next to true fish pathogens, aquatic ecosystems and fish can also act as a vector for human pathogens which can be transmitted via direct contact and handling of fish or via consumption (Novotny *et al.*, 2004; Austin, 2006). It has been shown for example, that consumption of filter feeders, like bivalves, is an important path to acquire human pathogens including *Vibrio fluvialis* and *Vibrio damsela* (Olafsen, 2001; Ramamurthy *et al.*, 2014). Human pathogens that are transmitted via direct contact include *Erysipelothrix rhusiopathiae*, *Photobacterium damsela*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio vulnificus* and *Mycobacterium* species, while pathogens known to be transferred by consumption of contaminated seafood comprise *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Aeromonas* and *Salmonella* species (Novotny *et al.*, 2004). Subsequent infections are dependent on the health

condition and eating habit (e.g. consuming uncooked food) of the consumer and pathogen load (Novotny *et al.*, 2004).

Toward pathogen control

A major challenge for aquaculture is to find sustainable methods to control infectious diseases that (re-)emerged in the last decades. Exploiting the 'good guys' in the microbiome to combat the 'bad guys', the pathogens, could be a way forward. However to do so, a profound insight in the composition and functioning of the microbiomes is not sufficient. We also need to learn more about the fundamental mechanisms underlying the intimate interactions between host and pathogen. Similar to animal and plant pathogens, aquatic pathogens use various strategies to invade their hosts. They can, for example, manipulate the metabolic processes in the host, as shown in Atlantic salmon where infection by *Piscirickettsia salmonis* resulted in a change in protein metabolism in muscle and liver (Tacchi *et al.*, 2011). Or they can avoid host immune defences or other biotic and abiotic perturbations, as shown in the late stage of amoebic gill disease, where the pathogen infects the host via evading the cell-mediated killing mechanisms or inducing an allergic reaction on the host (Benedicenti *et al.*, 2015). Also via co-evolution with hosts pathogens can acquire properties that enable them to avoid defence of their hosts. Another strategy exploited by for example *Staphylococcus aureus* and *Pseudomonas aeruginosa*, is destroying physical barriers of the host by producing proteases that lyse host proteins (Finlay and Falkow, 1997). Also other lytic enzymes, such as chitinases and lipases that are produced by fish pathogens, such as *Aeromonas* spp., are potential virulence factors that destroy the tissue of fish or the chorion and *zona radiata* of eggs (Olafsen, 2001; Defoirdt, 2014; Arslan and Küçüksari, 2015). Last but not least pathogens can compete with their hosts for chemical elements. This mechanism is for example exploited by *Vibrio anguillarum*, via sequestering iron from its hosts via siderophores, thereby limiting the iron availability for the host (Naka and Crosa, 2011; Defoirdt, 2014). A better fundamental understanding of these processes will be needed to identify critical steps in the disease cycle as potential targets for disease control.

How to use microbiome analyses to mitigate emerging diseases?

As part of the innate immunity, the microbiome harbours beneficial microbes that form the first line of defence in protection of their aquatic hosts. The microbial community composition is affected by external and intrinsic factors, such as feed ingredients, feeding regime, environment (water quality, temperature, pH, salinity, perturbation), genetics, stress level and density of the host (Landeira-Dabarca *et al.*, 2013; Merrifield and Rodiles, 2015). This was also shown for humans, where infections or changes of environment can substantially affect the gut microbiome by causing overgrowth of microbes that otherwise are present at low abundance (Stecher *et al.*, 2013), thereby boosting the propagation of specific beneficial or pathogenic microbes.

Microbiome analysis has evolved as an informative tool to understand the diversity, dynamics, richness, abundance and functions of the aquatic animal-associated microbes. The results from microbiome studies can, on the one hand, be used for decision making to control or prevent diseases, in particular by predicting potential disease outbreak based on the load and seasonal changes of (potential) native and non-native pathogens; and on the other hand, for evaluating the efficacy and toxicity of pharmaceutical or biological control treatments. Large multidisciplinary international programs should be launched to exchange experiences on farming and knowledge on disease control. Additionally, large-scale tests in farming conditions should accompany the culture-independent microbiome analyses to demonstrate the efficacy of microbiomes on disease control.

Aquaculture is sensitive to infectious pathogens and environmental perturbations. Therefore a suitable rearing system can maintain the beneficial microbes at a stable level. For example, recirculation aquaculture systems (RASs) are capable of stabilizing microbiome composition and maintaining microbial diversity within the rearing system, which subsequently inhibits the growth of harmful microbes and enhances the growth and survival of Atlantic cod (*Gadus morhua*) larvae (Attramadal *et al.*, 2012a; Attramadal *et al.*, 2012b; Attramadal *et al.*, 2014). To combat common and emerging aquatic diseases, a comprehensive understanding of the dynamics, richness, abundance and functions of microbiomes associated with their aquatic hosts and environment is pivotal to obtain the right bioaugmentation candidates for application.

Table 2 Examples of bacterial probiotics tested under *in vivo* conditions.

Bacterial genus	In vivo tests			Reference**
	Isolated from	Host	Method of application	Beneficial effects on
<i>Aeromonas</i>	Rainbow trout, fish farm effluent, carp	Trout, oyster, eel, perch, carp	Feed additive, incubation water, single or mixed*	<i>Aeromonas</i> , <i>Vibrio</i> , <i>Saprolegnia</i> , <i>Lactococcus</i> , <i>Streptococcus</i> , <i>Aeromonas</i> , <i>Ichthyophthirius</i> Survival, immune response
<i>Agarivorans</i>	Abalone	Abalone	Supplement in macroalgae, mixed	No pathogen tested Growth, survival, bacterial colonization and persistence in GI tract
<i>Alteromonas</i>	Seawater	Mussel, oysters, scallops	Incubation water, food supplement, single	<i>Vibrio</i> or no pathogen tested Growth, survival, quality of aquatic environment
<i>Arthrobacter</i>	Culture water, unknown	shrimp, haddock	incubation water, feed additive, single	<i>Vibrio</i> or no pathogen tested Growth, survival Immune responses, digestive enzyme disease resistance
<i>Bacillus</i>	Carp, tilapia, snook, abalone egg capsules, shrimp farm, pickles, prawn, shrimp, catfish, rohu, bream, yogurt, grouper, marron, microbial flakes, soil or unknown	Carp, tilapia, snook, trout, shrimp, abalone, seabream, prawn, catfish, rotifer, lobster, rohu, marron, founder, grouper	Feed additive, incubation water, single or mixed	<i>Aeromonas</i> , <i>Pseudomonas</i> , <i>Streptococcus</i> , <i>Yersinia</i> , <i>Vibrio</i> , <i>Edwardsiella</i> , white spot syndrome virus or no pathogen tested Growth, weight gain, feed utilization, immune response, survival, health status, disease resistance, water quality, increased number of beneficial bacteria, reduced pathogen colonization and load, stress tolerance, physiological condition
<i>Bifidobacterium</i>	Unknown	Rohu	Feed additive, mixed	No pathogen tested Survival, growth, weight gain
<i>Brevibacillus</i>	<i>Artemia</i> culture	<i>Artemia</i> , sea bass	Feed additive, incubation water, single	<i>Vibrio</i> or no pathogen tested Growth
<i>Brochothrix</i>	Trout	Trout	Feed additive, single or mixed	<i>Aeromonas</i> Survival, immune response
<i>Burkholderia</i>	Oyster	Oyster	Feed additive, incubation water, mixed	No pathogen tested Survival, growth
<i>Carnobacterium</i>	Cod, salmon, trout	Cod, trout,	Feed additive,	<i>Vibrio</i> , <i>Aeromonas</i> , Survival, disease

<i>Citrobacter</i>	Tilapia	salmon	single	<i>Yersinia</i>	resistance, immune responses	Mikkelsen, 1998; Jøborn <i>et al.</i> , 1999; Robertson <i>et al.</i> , 2000; Kim and Austin, 2006) (Aly <i>et al.</i> , 2008c)
		Tilapia	Feed additive, single or mixed	<i>Aeromonas</i>	Survival	
<i>Clostridium</i>	Chicken	Drum, trout	Feed additive, orally by incubation, single	<i>Vibrio</i> , <i>Aeromonas</i>	Survival, immune response, disease resistance, reduced pathogen load	(Sakai <i>et al.</i> , 1995; Pan <i>et al.</i> , 2008)
<i>Enterobacter</i>	Trout	Trout	Feed additive, injection, single	<i>Flavobacterium</i>	Survival, immune response	(Burbank <i>et al.</i> , 2011; LaPatra <i>et al.</i> , 2014)
<i>Enterococcus</i>	Fish, Carnivet® LBC or unknown	Trout, Shrimp, eel	Feed additive, single or mixed	<i>Saprolegnia</i> , <i>Vibrio</i> , <i>Edwardsiella</i>	Growth, feed utilization, survival	(Chang and Liu, 2002; Swain <i>et al.</i> , 2009; Frouzbakhsh <i>et al.</i> , 2014)
<i>Frondinhabitans</i>	Salmon eggs	Salmon eggs	Incubation water, single	<i>Saprolegnia</i>	Reduced <i>Saprolegnia</i> hyphal attachment to salmon eggs	(Liu <i>et al.</i> , 2014)
<i>Janthinobacterium</i>	Salamander	Frog	Incubation water, single	<i>Batrachochytrium</i>	Disease resistance and survival	(Harris <i>et al.</i> , 2009)
<i>Kocuria</i>	Trout	Trout	Feed additive, single	<i>Vibrio</i>	Survival, immune responses	(Sharfuzzaman and Austin, 2010a, b; Sharfuzzaman <i>et al.</i> , 2011)
<i>Lactobacillus</i>	Flounder, salmonids, seabream, scallop, human faeces, yogurt, unknown	Flounder, tilapia, seabream, trout, oyster, rohu	Feed additive, single or mixed	<i>Aeromonas</i> , <i>Pseudomonas</i> , <i>Streptococcus</i> , <i>Lactococcus</i> , <i>Edwardsiella</i> or no pathogen tested	Survival, growth, weight gain, immune responses, disease resistance	(Nikoskelainen <i>et al.</i> , 2001; Silv <i>et al.</i> , 2003; Carnevali <i>et al.</i> , 2004; Salinas <i>et al.</i> , 2005; Pirarat <i>et al.</i> , 2006; Rolo <i>et al.</i> , 2006; Luis Balcazar <i>et al.</i> , 2007; Aly <i>et al.</i> , 2008b; Vendrell <i>et al.</i> , 2008; Aguilar-Macias <i>et al.</i> , 2010; Campa-Córdova <i>et al.</i> , 2011; Liu <i>et al.</i> , 2013; Villamil <i>et al.</i> , 2014; Beck <i>et al.</i> , 2015; Jha <i>et al.</i> , 2015)
<i>Lactococcus</i>	Bean sprout, rotifer mass culture, salmonids, flounder	Flounder, rotifer, trout	Incubation water, feed additive, single or mixed	<i>Vibrio</i> , <i>Aeromonas</i> , <i>Streptococcus</i> or no pathogen tested	Growth, survival, weight gain, immune responses, disease resistance	(Harzevili <i>et al.</i> , 1998; Balcazar <i>et al.</i> , 2006; Balcazar <i>et al.</i> , 2007b; Balcazar <i>et al.</i> , 2007a; Luis Balcazar <i>et al.</i> , 2007; Vendrell <i>et al.</i> , 2008; Heo <i>et al.</i> , 2013; Beck <i>et al.</i> , 2015)
<i>Leuconostoc</i>	Salmonids	Trout	Feed additive, single	<i>Aeromonas</i> , <i>Lactococcus</i> or no pathogen tested	Survival, immune responses	(Balcazar <i>et al.</i> , 2006; Balcazar <i>et al.</i> , 2007b; Balcazar <i>et al.</i> , 2007a; Luis Balcazar <i>et al.</i> , 2007; Vendrell <i>et al.</i> , 2008)
<i>Microbacterium</i>	Cod	Cod	Incubation water, single	No pathogen tested	Survival	(Fjelheim <i>et al.</i> , 2010)
<i>Micrococcus</i>	Tilapia	Tilapia	Feed additive, single	<i>Aeromonas</i>	Growth, survival	(Abd El-Rhman <i>et al.</i> , 2009)
<i>Neptunomonas</i>	Seawater	Mussel, scallop, oyster	Incubation water, single	<i>Vibrio</i>	Survival, disease resistance, quality of aquatic environment	(Kesarodi-Watson <i>et al.</i> , 2009, 2010; Kesarodi-Watson <i>et al.</i> , 2012)
<i>Pediococcus</i>	Grass	Trout, sea bass, tilapia	Feed additive, single	No pathogen tested	Reduced disease, ossification, growth, immune response	(NCBI; Aubin <i>et al.</i> , 2005; Lamari <i>et al.</i> , 2013; Standen <i>et al.</i> , 2013)
<i>Phaeobacter</i>	Seawater, scallops, culture tanks of turbot	Scallops, oysters, cod	Feed additive, incubation water, single	<i>Vibrio</i>	Disease resistance, survival	(Ruiz-Ponte <i>et al.</i> , 1998; Ruiz-Ponte <i>et al.</i> , 1999; Helm <i>et al.</i> , 2004; Martens <i>et al.</i> , 2006; Porsby <i>et al.</i> , 2008; Kesarodi-Watson <i>et al.</i> , 2012; D'Alwaise <i>et al.</i> , 2013)

<i>Pseudalteromonas</i>	Abalone, cod, marine biofilm	Abalone, scallops, oyster, cod	Feed additive, incubation water, single	<i>Vibrio</i> or no pathogen tested	Growth, survival	(Leroy <i>et al.</i> , 2008; ten Doeschate and Coyne, 2008; Fjellheim <i>et al.</i> , 2010; Kesarcodi-Watson <i>et al.</i> , 2012)
<i>Pseudomonas</i>	Shrimp, perch, salmon eggs, trout eggs, brackish water, prawn, rohu and probiotic products	Oyster, perch, trout, prawns, salmon eggs, shrimp, rohu	Feed additive, incubation water, single or mixed	<i>Aeromonas</i> , <i>Vibrio</i> , <i>Saprolegnia</i> , <i>Flavobacterium</i> or no pathogen tested	Growth, survival, health, feed utilization, immune response, reduced <i>Saprolegnia</i> hyphal attachment to salmon eggs; disease resistance	(Gram <i>et al.</i> , 1999; Vijayan <i>et al.</i> , 2006; Gobeli <i>et al.</i> , 2009; Hai <i>et al.</i> , 2009; Aguilar-Macias <i>et al.</i> , 2010; Pai <i>et al.</i> , 2010; Campa-Córdova <i>et al.</i> , 2011; Giri <i>et al.</i> , 2011; Korkea-aho <i>et al.</i> , 2011; Giri <i>et al.</i> , 2012; Korkea-aho <i>et al.</i> , 2012; Liu <i>et al.</i> , 2015)
<i>Rhodopsudomonas</i>	Carp pond	Tilapia	Incubation water, single	No pathogen tested	Growth, immune response, weight gain	(Zhou <i>et al.</i> , 2010)
<i>Roseobacter</i>	Turbot hatchery tank	Turbot	Feed additive, single	<i>Vibrio</i>	Survival	(Planas <i>et al.</i> , 2006)
<i>Ruegeria</i>	Cod	Cod	Incubation water, single	No pathogen tested	Survival	(Fjellheim <i>et al.</i> , 2010)
<i>Shewanella</i>	Seabream, carp, abalone	Sole, seabream, carp, abalone	Feed additive, single or mixed	<i>Photobacterium</i> , <i>Aeromonas</i> , <i>Vibrio</i> or no pathogen tested	Growth, disease resistance, stress tolerance, survival, immune response	(Chabrillón <i>et al.</i> , 2005; Varela <i>et al.</i> , 2010; García de la Banda <i>et al.</i> , 2012; Jiang <i>et al.</i> , 2013; Wu <i>et al.</i> , 2015)
<i>Streptococcus</i>	Shrimp	Shrimp	Feed additive, single	<i>Vibrio</i>	Survival	(Swain <i>et al.</i> , 2009)
<i>Streptomyces</i>	Marine sediment, estuary sediment	<i>Artemia</i> , shrimp	Feed additive, incubation water, single	<i>Vibrio</i> or no pathogen tested	Growth, survival, water quality	(Das <i>et al.</i> , 2006; Das <i>et al.</i> , 2010)
<i>Synechocystis</i>	Seawater	Shrimp	Feed additive, single	<i>Vibrio</i> or no pathogen tested	Survival, reduced pathogen load	(Preetha <i>et al.</i> , 2007)
<i>Vagococcus</i>	Sole	Sea bass	Feed additive, single	<i>Vibrio</i>	Survival	(Sorroza <i>et al.</i> , 2012)
<i>Vibrio</i>	Trout, cod, macroalga, prawn egg, abalone, shrimp hatchery or unknown	Salmon, trout, cod, shrimp, abalone, prawn	Incubation water, feed additive, single or mixed	<i>Aeromonas</i> , <i>Vibrio</i> or no pathogen tested	Growth, survival, weight gain, immune response, water quality	(Austin <i>et al.</i> , 1995; Irianto and Austin, 2002a, b; Macey and Coyne, 2005; Fjellheim <i>et al.</i> , 2010; Majeed Rahman <i>et al.</i> , 2010; Thompson <i>et al.</i> , 2010; Silva-Acaries <i>et al.</i> , 2011; Fatmahanman <i>et al.</i> , 2015; Huddy and Coyne, 2015)
<i>Weissella</i>	Flounder	Flounder	Feed additive, single	No pathogen tested	Growth, disease resistance	(Byun <i>et al.</i> , 1997; Cai <i>et al.</i> , 1998)
<i>Yarrowia</i>	Unknown	Oyster	Feed additive, single	No pathogen tested	Growth, survival	(Aguilar-Macias <i>et al.</i> , 2010)
<i>Zooshikella</i>	Sea sediment	Flounder	Feed additive, single	<i>Streptococcus</i>	Growth, immune response, disease resistance	(Kim <i>et al.</i> , 2010)
Mixture of <i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Bifidobacterium</i> , <i>Enterococcus</i>	Unknown	Seabream	Feed additive (and incubation water), single	No pathogen tested	Growth	(Suzer <i>et al.</i> , 2008)
<i>Lactobacillus</i> , <i>Rhodopsudomonas</i> and <i>Bacillus</i>	Shrimp and carp ponds	Shrimp	Incubation water, single	No pathogen tested	Weight gain	(Wang and Gu, 2010)
Unidentified Gram-positive coccus A1-6	Trout	Trout	Feed additive, single or mixed	<i>Aeromonas</i>	Survival, immune response	(Irianto and Austin, 2002a, b)

*Mixed: in combination with other microbes or chemical compounds

** The prime sources of the contents and references of the table are Newaj-Fyzul *et al.* (2014), Hai (2015) and Verschuere *et al.* (2000).

Thesis outline

Among the emerging diseases, Saprolegniosis is causing substantial declines in fish and amphibian populations in natural ecosystems and aquaculture. The overall aim of the research described in this thesis was to decipher the microbiome of Atlantic salmon eggs and to investigate the diversity and activity of commensal microbes of fish eggs. This knowledge is instrumental for designing strategies to combat Saprolegniosis and other emerging diseases in aquaculture and natural habitats.

In this introductory chapter (**Chapter 1**) I gave an overview of the current status of common and emerging aquatic diseases and the current control measures. To mitigate emerging aquaculture diseases in a sustainable manner, there is an increasing interest in habitat bioaugmentation to protect fish against emerging pathogens. Understanding the impact of microbiomes on growth and health of their aquatic hosts might provide solutions to mitigate aquatic diseases. I have summarized the current state-of-the-art of fish microbiome research with a focus on the role of microbiomes in protection of fish and other aquatic animals against common and emerging diseases.

Chapter 2 presents the meta-taxonomic analyses of the oomycete, fungal, bacterial and archaeal communities associated with *Saprolegnia*-infected (diseased) and non-infected Atlantic salmon (*Salmo salar*) eggs from a commercial fish hatchery. While pathogenic *Saprolegnia* species were identified in both diseased and healthy salmon egg samples, a low incidence of Saprolegniosis was strongly correlated with a high abundance and richness of commensal Actinobacteria. Subsequent isolation of Actinobacteria and functional bioassays showed that an Actinobacterial isolate, classified as *Fron dihabitans* (Microbacteriaceae), significantly inhibited hyphal attachment of *S. diclina* to live salmon eggs while other bacterial genera were not able to provide protection.

Chapter 3 describes the results of a more comprehensive study aimed at unravelling the diversity, bioactivity and phylogeny of aquatic *Fron dihabitans* bacteria associated with healthy salmon eggs. The results showed that most *Fron dihabitans* isolates inhibited growth *in vitro* of *S. diclina*, formed biofilms and had extracellular protease and lipase activity at low temperature (5 °C). The *Fron dihabitans* sp. 762G35 that inhibited hyphal attachment of *S. diclina* to salmon eggs was selected for genome sequencing. An in-depth study was conducted to further analyse the genome, metabolite profile and bioactive compounds of *Fron dihabitans* sp. 762G35.

Since the meta-taxonomic analyses in chapter 2 revealed that the Proteobacteria (*Pseudomonas*) represented one of the most abundant bacterial genera associated with salmon eggs, the diversity and activity of aquatic *Pseudomonas* species and their metabolic potential to suppress Saprolegniosis on salmon eggs were investigated. As shown in **Chapter 4**, no distinct difference was observed in the frequencies of *Pseudomonas* isolates from diseased or healthy salmon eggs with *in vitro* activities against *Saprolegnia*. However, a significantly higher frequency of biosurfactant-producing *Pseudomonas* was found among the isolates from healthy salmon eggs. *Pseudomonas* isolate H6 consistently inhibited hyphal attachment of *Saprolegnia diclina* to live salmon eggs and significantly reduced salmon egg mortality caused by *Saprolegnia*. *Pseudomonas* H6 produces a viscosin-like biosurfactant, which inhibited hyphal growth of *S. diclina* and *S. parasitica* *in vitro*, but lacked significant effects *in vivo*.

Chapter 5 describes the isolation and characterization of salmon egg-associated fungi. The results showed that most isolates were closely related to *Microdochium lycopodium*/*Microdochium phragmitis*, and also to *Trichoderma viride*. The analyses revealed both qualitative and quantitative differences in the *Trichoderma* community associated with diseased and healthy salmon eggs, with a higher abundance on healthy salmon eggs. These differences were not observed for the *Microdochium* community and only few isolates showed *in vitro* antagonistic activity against *S. diclina*. For most *Trichoderma* isolates, the typical mycoparasitic coiling around and/or formation of papilla-like structures on *S. diclina* hyphae were observed, suggesting attachment of *Trichoderma* to *S. diclina* hyphae and mycoparasitism.

In **Chapter 6**, the major findings of this research are summarized and the potential application of beneficial microbes to mitigate Saprolegniosis and other emerging aquatic diseases is discussed.

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Chapter 2

Deciphering microbial landscapes of fish eggs to mitigate emerging diseases

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Abstract

Animals and plants are increasingly suffering from diseases caused by fungi and oomycetes. These emerging pathogens are now recognized as a global threat to biodiversity and food security. Among oomycetes, *Saprolegnia* species cause significant declines in fish and amphibian populations. Fish eggs have an immature adaptive immune system and depend on nonspecific innate defences to ward off pathogens. Here, meta-taxonomic analyses revealed that Atlantic salmon eggs are home to diverse fungal, oomycete and bacterial communities. Although virulent *Saprolegnia* isolates were found in all salmon egg samples, a low incidence of Saprolegniosis was strongly correlated with a high richness and abundance of specific commensal Actinobacteria, with the genus *Fronihabitans* (Microbacteriaceae) effectively inhibiting attachment of *Saprolegnia* to salmon eggs. These results highlight that fundamental insights into microbial landscapes of fish eggs may provide new sustainable means to mitigate emerging diseases.

Introduction

Fungal diseases that were previously not considered as major threats to ecosystem functioning are now causing severe ecological disruption (Fisher *et al.*, 2012). Among the fungi, *Fusarium solani* is causing mass mortality in eggs of endangered sea turtles in Cape Verde (Sarmiento-Ramirez *et al.*, 2010), and *Batrachochytrium dendrobatidis* and *Batrachochytrium salmandrivorans* are involved in major amphibian declines worldwide (Woodhams *et al.*, 2011; Martel *et al.*, 2013). Among the fungal-like oomycetes, *Aphanomyces astaci*, *Aphanomyces invadans* and *Saprolegnia* species are causing significant reductions in crayfish, fish and amphibian populations (Fernández-Benítez *et al.*, 2008; Phillips *et al.*, 2008; Krugner-Higby *et al.*, 2010; Bruno *et al.*, 2011; Van den Berg *et al.*, 2013), respectively. Saprolegniosis is a major disease problem in different wild and farmed fish species, including salmonid species such as Atlantic salmon, rainbow and brown trout, and non-salmonid species including perch, eels and catfish (Bruno *et al.*, 2011). This disease is characterized by white and grey patches of mycelial growth on the skin and fins of adult fish, and cotton-like filamentous mycelium on eggs. In fish, death often occurs from disruption of the osmotic balance (haemodilution) following destruction of large areas of the epidermis by massive, invasive hyphal growth (Bruno *et al.*, 2011). Fish eggs on the other hand are thought to be killed by hyphal breaching of the chorionic membrane regulating the osmosis of the embryo. *Saprolegnia* species can produce flagellated zoospores to disperse in the aquatic environment. *Saprolegnia* also forms secondary zoospores and secondary cysts that, for some species, contain both hooks that are

presumed to aid in attachment to the fish skin or promote floating (buoyancy) in water (Van den Berg *et al.*, 2013). Losses resulting from Saprolegniosis average 10% in eggs and young fish, but losses of up to 50% have been reported (Hatai and Hoshiai, 1992, 1994; Bruno *et al.*, 2011; Van den Berg *et al.*, 2013).

To control and prevent the spread of emerging pathogens, several conservation and disease mitigation strategies have been proposed because a singular solution is doubtful given the rich diversity of fish and amphibian habitats (Woodhams *et al.*, 2011). In aquaculture, immunization and chemical control are among the preferred approaches to mitigate diseases (Bruno *et al.*, 2011; Van den Berg *et al.*, 2013). For Saprolegniosis, however, no effective immunization is currently available and nonspecific chemical agents such as malachite green and formalin have been or will be banned, leading to a dramatic re-emergence of Saprolegniosis. Habitat bioaugmentation and introduction of protective microbiota have been proposed as potential strategies to rescue and protect fish and amphibians from emerging disease. Over the past years, several studies have unequivocally shown that microbes play a pivotal role in the protection of eukaryotes (humans, plants, insects) against pathogens (Round *et al.*, 2010; Mendes *et al.*, 2011; Weiss and Aksoy, 2011; Everard *et al.*, 2013; Stecher *et al.*, 2013; Turner *et al.*, 2013). For fish and amphibians, microbiome studies have only been initiated recently (Cahill, 1990; Hansen and Olafsen, 1999; Schulze *et al.*, 2006; McKenzie *et al.*, 2012). Several studies have indicated that specific bacteria can contribute to the protection of fish and amphibians against fungal and oomycete pathogens. This was shown for *Janthinobacterium lividum*, a bacterium that was isolated from frog skin and provided protection against *B. dendrobatidis* (Harris *et al.*, 2009).

Here, we deciphered the microbiome of Atlantic salmon eggs and determined how Saprolegniosis affects the structure (richness, evenness) of fungal, oomycete and bacterial communities, with the ultimate goal to identify commensal microbes that prevent or delay disease onset and/or development.

Materials and Methods

Salmon egg sample collection for microbial community analysis

Atlantic salmon (*Salmo salar* L.) eyed eggs and the incubation water from each of the tanks were collected separately from a commercial hatchery in Scotland. The eggs were spawned in November 2010 and incubated in a flow-through system at a flow rate of 25-30 l s⁻¹ at 3-5 °C. The water source for the incubation of the salmon eggs is a catchment area that is an upland peat moor with conifer plantations, grazing cattle, sheep and wild animals, ~3 km from the hatchery and is delivered via a hydroelectric scheme using a hill loch as a source. Before the water enters the egg incubators, it is filtered through a 10-µm filter and a carbon filter and is subsequently treated with ultraviolet light for a few seconds. Eggs received a 1-h 1000 p.p.m. formalin bath treatment weekly, and were collected on 14 February 2011. For more details on each of the 12 egg samples, see Table S1. The microbial community was obtained by shaking the eggs and incubator water at 180 r.p.m. at 6 °C for 12 h, and subsequent centrifugation of the water at 24 000 ×g at 4 °C for 20 min. Cell pellets were resuspended in sterile demineralized water, concentrating the original sample 30-80 times, and stored at -80 °C

before DNA isolation. One aliquot was stored at -80 °C in 20% (v/v) glycerol for bacterial isolation (Figure S1).

Scanning electron microscopy

A selection of salmon egg samples showing visual signs of *Saprolegnia* infection were collected and stored in a 1% glutaraldehyde and 4% formalin solution. Subsequently, samples were fixed in 2.5% glutaraldehyde and then treated with 1% osmium tetroxide for 1 h. Samples were dehydrated with alcohol and subsequently dried using hexamethyldisilazane. Dried samples were coated in gold using an EMitech Limited (Ashford, UK) K550 sputter coater. Coated samples were viewed in a Zeiss (Oberkochen, Germany) EVO MA10 scanning electron microscope at 10 kV.

Metagenomic DNA isolation and storage

DNA was isolated from the pellet suspensions (described above) in duplicate with PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Duplicate DNA extracts were pooled and an aliquot was analysed on a 0.8% (w/v) agarose gel with SmartLadder MW-1700-10 (Eurogentec, Seraing, Belgium) as a size marker. The DNA concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were stored at -80 °C.

PhyloChip analysis

PhyloChip analysis of the bacterial and archaeal community structure of each sample (~0.5 µg DNA) was conducted at Second Genome (South San Francisco, CA, USA). Sample and data processing and data reduction were performed as described previously (Hazen *et al.*, 2010). Annotation of operational taxonomic units (OTUs) was performed based on the Greengenes database (McDonald *et al.*, 2012). Cluster analysis with Bray-Curtis similarity measures was performed in Primer-E v6.1.13 (Primer-E Ltd, Ivybridge, UK). Nonmetric multi-dimensional scaling and analysis of similarity (Ramette, 2007) were performed in Paleontological Statistics freeware package (PAST v2.10, <http://folk.uio.no/ohammer/past/>) (Hammer *et al.*, 2001).

