Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Immersion Challenge of Atlantic Salmon (*Salmo salar*) yolk sac fry with *Flavobacterium columnare*

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
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Acknowledgments

This master project was conducted in the research group Analysis and Control of Microbial Systems (ACMS) at the Department of Biotechnology and the Food Science at Norwegian University for Science and Technology (NTNU). Many thanks to Amalie Horn Mathisen, Eirik Degré Lorentsen, Madeleine Julia Gundersen, and Jenny Poppe for their much-needed assistance in the lab during this project.

I would like to thank my amazing supervisor during this project, Ingrid Bakke. Thank you for giving me the opportunity to be part of an incredibly exciting and fulfilling project, thank you for your knowledge and passionate guidance, and for the patience and unending kindness you have shown me. Special thanks to my co-supervisor PhD-candidate Alexander Willi Fiedler for his guidance in laboratory work, you have been a great teacher, and an even better lab-companion and friend. We shared many laughs together, and you made long days at the lab flash by in an instant. There is no one else I'd rather be working with in a cold fish room for hours other than you.

I would like to thank COVID-19 for keeping me home and away from the lab during some of the most important parts of this project. Lastly, I would like to thank my dearest friends and loved ones for their support and company during my years spent studying, especially Lea Marie Anfinset, Victor Jankowski, Martin Eide Lien, Haakon Zhang Moene, Henrik Dybedahl and Erlend Gløer Teigset. Without you I would have probably finished writing on this project considerably sooner, nevertheless, I still love you.

Abstract

The microbiota is regarded as an essential part for most organisms' development and health, especially through protection against pathogens, digestion and nutrient intake, and development of the immune system. However, the mechanics for such interaction are not fully understood yet. The ability of phages to infect and lyse certain target host bacteria has been used as a treatment against pathogens as well. However, there is only a limited number of studies on the protective effect of the microbiota against pathogens and phage therapy, and even less studies have been conducted in these fields in regard to fish such as *Salmo salar*. If the potential of phage therapy shows to be a promising tool against pathogens, it could be used as an alternative to antibiotics. In recent years, there has been an increasing focus on the emergence of antibiotics resistant bacteria, which pose a great threat in the medical and industrial fields of society. Gaining deeper understanding of microbiota host interactions and phage therapy could provide tools to further protecting animals, such as fish in aquaculture, from pathogens.

To achieve this, a reproducible immersion challenge of Atlantic salmon yolk sac fry with a pathogen needed to be established. One of the goals of this project is to examine the pathogen *Flavobacterium columnare* strains UCD Fc7 and FCO-F2 as candidates for immersion challenge of the fry. Additionally, once an immersion challenge has been established, the potential protective effects of the microbiota and the viability of phage therapy was tested in immersion challenges. To examine the protective effects of the microbiota, germ-free fish and fish with a microbiota present were immersion challenged with *F. columnare* Fc7 and FCO-F2, additionally some fish were treated with FCL-2, a phage targeting *F. columnare* Fc7, and the mortality between groups were compared. It was shown that conventionalized fish were noticeably more resistant against the pathogen than germ-free fish, and that fry receiving phage treatment had considerable increased resistance to the *F. columnare* Fc7 pathogen.

In conclusion, this project was successful in demonstrating the protective effects of the microbiota against pathogens and showed that phage therapy could be a viable alternative to antibiotics in treatment against pathogens.

Abbreviations

CFU	colony forming unit
CVZ	conventionalized
GF	germ-free
DNA	deoxyribonucelic acid
dsDNA	double-stranded deoxyribonucelic acid
ssDNA	single-stranded deoxyribonucelic acid
RNA	ribonucelic acid
dNTP	deoxyribonucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
M	molar (mol/L)
MilliQ H_2O	Pure water produced by a MilliQ® device
OD	optical density
PCR	polymerase chain reaction
RAS	recirculating aquaculture system
RDP	ribosomal database project
SGM	salmon gnotobiotic media
TAE	tris-acetate-EDTA
TSA	tryptic soy agar
TSB	tryptic soy broth
TYES	Tryptone Yeast Extract Salts
dpi	days post infection
dph	days post hatch

Contents

1. Introduction

Background

The work presented in this thesis is a part of the PhD thesis work of Alexander Willi Fiedler on understanding how the microbiome affects its host, and how it might contribute to combat pathogen infections. The thesis is also the continuation of the work of previous ACMS master students on "Immersion Challenges with Larvae of Atlantic salmon (*Salmo salar*)"[57] and "Immersion Challenge of Atlantic Salmon *(Salmo salar*) yolk sac fry with *Yersinia ruckeri* "[22]. So far, no reliable protocol for bath infection of Atlantic salmon fry has been established, which is crucial to test the microbiome's protective effect. Previous work in the group tried to establish an immersion challenge protocol using *Yersinia ruckeri* as a pathogen, however these trials were unsuccessful.[22,57]

Aquaculture

Aquaculture is a relatively new area in modern fishing-industry. The core concept of aquaculture is acquiring and rearing fish from eggs, all the way until they are mature for consumption, instead of capturing wild fish. One form of aquaculture are recirculating Aquaculture Systems (RAS), where the water is recycled, and thus have the highest degree of enclosure and control for the fish being reared, while traditional flow-through systems with a continuous flow of fresh external water are more open.

The implementation and construction of aquaculture-facilities is increasing, especially in Norway. In 2019, Norway produced 1.5 million tons of fish, mainly salmon and-, rainbow trout, from aquaculture facilities with a first-hand value of 71,7 billion NOK, equaling an increase of 5.7 percent in value compared to the previous year.[11] Sea catches of salmon and sea trout, on the other hand, amounted to only 171 tons in 2021 and between 2019 and 2021, there has been a decrease in sea catches by 27 percent.[11] Aquaculture production has therefore long eclipsed traditional fishing in Norway in terms of size and value.

Outbreaks in aquaculture

Pathogen outbreaks are still a major threat to this industry. Due to the high stocking density of the fish reared, transmission of infections occur very rapidly once a system has been

compromised.[1,25] High stocking density can also cause more stress for the fish, which renders them more susceptible to infections.[14] Diseases such as pasteurelloses, winter ulcers and yersiniosis were in total detected in at least 450 fish localities in Norway in 2021.[59] As the industry keeps growing, it becomes more important to thoroughly understand how to minimize risk in aquaculture systems and maximize productivity and sustainability by developing and improving methods in the industry.

To minimize the danger of pathogens, vaccination can be used preventatively, whilst antibiotics are often used post infection. Millions of fish are vaccinated yearly, and it has been shown that vaccines work at preventing bacterial and viral diseases.[23] In Norway for example, there has been a heavy reduction in antibiotics use since the introduction of vaccines.[24] A drawback of vaccines is that each fish must be physically injected with the vaccine, usually through an automated system.[2] This process is both very time consuming and stressful for the fish, and require the fish to have reached a certain size. In addition, there are diseases that do not have a vaccine yet and developing them can be very expensive.[24] Therefore, there is currently still a need to use antibiotics in aquaculture today, due to the problems mentioned with vaccines, and due to it still being an efficient way to deal with pathogen outbreaks. One of the problems with using antibiotics is, however, that some bacteria are or become resistant, rendering treatments ineffective.[3] This has been of great focus lately as the rampant use of antibiotics in many industries and healthcare is contributing to increasing the appearance of antibiotic-resistant bacteria.[4] It's therefore important to seek out other treatment options and find ways to reduce the amount of antibiotics used currently.

Salmo salar

The most important aquaculture species in Norway is *Salmo salar*, commonly known as Atlantic salmon. Of the fish produced in Norway in 2019, 94 percent consisted of Atlantic Salmon, making the species the majority of Norwegian production and fish export.[11] Atlantic salmon are mainly located in the northern Atlantic Ocean. They have a migratory lifecycle, with the eggs being laid and hatched in freshwater. Afterwards, the young fish return to the sea to feed and grow rapidly, before they return to their rivers to spawn.[32] In the first stage of their life cycle, the fish from newly hatched eggs are called alevins. An important characteristic of alevins is that they hatch with a yolk sack.[33] In the first weeks of their life, the alevins feed on this yolk as they

hide in gravel on the riverbed. At this stage they do not require external feeding to sustain themselves.[33,34] This stage can last between 20-120 days depending on the temperature, with warmer temperatures causing a shorter alevin-stage.[34] Once the absorption of the yolk sack is nearly complete, they emerge from the riverbed as fry.[34] In this stage, they need to actively feed, and the fry start dispersing from each other.[34] At the later stages, they continue to grow, and after 1-3 years, the salmon start smoltifying and travel out of rivers into the ocean where they grow into adult salmon.[34]

The production of Atlantic Salmon in aquaculture begins with fertilized fish roe, usually supplied by an independent roe producer, being hatched in freshwater-incubators in hatcheries.[60] Once hatched, they are kept in the hatchery until they reach the smolt phase.[60] In this phase, the salmon weigh 80-120 grams, and have gone through smoltification.[34] Post-smolt, they can be moved out of the hatcheries to aquafarms consisting of cages in sea water or put into specialized tanks in RAS-facilities where they grow out in 18–24 months.[60]

Microbiota

The term microbiota has slightly varying definitions. Julian R. Marchesi and Jacques Ravel define it as “The assemblage of living microorganisms present in a defined environment”[5], which is the definition this thesis will be following. All multicellular organisms have a microbiota that can change over time.[27] The composition of vertebrate microbiota often differ between individuals of the same species or group, and factors such as age, gender, and weight may all contribute to these differences.[6] For many organisms, the development of a microbiota starts quickly after birth or hatching.[7] With humans, bacterial colonization begins as the fetus is in the lower uterus, whilst a gut microbiota is established through the consumption of breast milk during the weaning period.[7]

For fish, as the egg is a physical barrier between the fish and the environment, it is unsure if proper bacterial colonization can take place before hatching. There has been some evidence found of pathogen bacteria being present already in the egg,[29] however, it is assumed that chorion-associated bacteria are the first to colonize the fish’ surfaces after hatching.[28] It is believed that further colonization of the fish’s gut and diversification of the microbiota occurs days after hatching, when the fish start opening their mouths to control osmoregulation.[7] A new

factor for changing the gut microbial composition is introduced when the fish start feeding.[7] Studies have shown clear signs that microorganisms in the microbiota have a mutualistic host-microbe relationship.[30] The gut microbiota can assist with helpful functions for the host, such as metabolizing hard to digest polysaccharides, detoxifying harmful compounds, and contribute to developing the immune system of the host.[8] Further, the microbiota is believed to protect the host from pathogenic attacks mainly in two ways.[9] One way is indirect inhibition, through competitive exclusion of nutrients, chemicals, and attachment space, serving as a barrier between the host and pathogens.[31] Secondly, some organisms are also capable of producing extracellular products that are harmful to potential pathogens. Lactic acid bacteria for example, which are often a large part of the fish microbiota, excrete lactic acid in combination with other compounds that deter harmful pathogens from colonizing.[9] In studies, it was shown through experimentation with rainbow trout- and zebrafish-fry that the microbiota of the fish was successful in protecting against pathogen infections,[20,77] possibly through the mechanics described.

As mentioned previously, a healthy and stable microbiota has been shown to protect against infections. It could therefore be possible to use the microbiota as a preventative measure against infection in many ways, including in aquaculture. [10] The practice for aquaculture facilities to use antibiotics post infection, especially in their feed, may disrupt the fish' microbiota and render them vulnerable to be colonized by pathogens or opportunistic antibiotics-resistant bacteria.[10]

Columnaris disease

Columnaris disease is an infection caused by *Flavobacterium columnare* which targets both cultured and wild freshwater fish, including salmonids (salmon and trout), catfish, and tilapia which are produced in eastern Asia, India, USA, Thailand and Brazil, making it a global problem.[12,35,36,12,35,37,38] The disease has been a bigger problem in warmer climates, especially in the summer, but considering the geographical span of columnaris infections reported,[39] outbreaks of columnaris disease could occur in almost any part of the world, causing massive economic losses in facilities with outbreaks. Finding new ways to control and treat columnaris disease would contribute a great deal to further improving the security, profits, and fish health in aquaculture.

The bacterium responsible for causing columnaris disease is *Flavobacterium columnare*, which are Gram-negative rod-shaped bacteria mostly found in freshwater in warmer climates. However, F. *columnare* strains have been isolated from Finnish aquaculture facilities and have been causing outbreaks in water as cool as 12–14 °C.[14,38] They form yellow colonies when grown on plates, and there are some varieties in virulence and morphology between strains.[40]

The mode of infection of F. *columnare* begins with the bacteria being present in the water, and entering the fish through the gills, mouth, or wounds, adhering mainly to the gills.[12,13] Once an infection develops, it may cause erosion, gill necrosis, and skin lesions in the fish, leading to a high mortality rate among them, especially in young fish. In addition, F. *columnare* contribute to forming biofilms on the surface of eggs, which causes membrane degradation.[15] They can cause disease under normal rearing conditions but are more likely to do so when the fish are stressed, such as when experiencing suboptimal temperature, pH, salinity, or fish density.[14]

F. Columnare outbreaks are treated with antibiotics such as oxytetracycline or florfenicol, which are broad spectrum antibiotics.[12] Other antibiotics such as oxolinic acid were previously used, but are discontinued due to large amounts of them contaminating water outlets and nearby water bodies.[16] The standard treatment with antibiotics can however disrupt the microbiome of the fish as described above, causing a decrease in microbiome-mediated disease resistance.[58] This could render the fish more susceptible to infection from other pathogens, or re-infection of *F. columnare.*

Pathogenic immersion challenge

The potential protective effect of a healthy microbiota in fish, especially *Salmo Salar,* could be of great use in aquaculture to combat diseases, and should be investigated. To achieve this, a reproducible challenge experiment of the fish with pathogens of interest can be used. Pathogenic challenges of fish are in general practiced in two ways: injections of the respective pathogen directly into the fish to initiate an infection, or through an immersion challenge where the pathogen is either added to the fish's environment, or the fish are bathed in pathogen-rich water for a certain time.[62] In general, injection challenges give more success of infection, and a higher degree of reproducibility.[64] With smaller fish however, injections are not very suitable, while an immersion challenge is not limited by the fish' size, meaning the method works for fry as well.[63]

In addition, an infection by injection will bypass a lot of the fish' outer defense, e.g. the skin, and the microbiota present in the rearing water and associated with the fish skin.[64] By doing so, the possible influence of the water microbiota to combat infections could not be investigated. This makes an immersion challenge the only viable option for testing the protective effects of the microbiome in smaller fish.

A problem with the immersion challenge model is that many experiments incorporating it have experienced too varying infection success and low reproducibility.[65] One way to increase the likelihood of a successful infection is by increasing the rearing temperature. The fish are more stressed in sub-optimal rearing temperatures, decreasing their defense against pathogenic threats.[14] In addition, based on the bacterial strain, an increase in temperature can promote faster bacterial growth, making them more infectious.[66]

Bacteriophages

Bacteriophages, or simply phages, are a type of virus which targets only bacterial cells. It is estimated that there are 4.8×10^{31} phage particles in total on the planet, outnumbering all other organisms in quantity and therefore making bacteriophages the most abundant entities on this planet.[17] Phages can be found in all environments where bacteria are present and grow, contributing greatly to the dynamics of the ecosystems on a microbial level.[17]

Like other viruses, phages consist of a core with nucleic acid, either single- or double-stranded DNA or RNA.[41] This nucleic acid is surrounded by a protein capsid.[17] There are three structural forms of bacteriophages: icosahedral head with a tail, icosahedral head without a tail, and a filamentous form. The majority of known phages are tailed phages from the order *Caudovirales*.[41] In general, they consist of a capsid head where the phage genome is located, and a tail part, which is often used to inject the phage genome into the host cell (through the spike) on the baseplate or eject the genome once the whole virus has entered the cell.[41]

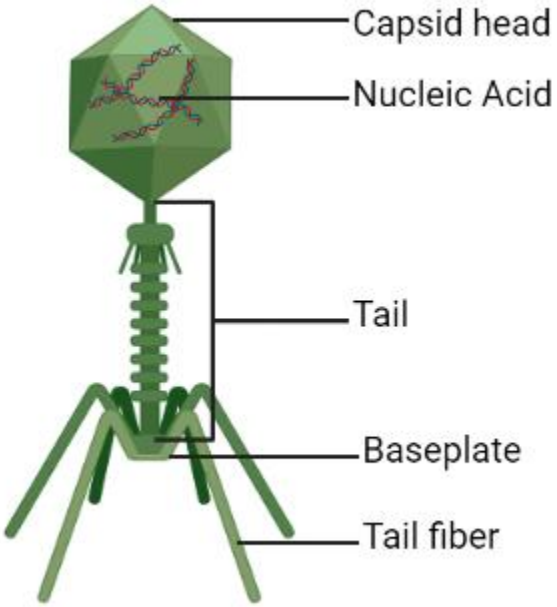


Figure 1: Tailed bacteriophage with parts of their structure highlighted.

There are two types of phages: lytic, and lysogenic .[17] For this work, only the lytic phages are of relevance and therefore only their life cycle is explained: The mode of replication for lytic phages starts with them attaching to their target host cell through interaction between cell surface receptors of the host, and receptor binding proteins of the phages, usually present at their tail end.[17]

For phages with contractile tails, once a phage binds to the host, the phage's baseplate undergoes a conformational change, resulting in sheath contraction and injection of the phage's nucleic acid into the host cell. The form of the nucleic acid varies between phages.[19] The purpose of injecting viral nucleic acids into the host is to use their metabolic pathways to replicate, creating copies of its viral genome, packaging the genomes into virions, and exiting the cell as new phages to infect new hosts. The mechanisms for achieving this is different between phages, especially for those with different forms of viral genome. Some phages use enzymes present in the host while some bring their own enzymes in addition to their virions. RNA viruses replicate their genomes either by RNA-dependent RNA synthesis, or DNA synthesis through reverse transcription, subsequently followed by DNA replication and transcription.[19] DNA-viruses only need to transcribe mRNA from the dsDNA, and then replicate the dsDNA or ssDNA in the cell nucleus.

As a final step, the newly assembled phages need to exit the host cell to continue their infection and reproduction cycle. To achieve this, the phages must lyse the cells in some way. Tailed phages use the phage-encoded enzyme endolysin to destroy the cell wall from within.[17] Endolysins are enzymes that degrade peptidoglycan, which are an integral part of the plasma membrane structure of most bacteria. These endolysins are expressed during the later stages of the lytic cycle, after a sufficient number of phages have been assembled.[17] The release of viral particles via lysis of the bacterial cell usually leads to death of the cell.

An infection requires the host to have, among other things, a certain morphology, surface compatible receptors, and the correct enzymatic pathways for the phage to successfully carry out the necessary steps for infection and replication.[17,18,19] This means that a single phage strain has a very definite selection of potential hosts, and their range can often be narrowed down to a specific species or strain of bacteria.

