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Alexander Fiedler

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Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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Department of Biotechnology and Food Science

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Trondheim, November 2023

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Let us finish it.

- Tomás Verde

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Summary

Sustainable production of aquatic species such as Atlantic salmon is gaining more importance as a growing human population needs to be supplied with high quality protein sources. Both for animal welfare and for increasing production volumes, good control of the biology in aquaculture facilities is crucial. In the recent years, control of the microorganisms in these facilities have gained more interest and we are getting more aware of their importance, both in respect to prevention of disease outbreaks and in increasing the water quality. Our understanding of these microbial communities has however just begun, and a lot of work remains.

The topic of this thesis was therefore to investigate the microbiota of the important aquaculture species Atlantic salmon (*Salmo salar*). By using a recently developed protocol to generate germ-free yolk sac fry of Atlantic salmon, the development of both the gut and skin microbiota of the fish was followed throughout the whole yolk sac stage. It was further investigated to which extent the microbial communities present at hatching influence the microbiota of the fish and how it reacts to invasions of pathogenic or commensal bacterial strains.

Apart from characterizing the microbiota, also its functions were investigated. For this, an immersion challenge protocol was developed that allowed to lethally infect the salmon with the bacterial pathogen *Flavobacterium columnare*. By comparing the mortality induced by *F. columnare* between germ-free fish and fish colonized with bacteria it was possible to demonstrate a protective effect of the microbiota.

Furthermore, phage therapy was explored as a tool to treat the fish after infection with *F. columnare*. Here, phage therapy was compared to classical antibiotic treatment in order to examine the effect of each treatment on both the fish and the water microbiota. By characterising the water microbiota both in the presence and absence of fish and in the presence and absence of the bacterial host of the phages, it was possible to show that phage therapy does not disturb the microbiota in contrast to classical treatment with antibiotics.

With this, this thesis expands our knowledge about the complex interplay of the water and fish microbiota, the animal host, bacterial pathogens threatening the health of the fish and treatment strategies against pathogenic bacteria.

Definitions

Conventionalized: This refers to animals that were hatched under germ-free conditions but were then re-colonized with bacteria after axenity was confirmed. Conventionalized animals can be used as control to account for effects induced by the derivation process or as gnotobiotic animals, when defined bacterial communities are added.

Derivation: The process of generating germ-free research animals. This involves removal of all microorganisms, often by a combination of antibiotic usage and other chemical disinfectants.

Dysbiosis: Dysbiosis is often not clearly defined in the literature. In this work, it means negative changes and disturbances in the microbiota that deviate it from its normal state, often concomitant with a decrease in diversity and evenness in the community.

Germ-free: Absence of any microorganisms, also called axenity. In this work, axenity was confirmed by flow cytometry and culturing, thus only absence of bacteria and archaea was confirmed, but not absence of viruses.

Gnotobiotic: This means that only defined bacterial communities are present on an animal that are known to the researcher. Gnotobiotic animals are therefore often animals that are mono-colonized with only one bacterial strain or a consortium of few bacterial strains.

Microbiota: There are many ways to define microbiota, but in this work, it is defined as all bacteria populating a certain space at a certain time. The microbiota also includes viruses, archaea and single-celled eucaryotes, which were however not examined in this thesis.

Yolk sac fry: The developmental stage in fish right after hatching when the fish are feeding on their yolk sac. This stage corresponds to the alevin stage in Atlantic salmon before the fry stage.

List of dissemination

List of scientific articles

Paper I:

Fiedler AW, Drågen MK, Lorentsen ED, Vadstein O, Bakke I

The stability and composition of the gut and skin microbiota of Atlantic salmon throughout the yolk sac stage.

Published in *Frontiers in Microbiology*

Paper II:

Gundersen MS, Fiedler AW, Bakke I, Vadstein O

The impact of phage treatment on bacterial community structure is negligible compared to antibiotics.

Under review at *Scientific Reports*

Paper III:

Fiedler AW, Gundersen MS, Vo T, Almaas E, Vadstein O, Bakke I

Phage therapy minimally affects the water microbiota in an Atlantic salmon (Salmo salar) rearing system while still preventing infection.

Under review at *Scientific Reports*

List of conference dissemination

Fiedler AW, Drågen MK, Gomez de la Torre Canny S, Lorentsen ED, Vadstein O, Bakke I (2022) – *The early gut and skin microbiota of Atlantic salmon yolk sac fry originating from two different microbial source communities* – NBS (Norsk biokjemisk selskap) contact meeting – Oral talk

Fiedler AW, Gomez de la Torre Canny S, Drågen MK, Lorentsen ED, Vadstein O, Bakke I (2022) – *The early gut and skin microbiota of Atlantic salmon yolk sac fry originating from two different source communities* – ISME 18 – Poster presentation

Fiedler AW, Gomez de la Torre Canny S, Drågen MK, Lorentsen ED, Vadstein O, Bakke I (2021) – *The early gut and skin microbiomes of Atlantic salmon yolk sac fry originating from two different microbial source communities* – 3rd Fish Microbiota Workshop (Digital conference) – Digital talk

Other dissemination

Fiedler AW (2022) – *First impressions matter: The role of the bacteria present at hatching for the microbiome of fish* – Newsletter of the Aquaculture Engineering Society – Scientific spotlight column

Fiedler AW (2023) – PhD Grand Prix of the NV faculty at NTNU

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1. Introduction and background

1. 1 Motivation

Treatment of bacterial diseases is essential for us humans, both for treatment of our livestock and for treating human patients. For this, we heavily rely on antibiotics, however, usage of antibiotics faces several challenges such as emergence of multidrug-resistant bacteria and stagnating development of new classes of antibiotics. Further, the untargeted nature of antibiotics leads to a damaging of the microbiota in and on the bodies of the treated animals, which mainly include commensal, non-pathogenic bacteria. We more and more appreciate the various beneficial functions of these commensals and are getting more aware of the grave implications a damaged microbiota has on host function. We therefore urgently need to find treatment alternatives to antibiotics that do not damage the commensal microbiota, but instead specifically target pathogenic bacteria. This is especially relevant for aquaculture, an industry with high animal densities that provides animal protein to an increasing number of humans. One of such alternative treatments is bacteriophage therapy. Even though the therapeutic effect of phage therapy is well-documented nowadays, the effect of phage therapy on the microbiota is still not well investigated. In this thesis, it was therefore examined what role the commensal microbiota plays in protection of the important aquaculture species Atlantic salmon against bacterial infection and how the microbiota develops throughout the yolk sac stage. Further, the effect of phage therapy on the surrounding water and fish microbiota was examined.

1. 2 Atlantic salmon and aquaculture

Aquaculture is the fastest growing food-producing sector worldwide and is expected to provide animal protein to a significant amount of people all around the globe (FAO, 2022). As catching rates are stagnant and the population of the world is increasing, aquaculture needs to expand to meet the increasing demand of sea-food produce (FAO, 2022). However, intensification of aquaculture bears risks, mainly in form of disease-driven losses caused by bacteria, viruses, fungi, and parasites (Lafferty *et al.*, 2015, Sommerset *et al.*, 2023). Traditionally, bacterial diseases are addressed by usage of antibiotics or vaccinations (Chen *et al.*, 2020), however, nowadays also new treatment methods like bacteriophage therapy and better preventative biosecurity measures help to decrease disease incidences (Defoirdt

et al., 2011). Here, the development of land-based systems, especially recirculating aquaculture systems (RAS), enables a high degree of control over the rearing water and the pathogenic organisms in it. In modern RAS, the inlet water gets filtered and/or UV-irradiated or treated with ozone to disinfect it, which reduces introduction of pathogenic organisms. Often the water is also disinfected in the recirculation loop, however, this has been suggested to have negative consequences for fish-microbiota interactions in the rearing water (Attramadal *et al.*, 2021). The high reuse rate of the water in these systems (up to 99 %) is enabled by biological filters that remove ammonia and nitrate from the water. These biological filters contain nitrifying or denitrifying biofilms that constantly shed bacteria into the rearing water. This is suggested to create relatively stable bacterial communities in RAS (Dahle *et al.*, 2022), which are assumed to have a positive effect on pathogen suppression (Vadstein *et al.*, 2018). The microbiota of RAS therefore plays an important role in protecting the fish from infection and stable microbial communities in RAS are highly desirable.

Atlantic salmon as aquaculture species

One of the most important commercial aquaculture species is Atlantic salmon (*Salmo salar*), a high-value aquaculture species with more than 2.7 million tonnes being produced in 2022 (FAO, 2022), mainly by Norway and Chile. Atlantic salmon is an anadromous fish, that spawns in rivers but spends its adult life in the sea (Fig. 1). After hatching in freshwater, the freshly hatched yolk sac fry (also called alevin) hides in gravel on the ground of the river and lives for the first weeks off its particularly large yolk sack. When the yolk sac is consumed, the salmon emerges from the gravel as fry and starts feeding on small invertebrate animals. The fry then develops into parr, which undergoes smoltification in order to adapt to seawater conditions and migrates downstream towards the sea. After smoltification, the smolt matures into adult fish in the sea, where they gain most of their weight. For spawning, they return to their natal rivers. Atlantic salmon are iteroparus, which means that they can spawn more than once (Mobley *et al.*, 2021). When Atlantic salmon are grown for aquaculture, they spend their freshwater life until the smolt stage in land-based aquaculture facilities, such as RAS facilities. When they are big enough, smoltification is induced and the smoltified fish are transferred to sea cages, where they are grown out. Nowadays however, also completely land-based

solutions are becoming available, where fish are kept in saltwater-RAS facilities during the grow-out stage.

Due to its value for aquaculture, research on Atlantic salmon has mainly been focused on disease prevention and developmental traits. Recently however, a protocol was developed to generate germ-free Atlantic salmon (Canny *et al.*, 2023) which in combination with its particularly long yolk sac stage of up to 13 weeks makes Atlantic salmon an ideal model for performing host-microbe interaction studies on gnotobiotic fish.

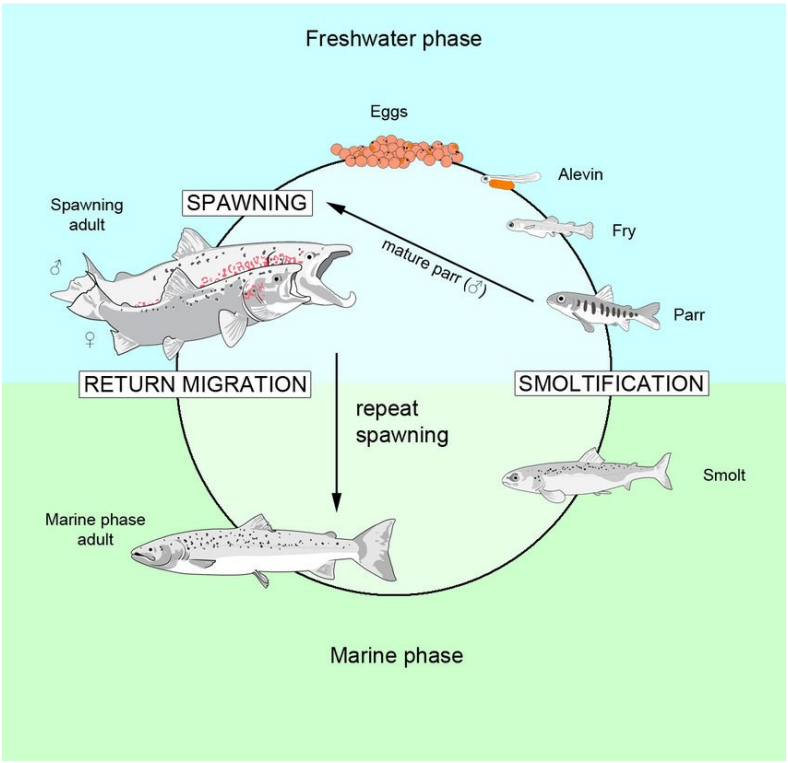


Fig. 1: Lifecycle of Atlantic salmon (*Salmo salar*). After hatching in freshwater, the fish undergoes smoltification and spends its adult life in the sea. For spawning, it migrates back into its natal river. Figure taken from Mobley *et al.*, 2021.

1. 3 Host-microbiota interaction

All naturally living animals harbour a distinct microbiota that, especially in more complex animals, affect virtually all aspects of their lives. A whole plethora of studies about how the microbiota interacts with the host has emerged the last decades and the possibilities of crosstalk and influence between host and microbiota seem infinite. A great deal of what we

know about host-microbe interactions stems from work with germ-free research animals (Al-Asmakh & Zadjali, 2015). Germ-free animals have been established for several model and non-model organisms, such as mice (Martin *et al.*, 2016), zebrafish (Melancon *et al.*, 2017), rainbow trout (Pérez-Pascual *et al.*, 2021) and, recently, Atlantic salmon (Canny *et al.*, 2023). Germ-free studies repeatedly showed that animals without a microbiota perform worse than animals that harbour a diverse microbiota, especially in respect to digestion, gut development and immune response (e.g. Dillon *et al.*, 2005, Galindo-Villegas *et al.*, 2012, Costa *et al.*, 2016, Stressmann *et al.*, 2021). Usually, the more diverse these microbiota are, the better they can serve their hosts (Dillon *et al.*, 2005, Endt *et al.*, 2010), even though it has also been shown that even mono-colonization with a single bacterial strain can restore many of the traits of a normal microbiota (Dillon *et al.*, 2005, Reeves *et al.*, 2012)

Well-studied is also how the microbiota is contributing to digestion and nutrition. This becomes especially obvious in animals that rely completely on microorganisms to digest their food such as ruminants (Liu *et al.*, 2021). However, not only the mere digestion of food is an area bacteria are involved in, but bacteria also provide their host with essential nutrients like vitamins and other metabolites (Zhang *et al.*, 2023). The microbiota seems also to be heavily involved in regulating the metabolism of its host, influencing weight loss or -gain of the host (Vrieze *et al.*, 2012, Graham *et al.*, 2015, Bouter *et al.*, 2017). Apart from nutrition, the microbiota also enables proper host development (Stappenbeck *et al.*, 2002, Splichalova *et al.*, 2018) and communicates with its host via diverse metabolites (Wang *et al.*, 2020).

The major aspect of host-microbiota interactions that will be of importance for this work is protection of the host against pathogenic organisms by its microbiota (Becattini *et al.*, 2017, Kissoyan *et al.*, 2019, Pérez-Pascual *et al.*, 2021, Stressmann *et al.*, 2021). This protection is inferred both directly via antagonistic actions against potential pathogens like production of inhibitory substances or competition for resources (Litvak *et al.*, 2019) and by training and support of the hosts immune system (Abt & Pamer, 2014, Tomkovich & Jobin, 2016, Brown *et al.*, 2017, Ubeda *et al.*, 2017). By these mechanisms, the microbiota is helping the host to reduce the numbers of potential pathogens, preventing that pathogens can establish themselves in, on and around the host and to prevent sickening of the host. Even though the protective effect of the microbiota is well-accepted nowadays and often presented as a fact, most of the research on it has been conducted in few mammalian model species and for

example until few years ago, this effect had not been demonstrated in fish (Pérez-Pascual *et al.*, 2021, Stressmann *et al.*, 2021). It is therefore important to investigate this protective effect and also show it in other species in order to examine whether it really is a universal feature of the host's microbiota.

Bacteria sana i corpore sano

It is important that the microbiota is as undisturbed and healthy as possible so that it can infer optimal protection to its host, even though it is not clearly defined what "undisturbed" and "healthy" in this context actually means (Vonaesch *et al.*, 2018). It has for example been shown that animals treated with antibiotics have a disturbed microbiota and are more susceptible to bacterial infections afterwards (van der Waaij *et al.*, 1971, Croswell *et al.*, 2009, Zhou *et al.*, 2018). This however is also valid the other way round, in the sense that an "unhealthy" host often has an altered microbiota (She *et al.*, 2017, Legrand *et al.*, 2018, Legrand *et al.*, 2020). While it is often impossible in these situations to disentangle what is cause and what is consequence, it has been shown that at least in some situations the microbiota is indeed changed by a decrease in host health (Llewellyn *et al.*, 2017, Mathieu-Begne *et al.*, 2023).

Due to its importance, mankind also tries to positively influence the microbiota. Here, the target is mainly the gut microbiota as it is often the most diverse microbiota of the host with the most potential for beneficial modifications (McCarville *et al.*, 2016, Hitch *et al.*, 2022). Improving the microbiota can for example be achieved by usage of probiotics or prebiotics. Probiotics are live bacteria that are consumed or applied to the animal and that are beneficial for its host (Hill *et al.*, 2014). These are mostly butyrate- or lactic acid producing bacteria that have a positive effect on the gut microflora (Zhang *et al.*, 2019, Rastogi & Singh, 2022). However, efficiency of probiotics is still debated and it is hard to permanently establish the probiotics in the bacterial flora as most of the times the probiotics are only transiently present in the bacterial communities and disappear when application is ceased (Skjermo *et al.*, 2015, Suez *et al.*, 2019). A reason why permanently establishing probiotic bacteria in a community is often difficult could be that undisturbed, productive microbial communities usually are very resistant towards invasion (Jones *et al.*, 2021). The probiotic therefore has to either be a strong competitor, be added continuously or needs to be introduced to the microbiota at a

stage where it can easily be included in the present bacterial communities. Prebiotics on the other hand are nutrients that are not degraded by the animal host but can be utilised by members of the microbiota and that confer a health benefit (Gibson *et al.*, 2017). Here the idea is to not add beneficial microbes but instead to selectively support the growth of beneficial microbes, especially in the gut microbiota of the host. Pre- and probiotics can also be used simultaneously, which is then referred to as synbiotics (Swanson *et al.*, 2020).

While most of these host-microbiome interactions were mainly investigated in mammalian hosts, it seems as if the functions inferred by the microbiota are present also in other groups of animals such as insects (Dillon *et al.*, 2005) and fish (Rawls *et al.*, 2004, Pérez-Pascual *et al.*, 2021, Stressmann *et al.*, 2021), however, there is a profound lack of studies that have actually shown these in non-model organisms.

1. 4 The microbiota of fish

In contrast to terrestrial animals, fish live in very close contact to high densities of bacteria in the surrounding water. They are therefore strongly exposed to bacteria all of the time and need efficient selective barriers to select their microbiota. The gut, skin and gills are those areas of the fish that are exposed to the surrounding water. They are covered with mucus that provides immunologically active substances and acts as first line of defence against pathogens (Ángeles Esteban, 2012, Peatman *et al.*, 2015). Further, the mucus is populated by commensal microbes and fish are selecting their commensals partially through their mucus layer (Benhamed *et al.*, 2014) so that distinct microbiota are developing on these sites (Lowrey *et al.*, 2015). There are large differences in the microbial compositions between fish species (Zhang *et al.*, 2019), and also other factors such as developmental status of the fish, diet, age and environmental conditions strongly influence the composition of the microbiota (summarized by Legrand *et al.*, 2020).

Fish hatch from their (generally) sterile eggs and it is assumed that they then get colonized by the bacteria of the surrounding water or their eggs (Taniguchi *et al.*, 2022). In adult fish, clear distinctions can be seen between the microbiota of the skin and gut, however it is not clear when in the larval or juvenile stage these microbiota begin to diverge (Guivier *et al.*, 2018, Reinhart *et al.*, 2019, Sylvain *et al.*, 2020). This is due to the fact that few studies have investigated the development of the early microbiota in fish larvae and most of those have

focused on either the whole fish or the gut, but not on the skin (Borges *et al.*, 2021). Investigation of the larval microbiota is however important as especially the young developmental stages of fish are very vulnerable and their immune system is not developed yet (Zapata *et al.*, 2006). The microbiota's protective effect might therefore play a large role in fish health in that early stage. Further, the microbiota is also important for enabling a proper development of the fish (Bates *et al.*, 2006) and it is therefore important to investigate how the early microbiota is affected both by external and internal factors.