Clone library sequencing of oomycete and fungal communities

The internal transcribed spacer (ITS) region of fungal and oomycete rRNA genes was PCR-amplified with primers ITS1F and ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993), and Oom-Up5.8S (5'-TGCGATACGTAATGCGAATT-3') and Oom-Lo28S (5'-ACTTGTTCGCTATCGGTCTCGCA-3') (Mazzola *et al.*, 2002; Tambong *et al.*, 2006), respectively. Amplification reactions were conducted in 50 µl volumes with Taq DNA polymerase (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions with 1 µl DNA (5-10 ng), 1 cycle at 94 °C for 3 min, 40 cycles at 94 °C for 1 min, 50 °C for 45 s, 72 °C for 1 min and 1 cycle at 72 °C for 7 min. The dominant representatives of fungal and oomycete populations were determined by sequencing clone libraries. Amplicons were cloned into the TOPO-TA vector and transformed into chemically competent *Escherichia coli* Top10 cells according to the manufacturer's protocol (Invitrogen). Inserts were PCR amplified from intact cells using primer pair M13F/M13R and purified with ExoSAP-IT (USB, Cleveland, OH, USA) according to the manufacturer's instructions. A total of 20 and 5-6 clones per sample were selected for fungal and oomycete amplicon sequencing, respectively, with primer T7 and GenomeLab DTCS Quick Start Kit using the Beckman Coulter CEQ 8000 Genetic Analysis system (Beckman Coulter, Brea, CA, USA). Sequences of at least 400 bp were analysed by BLASTn (Altschul *et al.*, 1990) on the National Center for Biotechnology Information (NCBI) database website (www.ncbi.nlm.nih.gov/). A cutoff of >93% identity and a 400-bp alignment length were used for identification (Pounder *et al.*, 2007; Nilsson *et al.*, 2008).

T-RFLP analysis of fungal community

Fluorescently labelled PCR products of the ITS region were generated using the D4-labelled ITS1F primer in conjunction with D3-labelled ITS4 primer. PCR was performed in quadruplicate 25 µl reactions containing 1× Promega PCR Master mix (Promega, Madison, WI, USA), 200 mM primer, 4 µg µl⁻¹ bovine serum albumin and 0.1-0.5 µl DNA template (0.5-5 ng) with above-mentioned cycling conditions. PCR products were digested in duplicate reactions with 10 U of the restriction endonucleases *Hae*III and *Hha*I at 37 °C for 6 h. Restriction enzymes were heat inactivated at 80 °C for 10 min. Restriction fragments were purified by ethanol precipitation and fragments were resuspended in 40 µl of sample loading solution and 0.25 µl 600 size standards. Terminal restriction fragment length polymorphism (T-RFLP) fragments were separated using the CEQ 8000 Genetic Analysis System with denaturation for 120 s at 90 °C, injection for 15 s at 2 kV, separation for 90 min at 4.8 kV and a capillary temperature of 50 °C. T-RFLP profiles were analysed using the Fragment Analysis Module of the CEQ 8000 Genetic Analysis Software, with a slope threshold of 10.1% relative peak height threshold, 95% confidence level, quartic calibration curve with PA, ver. 1, dye mobility calibration and calculated dye spectra. The baseline value was set to exclude background peaks that commonly occurred in tandem with the DNA standard peaks. Fragments were binned with a 1-bp margin using the AFLP tool in the Fragment Analysis software. Profiles that consisted of mean fragment length and fluorescence intensities were generated for each sample. As different amounts of DNA were used for amplification between samples, no accurate analysis of T-RF abundance could be carried out and we analysed the data only on the presence/absence of T-RF peaks. Cluster analysis was performed as mentioned above.

Isolation of oomycetes and fungi from salmon eggs

Per sample, one to two salmon eggs were placed on potato dextrose agar (PDA, Oxoid, Hampshire, UK). The cultures were incubated for 2 weeks at room temperature (20±1 °C) and for 17-18 days at 18 °C. The oomycete and fungal isolates were purified by subculturing hyphal tips three times on fresh PDA supplemented with 100 µg ml⁻¹ streptomycin and 50 µg ml⁻¹ tetracycline.

ITS sequencing and phylogenetic analysis of oomycete isolates

For genomic DNA extraction, hyphae were harvested in 90 µl of 0.5 M NaOH and disrupted by Mixer Mill MM400 (Retsch, Haan, Germany) at a frequency of 30/s for 35 s in the presence of one sterile glass bead. The tube was placed at room temperature for 30 min to 24 h until complete cell wall disruption. The hyphal suspension was diluted with sterile 0.1 M Tris-HCl (pH 7.0) and ITS rRNA genes were amplified with ITS1 and ITS4 primers in 25 µl volumes, each consisting of 2 µl of diluted DNA template with GoTaq DNA polymerase (Promega) according to the manufacturer's protocol. The amplification program consisted of 1 cycle at 94-95 °C for 2-5 min, 30-35 cycles at 94 °C for 1 min, 55 °C, 60 °C or 65 °C for 30-60 s, 72 °C for 30-120 s and 1 cycle at 72 °C for 5-10 min. PCR products were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Contigs were created from the sequence fragments using Geneious v6.1.4 (Kearse *et al.*, 2012) and aligned using MAFFT v7.0 with G-INS-i algorithm using default settings (Katoh and Standley, 2013), including previously identified reference sequences (Sandoval-Sierra *et al.*, 2013). Two different approaches were applied: Bayesian inferences and Maximum Likelihood inference. Bayesian inference analysis with Markov-chain Monte Carlo, MrBayes v3.2.1 (Ronquist *et al.*, 2012), was run at temperature parameter of 0.2, sample frequency of 1000, and three independent runs with two hot and six cold chains each for 20 million generations. Estimation of the effective samples size in the Markov-chain Monte Carlo and determination of a suitable burn-in time was checked with Tracer v1.5 (Rambaut and Drummond, 2007). A burn-in of 2 million generations was discarded after checking for stability on the log-likelihood curves, and the remaining trees from the independent runs were combined to build a 50% majority-rule consensus tree. The evolutionary model used for the molecular data in MrBayes was HKY+G and was obtained by running the data sets in jModelTest 2 (Darriba *et al.*, 2012). Maximum Likelihood inference analysis was carried out with RaxmlGUI v7.4.2 (Silvestro and Michalak, 2012) with a random starting tree, included the GTRGAMMA option and employed the rapid hill-climbing algorithm (Stamatakis *et al.*, 2007). Clade support was assessed with 1000 bootstrap replicates, with the rapid-hill climbing algorithm (Stamatakis *et al.*, 2008). Phylogram trees were visualized with FigTree (tree.bio.ed.ac.uk/software/figtree/).

Isolation, *in vitro* activity and identification of Actinobacterial isolates

Cell pellets obtained from the salmon eggs samples and stored in 20% glycerol at -80 °C were resuspended in sterile demineralized water and dilution plated on humic acid-vitamin (HA) and starch agar (GA) modified from (Zhang, 1990). Plates were incubated at 30 °C and bacterial colony-forming units were determined after 6 weeks. HA medium, modified from Hayakawa and Nonomura (1987), contained 1.0 g l⁻¹ humic acid (dissolved in 10 ml of 0.2 N NaCl), 0.5 g l⁻¹ Na₂HPO₄, 1.71 g l⁻¹ KCl, 0.05 g l⁻¹ MgSO₄·7H₂O, 0.01 g l⁻¹ FeSO₄·7H₂O, 0.02 g l⁻¹ CaCO₃ and 18.0 g l⁻¹ agar. Starch agar (GA) (Zhang, 1990) contained 15 g l⁻¹ potato starch (soluble starch), 0.5 g l⁻¹ KNO₃, 0.5 g l⁻¹ K₂HPO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 5.0 g l⁻¹ NaCl, 5.0 g l⁻¹ KCl, 0.01 g l⁻¹ FeSO₄·7H₂O and 18.0 g l⁻¹ agar. Both HA and GA were pH adjusted to 7.0-7.4 and were supplemented with 1 ml l⁻¹ of B vitamin solution, 20 µg ml⁻¹ nalidixic acid, 20 µg ml⁻¹ Trimethoprim and 100 µg ml⁻¹ Delvocid after autoclaving. B vitamin solution (Nonomura and Ohara, 1969) (suspended in Milli-Q water) included 0.5 mg ml⁻¹ of thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine hydrochloride, *myo*-inositol, calcium pantothenate and 4-aminobenzoic acid and 0.25 mg ml⁻¹ D(+)-biotin. From each sample, 40 single colonies were randomly isolated at different time points from both media and purified on GA or tryptone soya broth (Oxoid) supplemented with 10% sucrose (TSBS) with or without 1.8% (w/v) agar. Isolates were stored at -20 °C and -80 °C in 20-40% glycerol. *In vitro* antagonistic activity of the isolates against *Saprolegnia diclina* 1152F4 was determined by placing an agar plug of each Actinobacterial isolate at the edge of 1/5th strength PDA plates. After incubation at 30 °C for 7-10 days, an agar plug from a fresh *S. diclina* 1152F4 culture was placed in the centre of the plate. Plates were incubated for 4-5 days at 25 °C and inhibition of hyphal growth was scored. For DNA isolation, Actinobacterial cells were disrupted by incubation of 50 µl TSBS culture at 95 °C for 10 min and PCR was performed with Actinobacterial-specific primers Com2xf (5'-AAACTCAAAGGAATTGACGG-3') and Ac1186r (5'-CTTCCTCCGAGTTGACCC-3') (Schäfer *et al.*, 2010). Amplification reactions were conducted in 25 µl volumes using GoTaq DNA polymerase (Promega) according to manufacturer's instructions, with 1 cycle at 95 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 1 cycle at 72 °C for 15 min. All isolates positive for the amplified fragments were sent for 16S rRNA sequencing by Macrogen Inc. Sequence identity was obtained by comparison with sequences deposited in the Greengenes database (McDonald *et al.*, 2012) (<http://greengenes.lbl.gov/>) and Ribosomal Database Project (Cole *et al.*, 2009) (RDP, <http://rdp.cme.msu.edu/>). Among the 526 Actinobacterial OTUs with significant differential abundance based on the PhyloChip analyses, the 16S rRNA sequences (≥850 bp) of 413 OTUs were downloaded from the Greengenes database. These sequences were subjected to phylogenetic analysis with ClustalW in MEGA5 (Tamura *et al.*, 2011) to generate a neighbour-joining (Saitou and Nei, 1987) consensus tree with 1000 bootstrap replicates (Felsenstein, 1985). The evolutionary distances were analysed by Tamura-Nei model (Tamura and Nei, 1993) and the evolutionary rates among sites were modelled by a gamma distribution.

Salmon eggs for *in vivo* experiments

Atlantic salmon (*Salmo salar* L.) eyed eggs of strain AquaGen Atlantic QTL-innOva IPN from AquaGen AS (Trondheim, Norway) were used for the *in vivo* experiments. Salmon egg ages at the day of shipment were as follows; for the pathogenicity assay of *Saprolegnia* isolates, the eggs were 385 degree-days; for the *in vivo* assays with the Actinobacterial isolates, the eggs were 320 degree-days. All egg batches were disinfected during incubation and before transport with buffodine (1:100, 10 min) and treated with formalin according to Aquagen's in-house protocols. Upon arrival, blank, pin-eyed and white eggs were removed if observed.

Preparation of *Saprolegnia* inocula and pathogenicity assays

Live eggs were placed in 97-98 °C sterile distilled water or well water and incubated for 80-150 s. The resulting dead eggs were drained and placed on PDA agar plates overgrown with *Saprolegnia* isolates and incubated overnight at 25 °C. Each egg was then transferred into a well of a 24-well cell-culture plate (Greiner Bio-One, Kremsmünster, Austria) and further incubated for 1 day at 15-25 °C until *Saprolegnia* hyphal colonization was visible. The slow-growing *Saprolegnia* isolates 746F3 and 736F2 were further incubated for 2 and 4 days, respectively. After overnight acclimatization of the live eggs, two *Saprolegnia*-infected dead salmon eggs (inocula) were added to each cup containing 30 live eggs in a similar setup as described in Figure 4c, with the exception that here 3 l of dechlorinated Norwegian

tap water, and not well water, was used. Isolate 736F2 was added to the salmon eggs 2 days later as this isolate was a slow grower. Each treatment consisted of an incubator containing three cups with 30 salmon eggs each. In the control treatment no *Saprolegnia* was added. The water temperature was maintained at 10 ± 1 °C by adding ice blocks into each incubation unit every 12 h. After 8 days post inoculation (6 days post inoculation for isolate 736F2), the mortality percentage of the salmon eggs was determined visually.

In vivo bioassays to test disease suppression by Actinobacteria

The incubation setup (Figure S4) was located in a ventilated room of constant 15 °C ambient temperature. The egg incubation units, containing 2 l of well water (Table S6), were randomly distributed in an incubation tank with circulating water of 5-7 °C. Polystyrene panels were used to cover the incubation tank to maintain darkness and insulation without blocking aeration. Each incubation unit was aerated with an air stone connected to an air pump. Dissolved oxygen levels and temperature, measured with a pH/Oxi 340i multi-parameter instrument (WTW GmbH, Weilheim, Germany), ranged from 101.8-103.4% and from 5.7 °C to 6.4 °C, respectively. Within each egg incubation unit, 51 ± 1 live eggs were placed in perforated plastic cups in triplicate. The cups were stabilized by placing a petri dish (145 mm diameter) and a weight on top. The eggs were introduced into each incubation unit immediately after arrival from Norway and allowed to acclimatize and rehydrate overnight.

Actinobacterial isolates were inoculated in TSBS and incubated at 30 °C at 200 r.p.m. for 2-4 days. Cultures were centrifuged at $3400 \times g$ for 5-15 min at room temperature. The cell pellets were washed and resuspended in sterile well water and OD₆₀₀ values determined by spectrophotometry for the *Fronidhabitans* sp. and *Arthrobacter* sp. For the *Streptomyces* isolates, the OD₆₀₀ could not be determined because of aggregation of cells. Approximate equal amounts of cells were determined based on an estimate of the size of the cell pellet after centrifugation. On day 1, the *Fronidhabitans* sp. and *Arthrobacter* sp. were added to the incubation units at a final concentration of 10^7 cells per ml or 0.5 ml and 1.5 ml of *Streptomyces* sp. 1 and 2, respectively. In the consortium of all four isolates, one quarter of the cell suspensions of each bacterium was added. After 3 days of incubation, two *S. diclina* 1152F4 inocula were placed at opposite sides in each cup. As control treatments, salmon eggs were treated with *S. diclina* only or with malachite green and *S. diclina*. Each treatment was conducted in duplicate, that is, two independent experimental incubation units with three technical replicates per incubation unit. For those eggs that received malachite green treatment, the eggs were placed in 1.5 l of 2 p.p.m. malachite green solution for 60-70 min with aeration. Subsequently, eggs were gently rinsed in 500 ml fresh well water and placed back into their incubation units. This was repeated every 4 days, replacing the incubation unit water with fresh well water. Hyphal expansion was photographed and assessed every other day. Based on the photos of each group of eggs, ImageJ v1.44n (Schneider *et al.*, 2012) was used to measure the diameter of hyphal expansion. As a proxy for infection of salmon eggs by *Saprolegnia*, we determined hyphal attachment by lifting infection inocula with a pair of tweezers and counting the number of eggs attached to the hyphal patch (Movie S1). Hyphal attachment was determined at day 14 (10 days post inoculation) when attachment in the control, *S. diclina* only, was >50% (Table S5).

Nucleotide sequence accession numbers

The sequences of the 16S rRNA sequences of *Fronidhabitans* sp., *Arthrobacter* sp. and *Streptomyces* sp. 1 and 2 have been deposited in GenBank under accession numbers KF741274, KF741275, KF741276 and KF741277, respectively.

Colonization of Actinobacteria on the egg surface

Colonization of the applied Actinobacterial isolates on the salmon egg surfaces was determined by harvesting one to two eggs from each cup at 0 and 10 days post inoculation, rinsing with 1 ml of sterile well water three times, draining briefly on sterile filter paper and rolling gently over the surface of HA and GA agar plates (Movie S2). All plates were incubated at 30 °C for 5-6 weeks, and 2-8 colonies that were morphologically similar to each of the four isolates used for inoculation were purified and subjected to 16S rRNA sequencing.

Statistical analyses

For each experiment, obtained data were tested for normal distribution and homogeneity of variances before conducting parametric or nonparametric statistical analyses. Disease incidence of salmon egg samples was analysed with a Mann-Whitney *U*-test ($P<0.05$). The pathogenicity of *Saprolegnia* isolates were compared with one-way analysis of variance and with *post hoc* Dunnett analysis ($P<0.05$). Cluster analysis and nonmetric multidimensional scaling and analysis of similarity of T-RFLP-derived data for the fungal community and PhyloChip-derived data for the bacterial and archaeal communities were conducted using Bray-Curtis similarity measures in Primer-E and in PAST. The differences in total number of bacterial and archaeal OTUs associated with diseased versus healthy eggs was analysed by Student's *t*-test ($P>0.05$). Phylogenetic groups containing OTUs that have a significant differential abundance between diseased and healthy eggs were identified with Student's *t*-test ($P<0.005$) in Microsoft Excel. Percentage of hyphal attachment was analysed with Kruskal-Wallis one-way analysis of variance ($P<0.05$). Area under hyphal expansion curve and area under hatching percentage curve of different treatments in the bioassay were compared with Kruskal-Wallis one-way analysis of variance ($P<0.05$). All statistical analyses were performed in IBM SPSS Statistics v20 (Armonk, NY, USA), unless mentioned otherwise.

Results and discussion

We conducted a meta-taxonomic microbiome analysis of 12 salmon egg batches from a Scottish salmon hatchery (Figure S1, Supplementary Methods). In hatcheries, certain salmon egg batches become infected with *Saprolegnia*, whereas others remain unaffected. Here, six of the sampled egg batches were selected based on a high incidence of Saprolegniosis and six based on a low disease incidence (Figure 1 and Figure S1). From this point onwards, these two distinct sample sets are referred to as 'diseased' and 'healthy', respectively. Both sets, containing eggs from the same broodstock, received water from the same source, were all incubated at average temperatures ranging from 3 °C to 5 °C, and were all sampled at ~85 days after fertilization (Table S1). Total DNA was extracted from the microbiota present in the pellets obtained from each of the 12 egg samples and subjected to meta-taxonomic analyses (Figure S1 and Supplementary Methods).

Clone library sequencing of oomycete-specific ribosomal ITS amplicons showed the presence of *Saprolegnia*, *Achlya* and *Aphanomyces* (all belonging to the order Saprolegniales) in both diseased and healthy samples (Figure 2a). *Achlya* species have been described for diseased fish in natural environments (Jeney and Jeney, 1995; Kales *et al.*, 2007; Sosa *et al.*, 2007), and *A. invadans* is a notorious pathogen of different natural and farmed fish species worldwide, except in Europe and South America (Oidtmann, 2012). Both *Achlya* and *Aphanomyces* spp. have been isolated from eggs of sea trout (*Salmo trutta* m. *trutta*) in Polish rivers (Czeczuga *et al.*, 2005) and from eggs of salmonids in Japan (Kitancharoen and Hatai, 1997). To date, however, no reports have indicated that *Achlya* or *Aphanomyces* are pathogenic for Atlantic salmon. *Achlya* or *Aphanomyces* were not present among the population of oomycetes isolated from the salmon eggs. Therefore, their effects on salmon eggs could not be resolved.

Figure 1 Saprolegniosis in Atlantic salmon (*Salmo salar* L.) eggs. **(a)** Diseased and healthy salmon eggs collected from a hatchery ($N=6$). **(b)** Close-up picture of diseased and healthy salmon egg. **(c)** Scanning electron microscopy (SEM) image of *Saprolegnia*-infected salmon egg showing *Saprolegnia* hyphae (white arrows) and bacteria (orange arrows) covering the salmon egg surface. **(d)** Disease incidence of salmon egg samples collected in the hatchery. Error bars indicate s.e.m. ($N=6$). *Statistically significant difference compared with the diseased samples.

For *Saprolegnia* species, a similar number of sequenced clones was found in diseased and healthy eggs (52% and 50%, respectively; Figure 2a). To better characterize the *Saprolegnia* species, eggs from both sample sets were placed on agar media to allow outgrowth of the oomycetes. Subsequent purification and ITS sequencing of 31 randomly selected oomycete isolates showed that the majority were *S. diclina*, with no distinct phylogenetic differences between the *Saprolegnia* isolates from diseased and healthy samples (Figure 2b). Furthermore, when *Saprolegnia* isolates from the different phylogenetic clades were tested for pathogenicity on salmon eggs, equal levels of mortality were observed for eggs inoculated with *Saprolegnia* isolates from diseased or from healthy egg samples (Figure 2b). Collectively, these results indicate that the difference in incidence of Saprolegniosis between the two sets of salmon egg samples from the commercial hatchery cannot be explained by differences in population structure or pathogenicity of the *Saprolegnia* species present in both sample sets.

Hierarchical cluster analyses of T-RFLP data revealed no distinct differences between the fungal community from diseased or healthy salmon eggs (Figure 3b, Figure S2a). Further ITS-clone library sequencing showed that the fungal community associated with diseased and healthy salmon egg samples was dominated by the Ascomycota (Figure 3a), mainly *Microdochium* (Table S2). *Microdochium* species, known as snow moulds (Matsumoto, 2009), are pathogenic to cereals but also contain endophytic members that may have beneficial effects on plants (Ernst *et al.*, 2011). For aquatic ecosystems, however, no records exist of the presence of *Microdochium*. *Chytriumyces* was only detected in the diseased egg samples, and *Mortierella* and *Microdochium* species were more abundant in diseased samples (Table S2). These fungi have not been previously associated with salmon eggs or with Saprolegniosis. In addition, *Spirosphaera* and *Saccharicola*, the fungal genera that were only detected in healthy egg samples, have not yet been described for salmon eggs. *Spirosphaera* spp. are known to decompose submerged plant litter (Voglmayr, 2004), whereas some *Saccharicola* species (*S. bicolor*) cause disease in sugarcane (Eriksson and Hawksworth, 2003).

PhyloChip (DeAngelis *et al.*, 2009; Hazen *et al.*, 2010)-based meta-taxonomic analysis of the bacterial community revealed a total of 31 278 bacterial and 3 archaeal OTUs. Although 16S rDNA amplicon pyrosequencing is less biased towards detecting unknown species (DeSantis *et al.*, 2007; Kunin *et al.*, 2010), the PhyloChip technique allows detection of a large number of bacterial taxa including less abundant taxa. A study on lignin-degrading microbes showed that the microbial community profiles obtained by the PhyloChip technique and pyrosequencing were well comparable (DeAngelis *et al.*, 2011). The overall distribution of the four most dominant bacterial phyla for the salmon egg samples ranged from 9% for the Bacteroidetes to 12%, 24% and 44% for the Actinobacteria,

Figure 3 Fungal, bacterial and archaeal community analyses by clone library sequencing, T-RFLP and PhyloChip analyses. **(a)** Relative abundance of fungal phyla from *Saprolegnia* diseased and healthy salmon eggs as determined by sequence analysis of clones containing inserts of the ITS region. **(b)** Cluster analysis (Bray-Curtis similarity) of T-RFLP-derived data for the fungal community associated with diseased (●) and healthy (○) salmon eggs. **(c)** Number of OTUs of bacterial and archaeal taxa in diseased and healthy salmon egg samples as determined by number of OTUs passing stage 1 and 2 PhyCA. Error bars indicate s.e.m. ($N=6$). **(d)** Cluster analysis (Bray-Curtis similarity) of bacterial and archaeal OTUs associated with diseased (●) and healthy (○) salmon egg samples ($N=6$). **(e)** Phylogenetic groups containing OTUs that have a significant differential abundance between diseased and healthy salmon eggs.

Firmicutes and Proteobacteria, respectively (Figure 3c). A number of OTUs (4684) were exclusively found in healthy samples, especially taxa belonging to the phylum Actinobacteria, that is, Streptomycetaceae, Microbacteriaceae and Micrococcaceae (Figure 3c and Table S3). Along with differences in richness, the relative abundance of the detected bacterial taxa also resulted in a clear discrimination between the diseased and healthy samples, except for sample 12 (healthy) that appeared to be more distinct from all other (healthy) samples (Figure 3d and Figure S2b). A total of 4029 OTUs were significantly different in abundance between the diseased and healthy egg samples (Figure 3e). OTUs that were significantly more abundant in the diseased samples belonged to the Enterobacteriales, Clostridiales, Alteromonadales, Vibrionales and Aeromonadales (Figure 3e) and comprised several bacterial pathogens, including *Vibrio* spp. (Frans *et al.*, 2011), *Aeromonas* spp. (Beaz-Hidalgo and Figueras, 2013) and *Yersinia* spp. (Tobback *et al.*, 2007) that are common to salmon and other fish species. It is generally believed that *Saprolegnia* behaves as an opportunistic pathogen, infecting upon injury, stress or other infections (Stueland *et al.*, 2005; Bruno *et al.*, 2011). However, several papers have also reported that *Saprolegnia* acts as a primary infection agent in eggs and adult fish (Bruno *et al.*, 2011). Thus, whether these potentially pathogenic bacterial taxa were initially present and predisposed the eggs for *Saprolegnia* infection or infection of the salmon eggs by *Saprolegnia* led to proliferation of these opportunistic pathogenic bacteria remains to be resolved.

Most of the OTUs that were significantly more abundant in healthy samples belonged to the orders Actinomycetales and Burkholderiales (Figure 3e). In fungal gardens of ants, Actinobacteria were identified that protect the ant colony from a parasitic black fungus (*Phialophora* sp.) (Caldera *et al.*, 2009). Actinobacteria are prolific producers of an array of antimicrobial compounds and have been suggested as probiotic agents in aquaculture (Verschuere *et al.*, 2000; Das *et al.*, 2008; Dharmaraj, 2010). To study the potential role of the differentially abundant Actinobacterial taxa in protection of salmon eggs against *Saprolegnia* infections (Figure 3e), a total of 354 Actinobacteria were isolated from the diseased and healthy egg samples on two different media that are semiselective for Actinobacteria (Table S4). Isolates were subcultured, purified, characterized by 16S rRNA sequencing and tested for *in vitro* and *in vivo* activity against *S. diclina*. Most of the cultured isolates belonged to the families Microbacteriaceae, Micrococcaceae and Streptomycetaceae and, considering their 16S rRNA-based phylogeny, were in the same clades as the differentially abundant OTUs detected by the culture-independent PhyloChip analysis (Figure S3). OTUs detected only by PhyloChip and not by isolation belonged to Corynebacteriaceae,

Dermabacteraceae, Thermomonosporaceae and Streptosporangiaceae among others.

The results of the *in vitro* dual culture plate assays showed that 6.9% and 12.3% of the Actinobacterial isolates from diseased and healthy samples, respectively, inhibited hyphal growth of *S. diclina*. For the *in vivo* assays with salmon eggs, we selected four bacterial isolates from the obtained Actinobacterial collection belonging to clades with OTUs that were significantly more abundant in the healthy salmon egg samples (Figure 3e and Figure S3). Two isolates belonged to the genera *Fron dihabitans* (Microbacteriaceae) and *Arthrobacter* (Micrococcaceae), and two isolates to *Streptomyces* (Streptomycetaceae) (Figure 4a). The two *Streptomyces* isolates inhibited *in vitro* hyphal growth of *S. diclina*, whereas *Arthrobacter* and *Fron dihabitans* were not inhibitory (Figure 4b). For the *in vivo* assays, salmon eggs were transferred to aerated cups inside a unit cooled to 5-7 °C and preincubated for 3 days with each of the bacterial isolates at an initial density of $\sim 10^7$ cells per ml. Two heat-killed eggs overgrown with *S. diclina* were then placed in each cup serving as the inoculum source for pathogen outgrowth and attachment (Figure 4c and Figure S4). After 10 days of incubation, none of the four selected Actinobacterial isolates reduced radial outgrowth of *S. diclina* from the inoculum source (Figure S5a). However, the *Fron dihabitans* isolate significantly inhibited hyphal attachment to the salmon eggs (Figure 4d and Movie S1), even under high disease levels where malachite green treatment did not provide 100% control (Figure 4d). This suppressive effect was not observed for the *Streptomyces* and *Arthrobacter* isolates. When a consortium of all four isolates was tested, hyphal attachment was inhibited to the same level as obtained with the *Fron dihabitans* isolate alone (Figure 4d). Subsequent analysis of the bacterial community associated with the salmon eggs (see movie S2) showed that, based on colony morphology and 16S rRNA sequencing, the majority of the obtained colonies were indeed the Actinobacterial isolates initially applied to the salmon eggs. In the absence of *S. diclina*, stress-associated hatching (Czerkies *et al.*, 2001) of the salmon eggs was observed for one of the *Streptomyces* isolates but not for *Fron dihabitans*, *Arthrobacter* or other *Streptomyces* isolate (Figures S5b and c). None of the bacterial isolates exhibited adverse effects on alevin survival or morphology (data not shown). Collectively, these results highlight that insights into the microbial landscapes of salmon eggs allows for a taxon-targeted selection of specific commensal bacteria from fish eggs with the potential to mitigate Saprolegniosis.

Fron dihabitans (previously named *Fron dicola*) was originally isolated from fallen leaf litter from a pine forest in Australia (Zhang *et al.*, 2007; Greene *et al.*, 2009) and has also been isolated from lichens (Cardinale *et al.*, 2011) and the rhizosphere of *Peucedanum japonicum* (Lee, 2010). However, association with

Figure 4 Characteristics and *in vivo* testing of selected Actinobacterial isolates to control Saprolegniosis on salmon eggs. **(a)** Morphology of *Frondihabitans*, *Arthrobacter* and two *Streptomyces* isolates. **(b)** *In vitro* antagonism against *Saprolegnia diclina* 1152F4. **(c)** Setup of *in vivo* bioassay. Eggs were inoculated with bacteria and after 3 days of incubation, two *S. diclina*-infected salmon eggs were added at opposite ends of each cup. The diameter of hyphal expansion was determined every 2 days as indicated by the double arrow. **(d)** Percentage of eggs to which *Saprolegnia* hyphae were attached at 10 days post inoculation of the pathogen (see Movie S1). Error bars indicate the s.e.m. ($N=6$). * Statistically significant difference with the control, *S. diclina* only (Kruskal-Wallis, $P<0.05$).

freshwater environments has not previously been reported. The means by which *Frondihabitans* limits hyphal attachment of *S. diclina* to the salmon eggs is not known. The observation that hyphal expansion of the pathogen from the inoculum sources was not inhibited by the *Frondihabitans* isolate (Figure S5a) and that the bacterium did not show *in vitro* antagonism (Figure 4b) suggests that antibiosis via (water-soluble) secondary metabolites is most likely not a key mechanism of

pathogen control. Microscopic analysis further demonstrated that zoospores of *S. diclina* were not negatively affected by the presence of *Fronidhabitans* sp. However, the role of zoospores in pathogenesis is unclear as no zoospore-initiated secondary infection was observed throughout the experiment. Successful colonization of salmon egg surfaces by the *Fronidhabitans* isolate may point to competitive niche exclusion as one of the underlying mechanisms of disease suppression.