The receptors recognizable for phages include lipopolysaccharides, teichoic acids, pili, flagella, and capsular polysaccharides.[17,18] These molecules often serve essential roles in the bacteria cell, such as transport and movement, making deletion or downregulation of them very costly for the cells.[18] Bacteria may develop mechanisms to avoid being infected. Some can still change or lose surface receptors despite the drawbacks,[42] while some can secrete substances that inhibit viral adhesion to the bacteria surface, or block phage genome injection into the cell, or inhibit phage replication and release.[42]

Phage therapy

The specificity of the phages, and their ability to kill their hosts serves as an alternative way for therapeutic treatment against pathogenic infections from bacteria, such as *Flavobacterium columnare*. This application of phages is called phage therapy. Instead of broad-spectrum antibiotics, bacteriophage therapy, could be a potential alternative to antibiotics, as it would not harm the naturally occurring microbiome in the environment.

Since phages are so abundant, they are relatively easy to isolate. To isolate a bacteriophage from nature, water containing high loads of bacteria, and locations where the target bacterium is naturally occurring are the most likely to contain the wanted phage. The phages present in water samples from nature usually are of very low concentrations and increasing their numbers through

enrichment make them easier to detect when screening for phages. Phages targeting specific hosts can be isolated by plating the target bacteria on plates, mixed with some of the supernatant from the enrichment mix. If there are phages targeting the bacteria present, they will create visible plaques on the lawn of bacteria, as the phage will lyse the bacteria in a small radius around it.

The first use of phages in a therapeutic context occurred in 1919, when Felix d'Herelle, a microbiologist at the Institute Pasteur in Paris gave a 12-year-old boy with dysentery a phage cocktail, which resulted in a rapid recovery within days for the boy.[43] Despite the potential usefulness of phage therapy, it is not widely approved in the US or Europe for use in the medical field, mainly due to the availability of antibiotics, and more research being needed for it to be commercially safe[44]. There have, however, been some experimental uses of phage therapy, especially in East-Europe,[74] and in the west during emergency cases. As an example, a bacteriophage isolated at Yale university from a local pond was used to treat an 80-year-old man's chest infection in 2016.[45,46]

When compared with antibiotics, phage therapy has some advantages of notice. Some antibiotics are bacteriostatic, i.e., they inhibit growth, as for example: Tetracycline, a commonly used antibiotic in aquaculture. This means that the antibiotic does not kill bacteria completely.[47] This, in a way, may direct the bacteria into an evolutionary path of gaining antibiotics resistance.[52] By using lytic phages, infected bacteria are guaranteed to be eliminated.[52] When the phages eliminate their host, it is performed as a part of their reproduction.[19,17,47] This means that as phages continue to infect and kill their host, there is a form of auto-dosing or renewal of phages in the treated environment.[52] When there is no target bacterium left, the number of phages will decrease, terminating the treatment automatically. In contrast, antibiotics usually degrade through biodegradation, photodegradation or other ways over time, and need to be re-applied to maintain the effect.[48]

Most antibiotics have a wide range of effect, and usually affect a large part of the present microbiota when used for treatment. Due to phages being host specific and consisting mainly of encapsulating proteins and nucleic acid, they are relatively non-toxic and is assumed to not significantly disrupt the normal microbial flora.[49,50,51]

Further, bacterial adaptations for resistance against phage infections sometimes diminishes their virulence, as they might lose pathogenicity-related phage receptors as part of the adaptations.[52,53.54] In addition, some studies have shown that as bacteria gain phage resistance, their sensitivity to antibiotics increased, which would greatly improve treatment when combining both.[55] Phages against many pathogenic bacteria are relatively easy to discover from locations with high bacterial numbers, such as sewers.[47] Unlike antibiotics that remain static compounds, phages are able to adapt to new obstacles presented by their hosts, as there has been an evolutionary arms race between phages and their hosts since they started appearing.[56] This would mean that newly evolved strains of phages could potentially be isolated that are effective against a pathogen, despite their evolved phage-resistance.

There are however some disadvantages and problems with phage therapy. Firstly, not all phages are suitable to be used in phage therapy, and their characteristics need to be uncovered through testing or sequencing. Preferably, they would need to be obligatory lytic, not carry toxic genes, be reasonably stable during storage, and have low potential for transduction. Some phages have low virulence, possibly due to poor replication, poor absorption, or few adaptations to bacterial defenses, meaning application of the phage in therapy would not amount in any significant enough decrease in pathogen activity.[49] Although the specificity of phages is good in some ways, it also makes it very difficult to use it preventatively against general pathogen infections.[18] To use phages in this way would require a “cocktail” consisting of several different phages to be applied in treatment, but they would generally still have a smaller spectrum than a broad spectrum antibiotic.[47]

Despite these disadvantages, their other mentioned properties make bacteriophages a very promising alternative to traditional antibiotics.

Aim of the experiment

The ACMS group has previously tried to study the potential protective effects of the microbiota, but the immersion challenges attempted previously were not successful due to the pathogens trialed being incapable of reproducibly and lethally infecting the fish. The main aim of this study will be to continue the work on studying the protective effects of the microbiota.

The subgoals established to achieve this aim are:

- Establish a reproducible protocol for infection challenge of Atlantic salmon fry. For this project, *Flavobacterium columnare* strains UCD Fc7 and FCO-F2 will be tested as candidate pathogens for an infection challenge with Atlantic Salmon fry.
- After a successful infection challenge is established, the potential protective effect of the microbiota will be investigated through immersion challenges with germ-free and conventionally raised fish.

A second aim will be to test phage therapy as a viable measure to counteract an immersion challenge. The subgoals established to achieve this are:

- Isolating a lytic phage from nature against *F. columnare* strain UCD Fc7 or *F. columnare* strain FCO-F2 if the immersion challenges are successful.
- Examine if treating the fish by adding respective phage post-infection yields increased resistance to the infection.

2. Methods

Bacterial strains

Flavobacterium. sp. TRD was isolated in the ACMS group from eggs received from AquaGen.

The *Flavobacterium columnare* strain UCD Fc7 was kindly supplied by David Pérez-Pascual from the Institute Pasteur in Paris.[20]

Flavobacterium columnare strain FCO-F2 was kindly supplied by Lotta-Riina Sundberg from the University of Jyväskylä .[21]

Janthinobacterium sp. MM5 was isolated in the ACMS group from the rearing water of an aquaculture facility.

Bacterial cultivation

Liquid bacterial cultures were grown by picking single colonies from agar plates into 3 mL of sterile liquid media in 15 mL glass tubes. For subculturing of liquid cultures, 5% (v/v) of an outgrown overnight culture was used as inoculum. All bacteria used were grown at 18°C or room temperature (ca. 22 °C) in incubators with horizontal shaking (120 rpm) under aerobic

conditions. *F*. sp. TRD and *J.* sp. MM5 were grown in Tryptic-soy-broth (TSB, Appendix A.5), or on tryptic soy agar-plates (TSA-plates, Appendix A.5) when solid growth medium was needed. The *F. columnare* strains were grown in TYES (tryptone yeast extract salts, Appendix A.5), or on TYES-plates (Appendix A.5).

Cryostocks were prepared by adding 500 µl of a late exponential phase bacterial culture to 500 µL sterile glycerol (50%) in a 2 mL screw top tube and were stored at -80 °C.

Antibiotic resistance test

The antibiotic resistance of bacterial strains was tested by adding 100 µl late exponential phase bacteria on agar plates and spreading them by adding and shaking sterile glass beads on the plate surface. After plating out the bacteria, 50mm^2 filter papers were placed on the plates. Different antibiotic solutions with different concentrations (20µl; see Table 4) were pipetted on the filter papers, and the plates were incubated at RT until a bacterial lawn became visible. Susceptibility or resistance of the bacterial strains towards the antibiotics used was determined by evaluating the size of inhibition zones on the plate.

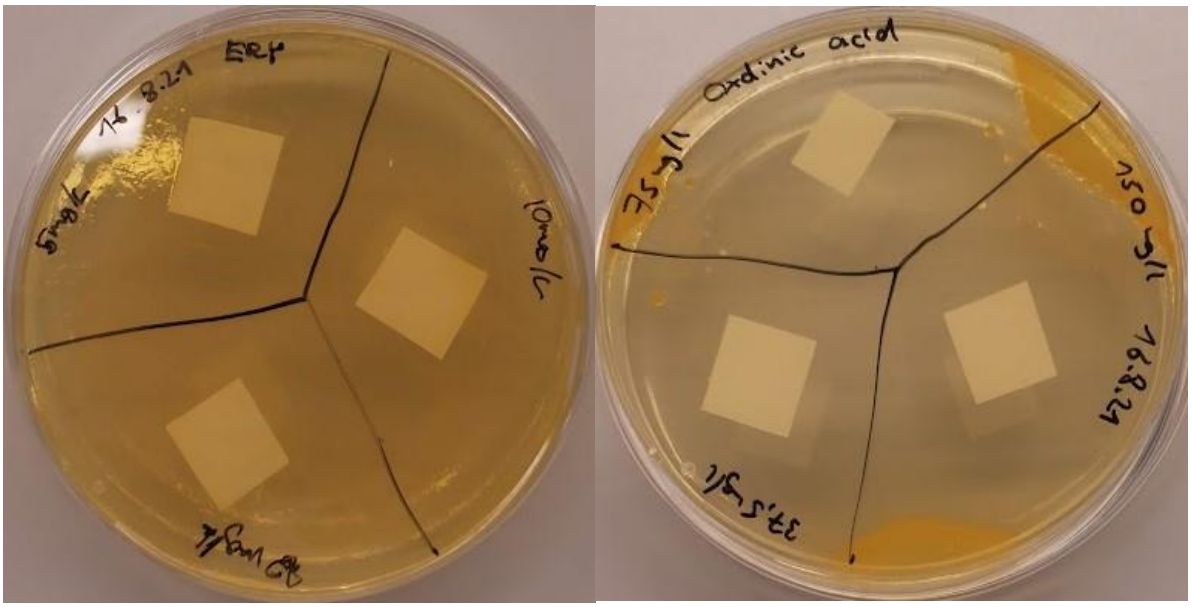


Figure 2: Antibiotics test performed by applying filterpapers with antibiotics solutions onto agar plates newly plated with bacteria. No visible inhibition zones (left) indicated resistance to the antibiotic tested, whereas clear inhibition zones (right) indicated susceptibility of the bacterial strain to the antibiotic.

DNA extraction and partial sequencing of the 16s rRNA gene

Preparation of cell lysate for polymerase chain reaction

Cells were lysed either from bacterial colonies that were picked into 30 µL PCR grade water, or by using outgrown liquid bacterial cultures (500 µL), using the DNeasy® Powersoil® DNA extraction kit (Qiagen) by following the manufacturer's protocol (Appendix B.1). DNA was eluted in PCR-grade water and stored at -20°C.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify the 16S rRNA region in bacterial DNA. An adequate amounts of PCR master mix was prepared following the contents of Table 1. For each sample, 24 µL of master mix was mixed with 1 µL of template (either DNA or a lysate prepared by boiling a bacterial colony from an agar plate in 30 ul water for 10 minutes followed by a quick centrifugation). Eub8F (Table 3) was used as the forward primer, and 1492R (Table 3) as the reverse primer. A T100TM Thermal Cycler (BioRad) was used for PCR amplification, and the program used is listed in Table 2.

Table 1: Contents of PCR master mix for 1 PCR-tube reaction.

Component	Volume [µL]
PCR grade water	17.5
5x Phusion buffer	5
Forward primer (10 µM)	0.375
Reverse primer (10 µM)	0.375
dNTP (10 µM each)	0.625
Phusion hot start DNA polymerase (2 units/µL)	0.1875
Total:	24

Table 2: PCR program for cycling conditions used to amplify DNA-regions from bacterial samples.

Step	Reaction	Temperature [°C]	Time [min:sec]
1	Denaturation	98	1
2*	Denaturation	98	0:15 (x37)

3*	Annealing	55	0:20 (x37)
4*	Elongation	72	0:20 (x37)
5	Final elongation	72	5:00
6	Cooling down	4	1:00
7	Storage	10	∞

*Step 2-4 were repeated 37 times

Table 3: The sequence and target gene for primers used in the experiment

Primer	Sequence	Target gene
Eub8F	5'-AGAGTTTGATCMTGGCTCAG-3' 1	16S rRNA (whole gene)
1492R	5'-GGTTACCTTGTTACGACTT-3'	16S rRNA (whole gene)

Agarose Gel electrophoresis

To examine whether the PCR was successful, agarose gel electrophoresis was used.

The agarose gel consisted of 1 %(w/v) agarose dissolved in 1x TAE buffer (4.84 g Tris base, 1.142 mL glacial acetic acid, 2 mL 0.5 M EDTA pH 8.0, 0.997 L MilliQ H2O). The agarose was dissolved into the TAE buffer by heating in the microwave owen. To visualize amplified DNA in the PCR products, 5 µl of 10,000x GelRed (Biotium) were added to 100 ml agarose gel. The agarose solution was poured into a gel electrophoresis container where it solidified for 15-30 minutes at room temperature. The gel was covered with 1x TAE buffer (Appendix A.6) and wells on the gel were loaded with samples. The samples were prepared by mixing 5 µL of PCR product and 1 µL 6x DNA loading dye (Thermo Scientific). 5 µL of GeneRuler^{TM} 1kb Plus ladder was added in addition to measure DNA lengths of PCR product. The gel was run at 110 V for 45-70 minutes for proper separation of the DNA bands. The gel was illuminated in a UV-light cabinet for evaluation.

Sanger sequencing of 16S rDNA PCR products

After a successful PCR had been confirmed, the PCR products were purified for Sanger sequencing using the QIAquick® PCR Purification Kit (Qiagen) following the manufacturer's protocol (Appendix. B.2). For sequencing, 5 µL of purified PCR product was mixed with 5 µL of

one of the primers (either Eub8F or 1492R, 5 µM). After mixing, the samples were sent to Eurofins Genomics for Sanger sequencing.

Phage work

The phage targeting *F. columnare* strain UCD Fc7 used in this project, called FCL-2, was kindly provided by Lotta-Riina Sundberg from the University of Jyväskylä,[21] who isolated the phage from a Finnish aquaculture facility.[21] It was further tried to isolate lytic phages against *F. columnare* from water samples (described below).

All phages were stored in SM-buffer (Appendix. A.3) at approximately 4°C.

Phage isolation against *F. columnare* Fc7

Water was sampled from the river Nidelva, a salmon hatchery of the company AquaGen, lake Jonsvatnet, the communal wastewater treatment plant of Trondheim at Trolla, as well as from the Trondheimfjord at Leangbukta and the beach Korsvika. Water samples were centrifuged at 5000 xg for 10 min to sediment bacteria and eucaryotes in the sample and the supernatant was used to enrich potential phages.

Potential phages in the natural water samples were enriched by mixing 50 mL of supernatant of sample water with 50 mL of 2X TYES and adding 5% (v/v) of a *F. columnare* Fc7 culture in exponential phase in a sterilized 250 mL Erlenmeyer flask. The mixture was incubated for 1-2 days to let the phages multiply sufficiently. Afterwards, the mixture was centrifuged at 5000 xg for 10 minutes, and the supernatant was filtered through a 0.2 µm membrane filter.

Cultivation for bacteriophage work

In phage experiments, the bacteria were grown on plates with the soft-agar overlay technique. The technique involves growing bacteria inside soft-agar (agar medium with only half the amount of agar compared to the standard) on top of regular agar. It was performed by warming 3 mL of soft-TYES or soft-TSA in 13 mL glass tubes in a heating block at 51°C. While the agar was still approximately 50°C, 1 mL exponential or late exponential phase bacterial culture was added to the tube, and it was vortexed for approximately 3 seconds. Afterwards, the soft-agar mix was poured on top of a room temperate regular agar-plate and left to solidify for 2 minutes. After 1-2 days, a bacterial lawn had grown inside the soft-agar.

Plating and isolation

Plating and isolation of phages was conducted by using the soft-agar overlay method and adding 200 µl of centrifuged enrichment in addition to 1 mL of Fc7 culture in exponential phase in TYES-soft-agar before vortexing and pouring on regular-TYES-plates. The plates were left to solidify for 2 minutes, moved to an incubator at room temperature and the bacteria were left to grow overnight. When bacterial lawns had grown in the soft agar overnight, circular areas with diminished density on the lawn, so-called plaques, were formed from growing bacteriophages targeting the plated bacteria present in the water samples (Figure 3).

To make phage stocks of the plaques, they were picked and suspended SM-buffer (2 mL) in Eppendorf tubes. Plaques of different sizes or transparency may be caused by multiple different strains of phages being present and each was isolated into their own tube. After suspending the plaque in SM-buffer, the tubes were vortexed to disperse the phages in the agar into the SM-buffer. The phage stock was then filtered through a 0.2 µm filter to remove bacteria and other contaminants from the stock. The phage stock was stored at 4-6°C. For longer storage, 50% glycerol with 50% phage suspension was be mixed and frozen at -80°C.

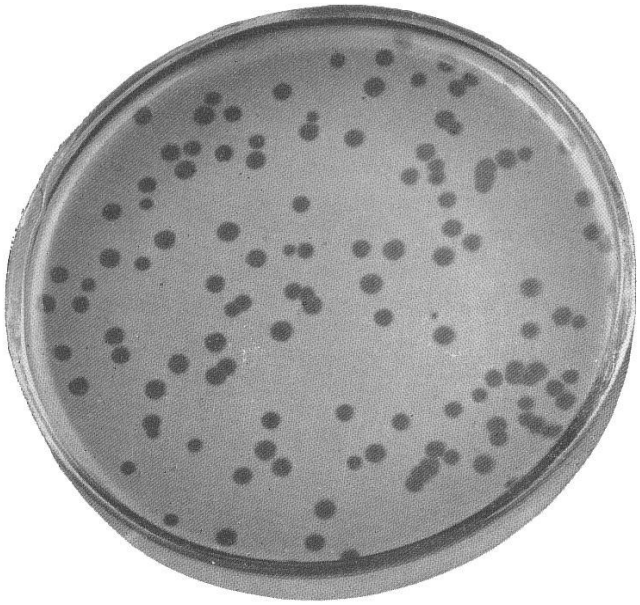


Figure 3: Example of plaque formation a bacterial lawn on plate.[75]

Determining titer

To test the concentration of the phage stock, the so-called titer, a spot test was performed. The soft-agar overlay technique was used to grow target bacteria for the spot test. The phage stock was diluted with SM-buffer in a 10-fold dilution series before being pipetted (5-10 µl) on soft agar plated with the overlay method. The agar plate was divided in 4 to 8 areas, each for 1-2 drops from a dilution. The plates were incubated overnight for the formation of plaques (Figure 4). The plaques were counted, and the plaque forming units per mL (PFU/mL) was calculated.