The microbial environments in aquaculture facilities differ strongly from that in the natural habitat of the fish and it is therefore important to develop a beneficial microbiota in aquaculture facilities to prevent detrimental fish-microbe interactions. Further, rearing fish in aquaculture facilities that reuse their rearing water is increasingly becoming more common. Due to the reuse of water and usage of biofilters for increased water quality, these facilities harbour diverse microbial communities (Dahle *et al.*, 2022, Dahle *et al.*, 2023). These bacterial reservoirs can both be a threat to the fish as pathogenic bacteria can increase in numbers, but it can also be a chance for increased fish welfare (De Schryver & Vadstein, 2014). It has further been shown that the microbiota of the rearing environment is influencing the microbiota of the fish (Giatsis *et al.*, 2015, Vestrum *et al.*, 2018, Razak *et al.*, 2019, Vestrum *et al.*, 2020, Bugten *et al.*, 2022) so keeping beneficial bacteria in the rearing water could be a great chance to provide the fish microbiota with e.g. probiotic commensals.

1. 5 Negative impacts of antibiotics on the microbiota

The discovery of antibiotics undoubtedly revolutionized medicine and saved uncountable lives. Their easy application and often broad coverage of affected bacterial groups made antibiotics the default treatment for bacterial diseases all around the world. Usage of antibiotics is however not without challenges. Nowadays, the probably biggest challenge concerning antibiotic usage is the spread of antibiotic resistance that renders more and more antibiotics useless against important pathogens (Kumar *et al.*, 2021). That resistance against antibiotics will spread was already predicted early on by Alexander Fleming, who discovered penicillin in 1928 (Fleming, 1945). It was estimated that antimicrobial resistance was responsible for almost five million deaths in 2019, a number that could double until 2050 (O'Neill, 2016, ARC, 2022). The gravity of antimicrobial resistance was also acknowledged by

the World Health Organisation and an action plan was endorsed in 2015. Antibiotic resistance will however not be the major focus of this work. Instead, this thesis focuses on another challenge associated with antibiotic usage which is ironically associated to one of their biggest strengths, their broad spectrum. As they are effective against many different bacterial groups, antibiotics often do not only affect the target pathogen, but also all other commensal bacteria of the patient or the system where they are applied. This disturbance of the microbial communities is often characterized by a decrease in diversity in the samples (α-diversity) and changes in the composition of the microbiota (Narrowe *et al.*, 2015, Pindling *et al.*, 2018, Wang *et al.*, 2019, Legrand *et al.*, 2020). The altered state that the microbiota then enters is often referred to as "dysbiosis". Dysbiosis induced by antibiotics has been shown to reduce the colonisation resistance of the microbiota, leaving the host more vulnerable to infections with pathogenic bacteria (Croswell *et al.*, 2009, Carlson *et al.*, 2017, Zhou *et al.*, 2018). Changes in the microbiota induced by antibiotics have further been shown to only partially be reverted, meaning that the microbiota often stays permanently disturbed after antibiotic exposure (Schokker *et al.*, 2015, Becattini *et al.*, 2016). This is especially worrisome for us humans that live in a world that is more and more hygienic and aseptic leading to a two-fold attack on the diversity of our microbiota by both antibiotics and our lifestyle (Blaser, 2016). Nevertheless, we are so far heavily dependent on antibiotic usage in our modern world, and therefore need alternatives to antibiotics that will take over the same functions without their deleterious side effects. One of these alternatives is bacteriophage therapy, which is in the focus of this thesis.

1. 6. Bacteriophage therapy

Bacteriophages (short, phages) are viruses that specifically target bacteria. They were independently discovered by Felix D'Herelle (D'Herelle, 2007) and Frederik Twort (Twort, 1915) in the beginning of the 20th century, even before the discovery of penicillin. Just like other viruses, phages infect their host, hijack its cellular machinery to reproduce themselves and in the end lyse the host cells to release their offspring. The life strategy of phages can be broadly separated into two categories, the lytic or the lysogenic life cycle (Fig. 2). Lytic phages infect a bacterial cell and immediately start to produce new prophages until the bacterial cell is lysed and the phages are released. In contrast to that, temperate phages follow a lysogenic

life cycle where they first integrate their genetic material into the genome of the host after they infected a bacterial cell. Only later, after induction by e.g. nutrient limitation, new phages are produced and the host cell is lysed (Salmond & Fineran, 2015).

Especially the lytic life cycle of phages has been used already early on in what is now known as "(Bacterio)phage therapy" (Sulakvelidze *et al.*, 2001, Nobrega *et al.*, 2015). In phage therapy, a specific phage or a phage cocktail is used for treating a bacterial disease. Phage therapy was successfully performed already few years after the discovery of bacteriophages (Chanishvili, 2012), however, bacteriophage therapy was abandoned in western medicine after the discovery of antibiotics, mainly because the causative bacterial agent needs to be known for treating a disease with phages due to their high specificity (Loc-Carrillo & Abedon, 2011). Antibiotics instead often cover a broad spectrum of bacterial groups and can therefore be used to treat diseases without identifying the underlying bacterial pathogen. Even though phage therapy was quickly abandoned in the western medicine, it was still used in the former soviet republic and e.g. in Brazil (Almeida & Sundberg, 2020).

Nowadays, phage therapy is making its comeback due to several reasons: First, antibiotic resistance is on the rise and the development of new antibiotics is stagnating (Watkins & Bonomo, 2016, Murray *et al.*, 2022). Second, phage therapy has several major advantages over chemotherapy with antibiotics (Loc-Carrillo & Abedon, 2011, Lin *et al.*, 2017): There is an evolutionary arms race between phages and bacteria, meaning that whenever a bacterium gets resistant against a phage, new phages will evolve that can target the resistant bacterium. This is in contrast to antibiotics, that are static and are not evolving when resistance is spreading. Further, phages are self-amplifying but also self-limiting. As long as there are bacterial hosts present for the phages to infect, they will produce new offspring and will multiply. When there are no more host cells present (i.e., the infection is cleared), the phages will also not be able to propagate and will eventually disappear from the system. One of the biggest advantages of phage therapy, which will also be the major focus of this thesis, is, however, that phages have a very narrow host spectrum, usually infecting only specific bacterial strains. This should in theory not harm the commensal bacteria that are not contributing to the disease, in contrast to antibiotics that lead to dysbiosis in the system they are employed in.

It has been repeatedly shown in different animal models with different pathogens that phage therapy is a well-working treatment option against bacterial diseases (e.g. Laanto *et al.*, 2015, Brady *et al.*, 2017, Dedrick *et al.*, 2019), however, regulatory hurdles and a lack of experience with controlled clinical trials in humans so far inhibited wide-spread usage of phage therapy for treating humans in the western world (Furfaro *et al.*, 2018, Pires *et al.*, 2020, Suh *et al.*, 2022). One of the hurdles that needs to be overcome is to determine the effect phage therapy has on the microbiota when it is administered (both prophylactically and therapeutically), which will be investigated in this thesis.

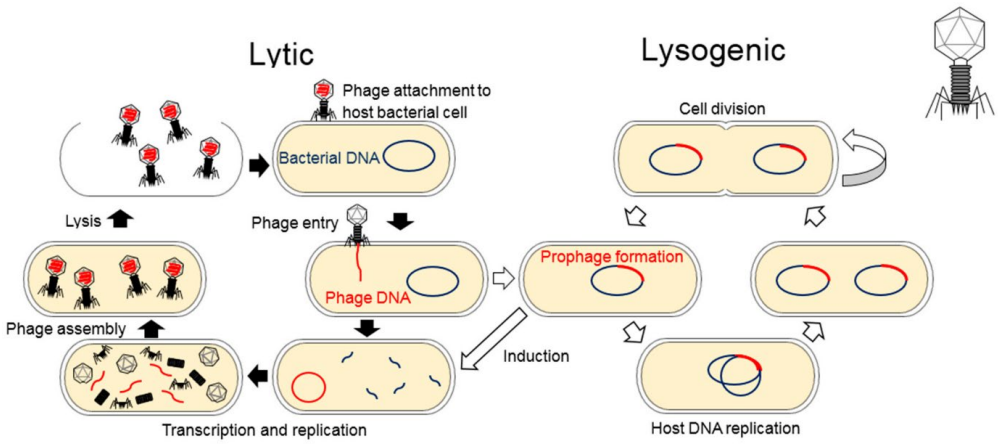


Fig. 2: Comparison of the lytic and lysogenic life cycle of phages. Lytic bacteriophages start replicating immediately when they enter the cell until the host cell gets lysed, whereas temperate phages, using the lysogenic life cycle, first integrate their genomic material into the genome of the bacterial host. The phage's genome thus gets replicated together with the bacterial host until transcription of the viral genes is induced, leading to replication of the virus and lysis of the bacterial host. Figure taken from Batinovic *et al.*, 2019.

The impact of bacteriophage therapy on the microbiota

As mentioned above, bacteriophages have a very narrow host range, therefore infecting only few hosts and theoretically not exerting damage to the surrounding microbiota. However, this concept might be overly simplified: Phages can indeed have a larger spectrum of hosts and thus infect also more than just the target species (Ross *et al.*, 2016). Phages used in phage

therapy are however usually selected for infection of only one bacterial species and it is therefore unlikely to also target other bacteria present in the community by chance (Hyman, 2019).

Another way in which phages can potentially affect the microbiota is by secondary effects due to lysis of the target bacterium. These could be 1) that when the target bacterium was an important part of the microbial interaction network of the system, removal of that target species can lead to downstream effects on the other species (Hsu *et al.*, 2019) and 2) lysis of the target species can lead to a liberation of nutrients, which can then be used by other bacteria (Middelboe *et al.*, 2003).

It is therefore indeed possible and has also been shown that phages influence the microbiota (Hsu *et al.*, 2019, Donati *et al.*, 2022, Zhao *et al.*, 2022). However, it is not expected that phage therapy leads to a widespread dysbiosis in the microbiota in the same way as antibiotics do. Antibiotics haven been repeatedly shown to negatively affect microbial communities by e.g., reducing α-diversity and shifting the bacterial communities towards non-stable communities, which supports growth of opportunistic pathogens (see chapter above). This is however usually not observed when phage therapy is employed. No negative effects on α-diversity and community structure were for example observed in mice (Bao *et al.*, 2018, Titecat *et al.*, 2022), chicken (Richards *et al.*, 2019, Clavijo *et al.*, 2022) or humans (Febvre *et al.*, 2019, Mu *et al.*, 2021), even in the presence of the host. Some studies even found that phage therapy increased the bacterial richness in the communities (Tetz *et al.*, 2017, Lorenzo-Rebenaque *et al.*, 2022). It is therefore still debated to which extend phage therapy affects the microbiota, which factors are influencing this (e.g., presence or absence of the bacterial host) and also what implications this has on the functionality of the commensal microbiota. This thesis therefore investigated to which extend phage therapy affected the water bacterial communities in a freshwater system and which role the presence of the phage's host is playing.

2. Aims and objectives

The overall aim of this work was to investigate the triangular relationship between host, microbiota, and bacterial pathogens during a bacterial infection challenge and to examine the effect of different treatment strategies on the model system. A model system with germ-free yolk sac fry of Atlantic salmon was therefore employed, with Atlantic salmon as animal host, *Flavobacterium columnare* and *Yersinia ruckeri* as bacterial pathogens and bacteriophage FCL-F2 for phage therapy.

The first objective was to investigate whether there is a protective effect of the microbiota in Atlantic salmon yolk sac fry. For that, a protocol for an immersion challenge had to be developed which would allow a reproducible lethal infection of the fish with a bacterial pathogen. Establishment of such a protocol then allowed to examine the presence of a protective effect by comparing germ-free fish with fish that were colonized by a microbiota.

For fish larvae, little is known about the development of the gut and skin microbiota before the start of active feeding, and therefore a second aim of this thesis was to investigate how the gut and skin microbiota of Atlantic salmon is developing throughout its yolk sac stage. Another objective was to examine which role the water microbiota present at hatching is playing for the microbiota development and also whether the microbiota can be influenced by addition of large amounts of defined bacterial strains that were previously absent in the bacterial communities.

The last objective of this thesis was to determine the influence of phage therapy on the surrounding microbiota and survival of the fish, comparing it to classical antibiotic treatment. It is known that antibiotics negatively influence the microbiota and in this thesis the aim was to investigate whether phage therapy has any comparable negative effects on the fish and water microbiota. A second question addressed in this regard was how the amount of bacterial host for the phages is influencing these potential effects on the microbiota, i.e. whether stronger effects are observed when more host is present for the phages to lyse.

3. Summary of publications

Paper I: The stability and composition of the gut and skin microbiota of Atlantic salmon throughout the yolk sac stage

In paper I, Atlantic salmon yolk sac fry was raised under two different microbial conditions, either with a microbiota derived from their eggs (egg-derived microbiota; EDM) or the eggs were disinfected, and the fish were then recolonized by bacteria originating from a freshwater lake (lake-derived microbiota; LDM). In a first experiment, the development of the gut and skin microbiota of the fish was followed by sampling at 6 weeks post hatching (wph), 9 wph and 13 wph. In a second experiment, the fish were exposed to large numbers of either the bacterial pathogen *Yersinia ruckeri* or the fish commensal *Janthinobacterium* sp. 3.108 at 6 wph, and the microbiota of the gut and skin of the fish as well as of the surrounding water was examined at 8 wph. It was found that the origin of the microbiota (egg-derived or lake-water derived) had a strong influence on both the gut and skin microbiota of the fish as well as on the water microbiota. Further, differences between the gut and skin microbiota were observed already in the yolk sac stage, before the fish starts active feeding. Here, the skin microbiota was very similar to the water microbiota. Neither addition of *Y. ruckeri* nor of *J.* sp. 3.108 strongly influenced the microbiota, even though both strains colonized the fish. It was therefore concluded that manipulation of the fish's microbiota might be achieved best when desirable bacterial strains are already present in the rearing water at hatching.

Paper II: The impact of phage treatment on bacterial community structure is negligible compared to antibiotics

In the second paper, we added three different amounts of the bacterial fish pathogen *Flavobacterium columnare* Fc7 (no, low or high concentration) to freshwater microcosms and consecutively added either the bacteriophage FCL-2 against *F. columnare* Fc7 or the antibiotic penicillin or applied no treatment (3x3 factorial design). We sampled the water communities for seven days for flow cytometry analysis and for analysing the microbiota using 16S rDNA gene amplicon sequencing. As expected, we found that antibiotics treatment always induced changes in the community characteristics we examined (absolute number of cells, α-diversity and community dynamics/composition), independent on the amount of *F. columnare* Fc7

present. In contrast, we did not see any effect of phage therapy on these characteristics in the presence of the host of the phage (*F. columnare* Fc7), independently on whether high or small amounts were added. In contrast to what we expected, we saw however changes in the bacterial community compositions when the phage was added in the absence of its host. The absolute bacterial numbers and α-diversity were however not affected also in the absence of the phage's host. In this paper we could thus show that phage therapy is not negatively affecting the bacterial communities both in the presence and absence of the host, in contrast to antibiotic treatment that always resulted in profound changes and a decrease in diversity.

Paper III: Phage therapy minimally affects the water microbiota in an Atlantic salmon (*Salmo salar*) rearing system while still preventing infection

In the third paper, germ-free or microbially colonized Atlantic salmon yolk sac fry were challenged with *F. columnare* Fc7 in order to investigate the presence of a protective effect of the microbiota on the hosts. Further, phage therapy using phage FCL-2 against *F. columnare* Fc7 was employed and its effect on fish survival and the water microbiota was investigated in comparison to the antibiotic oxytetracycline. We could show that the microbiota of the fish was indeed protecting the fry against infections, as mortality of colonized fish was significantly lower than that of germ-free fish. Further, also phage therapy and oxytetracycline treatment protected the fish against mortality. When phage therapy was employed in the absence of *F. columnare* (no host present for the phage), no significant effects on the water microbiota were observed. When the phage's host was present, phage therapy effectively reduced its relative abundance in the microbial community, which led to slight changes in the microbiota. The α-diversity was not significantly affected by phage therapy both in the absence and presence of the host. Surprisingly, oxytetracycline did not reduce the relative abundance of *F. columnare* in the bacterial communities and did further not disturb the microbiota or reduce the α-diversity. Here, we could therefore show that both phage therapy and the presence of microbes protected the fish against lethal infections *with F. columnare* Fc7. We further showed that phage therapy is not negatively affecting the water microbiota in our fish rearing system, especially in the absence of the phage's host.

4. Unpublished results

4. 1 Establishing a protocol for a successful immersion challenge of Atlantic salmon with a bacterial pathogen

An overview over all challenge experiments conducted is shown in appendix IV. Most of the challenges experiments were conducted using the bacterial pathogen *Yersinia ruckeri*. Here, different strains haven been used (NVI-11025, NVI-10705, both provided from the Norwegian Veterinary Institute; 06059 and 06060, both provided by Tim Wallis) and also different temperatures between 6 and 16 °C. Further, different concentrations of the pathogens have been tested, however, for most experiments a final concentration of the pathogen of 10^7 CFU (colony forming units)/ml in the water was used. Apart from that, also different stressors have been used, such as exposing the fish to 200 mg/ml H_2O_2 for 1 h or 50 mg/ml formaldehyde for 30 min prior to challenge or cutting off their caudal fins. We further challenged both germ-free fish and fish that harboured a microbiota but were never able to induce mortality caused by *Y. ruckeri*. In experiment AM1 (see appendix IV) were therefore tested also other bacterial fish pathogens (*Aeromonas salmonicida* ssp. *salmonicida* and *Flavobacterium psychrophilum*), which however also did not induce mortality. Another approach was to grow the bacterial strains in the presence of an iron chelator (bipyridyl) in an attempt to increase their virulence (experiments AM1-AM3), which however also proved futile. Indeed, we observed mortality when we infected the fish at 16 °C with *Y. ruckeri* 06059 grown in medium containing bipyridyl, however it turned out that mortality also appeared in unchallenged flasks.

The only approach that led to mortality in only challenged flasks was when *Flavobacterium columnare* was used as bacterial pathogen in experiments TM1-TM5 (see appendix IV), however, especially at 10 °C with varying success. Only infecting the fish with 10^7 CFU/ml of *F. columnare* at 14 °C allowed reproducible infection success in the Atlantic salmon yolk sac fry.

