Master's thesis

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

Anders Skovly

Immersion Challenges with Larvae of Atlantic salmon (*Salmo salar*)

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Alexander Fiedler
May 2021

NTNU
Norwegian University of
Science and Technology

Anders Skovly

Immersion Challenges with Larvae of Atlantic salmon (*Salmo salar*)

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Alexander Fiedler
May 2021

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

NTNU
Norwegian University of
Science and Technology

Abstract

It is known that commensal microbiomes can provide their animal hosts with extra resistance to bacterial infections through mechanisms such as nutrient competition, secretion of anti-microbial compounds, and interaction with the host's immune system. Recent studies using Zebrafish and Rainbow trout have shown that these fish, when raised in germ-free conditions and therefore lacking commensal microbiomes, are more susceptible to infection by *Flavobacterium columnare* compared to conventionally raised fish. This shows that the microbiome can have a protective function also in fish. Currently, there exist no such studies on Atlantic salmon. We have therefore performed three immersion challenge experiments with larvae of Atlantic salmon (*Salmo salar*), in order to test whether the presence of a microbiome in these fish provides increased resistance to bacterial pathogens. In the first immersion challenge we attempted to infect conventionally raised larvae, using two strains of *Aeromonas salmonicida ssp. salmonicida* and two strains of *Flavobacterium psychrophilum*. The strains were cultured in medium containing the iron chelator 2,2'-bipyridyl, which is thought to increase expression of certain virulence-genes. The two strains of *A. salmonicida* were cultured both in tryptic soy broth and in brain heart infusion, in order to assess whether the growth medium could affect the pathogenicity of the strains. The results of this challenge were hard to interpret as there was large variation in mortality between replicate groups and large mortality was also observed in some replicates of the control groups, thought it seemed like none of the strains could cause mortality in the larvae under our experimental conditions.

In the second immersion challenge, germ-free (GF) and conventionalized larvae were challenged with *Yersinia ruckeri* 06059, cultured both with and without bipyridyl. Among GF larvae challenged with *Y. ruckeri* cultured in bipyridyl we observed over 60 % mortality in all three replicate groups, compared to minimal mortalities in the GF control groups. However, the other groups in the challenge had results that were as hard to interpret as those of the previous challenge, with high variance between replicates and with many deaths in the controls.

In the third immersion challenge we attempted to reproduce the seemingly successful infection with *Y. ruckeri* that was obtained among the GF larvae in the second challenge. However, this time small to moderate mortalities (<50 %) were observed among GF larvae both in the challenged group and the control groups. The attempt at reproducing the previous result was therefore unsuccessful.

In addition to the immersion challenges, we tested whether the amount of *Y. ruckeri* cells in tissue samples from Atlantic salmon larvae could be quantified using qPCR. This was done by using primers specific for a region of the hom7 gene, which was assumed to not be present in bacteria other than *Y. ruckeri*. We found that sample triplicates from individual qPCRs sometimes resulted in values with very large standard deviations, and that the mean values of sample triplicates from different qPCRs sometimes were very different from each other. At best, qPCR could be used to determine the amount of *Y. ruckeri* semi-quantitatively.