Figure 4: Titer test. Agar plate with bacterial lawn in soft agar applied with a phage stock in a dilution series, spanning 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}-dilutions. The density of plaques appearing diminishes with dilution. At 10^{-2} and 10^{-3} dilution, the single plaques are countable in this example.

Enrichment of FCL-2

The FCL-2s' virulence was tested against Fc7 and FCO-F2 by applying 5 µL of the phage stock received onto plates of freshly plated bacteria using the described soft-agar overlay method and examining for the formation of plaques on the bacterial lawns.

For enriching FCL-2, the soft-agar overlay technique was used. However, instead of enriched water samples, 100 µl of phage stock with higher phage titer was added, in addition to 1 mL of exponential Fc7 culture in the soft agar before pouring on agar-plates. Once plated and left for 1

day, the soft agar was scraped off and suspended in SM-buffer. For each plate, approximately 3mL of SM-buffer was used. Vacuum-filtration through a 0.2 µm filter was performed to remove bacteria and contaminants. The titer of all phage stocks made were determined and the titer was determined as described above.

Salmo salar experiments

Yolk sac fry of *Salmo salar* (Atlantic salmon) were used in the immersion challenges with pathogens in this project. Salmon eggs were supplied by AquaGen (Kyrksæterøra, Norway). The eggs were 80% developed at arrival at our laboratories and had been sterilized in buffodine solution before being sent.

Sterilization of salmon eggs

At arrival, the eggs were transferred into a dark room with a constant temperature of 6°C ± 0,4 °C (Fish room temperature) and distributed into large petri dishes with a diameter of 140 mm, with approximately 100 eggs in each petri dish. Salmon gnotobiotic medium (SGM; Appendix A.1) was added to each petri dish until the eggs were completely covered. The eggs were left in the petri dishes for 24 hours. The number of bacteria in the SGM in the dishes was estimated by taking water samples and making dilution series of 10-2, 10-3, 10-4 before plating them (100 µl) on TSA-plates with glass beads. The plates were incubated at room temperature for up to two weeks and the colonies that formed were counted.

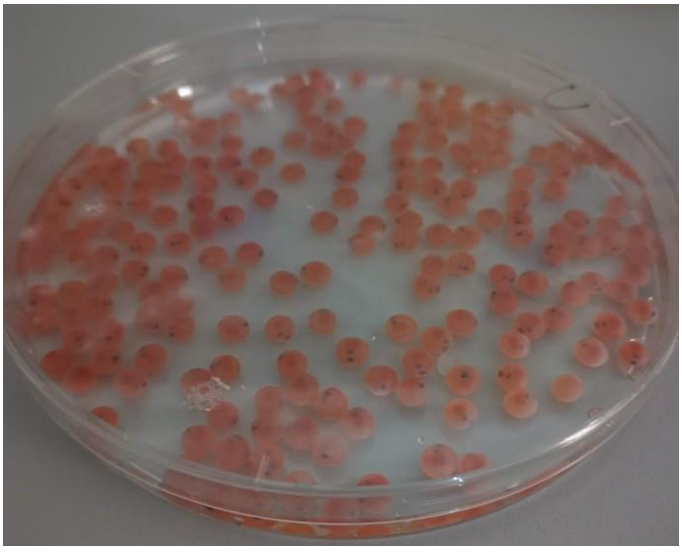


Figure 5: Newly received salmon eggs submerged in SGM in large petri dish.

Sterilization protocol

After the newly received eggs had been immersed in SGM for 24 hours, the SGM was removed with a 50mL serological pipette, and the eggs were treated with an SGM-antibiotics cocktail (80 mL) composed of the antibiotics listed in table 4.

Table 4: Contents of antibiotics cocktail used in the standard sterilization procedure of fish eggs.

Antibiotic	Working concentration
Rifampicin	10 mg/l
Erythromicin	10 mg/l
Kanamycin	10 mg/l
Ampicillin	100 mg/
Amphotericin B	250 ug/l
Penicillin	150 mg/l
Oxolinic acid	75 mg/l

* For some experiments (as indicated with the results), the amount of oxolinic acid was ten times higher (750 mg/l)

The eggs were incubated in the antibiotic cocktail for 24 h at FRT. The eggs were taken out of the antibiotic solution and 17 eggs were placed in a sterile 50 ml falcon tube. A diluted buffodine solution (100 mg/L) was added to the eggs and the eggs were incubated in the iodine solution for 30 min at FRT. After 30 min, the iodine solution was removed, and the eggs were washed 4 times with ~40 ml of SGM. Afterwards, the eggs were placed in 250 ml cell culture flasks filled with 100 ml sterile SGM.

Rearing conditions

The eggs and hatchlings were kept in the 250 mL cell culture flasks for the remainder of the experiments, and the hatching rates of eggs in each flask was recorded during rearing. The hatching day was defined as the day when 70% of all eggs have successfully hatched. All unhatched eggs were removed at 7 dph (days post hatching) In order to maintain good water quality, 60 mL of the rearing water in each flask was replaced 3 times a week with new sterile SGM. Deceased fish were removed with a serological pipette immediately upon detection. One

week before the bacterial challenge, the number of fish was adjusted to 15 fish per flask by removing fish with a sterile pipette

For some hatched fish the temperature in the room was gradually increased to 10 and 14 °C, respectively, one week before immersion challenge (explained further below). The timing for bacterial challenge varied between 20- and 54-days post hatching (dph). All handling of the fish, e.g during SGM-change, was performed under sterile conditions in a laminar flow cabinet with ethanol-washed and UV-irradiated equipment.

Figure 6: Salmon eggs placed in 250 mL cell culture flasks after sterilization protocol.

Conventionalization

In some of the immersion challenge experiments, some of the fish were conventionalized (colonized by commensal bacteria) at 7 dph. This was performed during the regular water change, but instead of adding SGM, untreated water from lake Jonsvatnet, Trondheim, was added to inoculate the fish with the natural lake microbiota. The water was received from

Vikelvdalen water treatment facility in Trondheim. After conventionalization, the flasks had their water changed with SGM upon the water exchanges.

Sterility Check

Sterility checks were performed for flasks with germfree eggs on the day of hatching, before conventionalization, and before a bacterial challenge. Flasks that were contaminated pre-conventionalization were conventionalized. The sterility check was performed in 3 ways; by transferring 100 µl from each germ-free flask into four different types of liquid media (nutrient broth, brain heart infusion, sabouraud-2% dextrose broth and glucose yeast extract broth, Appendix A.4), by plating 100 µl on TSA, and sometimes TYES-plates. Positive controls were added from conventionalized flasks or bacteria cultures. Liquid media and agar plates were incubated at both room temperature and fish room temperature, and regularly examined for bacterial growth over 2-4 weeks.

Immersion challenge

The immersion challenges of the fish were performed three to six weeks post hatching. Fresh cultures of Fc7 or FCO-F2 in late exponential phase, grown overnight in TYES-medium, were used for the challenges. The bacteria cultures were measured and adjusted to have an OD of approximately 1, which roughly equals a CFU of 10^9/mL. The bacterial cells were washed once with SGM by centrifuging the bacteria and resuspending them in SGM. For the immersion challenge, 1 mL of bacteria resuspended in SGM was added into each fish flask after water change. The final concentration of pathogenic bacteria in the fish flask was approximately 10^7 CFU/mL. Some flasks were exposed to a fish commensal strain (*J.* sp. MM5), which was prepared the same way as the *F. columnare* strains. During the immersion challenge, the flasks were kept in a horizontal position to increase oxygen diffusion. They were examined 1-2 times daily for deaths occurring in the flasks, which were recorded, and deceased fish were removed immediately. After the immersion challenge experiment was finished, the salmon fry were euthanized by submerging them in a tricane solution (5,2 g/l, Appendix A.2).

Testing new sterilization protocol on *Flavobacterium. sp. TRD*

A new sterilization antibiotics cocktail in the sterilization protocol was developed after antibiotics resistance tests were conducted on a contaminant which survived sterilization (*F*. sp. TRD). The new protocol was tested on this contaminant to measure it's efficiency.

Approximately 500 eggs were received and distributed in 5 petri dishes. Two of the dishes were added 1 mL of an OD 1 *F.* sp. TRD culture in late exponential phase. Two dishes were left untreated, and the eggs in the last petri dish were immersed in Jonsvatnet-water instead of SGM. One of the petri dishes that received the TRD strain, and one untreated petri dish was treated with the 10X oxolinic acid antibiotics cocktail. The rest of the dishes were treated with the normal antibiotic cocktail (Table 4). Afterwards, the eggs were sterilized as described above. CFUs in the rearing water were measured in all petri dishes by plating out 100 µl on TYES and TSA plates. The CFUs on the eggs' surfaces was also measured by moving an egg immersed in the SGM and rolling in on a TSA-plate. The measurements of CFUs were conducted immediately after the eggs were immersed in SGM, before antibiotics treatment, and before iodine treatment.

Immersion challenge experiments

Challenge experiment 1: Establishing optimal temperature for immersion challenge with *F. columnare* Fc7

The aim of the experiment was examining if *F. columnare* Fc7 was suitable for use in immersion challenges with yolk sac fry by inducing lethal infections in the fish. Further, it was tested if the virulence of Fc7 could be increased conducting the immersion challenge at higher temperature, making the bacterium more active and exposing the fish to thermal stress.

In the experiment, 550 eggs were sterilized and distributed into cell culture flasks as described above. The flasks were challenged with Fc7 at 42 dph. For that germ-free (GF) flasks, containing 15 fish each, were divided into three groups, where they would be challenged at different temperatures, 6°C, 10°C, and 14°C. Each temperature group contained three flasks that were challenged with Fc7, with a final concentration of approximately 10^7 CFU/mL in the rearing water, as described in the infection protocol, one flask exposed to *J.* sp. MM5 (10^7 CFU/mL final concentration), and one uninfected flasks. For the groups challenged at higher temperatures than 6°C, the temperature was gradually increased from 35 dph on until the immersion challenge at 42

dph. Water changes were performed as usual. The immersion challenge was concluded 11 dpi (days post infection, 53 dph).

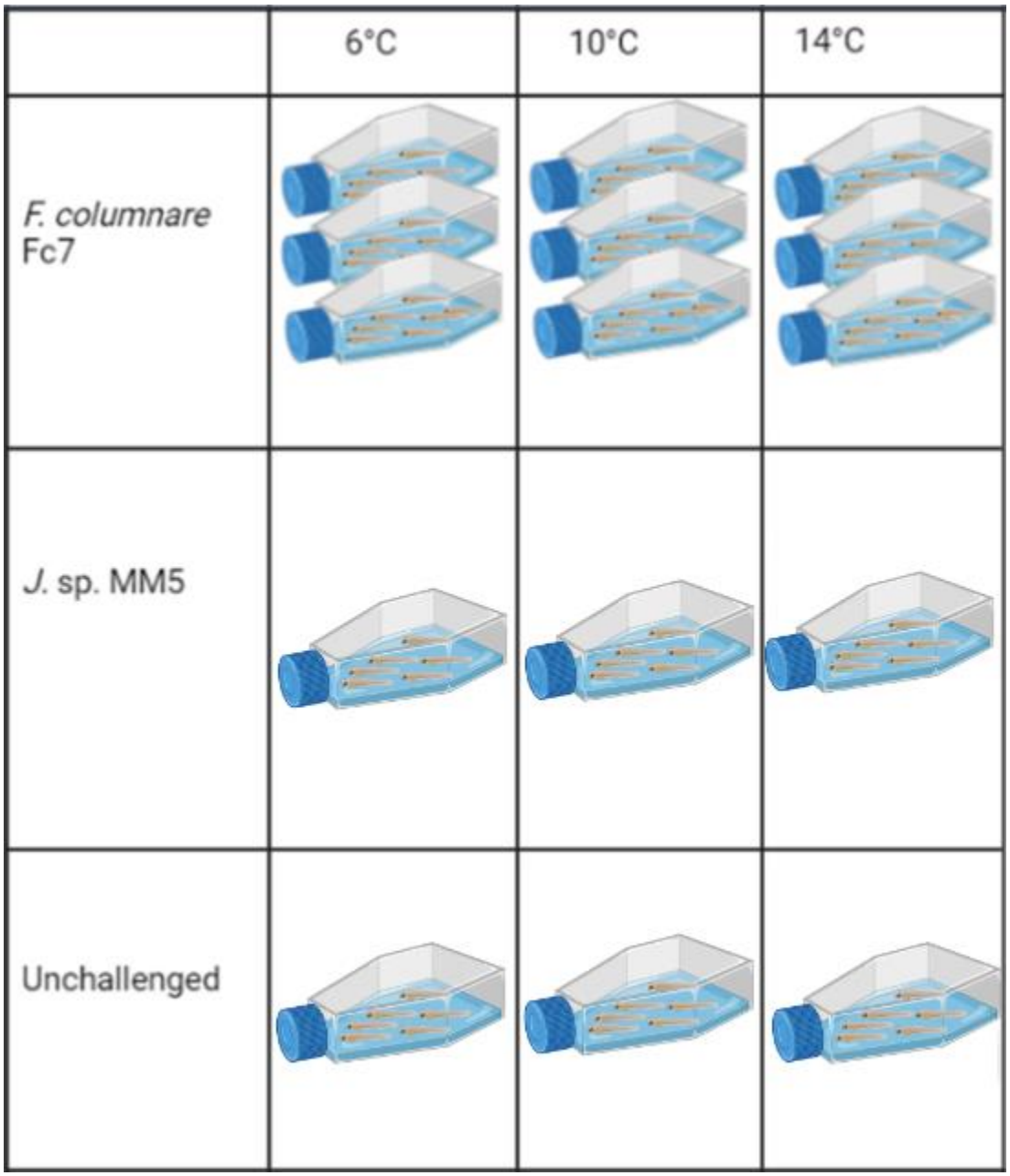


Figure 7: Experimental design of Challenge experiment 1. Triplicate germ-free fish flasks were challenged with *F. columnare* Fc7 at 6°C, 10°C, and 14°C 42 dph. Additional triplicates of flasks were added *Janthinobacterium* sp. MM5 or left unchallenged at the same temperatures at the challenged flasks.

Challenge exp 2: Examining commensal microbiome with earlier immersion challenge and lower infection dose of *F. columnare* Fc7

After establishing that immersion challenges with *F. columnare* Fc7 were viable at 10°C, it was examined whether the microbiota had a protective effect against pathogenic attacks. In addition, it was also tested whether the infection dosage of *F. columnare* Fc7 during the challenge could be decreased to slow the rate of deaths, and if challenging the salmon fry earlier (at 20 dph) was as effective as in Challenge experiment 1.

For the second immersion challenge experiment, 500 eggs were used. They were sterilized, distributed in flasks, sterility checked, and conventionalized like in challenge experiment 1. The fish were reared at 6°C until infection at 20 dph. The number of fish per flask was adjusted to 15 fish in each flask at 13 dph. All flasks were kept at 10°C during the experiment, and the fish were acclimated to the temperature from day 13 post hatching. Both the germ-free and CVZ flasks were tested with identical treatments. Both groups contained 12 flasks. In each group, the flasks were split into four treatments (three flasks per treatment): High concentration of *F. columnare* Fc7 infection (10^7 CFU/mL), low concentration *F. columnare* Fc7 infection (10^6 CFU/mL) exposed to the non-pathogenic *J.* sp. MM5 (10^7 CFU/mL), or uninfected. The fish were examined 1-2 times daily for deaths occurring in the flasks, and deceased fish were removed immediately. Water changes were performed as usual. The immersion challenge was concluded at 10 dpi.

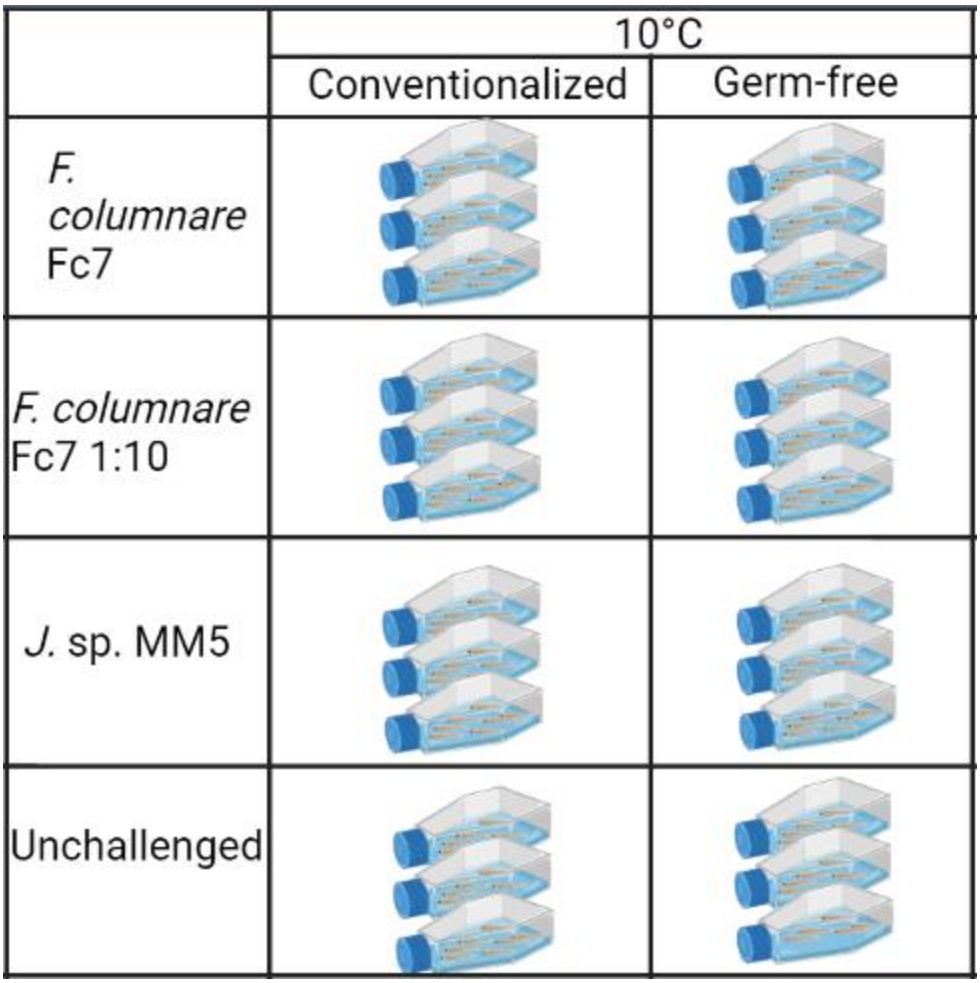


Figure 8: Experimental design of Challenge experiment 2. Triplicate germ-free and conventionalized fish flasks were challenged with *F. columnare* Fc7 (either 10^7 or 10^6 CFU/mL final concentration) at 10°C 20 dph. Additional triplicates of fish flasks were added *Janthinobacterium* sp. MM5 or left unchallenged.