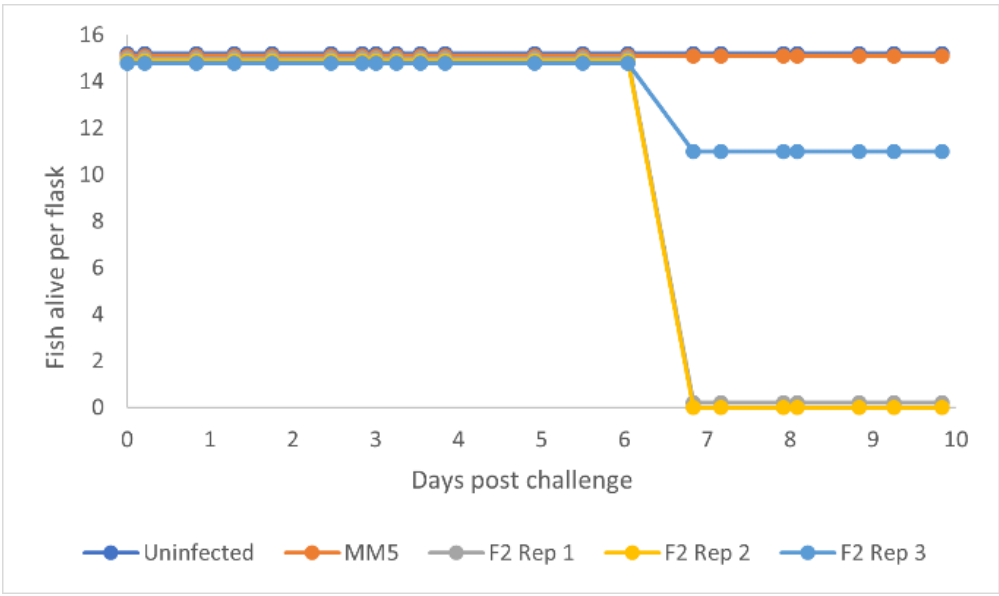
Further details about the results of all challenge experiment can also be found in the following master theses: Drågen, 2020, Skovly, 2021, Vo, 2022.

4. 2 Infection experiment with *Flavobacterium columnare* FCO-F2

We conducted an infection experiment where Atlantic salmon yolk sac fry was challenged with *F. columnare* strain FCO-F2 (corresponding to challenge experiment 3 in the master thesis of Toan Vo, 2022). Here, conventionalized (germ-free fish that were recolonized with microbes), and germ-free Atlantic salmon yolk sac fry was reared as described in paper I and paper III until 5 weeks post hatching. Then, the number of fish per flask was adjusted to 15 and the temperature was increased from 6 °C to 10 °C over the course of one week. At 41 days post hatching, three replicate flasks of each conventionalized and germ-free fish were infected with 10^7 CFU/ml (final concentration in the rearing water). As controls, to one flask the commensal *Janthinobacterium* sp. MM5 (corresponding to strain 3.108 in Paper I) was added and one flask was kept as unchallenged control. The survival was recorded twice a day and after 10 days all fish were euthanized as described in paper I and III.

The survival in each flask is shown in Fig. 3. In germ-free flasks, all challenged fish were dead after three to four days, whereas mortality occurred around three days later in the conventionalized flasks. Further, mortality reached 100 % in only two of the three challenged conventionalized flasks whereas 11 out of 15 fish survived in the last flask. No mortalities occurred in the controls to which no pathogenic bacteria were added. This shows that *F. columnare* FCO-F2 induced mortality in our yolk sac fry at 10 °C and indicates that the presence of a microbiota protected the fish against mortality caused by the infection.

A)



B)

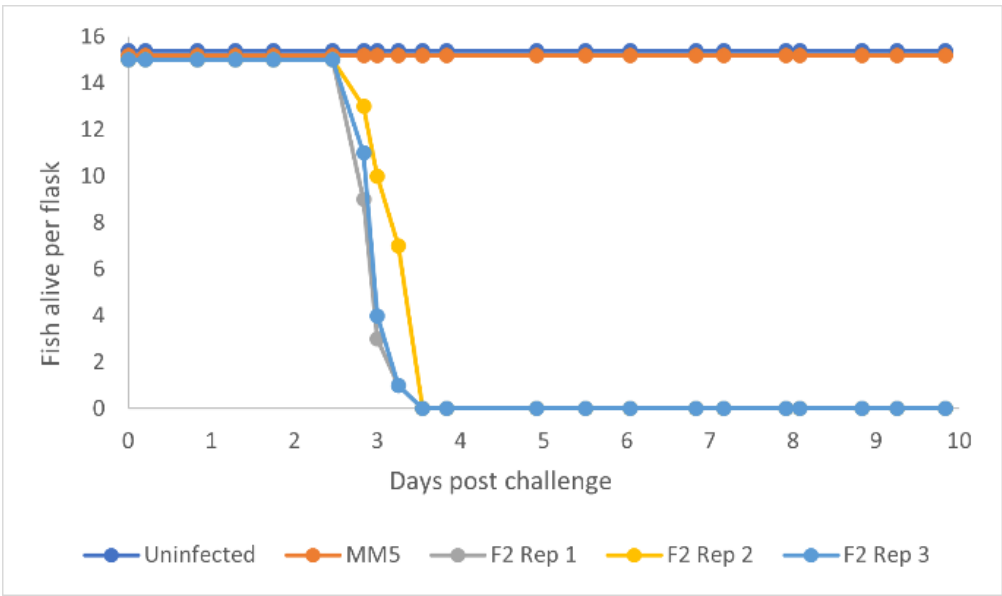


Fig. 3: Surviving fish per flask after challenge with *F. columnare* FCO-F2 in A) conventionalized and B) germ-free flasks. Dark-blue lines indicate unchallenged control flasks (Uninfected), orange lines mock-infected flasks (MM5) and the other colours represent flasks that were challenged. More details about this experiment can be found in (Vo, 2022) where it is referred to as Challenge Exp. 3.

4. 3 Effect of phage therapy on the microbiota of Atlantic salmon yolk sac fry

During Exp.2 described in paper III, we also took fish samples to analyse the fish microbiota in addition to the water microbiota. For that, we euthanized three fish per flask and sampling time point in sterile tricaine solution and washed each fish in sterile SGM once before transferring it to a BeadBashing tube of the ZymoBIOMICS™ 96 MagBead DNA kit (Zymo), snap-freezing it in liquid nitrogen and storing it at -80 °C until DNA extraction. For extracting DNA, we used the ZymoBIOMICS™ 96 MagBead DNA kit (Zymo) and a KingFisher Flex instrument. The fish were homogenized by adding 750 µl (450 µl for 0 dpi samples) lysis buffer from the DNA extraction kit and running two cycles à 30 seconds at 5500 rpm in a Precellys 24 (Bertin Technologies) and the DNA was extracted the same way as described for the water samples in paper III. The v3+v4 region of the 16S rRNA gene was amplified as described for the water samples with the exception that 38 cycles were used during PCR. Further preparation of the library and analysis of the resulting reads was performed the same way as described for the water samples. The resulting ASV table containing both the water and the fish samples was normalized to 52,863 reads per sample.

Unfortunately, we only managed to amplify the bacterial 16S rDNA from 70 of the 216 fish samples taken. As these 70 samples were also unevenly distributed over the treatment groups which strongly limited our possibilities to statistically assess our hypotheses, we decided to exclude them from the analyses published in paper III. Here, however, I would like to take the opportunity to also briefly present the data obtained from the 70 samples where we managed to amplify the v3+v4 region of the bacterial 16S rDNA gene. A principal coordinate analysis (PCoA) of both the fish and the water samples is shown in Fig. 4. Here, we can see that the fish samples generally cluster together with the water samples from the same replicate flask and sampling timepoint. Further, our analyses show that the fish microbiota samples behave the same way as the water samples, i.e., we see changes in the microbiota after addition of *F. columnare* Fc7, whereas no temporal changes can be seen in the unchallenged group.

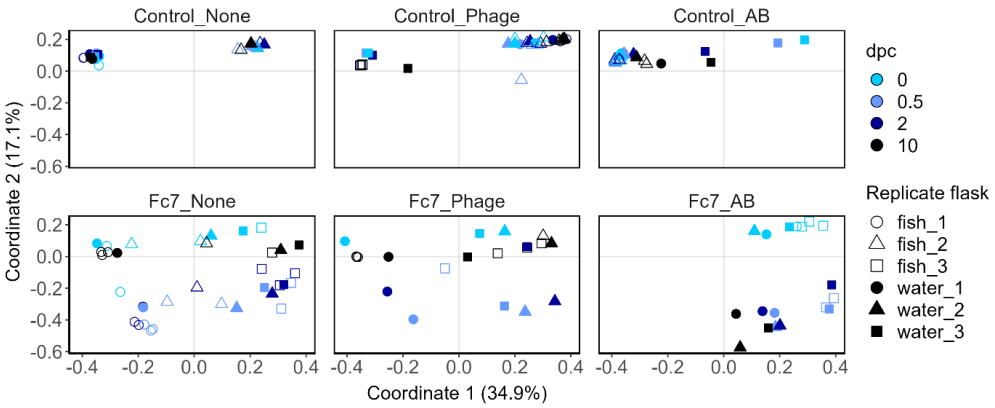
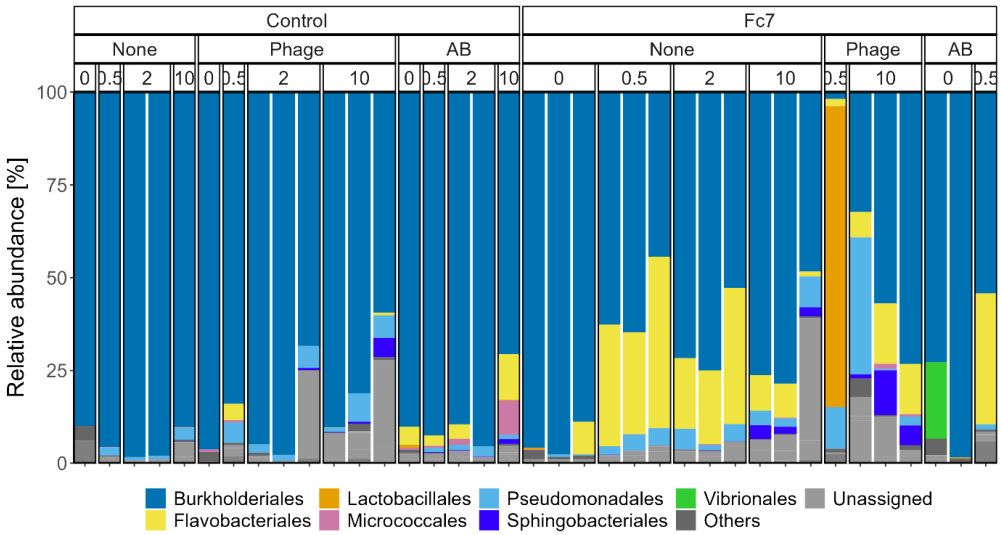


Fig. 4: PCoA of the Bray-Curtis dissimilarities of all samples taken from Exp.2 in paper III. Fish samples are shown in empty symbols, whereas water samples are filled. We can see that the fish samples in general follow the same patterns as the water samples. Note that different numbers of fish samples are available for each flask at each sampling timepoint.

The fish microbiota generally resembled the water microbiota at the order level (Fig. 5). Even though we did not have fish samples from all experimental groups for all timepoints, it still appears as if the fish samples were very similar to the water samples. This indicates that the findings drawn from the water microbiota in paper III can also be transferred to the fish microbiota (see corresponding paragraph in the Discussion).

A)



B)

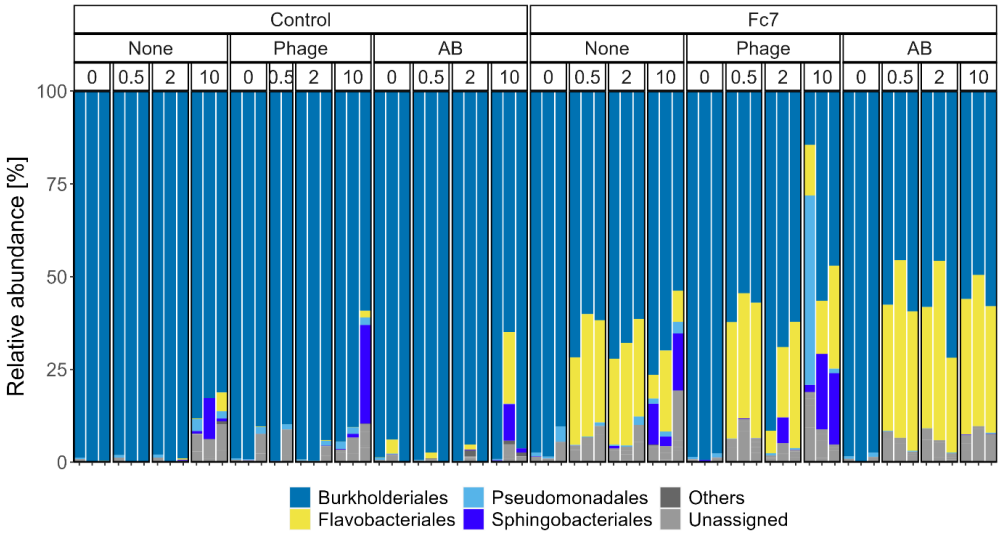


Fig. 5: The bacterial composition of samples from each replicate flask for each timepoint on the order level. In A), the microbiota of fish samples is shown, whereas in B) only the water samples are shown (the data from B is corresponding to Fig. 3 from paper III). All orders that are not appearing with > 5% in at least one of the samples are summarized as “Others”.

5. Discussion of results

This work aimed to deepen our knowledge about how the microbiota protects its host, is developing throughout the yolk sac stage of Atlantic salmon and furthermore how phage therapy can be used as an alternative to antibiotics. This was mainly investigated in a setting related to aquaculture, as Atlantic salmon were used in papers I and III and the important bacterial fish pathogen *Flavobacterium columnare* was used in papers II and III. The aquaculture industry is suffering from disease outbreaks where the natural microbiota is often damaged when antibiotics are administered for treatment. This negatively influences the stability of the microbiota in aquaculture facilities, and it is therefore important to investigate I) how the microbiota of the fish is developing and affected by the surrounding water microbiota II) how it is contributing to protecting the fish against disease and III) find treatment solutions against bacterial diseases as an alternative to antibiotics that do not disturb the microbiota.

5. 1 The challenges with immersion challenges

The first question that was addressed when starting this thesis was to examine whether a protective effect by the microbiota is present in fish larvae, which had not yet been shown when this thesis was started. To address this, we first had to establishing an adequate challenge system in which Atlantic salmon yolk sac fry was lethally infected with a bacterial pathogen. However, this task turned out to be rather challenging and a considerable portion of the whole timespan of this thesis was used on this topic. Generally, injection is the most commonly used infection method for these kinds of experiments and is often giving reproducible results (Nordmo *et al.*, 1997, Madsen & Dalsgaard, 1999). This was however not possible here as the fish were too small and, more importantly, injecting the pathogen into the fish is bypassing the first lines of defence, the epithelia and the microbial communities inhabiting them. As an objective of this thesis was to investigate a protective effect of the microbiota, bath challenge or co-habitation challenge had to be used.

Unfortunately, we were not able to inflict mortality on the yolk sac fry with the bacterial pathogen *Yersinia ruckeri* even though several different approaches were used to induce mortality during the immersion challenge: Different strains of *Y. ruckeri* (NVI-10705, NVI-

11025, 06059 and 60606) were used that all were isolated from diseased fish and some of them had also been used earlier for successful infections (Haig *et al.*, 2011). Another approach was to apply stress to the fish in several ways, e.g. by exposing them to H_2O_2 or formaldehyde (inspired by Maria *et al.*, 2013 and Madsen & Dalsgaard, 1999 or by cutting off their tails to generate entry routes for the bacteria (Madetoja *et al.*, 2000, Bader *et al.*, 2003, Long *et al.*, 2014). Further, several different infection temperatures were tested between 6 and 16 °C as Haig and co-workers found higher mortality using *Y. ruckeri* 06059 at 16 °C than at 12 °C (Haig *et al.*, 2011). While mortality in challenged flasks was sometimes observed at 16 °C, it became obvious that this temperature is too high for the yolk sac fry, as spontaneous deaths due to oxygen deprivation in the (non-oxygenated) flasks started occurring already at 14 °C and became very common at 16 °C. We were therefore not successful in establishing a reproducible infection protocol with *Y. ruckeri*.

Therefore, also other pathogenic bacteria were tested in challenge experiments. Bacterial strains that were tested for infection were *Flavobacterium psychrophilum* NCIMB 13383 (Lorenzen *et al.*, 1997) and DSM 3660 (Aoki *et al.*, 2005) as well as *Aeromonas salmonicida* ssp. *salmonicida* strains VI-88/09/03175 and 11540 (provided by the Norwegian Veterinary Institute). However, also those strains did not induce mortality when six-week old fish were challenged with them at 12 °C.

The last bacterium tested was *Flavobacterium columnare* and here we were finally able to induce high mortality in the challenged groups as reported in paper III. However, the reproducibility of mortality measures between challenge experiments with *F. columnare* Fc7 was low at 10 °C and only at sub-optimally high temperatures (14 °C) we were able to reproducibly infect the fish with this strain with a lethal outcome. We also observed mortality at 10 °C using another strain of *F. columnare* (strain FCO-F2), due to time limitations we were however not able to confirm these findings in replication experiments.

These experiences with bath infection experiments reflect the general challenges of these kind of experiments. In comparison to injection challenges, bath challenges often have lower reproducibility and also lead to lower mortalities (Garcia *et al.*, 2000) if mortalities are observed at all (Decostere *et al.*, 2000). There are many factors that have an influence on infection success in immersion challenges, such as infection dose, temperature and how the pathogens are grown prior to the challenge (Avila *et al.*, 2022), an important factor is however

age of the fish (Ronneseth *et al.*, 2007). This could be the main reason why no mortality was inflicted by challenge with *Y. ruckeri* that mainly infects through the gills (Guijarro *et al.*, 2018). Especially in the first weeks after hatching the fish mainly breath through their skin and the gills are not properly developed yet (Wells & Pinder, 1996), which could have prevented successful infection of the fish with *Y. ruckeri*. The strain that we mainly used *(Y. ruckeri* 06059) has already successfully been used earlier under experimental conditions similar to ours (Haig *et al.*, 2011), with a major difference being only the age of the fish (0.4 g in our experiments and 5-10 g in the work of Haig *et al.*, 2011. We therefore concluded that the age of the fish was likely the main reason why we did not observe mortality in our yolk sac fry following challenge with *Y. ruckeri*.

For the challenge experiments with *F. columnare*, we saw a clear trend that a higher mortality is induced at higher temperatures (14 °C over 10 °C). This is in line with what would be expected from the literature as *F. columnare* is known as a warm-water pathogen that usually infects at higher temperatures (Declercq *et al.*, 2013). Unfortunately, 14 °C is a suboptimal temperature for the salmon yolk sac fry leading to mortalities also in uninfected control flasks. As mortality was only observed with *F. columnare* as pathogen and only at sub-optimally high temperatures, I conclude that the challenge system using Atlantic salmon yolk sac fry might not be ideal to investigate lethal infections induced by bacterial pathogens.