The impact of the microbiota on host health and development is gaining increasing attention. For humans, environmental changes or infections can substantially influence the gut microbiome by causing blooms of microbes that are otherwise present at low abundance (Stecher *et al.*, 2013). Human gut microbiomes can significantly drive or suppress disease development (Round *et al.*, 2010; Everard *et al.*, 2013). Although less diverse, the gut microbiome of insects also influences host development by increasing nutrient uptake and stimulating host immunity maturation (Weiss and Aksoy, 2011). Similarly for plants, endophytic, rhizospheric and phyllospheric microbiota play an important role in the protection against biotic and abiotic stress factors (Vorholt, 2012; Bulgarelli *et al.*, 2013; Mendes *et al.*, 2013; Turner *et al.*, 2013). Here we showed a significant correlation between the incidence of Saprolegniosis and the richness and evenness of specific bacterial genera associated with salmon eggs. These results indicate that along with established factors influencing disease susceptibility, such as genetic inheritance (broodstock effect), developmental stage of the salmon eggs, environmental and other stress conditions (Hansen and Olafsen, 1999), the microbiota are also a determining factor in health and disease of fish eggs that have an immature adaptive immune system and depend on other defences for protection. In terms of genetic inheritance, components of the complement immune system were shown to be maternally transferred to the eggs for rainbow trout, zebrafish and amphibian eggs (Lovoll *et al.*, 2006; Poorten and Kuhn, 2009; Wang *et al.*, 2009; Walke *et al.*, 2011) and a paternal genetic component contributed to resistance against *Saprolegnia* in frog eggs (Sagvik *et al.*, 2008). The relative contribution of the genetic inheritance, the fish egg microbiome or their interplay to protect against Saprolegniosis is not clear yet. Hence, our current and future experiments on Saprolegniosis focus on the temporal dynamics of microbial communities on salmon eggs of different broodstocks and how these community changes affect egg and disease development. These studies will give insight into the recruitment of beneficial commensal bacteria, like *Fronidhabitans* and other genera, during egg development and the transfer of specific microbiota via the mother during spawning.

Aquaculture has emerged as the fastest growing animal food-producing sector, in part as a response to regulations that seek to prevent overfishing of wild oceanic

populations (Bruno *et al.*, 2011). Environmental regulations have restricted the use of chemical control agents (malachite green, formalin) in aquaculture production systems and alternative treatments such as hydrogen peroxide, NaCl and seawater flushes are not as effective (Bruno *et al.*, 2011; Van den Berg *et al.*, 2013). Given the importance of aquaculture for long-term food security, there is a strong need for sustainable means to mitigate Saprolegniosis and other emerging diseases. Our results provide a strong basis for the selection of beneficial microorganisms that can act as a first line of defence. Similar strategies can be used to mitigate other diseases including those that threaten wild populations of fish and amphibians.

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Author contributions

YL, IdB., MvdV and JMR designed the project. YL set up experiments, performed DNA extractions, strain isolations and characterization, Phylochip data analysis and *in vitro* and *in vivo* bioassays. MvdV and RM performed the PhyloChip data analysis. ALHJ contributed to the *in vivo* bioassay data collection and analysis. Salmon eggs were sampled from a Scottish hatchery by KD and AHvdB. Scanning electron microscopy analysis was performed by AHvdB and performed in the laboratory of PvW. Pathogenicity assays with the *Saprolegnia* isolates were designed and performed by YL, ET and IS and performed in the laboratory of IS. Phylogenetic analysis of oomycete isolates was performed by VS-S in the laboratory of JD-U. MM performed fungal and oomycete T-RFLP analysis and clone library sequencing. YL, IdB, MvdV, ALHJ, VS-S, and MM analysed data and created figures. IdB, YL and JMR wrote the manuscript with input from all co-authors.

Conflict of interest

All authors declare no conflict of interest.

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Supplementary Figures:

Figure S1 Overall strategy used to decipher the salmon egg microbiome and to identify commensal microorganisms that can mitigate Saprolegniosis.

Figure S2 Non-metric multi-dimensional scaling (NMDS) plots of **a**, Fungal community T-RFLP analysis based on the presence/absence of individual T-RFs, and **b**, Bacterial and archaeal operational taxonomic units (OTUs) in PhyloChip analysis. Ellipses indicate 95% confidence intervals for each treatment. Closed circles: diseased eggs; open circles: healthy eggs. R and p values along with mean ranks of all distances within and between treatments reported for analysis of similarity (ANOSIM) based on 10 000 permutations. No significant difference found between fungal community in diseased and healthy egg batches ($P=0.5802$). Although some overlap was observed ($R = 0.65$), bacterial community were significantly different between diseased and healthy batches of eggs ($P=0.0031$).

Figure S3 Phylogenetic tree of Actinobacterial 16S rRNA sequences (≥ 850 bp) of i) 354 isolates obtained by plating on semi-selective media and ii) 413 PhyloChip detected OTUs with a significant differential abundance between diseased and healthy egg samples. The bootstrap values indicated at the nodes are based on 1 000 bootstrap replicates. Branch values lower than 50% were hidden. Clades indicated in black contain OTUs detected by PhyloChip and isolates obtained by plating on semi-selective media. Clades indicated in red contain OTUs detected by PhyloChip only.

Figure S4 Set-up of the *in vivo* bioassay to determine the disease suppressive activity of the commensal Actinobacterial isolates. White arrows indicate the directions of water recirculation.

Figure S5 a, *Saprolegnia* hyphal expansion from infected salmon eggs in the *in vivo* bioassay. Area under hyphal expansion curve (AUHEC) was calculated and only the *Streptomyces* sp. 2 treatment was significantly different than the control, *S. diclina* only. **b** and **c**, Hatching percentage of salmon eggs. In **b**, eggs are non-treated, treated with only *Saprolegnia*, or with the combination of *Saprolegnia* and bacteria. None of the area under hatching percentage curves (AUHPCs) were statistically significant different compared to the control *S. diclina* alone. In **c**, eggs were non-treated or treated with bacteria. The AUHPC of *Streptomyces* sp. 2 treatment was significantly higher than the non-treated eggs. Statistical analyses were performed with Kruskal-Wallis one-way analysis of variance, $P < 0.05$. The addition of *S. diclina*, bacterial suspensions or the combination of bacterial suspensions and *S. diclina* did not lead to stress-related hatching of salmon eggs in our experimental set-up, except for treatments with isolate *Streptomyces* sp. 2. The survival of eggs treated only with bacteria at 10 dpi ranged between 99.7-100%, indicating that the Actinobacteria tested did not adversely affect the salmon eggs.

Supplementary Tables

Table S1 Details of the salmon egg samples of each incubator on the most recent formalin treatment, incubation temperature, and fertilization day before sampling.

Incubator ID	Health status	Days from last formalin treatment prior to sampling	Average incubation temperature per sample ($^{\circ}\text{C} \pm \text{standard deviation}$)	Average incubation temperature ($^{\circ}\text{C} \pm \text{standard deviation}$)	Days from fertilization (start of incubation) prior to sampling	Average days from fertilization prior to sampling (days $\pm$ standard deviation)
1	Diseased	6	4.1 $\pm$ 1.5	4.4 $\pm$ 1.8	103	86 $\pm$ 9
2		6	3.6 $\pm$ 0.7		87	
3		5	4.5 $\pm$ 1.9		87	
4		13	4.8 $\pm$ 2.0		80	
5		13	4.8 $\pm$ 2.0		80	
6		12	4.8 $\pm$ 2.0		80	
7	Healthy	6	3.5 $\pm$ 0.7	3.5 $\pm$ 0.7	83	84 $\pm$ 2
8		6	3.5 $\pm$ 0.7		83	
9		6	3.5 $\pm$ 0.7		83	
10		6	3.6 $\pm$ 0.7		87	
11		6	3.5 $\pm$ 0.7		83	
12		6	3.5 $\pm$ 0.7		83	

Table S2 Number and classification of fungal clones detected by clone library analysis. Sequences of at least 400 bp were analysed by BLASTn in the NCBI database. As identification criteria, hits should have at least 93% identity and a 400 bp alignment length.

Kingdom	Phyla	Genera	Diseased	Healthy
Fungi	Ascomycota	<i>Microdochium/Monographella</i>	91	48
		<i>Clathrosphaerina</i>	2	-
		<i>Cadophora</i>	1	-
		<i>Geomyces</i>	1	1
		<i>Spirosphaera</i>	-	5
		<i>Saccharicola</i>	-	5
	Zygomycota	<i>Mortierella</i>	14	4
		Zygomycete genus	1	9
	Chytridiomycota	<i>Chytriumyces</i>	13	-
	Basidiomycota	<i>Heterobasidion</i>	1	-
		<i>Cryptococcus</i>	-	3
		<i>Deconica</i>	-	2
	Unknown fungi	-	-	6
Animalia	Arthropoda	<i>Eubosmina</i>	-	1
Unknown organisms	-	-	-	1
Total			124	85

Table S3 Unique and shared number of operational taxonomic units (OTUs) in diseased and healthy salmon egg samples. PhyloChip analysis detected 31 278 bacterial and 3 archaeal OTUs in diseased and healthy salmon egg samples.

Phyla	Families	No. of OTUs		
		Total detected	Diseased only	Healthy only
<u>Unique</u>				
Actinobacteria	Glycomycetaceae	1	1	0
Proteobacteria	Celerinatantimonadaceae	1	1	0
Synergistetes	Thermovirgaceae	1	1	0
Other phyla	unclassified	3	3	0
Actinobacteria	Dermacoccaceae	3	0	3
	Williamsiaceae	2	0	2
	Actinospicaceae	1	0	1
	Dermatophilaceae	1	0	1
	Rarobacteraceae	1	0	1
Cyanobacteria	Scytonemataceae	2	0	2
Firmicutes	Thermodesulfobiaceae	3	0	3
Other	Rs-045	1	0	1
Others	unclassified	21	0	21
<u>Shared</u>				
Proteobacteria	Enterobacteriaceae	1 711	1 628	1 438
	Comamonadaceae	1 464	1 398	1 445
	Pseudomonadaceae	1 207	1 136	1 185
	Burkholderiaceae	472	437	468
	Rhodobacteriaceae	447	345	413
	Aquabacteriaceae	408	390	399
	Sphingomonadaceae	400	338	396
	Rhodospirillaceae	320	268	316
	Rhizobiaceae	320	260	317
	Xanthomonadaceae	313	220	299
	Moraxellaceae	255	178	248
	Bradyrhizobiaceae	241	224	240
	Oxalobacteraceae	236	222	235
	Chromatiaceae	211	175	198
	Pelagibacteraceae	208	139	193
	Phyllobacteriaceae	203	158	197
	Vibrionaceae	202	159	142
	Hyphomicrobiaceae	197	155	192
	Alcaligenaceae	195	183	185
	Neisseriaceae	192	159	177
	Rhodocyclaceae	191	157	181
	Acetobacteraceae	165	119	157
	Pasteurellaceae	159	140	151
	Halomonadaceae	155	121	142
	Aeromonadaceae	151	142	114
	Caulobacteraceae	129	121	129
	Sinobacteraceae	123	87	120
	Helicobacteraceae	123	84	115
	Alteromonadaceae	105	84	94
	Desulfobacteraceae	101	70	94
	Others	3 275	2 624	3 050
	Subtotal	13 879	11 921	13 030
Firmicutes	Lachnospiraceae	2 188	1 495	1 855
	Ruminococcaceae	1 216	912	1 010
	Bacillaceae	804	522	713

	Streptococcaceae	705	277	676
	Clostridiaceae	508	465	391
	Staphylococcaceae	367	192	341
	Veillonellaceae	317	226	293
	Lactobacillaceae	284	256	188
	Clostridiales Family XI. Incertae Sedis	172	140	159
	Planococcaceae	164	124	145
	Paenibacillaceae	132	84	119
	Enterococcaceae	110	76	92
	Others	600	471	547
	Subtotal	7 567	5 240	6 529
Bacteroidetes	Flavobacteriaceae	926	798	894
	Rikenellaceae	492	420	395
	Prevotellaceae	397	155	390
	Chitinophagaceae	173	157	164
	Flexibacteraceae	104	89	102
	Sphingobacteriaceae	104	93	100
	Porphyromonadaceae	102	71	92
	Others	416	325	381
	Subtotal	2 714	2 108	2 518
Actinobacteria	Corynebacteriaceae	690	549	652
	Streptomycetaceae	414	178	392
	Microbacteriaceae	359	302	346
	Micrococcaceae	336	270	332
	Mycobacteriaceae	188	78	185
	Micromonosporaceae	140	84	139
	Nocardiaceae	121	41	119
	Others	1 355	892	1 323
	Subtotal	3 603	2 394	3 488
Acidobacteria	Acidobacteriaceae	266	202	264
	Solibacteraceae	190	130	189
	Others	214	161	208
	Subtotal	670	493	661
Verrucomicrobia	Verrucomicrobiaceae	223	166	195
	Others	170	121	168
	Subtotal	393	287	363
Planctomycetes	Planctomycetaceae	163	110	159
	Others	116	89	112
	Subtotal	279	199	271
Tenericutes	Erysipelotrichaceae	139	90	119
	Others	179	148	153
	Subtotal	318	238	272
Chloroflexi	Anaerolinaceae	119	83	111
	Others	163	109	159
	Subtotal	282	192	270
Spirochaetes	Spirochaetaceae	114	68	108
	Others	61	53	52
	Subtotal	175	121	160
Others		1 360	1 009	1 295
Total		31 281	24 208	28 892

Table S4 Number of Actinobacterial isolates from each of the 12 salmon egg samples. To isolate Actinobacteria, incubation water of the salmon egg samples was spread plated on two semi-selective agar media, humic acid agar (HA) and starch agar (GA). On average 40 bacterial isolates were randomly picked from each culture medium of each sample. All the isolates were screened by an Actinobacterial specific primer pair. All isolates showing the expected amplification product for the Actinobacterial specific genes were sent for 16S rRNA sequencing. Sequence identity was obtained by comparison to sequences deposited in the Greengenes database and Ribosomal Database Project (see supplementary materials and methods).

Treatments	Sample ID	No. of Actinobacterial isolates	
		HA	GA
Diseased	1	3	9
	2	4	17
	3	17	26
	4	16	14
	5	12	18
	6	2	21
Healthy	7	11	25
	8	12	20
	9	4	15
	10	15	26
	11	14	27
	12	8	18

Table S5 Time line and experimental details of the *in vivo* bioassays with salmon eggs to assess the activity of four Actinobacterial isolates against *Saprolegnia diclina*.

Day	dpi	Procedures
-3		Cultures of isolates <i>Arthrobacter</i> sp., <i>Streptomyces</i> sp. 1 and <i>Streptomyces</i> sp. 2 were incubated at 30°C
-1		Cultures of isolate <i>Frondihabitans</i> sp. were incubated at 30°C
0		Eggs arrived and were placed into incubation units
1		Incubation units inoculated with bacteria
2		Boiled eggs were placed on <i>Saprolegnia</i> hyphal culture and incubated at 25°C
3		<i>Saprolegnia</i> colonized eggs were transferred to 15°C
4	0	Eggs were plated on HA and GA media, <i>Saprolegnia</i> inocula were introduced into incubation units, malachite green treatments, incubation units photographed
6	2	Incubation units photographed
8	4	Malachite green treatments, incubation units photographed
10	6	Incubation units photographed
12	8	Malachite green treatments, incubation units photographed
14	10	Incubation units photographed, determination of hyphal attachment percentage, eggs were plated on HA and GA media, hatching rate monitored
16	12	Hatching rate monitored
18	14	Hatching rate monitored
21	17	Hatching rate monitored, bioassay terminated

Table S6 Composition of the well water (after iron removal) used in the *in vivo* bioassays at the research facility 'Carus' of the Department of Animal Sciences, Wageningen University.

Elements	Concentration
Cl	11.7 mg/l
Al	0.02 mg/l
Ca	28.4 mg/l
Fe	0.02 mg/l
K	0.5 mg/l
Mg	1.97 mg/l
Mn	0.010 mg/l
Na	5.7 mg/l
P	0.04 mg/l
S	3.68 mg/l
Zn	0.010 mg/l
As	1.47 µg/l
Mo	0.36 µg/l
Pb	0.07 µg/l
Sn	0.5 µg/l
Ti	0.08 µg/l

Supplementary Movies

Movie S1 *Saprolegnia* hyphal attachment was determined by lifting infection inocula with a pair of tweezers and counting the number of eggs attached to the hyphal patch.

Movie S2 Bacterial colonization of salmon egg surfaces was determined by rinsing the eggs with 1 ml of sterile well water three consecutive times, draining briefly on sterile filter paper and rolling gently over the surface of semi-selective media. Colonies that were morphologically similar to the Actinobacterial isolates used for inoculation were subjected to 16S rRNA sequencing.

Supplementary movies accompany this paper on The ISME Journal website (<http://www.nature.com/ismej>)

Chapter 3

Phenotypic, genomic and metabolic characterization of aquatic *Fronidhabitans* with activity against the oomycete fish pathogen *Saprolegnia diclina*

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Abstract

Among the aquatic oomycete pathogens, *Saprolegnia* species cause declines in populations of amphibians and fish. Understanding the diversity, dynamics and activities of microbiomes of aquatic ecosystems may provide alternative means to mitigate diseases caused by *Saprolegnia* and other emerging pathogens. Previously we studied the microbiome of salmon eggs and showed that *Fronidhabitans* sp. 762G35 (Microbacteriaceae) significantly decreased attachment of *Saprolegnia* to salmon eggs (Liu *et al.*, 2014). Here, phenotypic and phylogenetic analyses of twelve *Fronidhabitans* isolates from healthy salmon eggs showed that they were closely related to known *Fronidhabitans* and *Frigoribacterium* species and that they possessed lipase, protease, biofilm formation activities, and inhibited hyphal growth of *Saprolegnia* at low temperature (5 °C). Subsequent analysis of the genome (3.4 Mb) of *Fronidhabitans* sp. 762G35 and another publically available *Fronidhabitans* genome sequence showed that only 57% of the genes were shared between the two genomes. In addition to a type III polyketide synthase gene cluster, two terpene biosynthesis gene clusters were identified, one of which was unique to aquatic *Fronidhabitans* sp. 762G35. Subsequent liquid chromatography-mass spectrometry (LC-MS) of the extracellular cell-free culture supernatant of aquatic *Fronidhabitans* sp. 762G35 identified accumulated furancarboxylic acid-derived metabolites which might be the potential active compounds involved in hyphal growth inhibition of *Saprolegnia*.

Introduction

Emerging fungal and oomycete diseases have detrimental effects on natural ecosystems and biodiversity as well as on food productivity in agriculture, horticulture and aquaculture (Fisher *et al.*, 2012; Gozlan *et al.*, 2014). For example, *Fusarium solani* and *Fusarium falciforme* cause high mortality of endangered sea turtle eggs (Fisher *et al.*, 2012; Sarmiento-Ramírez *et al.*, 2014) and *Batrachochytrium dendrobatidis* and *Batrachochytrium salmandrivorans* significantly reduce amphibian populations around the world (Woodhams *et al.*, 2011; Martel *et al.*, 2013). Among the oomycetes, *Saprolegnia* and *Aphanomyces* species cause a reduction in populations of amphibian, fish and crayfish in natural ecosystems and in aquaculture (van West, 2006; Fernández-Benítez *et al.*, 2008; Phillips *et al.*, 2008; Krugner-Higby *et al.*, 2010; Bruno *et al.*, 2011; Fisher *et al.*, 2012; van den Berg *et al.*, 2013). Infections by *Saprolegnia* species cause Saprolegniosis, a wide spread aquatic disease characterized by white or grey cotton-like hyphal mats on the skin of amphibians, fish and fish eggs (van West, 2006). In aquaculture, *Saprolegnia* species frequently infect salmonids, like trout

and salmon, during their freshwater life stage, but also non-salmonid fish including carp, catfish and perch (Bruno *et al.*, 2011; Das *et al.*, 2012). In Japan, *Saprolegnia* infection induced annual mortality of at least 50% in Coho salmon (Hatai and Hoshiai, 1992; Hatai and Hoshiai, 1994; Bruno *et al.*, 2011). In the USA, an economic loss of around \$40 million in channel catfish was caused by *Saprolegnia* infection, referred to as 'winter kill' (Bruno *et al.*, 2011).

To control Saprolegniosis, formalin is widely applied in aquaculture but its use is expected to become restricted in the near future because of harmful effects on the environment (Bruno *et al.*, 2011). Various efforts have been made to find effective and sustainable alternatives. For example, Pyceze[®] (bronopol), sea water flushes and NaCl applications are some of the recommended methods to suppress Saprolegniosis (Taylor and Bailey, 1979; Kitancharoen *et al.*, 1997; Bruno *et al.*, 2011). So far, none of these alternative strategies are as effective as the banned chemical malachite green (Bruno *et al.*, 2011). A vaccine is not yet available for Saprolegniosis (van den Berg *et al.*, 2013). Therefore, other sustainable remedies are needed to suppress *Saprolegnia* infections in aquaculture and natural ecosystems.

The use of protective microbes, such as *Aeromonas* and *Pseudomonas*, has been proposed as a promising measure to inhibit Saprolegniosis and other emerging diseases (Hatai and Willoughby, 1988; Bly *et al.*, 1997; Hussein and Hatai, 2001; Lategan and Gibson, 2003; Lategan *et al.*, 2004a, b; Carbajal-Gonzalez *et al.*, 2011; Liu *et al.*, 2014; Liu *et al.*, 2015). In our previous work, 31,281 bacterial and archaeal operational taxonomic units (OTUs) were detected in *Saprolegnia*-infected (diseased) and non-infected (healthy) salmon egg samples from a commercial fish hatchery (Liu *et al.*, 2014). The commensal Actinobacteria were significantly more enriched in healthy salmon egg samples and in particular *Frondihabitans* sp. 762G35 (Microbacteriaceae) effectively reduced hyphal attachment of *Saprolegnia* to live salmon eggs (Liu *et al.*, 2014). Here we further characterized the commensal *Frondihabitans* strains isolated from healthy salmon eggs. Various phenotypic, genomic and metabolic traits of *Frondihabitans* sp. 762G35 were studied in detail.

Materials and methods

Isolation, identification and phylogeny of Frondihabitans isolates

Frondihabitans sp. were isolated by plating the bacterial cell suspension retrieved from salmon eggs on humic acid-vitamin agar and starch-vitamin agar media and incubated at 30 °C. The isolates were selected at different time points within 6 weeks (Liu *et al.*, 2014). Phylogenetic analyses of the 16S rRNA sequences, including sequences of 12 *Frondihabitans* isolates from healthy salmon eggs and 204 strains including 35 type strains from Microbacteriaceae, were conducted with a similar method as

described in Liu *et al.* (2015). The 16S rRNA sequences (≥ 1200 bp) were downloaded from the Ribosomal Database Project (RDP) (Cole *et al.*, 2014).

Phenotypic characterization of Frondihabitans isolates

The 12 *Frondihabitans* isolates obtained from healthy salmon eggs were grown, washed and resuspended in well water (Liu *et al.*, 2014) to a final cell density of $1\text{--}3 \times 10^9$ cells ml^{-1} . For each assay described below, 2–3 μl of bacterial suspension was inoculated and performed in triplicate, unless mentioned otherwise.

The lipase indicator medium (Sierra, 1957) consisted of tryptone soya broth (TSB, Oxoid Ltd., Hampshire, England) supplemented with 1.5–2% agar (TSA; Merck KGaA, Darmstadt, Germany), 1% Tween[®] 80 (Merck Schuchardt OHG, Hohenbrunn, Germany) and 0.01% CaCl_2 . The cell suspension of each *Frondihabitans* was spot-inoculated in the middle of the plate and the plates were incubated at 5 and 25 °C up to four months with regular observation. The lipase activity was determined by observation of crystals around the inoculum.

The glucanase indicator medium consisted of TSA supplemented with 0.5% laminarin in a 24-wells plate (Cellstar[®], Greiner bio-one, Frickenhausen, Germany) and the cell suspension of each *Frondihabitans* was spot-inoculated into each well. After incubation at 5 and 25 °C for about one month, the bacterial cells were removed by water and the medium was stained by congo red for 1–2 min and de-stained with water. The positive and negative glucanase activity was determined by observation of orange (reflecting degradation of laminarin) and red colour, respectively. This assay was conducted in duplicate per incubation temperature.

The protease indicator medium consisted of TSA supplemented with 1.5% skim milk powder and the cell suspension of each *Frondihabitans* was spot-inoculated at three different spots of the plate. After incubation at 5 and 25 °C for about 3–4 month, the protease activity was determined by observation of a clearing halo around the inoculum.

The chitinase indicator medium was prepared as follows: 10 ml of TSA was poured in a petri dish and allowed solidification. 10 ml of TSB supplemented with 0.2% (w/v) colloidal chitin (Hsu and Lockwood, 1975; de Bruijn *et al.*, 2015) and 1.2% agar was poured on top of the solidified TSA. The cell suspension of each *Frondihabitans* isolate was spot-inoculated at three different places of the plate. After incubation at 5 and 25 °C for about 2–4 weeks, the chitinase activity was determined by observation of clearing halo around the bacterial inoculum.

The *in vitro* activity of the *Frondihabitans* isolates against *Saprolegnia diclina*, was tested on 1/5th strength TSA, 1/5th strength potato dextrose agar (Difco[™], Becton, Dickinson and company, New Jersey, USA) and *Pseudomonas* minimal medium (2 gl^{-1} glucose; 1 gl^{-1} $\text{NH}_4\text{H}_2\text{PO}_4$; 0.2 gl^{-1} KCl; 0.2 gl^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) dissolved in salmon egg water (50 salmon eggs were shaken in one litre of well water (Liu *et al.*, 2014)) and 1/5th strength malt extract broth (Difco[™], Becton, Dickinson and company, New Jersey, USA) supplemented with 1.6% agar (1/5MA). In addition, the activity of *Frondihabitans* sp. 762G35 was also tested on *Pseudomonas* agar F (Difco[™], Becton, Dickinson and company, New Jersey, USA), TSA and salmon egg agar (20, 50 or 100 homogenized salmon eggs per litre of well water and 1.5–2% agar). Each isolate was tested in duplicate or triplicate on each medium at 5 and 25 °C. In the preliminary test on different media, each bacterial isolate was spot-inoculated at one spot of the petri dish and incubated for about one week at 25 °C and four weeks at 5 °C prior to inoculation of a plug of *S. diclina* in the centre of each plate. For the tests on 1/5MA each bacterial isolate was spot-inoculated at four different corners of the plate and incubated for about two week at 25 °C and four weeks at 5 °C prior to inoculation of *S. diclina*. Volatile activity was tested in two-compartment petri dishes (94x15, Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) with both compartments containing 1/5MA and each bacterial isolate was spot-inoculated at four spots in one compartment and incubated for about two weeks at 25 °C and four weeks at 5 °C, respectively, prior to inoculation of a

plug of *S. diclina* in the other compartment. Hyphal growth inhibition was monitored for all bacterial isolates during incubation for 4-7 days at 25 °C and 22-43 days at 5 °C.

The biofilm assay was conducted in 24-wells plates. 60 µl of the aforementioned cell suspension of each isolate was added in 1140 µl of TSB supplemented with 10% sucrose (TSBS) to reach the density of 1.5×10^8 cells ml⁻¹. The standing cultures were incubated for 14 days at 5 °C and 56 days at 25 °C. The biofilm formation activity was determined by observation of cell attachment on the side or at the bottom of solid surface.

The swimming and swarming media were prepared in TSB supplemented with 0.3% (w/v) and 0.6% (w/v) of agar, respectively. In the centre of each plate, 5 µl of the bacterial cell suspension of each isolate was spot-inoculated. After incubation at 5 and 25 °C for one to four weeks, the swimming and swarming activities were determined by observation of motile outgrowth from the inoculum point. This assay was conducted with one replicate per incubation temperature.

To test if the isolates grew at 37 °C, 2 µl of the bacterial cell suspension of each isolate was inoculated in 2 ml fresh TSB and shaken for 11 days at 37 °C at 200 rpm before the cell density was measured by spectrophotometer at 600 nm.

To test the growth of each isolate at pH ranges of 3-9, the International *Streptomyces* Project (ISP) medium 2 (Shirling and Gottlieb, 1966; Cardinale *et al.*, 2011) was used. The medium contained 4 g l⁻¹ yeast extract, 10 g l⁻¹ malt extract, 4.4 g l⁻¹ D(+)-Glucose Monohydrate with the buffering system consisting of 0.05 M NaH₂PO₄/ Na₂HPO₄. The pH values of ISP medium 2 were adjusted to 3, 5, 7 and 9 by addition of HCl or NaOH and filter-sterilized through a 0.2 µm filter (Whatman™) before use. 2 µl of each cell suspension was added into each well of 24-wells plates containing 1 ml of ISP medium 2. The standing cultures were incubated for 4 weeks at 5 °C and 11-12 days at 25 °C before the cell density was measured by spectrophotometer at 600 nm.

Growth curve and supernatant activity of Frondihabitans sp. 762G35

To determine the growth and activity of *Frondihabitans* sp. 762G35, a pre-grown cell suspension was added to fresh 1/5MB with a final density of 10^6 cells ml⁻¹. The cultures were incubated at 300 rpm at 5 °C and the cell density was determined at different time points by spectrophotometer at 600 nm. The cultures of day 7, 14, 21 and 27 were filter-sterilized through 0.2 µm filters (Whatman™), lyophilized, re-dissolved in 1/5MB to 2.5 times concentrated level than the original culture and filter-sterilized again. Subsequently, 300-600 µl of this solution was added to a well of 24-wells or 48-wells plates (Cellstar®, Greiner Bio-One GmbH, Frickenhausen, Germany). One *S. diclina* 1152F4 hyphal plug of about 2 mm² pregrown on 1/5MB with 1.5-2% agar was added into each well and the culture was incubated at 5 °C. The effect on hyphal growth of *S. diclina* was monitored after 7-18 days and the diameter of hyphae was measured. Statistical significant differences were determined by independent-samples *t*-test ($P < 0.05$).