Challenge exp 3: Testing immersion challenge with *F. columnare* FCO-F2 and Fc7

A new attempt at examining the protective effects of the microbiota was conducted by challenging salmon fry with *F. columnare* Fc7 and a newly received pathogen, *F. columnare* FCO-F2.

Like previously, 500 eggs were reared, sterilized, distributed in flasks, sterility checked, and conventionalized in accordance with the protocol previously explained. Flasks were redistributed to contain 15 fish in each before the infection challenge at 41 dph.

The fish were challenged with FCO-F2 and Fc7. Both the germ-free and CVZ flasks were tested with identical treatments. Both groups contained 12 flasks. In each group, the flasks were split into four treatments (three flasks per treatment): Fc7 infection with 10^7 CFU/mL in the rearing water, FCO-F2 infection with 10^7 CFU/mL in the rearing water, exposed to the non-pathogenic *J*. sp. MM5 (10^7 CFU/mL in the rearing water), and uninfected. All flasks were kept at 10°C during the experiment, and the fish were acclimated to the temperature from 34 dph to 41 dph. The fish were examined 1-2 times daily for deaths occurring in the flasks, and deceased fish were removed immediately. The immersion challenge was concluded at 10 dpi.

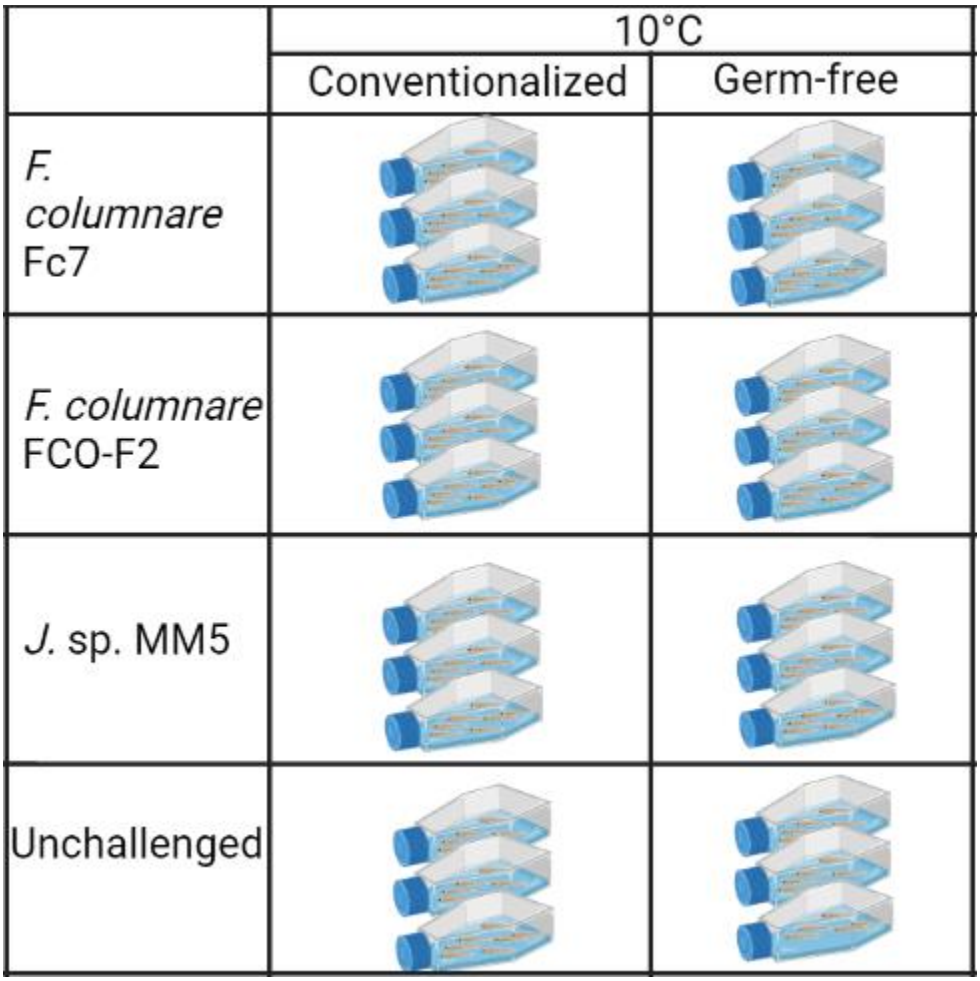


Figure 9: Experimental design of Challenge experiment 3. Triplicate germ-free and conventionalized fish flasks were challenged with *F. columnare* strains Fc7 or FCO-F2 at 10°C 41 dph. Additional triplicates of fish flasks were added *Janthinobacterium* sp. MM5 or left unchallenged.

Challenge exp 4: Fc7 immersion challenge with *F. columnare* Fc7 with FCL-2 phage- and antibiotic treatment

In this experiment the viability of phage therapy (using bacteriophage FCL-2) to protect the salmon fry against a *F. columnare* Fc7 immersion challenge was examined and compared to antibiotics treatment with oxytetracycline.

Approximately 800 eggs were received and distributed in 57 flasks. Like previously, the eggs were reared, sterilized, sterility checked, and conventionalized in accordance with the protocol explained previously. Flasks were redistributed to contain 15 fish in each before the infection challenge at 44 dph.

Equal numbers of germ-free and CVZ flasks were tested with identical treatments. Each of the groups were divided into two subgroups, uninfected and challenged with Fc7, with 9 flasks in each. Each subgroup was subjected to 3 different treatments (three flasks per treatment): FCL-2 phage-treated, oxytetracycline-treated (40 mg/l), or negative control. All flasks were kept at 10°C during the experiment, and the fish were acclimated to the temperature from 37 dph to 44 dph.

The flasks were infected with 10^7 CFU/mL Fc7 in the rearing water after water change. The flasks with phage treatment were added 100 µl of FCL-2 phage stock with $4*10^9$ PFU/mL, with a final phage-concentration of 10^6 PFU/mL in the flask. The flasks treated with oxytetracycline were added 10 mL of 0,4 g/L from an oxytetracycline stock, giving a flask concentration of 0,4 g/L. Both the phage and oxytetracycline were added daily. The fish were examined 1-2 times daily for deaths occurring in the flasks, and deceased fish were removed immediately.

For experiments not discussed in this thesis, fish from the conventionalized group were removed gradually for microbiome sampling and analysis. The fish were challenged at 10°C for 1-10 dpi. The temperature was later increased to 14°C 10-14 dpi for the remaining GF fish. The fish were examined 1-2 times daily for deaths occurring in the flasks, and deceased fish were removed immediately.

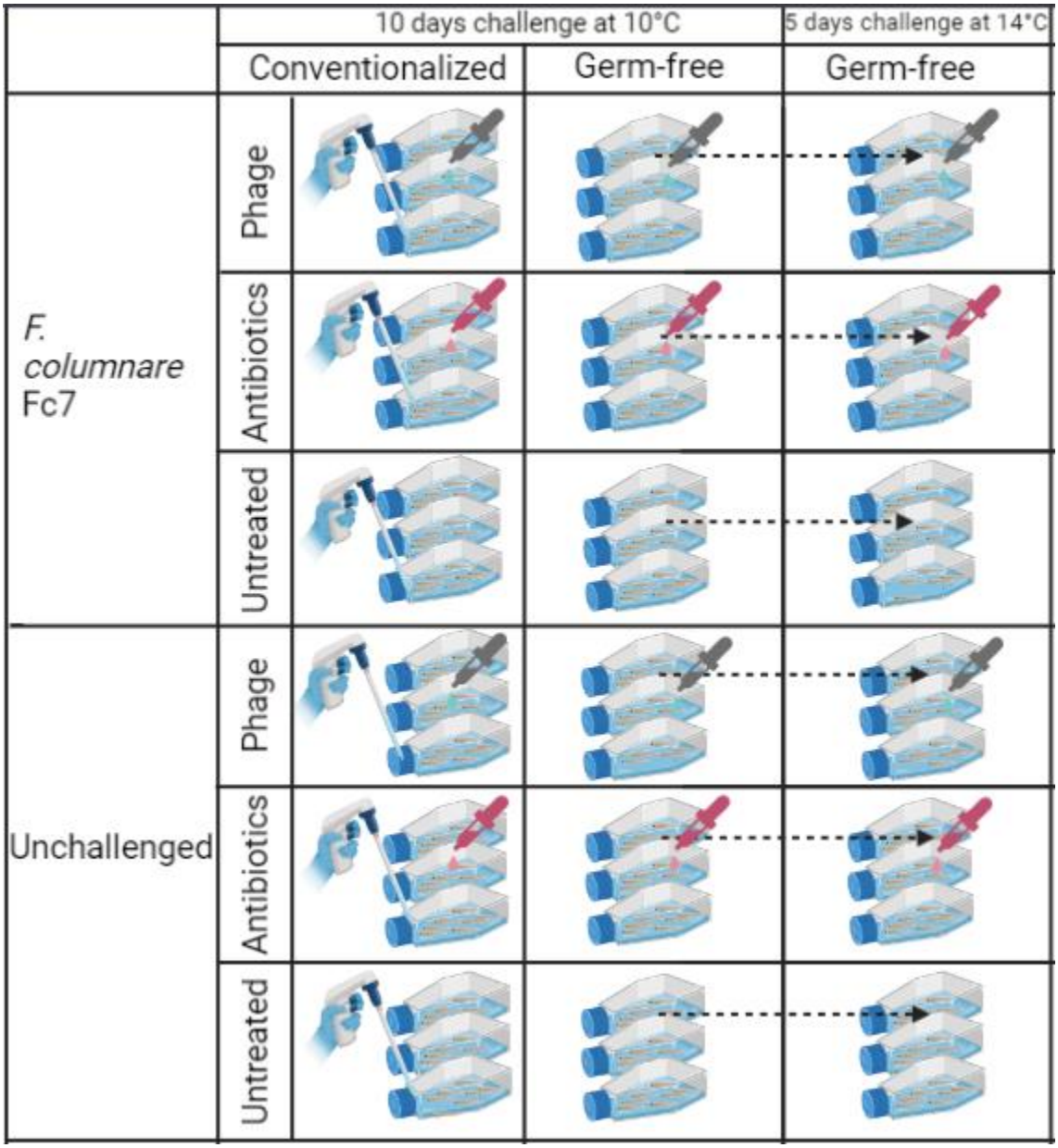


Figure 10: Experimental design of Challenge experiment 4. Germ-free and conventionalized fish flasks were challenged with *F. columnare* Fc7 at 10°C 44 dph. Additionally, some fish flasks were left unchallenged. Triplicate germ-free and conventionalized flasks (both challenged and unchallenged) were treated with FCL-2 (bacteriophage treatment), oxytetracycline (antibiotics treatment), or left untreated. Conventionalized fish were removed for sampling 2-3 times a week for an unrelated project (3 fish removed at a time per flask). After 10 days into the challenge, all conventionalized fish had been removed for sampling, and the temperature was increased to 14°C for the remaining germ-free fish.

Challenge exp 5: Immersion challenge with *F. columnare* Fc7 at 14°C

An immersion challenge with *F. columnare* Fc7 on salmon fry was conducted at 14°C due to low mortality observed in previous Challenge experiments with *F. columnare* Fc7 at 10°C.

This experiment was conducted by using the uninfected and untreated germ-free- and CVZ fish flasks from fish experiment 3 and 4 after their experiments were concluded. The fish were then at 54 dph. The germ-free group consisted of 4 fish flasks challenged with *F. columnare* Fc7 (10^7 CFU/mL) and 2 flasks were exposed to the non-pathogenic *J.* sp. MM5 (10^7 CFU/mL) All CVZ flasks, 3 in total, were also infected with 10^7 CFU/mL in the rearing water. The experiment was conducted at 14°C for 4 dpi. The fish were examined 1-2 times daily for deaths occurring in the flasks, and deceased fish were removed immediately.

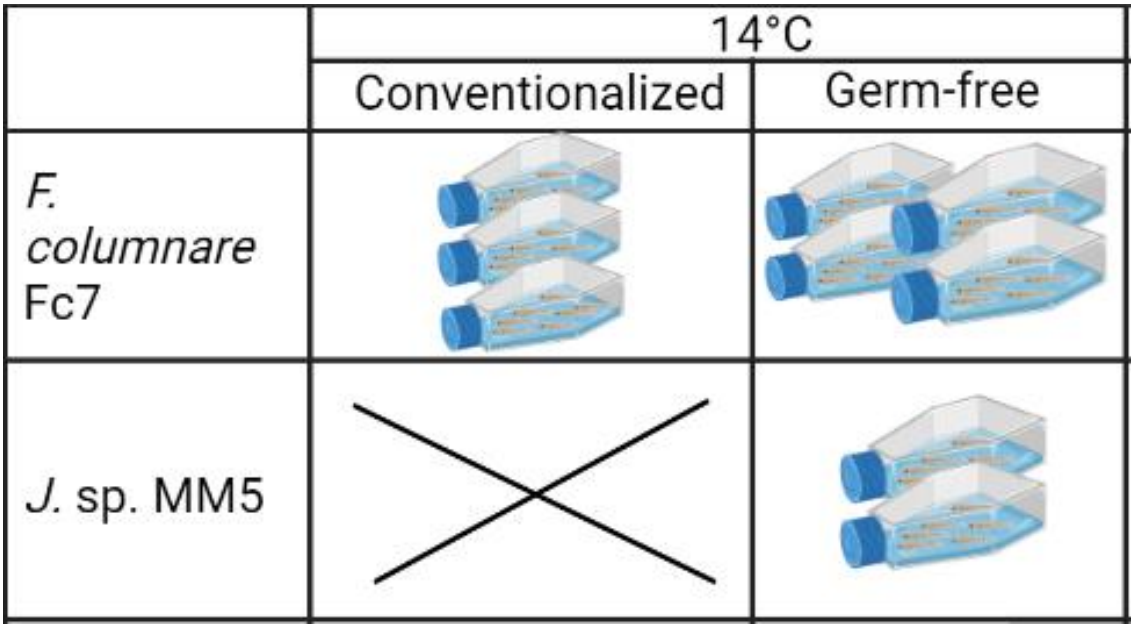


Figure 11: Experimental design of Challenge experiment 5. Four germ-free and three conventionalized fish flasks were challenged with *F. columnare* strains Fc7 at 14°C 54 dph. Additional two germ-free fish flasks were added *Janthinobacterium* sp. MM5.

3. Results:

1. *F.* sp. TRD

In some of the experiments conducted within this thesis, and additionally in previous experiments conducted in the ACMS group, a contaminant was repeatedly observed in germfree flasks. The bacterium was isolated, DNA was extracted and its 16S rRNA gene was sequenced. By using rdpi classifier v. 18,[67] the strain was determined to be a *Flavobacterium sp.*, and by consequently using a sequence matching tool in RDP,[67] the isolated bacterium found was assumed to not represent any existing *Flavobacterium* type strains, and the bacterium was termed *Flavobacterium*. sp. TRD

It was assumed that the *Flavobacterium* contaminant was present on the salmon eggs and was able to survive the sterilization protocol of the salmon eggs used. However, for the subsequent experiments with germ-free fish, it was critical to have completely axenic fish to compare the lethality of germ-free fish and conventionalized fish during immersion challenges. *F.* sp. TRD could potentially survive the sterilization and become a contaminate in fish flasks, which would compromise the following experiments. It was therefore investigated whether *F*. sp. TRD was indeed able to survive the standard derivation procedure and how this procedure needed to be adapted to eliminate it. Therefore, the antibiotic's resistance of the *F. sp.* TRD was characterized.

Antibiotics resistance test of *Flavobacterium.* sp. TRD

The resistance of *F. sp. TRD* against the antibiotics used in the sterilization was tested by examining growth on agar plates in the presence of antibiotics.

The test showed that *F.* sp. TRD had a high degree of resistance to many of the antibiotics tested on. Rifampicin, penicillin, and erythromycin had a very small inhibitory effect, being notable only at 20 mg/l (2x the working concentration) (Table 6). The rest of the antibiotics, kanamycin, ampicillin, amphotericin B, showed no inhibitory effect on *F*. sp. TRD on any of the applied concentrations. A large inhibition of growth occurred only when the bacteria was exposed to oxolinic acid at all concentrations (Table 6 and Figure 12). This implied that the oxolinic acid was the only antibiotic in the cocktail that could inhibit *F*. sp. TRD but was currently applied at too low concentrations to fully neutralize the contaminant.

Table 6: Antibiotics test of *F*. sp. TRD with different antibiotics at different concentrations. Concentrations of antibiotics used were relative to concentrations in antibiotics cocktail used in sterilization protocol listed in table 4 (at half, regular, and double the working concentrations). Filter papers soaked in antibiotics solution were applied onto agar plates with newly plated F.sp TRD and the levels of inhibition were measured by evaluating the inhibition zone of growth; no inhibition -, noticeable inhibition +, very inhibitory ++.

Antibiotic	½ x working concentration	1 x working concentration	2x working concentration
Rifampicin	-	-	+
Erythromycin	-	-	+
Kanamycin	-	-	-
Ampicillin	-	-	-
Amphotericin B	-	-	-
Penicillin	-	-	+
Oxolinic Acid	++	++	++

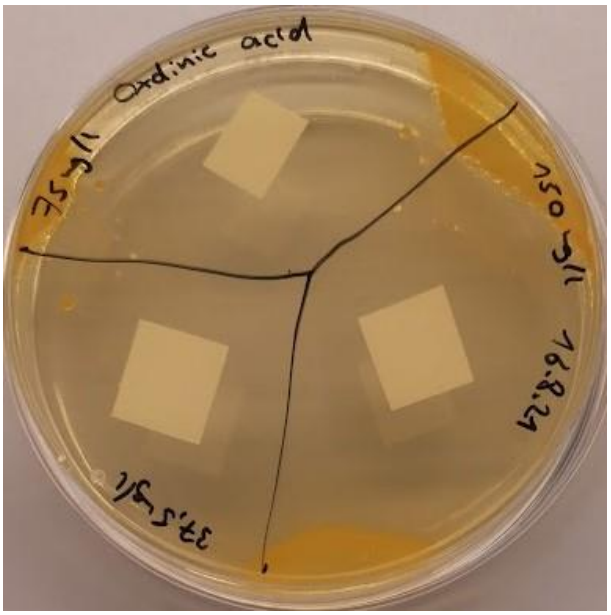


Figure 12: Antibiotics test of *F*.TRD with oxolinic acid. Inhibition of *F*.sp.TRD growth on TSA-plate with oxolinic acid (20µl) applied onto filter papers with concentrations of 37,5 mg/l, 75 mg/l and 150 mg/l.