5. 2 The protective effect of the microbiota

Even though bacterial challenge of the yolk sac fry turned out to be challenging, we still managed to address one of the major objectives of this thesis, whether the microbiota in fish is providing protection against pathogenic infections. It is now often stated as a general fact that the microbiota of animals protect them against diseases, however, this protective effect has rarely been shown in animals so far and by the start of this thesis in 2019, no such protective effect has been demonstrated in fish (Situmorang *et al.*, 2014). In 2021, the group of Jean-Marc Ghigo at the Institut Pasteur in Paris used germ-free zebrafish and rainbow trout to convincingly demonstrate a protective effect of the microbiota for the first time in fish (Pérez-Pascual *et al.*, 2021, Stressmann *et al.*, 2021). This thesis now expands upon this by also reporting a protective effect in Atlantic salmon yolk sac fry. In paper III we could show that after challenging the yolk sac fry with *F. columnare* Fc7 the mortality was significantly

lower in colonized fish in comparison to GF fish. Results from an unpublished experiment where we also found higher survival of challenged fish when a microbiota was present is shown in Fig. 5. In this experiment, the fish were challenged with a different strain of *F. columnare* (FCO-F2; kindly provided by Lotta-Riina Sundberg; Runtuvuori-Salmela *et al.*, 2022) and again, bacterially colonized fish survived longer than germ-free fish and also more fish survived the challenge in the colonized group.

In this thesis, it was therefore demonstrated that the microbiota of Atlantic salmon yolk sac fry protects the fish against lethal infection with *F. columnare*. The results support the idea that the protective effect of the microbiota is present throughout all animal groups (Dillon *et al.*, 2005, Becattini *et al.*, 2017, Kissoyan *et al.*, 2019) and that it is a general feature of microbial colonization of animal hosts.

5. 3 Development of the gut and skin microbiota of Atlantic salmon yolk sac fry and differences between replicates

Even though we begin to know more and more about the microbiota of juvenile and adult fish, less is known about the colonization and development of the microbiota of fish larvae. Here, especially little is known about the skin microbiota, as most studies focus on the gut microbiota (Borges *et al.*, 2021). In adult fish, the skin and the gut microbiota are distinctly different from each other, however this has not been properly investigated in fish larvae. In paper I we therefore examined the gut and the skin microbiota of Atlantic salmon throughout the yolk sac stage. We found a very strong inter-individual variation and also profound differences in the microbiota between the replicate flasks. These differences in the microbiota between replicate flasks has also been found previously (Bakke *et al.*, 2013, Schmidt *et al.*, 2016) and was also observed by us in paper III. The fish rearing flasks received a common microbiota to start with (the lake-water microbiota), however, no microbial transfer was possible between the flasks for the rest of the experiment and the microbiota in each flask thus had weeks to develop itself independently from the other replicate flasks. Stochastic processes such as drift then probably led to variation in the microbiota between replicates, as it as for example been shown for cod larvae by Vestrum *et al.*, 2020. It is important to be aware of these differences, both between individual fish and individual replicate flasks, especially when analysing microbial datasets. These differences can both hide

treatment effects or generate apparent effects of treatments, and further drastically reduce the reproducibility of experiments, however to my knowledge this has not yet been addressed systematically in the literature.

Apart from these differences between individual fish and individual replicate flasks, we also observed differences between the gut and the skin microbiota in the salmon larvae, several weeks before the onset of active feeding. This indicates that even before the larvae start feeding and the feed starts to influence the gut microbiota, selective pressures are already in place in the gut to select for a distinct gut microbiota in the fish larvae.

It further became visible that both the skin and the gut microbiota were highly diverse and dynamic throughout the yolk sac stage. Pronounced changes of the gut microbiota over time were also observed by (Lokesh *et al.*, 2019) that followed the microbiota of Atlantic salmon until 80 weeks post hatching, however with only one sample in the yolk sac stage. Both our and their study found that already the early gut microbiota is very diverse, however, whereas we could show that the α-diversity was increasing over time, they found it to be static over the first weeks after hatching (Lokesh *et al.*, 2019). Also in other fish, the early gut microbiota has been shown to be dynamic, however, most fish have only a short yolk sac stage and need to be fed soon, which then might have a great influence on the microbiota (Sun *et al.*, 2015, Bledsoe *et al.*, 2016, Stephens *et al.*, 2016, Taniguchi *et al.*, 2022). It was however also shown that the diet has little effect on the larval gut microbiota (Bakke et al., 2013). Nevertheless, feeding is most likely one of the factors that drive a divergence of the skin and gut microbiota and our gnotobiotic Atlantic salmon model used in this thesis avoids the necessity to feed for up to 13 weeks (Canny *et al.*, 2023) and therefore serves as a great tool to investigate the larval fish microbiota. The findings from paper I therefore greatly enhance our understanding of the larval microbiota and especially how the gut and skin microbiota develop independently from each other prior to the onset of feeding.

5. 4 Possibilities to influence the microbiota

As this thesis and lots of other studies could show that the microbiota is important for host protection, it was further examined in paper I how the microbiota could be influenced, for example in order to include beneficial bacterial strains in the microbial communities. The early microbiota of fish larvae is not yet very stable and therefore temporally variable, as we

could show in paper I and as it also has been shown earlier (Xiao *et al.*, 2022). Previous works have therefore tried to establish probiotic strains in the gut microbiota of the fish by adding it to the rearing water or feed of the larvae (e.g. Skjermo *et al.*, 2015, Giatsis *et al.*, 2016, Xia *et al.*, 2019). These studies found however, that even though the probiotic strains were found in the gut microbiota as long as they were added to the fish, they quickly disappeared as soon as addition was ceased. When we added bacterial strains to fish flasks with yolk sac fry at 6 wph, we observed that both strains were able to colonize the fish, however, the commensal that we added (*Janthinobacterium* sp. 3.108) was already present in the communities before we added it. We could still detect the pathogenic strain we added (*Y. ruckeri* 06059) two weeks after addition which indicates that it could establish itself in the microbiota at least to some extent. Our study design allowed however not a conclusive judgement on to which extend the two strains were stably integrated into the microbiota. For future studies it could be beneficial to add potential probiotic strains shortly after hatching and then follow the bacterial communities over time to investigate how the relative abundances of these strains develop.

The designs of the experiments in Paper I aimed however more to investigate the influence of the microbial community present in the rearing system at hatching, not that of addition of individual strains. Earlier work has shown that the rearing system has a great influence on the microbiota of the fish (Deng *et al.*, 2021). Deng and co-workers raised tilapia larvae under two different rearing systems which resulted in different gut microbiota of the fish. When they however took fish from both rearing systems and then raised them in a common system, these differences soon disappeared and thus no legacy effect was observed. Similar observations were made by Vestrum *et al.*, 2018 and Vestrum *et al.*, 2020, that compared the microbiota of cod larvae reared in two and three different rearing systems, respectively (flow-through vs. RAS vs. MMS (microbially matured system)). They also found that the larval microbiota was different between the rearing systems, however, they did not check whether these differences would prevail if the fish were transferred to a common rearing system. We adopted a slightly different approach in our work, where we raised the salmon larvae under identical rearing conditions, however with different source microbiota. Here, one source bacterial community originated from the egg microbiota, whereas the other one originated from a freshwater lake bacterial community. As we saw profound differences in both the gut

and skin microbiota between these two groups, we concluded that the source community that is present at hatching greatly influences the larval microbiota. Unfortunately, we did not transfer the two groups to a common rearing system at one point to see which effects this would have had on the microbiota. Based on the findings of paper I and the existing literature, it therefore appears as if that the best way of modifying the fish microbiota in aquaculture is not by punctual introduction of probiotic strains, but more by controlling the microbial communities in the whole rearing system.

5. 5 Treatment success of phage therapy

This thesis did not have a major focus on whether phage therapy is successful as a treatment strategy against bacterial diseases as the success of phage therapy in treating bacterial infections has already been shown in a plethora of publications dating back all the way to the first experiments by D'Herelle (Chanishvili, 2012). Nevertheless, we followed the relative abundance of *F. columnare* Fc7 in the bacterial communities of the experiments from Paper II and III over time in order to examine whether phage treatment decreases the relative abundance of the bacterial pathogen in the bacterial communities. The experimental setup in these two papers was similar, with the major difference between them being that no fish were present in the flasks used in paper II, whereas Atlantic salmon yolk sac fry were present in the flasks of paper III. Unfortunately, it turned out that strain Fc7 was not very successful in establishing itself in the water microbiota of the flasks of both Paper II and III and its numbers decreased rapidly also in the untreated control groups. Nevertheless, we could show that in our experiments phage treatment reduced the relative abundance of strain Fc7 faster and to a stronger extend than observed in the untreated control groups. In paper III, we could further show that phage treatment not only reduced the amount of *F. columnare*, but also significantly increased the survival of the fish. In this thesis it was therefore confirmed that phage therapy is indeed a viable option for treating bacterial disease in aquaculture systems as it has also been shown earlier (Laanto *et al.*, 2015, Liu *et al.*, 2022).

5. 6 Effect of phage therapy on the water microbiota

The main focus of Papers II and III was lying on the question whether phage therapy affects the microbiota and, if so, whether these effects are detrimental for the microbiota, leading

to dysbiosis. Part of the problem here lies in how to define “negative ways”, since we often do not know what a “good” microbiota is and whether effects are negative of positive (Shanahan *et al.*, 2021). In these two papers we have therefore tried to answer this question by mainly addressing three different characteristics of the community: 1. Is the absolute number of bacterial cells affected? 2. Is the α-diversity of the microbiota affected? and 3. Are the compositions and dynamics of the microbiota affected? These three questions will be looked at in more detail in the following paragraphs:

1. Is the absolute number of bacterial cells affected?

The easiest way to address the effect of phage therapy on the microbiota is to simply see how the abundance of bacterial cells is changing over time. In non-specific treatments, like antibiotic treatment, the overall number of bacterial cells is reduced as also non-target bacteria are hit. Indeed, in paper II we observed that this was the case, as treatment with penicillin reduced the number of bacterial cells in the water to a greater extent than in the control. In paper III, however, oxytetracycline (OTC) did not decrease the bacterial abundance to a great extent. This might be due to the fact that OTC is bacteriostatic, in contrast to the bactericidal penicillin. Nevertheless, in both papers we did not see a significant reduction of bacterial cells due to phage therapy. Instead, for some groups we even saw tendencies of a slight increase in bacterial cells after phage therapy in comparison to the untreated control. This has also been observed earlier by Noble and co-workers (Noble *et al.*, 1999). The same authors could also show that phages and their lysis products are used for growth by the surrounding bacterial communities (Noble & Fuhrman, 1999). As we added quite high amounts of phage particles (10^5-10^7 PFU/ml), this could be an explanation for the slight increase in bacterial numbers observed in our experiments. In conclusion, experiments from both paper II and III showed that phage therapy does not lead to a decrease in bacterial numbers and therefore is not negatively affecting the microbial communities in this respect.

2. Is the α-diversity of the communities affected?

Generally, it is assumed that a bacterial community is benefiting from a high diversity e.g. by exploitation of more nutrients and more functions of the community, such as production of secondary metabolites (Cardinale *et al.*, 2002, Gonzalez *et al.*, 2011). Further, a high diversity provides resilience in case of a disturbance as chances are higher that another population can

take over the functions of a population that got lost during an extinction event (Elmqvist *et al.*, 2003, Mori *et al.*, 2013). A higher diversity of species is also counteracting dominance of one or few fast-growing populations, which often include opportunistic pathogens. It is well-known that antibiotic treatment reduces α-diversity due to its untargeted nature resulting in common side effects of antibiotics such as susceptibility to secondary infections (Nogueira *et al.*, 2019). This was again observed by us in paper II, where we saw a decrease in ASV richness over time in the penicillin-treated groups. Oxytetracycline did however not lead to such an effect in paper III, a finding that is still puzzling. Both paper II and III agree however in their findings that phage therapy did not reduce the α-diversity in the microbial communities. This was expected due to the selective nature of the phage treatment and has also been observed in other studies (Bao *et al.*, 2018, Ahasan *et al.*, 2019, Jakobsen *et al.*, 2022). Phage therapy therefore does not harm the bacterial diversity of the water communities to which the phages are added.

3. Are the compositions and dynamics of the microbiota affected?

This question is the most difficult to answer, as microbial communities are constantly changing and it is often hard to determine whether an increase or a decrease of specific bacterial populations has a positive or negative effect on the community or was caused by the treatment or by something else. Further, there is a plethora of methods how to compare bacterial communities, both over time and between groups. The approach we used in both paper II and III was based on calculating the Bray-Curtis similarities between samples and ordinating those using PCoA to detect patterns. Further, stacked bar graphs of the bacterial communities on order level were generated to see changes in the most abundant bacterial orders. For the PCoAs we could see that the bacterial communities in the phage-treated groups did mainly not change to a stronger extend than observed in the control group. This was however dependent on the presence or absence of the phage's host, which is further addressed in the paragraph below. Also when looking at the bacterial community composition on order level we saw no strong differences between the untreated and the phage-treated groups, except for an increase in cytophagales in paper II when the pathogen was not present (further addressed below). As expected and in contrast to phage therapy, antibiotic treatment disturbed the bacterial communities, as antibiotic treatment samples clustered and moved differently from the control- and phage treated samples, especially in paper II.

We therefore conclude from these two papers that the bacterial communities were not negatively disturbed by the phage therapy, which has also been shown in earlier studies (Mai *et al.*, 2015, Febvre *et al.*, 2019, Clavijo *et al.*, 2022, Donati *et al.*, 2022, Jakobsen *et al.*, 2022). This shows that phage therapy is a non-disruptive alternative to antibiotic treatment, which usually disturbs the microbiota.

5. 7 Which role does the presence of the phage's host play?

Another very important aspect that needs to be taken care of when assessing the effect of phage therapy on the microbiota is whether or not the bacterial host of the phage is present in the communities or not. Even though phages usually only remove one population from a bacterial community, this can still have an influence on the community due to two mechanisms. First, lysis of bacterial cells can liberate nutrients that can then be used by bacterial populations (Middelboe *et al.*, 2003). Here it is important to consider how many of the target bacterial cells are present. If high numbers of target cells are present, more nutrients can get liberated and thus have a stronger effect on the microbiota. Second, removal of the target bacterium also leads to loss of all the function that this population had in the interaction network (Mu *et al.*, 2021). Here, the more connections it had to other populations, e.g. by providing essential nutrients or being in an antagonistic relationship, the more drastic the effect on the microbiota upon removal of the target bacterium. The target bacterium in paper II and III was *F. columnare*, which was not detected in the bacterial communities before we added it. The phages were added immediately after the target bacterium, so the target bacterium probably did not establish functions in the microbial ecosystems. We assume therefore that in our system only the first mechanism, providing nutrients by lysis of the target cells, was playing a role. While we only tested the presence or the absence of the target bacterium in paper III, we investigated two different concentrations of *F. columnare* in paper II, that differed by one magnitude (10^5 and 10^6 PFU/ml). The theoretical phage concentration was one magnitude higher in paper III (10^7 PFU/ml).

Interestingly, our findings on the influence of the target bacterium's presence are different between paper II and III. In paper II, we saw the strongest influences of phage therapy on the absolute bacterial abundance and the bacterial community composition in the absence of the

target bacterium. This was contradicting our hypothesis that more effects on the microbiota will be present the more of the target bacterium is present. The changes in the community composition were mainly visible as an increase in a single ASV classified as *Aquirufa* sp. The *Aquirufa* genus has only recently been discovered (Pitt *et al.*, 2019) and belongs to the family of Cytophagaceae. Members of this family are wide-spread in aquatic environments where they play important roles in digestion of organic matter (Kirchman, 2002) and it therefore appeared to us that the increase of this bacterial ASV might be due to the fact that it fed on the decaying phage particles that we added to our microcosms, a mechanism proposed earlier (Middelboe *et al.*, 2003). Even though we therefore saw differences in the bacterial community compositions between the different treatment groups, no differences between the no, low or high group of added *F. columnare* were observed in terms of ASV richness in paper II.

The findings of paper III on that topic were however different from that of paper II, as no significant influences on the microbiota were observed in the absence of the pathogen, however instead slight effects were observed when the target bacterium was present. For example, the microbiota of the phage-treated groups changed to a stronger extend over time when *F. columnare* was present than when it was absent. We could therefore show that the abundance of the target bacterium indeed plays a role when the influence of phage therapy on the microbiota is assessed, however, the findings were inconclusive on whether these effects on the microbiota are stronger in the presence or absence of the target bacterium. Both our studies agreed however in the finding that phage therapy never had an effect on α-diversity, independently on the abundance of *F. columnare*.

5. 8 Concluding on the effect of phage therapy on the fish microbiota from the water microbiota

Originally, it was planned to assess the effect of phage therapy on the water microbiota in paper II and on both the fish and water microbiota in paper III. In paper III, we therefore sampled three fish per rearing flask for each timepoint in addition to one water sample for microbiota analysis (216 fish samples and 72 water samples). Unfortunately, we were not able to generate PCR amplicons from around 70% of all fish samples and therefore had to discard all fish samples from the analysis. It seems that amplification of 16S rDNA material from

samples containing fish tissue is generally challenging, as this is a reoccurring problem in our group and also an issue I have talked about with colleagues from other universities during conferences. In paper I, we could however show that for our yolk sac fry especially the skin microbiota is very similar to and strongly influenced by the water microbiota and also others have found that the fish microbiota is strongly affected by the water microbiota (Minich *et al.*, 2020, Bugten *et al.*, 2022). Further, the fish samples where we managed to generate PCR amplicons are very similar to the water samples and also react to our treatments the same way as the water samples did (See chapter 4.2.). I am therefore confident that the findings from paper III, even though obtained from water samples can also be transferred to the fish microbiota.

6. Conclusions and future perspectives

In this thesis the importance of the microbiota for protecting its host was demonstrated and that we need to have a stronger focus on not harming it when treating bacterial infections. Even though this work was focusing on an aquacultural setting, the findings gained in this thesis are valid also for other fields, such as human medicine and rearing of other farmed animals. It was shown that there is a complex microbiota present in Atlantic salmon larvae already shortly after hatching and before the onset of active feeding. This microbiota is strongly influenced by the source microbiota that is present at hatching and it is dynamic over time. Further, there are differences present between the gut and the skin microbiota even before the onset of active feed. It was further concluded that this microbiota is protecting the fish the same way as it has already been shown for other animal species. This underlines the importance of a healthy, intact microbiota for animal welfare and therefore treatment methods against bacterial diseases that do not harm the microbiota were explored. Here I conclude that phage therapy is a well-suited alternative to antibiotics, as phage therapy provided the same protection against bacterial pathogens as classic antibiotic treatment. Furthermore, phage treatment did not damage the commensal microbiota, independent on whether the phage's host was present or not. Phage therapy therefore appears to me as a promising solution to replace antibiotic therapy in the near future.

For this, more research should be done on how phages affect the bacterial communities in the systems where they are applied. Here, I think it is especially important to also explore prophylactic phage therapy, and to examine the effect of phage therapy on the microbiota in the absence of bacterial pathogens. Prophylactic antibiotic therapy has been prohibited in many countries for animal rearing and phage therapy could close this gap as a non-harmful alternative to antibiotics. Gnotobiotic animal systems as the one that was used in this thesis can be used for this purpose, as they are very flexible in how they can be applied. Using these models it can be investigated how phage therapy is affecting both the host and the environmental microbiota in the absence or presence of the phage's target and also in the absence or presence of either a natural microbiota or just defined bacterial communities. Also microcosm experiments as the one that was performed in paper II provide powerful tools for investigating the impact on phage therapy on the environmental microbiota. These microcosm systems can also be used to address another potential advantage of phage

therapy over antibiotics treatment, the lower risk of resistance development in the bacterial pathogen. Further, regulatory laws and practices should be set in place in order to facilitate the development of phage products and to ease the start-up of commercial phage products. This is however not a task for scientist, but for policymakers.