Genomic DNA isolation and genome sequencing

A single colony of a pregrown culture of *Frondihabitans* sp. 762G35 was inoculated in TSBS and the culture was incubated at 200 rpm for 2 days at 25 °C. 2 ml of the culture was centrifuged at 18 400 ×g for 2 min at room temperature and the cells were washed three times with sterile Milli-Q water. Subsequently, the genomic DNA was extracted by resuspending the cells in 660 µl solution of Tris-EDTA buffer and lysozyme (10mM Tris, 10mM EDTA, 1.82 mg ml⁻¹), and incubation for 30 min by shaking at 300 rpm at 37 °C. SDS and proteinase K were added to the final concentration of 1.2% and 0.1 mg ml⁻¹, respectively, mixed the contents by flipping the tube, and incubation for 15 min at 65 °C at 300-450 rpm. Proteins were precipitated by adding NaCl to a final concentration of 0.7 M, mixing by flipping the tube, and CTAB/NaCl to a final concentration of 1% CTAB (w/v) and 0.7 M NaCl and mixing by flipping the tube and incubation for 10 min at 65 °C at 300 rpm. The contaminating RNA was

removed by adding DNase free RNase (dissolved in 10 mM Tris (pH 7.6) and 15 mM NaCl) to the final concentration of 0.1 mg ml⁻¹. 90% volume of chloroform:isoamylalcohol (96:4, v/v) was added and mixed by flipping the tube for at least 15 sec, and the tubes were centrifuged at 18 400 ×g for 10 min at room temperature. The aqueous layer was transferred to a new tube and the DNA was precipitated with an equal volume of isopropanol and mixed by inverting several times, and subsequent centrifugation at 18 400 ×g for 15 min at 4 °C. The supernatant was removed, 1 ml ice cold 70% ethanol was added and the samples were centrifuged at 18 400 ×g for 10 min at room temperature. The ethanol was removed and the DNA pellet was air dried and dissolved DNA in sterile Milli-Q water by incubation overnight at 4 °C. Subsequently, the DNA solution was stored at -20 °C. The genomic DNA of *Fron dihabitans* sp. 762G35 was sequenced by the Pacific Biosciences RS II sequencing technology (Pacific Biosciences). A 10-kb insert library was prepared and sequenced using P4-C2 chemistry for 3 single-molecule real-time (SMRT) cells. Sequences were filtered using SMRT Analysis server v2.3.0.140936 using default settings. The RS_HGAP Assembly.2 (HGAP2) protocol (Chin *et al.*, 2013) was used to assemble the filtered reads resulting in a single contig. The resulting assembly was subjected to read remapping using the RS_Resequencing.1 module for sequence polishing up to 99.9999% consensus concordance with a mean coverage of 142.48. The final assembly was circularized by overlapping the terminal repeats and rearranged to start at the *dnaA* gene in the forward direction as proxy for the origin of replication (ORI) resulting in a chromosome of 3409251 nt and G+C content of 70.43%. There was no evidence for plasmids in the sequence data.

Whole genome phylogeny and bioinformatics analyses of Fron dihabitans sp. 762G35

Annotation was performed with a modified version of Prokka (Seemann, 2014). A total of 3278 genes, 3212 CDSs, 50 tRNAs, 6 rRNAs and 1 tmRNA were annotated. No clustered interspaced short palindromic repeats (CRISPR) arrays were found. The annotation was manually curated and enriched by adding X prophages predicted by PHAST (Zhou *et al.*, 2011). COG annotations for the predicted proteins were determined using KOGnitor (<http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html>) (Snel *et al.*, 2002). Pan-core genome analyses were performed by clustering together the protein coding genes from *Fron dihabitans* sp. Leaf304, *Frigoribacterium* sp. Leaf415 and *Fron dihabitans* sp. 762G35, with the protein coding genes of *Clavibacter michiganensis* subsp. *sepedonicus* ATCC33113 as an outgroup, using cd-hit (Huang *et al.*, 2010) with word length 3 (-n 3), global identity (-G 1 and a minimal alignment coverage of 60% for the shortest protein (-aS 0.6). Cd-hit clusters were parsed into a presence-absence matrix from which the pan, core and variable genomes were parsed using custom scripts.

Analyses of genes and/or gene clusters encoding antibiotics and secondary metabolites were conducted in antiSMASH 3.0 (Weber *et al.*, 2015).

For whole genome phylogeny, only those reference genomes of Microbacteriaceae with complete and/or scaffold level sequences from all the type strains, all *Microbacterium* strains characterized to species level and all strains from aquatic environments were downloaded from GenBank. Per genus, at least one strain was included. This resulted in a total of 24 genome sequences. Whole genome comparisons were conducted in Gegenees (Ägren *et al.*, 2012). In this program, the genome sequences are fragmented in 200 bp fragments using overlap size of 100 bp, and all-against-all BLASTn comparison with a 20% cutoff threshold for non-conserved material is conducted. From the generated matrix a phylogenetic tree was made and visualised with Splitstree. Phylogenetic analyses of the housekeeping gene RpoB (DNA-directed RNA polymerase subunit beta) protein sequences including the sequence of *Fron dihabitans* sp. 762G35 (geneID: FR762G35v2_01370) and the 24 Microbacteriaceae strains were conducted with a similar method as described in Liu *et al.* (2014). A maximum likelihood consensus tree was generated with the Whelan And Goldman model (Whelan and Goldman, 2001). The RpoB protein sequences of 24 Microbacteriaceae strains were approximately 1160 aa and downloaded from GenBank (Benson *et al.*, 2013).

Microbacteriaceae or Actinobacteria genes encoding protease, lipase or genes involved in biofilm formation were downloaded from GenBank and similar protein sequences in *Frondihabitans* sp. 762G35 were identified by BLASTp (e-value $<10^{-5}$). Next to the blast searches, the gene descriptions of *Frondihabitans* sp. 762G35 were searched for the terms 'protease', 'lipase' and 'biofilm'. The presence or absence of the identified genes in the *Frondihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 genome sequences was also determined.

HPLC fractionation and activity of fractions

Frondihabitans sp. 762G35 was pregrown in 1/5MB for 21 days at 5 °C at 250 or 300 rpm. The culture was filter-sterilized through a 0.2 µm filter (Whatman™), lyophilized and dissolved in 50% methanol. High Performance Liquid Chromatography (HPLC) analysis was performed by injection of 50 µl sample on a HPLC (Thermo scientific, Dionex UltiMate 3000 including pump, autosampler, detector, RF2000 Fluorescence detector and automated fraction collector) equipped with a C18 reversed-phase column (150 x 4.6 mm, 3 µm, Alltima™). Samples were analysed at a flow rate of 0.5 ml/min in a gradient running from 5-100% acetonitrile acidified with 0.1% (v/v) trifluoroacetic acid in 45 min. The HPLC fractions were collected every 5 min and subsequently 9 fractions were collected. Each fraction was air-dried, lyophilized, dissolved in 50% methanol and added to fresh 1/5th strength malt extract broth at 2.5 times concentrated level than the original culture. The activity of the extracts was tested by adding 300-500 µl of the concentrated extract to 48-wells plates. One *S. diclina* 1152F4 hyphal plug of about 2 mm² was added into each well and the culture was incubated at 5 °C. The effect of HPLC fractions on hyphal growth of *S. diclina* was determined after 7-18 days.

Metabolite extraction and liquid chromatography mass spectrometry (LC-MS) analyses

24 cultures of 5 ml 1/5MB were inoculated with 10^6 cells ml⁻¹ *Frondihabitans* sp. 762G35 and incubated at 5 °C at 250-300 rpm in each of the two different shakers. Six cultures of each shaker were collected on day 7, 14, 21 and 27, filter-sterilized, lyophilized and stored at -20 °C. The lyophilized compounds of each tube was dissolved in 1 ml of 50% methanol spiked with 0.1% formic acid, sonicated for 10 min at room temperature and centrifuged for 10 min at 21,000 ×g at 15 °C. After centrifugation, the supernatant of each sample was transferred to a new tube. Samples from both shakers were pooled, dried by nitrogen air, redissolved in 1 ml of 50% methanol spiked with 0.1% formic acid and filtered through a Minisart SRP 4 syringe filter (PTFE-membrane, PP-housing, 0.45 µm membrane, Sartorius Stedim Biotech GmbH, Goettingen, Germany). In total six replicates per time point were considered for LC-MS analysis.

The LC-PDA-LTQ-Orbitrap-FTMS instrument consisted of an Accela HPLC system (Accela photodiode array (PDA) and autosampler), interfaced to an LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) that was equipped with an ESI source (van der Hooft *et al.*, 2011). A HS F5 analytical column (Sigma-Aldrich) was used for chromatographic separation. The mobile phase consisted of a binary eluent solvent system of degassed ultra-pure water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v formic acid. The sample injection volume was 5 µl, while keeping the column temperature at 40 °C. The HPLC gradient was kept at 0% acetonitrile and linearly increased to 35% acetonitrile in 25 min with flow rate of the eluents at 0.1 ml/min. The column was re-equilibrated for 18 min following the separation of each sample. The mass chromatograms that were generated by the Orbitrap-FTMS were processed (peak picking and baseline correction) using the MetAlign software package (Lommen, 2009). Extraction and reconstitution of compound mass spectra were performed according to the method described by (Tikunov *et al.*, 2012). 1/5MB cultures without *Frondihabitans* inoculation were included as a control for determining the background.

Nucleotide sequence accession numbers

All sequences of the *Fron dihabitans* sp. isolates have been deposited in GenBank. The accession numbers for the 16S rRNA sequences of isolates 762G35, 762H6, 734G14, 734G19, 734G114, 746H33, 746G4, 746G130, 746G131, 749G138, 749G139 and 762G34 are: KF741274, KT803428, KT803404, KT803158, KT803370, KT803275, KT803218, KT803179, KT803180, KT803170, KT803171 and KT803366, respectively. The accession number of the genome sequence of *Fron dihabitans* sp. 762G35 is CP014619.

Results and Discussion

Identification and phylogeny of *Fron dihabitans* isolates

From healthy salmon egg samples, we isolated several Actinobacterial genera of which 12 isolates were identified as *Fron dihabitans* sp. based on 16S rRNA gene sequencing and phylogenetic analysis (Figure 1A). We previously showed that one of these aquatic isolates, designated *Fron dihabitans* sp. 762G35, significantly reduced attachment of *Saprolegnia diclina* to salmon eggs (Liu *et al.*, 2014). Nine of the twelve *Fron dihabitans* isolates clustered phylogenetically with *Fron dihabitans australicus* E1HC-02 and three to *Fron dihabitans peucedani* RS-15 (Figure 1A). *Fron dihabitans* (formerly named *Fron dicola*) was originally isolated from leaf litter from a pine forest in Australia and belongs to the family Microbacteriaceae (Zhang *et al.*, 2007; Greene *et al.*, 2009). Subsequently it was also isolated from the rhizosphere of *Peucedanum japonicum* in Korea (Lee, 2010), from lichens in the Austrian Alps, Antarctic and Arctic areas (Cardinale *et al.*, 2011; Lee *et al.*, 2014; Vasileva-Tonkova *et al.*, 2014), from sap of *Acer mono* in Korea (Kim *et al.*, 2014), and from acid mine-impacted soil in Germany (Mayanna *et al.*, 2015). Among relatives from the Microbacteriaceae family, *Microbacterium* has been reported to be associated with cod, yellowtail and bleak, and enhancing survival of cod larvae (Reid *et al.*, 2009; Fjellheim *et al.*, 2010; Ozaktas *et al.*, 2012; Aguilera *et al.*, 2013; Skjermo *et al.*, 2015). To date, however, little is known about the ecology, genomics and metabolomics of *Fron dihabitans* and their function(s) in aquatic ecosystems.

Phenotypic characterization of *Fron dihabitans* isolates

For all 12 *Fron dihabitans* isolates a range of *in vitro* assays were conducted to identify different phenotypic traits and to evaluate the effect of temperature on their activity. None of the 12 *Fron dihabitans* isolates grew at 37 °C (Table 1). All isolates grew at pH5 and pH7 at both 5 °C and 25 °C, whereas none of the isolates grew at pH3 and pH9 (Table 1). None of the isolates showed swimming or swarming activities under the growth conditions tested (Table 1), which is consistent with

Figure 1 Phylogenetic trees of *Frondihabitans* and Microbacteriaceae isolates and reference strains. (A) Phylogenetic tree of 16S rRNA sequences of 12 *Frondihabitans* isolates from healthy salmon eggs and 204 Microbacteriaceae sequences including 35 type strains. The 16S rRNA sequences (≥ 1200 bp) of the reference strains were downloaded from the Ribosomal Database Project (RDP) (Cole *et al.*, 2014). Each genus name is followed by the number of strains included in the same collapsed branch. Only the

Fron dihabitans subtree is expanded and displayed. Strain names of the two reference strains of *Fron dihabitans* are preceded by the accession numbers. (B) Phylogenetic tree of RpoB protein sequences of aquatic *Fron dihabitans* sp. 762G35 and 24 Microbacteriaceae strains. The sequences of strains approximately 1160 aa were downloaded from GenBank; the strain names are preceded by the accession numbers. (C) Phylogenetic tree based on whole genome sequences of *Fron dihabitans* sp. 762G35 and other Microbacteriaceae strains. Only those reference genomes of Microbacteriaceae with complete and/or scaffold level sequences from type strains, *Microbacterium* strains characterized to species level and all strains from aquatic environments were downloaded from GenBank. Per genus, at least one strain was included. This resulted in a total of 24 strains. Whole genome comparisons were performed by Gegenees (Ågren *et al.*, 2012) with fragmented alignment in BLASTn mode with settings 200 bp fragments, 100 bp overlap and a 20% cutoff threshold for non-conserved material. Black, blue and pink colours indicate strains isolated from terrestrial/plant, aquatic, or both sources of isolation, respectively. The scale bars in (A), (B) and (C) indicate an evolutionary distance of 0.005, 0.05 or 0.1 nucleotide substitution per sequence position, respectively. The bootstrap values indicated at the nodes in (A) and (B) are based on 1 000 bootstrap replicates and branch values lower than 50% were hidden.

previous results for *F. australicus*, *F. peucedani* and *Fron dihabitans cladoniiphilus* (Zhang *et al.*, 2007; Greene *et al.*, 2009; Lee, 2010; Cardinale *et al.*, 2011). In contrast, *Fron dihabitans sucicola*, isolated from sap of *Acer mono*, was reported to be motile (Kim *et al.*, 2014).

We previously showed that *Fron dihabitans* sp. 762G35 did not have *in vitro* activity against *S. diclina* in dual culture assays on 1/5th strength potato dextrose agar at 25 °C (Liu *et al.*, 2014). Here, results showed that this isolate and almost all other *Fron dihabitans* isolates could inhibit hyphal growth of *S. diclina* when grown on 1/5th strength malt agar (1/5MA) but only when incubated at 5 °C and not at 25 °C (Table 1). For most isolates, this activity was not due to the production of volatile compounds, as only one isolate was active against *S. diclina* at 5 °C in a split-plate assay where *Fron dihabitans* was physically separated from *S. diclina* (Table 1).

To further elucidate this inhibitory activity, we tested the isolates for specific extracellular enzyme activities. None of the *Fron dihabitans* isolates showed chitinase activity at the two temperatures tested (Table 1). However, most of the *Fron dihabitans* isolates showed extracellular protease activity at both 5 and 25 °C, and glucanase activity at 25 °C. Lipase activity of the *Fron dihabitans* isolates was mostly observed at 5 °C (Table 1). Extracellular lipase of *Fron dihabitans* isolated from Arctic and Antarctic lichens, was produced at 10-20 °C and 20 °C, respectively, but not at 4 °C; the extracellular protease of one Arctic *Fron dihabitans* isolate was produced at 10-20 °C (Lee *et al.*, 2014). Enzymes active at cold and temperate conditions are potentially of great interests for industrial, medicinal, and biotechnological applications. Bacterial-derived lipases and proteases, such as proteases of *Bacillus*, *Pyrococcus*, *Staphylothermus*, *Thermococcus* and *Thermotoga*, have optimal temperatures ranging from 45-100 °C and are used as detergents in the food industry and leather production (Haki and Rakshit, 2003).

Table 1 Phenotypic characterization of twelve aquatic *Fronidhabitans* isolates obtained from healthy salmon eggs. Isolate 762G35 that reduced hyphal attachment of *Saprolegnia diclina* to salmon eggs (Liu *et al.* 2014) and from which the genome was sequenced. A range of *in vitro* assays were conducted and the effect of the temperature on the activity was evaluated. The images show examples of positive (+) or negative (-) activity.

	Positive	Negative	Temperature	762G35	762H6	734G14	734G19	734G114	746H33	746G4	746G130	746G131	749G138	749G139	762G34
Lipase			5°C	+	+	+	+	+	+	+	+	+	+	+	+
			25°C	+	-	-	-	-	+	+	-	-	-	-	-
Protease			5°C	+	+	+	+	+	+	+	+	+	-	-	-
			25°C	-	+	+	+	+	+	+	+	+	-	-	+
Glucanase ^[1]			5°C	+	+	+	+	-	-	+	-	-	-	-	-
			25°C	+	+	+	+	+	+	+	+	+	-	-	+
Chitinase			5°C	-	-	-	-	-	-	-	-	-	-	-	-
			25°C	-	-	-	-	-	-	-	-	-	-	-	-
Diffusible compounds against <i>S. diclina</i>			5°C	+	+	+	+	-	+	+	+	+	+	+	+
			25°C	+[3]	-	-	-	-	-	-	-	+[3]	-	-	-
Volatile-mediated activity against <i>S. diclina</i>			5°C	-	-	+	-	-	-	-	-	-	-	-	-
			25°C	-	-	-	-	-	-	-	-	-	-	-	-
Biofilm formation (bottom)			5°C	+	+	+	+	+	+	+	+	+	+	+	+
			25°C	+	+	-	-	-	+	+	+	+	-	-	-
Biofilm formation (pellicle)			5°C	+	+	+	-	-	+	+	+	+	-	-	-
			25°C	+	-	+	-	-	+	+	+	+	+	+	-
Swimming +swarming			5°C	-	-	-	-	-	-	-	-	-	-	-	-
			25°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH 5 or 7			5°C	+	+	+	+	+	+	+	+	+	+	+	+
			25°C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 3 or 9			5°C	-	-	-	-	-	-	-	-	-	-	-	-
			25°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth			37°C	-	-	-	-	-	-	-	-	-	-	-	-

^[1] When the colour of the medium was in-between orange and red after destaining, a negative glucanase activity was recorded.

^[2] '---' no picture available.

^[3] For the two *Fronidhabitans* isolates 762G35 and 746G131 that inhibited hyphal growth of *S. diclina* by diffusible compounds at 25 °C, the inhibition was not consistent between replicates.

^[4] The images were taken from experiments conducted in a glass tube (Ø15 mm). The left image shows biofilm formation containing 2 ml tryptone soya medium and inoculated with *Fronidhabitans* sp. 762G35 to an initial cell density of 10⁷ cells ml⁻¹, incubated for 6 weeks at 5 and 25 °C. The right image shows the negative control, i.e. the same medium incubated for 6 weeks but not inoculated.

Microbial lipases from marine bacteria can be used to disrupt biofilm of pathogenic bacteria and remove corn oil from fabrics (Nerurkar *et al.*, 2013; Seghal Kiran *et al.*,

2014). This indicates that the lipases and proteases produced by our aquatic *Frondihabitans* isolates may have potential for application at low temperature, as was suggested for marine bacteria (Kennedy *et al.*, 2011).

In our previous work we postulated that one of the mechanisms by which *Frondihabitans* sp. 762G35 could suppress *S. diclina* disease may be via competitive niche exclusion (Liu *et al.*, 2014). One potential mechanism contributing to competitive exclusion is the formation of biofilms. There are reports showing that bacterial biofilms can clean up organic wastes in water, protect the fish host against invasion by pathogenic species, improve attachment and aggregation of other community members, prevent desiccation and facilitate nutrient uptake (Burmølle *et al.*, 2006; Ortega-Morales *et al.*, 2007; Iijima *et al.*, 2009; Flemming and Wingender, 2010; Wesseling, 2015). The majority of the twelve aquatic *Frondihabitans* isolates that we characterized formed a biofilm at 5 °C at the bottom of the microtiter plate well whereas at the air-water interphase, biofilm (pellicle) formation varied between isolates and temperatures (Table 1). Biofilm-forming *Microbacterium* species were found in various environments, including drinking and recirculating waters, marine algae and seagrass, and diatom-associated biofilm in rivers (Makk *et al.*, 2003; Burmølle *et al.*, 2006; Ortega-Morales *et al.*, 2007; Silbaq, 2009; Liu *et al.*, 2011). Whether our aquatic *Frondihabitans* isolates also form biofilms on the salmon egg surface, thereby protecting against *Saprolegnia* invasion, remains to be investigated.

Genome sequencing and bioinformatics analyses

Since *Frondihabitans* sp. 762G35 significantly suppressed hyphal attachment of *S. diclina* to salmon eggs (Liu *et al.*, 2014) and represents the largest phylogenetic group among the 12 *Frondihabitans* isolates (Figure 1A), we selected this isolate for genome sequencing. By PacBio sequencing and subsequent assembly we obtained a closed genome sequence of 3.4 Mb with a GC content of 70% and 3212 coding DNA sequences (CDSs) (Table 2). The three largest groups of CDSs belonged to carbohydrate transport and metabolism (9.5%), amino acid transport and metabolism (8.2%), and transcription (8.3%) (Figure 2).

Based on the housekeeping protein RpoB (Figure 1B) and whole genome (Figure 1C) phylogeny, *Frondihabitans* sp. 762G35 clustered closely with *Frondihabitans* sp. Leaf304, an isolate from the leaf of *Arabidopsis thaliana* (Bai *et al.*, 2015), and *Frigoribacterium*, a bacterium isolated from diverse environments including air (Kämpfer *et al.*, 2000; Murayama *et al.*, 2010), soil (Dastager *et al.*, 2008), plants (Nejad *et al.*, 2006; Rijavec *et al.*, 2007; Shen and Fulthorpe, 2015; Wang *et al.*, 2015), animals (Rudolf *et al.*, 2009), earthworm (Kim *et al.*, 2011) and aquatic environments such as seaweed (Moore *et al.*, 2002), nonsaline alkaline

Table 2 Assembly statistics and features of the genome sequence of *Frondihabitans* sp. 762G35.

<i>Frondihabitans</i> sp. 762G35 genome sequence	
Sequencing technology	PacBio P4-C2
<u>Assembly statistics</u>	
Genome size (Mb)	3.4
# scaffolds	1
# contigs	1
# reads	89853
Mean subread length (bp)	5476
N50 read length (bp)	7920
GC%	70
<u>Genomic features</u>	
# coding DNA sequences (CDSs)	3212
# hypothetical proteins	1123
# rRNA genes	6
# tRNA genes	50

Figure 2 Prediction of clusters of orthologous groups (COGs) in the genome of aquatic *Frondihabitans* sp. 762G35. In brackets the number of coding DNA sequences. COG annotations for the predicted proteins were determined using KOGnitor (<http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html>) (Snel *et al.*, 2002).

groundwater (Tiago *et al.*, 2004) and atmospheric water masses collected at the summit of a lava dome (Amato *et al.*, 2007). *Frigoribacterium* harbours both (potential) pathogenic and beneficial species (Nejad *et al.*, 2006; Rudolf *et al.*, 2009; Pereira and Castro, 2014). For example, the psychrophilic *Frigoribacterium faeni* was pathogenic to willow because of its ice-nucleation activity (Nejad *et al.*, 2006). Whether or not this activity is present in *Frondihabitans* still needs to be verified. Temperatures for optimal growth of *Frigoribacterium* can range from 2 to 10 °C (Kämpfer *et al.*, 2000; Nejad *et al.*, 2006) or from 25 to 30 °C (Wang *et al.*, 2015).

Pan-core genome analysis of *Frondihabitans* sp. 762G35, *Frondihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 with the plant pathogen *Clavibacter michiganensis* subsp. *sepedonicus* ATCC33113 as an outgroup, revealed 1292 core orthologous groups, representing 40-41% of each genome, and 5955 pan genome orthologous groups (Figure 3A). Only 57% of the number of CDSs was shared between the genome sequence of *Frondihabitans* sp. 762G35 and *Frondihabitans* sp. Leaf304, of which 529 orthologous groups were unique to the two *Frondihabitans* strains only, representing 16% of each genome. The number of unique orthologous groups for *Frondihabitans* sp. 762G35 was 1206, comprising 1213 CDSs and representing 37% of its genome. Among the unique orthologous groups, COGs could only be assigned to 52% of the CDSs and the three largest groups included transcription, carbohydrate transport and metabolism, and amino acid transport and metabolism, accounting for 8%, 7% and 4% of the total CDSs, respectively (Figure 3B).

Genome mining for bioactive compounds

Since *Frondihabitans* sp. 762G35 secretes compounds that inhibit hyphal growth of *S. diclina* (Table 1), we mined the annotated genome for genes or gene clusters encoding bioactive compounds. Several genes encoding proteases or lipases, or genes associated with biofilm formation were identified in the *Frondihabitans* sp. 762G35 genome sequence, several of which were also found in the *Frondihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 genome sequences (Table S1). Whether these genes are encoding the enzymes involved in extracellular lipase or protease activity or contribute to biofilm formation remains to be investigated.

Figure 3 Pan-core genome analysis of *Frondihabitans*. (A) Number of core, pan, unique and shared orthologous groups among aquatic *Frondihabitans* sp. 762G35, *Frondihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415. The number of shared and strain-specific gene clusters depends on which strain was added to the comparison (x-axis). Core gene clusters (white bars) appear in all strains, variable gene clusters (black bars) appear in more than one but not all strains and singleton gene clusters (grey bars) appear only in one strain. (B) Overview of the different clusters of orthologous groups (COGs) assigned to the coding DNA sequences (CDSs) that were unique for aquatic *Frondihabitans* sp. 762G35. In brackets the number of CDSs.

Using antiSMASH (Weber *et al.*, 2015), two gene clusters encoding terpene biosynthesis (Figure 4) were identified. The first terpene gene cluster appears to be unique to *Frondihabitans* sp. 762G35, while the second terpene gene cluster is partially present in the genome of *Frondihabitans* sp. Leaf304. Terpenes were originally identified in plants and later also in bacteria and animals (Gershenzon and Dudareva, 2007; Rabe *et al.*, 2013), including actinomycetes (Actinobacteria) and cyanobacteria (Nagarajan *et al.*, 2013; Rabe *et al.*, 2013). Terpenes have low molecular weight and each of them consists of several isoprenes (Bakkali *et al.*, 2008). Many of them, like gibberellic acid, artemisinin and altemicidin, are active in promoting plant growth, suppressing malaria and cancer, and protecting marine organisms from pathogen infection (Paul *et al.*, 2006; Gershenzon and Dudareva, 2007; Olano *et al.*, 2009; Rabe *et al.*, 2013). Marine actinomycetes, mainly *Streptomyces*, *Salinispora* and *Micromonospora* species, are capable of producing various antitumor terpenes (Olano *et al.*, 2009), and yellow to orange or yellow-red coloured terpenoids, called carotenoids, including canthaxanthin, lycopene, zeaxanthin (Romero *et al.*, 2012). Collectively, these studies suggest a potential role of terpenes in the activity of *Frondihabitans* isolate 762G35 against *S. diclina*.

Beside the terpene clusters, a type III polyketide synthases (T3pks) gene cluster was identified in the genome of *Frondihabitans* sp. 762G35, which was partially present in the genome of *Frondihabitans* sp. Leaf304 (Figure 4). Polyketides comprise a wide variety of bioactive compounds, such as phenolic lipids, 1,3,6,8-tetrahydroxynaphthalene, the anticancer compound giganin, the anti-cholesterol compound herboxidiene, and the antibiotics balhimycin, tetracyclines, vancomycin and teicoplanin (Rohr, 1992; Fang *et al.*, 1993; O'Hagan, 1995; Funa *et al.*, 1999; Chen *et al.*, 2001; Li *et al.*, 2001; Pfeifer *et al.*, 2001; Funa *et al.*, 2002; Funa *et al.*, 2006; Funabashi *et al.*, 2008; Pokhrel *et al.*, 2015). To elucidate which compound(s) the T3pks gene cluster and the terpene gene clusters are encoding and if these play a role in the activity of *Frondihabitans* sp. 762G35 against *Saprolegnia*, site-directed mutagenesis of the genes should be conducted in future studies, followed by chemical identification and *in vivo* bioassays to study gene transcription and the activities of T3pks- or terpene-deficient mutants should be compared to the wild type.

Cluster	ORF	<i>Frondihabitans</i> sp. 762G35 gene ID	bp	Gene description	Present in genome	
					<i>Frondihabitans</i> sp. Leaf304	<i>Frigoribacterium</i> sp. Leaf415
Cluster 1 Terpene	ORF1	00860	1115	Unclassified	-	-
	ORF2	00880	1415	Amidase	-	-
Cluster 2 Terpene	ORF3	14395	1607	Dehydrogenase	-	-
	ORF4	14400	893	Unclassified	+	+
	ORF5	14405	1229	Polyprenyl synthetase	+	-
	ORF6	14425	1067	Crotonyl-CoA reductase/alcohol dehydrogenase	+	+
Cluster 3 T3pks	ORF7	10860	1217	Aminotransferase	+	+
	ORF8	10885	1145	Monooxygenase FAD-binding	+	+
	ORF9	10895	1298	Chalcone and stilbene synthase domain protein	-	-
	ORF10	10920	974	Aldo/keto reductase family oxidoreductase	+	-
	ORF11	10930	704	Aldo/keto reductase family oxidoreductase	+	+
	ORF12	10935	1724	Alpha-glucosidase	-	-
	ORF13	10945	803	Inositol monophosphatase	+	-

Figure 4 Gene clusters in the *Frondihabitans* sp. 762G35 genome predicted to encode antibiotics and secondary metabolites. The genome was analysed by antiSMASH 3.0 (Weber *et al.*, 2015). Specific open reading frames (ORFs) of biosynthetic genes were numbered and described by gene ID, size (bp) and a short description. The presence ('+') or absence ('-') of these ORFs in the genome of *Frondihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 is also indicated. The marker on top indicates the size (bp) of the gene clusters.