Sammendrag

Det er kjent at kommensale mikrobiomer kan bidra med å beskytte deres vert mot bakterielle infeksjoner ved å bruke opp næringsstoffer, sekretere antimikrobielle forbindelser, og interagere med vertens immunsystem. Nylige studier på Zebrafisk og Regnbueørret har vist at når disse fiskene holdes i sterile miljø helt fra klekkestadiet så er de mer mottakelige for infeksjon fra *Flavobacterium columnare*, sammenlignet med fisk holdt i konvensjonelle ikke-sterile miljø. Dette viser at mikrobiomet kan ha en beskyttende effekt også i fisk. Det finnes for øyeblikket ingen slike studier av Atlanterhavslaks. Vi har derfor utført tre smittebad-forsøk med yngel av Atlanterhavslaks (*Salmo salar*), for å teste om mikrobiomer i disse fiskene gir økt beskyttelse mot bakterielle patogener. I det første smittebad-forsøket prøvde vi å smitte yngel holdt på konvensjonelt ikke-sterilt vis, med to stammer av *Aeromonas salmonicida* og to stammer av *Flavobacterium psychrophilum*. Stammene ble dyrket i medium med jernkilatoren 2,2'-bipyridyl, som antas å øke uttrykkelsen av virulens-gener. De to stammene av A. salmonicida ble dyrket både i tryptikase-soya-buljong og i hjerne-hjerte-infusjon, for å teste om vekstmediet kunne påvirke patogeniteten til stammene. Resultatet fra forsøket var vanskelig å analysere da det var høy variasjon i dødelighet mellom replikatgrupper, og stor dødelighet i noen av kontrollgruppene, som var ment å ha minimal dødelighet. Det så mest ut som om ingen av stammene hadde klart å forårsake død blant fiskene.

I det andre smittebad-forsøket ble både bakteriefri yngel og konvensjonalisert yngel forsøkt smittet med *Yersinia ruckeri* 06059, dyrket både med og uten bipyridyl. Bland den bakteriefrie yngelen som ble forsøkt smittet av *Y. ruckeri* dyrket med bipyridyl observerte vi over 60 % mortalitet i alle replikatgrupper, sammenlignet med minimal dødelighet i de bakteriefrie kontrollgruppene. De andre forsøksgruppene gikk det dårligere med, med høy varians mellom replikatgrupper og mange dødsfall i kontrollgruppene, som vi også hadde problemer med i det første forsøket.

I det tredje smittebad-forsøket prøvde vi å reprodusere det som hadde sett ut til å være en suksessful *Y.ruckeri*-infeksjon blant bakteriefri yngel i det andre forsøket. Men i dette forsøket observerte vi liten til moderat grad av dødelighet (<50 %) i bakteriefri yngel, både i smittegruppene og i kontrollgruppene. Vi fikk derfor ikke til å reprodusere det tidligere resultatet.

I tillegg til smittebad-forsøkene så testet vi også om mengden *Y. ruckeri*-celler i vevsprøver fra lakseyngel kunne kvantifiseres med qPCR. Dette ble utført ved å bruke primere spesifikke for en region av hom7-genet, som ble antatt å ikke være til stede i andre bakterier enn Y. ruckeri. Vi fant at prøvetriplikater fra individuelle qPCR noen ganger resulterte i verdier med veldig store standardavvik, og at gjennomsnittsverdiene av prøvetriplikater fra ulike qPCR noen ganger var veldig forskjellig fra hverandre. I beste fall kan qPCR brukes til å bestemme antall *Y. ruckeri* semi-kvantitativt.

Acknowledgements

The work in this project was performed with help of PhD candidate Alexander W. Fiedler. A huge thanks to Alexander for giving me a hand when work loads were heavy, for using time to provide good answers for every one of my questions, and for giving detailed feedback on the draft for the thesis. Thanks to my superviser, Professor Ingrid Bakke, for providing the opportunity to do this project, and for making sure things were going well. Thanks to Professor Olav Vadstain for his input on selection of pathogenic strains for the first immersion challenge experiment. And thanks to everyone in the research group for being great people to work together with.

Contents

Abbreviations and Definitions

Milli-Q water: Type 1 water purified by a Direct-Q 8 UV water purification system.

Buffodine: An iodine-solution used for disinfecting fish eggs.

Bipyridyl: 2,2'-Bipyridyl, also referred to as 2,2'-dipyridyl, is an iron chelator.

SGM: Salmon Gnotobiotic Medium: Artificial freshwater used in the rearing of our fish.

TSB: Tryptic Soy Broth.

TSA: Tryptic Soy Agar.

TYES: Tryptone Yeast Extract Salts.