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8. Appendix

Paper I:

The stability and composition of the gut and skin microbiota of Atlantic salmon throughout the yolk sac stage

Alexander W. Fiedler, Martha K. R. Drågen, Eirik D. Lorentsen, Olav Vadstein and Ingrid Bakke

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The stability and composition of the gut and skin microbiota of Atlantic salmon throughout the yolk sac stage

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The bacterial colonization of newly hatched fish is important for the larval development and health. Still, little is known about the ontogeny of the early microbiota of fish. Here, we conducted two independent experiments with yolk sac fry of Atlantic salmon that were (1) either reared conventionally, with the eggs as the only source for bacteria (egg-derived microbiota; EDM) or (2) hatched germ-free and re-colonized using lake water (lake-derived microbiota; LDM). First, we characterized the gut and skin microbiota at 6, 9, and 13 weeks post hatching based on extracted RNA. In the second experiment, we exposed fry to high doses of either a fish pathogen or a commensal bacterial isolate and sampled the microbiota based on extracted DNA. The fish microbiota differed strongly between EDM and LDM treatments. The phyla Proteobacteria, Bacteroidetes, and Actinobacteria dominated the fry microbiota, which was found temporarily dynamic. Interestingly, the microbiota of EDM fry was more stable, both between replicate rearing flasks, and over time. Although similar, the skin and gut microbiota started to differentiate during the yolk sac stage, several weeks before the yolk was consumed. Addition of high doses of bacterial isolates to fish flasks had only minor effects on the microbiota.

KEYWORDS

Atlantic salmon, initial colonization, microbiota, yolk sac fry, 16s sequencing

1. Introduction

All naturally living animals harbor a complex community of microorganisms, termed the animal's microbiota. The microbiota as assemblages of commensal and pathogenic bacteria have mainly been studied in mammalian species like mice and humans, and it has been shown that the microbiota serves its host in a multitude of ways, e.g., by providing nutrients, protecting against pathogens and enabling a proper development (Lynch and Hsiao, 2019; Zheng et al., 2020). The early bacterial colonization is crucial for the host's later development, influencing all aspects of adult life, like immune responses (Sevelsted et al., 2015), cognitive functions (Carlson et al., 2021), and nutrition (Huh et al., 2012).

These mechanisms are conserved between fish and mammals (Rawls et al., 2004; Phelps et al., 2017; Legrand et al., 2020; Borges et al., 2021). Fish live in close contact with bacteria in the surrounding water, which leads to an intimate relationship between fish and their surrounding bacteria. Fish therefore need strong barriers to protect themselves against unwanted microbes. The mucosal surfaces that cover the fish act as a selective barrier with antagonistic

properties against pathogens by providing nutrients and colonization space for the surrounding bacteria (Merrifield and Rodiles, 2015; Gomez and Primm, 2021). These mucosal surfaces on both skin and digestive tract are therefore the primary interaction site between the fish, its microbiota, and the microbes of the surrounding water (Li et al., 2019).

Generally, fish are microbe-free when they are in their eggs and immediately get colonized by the water microbes after hatching, making bacteria in the water an important source of the fish's microbiome at this stage (Llewellyn et al., 2014). This contrasts with mammals, where the initial microbiota mainly originates from the mother's microbiome (Ferretti et al., 2018). Generally, the skin of fish is colonized by bacteria immediately after hatching, and consensus is that the gut is colonized after the opening of the mouth (Reitan et al., 1998; Lokesh et al., 2019; Nikouli et al., 2019). Detailed studies of the early fish microbiota have been conducted in only a few species, and with a focus on the gut (reviewed by Borges et al., 2021). These few studies indicate that the initial fish gut bacterial community is dominated by Proteobacteria, Bacteroidetes, Firmicutes, and Actinomycetes and is increasing in diversity from early larval stages until the juvenile stage (Borges et al., 2021). Lokesh et al. (2019) investigated the ontogeny of the microbiota of Atlantic salmon and found that Proteobacteria were dominating the gut microbiota during the weeks before feeding. The onset of feeding seems to have a great influence on the composition of the gut microbiota (Ingerslev et al., 2014a,b; Michl et al., 2019). However, a well-established gut microbial community is probably already present before the fish start exogenous feeding (Ingerslev et al., 2014a,b; Sun et al., 2015; Stephens et al., 2016; Califano et al., 2017; Nikouli et al., 2019; Wilkes Walburn et al., 2019). Which factors influence these early microbial communities is, however, not thoroughly examined.

Further, not much research has been done on comparing the larval gut and skin microbiota and how they are assembled and interact (Dodd et al., 2020; Gomez and Primm, 2021). For adult fish, both the skin and gut microbiota are influenced by both abiotic factors (e.g., water temperature, salinity and diet) and biotic factors (e.g., sex, genetic background, and developmental stage) (Bakke et al., 2015; Dehler et al., 2017; Legrand et al., 2020). Viral and bacterial infections also influence the microbial community structures in the fish (Ingerslev et al., 2014a,b; Reid et al., 2017; Bozzi et al., 2021). It is further assumed that certain bacterial groups are selected for at the mucosal surfaces of the fish (Reitan et al., 1998; Lokesh et al., 2019).

Understanding the factors that affect the community assembly in developing fish is important as it could be used to steer against the presence of pathogenic and opportunistic bacteria in aquaculture systems, and thereby counteract negative fish-microbe interactions (Verschuere et al., 2000; Vadstein et al., 2018). This is especially important in the early life stages, when the immune system is not fully developed (Zapata et al., 2006) and the fish are explicitly vulnerable (Vadstein et al., 2013). The early life stages therefore generally represent a bottleneck in aquaculture (Sifa and Mathias, 1987). It has further been suggested that the fish microbiota plays a crucial role in protecting especially fish in their early life stages against pathogens (Liu et al., 2014; Pérez-Pascual et al., 2021; Stressmann et al., 2021). The early life stages of fish are therefore especially interesting for treatments to promote positive host–microbe interactions to reduce mortality and sickness. However, this requires more fundamental knowledge of mechanisms involved in community assembly of early life stages.

Atlantic salmon (*Salmo salar*) is an important aquaculture species, with more than 2.4 million tonnes being produced per year (FAO, 2020). This species has a long yolk sac stage of around 500 day-degrees, and recently a protocol for raising germ-free salmon has been developed (Gomez de la Torre Canny et al., 2022). Thus, germ-free or gnotobiotic Atlantic salmon can be kept in their yolk sac stage for as long as 13 weeks at 6°C without feeding. This system is therefore an ideal model system for studying the initial colonization of a host and allows for complete manipulation of the colonizing bacterial communities. By using this experimental design, we recently showed that colonizing newly hatched salmon with fish distinct aquatic microbial communities resulted in distinct fish microbiota, which again influenced the skin mucosa, the somatic growth, and the utilization of the yolk (Gomez de la Torre Canny et al., 2022).

In this study, we used the gnotobiotic Atlantic salmon model system to investigate the initial bacterial colonization and the development of the gut and skin microbiota of Atlantic salmon throughout the yolk sac stage. We aimed to assess the influence of the composition of the bacterial source community present at hatching on the development of both the gut and skin microbiota. We hatched fish under germ-free conditions and exposed them to either their egg microbiota or to a lake water microbiota. Furthermore, we examined the potential for manipulating the early larval bacterial communities by exposing the fish to high concentrations of both a presumptive pathogenic (*Yersinia ruckeri* 06059) and a putative commensal bacterial strain (*Janthinobacterium* sp. 3.108). Finally, we compared skin and gut microbiota to the microbiota of the rearing water. Characterization of the host microbiota was done by extracting RNA (Exp.1) or DNA (Exp.2) from gut and skin samples (and water samples for Exp.2) and sequencing the v3 + v4 hypervariable region of the 16S rRNA (gene) using the Illumina platform. The microbiota analysis of Exp.1 was based on extracted RNA instead of DNA as the original intend with these samples was to investigate gene expression in the fish. Here, we used the extracted RNA to characterize the microbiota of the yolk sac fry.

2. Materials and methods

2.1. Experimental design

Two independent fish experiments were conducted from October 2019 to January 2020 (Exp.1) and from February to April 2020 (Exp.2).

For both experiments, Atlantic salmon yolk sac fry were raised under two microbial conditions: (1) Fish were raised under conventional microbial conditions, i.e., the eggs were not sterilized after arrival to the laboratory. However, they were hatched and reared in a sterile freshwater medium so that the only source of bacteria for colonization after hatching was bacteria originating from their eggs (egg-derived microbiota, EDM). (2) Alternatively, eggs were hatched under germ-free conditions and then exposed to bacteria by adding untreated lake water to the sterile rearing flasks (lake-derived microbiota, LDM). In the following, the terms "EDM flasks" and "LDM flasks" are used to refer to these two experimental groups, and

the terms "EDM samples" and "LDM samples" are used to refer to samples taken from EDM and LDM rearing flasks, respectively.

For Exp.1, samples were collected during the experiment described in Figure 4A in Gomez de la Torre Canny et al. (2022) from conventionally raised fish (corresponding to the EDM experimental group in the present study) and conventionalized fish (corresponding to the LDM experimental group in the present study). Gomez de la Torre Canny et al. (2022) included the analysis of the skin and gut at 13 weeks post hatching (wph). Here, we extended the analyses of the gut and skin microbiota at 13 wph and also included samples taken at 6 and 9 wph from two replicate flasks per sampling time. Characterization of microbial communities was performed by Illumina sequencing of the v3 + v4 16S rRNA amplicons, based on RNA extracts from gut and skin samples (for details, see below). Total RNA was extracted from fish samples in Exp.1 instead of DNA, because the samples were originally planned to be used to study gene expression in the fish by qPCR. Here, we used the extracted RNA to analyze the microbial communities.

Exp.2 was originally designed as a challenge experiment, with *Y. ruckeri* as the pathogen, and the commencal *Janthinobacterium* sp. 3.108 as a control, representing a non-pathogenic bacterium. The design originally included both germ-free fish, and colonized fish (EDM and LDM). However, the *Y. ruckeri* strain did not induce mortality in the fish, and we were therefore not able to investigate the potential protective role of the fish microbiota in pathogenic infection. Here, we used the fish and water samples that were collected to examine the effect on the fish and water microbiota of the exposure to high doses of *Y. ruckeri* and *J.* sp. 3.108. The experiment had a factorial design, with source bacterial community (EDM vs. LDM) and addition of high quantities of two bacterial isolates (added vs. not added) as the two factors. Exp.2 included nine EDM and nine LDM flasks. At 6 wph, either the fish pathogen *Yersinia ruckeri* 06059 or the fish commensal *Janthinobacterium* sp. 3.108 was added to three replicate flasks for both EDM and LDM, whereas three flasks were left untreated. This resulted in six experimental groups. Characterization of bacterial communities by 16S rRNA gene amplicon sequencing was based on total DNA extracts from gut, skin and water samples taken at 8 wph.

2.2. Fish husbandry

In general, the derivation of Atlantic salmon eggs and their husbandry as described in Gomez de la Torre Canny et al. (2022) was followed for Atlantic salmon husbandry. Briefly, salmon eggs were obtained at around 80% developmental status from AquaGen AS (Hemne, Norway), transferred to a dark room, and kept at a constant water temperature of 5.8 ± 0.3 °C. The eggs were placed in petri dishes (13.5 cm Ø) at a density of 100 eggs/dish and covered with Salmon Gnotobiotic Medium (SGM). SGM contained 0.5 mM $MgSO_4$, 0.054 mM KCl, 0.349 mM $CaSO_4$ and 1.143 mM $NaHCO_3$ dissolved in MilliQ water and was sterilized by autoclaving prior to use (121 °C for 20 min). One day after arrival, the fish eggs were split into two groups. One group was surface-sterilized to obtain germ-free fish [for generating conventionalized fry; LDM group, corresponding to CVZ in the study by Gomez de la Torre Canny et al. (2022)], whereas the other group was not treated [for generating conventionally reared fry; EDM group, corresponding to CVR in the study by Gomez de la Torre Canny et al. (2022)]. Two days after arrival, all eggs were distributed into 250 ml cell culture flasks with vented caps and covered with 100 ml sterile SGM (17 eggs per flask). The eggs, and, after hatching, the fish, were reared in these flasks for the rest of the experiment. To maintain good water quality, 60% of the SGM in the fish flasks was exchanged three times a week and replaced with sterile SGM. Fish mortality was checked regularly, and dead fish were removed. For sampling and at the end of the experiment, fish were euthanized by a lethal dose of tricaine [5.2 g tricaine (20 mM final concentration) in 27.3 ml 1 M Tris buffer (pH 9), ad 1 L with SGM, sterilized by filtration through a 0.2 µm filter].

2.3. Sterilization of fish eggs and reintroduction of bacteria (conventionalization)

The sterilization procedure described by Gomez de la Torre Canny et al. (2022) was followed. Eggs were surface-sterilized 24 h after arrival at our laboratory. The eggs were submerged in an antibiotic cocktail (10 mgl^{-1} Rifampicin, 10 mgl^{-1}, Erythromycin, 10 mgl^{-1} Kanamycin, 100 mgl^{-1} Ampicillin, 250 $\mu g/l^{-1}$ Amphotericin B, 150 mgl^{-1} Penicillin, and 75 mgl^{-1} Oxolinic acid) and incubated at 6 °C for 24 h. Afterwards, groups of 17 eggs were incubated in a Buffodine® solution (FishTech AS) containing 50 mgl^{-1} available iodine for 30 min, washed four times in 50 ml SGM and were then transferred into 250 ml cell-culture flasks with vented caps containing 100 ml SGM. A sterility check was performed on the hatching day (hatching day defined as the day when 80% of all eggs have hatched) and regularly throughout the experiment by inoculating four different liquid media (Brain Heart Infusion, Glucose Yeast Broth, Sabourad-Dextrose Broth and Nutrient Broth) and Tryptic Soy Agar plates with 100 µl rearing water. The liquid media and TSA plates were incubated at room temperature for up to 3 weeks. If bacterial growth was observed in one of the media, the fish flask was considered contaminated and was removed from the experiment. One week after hatching, the axenic fish were conventionalized by removing 60 ml rearing water and adding 60 ml water from the lake Jonsvatnet (Trondheim, Norway). The water from lake Jonsvatnet was untreated and taken from a depth of 50 m in October 2019 (Exp.1) and March 2020 (Exp.2), respectively.

2.4. Isolation of *Janthinobacterium* sp. 3.108

Janthinobacterium sp. strain 3.108 was isolated from the skin of healthy Atlantic salmon fry in a commercial flow-through-system as follows: skin was scraped off both sides of an individual under aseptic conditions. The skin mucus was collected in a cryotube, added 500 µl glycerol (50%), snap-frozen on dry ice, and transported back to the laboratory. The sample was added Maximum Recovery Diluent (MRD), thawed and homogenized using a glass rod (MRD added step-wise to a total of 1 ml) and finally vortexed. The homogenate was serial diluted (1:10) in MRD and streaked on Plate Count Agar plates (PCA; 5 g tryptone, 2.5 g yeast extract, 1 g glucose and 12 g bacteriological agar per l). Single colonies were picked and resuspended in 50 µl MRD, serial diluted in MRD and streaked again on PCA plates. This was repeated two more times to ensure that the picked colony represented a single

bacterial isolate. The isolate was taxonomically assigned by PCR amplification of the 16S rRNA gene, followed by Sanger sequencing. PCR was performed using the primers Eub8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR reactions were run for 35 cycles (98°C 15 s, 55°C 20 s, and 72°C 20 s) with 0.2 mM of each dNTP, 0.3 µM of each primer, and Phusion Hot Start II DNA polymerase and reaction buffer (Thermo Scientific). As template, we used 1 µl of a lysate generated by boiling a colony of the relevant isolate for 10 min. The resulting PCR product was purified using the QIAquick® PCR Purification Kit (Qiagen) as described by the manufacturers. The purified PCR product (5 µl) were mixed with 5 µl sequencing primer (5 mM) and sent to Eurofins Genomics for Sanger sequencing. Three sequencing primers were applied: Eub8F, 1492R, and 805R (5′-ATTACCGCGGCTGCTGG-3′). For the resulting sequences, regions of poor quality in the 5′- and 3′-ends, as well as primer sequences, were trimmed off, and the sequences were assembled. The resulting sequence is provided in Supplementary Figure S7.

2.5. Bacterial exposure in Exp.2

At 5 wph, the number of fish was adjusted to 10 individuals per flask and the water temperature was gradually increased to 14.0 ± 0.1°C over the course of 7 days. Two bacterial isolates were used in this experiment: *Y. ruckeri* 06059 and *Janthinobacterium* sp. 3.108 (described above). A virulent strain of *Yersinia ruckeri* (strain 06059; Serotype O1) that was isolated from Atlantic salmon in the UK in 2006 was kindly provided by Tim Wallis (Ridgeway Biologicals Ltd., UK; Haig et al., 2011). At 6 wph, three EDM and LDM flasks were added *Y. ruckeri* and three *J.* sp. 3.108. Three EDM and three LDM flasks served as untreated control. *Y. ruckeri* and *J.* sp. 3.108 were grown in liquid TSB medium overnight at room temperature in an orbital shaker at 120 rpm under aerobic conditions and harvested at an OD_{600} of app. 1. One ml culture was centrifuged at 13.000xg for 1 min to obtain a bacterial pellet. The pellet was washed with SGM once, before it was resuspended in 1 ml of SGM and added to the fish flasks. This resulted in a theoretical final concentration of app. 10^7 $CFUml^{-1}$ of the respective strains in the fish flasks. After addition of bacteria, the fish were reared at 14°C for 2 weeks (until 8 wph) and then sampled.

2.6. Sampling

For Exp.1, three fish were sampled from each of two replicate flasks for both EDM and LDM fish per timepoint (12 fish sampled at 6, 9, and 13 wph), resulting in a total of 36 fish sampled. The flasks were removed from the experiment after sampling. Sampling of gut and skin at 13 wph in Exp.1 is described by Gomez de la Torre Canny et al. (2022) and samples from 6 and 9 wph of Exp.1 were prepared the same way. In brief, individual fish were transferred to individual wells of a 12-well plate prefilled with sterile SGM. The SGM was replaced with sterile tricaine solution for euthanization and each fry was rinsed three times with sterile SGM. Excess SGM was removed and fish were individually dissected in sterile petri dishes. Using sterile forceps, the yolk sac was removed and discarded. The gut was dissected out of the fish by pulling it out from esophagus to anus and was placed in screw-cap centrifuge tubes prefilled with 200 µl 1.4 mm zirconium beads and TRIzol (0.5 ml TRIzol for gut samples, 0.75 ml for skin samples from 6 and 9 wph and 1 ml TRIzol for skin samples from 13 wph). For samples from 6 and 9 wph, the remainder of the fish was used as an approximation for a skin sample, since the skin mucosa could not be dissected off the fish at these early stages, while for samples taken at 13 wph the skin was dissected off the fish.