Chemical analyses of *Frondihabitans* sp. 762G35

Frondihabitans sp. 762G35 was grown in 1/5MB at 5 °C and the supernatants were collected at several time points along the growth curve (Figure 5) and tested for activity against *S. diclina*. The supernatants of the lag phase (day 7), exponential phase (day 14) and stationary phase (days 21 and 27) showed increasing inhibitory activity on hyphal growth of *S. diclina* (Figure 5). As a first step to partially purify the bioactive compounds, HPLC analyses and fractionation were performed. Most compounds eluted in the first 15 min (Figure S1A and S1B). Among the nine fractions collected every 5 min over the 45-min gradient, only compounds in the second fraction (5-10 min elution) inhibited hyphal growth of *S. diclina* when it was concentrated 2.5 times (Figure S1C).

Figure 5 Growth of *Frondihabitans* sp. 762G35 at 5 °C and production of extracellular compounds inhibitory to hyphal growth of *Saprolegnia diclina*. (A) Growth curve of *Frondihabitans* sp. 762G35 over 41 days. A pregrown cell suspension was added to fresh 1/5th strength malt broth (1/5MB) to obtain the density of 10⁶ cells ml⁻¹. The cultures were incubated at 300 rpm at 5 °C and the cell density of different time points was determined by spectrophotometer at 600 nm. (B) Inhibitory activity of the cell-free culture supernatants on hyphal growth of *S. diclina*. The supernatants of day 7, 14, 21 and 27 were filter-sterilized through 0.2 µm filters (WhatmanTM), lyophilized, redissolved in 1/5MB to a 2.5 times concentrated level than the original culture and filter-sterilized again. Subsequently, 300-600 µl of this solution was added to a well of a 24-wells or 48-wells plate. One *S. diclina* 1152F4 hyphal plug of approximately 2 mm² was added into each well and the culture was incubated at 5 °C. The effect on hyphal growth of *S. diclina* was determined by measuring the diameter of hyphal mat after 11 days of incubation. Error bars indicate S.E.M. (N=2). An asterisk indicates a statistically significant difference compared to the medium control of the corresponding time point ($P<0.05$, independent-samples *t*-test).

To monitor the temporal change in polar and semi-polar metabolites produced during the growth curve of *Frondihabitans* sp. 762G35, samples collected on day 7, 14, 21 and 27 were subjected to LC-PDA-LTQ-Orbitrap-FTMS analysis in negative ionization mode. A total of 344 putative metabolites were detected across all the samples; 326 were significantly different at least under one of the conditions analysed ($P < 0.05$) and used for computing the hierarchical cluster analyses (Figure 6A). The metabolome profile of samples collected on day 14, 21 and 27 is more alike and clearly different from samples collected at day 7 and the control. The difference between these two groups is explained by different clusters of metabolites that showed variation in their abundance across the time points. The results suggest that metabolites in cluster I were consumed by *Frondihabitans* sp. 762G35 in the first week of culturing. Similarly, clusters II and III appear to be consumed in the second and third week of culturing, respectively. By contrast, metabolites in cluster IV showed accumulation from the second week onward. Metabolites in clusters I and II are depleted from the supernatant at the lag and exponential growth phases of *Frondihabitans* sp. 762G35 (day 7 and 14, respectively) and could be used for growth and normal metabolism of the microorganism. Instead, metabolites in cluster III showed drastic depletion at the stationary growth phase (day 21 and 27) and coincided with the accumulation of 65 metabolites in cluster IV. Based on this observation we assume that metabolites in cluster III might be used by *Frondihabitans* sp. 762G35 to produce metabolites in cluster IV.

The significance of the temporal accumulation of these 65 metabolites in cluster IV was assessed by comparing their accumulation in the supernatant obtained from different growth stages of *Frondihabitans* sp. 762G35 to the control using independent-samples *t*-test ($P < 0.05$) and fold-change in accumulation (fold-change > 2). This resulted in 29 metabolites that were significantly accumulated at least under one of the growth stages when compared to the control (Figure 6B, Table S2). There were 7 core-metabolites that showed significant accumulation in all growth stages of *Frondihabitans* sp. 762G35 when compared to the control. Similarly, 17 metabolites showed significant accumulation both at the early and late (day 21 and 28, respectively) stationary growth phase of *Frondihabitans* sp. 762G35 and 4 other metabolites showed significant accumulation only at the late stationary growth phase (day 27). Only 1 metabolite significantly accumulated in the exponential growth phase (day 14) of *Frondihabitans* sp. 762G35 (Figure 6B).

Figure 6 Hierarchical cluster analysis (HCA) of 344 metabolites detected from supernatant of *Frondihabitans* sp. 762G35 extract obtained on day 7, 14, 21 and 27 in 1/5th strength malt extract broth at 5 °C. (A) Heat map based on 326 metabolites identified by liquid chromatography mass spectrometry (LC-MS) that are significantly different in abundance at least under one of the conditions analysed. D7, D14, D21 and D27 correspond to the six samples collected on day 7, 14, 21 and 27, respectively. To differentiate metabolites that are associated with the medium from the ones that are produced by *Frondihabitans* sp. 762G35, a set of 1/5th strength malt extract broth cultures without *Frondihabitans* sp. 762G35 inoculation was included ('control'). There are four major clusters. Clusters I, II and III indicate metabolites in the medium that were consumed in the first, second and third week after inoculation, respectively. Cluster IV represents metabolites that accumulated from the second week onward. (B) Venn diagram showing the number and distribution of 29 significantly accumulated metabolites from cluster IV when their abundance is compared between the 4 growth stages (day 7, 14, 21 and 27) with the control ($P < 0.05$ and fold change > 2). (C) The temporal accumulation pattern of 9 metabolites that eluted between 3-10 minutes of chromatographic separation that were among the 29 metabolites in cluster IV of panel A that showed significant accumulation at least under one of the growth stages when compared to the control.

Supernatant obtained from the early and late stationary growth phase (day 21 and 27) of *Fron dihabitans* sp. 762G35 showed the highest inhibitory activity against *S. diclina* (Figure 5) and this coincides with the accumulation of these 29 metabolites in the extract. Moreover, using bioactivity-guided fractionation from the extract of supernatant obtained on day 21, fractions collected between 5 and 10 minutes of HPLC run showed the highest inhibitory activity against *S. diclina* (Figure S1). Hence, using the elution time window of 3-10 min we narrowed down the list of 29 metabolites to 9 potential candidates (Table S2). For these 9 metabolites, 3 were among the 7 core-metabolites (Figure 6B) and the others were evenly distributed among the 17 metabolites that are common to day 14, 21 and 27 and to those 4 metabolites that exclusively accumulated on day 27 (Figure 6B).

We used the accurate mass and the MS/MS (MS^3) fragmentation patterns of the parent ion of each of the selected 9 metabolites to give tentative identification. Out of the 9 metabolites only 3 were sufficient in abundance to be subjected for deep MS/MS analysis (Figure 6C). These 3 metabolites are represented by the parent ions $[M-H]^-$ m/z 111.0088, 173.0089 and 353.0721 (Table S2). Two of them (m/z 111.0088 and 353.0721) were among the 7 core-metabolites that showed accumulation under all the growth stages and were 24x and 43x more abundant at day 27 compared to day 7, respectively. M/z 173.0089 was among the 17 metabolites that showed significant accumulation on day 14, 21 and 27, which could not be detected at day 7, and was 2.7x more abundant at day 27 compared to day 14. Based on the accurate mass, the molecular formula $C_5H_3O_3$ was assigned to the ion peak at m/z 111.0088. MS/MS analysis of m/z 111.0088 revealed a dominant furan ion peak at m/z 67.0192 (C_4H_3O) with the loss of carboxyl group (COO). Both the accurate mass (sub-ppm accuracy) and the fragmentation pattern of m/z 111.0088 match with 2-furancarboxylic acid in the MassBank Database (<http://www.massbank.jp/index.html>). The ion peak at m/z 173.0089 gave major fragment peaks at m/z 111.0088 (100%), 131.3856 (60%) and 129.01913 (20%). Similarly, the ion peak at m/z 353.0721 is characterized by major fragment ion peaks at m/z 111.0088 (100%), 173.0092 (50%) and 131.3772 (15%). Therefore, based on the observed fragmentation pattern we assert that both m/z 173.0089 and 353.0721 are potentially derived from m/z 111.0088 because they showed a common fragment m/z 111.0088, corresponding to 2-furancarboxylic acid. The production of at least two of the three metabolites (m/z 111.0088 and 353.0721), even at the lag (day 7) and exponential (day 14) growth phases of *Fron dihabitans* sp. 762G35 (Figure 6B and C), is an indicator of the potential ecological role of these group of metabolites as antagonist against *Saprolegnia*. Interestingly, furancarboxylic acid-related compounds were previously described for the marine fungi *Chaetomium* and *Penicillium* and the plant-associated fungi *Cephalosporium* and *Penicillium* (Kobayashi and Ui, 1975; Abdel-

Lateff, 2008; Kimura *et al.*, 2009; Wang *et al.*, 2016) and showed antibiotic, antifibrotic and phytotoxic activities (Kobayashi and Ui, 1975; Almeida *et al.*, 2014; Wang *et al.*, 2016). Several Actinomycetales, including *Agromyces* and *Arthrobacter*, can degrade toxic furanic compounds (Trifonova *et al.*, 2008; Wierckx *et al.*, 2010). Such microbes may be used as biotransformers in industry (Wierckx *et al.*, 2011). Besides, furan-2-yl acetate produced by marine *Streptomyces* inhibited *in vitro* the replication of fish nodavirus in fish cells. Hence, this class of compounds may contain promising candidates to control nodaviral diseases in marine aquaculture (Suthindhiran *et al.*, 2011) and, based on the results of our study, Saprolegniosis of salmonids.

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Author Contributions

Conceived and designed the experiments: YL IdB JMR. Performed the experiments: YL. Analyzed data and created figures: YL DE IdB. Wrote the paper: YL IdB DE VdJ JMR. Performed the bacterial isolations and characterization, phylogenetic and genome analyses, *in vitro* and HPLC experiments: YL. Performed LC-MS analyses: DE YL. Contributed to *in vitro* enzymatic activity tests: VJC. Contributed to genome analysis: IdB VdJ VJC. Contributed to HPLC analysis: IdB. Contributed to review of the manuscript: all authors.

Competing Interests

The authors have declared that no competing interests exist.

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Supporting information

Table S1 Identification of genes encoding proteases and lipases and genes associated with biofilm formation in the genome of *Fronidhabitans* sp. 762G35. Microbacteriaceae or Actinobacteria reference genes were downloaded from GenBank and similar protein sequences in *Fronidhabitans* sp. 762G35 were identified by BLASTp (e-value $<10^{-5}$). For each blast hit, the percentage of identity to *Fronidhabitans* sp. 762G35 is indicated. Next to the blast searches, the terms 'protease' (including both intracellular and extracellular proteases), 'lipase' and 'biofilm' were searched in the gene descriptions of *Fronidhabitans* sp. 762G35 and the corresponding genes are indicated in the table. The presence ('+') or absence ('-') of these genes in the reference genomes of *Fronidhabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 is also indicated. NONE indicates gene name is not available.

Frondihabitan 762G35 gene ID	Gene name and description	Present in genome		Query (GenBank accession number, name and organism) used in BLASTp	% identity
		Frondihabitan sp. Leaf304	Frigoribacterium sp. Leaf415		
Lipase					
16115	nlhH_2 Carboxylesterase NihH	-	-	ACD89058 lipase [Brachybacterium tyrofermentans]	33,74
16045	nlhH_1 Carboxylesterase NihH	+	+	ACD89058 lipase [Brachybacterium tyrofermentans]	33,19
11900	lipR Putative acetyl- hydrolase LipR	-	-	ACD89058 lipase [Brachybacterium tyrofermentans]	26,81
11900	lipR Putative acetyl- hydrolase LipR	-	-	ACD89059 lipase [Rhodococcus erythropolis]	31,61
16045	nlhH_1 Carboxylesterase NihH	+	+	ACD89059 lipase [Rhodococcus erythropolis]	31,51
16115	nlhH_2 Carboxylesterase NihH	-	-	ACD89059 lipase [Rhodococcus erythropolis]	30,09
04655	NONE Lipase 2	-	-	-	-
13125	NONE Lipase 2	-	-	-	-
13425	ytpA_1 Phospholipase YtpA	+	+	-	-
15310	ytpA_2 Phospholipase YtpA	-	-	-	-
Protease					
04515	htrA_1 Putative serine protease HtrA	+	-	WP_025157174 serine protease [Leifsonia aquatica]	60,39
06550	htrA_2 Putative serine protease HtrA	+	+	WP_025157174 serine protease [Leifsonia aquatica]	48,75
00065	gluP Rhomboid protease GluP	+	+	-	-
00350	bepA Beta-barrel assembly-enhancing protease	-	-	-	-
00360	ftsH_1 ATP-dependent zinc metalloprotease FtsH	-	-	-	-
00720	ftsH_2 ATP-dependent zinc metalloprotease FtsH	+	+	-	-
00785	clpC1 ATP-dependent Clp protease ATP- binding subunit ClpC1	+	+	-	-
01725	paiA_1 Protease synthase and sporulation negative regulatory protein PAI 1	-	-	-	-
02055	clpS ATP-dependent Clp protease adapter protein ClpS	+	-	-	-
03850	isp Intracellular serine protease	+	+	-	-
04490	lon1 Lon protease 1	+	+	-	-
04515	htrA_1 Putative serine protease HtrA	+	-	-	-
04895	iga_1 Immunoglobulin A1 protease	-	-	-	-
05060	paiB Protease synthase and sporulation protein PAI 2	-	-	-	-
05100	clpP2_1 ATP-dependent Clp protease proteolytic subunit 2	+	+	-	-
05105	clpP2_2 ATP-dependent Clp protease proteolytic	-	+	-	-

05110	subunit 2 clpX ATP-dependent Clp protease ATP-binding subunit ClpX	+	+	-	-
05445	ptrB Protease 2	+	+	-	-
05530	prwW Protease PrsW	+	+	-	-
05610	NONE putative zinc protease	+	+	-	-
06280	iga_2 Immunoglobulin A1 protease	-	+	-	-
06550	htrA_2 Putative serine protease HtrA	+	+	-	-
06925	hflK Modulator of FtsH protease HflK	+	+	-	-
08795	rip1 Zinc metalloprotease Rip1	-	+	-	-
11620	NONE Protease 1	+	-	-	-
12485	clpC ATP-dependent Clp protease ATP-binding subunit ClpC	-	-	-	-
13830	paIA_2 Protease synthase and sporulation negative regulatory protein PAI 1	+	-	-	-
14815	ftsH_3 ATP-dependent zinc metalloprotease FtsH	-	-	-	-
<hr/>					
Biofilm					
10225	birA_1 Bifunctional ligase/repressor BirA	-	-	WP_022893652 biofilm PGA synthesis protein PgaB [<i>Agromyces subbeticus</i>]	41,61
10225	birA_1 Bifunctional ligase/repressor BirA	-	-	KRC52209 biofilm PGA synthesis protein PgaB [<i>Leifsonia</i> sp. Root227]	47,01
10225	birA_1 Bifunctional ligase/repressor BirA	-	-	KQR52152 biofilm PGA synthesis protein PgaB [<i>Leifsonia</i> sp. Leaf336]	45,35
10225	birA_1 Bifunctional ligase/repressor BirA	-	-	WP_022888292 biofilm PGA synthesis protein PgaB [<i>Agromyces italicus</i>]	44,14
10225	birA_1 Bifunctional ligase/repressor BirA	-	-	WP_022881549 biofilm PGA synthesis protein PgaB [<i>Gryllotalpicola ginsengisoli</i>]	50,17
09490	bigR Biofilm growth- associated repressor	-	-	-	-
10200	brpA_1 Biofilm regulatory protein A	-	+	-	-
13250	brpA_2 Biofilm regulatory protein A	+	-	-	-

Table S2 Chemical analyses revealed 29 metabolites that were significantly accumulated at least under one of the growth stages when compared to the medium control. Statistical significant different metabolites were determined by independent-samples *t*-test ($P<0.05$) and fold change in accumulation (fold change >2). Growth stages include growth of *Frondihabitans* sp. 762G35 on day 7, 14, 21 and 27.

Mass (Da) [M-H]	Retention time (min)	Average intensity				Independent-samples <i>t</i> -test			
		Day7	Day14	Day21	Day27	Day7	Day14	Day21	Day27
412.571	3.2	11462	14154	14871	14744	0	0	0	0
217.119	5	2057	7279	10092	11699	0	0	0	0
353.0727 ^[3]	5.1	4322	66159	138145	185688	3.50E-05	5.00E-06	0	0
111.008 ^[3]	5.1	10032	87117	178527	243188	5.00E-06	6.00E-06	0	0
302.971	5.2	ND ^[1]	ND	3371	5686	NS ^[2]	2.00E-06	0	0
459.016	5.3	ND	ND	3207	4179	NS	4.30E-03	0	0
465.05	5.7	ND	2164	4046	5051	NS	0	0	0
293.081	6.4	ND	5754	6845	9845	NS	0	0	0
173.009 ^[3]	6.4	ND	15761	33331	41357	NS	0	0	0
345.094	10.8	ND	12328	18295	17269	NS	0	0	0
284.125	19.7	4149	22947	29197	32026	5.20E-04	0	0	0
343.114	19.7	ND	11409	13926	15577	NS	0	0	0
335.092	20.6	ND	3108	4955	4891	NS	0	0	0
245.114	21.6	5073	10274	10718	10709	2.10E-02	0	0	0
287.125	22.4	ND	9117	11969	13425	NS	0	0	0
259.13	22.7	5164	12665	37918	52100	NS	0	0	0
375.131	23.1	ND	ND	2678	3239	NS	6.80E-03	3.30E-05	0
295.13	24.9	2131	5318	8739	16270	2.60E-05	0	0	0
281.114	25.2	ND	4643	7330	7581	NS	0	0	0
338.172	25.7	4032	14280	16094	17354	1.00E-06	0	0	0
349.161	26.6	ND	2859	3106	3222	1.10E-03	2.70E-05	0	0
145.087	26.9	ND	8196	15356	22828	NS	0	0	0
147.045	27.6	ND	5933	17795	23855	NS	0	0	0
164.072	28.4	5261	16896	23881	19135	8.40E-04	0	0	0
326.172	28.6	2724	6842	7836	8555	3.00E-05	0	0	0
480.246	30.1	ND	2194	2135	2163	7.70E-03	1.00E-06	0	0
411.167	30.1	2698	5823	6739	6800	1.00E-05	0	0	0
173.024	31.4	ND	3133	5488	8495	NS	0	0	0
491.251	33.1	ND	8325	ND	ND	NS	6.00E-06	NS	NS

^[1]ND, not detected

^[2]NS, not significant ($P<0.05$)

^[3]Metabolites that were subjected to MS³ analysis

Figure S1 HPLC analyses of cell-free culture fractions from *Frondihabitans* 765G35 and their effect on *Saprolegnia diclina* 1152F4. 3D field (A) and chromatogram (B) of HPLC analyses of cell-free culture from *Frondihabitans* 765G35 collected after 21 days of incubation. The chromatogram was visualized at wave length of 254 nm. (C) Effect of HPLC fractions on hyphal growth of *S. diclina* 1152F4. The HPLC fractions were collected every 5 min over 45 min, air-dried, lyophilized, dissolved in 50% methanol and added to fresh 1/5th strength malt extract broth at 2.5 times concentrated level than the original culture. The test was conducted with 300-500 μ l of the fractions in each well of a 48-wells plate. One *S. diclina* 1152F4 hyphal plug of about 2 mm² was added to each well and the culture was incubated at 5 °C. The effect of HPLC fractions on hyphal growth of *S. diclina* was determined after 7-18 days. The total lyophilized culture dissolved in 50% methanol and 50% methanol alone, were used as positive and negative controls, respectively.

Chapter 4

Diversity of aquatic *Pseudomonas* species and their activity against the fish pathogenic oomycete *Saprolegnia*

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Abstract

Emerging fungal and oomycete pathogens are increasingly threatening animals and plants globally. Amongst oomycetes, *Saprolegnia* species adversely affect wild and cultivated populations of amphibians and fish, leading to substantial reductions in biodiversity and food productivity. With the ban of several chemical control measures, new sustainable methods are needed to mitigate *Saprolegnia* infections in aquaculture. Here, PhyloChip-based community analyses showed that the Pseudomonadales, particularly *Pseudomonas* species, represent one of the largest bacterial orders associated with salmon eggs from a commercial hatchery. Among the *Pseudomonas* species isolated from salmon eggs, significantly more biosurfactant producers were retrieved from healthy salmon eggs than from *Saprolegnia*-infected eggs. Subsequent *in vivo* activity bioassays showed that *Pseudomonas* isolate H6 significantly reduced salmon egg mortality caused by *Saprolegnia diclina*. Live colony mass spectrometry showed that strain H6 produces a viscosin-like lipopeptide surfactant. This biosurfactant inhibited growth of *Saprolegnia in vitro*, but no significant protection of salmon eggs against Saprolegniosis was observed. These results indicate that live inocula of aquatic *Pseudomonas* strains, instead of their bioactive compound, can provide new (micro)biological and sustainable means to mitigate oomycete diseases in aquaculture.

Introduction

Emerging fungal and fungal-like diseases are causing severe ecological disruptions and are recognised as a global threat to biodiversity and food security (Fisher *et al.*, 2012; Gozlan *et al.*, 2014). For example, *Fusarium solani* is involved in mass mortality of eggs of the endangered sea turtles in Cape Verde (Sarmiento-Ramírez *et al.*, 2014), and *Batrachochytrium dendrobatidis* and *B. salamandrivorans* are causing major amphibian declines globally (Woodhams *et al.*, 2011; Martel *et al.*, 2013). Amongst oomycetes, *Aphanomyces* and *Saprolegnia* species are causing significant declines in crayfish, fish and amphibian populations (van West, 2006; Fernández-Benítez *et al.*, 2008; Phillips *et al.*, 2008; Krugner-Higby *et al.*, 2010; Bruno *et al.*, 2011; Fisher *et al.*, 2012; van den Berg *et al.*, 2013). *Saprolegnia* species are the causative agents of Saprolegniosis, a disease characterized by fluffy and filamentous white or grey mycelial patches on fish, fish eggs or amphibians (van West, 2006). In aquaculture, *Saprolegnia* species regularly infect freshwater cultured salmonids, including Atlantic salmon and rainbow trout, and non-salmonids like eel, perch, carp and catfish (Bruno *et al.*, 2011; Das *et al.*, 2012). In Japan, at least 50% annual mortality in Coho salmon

due to Saprolegniosis was reported (Hatai and Hoshiai, 1992; Hatai and Hoshiai, 1994; Bruno *et al.*, 2011). Also the 'winter kill' by *Saprolegnia* species in channel catfish in the USA resulted in a substantial financial loss of approximately \$40 million (Bruno *et al.*, 2011).

Formalin is now commonly used to control Saprolegniosis, but is expected to be banned soon due to adverse environmental effects (Bruno *et al.*, 2011). Several treatments have been tested to prevent Saprolegniosis, such as hydrogen cyanide, Pyceze® (bronopol), sea water flushes and NaCl, but none of these measures exerted control to a level similar as obtained with malachite green, a chemical banned due to its carcinogenic properties (Bruno *et al.*, 2011). Currently, no vaccination is available for Saprolegniosis (van den Berg *et al.*, 2013). Therefore, new sustainable measures are urgently needed. A potential approach to control Saprolegniosis and other emerging diseases involves the application of beneficial microbes. A limited number of bacterial genera and species, including *Aeromonas* and *Pseudomonas*, have been reported as potential anti-pathogen agents in aquaculture, but a comprehensive understanding of the microbiome composition of fish eggs and their protective potential is still limited (Hatai and Willoughby, 1988; Bly *et al.*, 1997; Hussein and Hatai, 2001; Lategan and Gibson, 2003; Lategan *et al.*, 2004a, b; Carbajal-González *et al.*, 2011).

In previous work, we detected 31,281 bacterial and archaeal operational taxonomic units (OTUs) on salmon eggs from a hatchery by PhyloChip metataxonomic analysis. The highest number of OTUs belonged to Proteobacteria (Liu *et al.*, 2014). Based on this large-scale metataxonomic analysis, the diversity of salmon egg-associated Proteobacteria and their functional potential to protect fish eggs against *Saprolegnia* were investigated in this study. We focused specifically on the Pseudomonadales and isolated several *Pseudomonas* strains, assessed their genotypic diversity and tested their inhibitory activity against *Saprolegnia* species both *in vitro* and *in vivo*. Given the zoosporicidal activity of biosurfactants produced by *Pseudomonas* species (De Souza *et al.*, 2003; Raaijmakers *et al.*, 2006; de Bruijn *et al.*, 2007), the isolates obtained from salmon eggs were also phenotypically screened for biosurfactant production. For the *Pseudomonas* isolate that provided the best protection against Saprolegniosis on salmon eggs, chemical profiling was performed by Nanospray Desorption ElectroSpray Ionization (NanoDESI) live colony mass spectrometry followed by MS/MS analysis for partial identification of the biosurfactant.

Materials and Methods

Isolation of bacteria associated with salmon eggs

Healthy and *Saprolegnia*-infected salmon eggs ($N=6$ for healthy and $N=6$ for diseased eggs) and their corresponding incubation water was collected from a commercial hatchery (Liu *et al.*, 2014). To release bacteria from the surface of eggs, approximately 30 eggs and 20 ml of incubation water of each sample was transferred into a glass tube, vortexed for one minute, sonicated for one minute and vortexed again for one minute. The total culturable bacteria were isolated and enumerated by plating on $1/10^{\text{th}}$ strength tryptone soya broth (Oxoid) with $15\text{--}20\text{ g l}^{-1}$ agar (1/10TSA) supplemented with $100\text{ }\mu\text{g ml}^{-1}$ Delvocide (DSM, Delft, Netherlands) to inhibit fungal growth. *Pseudomonas* strains were isolated and enumerated on semi-selective *Pseudomonas* agar F (PSA, Difco™) supplemented with $100\text{ }\mu\text{g ml}^{-1}$ Delvocide, $12.5\text{ }\mu\text{g ml}^{-1}$ chloramphenicol and $50\text{ }\mu\text{g ml}^{-1}$ ampicillin. Both media were incubated at 25°C for 4 days. From each replicate sample and each growth medium, approximately 40 bacterial isolates were randomly selected and stored, which resulted in a total of approximately 900 random bacterial isolates.

In vitro activity and biosurfactant production by the bacterial isolates

All bacterial isolates were tested for activity against *Saprolegnia diclina* strain VS20 and *Saprolegnia parasitica* strain CBS 223.65 (C65) according to Liu *et al.* (2014). Bacteria were spot-inoculated at the edge of plates of $1/5^{\text{th}}$ strength potato dextrose broth (Difco™) with $15\text{--}20\text{ g l}^{-1}$ agar (1/5PDA) and incubated for 2–4 days at 25°C prior to inoculation of a plug of *Saprolegnia* in the centre of a 1/5PDA plate. Hyphal growth inhibition was monitored for all bacterial isolates during incubation for 4–5 days at 18°C . All bacterial isolates were also screened for biosurfactant production by the drop collapse assay according to the method described by de Bruijn *et al.* (2007).

Identification and phylogeny of bacterial isolates

Pseudomonas isolates inhibiting *Saprolegnia* hyphal growth and/or producing biosurfactants were subjected to BOX-PCR fingerprinting using primer BOX A1R (Versalovic *et al.*, 1994; Rademaker *et al.*, 1998). Representative isolates from the BOX groups with at least 4 isolates for diseased or for healthy salmon egg samples were chosen for phylogenetic analysis. Therefore, a total of 27 representative isolates (S1 Table) were selected and identified by 16S rRNA sequencing. Phylogenetic analyses, including the 16S rRNA sequences of 29 known reference strains (Loper *et al.*, 2012), was performed according to Liu *et al.* (2014). The evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980).

In vivo bioassays to test disease suppression by Proteobacteria

The representative isolates were chosen for activity testing in salmon egg bioassays (Table S1). From the shared representative isolates, *Pseudomonas* isolates S1 and S2, which belonged to the largest shared BOX group and originated from both healthy and diseased salmon eggs were selected. Collectively, a total of 11 representative isolates were selected for activity testing in salmon egg bioassays (Table S1).

The experimental set-up of the *in vivo* bioassays was similar to that described by Liu *et al.* (2014). For bioassay 1 conducted in Norway in 2012, salmon eggs used in this bioassay were 385 degree-days at the day of shipment. Each treatment was conducted in a separate incubation unit containing three perforated cups with 30 live salmon eggs per cup. The 11 representative isolates described above were pre-grown on PSA for 2 days at 25°C , washed with sterile de-mineralized water and added to each salmon egg incubation unit to a final cell density of 10^8 CFU ml^{-1} . *S. diclina* 765F3 and bacteria were added to the incubation units with 2.5 litre of de-chlorinated Norwegian tap water on day 0 (0 day post inoculation) and egg mortality was scored on day 6 (6 days post inoculation, dpi) and expressed as a percentage of the total number of salmon eggs. To further study the activity spectrum of the *Pseudomonas* isolates, a similar experiment was conducted with *S. parasitica* 762F4 (Liu *et al.*, 2014). To check if the bacterial isolates alone were pathogenic to salmon eggs, a similar bioassay as

described above was conducted with the bacterial inoculum only. Also here, the percentage of egg mortality was determined at 6 dpi.

For bioassay 2 conducted in The Netherlands in 2014, the experimental design was adjusted to monitor the disease progress over a longer period of time. Therefore, the salmon eggs were 'younger' than those in bioassay 1, i.e. 321 degree-days at the day of shipment. Each treatment was conducted in two separate incubation units; each incubation unit contained three perforated cups with 51 ± 2 live salmon eggs per cup. Spontaneous rifampicin resistant mutants of the *Pseudomonas* isolates were generated to allow monitoring of their population dynamics. The *Pseudomonas* isolates were added to each salmon egg incubation unit filled with 2 litres of well water (Liu *et al.*, 2014) to the final cell density of 10^7 CFU ml⁻¹. *S. diclina* 1152F4 (Liu *et al.*, 2014) was used in bioassay 2. Salmon eggs not treated with the bacterial isolates or exposed to *S. diclina* only served as the controls. The egg incubation water of each incubation unit was sampled on day 1, day 4 (0 dpi) and day 24 (20 dpi) and dilution-plated on PSA+rifampicin to determine the density of the introduced isolates in the water. Colonization of the applied *Pseudomonas* isolates on the salmon egg surface was determined by rolling one to two eggs from each cup on PSA+rifampicin at 0 and 20 dpi as described by Liu *et al.* (2014). The percentage of salmon eggs to which hyphae of *S. diclina* were attached was determined at 20 dpi according to Liu *et al.* (2014).