Test of modified sterilization protocol against *F. sp. TRD*

The antibiotics test showed that *F*. sp. TRD was resistant against 5 of the 6 antibiotics used during the sterilization (for the concentrations used during sterilization). We therefore assumed that the contaminant might be able to survive the sterilization procedure and therefore wanted to test whether increasing the concentration of the only antibiotic that the strain was susceptible towards (oxolinic acid) could help in making the sterilization more effective against *F*. sp. TRD. Therefore, 500 eggs were ordered from Aquagen and evenly distributed into four petri dishes (100 eggs each; see also Material and Methods). To two dishes late exponential phase F. sp. TRD was added, and two dishes were left without addition of *F*. sp. TRD. One of each *F*. sp. TRD treated and untreated petri dish was treated with the standard antibiotics cocktail (Table 4), whilst the other was treated with the modified cocktail with increased oxolinic acid (10x of the normal concentration). In order to follow the number of culturable bacteria in the groups, CFU analysis was used to estimate the density of culturable bacteria in both the water and on the eggs' surfaces at arrival, before addition of the antibiotics and before iodine treatment (24 h after the antibiotics were added see; sterilization protocol in methods p.x).

Table 7: CFU counts of the rearing water during the test of a modified sterilization protocol. Triplicate CFU counts of *F*.sp.TRD were performed in each petri dish measured right after addition of *F*. sp. TRD, before antibiotics addition (24 h after F. sp. TRD addition), and 24 h after antibiotics treatment. The mean CFU/mL calculated from the triplicates for each dish is reported for each sampling timepoint with standard deviations.

	CFU/ml		
Treatment	**Initial challenge**	**Before AB addition**	**After addition**
Control modified treatment	0 (± 0)	0 (± 0)	0 (± 0)
Control standard treatment	0 (± 0)	0 (± 0)	0 (± 0)
F. sp. TRD added modified treatment	363 (± 38,00)	23500 (± 12735)	0 (± 0)
F.s p. TRD added standard treatment	360 (± 64,81)	20900 (± 14100)	33 (± 41)

The CFU analysis of rearing water (Table 7), showed there were little bacteria in the control group at arrival, and that the rearing water of the groups challenged with *F*. sp. TRD contained approximately 360 CFU/ml after addition of *F*. sp. TRD. As no bacteria were detected in the CFU analysis of the eggs in the control group, it was assumed that all or at least most of these CFUs represent *F*. sp. TRD. One day after addition of *F*. sp. TRD in the SGM, and prior to antibiotic treatment, the CFUs in both the challenged groups had increased to ~ $2{,}2*10^4$ CFU/mL, while the controls still had no colonies. After the antibiotic treatment, the CFUs in the rearing water had decreased drastically in both *F*. sp. TRD challenged groups. There were no remaining bacteria in the *F*. sp. TRD challenged group's rearing water treated with the modified antibiotics cocktail, whilst the rearing water of the *F*. sp. TRD challenged group treated with the standard antibiotics cocktail still had *F*. sp. TRD present, with a bacterial density of 33 CFU/ml.

Table 8: CFU counts on the salmon eggs during the test of a modified sterilization protocol. The CFUs per egg were determined before addition of *F*. sp. TRD, before antibiotics addition (24 h after *F*. sp TRD addition), and 24 h after antibiotics treatment. On some TSA-plates, the bacterial density was too high to be countable, and are listed as >1000. Five eggs were counted per group and sampling timepoint. Average CFU numbers with standard deviations are reported.

	CFU		
Treatment	**Before challenge**	**Before AB addition**	**After AB addition**
Control modified treatment	0 (± 0)	0 (± 0)	0 (± 0)
Control standard treatment	0 (± 0)	0 (± 0)	0 (± 0)
F.sp.TRD modified treatment	0 (± 0)	>1000	0 (± 0)
F.sp.TRD standard treatment	0 (± 0)	>1000	40,7 (± 11,6)

The number of CFUs per egg were determined by rolling the eggs on TSA plates. Initially, there were no culturable bacteria on the egg's surface. After 24 h, the *Flavobacterium* added to the rearing water had colonized the eggs, while no culturable bacteria were observed in the group where no additional bacteria were added. After the antibiotic's treatment, eggs treated with the

modified antibiotics cocktail containing more oxolinic acid had more culturable bacteria on their surfaces, whilst the eggs treated with the standard antibiotics cocktail still had bacteria present on their surfaces. As no CFU were observed in the group where *F*. sp. TRD was not added, it is assumed that all CFUs present on the eggs were strain *F*. sp. TRD (this was also supported by the morphologies of the colonies).

The CFU analysis of both the rearing water and egg surfaces before and after sterilization with antibiotics showed that the *F*. sp. TRD contaminant indeed can survive antibiotic treatment of the standard sterilization protocol as hypothesized but can be combated by using an antibiotics cocktail with 10 times higher concentration of oxolinic acid (750 mg/l) compared to the standard.

The standard iodine treatment was performed for all eggs and the sterility of the rearing flasks was determined at hatching day (see also Material and Methods). Four contaminated flasks (of 27 flasks in total) were identified during the sterility checks (Appendix D). Three flasks of the flasks without *F*. sp. TRD addition were contaminated (three with standard sterilization, zero with modified sterilization) and one flask from the eggs to which *F*. sp. TRD was added (only with standard sterilization). The hatchability of eggs (the rate that eggs hatched) was recorded for each flask and group (Appendix E), and there were no noticeable differences in hatching success between the differently treated eggs.

2. Bacteriophage isolation

During the project, attempts were made to isolate a bacteriophage with *F. columnare* Fc7 as target host for use in experiments testing bacteriophage therapy as protection against an infection. Water was sampled from Nidelva, AquaGen salmon hatchery rearing water, lake Jonsvatnet, the communal wastewater treatment plant of Trondheim at Trolla, Trondheimfjord at Leangbukta and the beach Korsvika to try isolating a phage (see Phage isolation against *F. columnare* in MM). We were unsuccessful in isolating a bacteriophage against Fc7 in the project. Instead, a bacteriophage targeting *F. columnare* Fc7, called FCL-2, was kindly provided by associate professor Lotta-Riina Sundberg from the University of Jyväskylä. The phage was isolated from a Finnish aquaculture facility in 2008.[76]

The bacteriophage was confirmed to be effective against strain Fc7 and was later used in an immersion challenge experiment with of salmon yolk sac fry.

Fish immersion challenge experiments

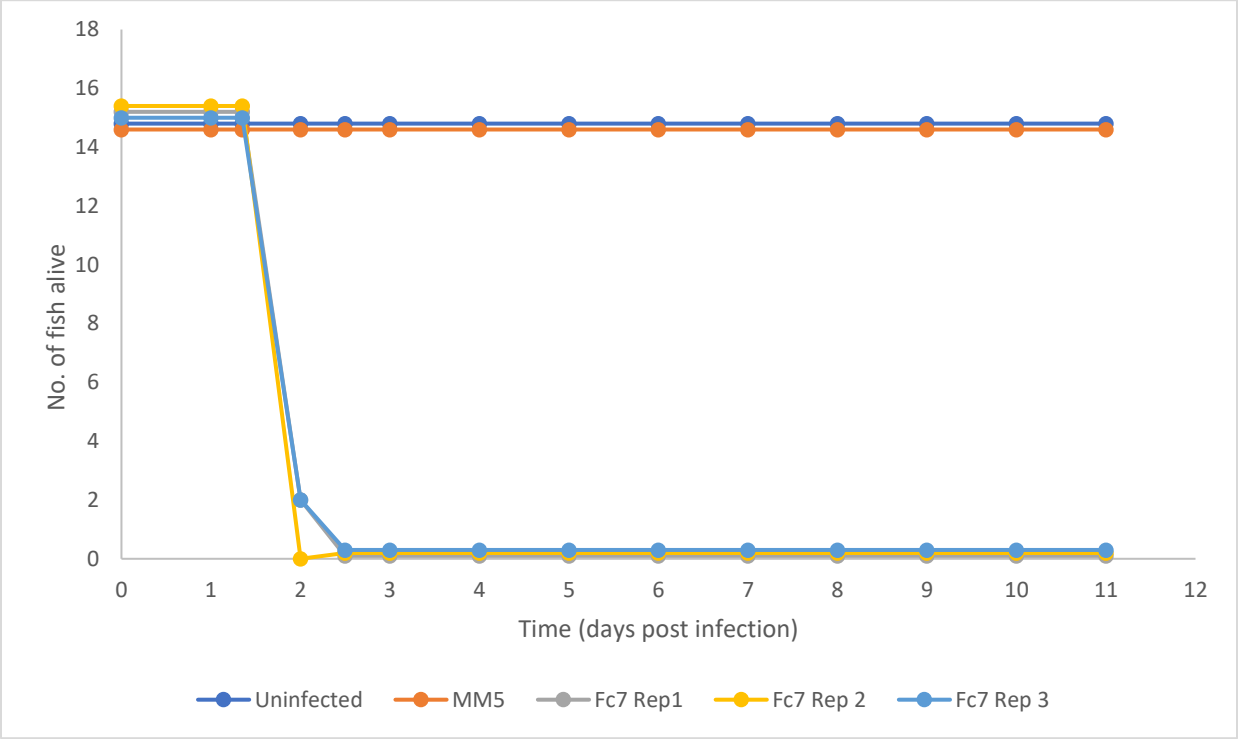
One of the aims of this thesis was studying the protective effects of the microbiota against bacterial infections of the fish host. To achieve this, the strategy was to challenge both axenic *Salmo salar* yolk sac fry, and fry with a commensal microbiome, to observe for differences in survival between the two groups. A critical part required for the experiment was a pathogen for *Salmo salar* fry with observable lethality. Previous experiments conducted were unsuccessful in establishing an infection protocol using the pathogens *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Aeromonas salmonicida*.[22,57] In this study, a new pathogen, *Flavobacterium columnare* strain Fc7 was used.

Challenge exp 1: Establishing optimal temperature for immersion challenge with *F. columnare* Fc7

The first fish challenge experiment was designed to test the potential of *F. columnare* Fc7 to be used in an immersion challenge with salmon yolk sac fry. It was tested if the infection success of *F. columnare* Fc7 could be improved by increasing the incubation temperature during the immersion challenge. The goal of the experiment was testing the deadliness of *F. columnare* Fc7 in an immersion challenge on germ-free salmon fry at the minimum (6°C), intermediate (10°C), and highest (14°C) temperatures.

The yolk sac fry were exposed to the *F. columnare* Fc7 pathogen at 42 dph by adding *F. columnare* Fc7 (final concentration 10^7 CFU/mL) to the rearing water in the fish flasks. The flasks were kept at either 6°C, 10°C or 14°C. For controls, some fish-flasks were infected with the non-pathogenic bacteria *Janthinobacterium* strain MM5, whilst some were kept germ-free during the experiment. All temperature groups contained three Fc7-infected flasks, one *J.* sp. MM5-added flasks, and one uninfected flasks. The survivability of each flask is reported in Figure 13.

(a)



(b)

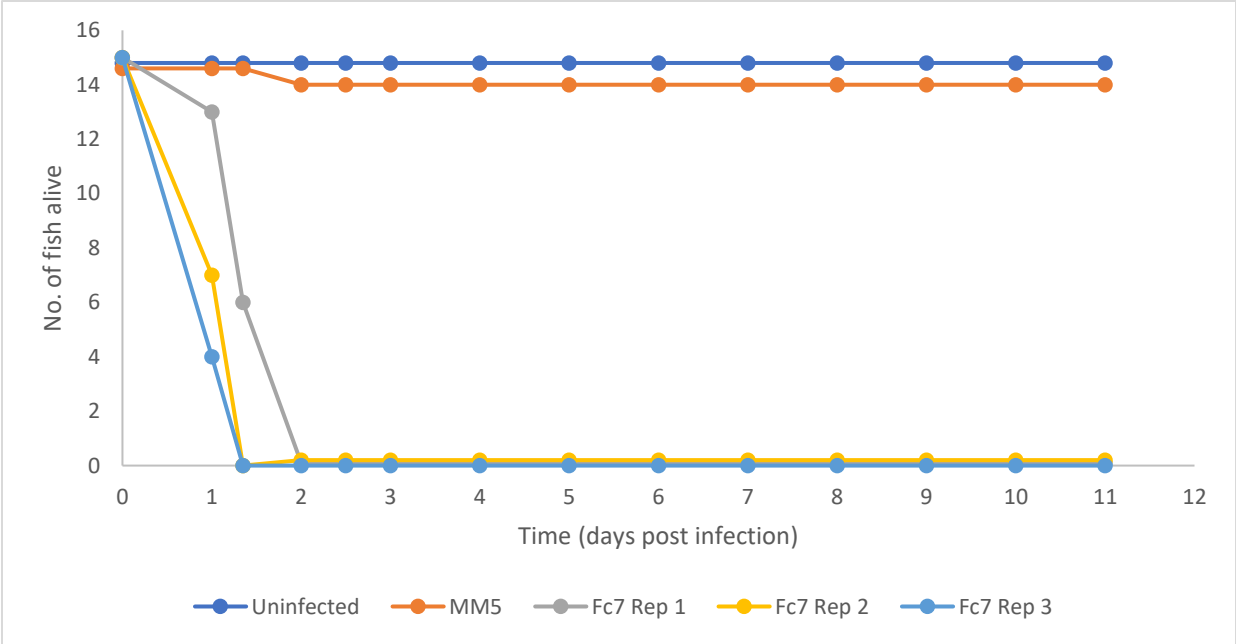


Figure 13: Survival of the yolk sac fry after challenging with F. columnare Fc7 at three different temperatures. All flasks started with 15 fish in each and each, and for each flask the survival is shown. (a) shows the number of surviving fish in each flask at 10°C, while (b) shows the surviving fish at 14°C. No deaths were observed at 6℃ (not shown).

The fish flasks incubated at 6°C experienced no lethality in neither the *F. columnare* Fc7 challenged flasks nor the controls. Of the flasks incubated at 10°C, those challenged with *F. columnare* Fc7 were observed to have a decline in surviving fish, starting 2 days post infection (dpi) (Figure 13, a). After 2,5 dpi, all individuals in the three *F. columnare* Fc7 infected replicate flasks had died, while no mortalities were observed in the controls.

Also at 14°C, deaths were observed in flasks exposed to *F. columnare* Fc7, however at this temperature the fish started dying already at 1 dpi, and all fish were dead within 2 dpi (Figure 13, b). In the *J.* sp. MM5 added flask, one fish died at 2 dpi, however no further fish died in the controls at 14°C.

The results show an evident correlation between the deadliness of *F. columnare* Fc7 against salmon yolk sac fry and higher temperatures. There occurred no deaths for fish challenged at 6°C, while the fish challenged at 10°C and 14°C experienced mortality days after infection, with deaths occurring noticeably earlier in the fish challenged at 14°C compared to 10°C. Based on these findings, for future experiments 10°C was used as the temperature for immersion challenges with *F. columnare* Fc7, as it is better suited for the salmon fry, and it was shown that *F. columnare* Fc7 was still able to lethally infect the fish at this temperature.

Challenge exp 2: Examining commensal microbiome with earlier immersion challenge and lower infection dose of Fc7

The second immersion challenge experiment had three aims: examining the influence of infection dose on mortality, examining the influence of the age of the fry on mortality, and examine the potential protective effects of a commensal microbiota against an Fc7 infection in salmon yolk sac fry. Both germ-free (GF) and conventionalized (CVZ; harboring a microbiome) fish were challenged with Fc7 at an earlier life stage than in challenge experiment 1, (infected at 20 dph instead of 42 dph) and some were challenged with a 10x lower *F. columnare* Fc7 concentration (see Challenge experiment 2 in MM).