BHI: Brain Heart Infusion.

OD_{600}: Optical Density measured at 600 nanometers.

CFU: Colony Forming Units.

PCR: Polymerase Chain Reaction.

qPCR: quantitative Polymerase Chain Reaction.

RPM: Rotations Per Minute.

ø: Diameter.

g: When used to specify the settings in a centrifuge, this is the symbol for gravitational acceleration: 9.81 m/s^2. Otherwise, this is the symbol for gram.

v/v: Volume per volume.

w/v: Weight per volume.

w/: With.

RT: Room temperature (22 ± 2°C).

DBC: Days Before Challenge

DPC: Days Post Challenge

1. Introduction

1.1 Aquaculture

Aquaculture, or fish farming, refers to breeding, rearing, and harvesting of fish (or other aquatic creatures) in controlled environments. Offshore aquaculture utilizes cages built from nets to keep the fish in place, whereas in onshore aquaculture the fish are kept in massive water tanks. It is primarily used for sustainable food production as an alternative to catching wild fish, especially when the wild catching in an area has reached maximum sustainable harvest. In fact, aquaculture is today responsible for over half of the fish products consumed globally (FAO, 2020). Salmon is one of the most important species of farmed fish, with a yearly production worth over 15 billion US dollars (Davidsen, 2018).

The greatest challenge currently facing aquaculture is disease, which constitute the main cause of economic loss in the industry. About 10 % of farmed fish succumb to infectious disease, equating a yearly loss of 10 billon US dollars (Adams, 2019).

Vaccination is one method for reducing losses due to disease. However, most vaccines must be administred by injection, and the injection process is expensive (Adams, 2019). Vaccines are not available against all pathogens and are also of no use in treating already infected fish. This can make alternative methods, such as application of antibiotics, more attractive (especially in poor countries). But it is now common knowledge that excessive use of antibiotics creates a selective pressure that drives the rise of antibiotic-resistant bacteria. For the sake of the future, it is therefore beneficial to restrict the use of antibiotics as a method of last resort. Other alternatives would therefore be useful to have at hand.

1.2 Microbiomes

A microbiota is the sum of every microorganism that lives in a particular environment. This in contrast to the term microbiome, which has been defined in somewhat varying ways. Merriam-Webster defines the microbiome as “a community of microorganisms (such as bacteria, fungi, and viruses) that inhabit a particular environment and especially the collection of microorganisms living in or on the human body”. This definition is identical to that of the microbiota.

On ScienceDirect the microbiome is defined as “a term that describes the genome of all the microorganisms, symbiotic and pathogenic, living in and on all vertebrates. The gut microbiome is comprised of the collective genome of microbes inhabiting the gut including bacteria, archaea, viruses, and fungi.” This definition emphasizes the genomes rather than the organisms themselves and restricts the term to specific environments: in or on a vertabrate host.

A third and broader definition comes from Whipps et al.: “[The microbiome] may be defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved

but also encompasses their theatre of activity". In their paper on the microbiome, Berg et al. interprets "theater of activity" as referring to the microbial environment itself along with all its microbial products, such as the macromolecules and metabolites. It also includes viruses and extracellular DNA that are present in the environment (Figure 1).