In Exp.2, three fish were sampled at 8 wph from three flasks each of both EDM and LDM flasks for the two bacteria-treated groups and the untreated control. In addition, a water sample was taken from each flask, resulting in a total of 54 fish samples and 18 water samples. Fish samples were prepared by replacing the rearing water of the sampled fish flasks with sterile tricaine solution. After euthanisation of the fish, individual fish were transferred to individual wells of a 6-well petri dish prefilled with sterile SGM. For rinsing, each individual was transferred to a new well prefilled with sterile SGM. The fish were removed from the wells using sterile forceps and excessive SGM was removed using Kimtech-Wipes, without the fish touching the wipes. Each fish was transferred to a sterile petri dish and was dissected under a stereoscope. Using sterile forceps, the yolk sac was removed and discarded and the gut was dissected from the fish by pulling it from anus to esophagus. The rest of the fish was used as skin sample. Gut and skin samples were each transferred into separate 2-ml empty sterile screw-cap centrifuge tubes. Water samples were taken by filtrating 45 ml of fish rearing water through a 0.2 µm filter (STERIVEX™, Millipore) and placing the filter in an empty sterile 2 ml screw-cap tube. All samples from both experiments were snap frozen in liquid nitrogen and stored at −80°C until DNA/RNA extraction.

2.7. DNA and RNA extraction and cDNA synthesis

For Exp.1, total RNA was extracted and cDNA synthesized as previously described (Gomez de la Torre Canny et al., 2022). In brief, gut and skin samples were homogenized and total RNA was extracted using the Purelink™ RNA Mini Kit (Invitrogen™), then treated with DNase (On-Column Purelink DNase Treatment; Invitrogen), and immediately frozen at – 80°C. The iScript™ cDNA Synthesis kit (Bio-Rad) was used for cDNA synthesis with 800 ng DNase treated RNA as template, following the manufacturer's instructions.

For samples collected in Exp.2, DNA was extracted from skin, gut and water samples using a KingFisher Flex instrument with the ZymoBIOMICS™ 96 MagBead DNA kit. First, all samples were homogenized and lysed in 750 µl lysis buffer from the kit by vortexing them horizontally in 2 ml screwcap tubes with 1.4 mm Zirconium beads for 45 min. DNA was extracted from 300 µl lysate following the kit's protocol for the KingFisher Flex (50 µl DNAse-free water was used for elution) and samples were frozen at −20°C until examination. For a few samples we could not generate 16S rRNA gene amplicons, here, the DNA extraction was repeated using the remaining 400 µl of the lysate.

2.8. Amplification of the v3-v4 region of the 16S rRNA gene

Two amplicon libraries were prepared, one for samples from Exp.1 and one for samples from Exp.2. For DNA extracts from Exp.2, the v3 + v4 region of the 16S rRNA gene was amplified using the primers

Ill-338F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG NNNN**ACTCCTACGGGWGGCAGCAG**-3′) and Ill-805R (5'-GT CTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNN**GACT ACNVGGGTATCTAAKCC**-3′), with the target sequences shown in bold (Nordgard et al., 2017). For the cDNA representing total RNA from the samples from Exp.1, we had problems with co-amplification of host DNA, and therefore designed a new forward primer that had lower similarity to the *Salmo salar* 18S rRNA gene (Ill-329F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNN**A CKGNCCWDACWCCTACGGG**-3′; Gomez de la Torre Canny et al., 2022). The same reverse primer as for Exp.2 was used for Exp.1.

The PCRs were performed in 25 µl total reaction volume with either 1 µl cDNA extracts (Exp.1) or 2 µl of 1:10 diluted DNA extracts (Exp.2) as templates. Each PCR reaction contained 0.3 µM of each primer (0.15 µM for Exp.2) and 0.25 µM of each dNTP as well as 0.4 U Phusion hot start polymerase and the respective buffer from Thermo Scientific. The PCRs were run with the following temperature cycling conditions: an initial denaturation step at 98°C for 60 s followed by 38–40 cycles (33 cycles for water samples) of 98°C for 15 s, 58°C for 20 s (55°C for the second experiment) and 72°C for 20 s. The final elongation step was 72°C for 5 min before the samples were cooled to 10°C. PCR products were evaluated by electrophoresis on 1.5% agarose gels containing 50 µM GelRed (Biotium) for 1 h at 110 V.

2.9. Amplicon library preparation and Illumina sequencing

PCR products of expected size and quantity were normalized using Sequal Prep™ Normalization plates (96 wells, Invitrogen) before they were indexed using the Nextera® XT Index Kit v2 Set A in a second round of PCR. Indexing PCR consisted of 2.5 µl normalized and purified PCR product as template, 2.5 µl of both indexing primers, 0.25 µM of each dNTP, 0.5 mM $MgCl_2$ (in addition to $MgCl_2$ contained in the buffer) as well as 0.4 U Phusion hot start polymerase and the respective buffer from Thermo Scientific in a total reaction volume of 25 µl. The indexing PCR was run with an annealing temperature of 58°C and 10 cycles, the other cycling conditions were as described above. The indexed PCR products were normalized using the Sequal Prep Normalization kit and then pooled and up-concentrated using an Amicon® Ultra 0.5 ml centrifugal filter (30 K membrane, Merck Millipore). The quality of the DNA of the amplicon libraries was determined using a NanoDrop™ One Microvolume Spectrophotometer (Thermo Scientific™). For Exp.1, the amplicon library included 93 samples, whereas for Exp.2 the amplicon library included 96 samples (both libraries included few samples not relevant for this study). The samples were sent to the Norwegian Sequencing Center using one run on a MiSeq v3 instrument for each amplicon library with 300 paired ends. The sequencing data was deposited at the European Nucleotide Archive (ERS14440101-ERS14440192).

2.10. Analysis of the Illumina sequencing data

The USEARCH pipeline (v.11) (Edgar, 2010) was used to process the data obtained from Illumina sequencing. For Exp.1, all data were processed together as described in Gomez de la Torre Canny et al. (2022), while the data obtained from Exp.2 were processed together using the same pipeline. In brief, the paired reads were merged, and primer sequences trimmed off using the Fastq_mergepairs command with a minimal length of 390 bp. The merged sequences were quality-filtered using the Fastq-filter function with the default error threshold value of 1. The reads were pooled, dereplicated and singleton reads removed. Zero-range OTUs (zOTUs, synonymous to amplicon sequence variants, ASVs) were generated using the Unoise3 command (Edgar, 2016b) with the default minimum abundance threshold of 8 reads in the total dataset. Taxonomical assignment of the ASVs was achieved using the Sintax command (Edgar, 2016a) with a confidence threshold of 0.8 and the ribosomal database project (RDP) reference dataset. RDP training set v16 was used for the data obtained from Exp.2 and training set v18 for the data obtained from Exp.1. A minor fraction of the reads was classified as eukaryotes and chloroplasts and were removed from the data set. A few ASVs that were highly abundant in negative controls for the DNA extraction, but less abundant in the samples, were considered to represent contaminating DNA associated with the DNA extraction kit and/or PCR reagents and were removed from the data sets. For Exp.2, ASV3 and ASV15 were combined to ASV3-15, since both ASVs corresponded to *Janthinobacterium* sp. 3.108, which has two highly similar 16S rRNA gene sequences, differing in only one base pair that corresponded to an ambiguous nucleotide position in the 16S rDNA sequence of *J.* sp. 3.108 (Supplementary Figure S7). After quality filtering, the 70 samples of Exp.1 contained a total of 1,562 ASVs and 4,777,641 reads (68,252 reads per sample on average). For Exp.2, the 91 samples consisted of 598 ASVs and 9,583,497 reads (105,312 reads per sample on average). The mean sequencing depth, as indicated by Chao-1 was 83.8% for Exp.1 and 83.4% for Exp.2 (Supplementary Tables S1, S2). The final ASV tables were normalized by scaling to 26,000 reads per sample (Exp.1) and 43,347 reads per sample (Exp.2), respectively. All statistical analyses were performed using the normalized ASV tables.

2.11. Statistical analysis

All statistical analyses were performed in R (v. 4.0.4)[1] using the packages Phyloseq (v. 1.34.0) and Vegan (v. 2.5.7). α-diversities were calculated as Hill's diversity numbers (Hill, 1973; Lucas et al., 2017) using the *renyi* function of vegan. The evenness was calculated by dividing Hill's diversity of order 1 (exponential Shannon index) by Hill's diversity of order 0 (richness). Ordination by principal coordinate analyses (PCoAs) were performed using the *ordinate* function from phyloseq for Bray–Curtis similarities, if not stated otherwise. For PCoAs based on weighted Unifrac analysis, phylogenetic trees were generated using the MEGA-X software. The trees were generated employing the maximum likelihood method using a Tamura-Nei model with 1,000 bootstrap replications. The trees were rooted by using the longest branch as root. To compare similarity in community composition between groups of samples, PERMANOVA analyses (Anderson, 2001) based on Bray–Curtis similarities (if not stated otherwise) were done using the *adonis*2

1 https://cran.rstudio.com/

function from vegan by running it in 100 iterations with 999 permutations each and the mean value of p of the 100 iterations was reported (mathematically lowest possible value of $p = 0.001$). Whenever the sample size allowed it, PERMANOVAs were run as nested PERMANOVAs with "replicate flask" as sublevel. For statistical univariate data (e.g., α-diversity indices or abundance of certain ASVs), the data was checked for normality using the Shapiro–Wilk test (*shapiro.test* function). Generally, the data were not normally distributed and therefore a Mann–Whitney U test was used for data with two groups (*wilcox.test* function) and a Kruskal–Wallis test (Kruskal.test function) was used when more than two groups were compared. A significant Kruskal–Wallis test was followed by a Bonferroni-corrected Dunn test (*dunnTest* function).

3. Results

3.1. Hatching rate and survival of the fish

The hatchability of eggs in Exp.1 was very high and has already been reported in Gomez de la Torre Canny et al. (2022). The hatchability of Exp.2 was equally high, being >90% in both LDM and EDM flasks. For Exp.2, none of the fish died after addition of *Y. ruckeri* and two fish died after *J.* sp. 3.108 was added to their replicate flasks. One fish died in the untreated control group. The bacterial fish pathogen *Y. ruckeri* did therefore not induce mortality in the 6-week-old Atlantic salmons under the experimental conditions applied in this study.

3.2. The influence of the source microbiota on the gut and skin microbiota of Atlantic salmon larvae

The fish in both experiments included in this study derived their bacterial communities from one of two source microbiota, either from their eggs (egg-derived microbiota, EDM, i.e., the eggs were not hatched germ-free, but in the presence of the microbiota associated with the eggs) or from lake water (lake-derived microbiota, LDM, lake water added to germ-free fry soon after hatching). All the bacteria in EDM flasks therefore originated from the fish eggs (EDM source microbiota) and all bacteria in the LDM flasks originated from the freshwater lake water (LDM source microbiota). Principal coordinate analysis (PCoA) based on the Bray–Curtis similarities for samples from Exp.1 showed that the fish microbiota differed considerably between EDM and LDM samples (Figure 1A). A nested PERMANOVA test with "replicate flask" as sublevel showed that the fish microbiota differed significantly between EDM and LDM samples at all sampling times (6, 9 and 13 wph; PERMANOVA, value of ps = 0.002, ≤0.001 and ≤0.001, respectively; gut and skin samples combined). Average Bray–Curtis similarities showed that the microbiota of the EDM and LDM became increasingly different with increasing age (Supplementary Figure S1).

Also for Exp.2, a PCoA corroborated this finding, and showed a clear separation of the fish microbiota between the EDM and LDM samples (Figure 1B). A nested PERMANOVA test with "replicate flask" as sublevel showed again that the fish microbiota differed significantly between LDM and EDM samples (value of $p \leq 0.001$). Interestingly, the separation between the microbiota of the EDM and LDM fish was less prominent in a PCoA based on weighted UniFrac distances (Supplementary Figure S2). However, for Exp.1 the differences were still significant for samples from week 9 and 13 (nested PERMANOVA, $p \leq 0.001$; gut and skin combined) but not for week 6 samples ($p = 0.464$). Also for Exp.2, the nested PERMANOVA showed that the difference between EDM and LDM microbiota was significantly different when UniFrac distances were used ($p = 0.019$). Altogether, these results show that the source microbiota had a major impact on the bacterial communities associated with the fish.

Pseudomonadales, Burkholderiales, Propionibacteriales, and Flavobacteriales were the dominant bacterial orders for all fish samples in both experiments (Figure 2). Interestingly, the order Pseudomonadales had a significantly higher relative abundance in the fish microbiota in LDM flasks, both in Exp.1 and Exp.2 (*t*-test, $p < 0.001$ and $p = 0.013$ for Exp.1 and Exp.2, respectively), and accounted on average for as much as 20–60% of the reads in the samples. Furthermore, Flavobacteriales was more abundant in the fish microbiota of EDM than the LDM samples in both experiments.

At ASV level, most of the abundant ASVs were exclusively present in either EDM or LDM samples (Figure 3 and Supplementary Figures S3, S4). Most samples, especially of the LDM group, were dominated by only a few ASVs that accounted for the majority of the reads (Supplementary Figures S3, S4). Accordingly, the evenness was significantly lower in LDM samples compared to EDM samples at 9 and 13 wph in Exp.1 (Mann–Whitney *U* test, $p = 0.022$ and 0.006 for 9 and 13 wph, respectively). There was however no significant difference in the evenness between EDM and LDM samples at 6 wph in Exp.1 and not in Exp.2.

We examined the α-diversity by determining the ASV richness (Hill's diversity of order 0) and Hill's diversity of order 1. The α-diversity between EDM and LDM samples was similar in both gut and skin microbiota (Figure 4). There was no significant difference in Hill's diversity of the order 0 (ASV richness) between the EDM and LDM microbiota in Exp.1 (Mann–Whitney *U* test, $p > 0.05$), except for samples taken at 9 wph (Mann–Whitney *U* test, $p < 0.001$), where LDM samples had a higher richness (Figure 4A). Further, the α-diversity measured as Hill's diversity of order 1 was very similar between the EDM and LDM gut and skin microbiota (Mann–Whitney *U* test, $p = 0.889$; Figure 4A). In Exp.2, this was the case for both order 0 (Mann–Whitney *U* test, $p = 0.610$) and 1 (Mann–Whitney *U* test, $p = 0.682$; Figure 4B). These results show that even though the source microbiota strongly influenced the bacterial composition of the early Atlantic salmon gut and skin microbiota, it had little influence on the *α*-diversity.

3.3. Temporal development of the gut and skin microbiota throughout the yolk sac stage

We used the samples collected in Exp.1 to examine the temporal development of the skin and gut microbiota of the fish. A PCoA indicated that the microbiota was dynamic throughout the yolk sac stage, especially for samples from the LDM flasks (Figure 1A). A nested PERMANOVA test with flasks as sublevels showed that for both EDM and LDM samples (gut and skin samples analyzed

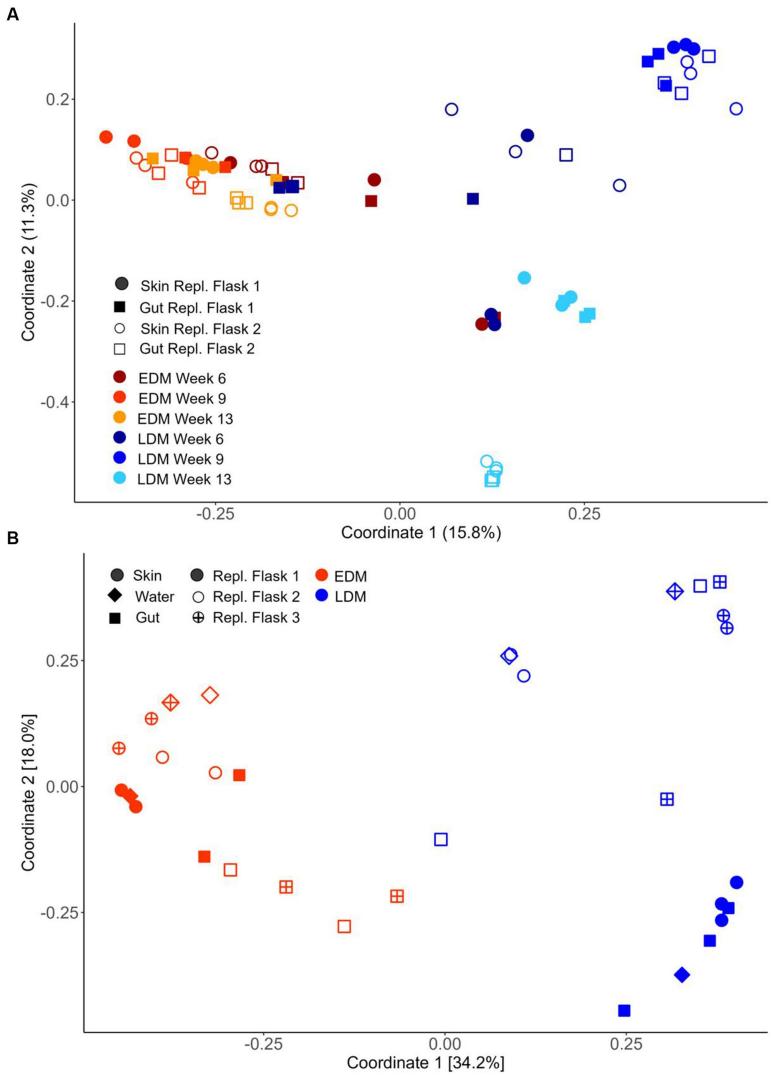

FIGURE 1
Ordination by PCoA based on Bray–Curtis similarities for skin, water (only Exp.2) and gut samples for groups receiving egg-derived (EDM) or lake-derived (LDM) microbiota. **(A)** Samples from Exp.1 based on 16S rRNA and **(B)** untreated samples from Exp.2 based on the 16S rRNA gene. For all samples of Exp.2, see Figure 6.

together), the microbiota changed significantly both from 6 to 9 wph and from 9 to 13 wph (value of $ps \leq 0.001$).