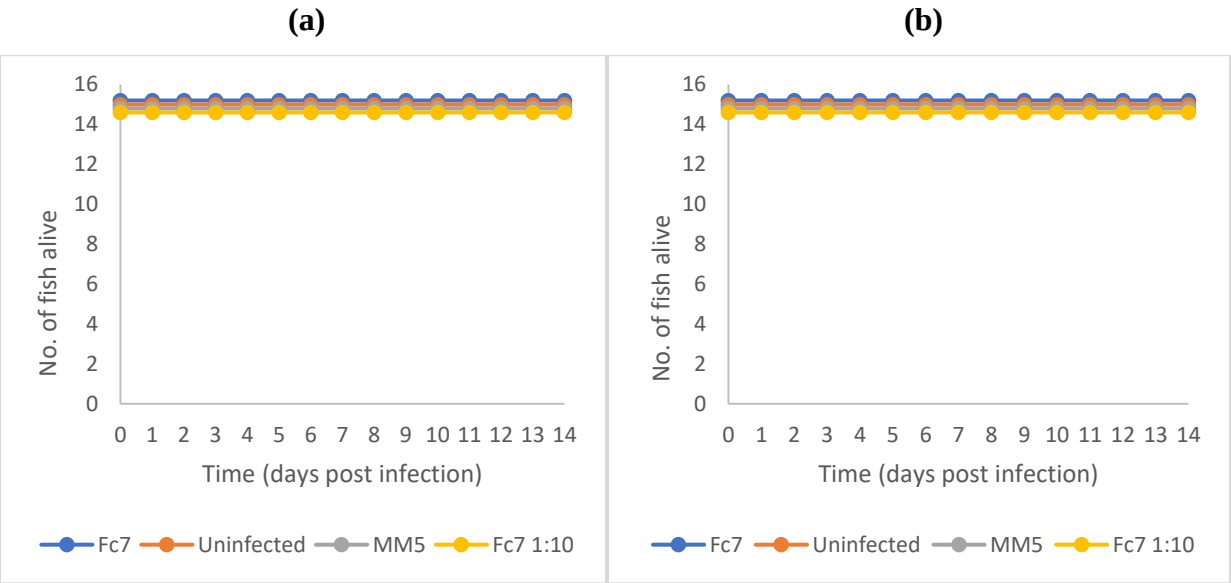
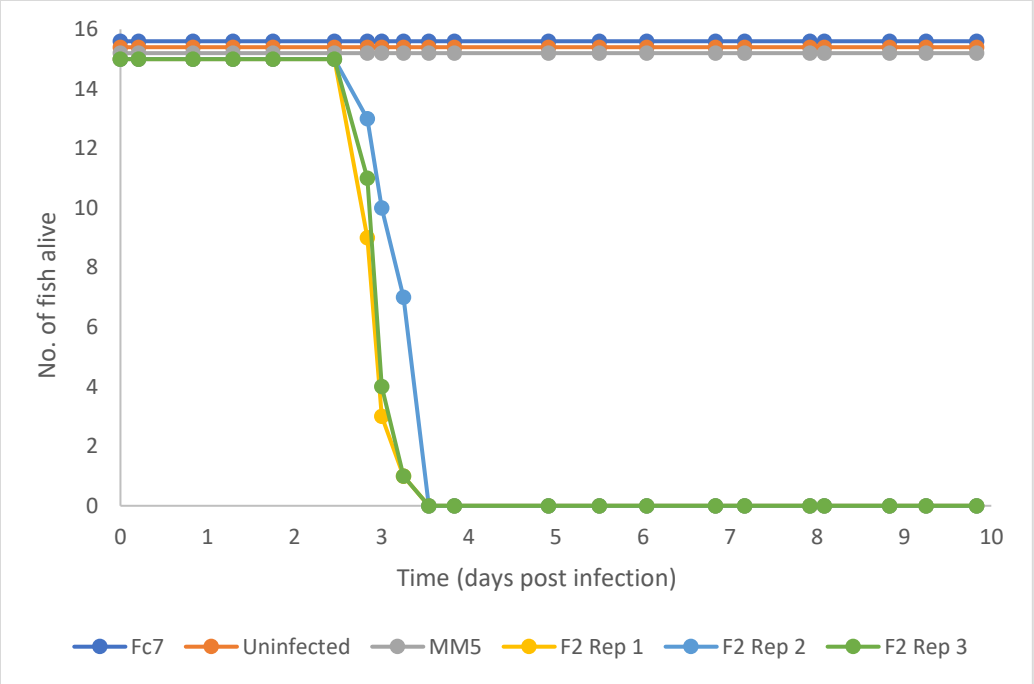


Figure 14: Earlier immersion challenge of Atlantic salmon fry with Fc7. The number of live fish in each flask from different groups is shown over the course of the challenge experiment. Each challenge group consisted of 3 replicate flasks containing 15 fish in each. (a) shows number of surviving fish in each GF flask during the immersion challenge. (b) shows the surviving CVZ fish during the immersion challenge. No deaths were observed throughout the experiment

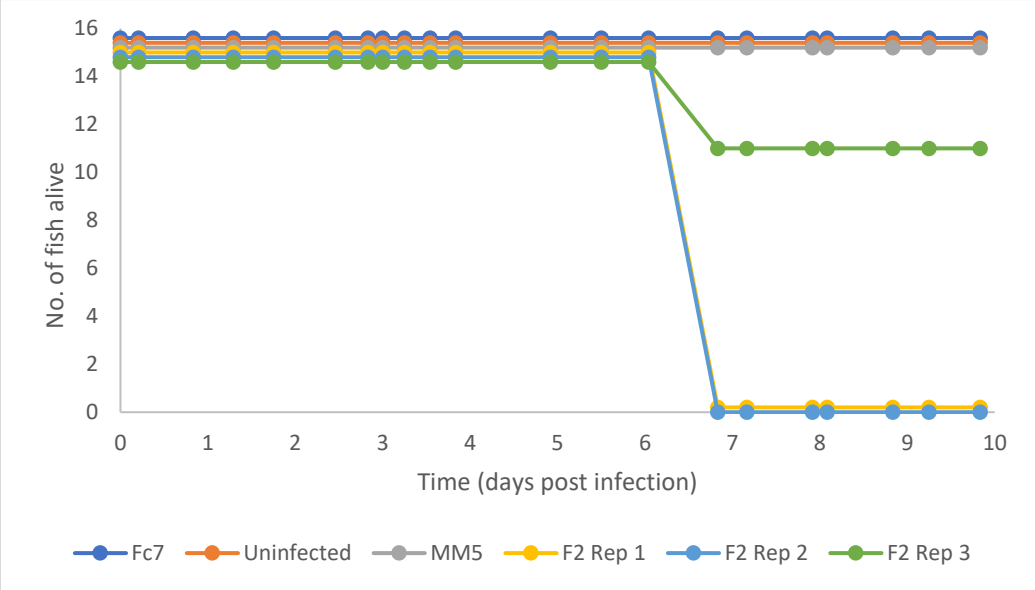
Very unexpectedly, no deaths occurred in any of the flasks for the entire duration of the experiment (Figure 14). The results indicate that some factor caused *F. columnare* Fc7 to be less pathogenic than previously observed in challenge experiment 1.

Challenge exp 3: Testing immersion challenge with FCO-F2 and Fc7

The third challenge experiment was designed with three aims: Further examining the limitations of immersion challenge Fc7 and finding the cause for the deviation in lethality observed between challenge experiment 1 and 2. Testing *Flavobacterium columnare* strain FCO-F2 (FCO-F2), a different *Salmo Salar* pathogen, for use in challenge experiments with salmon yolk sac fry. And lastly, examine the potential protective effects of a commensal microbiota against an FCO-F2 and Fc7 infection in salmon yolk sac fry by challenging GF and conventionalized fish with FCO-F2 and Fc7 at 10°C (see Challenge experiment 3 in MM).



(a)



(b)

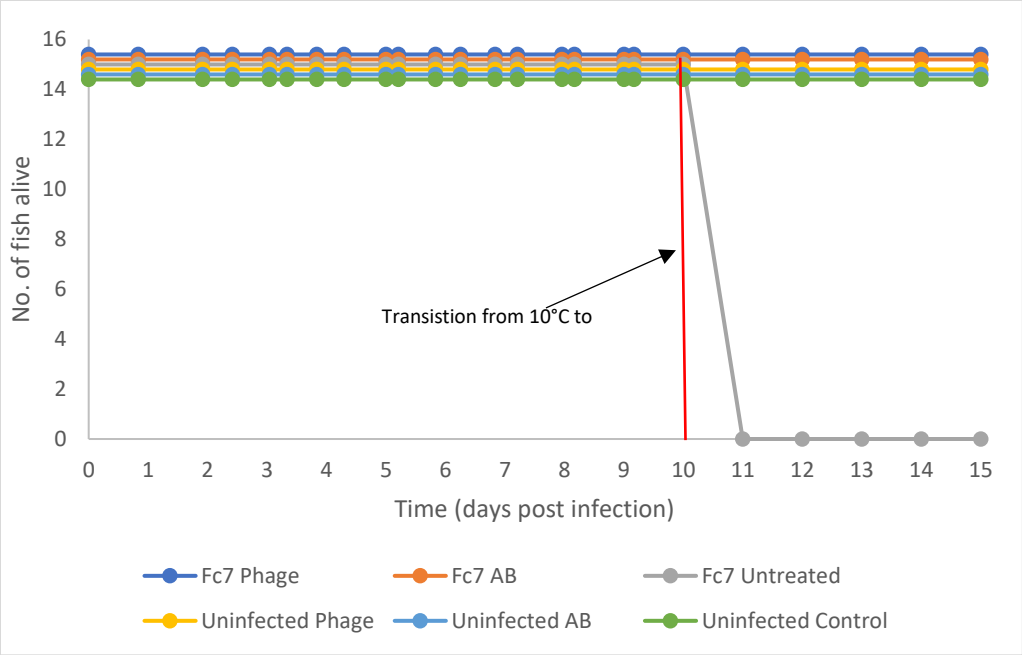
Figure 15: Immersion challenge of Atlantic salmon fry with FCO-F2 and Fc7. The number of living fish per flask in each challenge group is shown. Each challenge group consisted of 3 replicate flasks containing 15 fish in each, challenged at 41 dph. (a) shows number of surviving fish in each GF flask during the immersion challenge. (b) shows the surviving conventionalized fish during the immersion challenge. All flasks were incubated at 10°C. Groups with no deaths (Fc7, uninfected, MM5) are represented with one line instead of all replicates.

In the flasks challenged with *F. columnare* FCO-F2 all GF fish died during the experiment, whilst most CVZ fish died, except for a flask with 11 fish remaining. Deaths occurred noticeably earlier in GF fish (starting at 2,8 dpi, Figure 15), compared to conventionalized fish (starting at 6,8 dpi, Figure 15). For flasks exposed to *F. columnare* Fc7 in the immersion challenge, no mortality was observed in the GF or CVZ fish for the duration of the experiment. This was unexpected, as the conditions for Fc7 in this experiment were the same as in challenge experiment 1. No mortality was observed in the uninfected and MM5-challenged controls.

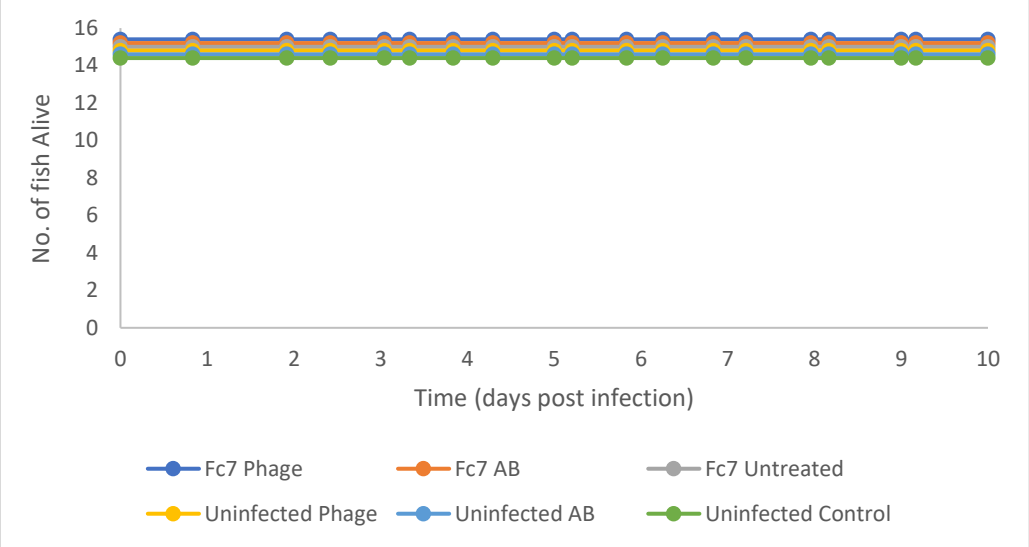
The results from this experiment indicate that *F. columnare strain* FCO-F2 is a more potent pathogen against Atlantic salmon yolk sac fry than strain Fc7. Further, it indicated that *F. columnare* Fc7's virulence was relatively low at 10°C, despite the observations from Challenge experiment 1.

Challenge exp 4: Fc7 immersion challenge with Fc7, with FCL-2 phage- and antibiotic treatment

Another aim of the project was examining whether phage-therapy is an alternative option to antibiotics for treatment against pathogens. The objective of this experiment was to achieve that by conducting an immersion challenge of salmon fry with *F. columnare* Fc7 and treat some of the fish with either a phage targeting *F. columnare* Fc7 (FCL-2), or the antibiotic oxytetracycline (see Challenge experiment 4 in MM). Oxytetracycline treatment was chosen due to being commonly used for antibiotic treatment in aquaculture, as well as being confirmed to be effective against *F. columnare* Fc7 in an antibiotic resistance test. Challenge experiment 4 was conducted before the conclusion from challenge experiment 3 was ready, and it was not yet determined that *F. columnare* FCO-F2 was more pathogenic against the salmon fry than *F. columnare* Fc7.



(a)



(b)

Figure 16: Immersion challenge of Atlantic salmon fry with Fc7 with phage- and antibiotic treatment. The number of living fish per flask in each challenge group is shown. Each challenge group consisted of 3 replicate flasks containing 15 fish in each. (a) shows number of surviving fish in each GF flask during the immersion challenge. (b) shows the surviving conventionalized fish during the immersion challenge. From 10 dpi, the temperature was increased to 14°C for the GF fish (a) until the end of the experiment at 15 dpi (represented by the solid red line) All flasks were incubated at 10°C. One line is used to depict each treatment group instead of all replicates, as all replicates always behaved the same.

During the first 10 days of the immersion challenge with *F. columnare* Fc7 at 10°C, neither the germ-free nor conventionalized fish experienced any deaths (Figure 16). After 10 days, the immersion challenge was ended for the conventionalized fish (explained in Challenge experiment 4 in MM). Since no deaths occurred in the first 10 days, it was examined whether the lethality of *F. columnare* Fc7 could be increased by increasing the incubation temperature. Therefore, the temperature was increased from 10°C to 14°C at 10 dpi for the germ-free fish and the challenge experiment was continued at 14°C for 5 days. All fish in the three *F. columnare* Fc7 challenged flasks with no treatment had died within 11 dpi, 1 day after the temperature increase (Figure 16, a). In flasks where the fish were treated with antibiotics or phage-treatment, no deaths were observed. There were also no observed deaths in any of the controls even after temperature increase.

These results show that the *F. columnare*. Fc7 was not pathogenic against Atlantic salmon yolk sac fry at 10°C. However, it caused a high degree of mortality in the untreated fish incubated at 14°C during the challenge. The results also indicate that both the phage treatment and antibiotics treatment was successful in preventing a lethal *F. columnare* Fc7 infection from occurring, as only the untreated group experienced any deaths at 14°C.

Challenge exp 5: Immersion challenge with Fc7 at 14°C

The fifth and final challenge experiment was designed to examine the protective properties of the fish microbiota against pathogens. This experiment used the new information regarding the optimal challenge temperature for *F. columnare* Fc7 for immersion challenge of salmon yolk sac fry. Based on the results from Challenge experiment 1, it was believed that 10°C was a sufficiently high temperature to induce deaths during the immersion challenge with *F. columnare* Fc7. As no deaths were observed at 10°C in neither experiment 3 and 4, it was assumed that the temperature at 10°C was too low for *F. columnare* Fc7 to be active enough to lethally infect the fish, and that the results observed in challenge experiment 1 was an unlikely occurrence (see discussion). The experiment was designed similarly to Challenge experiment 2, by challenging GF and conventionalized salmon fry with *F. columnare* Fc7, but this time at 14°C (see Challenge experiment 5 in MM) at 54 dph.

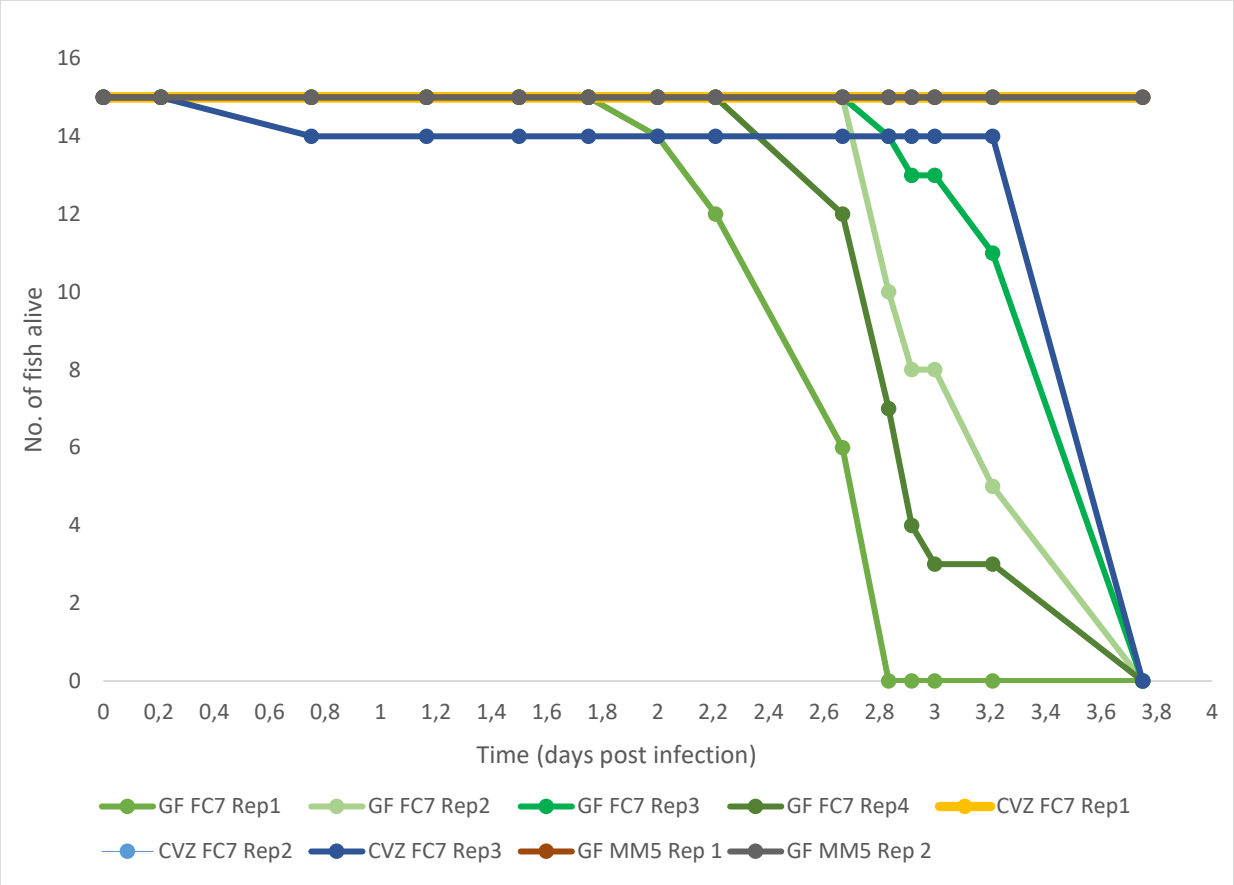


Figure 17: The number of living fish per flask in each challenge group recorded during the challenge experiment of 54 dph *Salmo Salar* fry with *F. columnare* Fc7 at 14°C. Each flask contained 15 fish at start.

For all germ-free flasks challenged with *F. columnare* Fc7, all the fish had died (starting 2,2 dpi) by the end of the experiment (Figure 17). In one of the conventionalized flasks challenged with *F. columnare* Fc7 (Figure 17, CVZ Fc7 Rep3), one fish died at 0,75 dpi, and 14 fish remained in the flask until 3,2 dpi. At 3,8 dpi however, all fish in that conventionalized flask had died. No further deaths occurred in the other conventionalized flasks and flasks with *J.* sp. MM5 added.

These results show that an immersion challenge with Fc7 on salmon fry at 14°C could induce mortality in the fish, and further showed a protective effect of the microbiome against infection with *F. columnare* strain Fc7.