S. diclina 765F3 from the healthy sample and *S. diclina* 1152F4 from the diseased sample were used as pathogen source in the bioassay 1 in 2012 and bioassay 2 in 2014, respectively. These two *S. diclina* isolates showed similar pathogenicity on salmon eggs in 2012 (Liu *et al.*, 2014).

Nucleotide sequence accession numbers

The 16S rRNA sequences of *Pseudomonas* strains D1, D2, D3, S1, S2, H1, H2, H3, H4, H5, H6 and S3-S18 have been deposited in GenBank under accession numbers KP890304-KP890314 and KT223371-KT223386, respectively.

Live colony NanoDESI MS/MS data acquisition and molecular networking

The most antagonistic *Pseudomonas* isolate H6 was subjected to chemical profiling by Nanospray Desorption ElectroSpray Ionization (NanoDESI) live colony mass spectrometry as described previously (Watrous *et al.*, 2012; Nguyen *et al.*, 2013). *P. fluorescens* SS101 and *P. fluorescens* SBW25 (Nguyen *et al.*, 2013) were used as references. *Pseudomonas* strains were cultured in Luria-Bertani broth by shaking overnight at 28°C. For *Pseudomonas* H6, four cultures (0.5 µl each) were spot-inoculated on ISP2 agar and incubated for 48 hours at 30°C (Nguyen *et al.*, 2013). *P. fluorescens* SS101 and *P. fluorescens* SBW25 were streaked on 1/5th strength NBY agar (1 g l⁻¹ glucose, 1.6 g l⁻¹ nutrient broth, 0.4 g l⁻¹ yeast extract and 15 g l⁻¹ agar) and cultured for 48 hours at 25°C. Data collection with a data-dependent MS/MS method was conducted on a hybrid 6.4T LTQ-FT (Thermo Electron) mass spectrometer according to Nguyen *et al.* (2013).

The MS/MS data of strains *Pseudomonas* H6, *P. fluorescens* SS101 and *P. fluorescens* SBW25 were combined with data obtained by Nguyen *et al.* (2013) for 18 other *Pseudomonads* and 42 *Bacilli*. Metabolic networks were generated by clustering as described previously (Pierce *et al.*, 2007) using the GNPS website (<http://gnps.ucsd.edu/>). Algorithms were the same as described by Nguyen *et al.* (2013). Networks were visualized using Cytoscape (v 3.1.1). The two plugins used for aiding data visualization and sequence tagging were described previously by Nguyen *et al.* (2013).

HPLC analysis

Pseudomonas H6, *P. fluorescens* SS101 and *P. fluorescens* SBW25 were pre-grown on PSA for 3-6 days at 25°C. Extraction of biosurfactants was conducted according to De Souza *et al.* (2003). After lyophilization, the biosurfactant was dissolved in Milli-Q water. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) analysis was performed by injection of 100 µl sample on a Waters 996 HPLC equipped with a Symmetry C18 Column (100 Å, 5 µm, 3.9 mm X 150 mm, Waters) as described previously (Cheng *et al.*, 2015). Samples were analysed at a flow rate of 0.5 ml/min for 50 min in an isocratic mobile phase of 45:40:15 acetonitrile:methanol:Milli-Q water with 0.1% (v/v) trifluoroacetic acid.

In vitro inhibitory activity of biosurfactants against *Saprolegnia* hyphal growth

The biosurfactant of *Pseudomonas* H6 was tested for activity against hyphae of *S. diclina* 1152F4 and *S. parasitica* C65 by adding the biosurfactant to 1 ml 1/5th strength potato dextrose broth (1/5PDB) in 24-well cell-culture plates (Greiner Bio-One, Kremsmünster, Austria) to final concentrations of 15, 40, 100, 200 $\mu\text{g ml}^{-1}$, respectively. The lipopeptide surfactant massetolide A produced by *P. fluorescens* SS101 was used as a standard. An agar plug of pre-grown *S. diclina* 1152F4 or *S. parasitica* C65 of approximately 0.2 cm^2 was added to each well and incubated at 25°C. Hyphal growth inhibition was scored after 3 days for *S. diclina* 1152F4 or 2 days for *S. parasitica* C65. Morphological abnormalities were monitored under an Olympus SZX12 stereomicroscope and image capture was accomplished using a Zeiss AxioCam MRc 5 camera with Zeiss AxioVision software (AxioVs40 V 4.8.2.0). ImageJ 1.47v (Schneider *et al.*, 2012) was used to measure the diameter of 10 hyphae in each treatment.

In vivo effects of biosurfactants on Saprolegniosis

The salmon eggs had been incubated at 0.92°C on average at AquaGen AS (Trondheim, Norway) and their age was 135 degree-days at the day of shipment. The set-up of the salmon egg bioassay was similar to that of bioassay 2 described above. The biosurfactants from *Pseudomonas* H6 and SS101 were tested at concentrations of $15 \pm 3 \mu\text{g ml}^{-1}$ and $40 \pm 8 \mu\text{g ml}^{-1}$. Malachite green ($2.5 \pm 0.5 \mu\text{g ml}^{-1}$ (ppm)) was used as a chemical reference. All treatments were performed with three separate incubation units (biological replicates), except for the treatments without *S. diclina* 1152F4 where one incubation unit with three incubation cups (technical reps) was used; each cup contained 51 ± 2 live salmon eggs. Application of biosurfactants or malachite green was conducted every 2-3 days by reducing the water level to 600 ± 100 ml and exposing the salmon eggs to the corresponding chemicals for 90-120 min with aeration. Afterwards, the treated water was removed. Each incubation unit was rinsed by 100-200 ml fresh well water and finally 2 litres of fresh well water was added. The percentage of hyphal attachment of *S. diclina* to the salmon eggs was determined on 18 dpi.

Results and Discussion

PhyloChip-based community profiling

Previously we analysed the bacterial community compositions of healthy salmon eggs and *Saprolegnia*-infected salmon eggs (referred to as diseased salmon eggs) by PhyloChip-based profiling (Liu *et al.*, 2014). Healthy eggs harboured more OTUs belonging to the Proteobacteria than diseased eggs (Liu *et al.*, 2014). Amongst the phylum Proteobacteria, the class Gammaproteobacteria was most represented (Figure 1A). Within the Gammaproteobacteria, most OTUs belonged to the orders Enterobacteriales and Pseudomonadales (Figure 1B), accounting for 6.57% and 6.71% of the total bacterial OTUs detected on average on salmon eggs, respectively. The majority of OTUs of the family Pseudomonadaceae belonged to *Pseudomonas*, representing 935 OTUs on average. No significant differences were found in the number of *Pseudomonas* OTUs between diseased and healthy salmon eggs. Given that *Pseudomonas* species (Pseudomonadales) are considered as potential aquaculture probiotics (Verschuere *et al.*, 2000), a more in-depth analysis was conducted here to unravel the genotypic and functional diversity of *Pseudomonas* species associated with healthy and diseased salmon eggs (Figure S1).

Figure 1 Proteobacteria (A) and Gammaproteobacteria (B) community analyses of diseased and healthy salmon eggs by PhyloChip-based analyses. Salmon eggs were sampled from a commercial hatchery (Liu *et al.*, 2014). Indicated is the average number of OTUs at different taxonomic levels: the phylum Proteobacteria (A) and the class Gammaproteobacteria (B). Error bars represent S.E.M (N=6).

Isolation, in vitro activity and phylogeny of aquatic Pseudomonas

The bacteria attached to the salmon eggs were released by vortexing and sonication in the incubation water from the hatchery. The total count of culturable aerobic bacteria was approximately 3×10^7 CFU ml⁻¹ for both healthy and diseased salmon eggs (Figure S2). Amongst 440 randomly selected isolates, 7% and 13% inhibited hyphal growth of *Saprolegnia diclina* VS20 and *Saprolegnia parasitica* C65, respectively. Drop collapse assays further revealed that 3% of the 1/10TSA isolates produced biosurfactants under the experimental conditions tested. For both the healthy and diseased salmon egg samples, the putative *Pseudomonas* population density enumerated on semi-selective PSA medium was approximately 10^6 CFU ml⁻¹ (Figure S2). Amongst 465 randomly selected *Pseudomonas* isolates, 60-69% of the isolates from the diseased salmon eggs and 72-80% of the isolates from the healthy salmon eggs inhibited hyphal growth of *S. diclina* VS20 and/or *S. parasitica* C65. No statistically significant difference was observed between diseased and healthy salmon eggs in the percentage of isolates with *in vitro* growth-inhibiting activities (Figure 2A). However, based on the results obtained in drop collapse assays, healthy eggs harboured a significantly higher frequency ($64 \pm 7\%$) of biosurfactant-producing *Pseudomonas* isolates than diseased salmon eggs ($34 \pm 8\%$) (Figure 2B). A marine biosurfactant-producing *Lactobacillus pentosus* provided protection for the crustacean *Artemia* against pathogenic *Vibrio alginolyticus*, suggesting a potential role of biosurfactants in disease suppression (Garces *et al.*, 2015).

Figure 2 Phenotypic and genotypic analysis of *Pseudomonas* isolates. The isolates were obtained from healthy salmon egg samples and salmon eggs infected with *Saprolegnia*. (A) Mean percentage of the number of bacterial isolates that were inhibitory to hyphal growth of *Saprolegnia diclina* VS20 and *Saprolegnia parasitica* CBS 223.65, tested by observation of an inhibition halo around the bacterial colony (right panel insert). (B) Mean percentage of the number of bacterial isolates that produce biosurfactants based on the drop collapse assay (right panel insert). Error bars represent the S.E.M. (N=6). The asterisk indicates a statistically significant difference ($P<0.05$, Student's *t*-test). (C) Genotypic BOX-PCR grouping of bacterial isolates that inhibiting *Saprolegnia* hyphal growth and/or producing biosurfactants. The Venn diagram (left panel insert) shows the total number of BOX groups obtained for isolates from either diseased, healthy or both samples. An example of the DNA profiles obtained by BOX-PCR is shown in agarose gel (right panel insert).

Genotypic profiling by BOX-PCR of the *Pseudomonas* isolates that inhibited *Saprolegnia* hyphal growth *in vitro* and/or produced biosurfactants, resulted in 131 BOX groups with 43 and 71 unique groups from diseased (D) or healthy (H) salmon egg samples, respectively, and 17 groups with isolates found in both diseased and healthy samples (referred to as 'shared' isolates (S)) (Figure 2C). Representative isolates from BOX groups that consisted of at least 4 isolates

unique for diseased (D) or healthy (H) samples, as well as 18 isolates from the 9 'shared' (S) BOX groups (Table S1) were subjected to phylogenetic analyses. No distinct differences were observed in the 16S rRNA-based phylogenetic delineation of *Pseudomonas* isolates from diseased or healthy salmon eggs. Most of the isolates belonged to the *P. fluorescens* clade, including the isolates from the shared BOX groups, except isolate S13 and S14 that clustered with *P. syringae* (Figure 3).

Bioactivity of aquatic Pseudomonas in vivo

When *Pseudomonas* isolates were applied at an initial density of 10^7 CFU ml⁻¹ to the incubation water *in vivo*, seven out of eleven *Pseudomonas* isolates significantly reduced hyphal attachment of *S. diclina* to salmon eggs (Figure 4). Strains originally isolated from healthy salmon egg samples showed a better control efficacy than those originally isolated from diseased salmon egg samples (Figure 4), suggesting that the healthy salmon eggs harbour *Pseudomonas* strains with stronger activity against *Saprolegnia*. The bacterial density in the incubation water decreased from 10^6 - 10^7 CFU ml⁻¹ on 0 dpi to 10^3 - 10^5 CFU ml⁻¹ on 20 dpi. When tested against another *S. diclina* isolate and *S. parasitica* isolate under different temperatures and bacterial densities, only *Pseudomonas* strain H6 showed the most consistent activity against *Saprolegnia* in all cases (Figure S3 and S4A). Hence, strain H6 was selected for further characterization.

Chemical profiling of aquatic Pseudomonas strain H6

To elucidate which compounds *Pseudomonas* strain H6 produces, live colony mass spectrometry and MS/MS analyses were performed with two phylogenetically related strains *P. fluorescens* SS101 and SBW25 as references. A Cytoscape network with spectra of in total 21 Pseudomonads and 42 Bacilli strains (Nguyen *et al.*, 2013) showed that *Pseudomonas* H6 produces a predominant compound with a parent mass-to-charge ratio of m/z 1148.65, which clustered together with the lipopeptides massetolide A (m/z 1162.69) and viscosin (m/z 1148.67) produced by strains SS101 and SBW25, respectively (Figure 5A). Further examination of the raw MS/MS data showed that mass shifts could be linked to specific amino acids, which created identical sequence tags of 87-113-87-113 Da (Ser-Leu/Ile-Ser-Leu/Ile) for the lipopeptide surfactants produced by *Pseudomonas* H6, *P. fluorescens* SS101 and SBW25 (Figure 5B) (Nguyen *et al.*, 2013). These results suggest that *Pseudomonas* H6 produces a lipopeptide surfactant with a peptide moiety that is, most likely, structurally similar to that of massetolide A and viscosin.

Figure 3 Phylogenetic tree of 16S rRNA sequences of *Pseudomonas* strains representative of 18 BOX-PCR groups. The 16S rRNA sequences were approximately 960 bp. The BOX-PCR groups were identified among the *Pseudomonas* isolates from healthy and diseased salmon eggs (Table S1). A total of 29 reference *Pseudomonas* species/strains were included to delineate the 27 aquatic *Pseudomonas* strains obtained in this study. Bootstrap values at the nodes are based on 1000 replications. Only those branch values higher than 80% are shown. Asterisks indicate the isolates selected for salmon egg bioassays.

Figure 4 *In vivo* activity of 11 *Pseudomonas* strains against *S. diclina* 1152F4. In bioassay 2, the mean percentage of eggs to which of *S. diclina* 1152F4 hyphae attached was determined at 20 days post inoculation (dpi) (Liu *et al.*, 2014). Bacterial strains were introduced at an initial cell density of 10^7 CFU ml^{-1} . The incubation temperature was 5-7°C. Error bars represent S.E.M. ($N=6$). Asterisks indicate statistically significant differences with the control, *S. diclina* only, based on a one-way analysis of variance and *post hoc* LSD analysis ($P<0.05$).

RP-HPLC analysis of the lipopeptide surfactants extracted from these three strains revealed a difference in retention time of the biosurfactants from *Pseudomonas* H6, *P. fluorescens* SS101 and SBW25 (Figure 5C), suggesting that the lipopeptide biosurfactant produced by *Pseudomonas* H6 is structurally not identical to massetolide A or viscosin. The small shift in retention could also be due to a structural difference in the lipid moiety.

Activity profiling of lipopeptide surfactant from aquatic Pseudomonas strain H6

We tested the effect of the purified lipopeptide surfactants of *Pseudomonas* H6 and *P. fluorescens* SS101 on hyphal growth of *S. diclina* 1152F4 (Figure 6A). Both surfactants showed growth-inhibitory activity at $15 \mu\text{g ml}^{-1}$, the lowest concentration tested. Hyphal growth was almost completely inhibited at $100 \mu\text{g ml}^{-1}$ for the biosurfactant of *Pseudomonas* H6 and at $200 \mu\text{g ml}^{-1}$ for massetolide A of *P. fluorescens* SS101 (Figure 6A). The biosurfactant of H6 also showed a stronger activity compared to massetolide A against hyphal growth of *S. parasitica* (Figure 7A). Lipopeptide surfactants are known to cause hyphal swelling, hyphal branching, zoospore lysis and inhibition of cyst germination of plant pathogenic oomycetes (de Bruijn *et al.*, 2007; de Bruijn *et al.*, 2008; van de Mortel *et al.*, 2009). When we

Figure 5 Live colony mass spectrometry and MS/MS networking. (A) Molecular network of *Pseudomonas* H6, *P. fluorescens* SS101, *P. fluorescens* SBW25 and 18 other *Pseudomonads* and 42 *Bacilli* (Nguyen *et al.*, 2013). The viscosin-like cluster was boxed and enlarged. (B) Selection of nodes of *Pseudomonas* H6, *P. fluorescens* SS101 and SBW25 for MS/MS raw spectra analyses and generation of amino acid sequence tag. The sequence tag is created by analysing the mass shifts between adjacent ions in the MS/MS spectra, which are corresponding to the mass of an amino acid. The value of the parent mass ions for *Pseudomonas* H6 and *P. fluorescens* SBW25 in Figure 5B is not completely matching to the parent mass ion as indicated in Figure 5A, because during network analyses a mass tolerance setting of 0.3 Da was used. (C) Reversed-phase HPLC chromatograms of cell-free culture extracts of lipopeptide surfactants from *Pseudomonas* H6, *P. fluorescens* SS101 and SBW25.

Figure 6 Effect of biosurfactants from *Pseudomonas* H6 and *P. fluorescens* SS101 on *S. diclina* 1152F4. (A) *S. diclina* 1152F4 hyphal plugs were incubated for 3 days in 1/5PDB supplemented with biosurfactants from *Pseudomonas* H6 or *P. fluorescens* SS101 (massetolide A) at concentrations ranging from 15 to 200 $\mu\text{g ml}^{-1}$. (B) The activity of the biosurfactants against *S. diclina* under *in vivo* condition was assessed by determining the mean percentage of salmon eggs to which *S. diclina* 1152F4 hyphae were attached at 18 dpi. Salmon eggs were treated with biosurfactants from *Pseudomonas* H6 and *P. fluorescens* SS101 at $15 \pm 3 \mu\text{g ml}^{-1}$ and $40 \pm 8 \mu\text{g ml}^{-1}$. Eggs exposed to *S. diclina* only were used as negative control. Eggs treated with $2.5 \pm 0.5 \mu\text{g ml}^{-1}$ (ppm) malachite green and *S. diclina* were used as the chemical reference. Error bars represent S.E.M. ($N=9$). The asterisk indicates a statistically significant difference from the negative control (*S. diclina* only) based on one-way analysis of variance and *post hoc* LSD analysis ($P<0.05$).

investigated the effect of the lipopeptide surfactants of strains H6 and SS101 microscopically, the diameter of *S. parasitica* hyphae grown in the presence of biosurfactants was larger (Table S2) and exhibited a higher number of branches compared to the control. This phenotypic effect intensified with increasing biosurfactant concentrations; furthermore, massetolide A induces more hyphal branching than the *Pseudomonas* H6 biosurfactant (Figure 7B).

The lipopeptide surfactants from *Pseudomonas* H6 and *P. fluorescens* SS101 were tested in an *in vivo* bioassay at concentrations of $15 \pm 3 \mu\text{g ml}^{-1}$ and $40 \pm 8 \mu\text{g ml}^{-1}$ of incubation water. Because the set-up of the *in vivo* bioassays involved large volumes and the yield of biosurfactants was relatively low, higher biosurfactant

Figure 7 Effect of biosurfactants from *Pseudomonas* H6 and *P. fluorescens* SS101 on *S. parasitica* CBS 223.65. The effect was according to hyphal growth (A) and hyphal morphology (B). *S. parasitica* hyphal plugs were grown for 48 hours in 1/5PDB supplemented with biosurfactants from *Pseudomonas* H6 or *P. fluorescens* SS101 (massetolide A) at concentrations ranging from 15 to 200 $\mu\text{g ml}^{-1}$. The bottom row shows the enlargement of the area indicated in the top row. White arrows indicate branching hyphae. The scale bars in the pictures of panel B represent 100 μm .

concentrations were not tested. At the concentrations tested, no significant reductions in attachment of *S. diclina* hyphae to salmon eggs were found, whereas the chemical control (malachite green) did significantly reduce hyphal attachment (Figure 6B). Although the lipopeptide surfactants were applied every 2-3 days for a duration of 90-120 min each time, instability (e.g. degradation) and/or a too short exposure time may have caused a lack of *in vivo* activity. Biosurfactants have been shown to be degraded in aquatic systems and non-sterile soil (Abd-Allah and Srorr,

1998; Nielsen and Sørensen, 2003; Abu-Ghunmi *et al.*, 2014) and the same may have occurred in the salmon egg incubation units. The fate of the biosurfactants could not be analysed by RP-HPLC analysis, since the applied concentrations were below the detection limit. Although the biosurfactants alone did not affect hyphal attachment by *Saprolegnia* *in vivo*, this does not exclude that these compounds may play a role in the activity of the producing bacterial strain, an aspect that remains to be further investigated. Lipopeptide surfactants are well-known for their role in biofilm formation (de Bruijn *et al.*, 2007; de Bruijn *et al.*, 2008) and may have enabled *Pseudomonas* H6 to colonize the salmon egg surface to form a protective biofilm that avoids hyphal attachment by *Saprolegnia*. Also the biosurfactant produced by *Lactobacillus pentosus* was suggested to facilitate the adhesion to the *Artemia* gut, thereby excluding the colonization of pathogenic *Vibrio alginolyticus* (Garces *et al.*, 2015). The bacterial biofilm provides an ecological niche in which the bacterial cells produce a protective extracellular matrix that is beneficial to their growth and development by excluding other microbes (Verschuere *et al.*, 2000). Whether the biosurfactants are actually produced in the egg incubation units or on the egg surfaces by the introduced *Pseudomonas* strains is not known. Recently, Song and colleagues (Song *et al.*, 2014) showed that massetolide A production by *P. fluorescens* SS101 increased at lower temperatures, indicating that the low temperatures (5-10°C) used in the salmon egg assays may be favourable for biosurfactant production. To provide more evidence for a role of the lipopeptide surfactant of *Pseudomonas* H6 in *in situ* production and protection against Saprolegniosis, site-directed mutagenesis of the biosynthetic genes should be performed in future studies, followed by *in vivo* bioassays where gene transcriptional analyses are conducted and activities of surfactant-deficient mutants are compared to those of the wild type strain H6. Additionally, even though *P. fluorescens* is recognized as a beneficial microbe against *Saprolegnia* (Bly *et al.*, 1997), it is also known to be pathogenic to a wide range of fish species, mostly to carps (Austin and Austin, 2007) but also to salmonids like rainbow trout (*Oncorhynchus mykiss*) (Austin and Austin, 2007) and Chinook salmon (*Oncorhynchus tshawytscha*) (Loch *et al.*, 2012). For some of the *Pseudomonas* isolates tested here, except H6, we indeed observed adverse effects on the salmon eggs (Figure S4B). Further studies should also look into effects of *P. fluorescens* H6 on the life-cycle of salmon post hatching.

Conclusions

Aquaculture is one of the fastest growing animal food sectors (FAO, 2014), partly as a response to the increasing demand for fish protein and regulations to prevent overfishing from wild populations (Bruno *et al.*, 2011). Considering the long-term

importance of aquaculture for food production and economic development, sustainable measures are urgently needed to mitigate emerging diseases including Saprolegniosis. Although the biosurfactant from *Pseudomonas* H6 did not show activity *in vivo*, the bacterial strain itself did provide promising antagonistic activity against *Saprolegnia* infections of salmon eggs. Our research provides a framework for selecting beneficial bacteria that can suppress Saprolegniosis and possibly other emerging diseases in aquaculture.

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Author Contributions

Conceived and designed the experiments: YL ER IdB MvdV VB JMR. Performed the experiments: YL ER MvdV CHW ET. Analyzed the data: YL ER CHW MvdV IdB. Wrote the paper: YL ER IdB MvdV VB JMR. Performed the strain isolations and characterization, genomic fingerprinting, phylogenetic analyses, *in vitro* and *in vivo* experiments: YL. Performed tests of massetolide A against *S. parasitica*: YL ER. Performed live colony mass spectrometry analysis: MvdV CHW PCD. Created Figures: YL ER CHW MvdV IdB. Contributed to HPLC analysis: MvdV. Contributed to design of bioassays: ET IS. Contributed to performance of bioassays: ET. Contributed to review of the manuscript: all authors.

Competing Interests

The authors have declared that no competing interests exist.

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Supporting Information

I Experimental set-up 1 <i>Saprolegnia</i> -infected salmon eggs (N=6) 2 Healthy salmon eggs (N=6)
II Bacterial and archaeal communities PhyloChip-based analysis and selection of target groups
III Functional analysis (<i>Pseudomonas</i>) 1 Phenotypic and genotypic characterisation (<i>in vitro</i> screening and phylogeny) 2 <i>In vivo</i> bioassays to test disease suppression by bacterial isolates 3 Chemical characterisation (LCMS, MS/MS, RP-HPLC)
IV Functional analysis (biosurfactant) 1 <i>In vitro</i> test against <i>Saprolegnia</i> hyphal growth 2 <i>In vivo</i> bioassays to test disease suppression by biosurfactant

Figure S1 Overall strategy used to decipher diversity of aquatic *Pseudomonas* species and their activity against the fish pathogenic oomycete *Saprolegnia*.

Figure S2 Colony count of salmon egg incubation water dilution-plated on 1/10TSA and PSA. Error bars represent S.E.M. (N=6).

Figure S3 *In vivo* activity of the 11 aquatic *Pseudomonas* strains against *S. diclina* on salmon eggs. The mean percentage of egg mortality caused by *S. diclina* 765F3 was determined at 6 days post inoculation (dpi) of this oomycete pathogen. *Pseudomonas* strains D1-D3 and S1 originated from diseased salmon eggs, whereas strains S2 and H1-H6 originated from healthy salmon eggs. All strains were introduced at an initial cell density of 10^8 CFU ml⁻¹. The incubation temperature was $10 \pm 1^\circ\text{C}$. Error bars represent S.E.M. ($N=3$). The asterisk indicates a statistically significant difference from the control (*S. diclina* only) based on a one-way analysis of variance and *post hoc* LSD analysis ($P<0.05$).

Figure S4 *In vivo* activity of 11 *Pseudomonas* isolates against *S. parasitica* and their effect on salmon egg mortality. In bioassay 1, the initial bacterial density was 10^8 CFU ml⁻¹ and incubation temperature was $10 \pm 1^\circ\text{C}$. (A) Mean percentage of egg mortality was determined at 6 dpi of *S. parasitica* 762F4 (Liu *et al.*, 2014). *Pseudomonas* H6 reduced ($P=0.062$) mortality compared to the control with *S. parasitica* only. (B) Mean percentage of egg mortality inoculated with cell suspensions of 11 *Pseudomonas* strains only. Error bars represent S.E.M. ($N=3$). Asterisks indicate statistically significant differences from the controls, *S. parasitica* only (A) or non-treated (B) based on one-way analysis of variance and *post hoc* LSD analysis on ArcSin square root transformed data ($P<0.05$).

Table S1 BOX-PCR genotypic grouping of bacteria isolated from diseased and healthy salmon eggs by plating incubation water on the *Pseudomonas* semi-selective medium PSA. Only the BOX groups that consisted of at least 4 isolates from either diseased or healthy salmon egg samples are shown. One representative isolate from each BOX group was selected for activity testing in salmon egg bioassays. From the shared representative isolates, *Pseudomonas* isolates S1 and S2, which belonged to the largest shared BOX group and originated from both healthy and diseased salmon eggs were selected.

BOX groups	No. of isolates		Isolates selected for phylogeny analysis	Positive against <i>Saproelgnia</i> hyphal growth	Capable of producing biosurfactants	Isolates selected for testing in salmon egg bioassays
	Diseased	Healthy				
1	9	0	D1	Yes	No	D1
2	7	0	D2	Yes	Yes	D2
3	6	0	D3	Yes	Yes	D3
4	0	23	H1	Yes	Yes	H1
5	0	7	H2	Yes	Yes	H2
6	0	5	H3 ^a	Yes	Yes	H3 ^a
7	0	5	H4	Yes	Yes	H4
8	0	4	H5	Yes	Yes	H5
9	0	4	H6	Yes	Yes	H6
10	30	15	S1, S2 ^a	Yes	No	S1, S2 ^a
11	10	9	S3, S4	Yes	No	
12	11	7	S5, S6	Yes	Yes	
13	10	4	S7, S8	Yes	Yes	
14	10	1	S9, S10	Yes	No	
15	3	8	S11, S12	Yes	S11: No; S12: Yes	
16	7	1	S13, S14	Yes	Yes	
17	5	1	S15, S16	Yes	No	
18	2	4	S17, S18	Yes	No	

^a Isolates H3 and S2 were obtained from 1/10TSA, not from PSA.

Table S2 Effect of biosurfactants from *Pseudomonas* H6 and *P. fluorescens* SS101 on hyphal diameter of *S. parasitica* CBS 223.65. Diameter of 10 *S. parasitica* hyphae of each treatment was measured by ImageJ 1.47v. Mean diameter and standard error of the mean are shown. Asterisks indicate statistically significant differences compared to the controls, based on a one-way analysis of variance and *post hoc* LSD analysis ($P < 0.05$).

		Control	15	40	100	200	($\mu\text{g ml}^{-1}$)
Hyphal thickness	H6	0.009 $\pm$	0.012 $\pm$	0.014 $\pm$	0.017 $\pm$	0.017 $\pm$	(mm)
		0.0005	0.0007*	0.0008*	0.0014*	0.0008*	
	SS101	0.007 $\pm$	0.011 $\pm$	0.013 $\pm$	0.011 $\pm$	0.013 $\pm$	(mm)
		0.0006	0.0008*	0.0007*	0.0008*	0.0007*	

Chapter 5

Elucidating the diversity of aquatic *Microdochium* and *Trichoderma* species and their activity against the fish pathogen *Saprolegnia diclina*

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Abstract

Animals and plants are increasingly threatened by emerging fungal and oomycete diseases. Amongst oomycetes, *Saprolegnia* species cause population declines in aquatic animals, especially fish and amphibians, resulting in significant perturbation in biodiversity, ecological balance and food security. Due to the prohibition of several chemical control agents, novel sustainable measures are required to control *Saprolegnia* infections in aquaculture. Previously, fungal community analysis by terminal restriction fragment length polymorphism (T-RFLP) revealed that the Ascomycota, specifically the genus *Microdochium*, was an abundant fungal phylum associated with salmon eggs from a commercial fish farm. Here, phylogenetic analyses showed that most fungal isolates obtained from salmon eggs were closely related to *Microdochium lycopodium*/*Microdochium phragmitis* and *Trichoderma viride* species. Phylogenetic and quantitative PCR analyses showed both a quantitative and qualitative difference in *Trichoderma* population between diseased and healthy salmon eggs, which was not the case for the *Microdochium* population. *In vitro* antagonistic activity of the fungi against *Saprolegnia diclina* was isolate-dependent; for most *Trichoderma* isolates, the typical mycoparasitic coiling around and/or formation of papilla-like structures on *S. diclina* hyphae were observed. These results suggest that among the fungal community associated with salmon eggs, *Trichoderma* species may play a role in *Saprolegnia* suppression in aquaculture.