From 9 to 13 wph, the microbiota of the LDM flasks changed to a significantly larger extent than the EDM microbiota (Mann–Whitney U test, $p < 0.001$), as indicated by lower Bray–Curtis similarities between the samples (Supplementary Figure S1). Thus, the microbiota of fish that had been colonized by the lake water was less stable over time than that of the fish that had been colonized by their egg microbiota. This temporal development is reflected in the community composition at the order level: for the EDM samples, the relative abundance of Pseudomonadales decreased, while that of Flavobacteriales and Burkholderiales increased with increasing age (Figure 2A). For the LDM samples, Pseudomonadales remained the dominant order for most samples, even at the end of the yolk sac stage (Figure 2A). The microbiota of the fish also underwent major changes at the ASV level (Supplementary Figure S3B). This was particularly profound for the microbiota of the LDM samples. For example, even though the genus *Pseudomonas* was highly abundant at all sampling times for the LDM samples, different ASVs (classified as *Pseudomonas*) accounted for this high relative abundance at different age (e.g., ASV7

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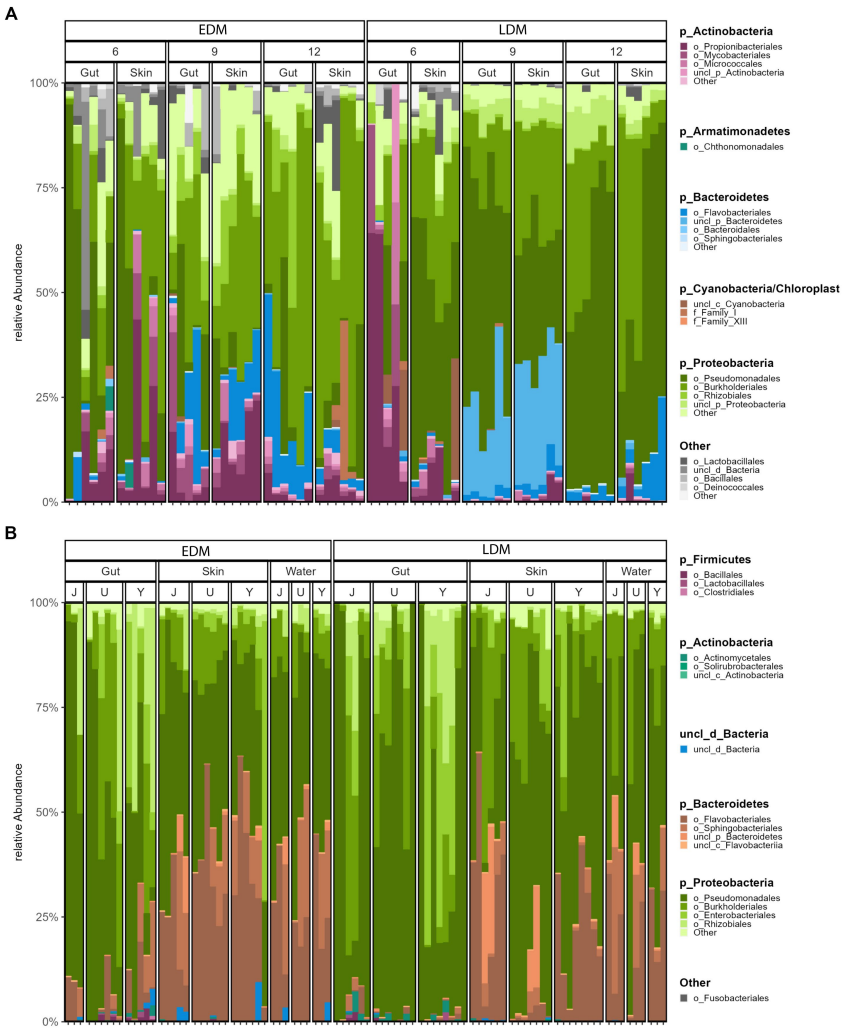

FIGURE 2
The relative bacterial community composition at the order level for all samples of Exp.1 **(A)** and Exp.2 **(B)**. For each phylum, the four most abundant orders are shown, others are summarized as "others." ASVs not classified at order level are shown at the highest taxonomic level.

and 15 on 6 wph, ASV11, and 26 on 9 wph, and ASV1 and 17 on 13 wph; Supplementary Figure S3B). The ASV richness increased significantly for both the EDM and LDM samples over time (Kruskal–Wallis test, value of $p = 0.019$ and 0.001 for EDM and LDM, respectively), while Hill's diversity of order 1 increased significantly only for the LDM samples (Kruskal–Wallis test, value of $p = 0.169$ and 0.008 for EDM and LDM, respectively; Figure 4A).

3.4. The effect of rearing flask on the larval microbiota

The PCoA for the fish samples from Exp.1 (Figure 1A) indicated that the skin and gut microbiota differed between replicate rearing flasks. This was particularly clear for the LDM flasks at 13 wph. A PERMANOVA test revealed that the fish microbiota differed significantly between the two replicate flasks for each timepoint for both EDM and LDM samples (value of ps <0.05), except for LDM samples from 6 wph ($p = 0.171$). Interestingly, average Bray–Curtis similarities suggested that the fish microbiota both differed more between replicate LDM flasks and was more alike within replicate flasks (Figure 5). This was more pronounced at the last sampling time at 13 wph (Figure 5 and Supplementary Figure S5A). Also in Exp.2, the fish microbiota differed between replicate flasks, and again, this was especially profound for the LDM samples (Figure 1B and Supplementary Figure S5B). A PERMANOVA tests confirmed a significant difference in the fish microbiota between the three LDM replicate flasks ($p \leq 0.001$), but not the EDM flasks ($p = 0.109$). Thus,

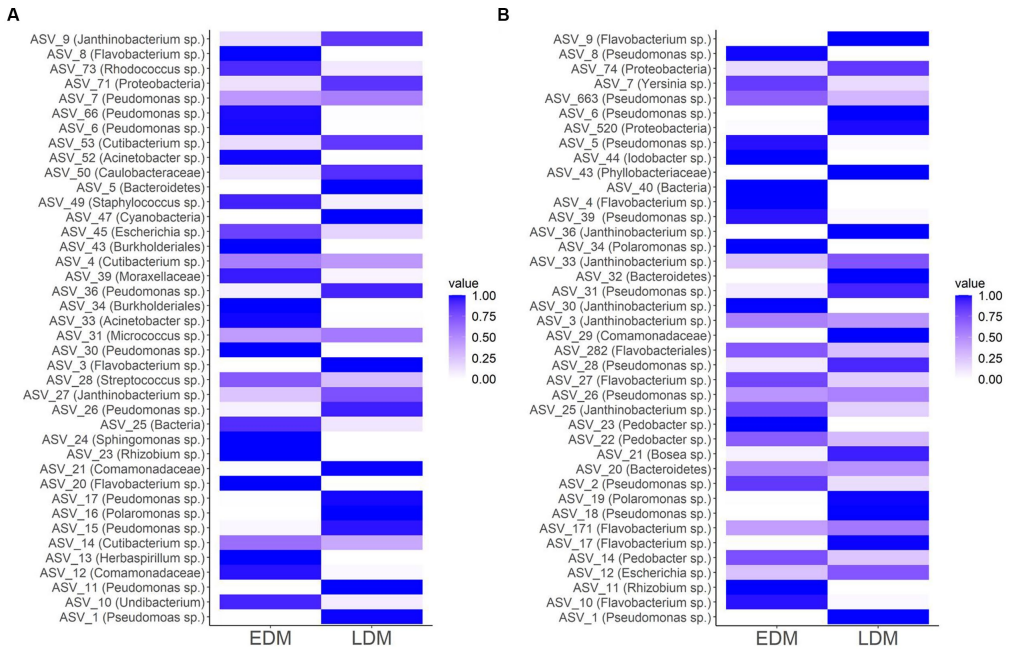

FIGURE 3
Heatmap showing the relative abundances of the Top 40 abundant ASVs in either EDM or LDM samples of Exp.1 **(A)** and Exp.2 **(B)**. Note that within this plot the ASVs are not sorted within their abundance but according to their number.

the microbiota of fish colonized by their egg bacteria was more stable between replicate rearing flasks than that of fish colonized by the lake microbiota.

3.5. Comparison of skin, gut, and rearing water microbiota

For Exp.1, we collected skin and gut samples, but did not sample the rearing water. The PCoA (Figure 1A) and the community composition at the order level (Figure 2A) indicated that the microbiota of gut and skin samples were relatively similar and PERMANOVA tests did not show significant differences (p-values >0.05) between the gut and skin microbiota at any of the sampling times for neither the EDM nor the LDM samples. To avoid potential biases due to the effect of the replicate flask on the microbiota, we compared the Bray–Curtis similarities of gut and skin samples within replicate flasks. This indicated that the gut and skin microbiota differed for fish in the LDM flasks, and in the EDM flasks at 13 wph (Supplementary Figure S6). A Mann–Whitney U test showed that for the LDM flasks at 9 and 13 wph, the Bray–Curtis similarities were significantly lower for gut-skin comparisons than for skin-skin and gut-gut comparisons ($p = 0.003$ and 0.002 for 9 and 13 wph, respectively). The microbiota of gut and skin samples did not significantly differ in Hill's diversity of order 0, 1 or evenness for any of the timepoints (Mann–Whitney U test, p-value >0.05; Figure 4A).

In Exp.2, we characterized the rearing water microbiota in addition to the gut and skin microbiota at 8 wph. Interestingly, the PCoA indicated that for the EDM rearing flasks, the skin and water microbiota seemed to be more alike to each other than to the gut microbiota (Figure 1B). For the LDM flasks, the samples clustered according to the replicate flask, and a potential higher similarity between skin and water samples was not obvious. Accordingly, a PERMANOVA test for the EDM samples showed that the gut microbiota differed significantly from both the skin and water ($p = 0.013$ and 0.003, respectively), whereas the microbiota of EDM skin and water samples did not differ significantly (PERMANOVA, $p = 0.261$). For the LDM samples, no significant difference was found between the microbiota of the different sample types (PERMANOVA, $p > 0.05$). However, the microbiota of both skin and water appeared to be characterized by a higher abundance of Flavobacteriales and Sphingobacteriales compared to the gut samples (Figure 2B). This might indicate differences in bacterial community compositions between gut and skin/water samples also for the LDM samples, even though this was not statistically significant in a PERMANOVA test. The α-diversity was highest for the water samples and lowest for the gut microbiota samples, both in terms of Hill's diversity of order 0 and 1 (Figure 4B). The differences in α-diversity between the gut and skin samples were however only significant for the EDM samples (Kruskal–Wallis test, $p = 0.020$ and 0.038 for order 0 and 1, respectively) but not for LDM samples (Kruskal–Wallis test, $p = 0.084$ and 0.202 for order 0 and 1, respectively).

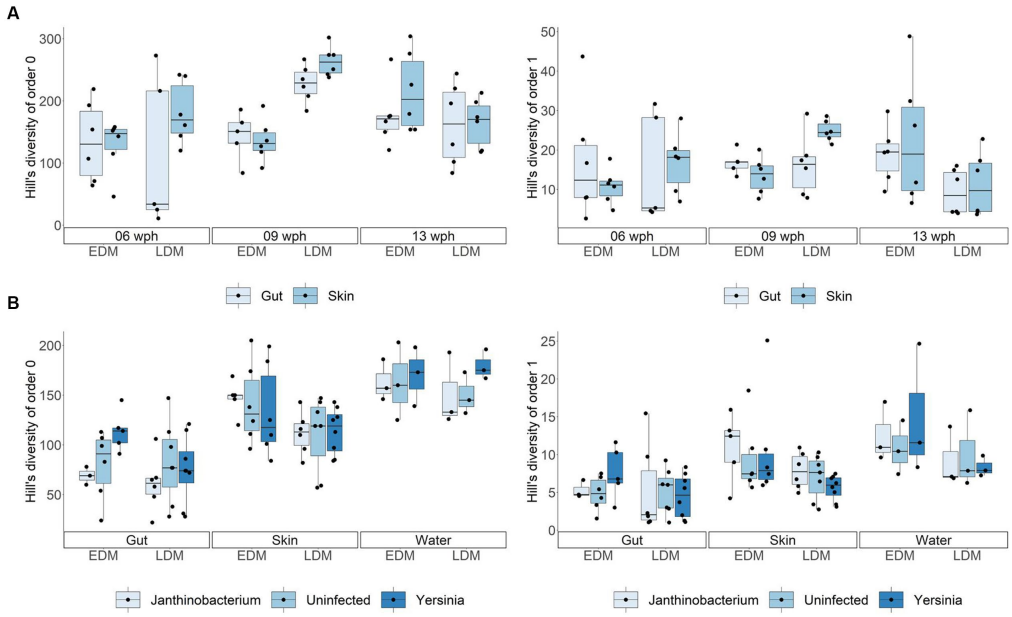

FIGURE 4
Hill's diversity of order 0 (ASV richness) and order 1 (exponential Shannon index) for all samples from Exp.1 **(A)** and Exp.2 **(B)**. The box in the boxplots represent the median of all samples as well as the upper and lower quartiles. Whiskers include all samples except for outliers.

These results indicate that while the skin microbiota was similar to the water microbiota, a distinctive gut bacterial community was developing already in the yolk sac stage, prior to the onset of external feeding. Still, the differences between the gut and skin microbiota were small compared to the differences we observed in the fry microbiota between replicate flasks and between LDM and EDM samples (Figure 1).

3.6. Potential for manipulating the larval microbiota through exposure to bacterial isolates

In Exp.2, we further examined the potential for manipulating the microbiota of the fish by adding high concentrations of either the fish pathogen *Yersinia ruckeri* 06059 or the fish commensal *Janthinobacterium* sp. 3.108 to the rearing water of both EDM and LDM flask at 6 wph (2 weeks prior to bacterial sampling). By comparing the 16S rRNA gene sequences of these two strains (Supplementary Figure S7) with the ASV sequences, we identified ASV7 as *Y. ruckeri* 06059 and ASV3 and ASV15 (combined to ASV3-15; see Methods, "*Analysis of the Illumina sequencing data*") as *J.* sp. 3.108.

Both strains successfully colonized the gut and skin of the salmon yolk sac fry (Supplementary Figure S8). As expected, ASV7 was generally not present in samples from flasks to which *Y. ruckeri* was not added and the relative abundance of ASV7 was significantly higher in samples taken from flasks to which *Y. ruckeri* 06059 was added (in both the EDM and LDM group; Mann–Whitney *U* test $p = 0.012$ and < 0.001, respectively). However, it varied strongly between individuals, from not observed for some samples and up to 50% in relative abundance for other samples, indicating that the colonization success for the *Y. ruckeri* isolate varied. Generally, the relative abundance of ASV7 was higher in gut samples than in skin or water samples (Supplementary Figure S8), however this was not significant (Kruskal–Wallis test $p = 0.944$ and $p = 0.969$ for EDM and LDM, respectively). Surprisingly, and in contrast to what we found for ASV7/*Y. ruckeri*, ASV3-15, representing *J.* sp. 3.108, was detected in considerable quantities in water, gut, and skin samples (on average around 5% in relative abundance), even for samples from flasks to which *J.* sp. 3.108 had not been added (Supplementary Figure S8). This was the case for samples from both EDM and LDM flasks, even though EDM and LDM samples were highly dissimilar in community composition at ASV level, with few highly abundant shared ASVs (Supplementary Figure S4). Furthermore, there was no significant difference in the relative abundance of ASV3-15 between samples from flasks that had been added *J. sp.* 3.108 and samples from flasks that had not been added this bacterial isolate (Mann–Whitney *U* test $p = 0.305$ and $p = 0.682$ for EDM and LDM, respectively). This means that the addition of *J.* sp. 3.108 to the fish flasks did not result in increased relative abundance of this strain in the gut and skin microbiota. Apart from ASV3, as many as 19 more ASVs were classified to the genus *Janthinobacterium* and these ASVs together had an average relative abundance of 10.3 ± 11.0% of all reads per sample. This, together with the fact that strain *J.* sp. 3.108 was originally isolated from the skin of salmon fry, might indicate a role of *Janthinobacterium* as a part of the commensal Atlantic salmon microbiota (see Discussion).

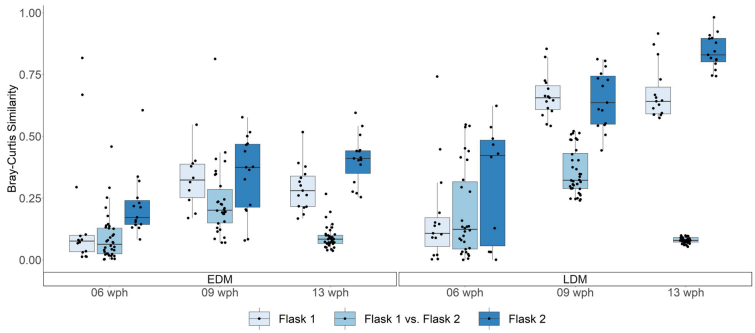

FIGURE 5
Boxplot showing Bray–Curtis similarities for comparisons of fish microbiota (both gut and skin) within and between replicate flasks for Exp.1. Each point represents a single comparison of two samples. The box in the boxplots represent the median of all samples as well as the upper and lower quartiles. Whiskers include all samples except for outliers.

A PCoA suggested that for EDM samples, there were no major differences in the fish's microbiota between flasks that had been added *Y. ruckeri* or *J.* sp. 3.108 and flasks that had not been added bacterial isolates (Figure 6). A PERMANOVA confirmed that there was no significant difference in neither the gut nor the skin microbiota between EDM flasks added bacterial isolates and control flasks, not added bacterial isolates ($p = 0.098$ and $p = 0.348$, for gut and skin samples of the *Yersinia*-treatment and $p = 0.468$ and $p = 0.225$ for gut and skin samples of *Janthinobacterium*-treated samples, respectively). For the LDM samples however, the PCoA plot indicated that the fish's microbiota was influenced by addition of *Y. ruckeri* and *J.* sp. 3.108 (Figure 6). A PERMANOVA test demonstrated that the skin microbiota, but not the gut microbiota, differed significantly between non-treated flasks and flasks that had been added bacterial isolates ($p = 0.007$ and 0.030 for *Y. ruckeri*-treated and *Janthinobacterium*-treated samples, respectively). However, a potential explanation for this observation could be the general difference in fish microbiota between replicate rearing flasks rather than the treatment with bacterial isolates *per se*. A nested PERMANOVA was performed to clarify this, however, due to the limited sample size, no conclusions could be drawn. Additionally, neither the richness, nor the exponential Shannon index were significantly affected by addition of *J.* sp. 3.108 or *Y. ruckeri* (Figure 4B).

Taken together, these findings suggest that neither addition of high loads of the fish pathogen *Y. ruckeri* nor of the presumed fish commensal *J.* sp. 3.108 to the rearing flasks lead to any major changes in the gut and skin microbiota of the Atlantic salmon yolk sac fry.

4. Discussion

The microbiota of fish is crucial for host health and development (Rawls et al., 2004), but little is known about the assembly of the fish microbiota just after hatching. In this study, we investigated the microbiota of the developing fish larvae that had been exposed to two different sources of microbiota present at hatching: either from the eggs of the fish (EDM; fish hatched under conventional, i.e., non-germ-free conditions) or from a freshwater lake (LDM; fish hatched under germ-free conditions and re-colonized). We found that the source microbiota had a strong influence on the skin and gut microbiota of the fish, as the microbiota differed significantly between the EDM and LDM group at all sampling timepoints. Interestingly, the microbiota of fish for which the source of bacteria was the egg (EDM) were more stable, both over time and between replicate rearing flasks, than fish colonized by lake water bacteria (LDM). A possible reason for this might be that egg-derived microbes were better adapted to colonizing the fish, whereas the bacterial populations in the lake water-microbiota were probably poorly adapted for colonization of the fish. This may have increased the significance of stochastic processes, such as ecological drift, that play an important role in the initial community assembly (Dini-Andreote et al., 2015; Gu et al., 2021). Vestrum et al. (2020) showed that drift was important for creating variation in the microbiota between individuals in rearing systems with Atlantic cod larvae. Thus, a stronger influence of drift on the community assembly could explain the divergence in the fish microbiota between replicate rearing flasks.

Previous studies of fish in larger rearing systems, for example for Atlantic cod larvae (Bakke et al., 2013) and Atlantic salmon (Schmidt et al., 2016; Minich et al., 2020), have also demonstrated that the fish microbiota differed between replicate rearing tanks. Interestingly, we observed that the effect was more prominent when comparisons were based on the abundance-based Bray–Curtis similarity than on the presence/absence-based Sørensen-Dice similarity (Supplementary Figure S9). This indicates that the effect arose rather due to differences in the relative abundances of ASVs than the presence of distinct ASVs. In Exp.2, we found that the water and fish microbiota was similar within each rearing flask, and that a distinctive system microbiota developed in each replicate rearing flasks, although the same lake water was used as source community, and that the same bacterial populations thus were present during the initial bacterial colonization of the fish. This indicates that the water microbiota has a stronger influence on the fish microbiota than the selection pressure in the gut and skin of the fry. Comparisons of water and fish microbiota throughout the yolk sac stage might bring new insight about the interrelationship between the water and fish microbiota during the establishment and development of the early fish microbiota.