Discussion

One of the main aims of this project was to examine the protective properties of the microbiota against pathogenic attacks. To achieve this, a subgoal was to first establish a reproducible protocol for immersion challenge of Atlantic salmon yolk sac fry with a bacterial pathogen. To easily detect successfully infected individuals, we decided that the use of a pathogen causing a lethal infection was the best option. The pathogen would be used to conduct an immersion challenge experiment on salmon fry with a commensal microbiome and germ-free fry to compare their survivability to the pathogen infection. The hypothesis was that if the microbiota did protect against pathogen attacks, the salmon fry with a commensal microbiome should be less affected during an immersion challenge. The expected observation would be that the fish with a microbiome would experience low mortality or die noticeably slower than germ-free fish during an immersion challenge with a lethal pathogen.

Immersion challenge, (challenging the fish by immersing them in water containing a pathogen) was chosen over other methods, e.g., injection challenge, because it more closely resembles the natural mode of infection of fish and also gives the external microbiome of the fish the opportunity to protect against the infection, which was partly what we wanted to observe. A problem that many experiments using immersion challenge models encountered was varying infection success and low reproducibility.[65]

The previous experiments to infect yolk sac fry of Atlantic salmon with *Yersinia ruckeri* performed in the ACMS group showed that they were unexpectedly unsusceptible to being lethally infected during immersion challenges under our laboratory conditions.[22] One possible explanation was that the rearing conditions in our lab were of too high quality, when compared to fish reared in aquaculture, which have higher stocking density and poorer water quality.[68,22] This could make the fish less stressed than under normal conditions, resulting in an increased resistance to infections.[68] The low mortality rate recorded in previous experiments could also indicate that the fish in the yolk sac phase are more resistant to infections. One solution to this could be exposing them to thermal stress during the immersion challenge, potentially weakening their resistance to infections and also increasing the pathogenicity of the pathogens.[14,62]

Unfortunately, there had not yet been identified a potent enough pathogen by the ACMS group which reliably induced mortality in salmon yolk sac fry in an immersion challenge under laboratory conditions. Previous projects had tried establishing an immersion challenge of salmon fry with, which were unsuccessful.[22] In this project, *Flavobacterium columnare* strains Fc7 and FCO-F2, which cause columnaris disease in salmonids and other fish,[73] were tested for immersion challenge of Atlantic yolk sac fry. *F. columnare* Fc7 had successfully been used previously in an immersion challenge of rainbow trout fry by David Pérez-Pascual,[20] whilst *F. columnare* FCO-F2 had been shown to be highly deadly when infecting rainbow trout and have been used in phage therapy experiments by other groups.[21,61] This made the *Flavobacterium columnare* strains good potential candidates for immersion challenges in this project.

Initially, we attempted to isolate a bacteriophage against *F. columnare* Fc7 for Challenge experiment 4 (where phage therapy-treatment was tested) from water samples taken from various locations (see: isolation of phage against Fc7 in MM). None of these attempts were successful as we never found any plaques on soft agar plates mixed with the preparate from water samples and *F. columnare* Fc7

As we were not able to isolate phages against *F. columnare*, we contacted professor Sundberg from the University of Jyväskulä, which is an in phages against *F. columnare.* She also informed us that all the bacteriophages she had isolated against *F. columnare* had been found during the summer, when the temperature is higher (personal communication). This is not surprising as *F. columnare* is a warm-water pathogen and outbreaks usually occur during summer, when water temperatures are high.[14] Our attempts at isolating bacteriophages against *F. columnare* Fc7 were conducted during the winter, which may explain our lack of phage discoveries.

The immersion challenges conducted during the project required germ-free fish for comparing lethality between germ-free fish and fish with a microbiome present during the challenge. Detection of *F.* sp. TRD on the eggs after being sterilized using our sterilization protocol (see; sterilization protocol in MM) proved to be problematic, as they could contaminate germ-free groups of fish, ruining the immersion challenge experiments. An antibiotics test of *F.* sp. TRD was conducted, where we observed that it was susceptible to oxolinic acid (a bacteriocide), but resistant against all other antibiotics used in the sterilization protocol. The antibiotic was already present in the antibiotics cocktail used in our sterilization protocol of salmon eggs, so we

theorized that it was used at a too low concentration or for too short time to effectively remove all the *F.* sp TRD contaminations. By exposing the bacteria to a cocktail with 10 times the normal concentration of oxolinic acid, we showed that it was possible to remove them completely from the eggs and the rearing water. For future immersion challenges, it could be beneficial to use more oxolinic acid in the antibiotics cocktail than standard, to ensure the sterility of the eggs received for immersion challenges. We also observed that at 6°C, some of the oxolinic acid precipitated from the antibiotics cocktail when oxolinic acid was added at a high concentration, implying an excessive amount of the antibiotic in the cocktail. In the future, it could be beneficiary to test if lower concentrations than 10 times the standard of oxolinic acid is sufficient to acquire the same decontamination effect on *F.* sp. TRD observed in this experiment, without inducing precipitation of oxolinic acid in the medium.

To make a reproducible immersion challenge protocol of Atlantic salmon yolk sac fry with *F. columnare* Fc7, it was necessary to test if it could reliably induce mortality with the bacterium. Additionally, we attempted to examine the effect of thermal stress on infection success. This was the background for the experimental design of Challenge experiment 1. The germ-free salmon fry were challenged at 6°C, 10°C and 14°C, to compare their responses to the pathogen when under thermal stress. In addition, *F. columnare* is a bacterium that thrives in warmer climates and increasing the temperature during the immersion challenge provided a more hospitable environment for the pathogen, possibly making it more active and infectious.[14] We hoped that the combination of thermal stress and favorable conditions for the pathogen was sufficient to induce mortality in the challenged salmon fry, which had previously been shown hard to achieve in experiments with other pathogens.[22,57]

The results from immersion challenge experiment 1 showed that *F. columnare* Fc7 was able to induce mortality in the 42 dph germ-free salmon fry at both 10°C and 14°C (Figure 13). The fry challenged at 10°C started dying 2 dpi, whilst the fry challenged at 14°C started dying only 1 dpi (Figure 13). The fry challenged at 6°C did not experience any mortality during the immersion challenge. This implies that *F. columnare* Fc7's pathogenicity against the salmon fry is dependent on the incubation temperature, and that the pathogen is more effective and causes quicker mortality at higher temperatures. This makes sense, as *F. columnare* is a warm water pathogen.[14] However, the infection lethality observed could also be caused by the thermal stress

the salmon fry were subjected to during the immersion challenge at higher temperatures. Because of the design of the experiment, it is difficult to ascertain which of the two, thermal stress or favorable environment for *F. columnare* Fc7 that had the most impact on lethality. However, it was shown that the immersion challenge required either the salmon fry to be thermally stressed or that Fc7 needed higher temperatures than 6°C to be active, or a combination of both, to successfully induce mortality in the fry.

This finding was used in subsequent immersion challenges 2 and 3, where the fish were kept at 10°C. The main reason for choosing to conduct the immersion challenges at 10°C over 14°C, was that from past experience, the fish flasks systems tend to be very unstable at 14°C and higher, with high risk of sudden deaths of entire fish population in flasks ocurring.[22,57] This was found to be caused by higher temperatures promoting bacterial growth too rapidly and consume all the oxygen in the flasks, leading to fish death.[22] Having such deaths occur would be problematic, as they could make the results far more difficult to interpret. It could also be somewhat difficult to discern whether fish were dying due to lack of oxygen-, or from being caused by the pathogen added. As observed in Challenge Experiment 1 (Figure 13) all the fish died very rapidly after the first deaths started occurring. This is similar to what happens when oxygen deficiency caused by bacterial growth occurs. It is generally easiest to identify deaths caused by oxygen deficiency by examining the unchallenged conventionalized (non-germfree) fish or fish that were added *J.* sp. MM5. Normally, these groups should have a low rate of mortality, but at higher rearing temperatures, the bacteria start consuming too much oxygen, killing the fish. If many fish from either of these groups start dying during an experiment, the temperature is probably too high for the outcome to be reliable. In Challenge Experiment 1, the fish exposed to *J.* sp. MM5 and conventionally reared fish did not die, indicating that the mortality was actually caused by the *F. columnare* Fc7 (Figure 13).

During the project however, we experienced some difficulties with replicating the mortality observed in Challenge experiment 1 with *F. columnare* Fc7. One of the goals of Challenge experiment 2 was to attempt the immersion challenge of salmon fry at 10°C with *F. columnare* Fc7 earlier in their lifespan (20 dph), as well as test if reducing the bacterial dosage (10% of the usual dosage) during the challenge would change the response of the fish. It was theorized that at 10°C combined with one tenth the infection dose, the infection would develop more slowly, and

the deaths would occur more gradually. As the challenged fish all died 1,5-2 dpi, by increasing the time between the immersion challenge and most of the mortality occurring, it would provide a larger window to sample data during the experiments. This would also make the experiment possible to conduct earlier in the rearing process, which would require less time and resources to be spent to keep the fish alive.

Unfortunately, in Challenge experiment 2, no lethality in the *F. columnare* Fc7 challenged fish was observed (Figure 14). It was believed at the time of the conclusion of Challenge experiment 2 that this was caused by the fry being at a younger life stage than in Challenge Experiment 1, as the experiment otherwise was conducted under similar conditions to immersion challenge experiment 1, at 10°C. There, *F. columnare* Fc7 was able to induce mortality to the fry. A study by Wells showed a correlation between the virulence of *Flavobacterium columnare* and the ability of the bacteria to adhere to the gill tissue of fish.[69] This implies that the gills are an important part of the mode of infection for *F. columnare*. In newly hatched salmon fry, a large part of their oxygen uptake is conducted through the body surface.[70] As the fish grow in size, their skin area-to-mass ratio decreases, which requires their gills to develop and be responsible for more of the oxygen intake.[70] At earlier stages in their life (20 dph), their gills could be too undeveloped for the *F. columnare* to properly adhere to the gills for infection compared to the 44 dph fry. This was the main theory for why no mortality was observed, and consequently, the following challenge experiments were still conducted at 10°C, but with fry of similar age to those in challenge experiment 1. However, in Challenge experiment 3, where the age of the challenged salmon fry were similar to those in Challenge experiment 1, there were still no deaths observed in the fish challenged with *F. columnare* Fc7 at 10°C (Figure 15).

These results indicate that the age of the fish was not the only reason for why *F. columnare* Fc7 did not lethally infect the fish, as previously believed based on the results from challenge experiment 2. It seemed like the established deadliness observed in Challenge Experiment 1 was hard to replicate, even with similar conditions to Challenge Experiment 1. The age of the fry could still have an effect on the virulence of *F. columnare* Fc7, but the results show that something else was influencing the immersion challenge. The OD measured of the *F. columnare* Fc7 cultures added in both Challenge experiments 1 and 3 were similar at infection. This implies

the bacterial density used was not the explanation for the lack of mortality induced by *F. columnare* Fc7 observed in Challenge Experiment 3.

Another possible explanation was that at 10°C with the current conditions seemed to be around the breakpoint of *F. columnare* Fc7 being lethal, and some unknown differences between the experiments could affect the lethality. These differences could for example be the 3 dph difference between the fish in challenge experiment 1 and 3, or the behavior of the *F. columnare* Fc7 culture. Yet another explanation could be that during the cultivation of the bacteria between the two experiments, *F. columnare* Fc7 was grown in an incubator at 18°C in experiment 1, but at room temperature (22°C) in experiment 2. This difference in cultivation temperature might have selected for more cold-resistant bacteria in challenge experiment 1.

In Challenge experiment 4, it was observed that GF fish challenged with *F. columnare* Fc7 did experience mortality, when the temperature was increased from 10°C to 14°C. Partly due observations made in Challenge experiment 4, and the un-reproducibility of challenge experiment 1, it was theorized that 10°C was probably too low of a temperature to mortally infect the salmon fry reliably with *F. columnare* Fc7, and that the results from Challenge experiment 1 was an outlier occurrence due to some of the reasons discussed earlier, or something else entirely unaccounted for. We wanted to examine if we could conduct an immersion challenge with *F. columnare* Fc7 salmon fry at 14°C for the entire duration. If the main reason for *F. columnare* Fc7's low virulence observed in challenge experiment 2 and 3 was the low temperature, as we believed, we should observe mortality in the challenged fry.

This was our reason for conducting the fifth challenge experiment. During the experiment, all the germ-free fry challenged with *F. columnare* Fc7 at 14°C died (Figure 17). This confirmed our hypothesis that *F. columnare* Fc7's low virulence observed in some of the Challenge experiments was probably caused by 10°C being too low of a temperature for the bacterium to reliably lethally infect the fish. These results show that increasing the temperature higher than 10°C, possibly to 14°C when conducting an immersion challenge with *F. columnare* Fc7 on Atlantic salmon yolk sac fry could be beneficial if a higher fish mortality is wanted.

As a successful immersion challenge with *F. columnare* Fc7 had somewhat been established, the second aim in this project was to observe the protective properties of the microbiota against pathogen attacks. This was examined in subsequent Challenge experiments, where

conventionalized (CVZ) salmon fry and germfree (GF) salmon fry were challenged with *F. columnare* strains, and their responses were compared.

Unfortunately, in Challenge experiment 2, no lethality in the *F. columnare* Fc7 challenged fish was observed (Figure 14), and therefore no observations of the protective properties of the microbiota could be made. Examination of the protective properties of the fish microbiota was retried in Challenge experiment 3. Additionally, for this experiment, we had received another infectious strain of *F. columnare* (strain FCO-F2) from professor Sundberg from the University of Jyväskylä, which we also wanted to test if could work in an immersion challenge with Atlantic salmon yolk sac fry. In this experiment germ-free- and conventionalized fry were challenged with either *F. columnare* Fc7 or FCO-F2 at 10°C, and their responses to the pathogen were compared. Neither the germ-free- or conventionalized fish challenged with *F. columnare* Fc7 experienced any mortality for the duration of the experiment (Figure 15).

F. columnare FCO-F2 showed a very higher degree of virulence compared to *F. columnare* Fc7, even killing CVZ fish at 10°C (Figure 15). There was a difference in mortality between the conventionalized and germ-free groups, as the amount of time needed for the infection to become deadly was longer for the conventionalized fish (approximately 4 days in difference). Based on these results, immersion challenges with *F. columnare* FCO-F2 could be more reliable in inducing mortality in the fry at lower temperatures, compared to *F. columnare* Fc7. For future designs of immersion challenges of Atlantic salmon yolk sac fry, *F. columnare* strain FCO-F2 might be a better choice of pathogen.

These results indicate that there is some protection against pathogens provided by the microbiota. In the case with the single CVZ flask with surviving fish, the microbiota was able to counteract the pathogen so much so that only 4 fish died in total, while the rest survived for the duration of the experiment.

In the fifth challenge experiment older unchallenged untreated GF and CVZ fish from challenge experiments 3 and 4 were challenged with *F. columnare* Fc7 at 14°C. The challenge experiment was only kept running for 4 days after addition of *F. columnare* Fc7. During the experiment, all the challenged GF salmon fry died, as predicted (Figure 17). Unexpectedly, there occurred one death in a conventionalized flask, and by the end of the experiment all the fish in that single CVZ flask had died. The other two CVZ flasks had no deaths occur. We believe that the cause for all

the fish dying in a CVZ flask was probably due to bacterial bloom and lack of oxygen, as the other flasks were unaffected. Since the experiment was only performed for 4 days and with only a few numbers of replicate flasks, there is a possibility that *F. columnare* Fc7 simply used more time to lethally infect the fish, and the other CVZ fish could have died, if the experiment was kept running for longer. However, based on the available information, the first cause seems more probable. Either way, if the latter possibility was true, we would have observed that *F. columnare* Fc7 took longer to lethally infect the fish with a microbiome present.

Both the results from Challenge experiment 3 and 5 showed that the microbiota provided protection against the *F. columnare* infections. These results coincide with other studies of the microbiota, where a protective effect was shown. A study conducted by Stressmann showed that microbiota-associated colonization resistance against *F. columnare* in larvae and adult zebrafish was provided by some of the bacteria present in the fish microbiota, such as *Chryseobacterium massiliae*.[77] Additionally, it was showed that some bacteria that do not individually protect against infections, could provide a community-level resistance when different species were combined.[77] By further understanding the mechanisms of microbiota protection, and identifying bacterial species involved in this process, microbial communities providing high resistance to pathogenic infections could be engineered and used to protect other animals, such as fish reared in aquaculture.

Immersion challenge experiment 4 was conducted to examine bacteriophage therapy as a good alternative to antibiotics for protection of salmon yolk sac fry, which one of the aims of this project. Additionally, we wanted to test the efficiency of phage therapy treatment on fish with and without a microbiota and compare the results with fish treated with antibiotics (oxytetracycline). Our hypothesis was that a microbiota combined with phage therapy would be more effective than oxytetracycline treatment with microbiota, as the antibiotic could damage the commensal bacteria, lessening their effect. Oxytetracycline was chosen as the antibiotic instead of an antibiotics cocktail, because it is common treatment for fish infected with *F. columnare* in aquaculture.[72] We wanted the experiment to be grounded in the procedures usually used in aquaculture.

As described in the introduction, lytic phages reproduce by infecting specific target hosts and using their replication and-protein production complexes to assemble new phages.[19] These newly

assembled phages generally kill the hosts upon exiting.[17] By using the specificity of the phages and their ability to kill target hosts, they could in theory be used in treatment against pathogens. This could be achieved by isolating a phage targeting said pathogen and applying it in a similar way to how antibiotics are currently used. Unlike of broad-spectrum antibiotics, bacteriophage therapy would in theory not harm the naturally occurring microbiome present.[42]

The experiment was conducted by challenging germ-free and conventionalized salmon yolk sac fry with *F. columnare* Fc7 at 10°C (increased to 14°C at 10 dpi). Some were left untreated or treated with phages or oxytetracycline. Our hypothesis was that if the phage treatment could combat the infection development of *F. columnare* Fc7, we should observe a lower mortality rate in the fish compared to those without treatment.

During the first 10 dpi, no deaths occurred in any of the fish flasks. As explained previously in the results, during these 10 days, as part of another experiment not included in this project, microbiome samples were taken to investigate whether phages really do not disturb the microbiome, sine CVZ fish were gradually removed for sampling during the immersion challenge, which influenced the experimental design. The fish flasks environment were required to be stable, which was partly why the challenge was conducted at 10°C. After the sampling of CVZ fish had concluded, the temperature was increased to 14°C for the germ-free fish.

As there were no deaths occurring during the first part of the immersion challenge at 10°C, and all the conventionalized fish were removed in the latter part of the immersion challenge at 14°C, we were not able to examine the protective effects of the microbiota when combined with phage treatment or antibiotics. This could be attempted in the future.

We observed that one day after the temperature increase, all the fish challenged with *F. columnare* Fc7 that were untreated had died. None of the uninfected, *J.* sp. MM5 added fish or fish challenged with *F. columnare* Fc7 that received oxytetracycline- or phage treatment experienced deaths for the duration of the experiment (lasting 5 days).