Introduction

Saprolegniosis, caused by *Saprolegnia* species, results in tremendous losses in wild and cultured fish species including salmonids such as salmon and trout, and non-salmonids such as tilapia, catfish, carp, and eel (Bruno *et al.*, 2011). The typical symptoms of Saprolegniosis are white or grey fungal-like hyphal mats on fish or their eggs (van West, 2006). Yield losses of 10% to more than 50% have been reported in eggs and young fish (Hatai and Hoshiai, 1992; Hatai and Hoshiai, 1993; Bruno *et al.*, 2011).

To control Saprolegniosis, formalin is now commonly applied but is expected to be banned soon due to adverse effects on the environment (Bruno *et al.*, 2011). A limited number of chemical and non-chemical alternative treatments have been tested to control Saprolegniosis, including hydrogen peroxide, sea water flushes and ultraviolet irradiation, but none of these are as effective as the banned malachite green (Bruno *et al.*, 2011). Also, no vaccine is currently available to control this disease (Bruno *et al.*, 2011; van den Berg *et al.*, 2013).

Bacterial genera such as *Bacillus*, *Enterococcus* and *Lactobacillus* have been shown to reduce specific diseases in aquaculture and several of these beneficial

bacteria are being commercialized (Chang and Liu, 2002; Irianto and Austin, 2002; Lara-Flores *et al.*, 2003; Taoka *et al.*, 2006; Gatesoupe, 2008). As a sustainable measure to combat Saprolegniosis, the bacterial genera *Aeromonas*, *Fronidhabitans* and *Pseudomonas* have been proposed (Hatai and Willoughby, 1988; Bly *et al.*, 1997; Hussein and Hatai, 2001; Lategan and Gibson, 2003; Lategan *et al.*, 2004a, b; Bruno *et al.*, 2011; Carbajal-González *et al.*, 2011; Liu *et al.*, 2014). Alike the probiotic bacteria, several beneficial fungi and/or their bioactive compounds are applied to control diseases. These fungal species are isolated either randomly or systematically (Campbell, 1947; Sandys-Winsch *et al.*, 1993; Sallenave-Namont *et al.*, 2000; Vargas Gil *et al.*, 2009; Mulaw *et al.*, 2013; Lopez *et al.*, 2014; Kasa *et al.*, 2015). Amongst commercialized fungi, *Aspergillus oryzae*, *Coniothyrium minitans*, *Phlebiopsis gigantea* and *Trichoderma* (teleomorph *Hypocrea* (Druzhinina *et al.*, 2011)) spp., are able to suppress diseases and promote the growth of various hosts, mainly terrestrial crops and some animals such as cattle (Kmet *et al.*, 1993; de Vrije *et al.*, 2001; Wolken *et al.*, 2003). For fish, *Trichoderma viride* enhanced body weight and reduced mortality of Nile tilapia exposed to *Saprolegnia* sp. (Abdelhamid *et al.*, 2007). The commercial product HetroNex, containing *Trichoderma viride* and *Trichoderma harzianum*, has been developed to control fungal and oomycete diseases caused by *Fusarium*, *Lagenidium* and *Saprolegnia* in aquaculture ponds of fish, prawn and shrimp (Neospark).

In a fungal diversity study of the marine sponge *Dragmacidon reticulatum*, *Trichoderma* represented one of the most abundant genera among the isolated fungi (Passarini *et al.*, 2013). To date, however, still little is known about the fungal community in aquaculture or aquatic environments (Dörr *et al.*, 2012; Liu *et al.*, 2014). Previously, we showed by clone library analyses that the oomycete community associated with *Saprolegnia*-infected (diseased) and healthy salmon eggs from a commercial fish hatchery were dominated by *Saprolegnia* with no difference in the number and pathogenicity of the *Saprolegnia* isolates present in either diseased or healthy salmon egg batches (Liu *et al.*, 2014). Based on terminal restriction fragment length polymorphism (T-RFLP) analysis and clone library sequencing, also no obvious differences were observed in the fungal community composition between the diseased and healthy salmon egg batches (Liu *et al.*, 2014). The clone library consisted of 209 fungal clones, the majority of which belonged to the Ascomycota. More specifically, 139 clones were classified as *Microdochium* (teleomorph *Monographella*) (Alford, 2008; Horst, 2008; Liu *et al.*, 2014). To elucidate the role of fungi in the protection of salmon eggs against Saprolegniosis, we isolated and (phylogenetically) characterized fungi from diseased and healthy salmon eggs. Their abundance in diseased and healthy salmon egg batches and their activity against *Saprolegnia diclina* were investigated here.

Materials and Methods

Phylogenetic analysis of Microdochium and Trichoderma isolates

Fungal isolation from salmon eggs was described previously by Liu *et al.* (2014). DNA isolation, internal transcribed spacer (ITS) rRNA sequencing and phylogenetic analyses of fungal isolates were conducted as described by Liu *et al.* (2014). For *Trichoderma* isolates, additional phylogenetic analysis was conducted with translation elongation factor 1 alpha (*tef1*) sequences. The *tef1* gene was amplified by primer set EF1-728F (Cortinas *et al.*, 2006) and TEF1LLerev (Nagy *et al.*, 2007). The evolutionary distances of the phylogenetic trees were computed using the Kimura 2-parameter method (Kimura, 1980) for *Microdochium* ITS sequences, Tamura 3-parameter method (Tamura, 1992) for *Trichoderma* ITS sequences and Tamura-Nei method (Tamura and Nei, 1993) for *tef1* sequences.

Culture filtrate activity of Microdochium and Trichoderma isolates

One agar plug of each *Microdochium* and *Trichoderma* isolate was pregrown in 6 mL 1/5th strength potato dextrose broth (1/5PDB, Difco™, Franklin Lakes, NJ, USA) for one week at 20–25 °C. Each culture was lyophilized, the pellet dissolved in 6 mL fresh 1/5PDB and filter-sterilized through a 0.2 µm filter (Whatman™, Freiburg, Germany). 1 mL culture filtrate solution was added into a well of 24 well suspension culture plate (Greiner bio-one, Cellstar®, Frickenhausen, Germany) and one agar plug of *Saprolegnia diclina* 1152F4 was added into each well. After incubation at 14–15 °C for four days the effect of the culture filtrates on hyphal growth of *S. diclina* was determined.

Dual culture assay

One agar plug of each of the *Microdochium* or *Trichoderma* isolates and one agar plug of *S. diclina* were placed at two opposite sides of 1/5th strength potato dextrose agar (1/5PDA). After incubation for 6 days at 20–25 °C, inhibition of hyphal growth of *S. diclina* was determined and plates were stored for one to two months at 4 °C until microscopic analyses were performed. Hyphal interactions were observed under a Nikon 90i epifluorescence microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) with brightfield settings and accomplished with Nikon NIS-elements.

Quantification of total fungi, Microdochium and Trichoderma in salmon egg incubation water

DNA extraction from salmon egg samples and storage was described in Liu *et al.* (2014). All DNA samples were thawed on ice and normalized to $5 \pm 1 \text{ ng} \cdot \mu\text{L}^{-1}$. To quantify total fungi in the water samples, ITS rRNA genes were amplified with ITS4 (White *et al.*, 1990) and ITS9 (Ihrmark *et al.*, 2012) primers in 12 µL volumes, each consisted of 5 µL of DNA template, 5.8 µL BIOLINE 2x SensiFAST SYBR No-ROX mix, 0.1 µL ITS4 primer (10 mM), 0.1 µL ITS9 primer (10 mM) and 1 µL BSA (0.1 mg·mL⁻¹). The quantitative PCR program consisted of 1 cycle at 95 °C for 5 min, 45 cycles at 95 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, 82 °C for 15 s. To quantify *Microdochium lycopodium*/*Microdochium phragmitis* in the water samples, MPF (5'-AAGGTACCCGAAAGGGTGCTGG-3') and MPR (5'-GAATTACTGCGCTCAGAGT ACGT-3') primers were designed and firstly the accuracy was verified by PCR using the genomic DNA isolated from *M. phragmitis* CBS 285.71 and *Microdochium nivale* var. *nivale* CBS 110.94 as template (Supplementary Material, Figure S2). The quantitative PCR program consisted of 1 cycle at 95 °C for 5 min, 45 cycles at 95 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s, 82 °C for 15 s. To quantify *Trichoderma* in the water samples, *Trichoderma* specific genes were amplified with ITS1TrF and ITS4TrR primers (Meincke *et al.*, 2010) and QIAGEN Rotor-Gene® SYBR® Green PCR Master Mix 2x was used. The quantitative PCR program consisted of 1 cycle at 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 51 °C for 10 s, 72 °C for 20 s, 82 °C for 10 s. Genomic DNA of *Trichoderma* isolates 1152F1 and 762F1b, and *Microdochium* isolates 736F1a and 749F1 was isolated with PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Dilution series of DNA of each strain was prepared at 5, 5×10^{-1} , 5×10^{-2} , 5×10^{-3} , 5×10^{-4} , 5×10^{-5} , 5×10^{-6} , $5 \times 10^{-7} \text{ ng} \cdot \mu\text{L}^{-1}$ and used as standards (Supplementary Material, Figure S3).

Nucleotide sequence accession numbers

All DNA sequences have been deposited in GenBank. The accession numbers for the internal transcribed spacer sequences of *Trichoderma*, *Microdochium* and *Mortierella* are KU202214-22, KU202223-31 and KU202232-33, respectively. The accession numbers for the sequences of translation elongation factor 1 alpha of *Trichoderma* isolates are KU202234-42.

Results and Discussion

Isolation of fungi from diseased and healthy salmon eggs

Previously, *Saprolegnia*-infected (diseased) and healthy salmon eggs and their corresponding incubation water were sampled from a commercial fish farm ($N = 6$ for diseased eggs and $N = 6$ for healthy eggs) (Liu *et al.*, 2014). Per sample, one or two salmon eggs were placed on potato dextrose agar to allow fungal outgrowth (Figure 1). We obtained and purified 20 fungal isolates in total and by ITS sequencing identified three different genera, *Microdochium*, *Trichoderma* (both Ascomycota) and *Mortierella* (Zygomycota). *Microdochium* and *Mortierella* were the most represented genera in our previous clone library sequence analysis. Interestingly, *Trichoderma* was not detected in the previous analysis, probably due to the limited number of sequenced clones, the specificity of the primers or the efficiency of the PCR reaction (Liu *et al.*, 2014). The two *Mortierella* isolates (BLAST identity of 100% in Genbank database) were isolated from only one healthy salmon egg sample. Here we aimed at comparing isolates obtained from multiple replicate samples of diseased and healthy eggs. Therefore, we focused on the Ascomycota isolates for the subsequent analyses described below.

Figure 1 Isolation of salmon egg-associated fungi and oomycetes on potato dextrose agar plates. One or two salmon eggs from a *Saprolegnia*-infected batch (diseased, replicate No. 1–6) or a healthy batch (replicate No. 7–12) were placed onto the agar plates to allow fungal outgrowth.

Microdochium

Microdochium species are known as snow molds, and some are pathogenic to plants (Cook, 1981; Mediha *et al.*, 1986; Daamen *et al.*, 1991; Simpson *et al.*, 2000; Hong *et al.*, 2008; Matsumoto, 2009; Xu and Nicholson, 2009; Rapacz *et al.*, 2014). *Microdochium nivale* and *Microdochium majus* are two of the main causative agents of *Fusarium* head blight (Xu and Nicholson, 2009), whereas *Microdochium lycopodium* and *Microdochium phragmitis* were isolated from plants without causing disease (Ernst *et al.*, 2011; Jaklitsch and Voglmayr, 2012). *M. phragmitis* was endophytic in common reed and was more present in flooded habitats than the closely related *Microdochium bolleyi* (Ernst *et al.*, 2011). Some *Microdochium* species were antagonistic to the plant pathogen *Verticillium dahliae* (Berg *et al.*, 2005). Amongst our nine *Microdochium* isolates, five were isolated from diseased and four from healthy salmon eggs (Table 1). The origin of our *Microdochium* isolates was possibly from the catchment area, which was the water source for the salmon egg incubators (Liu *et al.*, 2014). Based on the phylogenetic analyses of internal transcribed spacer (ITS) sequences, all the nine *Microdochium* isolates are closely related to *M. lycopodium* and *M. phragmitis*, and no distinct separation is observed between isolates from diseased or healthy salmon eggs (Figure 2). Quantitative PCR using *M. lycopodium*/*M. phragmitis* specific primers showed and confirmed that *M. lycopodium*/*M. phragmitis* was detected in equal amounts in total DNA samples obtained from diseased and healthy salmon egg samples (Figure 3). One *Microdochium* isolate (749F1) inhibited the hyphal growth of *Saprolegnia diclina* on 1/5th strength potato dextrose agar (1/5PDA) (Table 1, Figure 4a) suggesting the secretion of enzymes or other bioactive metabolites.

Table 1 *In vitro* activity of *Microdochium* or *Trichoderma* isolates against hyphal growth of *Saprolegnia diclina* 1152F4. The fungal isolates were retrieved from *Saprolegnia*-infected (diseased) or healthy salmon egg samples.

Genus	Strain no.	Salmon egg sample	Activity of culture filtrate	Dual culture assay on 1/5PDA	Hyphal interaction with <i>S. diclina</i> microscopically
<i>Microdochium</i>	41F2	Diseased	Not inhibitory	Not inhibitory	Not observed
	684F5	Diseased	Not inhibitory	Not inhibitory	Not observed
	736F1a	Diseased	Not inhibitory	Not inhibitory	Not observed
	736F1b	Diseased	Not inhibitory	Not inhibitory	Not observed
	1056F2	Diseased	Not inhibitory	Not inhibitory	Not observed
	765F1a	Healthy	Not inhibitory	Not inhibitory	Not observed
	765F1b	Healthy	Not inhibitory	Not inhibitory	Not observed
	749F1	Healthy	Not inhibitory	Inhibitory	Not observed
	749F2	Healthy	Not inhibitory	Not inhibitory	Not observed
<i>Trichoderma</i>	684F1	Diseased	Not inhibitory	Not inhibitory	Coiling, papilla-like structure
	1056F1	Diseased	Not inhibitory	Not inhibitory	Papilla-like structure
	1152F1	Diseased	Not inhibitory	Not inhibitory	Inconclusive
	762F1a	Healthy	Not inhibitory	Not inhibitory	Coiling
	762F1b	Healthy	Not inhibitory	Not inhibitory	Coiling
	762F2	Healthy	Not inhibitory	Not inhibitory	Papilla-like structure
	764F1	Healthy	Inhibitory	Not inhibitory	Papilla-like structure
	764F2	Healthy	Not inhibitory	Not inhibitory	Coiling, papilla-like structure
	764F3	Healthy	Not inhibitory	Not inhibitory	Coiling, papilla-like structure

Figure 2 Phylogenetic tree of ITS rRNA sequences of nine *Microdochium* isolates from salmon eggs and reference strains. The phylogenetic analyses were conducted in Mega 5 using the Kimura 2-parameter method (Kimura, 1980) to compute evolutionary distances. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. Branch values lower than 50% are hidden. Closed and open circles indicate *Microdochium* isolates from *Saprolegnia*-infected (diseased) or healthy salmon egg samples, respectively. Red, blue and black colors indicate strains from terrestrial/plant, aquatic and unknown sources of isolation, respectively. The scale bar indicates an evolutionary distance of 0.01 nucleotide substitution per sequence position. Twenty-five ITS sequences of good quality and at least 550 bp of reference strains of *Microdochium* were downloaded from GenBank; their strain names are preceded by the accession numbers.

To date, not much is known about *Microdochium* in aquatic environments, aquaculture or aquatic animals (Liu *et al.*, 2014). Also not much is known about the bioactive compounds produced by *Microdochium*. Bhosale *et al.* (2011) reported that the active compound cyclosporine A, extracted from an estuarine *M. nivale*, has the potential to be applied pharmaceutically to control diseases caused by some dermatophytes and *Aspergillus* species in human and animals (Bhosale *et al.*, 2011); Santiago *et al.* (2012) reported that the extract of *M. phragmitis*, which was isolated from Antarctic angiosperms, showed cytotoxic activity against a human tumoral cell line (Santiago *et al.*, 2012). Therefore, further experiments are needed to decipher the bioactive potential capacity of our *Microdochium* isolates, especially their interaction with pathogens from cold water environments, like *Saprolegnia* spp.

Figure 3 Detection of total fungi, *Microdochium* and *Trichoderma* in salmon egg samples by quantitative PCR. Total fungal community was detected using ITS4-ITS9 primers, *Microdochium lycopodium*/*Microdochium phragmitis* species were detected by MPF-MPR primers, and *Trichoderma* species were detected by ITS1TrF-ITS4TrR primers. The concentration of DNA template of each sample was normalized at $5 \pm 1 \text{ ng} \cdot \mu\text{L}^{-1}$. Closed and open circles indicate DNA samples extracted from *Saprolegnia*-infected (diseased) or healthy samples, respectively. The lane on the left is the size marker and the band size (bp) is indicated next to each band.

Trichoderma

Most *Trichoderma* species are applied in agriculture as biocontrol agents against various plant-associated bacterial, fungal and oomycete pathogens, such as *Clavibacter*, *Fusarium* and *Phytophthora* (Verma *et al.*, 2007; Schuster and Schmoll, 2010; Druzhinina *et al.*, 2011). *Trichoderma* species are capable of producing a range of extracellular compounds to suppress plant pathogens, such as enzymes, fungicidal compounds and antibiotics; they can also promote plant growth via symbiotic association with plant hosts (Schirmböck *et al.*, 1994; Harman *et al.*, 2004; Schuster and Schmoll, 2010; Druzhinina *et al.*, 2011; Harman, 2011). *Trichoderma* is commonly isolated from terrestrial environments, such as soil and wood (Druzhinina *et al.*, 2011), but also from aquatic environments like freshwater (drinking water) and marine water (Miao and Qian, 2005; Hageskal *et al.*, 2008; Gal-Hemed *et al.*, 2011; Zhou *et al.*, 2011; Passarini *et al.*, 2013; Wu *et al.*, 2014). Marine *Trichoderma atroviride* and *Trichoderma asperelloides* suppressed disease caused by *Rhizoctonia solani* on beans and enhanced defence responses against pathogenic *Pseudomonas syringae* pv. Lachrimans on cucumber seedlings (Gal-Hemed *et al.*, 2011). Some other marine-derived *Trichoderma* strains were capable of producing antagonistic compounds against cancer, diabetes, cancer cell lines or pathogenic *Staphylococcus epidermidis*; such compounds include tandukisins from *Trichoderma harzianum* OUPS-111D-4, pyridones from *Trichoderma* sp. MF106, and trichoketides from *Trichoderma* sp. TPU1237 (Wu *et al.*, 2014; Yamada *et al.*, 2015; Yamazaki *et al.*, 2015).

Figure 4 *In vitro* activities of *Microdochium* and *Trichoderma* isolates. (a) Dual culture of *Microdochium* isolate 749F1 or *Trichoderma* isolate 762F1b with *Saprolegnia diclina* 1152F4 on 1/5th strength potato dextrose agar (1/5PDA). *S. diclina* and the fungal isolates were pre-grown on 1/5PDA. A hyphal plug of *S. diclina* was inoculated on the left side of the fresh 1/5PDA and a hyphal plug of *Microdochium* isolate 749F1 or *Trichoderma* isolate 762F1b was inoculated on the right side. The dual cultures were incubated for six days at 20–25 °C. The black arrows indicate *S. diclina* plugs and the white arrows indicate *Microdochium* or *Trichoderma* plugs; (b) Microscopic pictures of the hyphal interaction between *Trichoderma* and *S. diclina*. The black arrows indicate hyphae of *S. diclina*. The white arrows indicate the coiling of *Trichoderma* hyphae around *S. diclina* hyphae (top pictures) or the formation of papilla-like structure of *Trichoderma* hyphae around *S. diclina* hyphae (bottom pictures). Scale bars represent 10 μm.

It was suggested that *Trichoderma* may have the potential to also control infectious diseases in aquaculture (Citarasu, 2012). Among our nine *Trichoderma* isolates, three were isolated from diseased and six from healthy salmon eggs (Table 1). Quantitative PCR with *Trichoderma*-specific primers showed that *Trichoderma* was present in higher abundance in total DNA samples of healthy than of diseased salmon eggs (Figure 3), although some variation in results were observed between replicated PCR reactions on the same DNA samples (Supplementary Material, Figure S1). The total fungal community did not differ in abundance between healthy and diseased salmon eggs (Figure 3). Collectively, these results suggest that *Trichoderma* is more enriched in healthy salmon egg samples than in diseased salmon egg samples. Phylogenetic analyses based on ITS sequences showed that all nine *Trichoderma* isolates belonged to the *Trichoderma* section (Druzhinina and Kopchinskiy) and no apparent separation was observed between isolates from diseased or healthy salmon eggs based on ITS sequences (Figure 5). However, phylogeny based on sequences of the translation elongation factor 1 alpha (*tef1*) clearly separated the *Trichoderma* isolates from diseased and healthy salmon eggs (Figure 6). Our nine *Trichoderma* isolates and the *Trichoderma viride* reference strains (Jaklitsch *et al.*, 2006; Jaklitsch, 2011) formed three clades of *Trichoderma viride*. These results suggest that next to a quantitative difference also a qualitative difference in *Trichoderma* populations from diseased and healthy salmon eggs.

In terms of extracellular activity, we observed that the culture filtrate of only one *Trichoderma* isolate showed inhibition of hyphal growth of *S. diclina* (Table 1). Dual culture assays did not show inhibition of hyphal growth of *S. diclina* by any of the *Trichoderma* isolates tested (Table 1, Figure 4a). However, the hyphae of most *Trichoderma* isolates coiled around or produced a papilla-like structure on the hyphae of *S. diclina* (Table 1 and Figure 4b) (Druzhinina *et al.*, 2011). The coiling and papilla-like structures suggest attachment of *Trichoderma* hyphae to *S. diclina* hyphae. Coiling is required for mycoparasitism but not all coiling leads to mycoparasitism (Harman *et al.*, 2004; Lu *et al.*, 2004; Druzhinina *et al.*, 2011; Harman, 2011). The formation of papilla-like structures in the interaction with *S. diclina* could indicate the start of mycoparasitic invasion by *Trichoderma*; these structures have been shown to induce hyphal breakdown of various hosts (Rocha-Ramírez *et al.*, 2002; Harman *et al.*, 2004; Chacón *et al.*, 2007; Druzhinina *et al.*, 2011; Harman, 2011).

Figure 5 Phylogenetic tree of ITS rRNA sequences of nine *Trichoderma* isolates and reference strains. The phylogenetic analyses were conducted in Mega 5 using the Tamura 3-parameter method (Tamura, 1992) to compute evolutionary distances. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. Branch values lower than 50% are hidden. Closed and open circles indicate isolates from *Saprolegnia*-infected (diseased) or healthy samples, respectively. Red, blue and black colors indicate strains from terrestrial/plant, aquatic and unknown sources of isolation, respectively. The scale bar indicates an evolutionary distance of 0.01 nucleotide substitution per sequence position. Outer labels describe section names based on the list of species in ISTH website (Druzhinina and Kopchinskiy) and only sections contained at least five strains are indicated. 104 ITS sequences of good quality and at least 550 bp of reference strains of *Trichoderma* were downloaded from GenBank; their corresponding strain names are preceded by the accession numbers. Strain names followed by "(T)" indicate type strains.

Figure 6 Phylogenetic tree of *tef1* sequences of nine *Trichoderma viride* isolates and reference strains. The phylogenetic analyses were conducted in Mega 5 using the Tamura-Nei method (Tamura and Nei, 1993) to compute evolutionary distances. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. Branch values lower than 50% are hidden. Closed and open circles indicate isolates from *Saprolegnia*-infected (diseased) or healthy samples, respectively. Red, blue and black colors indicate strains from terrestrial/plant, aquatic and unknown sources of isolation, respectively. The scale bar indicates an evolutionary distance of 0.01 nucleotide substitution per sequence position. 11 sequences of good quality and at least 1200 bp of reference strains of *Trichoderma* were downloaded from GenBank; their strain names are preceded by the accession numbers. Strain name followed by “(T)” indicates type strain.

Even though *Trichoderma* species are commonly considered beneficial fungi, some *Trichoderma* strains, including *T. harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma pseudokoningii* and *Trichoderma viride*, maybe pathogenic to human (Escudero Gil *et al.*, 1976; Loepky *et al.*, 1983; Jacobs *et al.*, 1992; Gautheret *et al.*, 1995; Seguin *et al.*, 1995; Tanis *et al.*, 1995; Campos-Herrero *et al.*, 1996; Guiserix *et al.*, 1996; Munoz *et al.*, 1997; Furukawa *et al.*, 1998; Guarro *et al.*, 1999; Richter *et al.*, 1999; Rota *et al.*, 2000; Chouaki *et al.*, 2002; Myoken *et al.*, 2002; De Miguel *et al.*, 2005). Some marine *Trichoderma* were associated to contaminated mussels and some were even toxic to aquatic animals, such as *Artemia* larvae (Sallenave-Namont *et al.*, 2000; Marrouchi *et al.*, 2013). Therefore, evaluations of the adverse effects of our *Trichoderma* isolates on the environment and humans are needed.

Previous work by Abdelhamid *et al.* (2007) indicated that *T. viride* can enhance body weight and reduce mortality of Nile tilapia treated with *Saprolegnia* sp. (Abdelhamid *et al.*, 2007). Our isolates belong to the *T. viride* clade and are, to our knowledge, the first characterized *Trichoderma* from salmon eggs. Collectively, our results pointed to both quantitative and qualitative differences in *Trichoderma* population between diseased and healthy salmon eggs. These analyses suggest a potential role of *Trichoderma* species in the protection of salmon eggs from *S. diclina*. Hence, our *Trichoderma* isolates and/or their metabolites, especially isolate 764F1 and its bioactive compounds, may have the potential to be applied in aquaculture. To this end, *in vivo* experiments should be conducted to determine the beneficial effects of our *Trichoderma* isolates in controlling Saprolegniosis and other aquaculture diseases.

Conclusions

Aquaculture has become one of the fastest developing animal food sectors (FAO, 2014), partly due to regulations to protect wild fish populations from overfishing and the increased demand for fish products (Bruno *et al.*, 2011). To support this increase in food demand, aquaculture production is gradually intensifying, but effective and sustainable strategies are needed to suppress emerging diseases including Saprolegniosis. Very few studies have demonstrated the beneficial activity of fungi against aquatic pathogens (Abdelhamid *et al.*, 2007; Woodhams *et al.*, 2014). Our study is, to our knowledge, the first to establish correlations between the frequency/occurrence of indigenous fungal communities (*Trichoderma* and *Microdochium* species) and the health status of salmon eggs in a commercial hatchery. Our study is also the first to assess the diversity among *Trichoderma* and *Microdochium* isolates from aquaculture samples. The traditional plate assays provided informative results showing the potential antagonistic activity of our *Trichoderma* isolates obtained from salmon eggs against the pathogen *Saprolegnia diclina*. These results demonstrated the basic characters of our *Trichoderma* isolates, which provide a good starting point for future analyses on the molecular basis of *Trichoderma*-*Saprolegnia* interactions. Further *in vitro* and *in vivo* tests are needed to confirm their beneficial protective activity *in situ*. The role of *Trichoderma* in *Saprolegnia* disease suppression is especially interesting, since *Trichoderma* was shown to be more abundant in healthy salmon eggs than in diseased ones and showed a mycoparasitic interaction with *Saprolegnia*. Our study provides a framework to isolate and monitor putative protective fungi in *Saprolegnia* control and possibly other emerging diseases in aquaculture.

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Author contributions

Conceived and designed the experiments: Yiyang Liu, Christin Zachow, Irene de Bruijn and Jos M Raaijmakers. Performed the experiments: Yiyang Liu. Analyzed the data: Yiyang Liu, Christin Zachow, Irene de Bruijn. Created figures: Yiyang Liu, Irene de Bruijn. Wrote the paper: Yiyang Liu, Irene de Bruijn, Jos M Raaijmakers. Contributed to phylogenetic analyses, microscopic observation and quantitative PCR design: Christin Zachow. Designed *M. lycopodium*/*M. phragmitis* specific primers: Yiyang Liu, Irene de Bruijn. Contributed to review of the manuscript: all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Figure S1 Reproducibility of quantitative PCR based detection of *Trichoderma* species in salmon egg samples. Eight independent quantitative PCRs were run for each salmon egg sample to show the variation of amplification. The concentration of DNA template of each sample was normalized at 5 ± 1 ng· μ L⁻¹. Closed and open circles indicate *Saprolegnia*-infected (diseased) or healthy salmon egg samples, respectively. The expected band size is around 500 bp.

Figure S2 Amplification of *Microdochium lycopodium*/*Microdochium phragmitis* specific gene by primer set MPF-MPR using *M. phragmitis* CBS 285.71 as positive control and *M. nivale* var. *nivale* CBS 110.94 as negative control.

Figure S3 Standards of the quantitative PCR to determine the detection limits. The genomic DNA of the indicated fungal strains was set at 5 ng·μL⁻¹ and diluted in 10-fold steps. Each dilution, indicated at the top, was used as template for the PCR using the primer sets ITS4-ITS9 (a), MPF-MPR (b) and ITS1TrF-ITS4TrR (c).

Chapter 6

Summarizing discussion and future perspectives

Emerging pathogens of plants and animals are increasingly threatening global ecosystem functioning, biodiversity and food security (Fisher *et al.*, 2012, Gozlan *et al.*, 2014). Introduction of protective microbes and habitat bioaugmentation have been proposed as novel strategies to protect wild and farmed aquatic animals from emerging disease. Both strategies need profound knowledge of the microbial communities associated with aquatic animals. In recent years, several studies have shown that microbiomes play a vital role in the protection against pathogens in a variety of eukaryotes including humans, insects and plants (Round *et al.*, 2010, Mendes *et al.*, 2011, Weiss & Aksoy, 2011, Everard *et al.*, 2013, Stecher *et al.*, 2013, Turner *et al.*, 2013). For aquatic animals, such as fish and amphibians, large-scale metagenomics-based microbiome studies have only been initiated recently (Cahill, 1990, Hansen & Olafsen, 1999, Schulze *et al.*, 2006, McKenzie *et al.*, 2012, Merrifield & Rodiles, 2015) and little is known on the diversity, richness, dynamics and functions of microbes associated with fish eggs (Hansen & Olafsen, 1999, Olafsen, 2001). Fish eggs have an immature adaptive immune system and depend on nonspecific innate defences for protection against pathogen infections. In this thesis, the salmon egg associated microbiomes (bacteria, oomycetes and fungi) were analysed by both classic and state-of-the-art 'omics technologies. Potential beneficial microbes were subsequently isolated and characterised for their genotypic, phenotypic and metabolic diversity as well as their protective activity on salmon eggs against the oomycete pathogen *Saprolegnia diclina*.