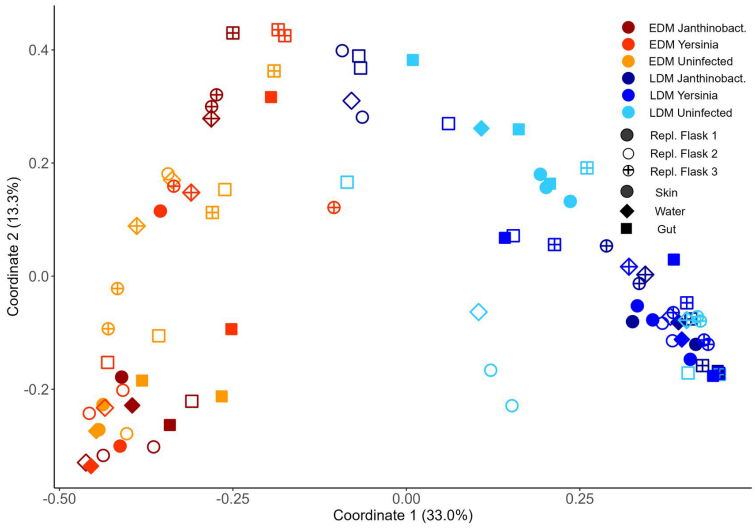

FIGURE 6
Ordination by PCoA based on the Bray–Curtis similarity of all samples from Exp.2, sampled 2 weeks after treatment.

However, unfortunately, we did not characterize the rearing water microbiota in Exp.1.

The finding that the source of bacteria present in the environment after hatching had a major impact on the composition and the stability of the fry's microbiota, points to the possibility for steering the microbiota of the yolk sac fry by manipulating the microbial environments upon hatching. This might have an applied potential in the aquaculture industry, where eggs are routinely disinfected prior to the distribution to hatcheries. In principal, this could be a strategy to counteract negative host – microbe interactions and to develop robust fry by, e.g., introducing probiotic strains. However, research is needed to identify strategies for obtaining this, and to investigate the consequences in terms of host responses.

The differences in the larval microbiota between the EDM and LDM flasks were more profound when PCoA was based on Bray–Curtis similarities than on the weighted UniFrac similarity, which also takes into account the phylogenetic distances between the ASVs. This indicates that the fish in EDM and LDM flasks were colonized by different bacterial populations, which represent related taxa, and thus, that certain phylogenetic groups were selected for on the mucosal surfaces of the fish. Proteobacteria, Bacteroidetes and Actinobacteria were the most abundant phyla of the yolk sac fry microbiota. These phyla were also found to be highly abundant in the microbiota of Atlantic salmon yolk sac fry in a study by Lokesh et al. (2019). They characterized the egg microbiota and followed the Atlantic salmon gut microbiota until the fish were fully developed but included only one sample between hatching and onset of active feeding. Both our and their study found that Proteobacteria was the dominating phylum in the yolk sac fry, and we further found that Actinobacteria were strongly present in the early timepoints and later decreased. These findings are in line with the conclusion of Borges et al. (2021), that summarized for different fish species that Proteobacteria, Bacteroidetes, Firmicutes and Actinomycetes are the dominant phyla in the fish larvae gut (Borges et al., 2021). The main bacterial phyla of the skin microbiota in juvenile and adult Atlantic salmon have been found to be Proteobacteria, Bacteroidetes and Firmicutes (Lokesh and Kiron, 2016; Minniti et al., 2017; Wynne et al., 2020; Bugten et al., 2022) and our study shows that they were highly abundant already at the larval stage.

In Exp.1, we observed that the gut and skin microbiota underwent major changes throughout the yolk sac stage. This was particularly profound at the ASV level and for LDM samples, and very few ASVs were highly abundant at all sampling timepoints. Interestingly, even though Pseudomonadales dominated the fish microbiota in LDM flasks at all sampling times, this phylum was represented by distinct ASVs at the three sampling times, indicating that distinct *Pseudomonas* populations colonized the fish at different ages. Also in other vertebrates and fish species, it has been observed that the early microbiome is dynamic, and is only stabilizing later in life (Schloss et al., 2012; Chen and Garud, 2022; Woodruff et al., 2022; Xiao et al., 2022). We further observed large interindividual variation in the microbiota, an observation often made in other aquatic larvae (e.g., Verschuere et al., 1999; Stephens et al., 2016; Vestrum et al., 2020), which has been suggested to be a consequence of ecological drift (Vestrum et al., 2020).

For adult fish, several studies have shown that the skin and gut of fish harbors distinct microbial communities (e.g., Lowrey et al., 2015; Sylvain et al., 2020). However, few studies have focused on the skin microbiota of fish larvae (e.g., Dodd et al., 2020), and little is known about the diverging development of the gut and skin microbiota in the early developmental stages, especially prior to onset of active feeding. Already at 7 dph, long before the fish starts to feed, the anus and the mouth of Atlantic salmon is opened and therefore available for bacterial colonization (Sahlmann et al., 2015). Here, we dissected out the fish guts and studied the development of both the gut and skin microbiota prior to the onset of external feeding. Both in Exp.1 and 2

we found indications that the skin and gut microbiota started to differentiate already at 8–9 wph, several weeks before the yolk sac was consumed. We observed however, that the gut was filled with yolk material (not shown), which might provide nutrients to the gut microbiota. Sahlmann et al. (2015) further observed that distinct gut organs and structures formed already from 7 dph on. This structuring of the gut might already provide colonization space for bacterial populations filling different niches, resulting in a distinct gut microbiota.

Moreover, Exp.2 showed that the microbial skin communities resembled the water microbiota, whereas the gut microbiota differed from the water and skin microbiota. In studies of Gilthead Sea Bream and Atlantic cod larvae the microbiota of the whole fish (no differentiation between gut and skin) was found to differ from the water microbiota (Bakke et al., 2015; Nikouli et al., 2019; Vestrum et al., 2020), indicating that the selection of bacterial populations differed between the water and mucosal surfaces of the larvae. In our study we could now show that in the larval stage it indeed appears to be only the gut microbiota that is different from the water microbiota, not the skin microbiota. Studies in adult fish report that the skin microbiota is distinctive and differs from the surrounding water microbiota (Razak et al., 2019; Gomez and Primm, 2021). It would be interesting to further investigate to which extent the skin and water microbiota diverge throughout the yolk sac stage.

In Exp.2, we further investigated the potential of manipulating the fish's microbiota by addition of one of two bacterial isolates in high densities (a theoretical final concentration of 10^7 CFUml^{-1}) 6 weeks after the fish had hatched. Either the fish pathogen *Y. ruckeri* or the presumed non-pathogenic fish commensal *Janthinobacterium* sp. 3.108 was added to EDM and LDM flasks. Surprisingly, the ASV corresponding to the *J.* sp. 3.108 isolate (or a strain with the same partial 16S rDNA gene sequence) was found in the microbiota of fish from all flasks, also those that had not been added the isolate. The relative abundances of that ASV varied extensively between individuals, but the average relative abundances did not increase in samples from flasks that had been added *J.* sp. 3.108. Accordingly, we found that addition of the commensal *J.* sp. 3.108 did not significantly change the microbiota. The fish pathogen *Y. ruckeri* was not present in significant amounts in flasks to which we did not add it. In flasks to which we added it, its abundance among individuals was highly variable and mainly present in low relative abundances. It further did not have a large impact on the skin and gut microbiota. These results may indicate that the microbiota of the larval Atlantic salmon is resistant to invasion by introduced bacterial strains. Skjermo et al. (2015) showed that none of the four probiotic candidate bacterial strains originally isolated from cod larvae were able to establish themselves as part of the microbiota of Atlantic cod larvae. Further, Puvanendran et al. (2021) found that their probiotic *Carnobacterium* isolate could not establish itself in Atlantic cod larvae. This shows that manipulating the microbiota of fish with, e.g., probiotic strains might be difficult to achieve already in the larval stage, when the fish's microbiota is still unstable. As discussed above, manipulating the microbial environments at hatching might be a better strategy for influencing the early fish microbiota.

Apart from the *Janthinobacterium* strain we added (strain 3.108), we also found several other ASVs classified as "*Janthinobacterium*" in high relative abundances. Strain *J.* sp. 3.108 was originally isolated from the skin of Atlantic salmon fry from a commercial RAS, and its 16S rRNA gene sequence is highly similar to the *Janthinobacterium lividum* type strain (99% similarity over the whole 16S rRNA gene, data not shown). *J. lividum* commonly occurs in freshwater (Pantanella et al., 2007) and is a commensal of both the amphibian (Brucker et al., 2008; Becker et al., 2009) and human (Grice et al., 2008; Ramsey et al., 2015) skin microbiota, and it has antagonistic properties against fungi and bacteria (Munakata et al., 2021). *Janthinobacterium* spp. have also been found in tank biofilms of fish farms for rainbow trout (Nakamura et al., 2002; Testerman et al., 2021). A *J. lividum* strain was shown to be capnophilic (Valdes et al., 2015), meaning it thrives under high concentrations of CO_2. The salmon larvae exchange gas mainly through the skin, and this could be an explanation for the presence of *Janthinobacterium* in the skin microbiota. As we also found high abundances of several ASVs classified as *Janthinobacterium* associated with the skin and gut samples in both experiments, we propose that strains from the genus *Janthinobacterium* are commensal bacteria for Atlantic salmon larvae. Members of *Janthinobacterium* have also been found in the intestine of adult Atlantic salmon (Wang et al., 2018).

In contrast to *Janthinobacterium*, *Y. ruckeri* is a well-known pathogen in later life stages of the salmon (e.g., Kumar et al., 2015). However, it appears as if no lethal disease was triggered by the addition of *Y. ruckeri*, even though the strain used in this study (*Y. ruckeri* 06059) has successfully been used to inflict mortality in Atlantic salmon fry (Haig et al., 2011). A possible reason for this might be that either the temperature used here was too low (14°C) or that the yolk sac fry was not developed enough for *Y. ruckeri* to induce mortality.

In this study, RNA extracts were available for characterization of the fish microbiota for the samples collected in Exp.1, while in Exp.2, the analyses of the microbiota were based on DNA. RNA-based microbiota analyses are assumed to reflect the actively growing populations in the microbial communities to a larger extent as compared to DNA-based analyses, which will also represent inactive bacterial cells. As these two experiments also differed in other parts of the methodology (e.g., in how the nucleic acids were extracted), it is not possible to compare these two datasets directly. We therefore analyzed the data from the two experiments separately and compared how they answered our research questions. We found that the key findings were shared for the two experiments, as for both experiments the fish microbiota varied between LDM and EDM flask and also between replicate rearing flasks. We further saw differences between gut and skin samples in both the RNA-based and DNA-based data. Therefore, even though different approaches were used in the two experiments, both answered our research questions in similar ways, which indicates that our findings are robust.

In conclusion, we showed that the skin and gut microbiota were similar, but started diverging during the yolk sac stage, several weeks before the yolk sac was consumed. The skin microbiota was more similar to the water microbiota than the gut microbiota. Furthermore, the microbiota differed profoundly between fish that had been conventionally reared, i.e., the egg microbiota was the only source of bacteria (EDM), and fish that had been made germ-free and were then colonized by using lake water as a source for bacteria (LDM). Proteobacteria, Bacteroidetes and Actinobacteria were the most abundant phyla in the fry microbiota. Both the skin and gut microbiota were highly dynamic and underwent major changes at

the ASV level throughout the yolk sac stage, and this was particularly evident for fry reared in LDM flasks. The fry microbiota differed profoundly between replicate rearing flasks, and again, this was particularly evident for the LDM flasks. Thus, the fry reared in EDM flasks had a more stable microbiota, both between rearing flasks and over time. Additions of high doses of the pathogen *Y. ruckeri* to fish flasks did not cause mortality. Addition of *Y. ruckeri* had only minor impact on the community composition. Finally, we exposed the fry to high doses of a *Janthinobacterium* sp. isolate and found no effects on the fry microbiota. An ASV sequence corresponding to the one for the added *J.* sp. isolate was abundant in most fry samples and indicated that this represented a commensal member of the early fry microbiota.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, ERS14440101–ERS14440192.

Ethics statement

Ethical review and approval was not required for the animal study because the Atlantic salmon yolk sac fry are not defined as living animals according to Norwegian legislation, therefore no ethical review was required.

Author contributions

MD and AF conducted the fish experiments. AF and EL prepared the 16S libraries and analyzed the resulting data. AF, OV, and IB conceived and designed the experiments. AF wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1177972/full#supplementary-material

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Paper II:

The impact of phage treatment on bacterial community structure is negligible compared to antibiotics

Madeleine S. Gundersen, Alexander W. Fiedler, Ingrid Bakke and Olav Vadstein

Under review at Scientific Reports

This paper is under review for publication and is therefore not included.

Paper III:

Phage therapy minimally affects the water microbiota in an Atlantic salmon (*Salmo salar*) rearing system while still preventing infection

Alexander W. Fiedler, Madeleine S. Gundersen, Toan P. Vo, Eivind Almaas, Olav Vadstein and Ingrid Bakke

Under review at Scientific Reports

This paper is under review for publication and is therefore not included.

Appedix IV: Summary of all challenge experiments where Atlantic salmon yolk sac fry was challenged with bacterial pathogens. The fish were raised as described in the Material and Methods section of papers I and III. All experiments also included uninfected control fish for all experimental groups. For more detailed information, see Drågen, 2020, Skovly, 2021 and Vo, 2022. Abbreviations: conc: concentration; CVZ: conventionalized; CVR: conventionally raised dph: days post hatching; exp: experiment; GF: germ-free; rep: Replicate; Temp: Temperature

Exp. ID	Fish age [dph]	Temp. [°C]	Pathogen	Final pathogen conc. per fish flask [CFU/ml]	GF/ CVZ/ CVR	Experimental groups	Rep. flasks	Comments	Length of exp. [days]	Experimental outcome
MP1	14 or 42	6.5	*Y. ruckeri* NVI-11025	$10^4 – 10^8$	CVR	Five different concentrations of the pathogen.	2	The pathogen was not washed before addition to the flasks, so it was added together with the TSB growth medium	7	No mortality in lower concentration. 100 % mortality at 10^8 CFU/ml final concentration. Mortality was not due to infection, but because of suffocation of the fish as 10 ml TSB were added to the fish flasks → massive growth of bacteria
MP2	42	6.5	*Y. ruckeri* NVI-11025 (and NVI-10705)	10^7	CVR	Five different treatments: Exposure to 200 mg/l H_2O_2 for 1 h; exposure to 50 mg/l formaldehyde for 30 min, increasing temperature to 14 °C for 24 h before and after challenge; adding a homogenized fish to the flasks; using strain NVI-10705 instead of NVI-11025 (no additional stressors)	2	The pathogen was not washed before addition to the flasks, so it was added together with the TSB growth medium	7	100% mortality when temperature was increased, however, temperature was increased from 6.5 to 14 °C abruptly → too much stress? High mortality in one of the two replicates each of the H_2O_2 and formaldehyde treatment, no mortality in the other replicates
MM1	35	10, 12, 14	*Y. ruckeri* NVI-11025	10^7	CVR	Six groups: Exposed to 10, 12 or 14 °C 24 h prior to exposure and then either put back to 6 °C or kept at the temperature for 24 h longer	2	Pathogen was washed in SGM before addition to the flasks. This was done in all further	5	No mortality observed

								experiments. Temperature increased abruptly		
MM2	42	6, 11, 14	*Y. ruckeri* 06059	10^7	CVR	Three different temperatures: 6, 11 and 14 °C. The flasks were kept at these temperatures for the whole two weeks the experiment was running after the challenge	3	Temperature increased over the course of one week, prior to challenge. This was done in all further experiments	14	No mortality observed
MM3	36	6, 14, 16	*Y. ruckeri* 06059	10^7	CVR	The fish were sedated, and their caudal fin was clipped off (also unclipped but sedated control included) at three different temperatures	n.a.	Fish were individually housed after challenge.	14	High mortality at higher temperatures, also in non-challenged controls. No increased mortality due to infection.
MM4	42	14, 16	*Y. ruckeri* 06059	10^7	GF/ CVR/ CVZ	Six groups in total: Two different temperatures (14 or 16 °C) and GF, CVR or CVZ fish	3		14	No mortality at 14 °C; high mortality in CVR and CVZ groups at 16 °C, also in groups that were not challenged → 16 °C is too high for the fish to thrive. No increased mortality in challenged flasks.
AM1	42	12	*A. salmonicida* 88/09/03175; *A. salmonicida* 11540; *F. psychrophilum* DSM 3660: *F. psychrophilum* NCIMB 13383	10^7	CVR	Fish were exposed to air for five minutes prior to the challenge. Six groups: Both *A. salmonicida* strains were either grown in BHI or TSB (100 µM bipyridyl each) and *F. psychrophilum* strains were grown in TYES medium (50 mM bipyridyl)	3	All bacterial strains grown in rich media containing 50 - 100 µM bipyridyl (iron-chelator) in an attempt to increase pathogenicity	21	Some mortality observed in *A. salmonicida*-challenged flasks around two weeks after challenge, but also mortality in uninfected controls.
AM2	42	16	*Y. ruckeri* 06059	10^7	GF/ CVZ	*Y. ruckeri* grown either with 100 µM bipyridyl added to its growth	3		21	High mortality in all CVZ flasks due to the high temperatures.

						medium or without. Both GF and CVZ flasks infected (four groups in total).				High mortalities in the challenged GF flasks
AM3	42	16	*Y. ruckeri* 06059	10^7	GF/ CVZ	*Y. ruckeri* grown in medium containing 100 µM bipyridyl. Both GF and CVZ flasks (two groups).	5	This experiment was conducted to confirm the promising results of exp. AM2	21	Again, high mortality in all CVZ flasks. No significantly increased mortality in challenged GF flasks.
TM1	42	6, 10, 14	*F. columnare* Fc7	10^7	GF	Three different challenge temperatures (6, 10 and 14 °C).	3		11	High mortality at both 10 and 14 °C after two to three days post challenge. No mortality at 6 °C or in the unchallenged controls.
TM2	20	10	*F. columnare* Fc7	10^6 or 10^7	GF/ CVZ	Two different concentrations of *F. columnare* Fc7 were tested in both GF and CVZ flasks (four groups in total).	3	Fish were challenged at 20 dph instead of 42 due to time constraints	10	No mortality observed.
TM3	41	10	*F. columnare* Fc7 *F. columnare* FCO-F2	10^7	GF/ CVZ	Two different strains of *F. columnare* were tested in both GF and CVZ flasks (four groups in total).	3		10	No mortality induced by strain Fc7. High mortality induced by strain FCO-F2 in GF fish, less mortality in CVZ fish. No mortality in the controls.
TM4	44	10	*F. columnare* Fc7	10^7	GF/ CVZ	Both GF and CVZ flasks were challenged and then either treated daily with oxytetracycline, phage FCL-2 against strain Fc7 or were left untreated (six groups in total).	3	TM4 was conducted at the same time as TM3, that is why Fc7 was used as pathogen instead of strain FCO-F2 even though FCO-F2 was shown to	10	No mortality observed

								perform better in TM3		
TM5	42	14	*F. columnare* Fc7	10^7	GF/ CVZ	Both GF and CVZ flasks were challenged and then either treated daily with oxytetracycline, phage FCL-2 against strain Fc7 or were left untreated (six groups in total)	3	Same experimental setup as TM4, but at 14 °C	10	See paper III

I finished it.

Everything’s all right

- Tommy Creo

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