The fish receiving both the antibiotic treatment and phage treatment therefore had increased resistance to *F. columnare* Fc7, with no deaths occurring. These results indicate that the phage treatment was as effective as the antibiotic therapy, and that FCL-2 could possibly be used in phage therapy against an *F. columnare* strain Fc7. We were not able to determine if either of the

treatments was more effective than the other, as fish from both treatments had identical mortality responses. The challenge at 14°C lasted only for 5 days due to time constraints, and differences to response may have been observable if the experiment had continued for longer. As the temperature was increased from 10°C to 14°C in the latter part of the immersion challenge, the results could be considered unreliable, and the experiment should probably be re-conducted at 14°C from the start to confirm the findings.

In a study conducted by professor Sundberg, phage therapy was able to increase the survivability in rainbow trout and zebrafish challenged with *F. columnare*.[13] The survivability of in zebrafish was increased to 100% (from 0%) and 50% for rainbow trout (8,3 %). Our results were similar, with a 0% survival rate in challenged GF salmon fry without treatment, whilst fry receiving phage-therapy treatment had a 100% survival rate. From our results and the results from Sundbergs study, it is evident that the effects of the phage therapy were very impactful, which indicates that phage therapy could be a good alternative to antibiotics. This is especially important, as there in recent time has been a larger focus on the emergence of antibiotics resistant bacteria, and how it has become increasingly challenging to manage these bacteria.[79]

The use of bacteriophage therapy has not always shown to be successful though, as a study conducted by Verner-Jeffreys showed no signs of protective effect in *Aeromonas salmonicida* challenged *Salmo Salar,* when treated with three different phages isolated against the bacteria.[78] In the study, bacteriophage resistant subspecies of *A. salmonicida* were isolated from the deceased fish.[78] This shows that even if phages are successfully isolated against a pathogen, they are not necessarily fit for use in phage therapy. Although a phage has a specific pathogen as target, they may be of too low virulence to significantly decrease the number of pathogens,[49] rendering their therapeutic effect negligible. Low virulence may be caused by the phage having slow replication, poor absorption into the host, or few adaptations to bacterial defenses.[49]

Additionally, as with antibiotic treatment, the emergence of phage resistant bacteria during phage treatment is a possible occurrence. However, it has been shown that bacteria with phage resistance adaptations can sometimes have diminished virulence, as they might lose pathogenicity-related phage receptors and gain a lower tolerance to antibiotics.[55] This means that it could be very beneficial to combine both antibiotics and phage treatment, to maximize the efficiency of treatment, and hindering the bacteria from developing immunity.

Conclusion

By running antibiotics tests of the *F. sp.* TRD contaminant present on the salmon eggs which previously had survived sterilization, we were able to develop a modified sterilization protocol that was shown to reliably remove the contaminant entirely from salmon eggs received.

Our challenge experiments showed that *F. columnare* Fc7 could be used in an immersion challenge with Atlantic salmon yolk sac fry, but need to be conducted at a higher temperature than 10°C. We observed that they can reliably induce lethality in the fry infected at 14°C, but there is some risk of deaths caused by oxygen deficiency in the flasks. To further optimize the immersion challenge protocol with *F. columnare* Fc7, it could be a good idea to test whether *F. columnare* Fc7 can cause lethality in the fry when challenged at a temperature between 10°C and 14°C, such as 12°C. We were unable to conclusively test whether the fry could be reliably challenged with *F. columnare* Fc7 at an earlier life stage than conducted in most of the Challenge experiments, as the pathogen turned out to have too low virulence at the temperature during the test.

We observed that that *F. columnare FCO*-F2 can be used in an immersion challenge with Atlantic salmon yolk sac fry, being more aggressive than the Fc7 strain under our immersion challenge conditions. There have also been bacteriophages isolated with FCO-F2 as target host, such as FCOV-F2,[61] which means immersion challenges with FCO-F2 with phage therapy treatments could be conducted on Atlantic salmon yolk sac fry in the future, if wanted.

Based on the results from the immersion challenges, we can conclude that the microbiome does provide a protective effect against pathogen infections, either by protecting the salmon fry entirely from being lethally infected, or slowing the infection rate down, causing deaths to occur at a later time.

We also observed that both bacteriophage therapy with FCL-2, and oxytetracycline treatment was effective in negating an infection of *F. columnare* Fc7 in germ-free Atlantic salmon yolk sac fry, although no comments on differences in efficiency of the two treatments could be made. We were unfortunately unable to observe the effects of those treatments combined with the presence of a microbiota, and this should be explored in the future.

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Appendix

A Media and solutions

A.1 Salmon Gnotobiotic Media (SGM)

Contents of Salmon Gnotobiotic Media used in rearing of Atlantic salmon yolk sac fry. The media was autoclaved at 121 °C for 60 minutes before use.

Compound	Stock concentration [g/l]	mL used for 1 l SGM
100x $MgSO_4 \cdot 7\ H_2O$	12,3	10
100x KCl	0,4	10
100x $NaHCO_3$	9,6	10
5x $CaSO_4 \cdot 2\ H_2O$	0,3	200
MilliQ H_2O	-	770

A.2 Tricane solution

Contents of tricane solution used to euthanize Atlantic salmon yolk sac fry after experiments. The solution was autoclaved at 121 °C for 60 minutes before use.

Compound	**Amount**
Tricane	5,2 g
Tris (1 M, pH = 9)	27,3 mL
SGM	972,7 mL

A.3 SM-buffer

Compound	**Amount**
NaCl	5,8 g
Tris (1 M, pH = 7,5)	50 mL
$MgSO_4 \cdot 7\ H_2O$	2 g
MilliQ H_2O	950 mL

A.4 Media for sterility check

Sterility checks of Atlantic salmon fry flasks were performed by inoculating some rearing water in the four following medias, and examining for bacterial growth. The medias were autoclaved at 121 °C for 60 minutes before use.

Nutrient broth (NB)

Compound	Amount
NB (VWR chemicals)	8 g
MilliQ H_2O	1000 mL

Brain heart infusion (BHI)

Compound	Amount
BHI (Merck KGaA)	37 g
MilliQ H_2O	1000 mL

Saboraud-2% dextrose broth (SD)

Compound	Amount
SD (Merck KGaA)	30 g
MilliQ H_2O	1000 mL

Glucose yeast extract broth (GY)

Compound	Amount
Glucose (Sigma Aldrich)	10 g
Yeast extract (Sigma Aldrich)	2,5 g
MilliQ H_2O	1000 mL

A.5 Bacterial growth media

Tryptic Soy Broth (TSB)

Compound	Amount
TSB (Sigma-Aldrich)	30 g
MilliQ H_2O	1000 mL

Tryptic Soy Agar (TSA)

Compound	Amount
TSB (Sigma-Aldrich)	30 g
Agar	15 g
MilliQ H_2O	1000 mL

Tryptone yeast extract salts broth (TYES)

Compound	Amount
$MgSO_4 \cdot 7\ H_2O$	0,5 g
$CaCl_2 \cdot 2\ H_2O$	0,2 g
Yeast extract	0,4 g
Tryptone	4,0 g
D-glucose	0,5 g
MilliQ H_2O	1000 mL

Tryptone yeast extract salts agar (TYES-agar)

$MgSO_4 \cdot 7\ H_2O$	0,5 g
$CaCl_2 \cdot 2\ H_2O$	0,2 g
Yeast extract	0,4 g
Tryptone	4,0 g
D-glucose	0,5 g
Agar	15,0 g
MilliQ H_2O	1000 mL

A.6 Tris-Acetate-EDTA (TAE) buffer (50x)

Compound	Amount
Tris base	242 g
Glacial acetic acid	57,1 mL
0.5 M EDTA pH 8.0	100 mL
MilliQ H_2O	Up to 1000 mL

B Protocols for DNA Extraction and Purification

B.1 DNeasy® Powersoil® DNA Isolation Kit (Qiagen)

Quick-Start Protocol June 2016

DNeasy® PowerSoil® Kit

The DNeasy PowerSoil Kit can be stored at room temperature (15–25°C) until the expiry date printed on the box label.

Further information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml collection tubes are provided.

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000–V1–24).
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml collection tube.
   Note: Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.

Sample to Insight QIAGEN

Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step.

8. Centrifuge the tubes for 1 min at 10,000 x *g*.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.

    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml collection tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through. Centrifuge again for 1 min at 10,000 x *g*.
18. Carefully place the MB Spin Column into a clean 2 ml collection tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no. 17000–10).
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.

    Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

B.2 QIAquick® PCR Purification Kit (Qiagen)

Protocol: QIAquick PCR Purification using a Microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions using the QIAquick PCR Purification Kit or the QIAquick PCR & Gel Cleanup Kit. For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb can be purified from primers, nucleotides, polymerases and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).
- Add 1:250 volume pH Indicator I to Buffer PB (i.e., add 120 µl pH Indicator I to 30 ml Buffer PB or add 600 µl pH Indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH Indicator I indicates a pH ≤7.5.
- Add pH Indicator I to entire buffer contents. Do not add pH Indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH Indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample, and then mix. It is not necessary to remove mineral oil or kerosene.

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH Indicator I has been added to Buffer PB, check that the mixture's color is yellow.

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.

   Collection tubes are reused to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 min.

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volumes are 48 µl from 50 µl elution buffer volume and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. Maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –30°C to –15°C because DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting it up and down before loading the gel.

    Loading Dye contains 3 marker dyes – bromophenol blue, xylene cyanol and orange G – that facilitate estimation of DNA-migration distance and optimization of the agarose gel run time. Refer to Table 2 (page 17) to identify the dyes according to migration distance and agarose gel percentage and type.

C 16S rRNA sequence of *Flavobacterium* sp. TRD

AAGTCGAGGGGTATATGTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTAC
CTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGCATTGCATGATGGCATCATCG
AGCAATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAGCTAGTTGGTAAGGTAACGGCTT
ACCAAGGCTACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCA
GACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGC
GTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGTACGAGAAGAAACACTCCTATGTATAGGA
GCTTGACGGTATCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATC
CAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAATAAGTCAGTGGTGAAAGCC
CATCGCTCAACGGTGGAACGGCCATTGATACTGTTAAACTTGAATTATTAGGAAGTAACTAGAATATG
TAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATACCAATTGCGAAGGCAGGTTACTACTAATGG
ATTGACGCTGATGGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGGATACTAGCTGTTGGGAGCAATCTCAGTGGCTAAGCGAAAGTGATAAGTATCCCACCTGGGG
AGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGTAGATTGACCGGTTTGGAAACAGATC
TTTCGCAAGACAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGT
CCTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACTCTAACAAGACTGCC
AGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCTACACAC
GTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGAGCGAATCTATAAAACCGGTCAC
AGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCTGGAATCGCTAGTAATCGGATATCAGCCATG
ATCCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGA
AGTCGGGACCGCAAGGAGC

D Sterility check results from Challenge experiment 2

Sterility check was performed on all fish flasks before conventionalization by inoculating rearing water in the sterility check media listed in A.4 and on one TSA plate. Flasks where bacterial growth was observed in a specific media is marked red. Overview of flasks and the treatment the eggs received before being placed in flasks during the *F*. sp. TRD sterilization experiment: Standard sterilization: Flasks 50-55, Modified sterilization: Flasks: 56-61, Modified sterilization with *F*. sp. TRD: 62-67, Standard sterilization with *F*. sp. TRD: 68-72, Standard sterlization with Jonsvatnet water added: 73-78.

	GY	NB	BHI	SR	TSA
50	-	-	-	-	-
51	-	-	-	-	-
52	-	-	-	-	-
53	-	-	-	-	-
54	-	-	-	-	-
56	-	-	-	-	-
57	-	-	-	-	-
58	-	-	-	-	-
59	-	-	-	-	-
60	-	-	-	-	-
62	-	-	-	-	-
63	-	-	-	-	-
64	-	-	-	-	-
65	-	-	-	-	-
66	-	-	-	-	-
68	-	-	-	-	-
69	-	-	-	-	-
70	-	-	-	-	-
71	-	-	-	-	-
72	-	-	-	-	-
73	-	-	-	-	-
74	-	-	-	-	-
75	-	-	-	-	-
76	-	-	-	-	-
77	-	-	-	-	-
78	-	-	-	-	-
Neg.	-	-	-	-	-
Pos.	+	+	+	+	+
SGM	-	-	-	-	-

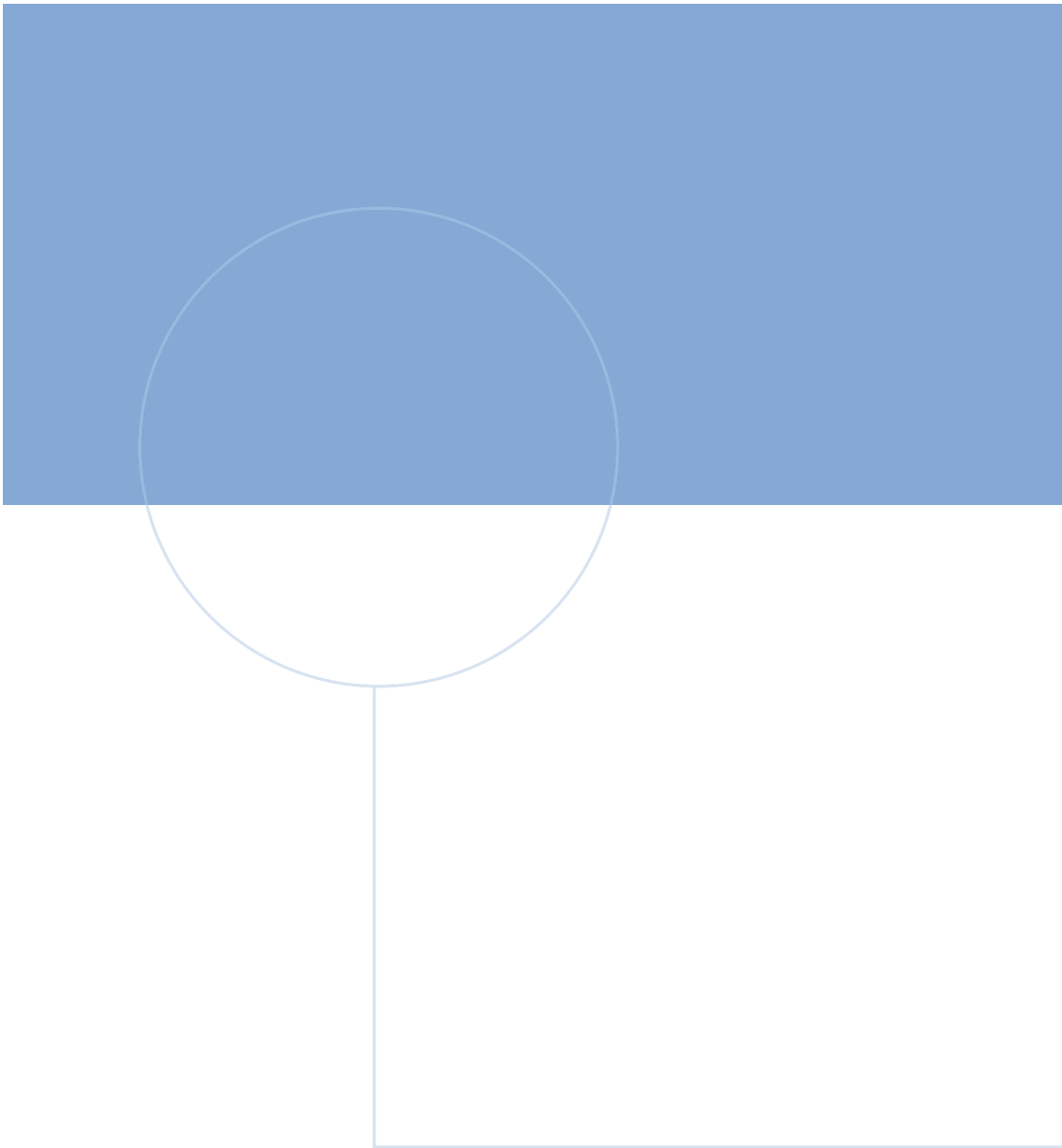
	GY	NB	BHI	SR	TSA
50	-	-	-	-	-
51	-	-	-	-	-
52	-	-	-	-	-
53	-	-	-	-	-
54	-	-	-	-	-
56	-	-	-	-	-
57	-	-	-	-	-
58	-	-	-	-	-
59	-	-	-	-	-
60	-	-	-	-	-
62	-	-	-	-	-
63	-	-	-	-	-
64	-	-	-	-	-
65	-	-	-	-	-
66	-	-	-	-	-
68	-	-	-	-	-
69	-	-	-	-	-
70	-	-	-	-	-
71	-	-	-	-	-
72	-	-	-	-	-
73	-	-	-	-	-
74	-	-	-	-	-
75	-	-	-	-	-
76	-	-	-	-	-
77	-	-	-	-	-
78	-	-	-	-	-
Neg.	-	-	-	-	-
Pos.	+	+	+	+	+
SGM	-	-	-	-	-

E Hatching rates of salmon eggs reared for Challenge experiment 2

Overview of flasks and the treatment the eggs received before being placed in flasks during the *F*. sp. TRD sterilization experiment: Standard sterilization: Flasks 50-55, Modified sterilization: Flasks: 56-61, Modified sterilization with *F*. sp. TRD: 62-67, Standard sterilization with *F*. sp. TRD: 68-72, Standard sterilization with Jonsvatnet water added: 73-78.

	50 (CVZ)	51 (CVZ)	52	53 (CVZ)	54	56 (CVZ)	57 (CVZ)	58	59	60	62 (CVZ)	63 (CVZ)	64	65
Date	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched
15.11.2021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19.11.2021	11	12	11	9	16	16	14	13	17	17	17	16	13	16
2021-11-22	15	17	13	15	16	16	17	16	17	17	17	16	17	16
2021-11-24	16	17	17	17	16	17	17	16	17	17	17	17	17	16
2021-11-26	17	17	17	17	17	17	17	16	17	17	17	17	17	16
2021-11-29	17	17	17	17	17	17	17	17	17	17	17	17	17	16

	66	68	69 (CVZ)	70 (CVZ)	71	72	73	74 (CVZ)	75 (CVZ)	76 (CVZ)	77	78
Date	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched	Hatched
15.11.2021	0	0	0	0	0	0	0	0	0	0	0	0
19.11.2021	9	5	9	13	10	11	10	12	11	13	15	13
2021-11-22	9	13	16	16	17	17	10	15	16	17	17	13
2021-11-24	17	13	16	16	17	17	12	17	16	17	17	14
2021-11-26	17	13	16	16	17	17	12	17	17	17	17	17
2021-11-29	17	13	17	16	17	17	12	17	17	17	17	17



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