The microbiome of Atlantic salmon eggs was unravelled by using various metataxonomic analyses including PhyloChip, clone library sequencing and terminal restriction fragment length polymorphism (T-RFLP; chapter 2). The results of these analyses showed that healthy and *Saprolegnia*-infected (diseased) salmon eggs are home to diverse oomycete, fungal, bacterial and archaeal communities. The oomycete community in both diseased and healthy salmon egg samples from a commercial fish hatchery, consisted mainly of *Saprolegnia*, *Achlya* and *Aphanomyces*, three genera in the order Saprolegniales. Even though *Achlya* and *Aphanomyces* have been described to be pathogenic on fish, no reports have described that these are pathogenic for Atlantic salmon. Virulent *Saprolegnia* isolates were found in both diseased and healthy salmon eggs, indicating that the difference in incidence of Saprolegniosis between the two sets of salmon egg samples cannot be explained by differences in population structure or pathogenicity of the *Saprolegnia* species present in both sample sets.

Analysis of the bacterial community composition revealed a total of 31,278 bacterial and 3 archaeal operational taxonomic units (OTUs). The most dominant bacterial phyla for the salmon egg samples were the Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (chapter 2). A low incidence of Saprolegniosis was correlated with a significant higher abundance and richness of

specific commensal Actinobacteria. Subsequent isolation of Actinobacteria and functional bioassays showed that an Actinobacterial isolate, classified as *Fron dihabitans* (Microbacteriaceae), significantly inhibited hyphal attachment of *S. diclina* to live salmon eggs (chapter 2).

The culturable *Fron dihabitans* population associated with healthy salmon eggs was further investigated (chapter 3) and results showed that most *Fron dihabitans* isolates inhibited growth *in vitro* of *S. diclina*, formed biofilms and had extracellular protease and lipase activities at low temperature (5 °C). When grown at 25 °C, the enzymatic activity was more variable between isolates and only one isolate inhibited growth of *S. diclina*. This suggests that *Fron dihabitans* is producing enzymes and antimicrobial compounds in a temperature-dependent manner. Enzymes active at cold-temperate conditions are potentially of great interest for industrial, medical, biotechnological and domestic applications. The *Fron dihabitans* sp. 762G35 that inhibited hyphal attachment of *S. diclina* to salmon eggs was selected for genome sequencing. Its genome is 3.4 Mb in size with a GC content of 70% and 3212 coding DNA sequences (CDS). An in-depth study was conducted to further analyse the genome of *Fron dihabitans* sp. 762G35. Among the clusters of orthologous groups (COGs), the categories carbohydrate and amino acid transport and metabolism, and transcription were the three largest COGs. Whole genome phylogeny showed that *Fron dihabitans* sp. 762G35 is related to *Fron dihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415, two bacteria isolated from leaves of *Arabidopsis thaliana* (Bai *et al.*, 2015). Pan-core genome analyses of *Fron dihabitans* sp. 762G35, *Fron dihabitans* sp. Leaf304 and *Frigoribacterium* sp. Leaf415 showed a core genome of 1292 orthologous groups, representing only 40% of each genome and with a pan genome of 5955 orthologous groups. More than one-third of the *Fron dihabitans* sp. 762G35 genome, comprising 1206 orthologous groups, was not found in the other two closely related bacterial strains. Mining for biosynthetic gene clusters by antiSMASH (Weber *et al.*, 2015) showed that *Fron dihabitans* sp. 762G35 harboured two terpene gene clusters and one type III polyketide synthase gene cluster. High performance liquid chromatography (HPLC) analysis and fractionation of the extracellular culture filtrate of *Fron dihabitans* sp. 762G35 resulted in a polar fraction that was capable of inhibiting *S. diclina* growth (chapter 3). Since this fraction still consisted of multiple compounds, liquid chromatography-mass spectrometry (LC-MS) was performed and this resulted in the identification of furancarboxylic acid-derived metabolites in the cell-free supernatant that might be involved in the activity of *Fron dihabitans* sp. 762G35 against *S. diclina*. Additional fractionations and liquid chromatography-tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) analyses are required to further identify these active compounds from

Fronidhabitans sp. 762G35. Such compounds may provide a basis for the further development of alternative measures to control Saprolegniosis in aquaculture.

Based on the PhyloChip-based metataxonomic analyses, *Pseudomonas* (Pseudomonadales) represented one of the most abundant bacterial genera associated with the salmon eggs (chapter 2). No distinct difference was observed in the 16S rRNA-based phylogenetic delineation of 440 *Pseudomonas* isolates from diseased or healthy salmon eggs, nor in the percentage of isolates with *in vitro* activities against *Saprolegnia* (chapter 4). However, a significantly higher frequency of biosurfactant-producing *Pseudomonas* was found among the isolates from healthy salmon eggs than among those from diseased eggs. Biosurfactants have been suggested to play a role in disease suppression (Raaijmakers *et al.*, 2010, Garces *et al.*, 2015). When tested against *S. diclina* and *S. parasitica*, only *Pseudomonas* strain H6 consistently inhibited hyphal attachment to live salmon eggs and reduced salmon egg mortality. Live colony mass spectrometry showed that *Pseudomonas* H6 produces a viscosin-like compound, a biosurfactant belonging to the class of cyclic lipopeptides. This biosurfactant inhibited hyphal growth of *S. diclina* and *S. parasitica* *in vitro*, but no significant effects were observed in protection of salmon eggs against *Saprolegnia* infection (chapter 4). Although the biosurfactant alone did not affect hyphal attachment of *S. diclina* to salmon eggs, this compound may still have a role in the activity and ecology of the producing bacterial strain, an aspect that remains to be investigated. For example, lipopeptide surfactants are well-known for their role in biofilm formation (de Bruijn *et al.*, 2007, de Bruijn *et al.*, 2008) and may have enabled *Pseudomonas* H6 to colonize the salmon egg surface to form a protective biofilm that prevents hyphal attachment by *Saprolegnia*.

Very few studies to date have addressed the beneficial activity of fungi against aquatic pathogens (Abdelhamid *et al.*, 2007, Woodhams *et al.*, 2014). Our analysis showed that the fungal community of the salmon eggs was dominated by Ascomycota, and mainly *Microdochium* species. Furthermore, no apparent difference was found in the fungal microbiome composition between diseased and healthy salmon eggs (chapter 2). Subsequent isolation and characterization of the salmon egg-associated fungi showed that most isolates were closely related to *Microdochium lycopodium*/*Microdochium phragmitis*, and also to *Trichoderma viride* (chapter 5). *Microdochium* species are known as snow molds and some are pathogenic to plants (Cook, 1981, Mediha *et al.*, 1986, Daamen *et al.*, 1991, Simpson *et al.*, 2000, Hong *et al.*, 2008, Matsumoto, 2009, Xu & Nicholson, 2009, Rapacz *et al.*, 2014). To date, not much is known about *Microdochium* in aquatic environments, aquaculture or aquatic animals (Liu *et al.*, 2014). Several *Trichoderma* species are applied in agriculture as biocontrol agents against various plant-associated bacterial, fungal and oomycete pathogens, such as *Clavibacter*,

Fusarium and *Phytophthora* (Verma *et al.*, 2007, Schuster & Schmoll, 2010, Druzhinina *et al.*, 2011). *Trichoderma* species are capable of producing a range of extracellular compounds to suppress plant pathogens, such as enzymes, fungicidal compounds and antibiotics, and they can also promote growth of plant hosts (Schirmböck *et al.*, 1994, Harman *et al.*, 2004, Schuster & Schmoll, 2010, Druzhinina *et al.*, 2011, Harman, 2011). *Trichoderma* is commonly isolated from terrestrial environments, such as soil and wood (Druzhinina *et al.*, 2011), but also from aquatic environments both fresh and marine water (Miao & Qian, 2005, Hageskal *et al.*, 2008, Gal-Hemed *et al.*, 2011, Zhou *et al.*, 2011, Passarini *et al.*, 2013, Wu *et al.*, 2014). In our study, quantitative PCR and phylogenetic analyses revealed both qualitative and quantitative differences in the *Trichoderma* community associated with diseased and healthy salmon eggs, differences that were not observed for the *Microdochium* community. Only few fungal isolates showed *in vitro* antagonistic activity against *S. diclina*. For most *Trichoderma* isolates, the typical mycoparasitic coiling around and/or formation of papilla-like structures on *S. diclina* hyphae were observed (chapter 5), suggesting attachment of *Trichoderma* hyphae to *S. diclina* hyphae. Coiling is required for mycoparasitism but not always leads to mycoparasitism (Harman *et al.*, 2004, Lu *et al.*, 2004, Druzhinina *et al.*, 2011, Harman, 2011). These results imply that among the commensal fungal microbiome of salmon eggs, *Trichoderma* species may be important to suppress *Saprolegnia* infection in aquaculture.

Overall, my research project demonstrated that the fundamental study of the microbiome of salmon eggs provides a framework for identifying beneficial microbes and their metabolites. This study together with other microbiome studies may ultimately be used to mitigate emerging diseases of aquatic animals in farmed and natural ecosystems. Future studies are needed to identify the genes and bioactive compounds involved in disease suppression and to unravel the underlying mechanisms. To this end, in-depth metabolomic analyses will help to understand the identities and activities of the bioactive compounds. Mutagenesis of biosynthetic genes encoding these compounds should be conducted, followed by *in vivo* bioassays complemented with transcriptomic analyses and activity profiling in which compound-deficient mutants are compared to wild type strains. Further large-scale tests under farming conditions are needed to assess the *in situ* activity of the beneficial bacterial and fungal isolates that were identified in this thesis.

Figure 1 Deciphering the microbial landscapes of Atlantic salmon eggs to mitigate the emerging disease Saprolegniosis. The studies described in this thesis were based on culture-independent analyses (highlighted in yellow) or a combination of culture-dependent and independent analyses (highlighted in cyan) and revealed potential mechanisms involved in suppressing *Saprolegnia* (highlighted in gray).

Future perspectives

Aquaculture is one of the fastest-growing food sectors in the world and important for the growing human population, human health and the economy by providing high value of animal proteins (FAO, 2014). Diseases threaten the development and productivity of aquaculture and need to be addressed in a sustainable manner. Amongst the existing and promising sustainable measures, probiotics and prebiotics are proposed as environmental friendly remedies to suppress diseases and to enhance productivity in aquaculture and agriculture. Our research provides a fundamental framework for the identification of the diversity and dynamics of microbial communities associated with fish eggs. This fundamental knowledge is needed to target specific microbial groups or microbial consortia for isolation, characterization and evaluation as protective microbiota for mitigation of emerging diseases in farmed and natural ecosystems. The bacterial and fungal isolates that we identified in this study, especially *Frondihabitans*, *Pseudomonas* and *Trichoderma* isolates, and their bioactive metabolites, are proposed as effective candidates to tackle the (re-)emerging disease Saprolegniosis. Although numerous studies have demonstrated the beneficial effects of microorganisms, very few are commercialized in aquaculture to date, largely due to concerns on long-term safety for the environment as well as other issues such as limited shelf-life, costs, stringent storage and transport conditions of the microbial inoculants. Also consistency of performance under different environmental conditions and their relatively narrow activity spectrum and compatibility with application processes are points of concern for the industry. International programs on disease control have been initiated with the aim to obtain more in depth knowledge and to gain experience with the application of probiotics (Verschuere *et al.*, 2000, Pandiyan *et al.*, 2013, Velivelli *et al.*, 2014). One example is the ParaFishControl program funded by the European Commission (<http://www.parafishcontrol.eu/>) in which there is strong focus on the underlying protective mechanisms of probiotics, the synergistic effects of probiotic strain mixtures, the population dynamics of the applied microbial strains and their effects on the resident microbial community. Additionally, the feasibility of applying beneficial microbes on a large industrial scale should be evaluated under strict guidelines and regulations (Verschuere *et al.*, 2000). Last but not least, the knowledge on probiotics should be accessible to fish farmers by conveying research findings and novel, sustainable technologies and products.

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Summary

Emerging diseases caused by oomycete, fungal, bacterial and viral pathogens increasingly threaten natural ecosystems, biodiversity and food security. In aquaculture, disease control is pivotal to the success of this industry. Recent studies in a variety of eukaryotes, in particular in humans, insects and plants, have demonstrated that their microbiomes play an essential role in protection against diseases. Therefore, habitat bioaugmentation and introduction of beneficial microbes have been suggested as novel sustainable measures to mitigate emerging diseases of fish and amphibians. Both measures require profound knowledge of the microbiomes associated with their aquatic hosts. However, large-scale metataxonomic studies for aquatic animals are still in its infancy and little is known on the diversity, ecology and functional potential of fish microbiomes. Fish eggs have an immature immune system and hence, for disease protection they likely rely on other defence mechanisms.

In my thesis, I studied the microbiome of Atlantic salmon (*Salmo salar* L.) eyed eggs and analysed the effects of infections by the oomycete pathogen *Saprolegnia* on the microbial architecture, in particular diversity and richness (chapter 2). By combining classic microbiological and state-of-the-art 'omics techniques, the composition and activities of oomycete, fungal, bacterial and archaeal communities associated with *Saprolegnia*-infected (diseased) and healthy salmon eggs were studied. A low incidence of Saprolegniosis was correlated with a relatively high abundance and richness of specific commensal Actinobacteria. Subsequent isolation of Actinobacteria and bioassays showed that an Actinobacterial isolate, classified as *Fron dihabitans* sp. 762G35 (Microbacteriaceae), significantly inhibited hyphal attachment of *Saprolegnia diclina* to live salmon eggs. The culturable *Fron dihabitans* population associated with healthy salmon eggs was further investigated (chapter 3) and showed that most *Fron dihabitans* isolates inhibited growth *in vitro* of *S. diclina*, formed biofilms and had protease and lipase activities at low temperature (5 °C). The genome of *Fron dihabitans* sp. 762G35 was sequenced and genome mining revealed gene clusters potentially involved in production of terpene and type III polyketide synthase. Chemical characterization of cell-free supernatant of *Fron dihabitans* sp. 762G35 by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) identified furancarboxylic acid-derived metabolites that may be involved in the *S. diclina* hyphal growth inhibiting activity.

Among the bacterial community, *Pseudomonas* (Pseudomonadales) represented one of the most abundant bacterial genera associated with the salmon eggs. No distinct difference was observed in abundance nor in *in vitro* activities against *Saprolegnia* spp. between *Pseudomonas* isolates from diseased or healthy salmon eggs (chapter 4). However, a significantly higher frequency of biosurfactant-producing *Pseudomonas* was found among the isolates from healthy salmon eggs. *In vivo* salmon egg bioassays revealed that *Pseudomonas* isolate H6 significantly inhibited hyphal attachment of *S. diclina* to salmon eggs and reduced salmon egg mortality. Live colony mass spectrometry showed that this isolate produced a viscosin-like lipopeptide surfactant. Although this biosurfactant inhibited hyphal growth of *Saprolegnia* spp. *in vitro*, no significant protective effect on salmon eggs against *S. diclina* was observed for this bacterial metabolite.

Among the fungal community, *Microdochium* (Ascomycota) represented the most abundant fungal genus associated with salmon eggs. Subsequent isolation and phylogenetic analyses showed that most fungal isolates obtained from salmon eggs were indeed closely related to *Microdochium lycopodium*/*Microdochium phragmitis* but also to *Trichoderma viride* (chapter 5). Phylogenetic and quantitative PCR analyses showed both a quantitative and qualitative difference in the *Trichoderma* population between diseased and healthy salmon eggs, which was not the case for the *Microdochium* population. Only few fungal isolates showed *in vitro* antagonistic activity against *S. diclina* and most *Trichoderma* isolates showed typical coiling around and/or formation of papilla-like structures on the hyphae of *S. diclina*. These results suggest that among the fungal community associated with salmon eggs, mycoparasitic *Trichoderma* species play a role in *Saprolegnia* suppression in aquaculture.

In conclusion, my PhD thesis provides a scientific framework for studying the diversity and dynamics of microbial communities associated with fish eggs. This fundamental knowledge is needed to target specific microbial groups or microbial consortia for evaluation as protective microbiota for mitigation of emerging diseases in aquaculture and natural ecosystems. The bacterial and fungal isolates, especially *Frondihabitans*, *Pseudomonas* and *Trichoderma*, and/or their bioactive metabolites, are proposed as effective candidates to control the emerging disease Saprolegniosis.

Samenvatting

Nieuwe ziektes veroorzaakt door (pseudo)schimmels, bacteriën en virussen zijn in opkomst en vormen niet alleen een bedreiging voor de biodiversiteit van natuurlijke ecosystemen maar ook voor onze voedselvoorziening. In de aquacultuur, de bestrijding van deze ziektes is van cruciaal belang en bepalend voor het succes van deze sector. Recente studies in onder andere mensen, insecten en planten hebben aangetoond dat de microbiële gemeenschappen die leven in associatie met deze organismen een belangrijke rol spelen bij de bescherming van de gastheer tegen ziekteverwekkers. Dit suggereert dat het toedienen van beschermende microben een duurzame methode kan zijn voor het onderdrukken van ziekteverwekkers in de aquacultuur. Dit vereist echter een grondige kennis van de diversiteit en ecologie van de natuurlijk aanwezige microbiële gemeenschappen in/op vissen en amfibieën en hun functies voor de gastheer. Viseieren hebben een onderontwikkeld immuunsysteem en zijn afhankelijk van andere manieren van verdediging tegen ziekteverwekkers. De rol van de microbiële gemeenschappen op viseieren is tot op heden niet uitgebreid bestudeerd.

In dit proefschrift beschrijf ik de samenstelling van de microbiële gemeenschappen, het zogenaamde microbioom, van zalmeieren. Door middel van klassieke en moderne technieken heb ik de effecten onderzocht van *Saprolegnia*-infecties op de diversiteit, samenstelling en activiteit van het zalmei-microbioom. Een lage ziekte incidentie was gecorreleerd met een verhoogde aanwezigheid van specifieke Actinobacteriën. Isolatie en activiteit-analyses toonden aan dat tenminste één Actinobacterieel isolaat, geclassificeerd als *Fron dihabitans* sp. 762G35 (Microbacteriaceae), de aanhechting van *Saprolegnia diclina* aan zalmeieren significant kon verminderen. Meerdere *Fron dihabitans* isolaten zijn vervolgens fenotypisch en genetisch gekarakteriseerd en hieruit bleek dat de meeste isolaten myceliumgroei van *S. diclina* kunnen remmen en een biofilm kunnen vormen, en dat ze bij lage temperaturen (5 °C) extracellulaire protease en lipase activiteit bezitten. Het genoom van *Fron dihabitans* sp. 762G35 werd vervolgens gesequenced en uit analyses bleek dat dit isolaat twee genclusters bevat die coderen voor de biosynthese van terpenen en één voor een type III polyketide synthase. Chemische karakterisatie van het cultuur supernatant van dit isolaat resulteerde in de identificatie van 9 stoffen die mogelijk betrokken zijn bij de activiteit tegen *Saprolegnia*.

Het genus *Pseudomonas* (Pseudomonadales) vormde één van de grootste groepen binnen de bacteriële gemeenschap geassocieerd met zalmeieren. Er waren overigens geen verschillen in diversiteit, aantallen of activiteit tegen *Saprolegnia* tussen de *Pseudomonas* isolaten afkomstig van gezonde of zieke zalmeieren. Echter, *Pseudomonas* isolaten afkomstig van gezonde zalmeieren hadden vaker het vermogen om oppervlakte spanning-verlagende stoffen (biosurfactants) te produceren dan die afkomstig van zieke zalmeieren. Uit chemische analyse bleek dat de biosurfactant een viscosin-achtig cyclische lipopeptide is. Ondanks de waarneming dat dit lipopeptide de myceliumgroei van *Saprolegnia in vitro* kon onderdrukken, was er geen beschermend effect van deze stof tegen infectie van *Saprolegnia* op de zalmeieren.

Ook de schimmelpopulatie werd bestudeerd en de Ascomyceet *Microdochium* bleek het meest abundant op basis van DNA analyse en isolatie. Verdere karakterisering van de schimmelisolaten liet zien dat de meeste isolaten sterk gerelateerd zijn aan *Microdochium lycopodium* of *Microdochium phragmitis*. Daarnaast werden ook meerdere *Trichoderma* isolaten gevonden. Fylogenetische en kwantitatieve PCR lieten een kwantitatief en kwalitatief verschil zien tussen *Trichoderma* populaties verkregen van zieke en gezonde zalmeieren, wat niet het geval was voor de *Microdochium* isolaten. Slechts enkele schimmel-isolaten lieten *in vitro* activiteit zien tegen *Saprolegnia*, terwijl de meeste *Trichoderma* isolaten wel de typische wikkeling rondom *Saprolegnia*-hyfen vertoonden en/of papilla-achtige structuren vormden op de hyfen. Deze resultaten suggereren dat mycoparasitaire *Trichoderma* soorten een rol kunnen spelen in de bescherming van zalmeieren tegen *Saprolegnia*.

Concluderend geeft mijn onderzoek een wetenschappelijk raamwerk voor microbiom-analyses van viseieren. Deze fundamentele kennis is nodig om de potentie te evalueren van specifieke microbiële groepen of consortia voor toepassing in een meer duurzame bescherming tegen opkomende ziektes in de aquacultuur en mogelijk in natuurlijke ecosystemen. De bacteriële isolaten *Frondihabitans* en *Pseudomonas* en hun bioactieve stoffen alsook de schimmel *Trichoderma* zijn potentiële kandidaten voor de bescherming van zalmeieren tegen *Saprolegnia*.

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Curriculum vitae

Yiyang Liu (劉懿瑩) was born in 1982 in Guangdong, China. She obtained a BSc degree in Biotechnology at Shaoguan University, China. In 2007, she started her MSc studies in Aquaculture at Ghent University, Belgium. In 2008, she was awarded with a Master Grant from Ghent University. Under the supervision of Dr. Tom Defoirdt and Prof. Peter Bossier, she conducted her MSc thesis research and published a paper entitled 'PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent vibriosis'. In 2010, she started her PhD research at Wageningen University, The Netherlands, in the Laboratory of Phytopathology, under the supervision of Dr. Irene de Bruijn, Prof. Jos M Raaijmakers and Prof. Francine PM Govers. In 2014 the research group of Jos M Raaijmakers moved to the Netherlands Institute of Ecology (KNAW-NIOO), also in Wageningen, where Yiyang continued her PhD project. The results of her PhD research are described in this thesis.

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Publications

Liu Y., Etalo D., de Jager V., Carrión V.J., Raaijmakers J.M. and de Bruijn I. Phenotypic, genomic and metabolic characterization of aquatic *Frondihabitans* with activity against the oomycete fish pathogen *Saprolegnia diclina*. (in preparation).

Liu Y., Raaijmakers J.M. and de Bruijn I. The fish microbiome: for better or for worse? (submitted).

Liu Y., Zachow C., Raaijmakers J.M. and de Bruijn I. (2016). Elucidating the diversity of aquatic *Microdochium* and *Trichoderma* species and their activity against the fish pathogen *Saprolegnia diclina*. International Journal of Molecular Sciences. 17, 140; doi:10.3390/ijms17010140.

Liu Y., Rzeszutek E., van der Voort M., Wu C.-H., Thoen E., Skaar I., Bulone V., Dorrestein P.C., Raaijmakers J.M. and de Bruijn I. (2015). Diversity of aquatic *Pseudomonas* species and their activity against the fish pathogenic oomycete *Saprolegnia*. PLoS ONE 10(8): e0136241. doi:10.1371/journal.pone.0136241.

Liu Y.*, de Bruijn I.*, Jack A.L.H., Drynan K., van den Berg A.H., Thoen E., Sandoval-Sierra V., Skaar I., van West P., Diéguez-Urbeondo J., van der Voort M., Mendes R., Mazzola M. and Raaijmakers J.M. (2014). Deciphering microbial landscapes of fish eggs to mitigate emerging diseases. The ISME Journal 8 (10), 2002–2014; doi:10.1038/ismej.2014.44.

Liu Y.*, De Schryver P.*, Van Delsen B., Maignien L., Boon N., Sorgeloos P., Verstraete W., Bossier P. and Defoirdt T. (2010). PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent vibriosis. FEMS Microbiology Ecology. 74: 196-204; doi:10.1111/j.1574-6941.2010.00926.x.

*Shared first author.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Yiying Liu**
Date: **13 June 2016**
Group: **Phytopathology (Wageningen UR) & Microbial Ecology (NIOO-KNAW)**
University: **Wageningen University & Research Centre**

1) Start-up phase		<u>date</u>
▶ First presentation of your project		
Title: Biological control of <i>Saprolegnia</i> species		Jan 10, 2011
▶ Writing or rewriting a project proposal		
Title: Biological control of <i>Saprolegnia</i> species		Jun-Sep 2011
▶ Writing a review or book chapter		
▶ MSc courses		
▶ Laboratory use of isotopes		
<i>Subtotal Start-up Phase</i>		<i>7.5 credits*</i>
2) Scientific Exposure		<u>date</u>
▶ EPS PhD student days		
EPS PhD student days, Leiden University		Nov 29, 2013
EPS Get2Gether, Soest (NL)		Jan 29-30, 2015
▶ EPS theme symposia		
EPS theme 4 symposium 'Genome Biology', Radboud University Nijmegen		Dec 07, 2012
EPS theme 4 symposium 'Genome Biology', Wageningen University		Dec 13, 2013
▶ Annual EPS Meetings (Lunteren) and other National Platforms		
Molecular Genetics meeting, Lunteren		Oct 06-07, 2011
WIAS Science Day 2013		Feb 28, 2013
NIOO Science Days 2015		Nov 11-12, 2015
▶ Seminars (series), workshops and symposia		
PSG Seminars: Angus Dawe, Rays Jiang, Peter C. Dorrestein		May & Oct 2011, Aug 2013
NIOO Seminars: Noah Fierer, Jos M Raaijmakers, Detmer Sipkema		Nov 2011, Jan 2014 & 2016
Farewell symposia: Pierre J.G.M. de Wit & J.A. Hans van Veen		05 & 27 June, 2014
Workshops: Citizen science & Writing for impact		Nov 11, 2015
Inaugural speeches: Shirley Pomponi, Jos M. Raaijmakers, Liesje Mommer		Sep 2013, Nov 2015, Feb 2016
▶ Seminar plus		
▶ International symposia and congresses		
EU SAPRO consortium meetings, Scotland and The Netherlands		Apr 11-12 & Oct 31, 2011
EU SAPRO mid-term review meeting, Norway		Oct 25, 2011
EU SAPRO consortium meetings, Spain and Sweden		Mar 26 & Sep 03, 2012
EU SAPRO consortium meeting, Norway		May 22, 2013
BE-Basic Symposium 2014		Feb 05, 2014
BE-Basic Symposium 2015		Mar 31-Apr 01, 2015
EU ParaFishControl kick-off meeting		May 26-29, 2015
Aquaculture Europe 2015 International Conference and Exposition (NL)		Oct 20-23, 2015
▶ Presentations		
SAPRO consortium meetings (Talks)		Apr 12 & Oct 31, 2011
Molecular Genetics ALW meeting (Poster)		Oct 06-07, 2011
SAPRO mid-term review meeting (Talk)		Oct 25, 2011
EPS Autumn School 'Host-Microbe Interactomics' (Poster)		Nov 01-03, 2011
SAPRO consortium meetings and Summer School (Talks)		Mar 26 & Sep 03, 2012
SAPRO consortium meeting (Talk)		May 22, 2013
PhD course Diseases in Salmonid Aquaculture, Norway (Poster)		May 23, 2013
Phytopathology Friday labmeetings (Talks)		Oct 2011 & May 2013
Microbial Ecology department meetings (Talks)		Apr 28, 2014 & Mar 14, 2016

BE-Basic Symposium 2014 & 2015 (Posters)	Feb 05, 2014 & Mar 31-Apr 01, 2015
ParaFishControl Kick-off meeting (Talk)	May 28, 2015
Aquaculture Europe 2015 International Conference and Exposition (Talk)	Oct 21, 2015
NIOO Science Days 2015 (Reused poster from BE-Basic Symposium 2015)	Nov 12, 2015
► IAB interview	
Meeting with a member of the International Advisory Board of EPS	Dec 03, 2014
► Excursions	
Field Trip-Trout Farm Visit, Spain	Mar 27, 2012
<i>Subtotal Scientific Exposure</i>	
<i>27.3 credits*</i>	

3) In-Depth Studies	<u><i>date</i></u>
► EPS courses or other PhD courses	
EPS Autumn School 'Host-Microbe Interactomics'	Nov 01-03, 2011
Initial Training Network SAPRO Summer School, Sweden	Sep 03-05, 2012
PhD course Diseases in Salmonid Aquaculture, Norway	May 23-24, 2013
► Journal club	
Literature discussion, Bacterial Ecology & Genomics Group of Phytopathology, Wageningen University	2011-2013
Literature discussion, Microbial Ecology Department meeting, NIOO-KNAW, Wageningen	2014-2016
► Individual research training	
Secondment in National Veterinary Institute, Norway	Jan-Apr 2012
<i>Subtotal In-Depth Studies</i>	
<i>6.9 credits*</i>	

4) Personal development	<u><i>date</i></u>
► Skill training courses	
WGS Course Ethics and Philosophy in Life Sciences	Jun 20-22, 2012
Career Orientation	Mar 01, 08, 15, 22, 28, 2013
Project and Time Management	Apr 02 & 18, May 14, 2013
BE-Basic Innovation Centre training course: Business Awareness Workshop	Mar 20, 2014
The last Stretch of the PhD Programme at Wageningen University	Sep 25, 2015
► Organisation of PhD students day, course or conference	
► Membership of Board, Committee or PhD council	
<i>Subtotal Personal Development</i>	
<i>3.6 credits*</i>	

TOTAL NUMBER OF CREDIT POINTS*	45.3
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Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.

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Front cover: salmon eggs decorated with the structures of *cis*-aconitic acid (left) and 2-furancarboxylic acid (right).

Back cover: salmon eggs decorated with the structure of the lipopeptide viscosin.

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