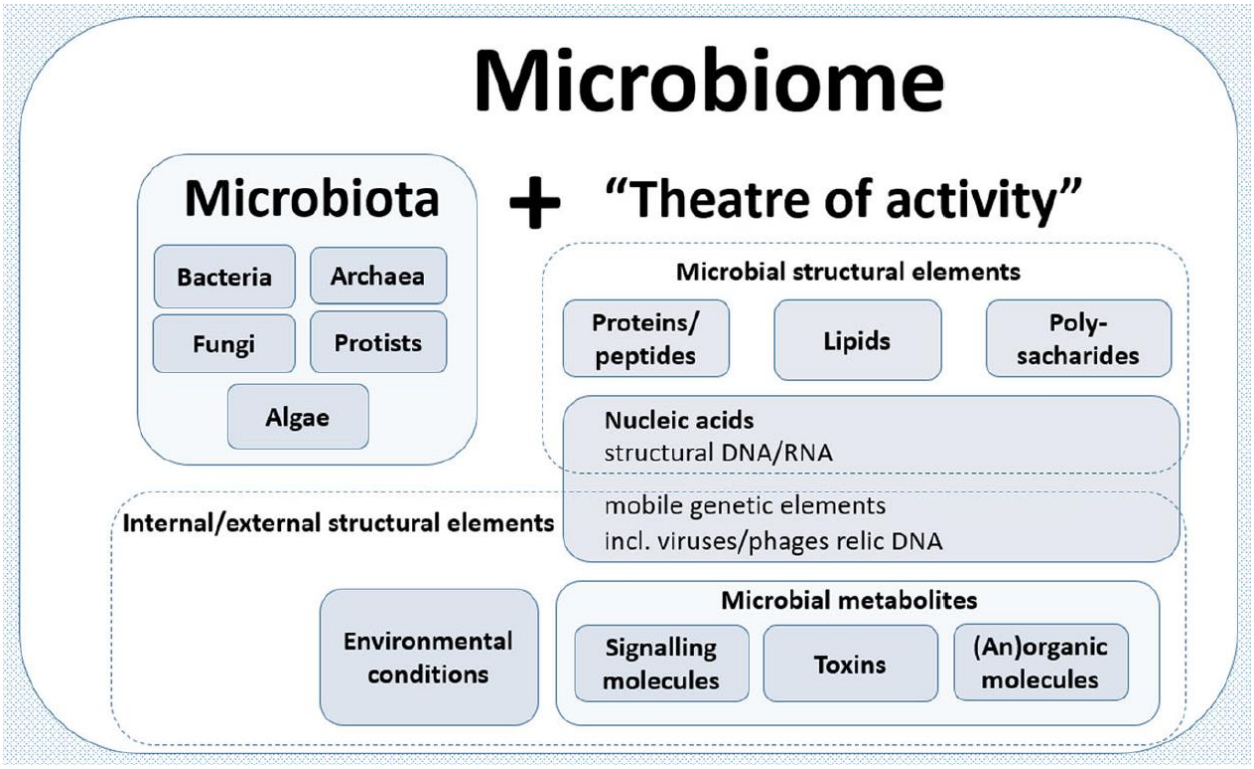

Figure 1: Illustrates all the components of a microbiome. The figure was found in the paper "Microbiome definition re-visited: old concepts and new challenges" (Berg et al., 2020)

Most animals, if not all, have a microbiota. The microbiota's effect on host fitness may be a major one, as in the case of ruminants and termites that rely on microorganisms for their digestion of complex carbohydrates, because the ruminants and termites lack the ability to produce the necessary enzymes themselves. Alternatively, there may be little or no effect on host fitness, as appears to be the case in for example the red pandas. Certain species of caterpillars and ants are found to contain either just a few permanently residing bacteria, or none at all (Hammer et al., 2019).

The microbiota can help protect its host through various mechanisms. One mechanism is nutrient competition: when commensal microorganisms consume the same limited nutrients as a pathogen, the pathogen will have less nutrients available for growth. Another mechanism is stimulation of the immune system. Certain microbes have been found to stimulate the

production of IgA from lymphoid tissue in the gut, which can bind to and inhibit pathogens. And some microbes can influence the host's T-cells into mounting an inflammatory reaction, which would aid in clearance of pathogens. A third mechanism is the inhibition of pathogen's biofilm development through use of proteases. And a fourth mechaism involves production of a variety of antimicrobial compounds, including acids that lower pH to restrict pathogen growth (Chiu et al., 2017). Microorganisms capable of butyrate fermentation can maybe aid in pathogen defense, as butyrate has been found to stimulate production of mucus in the colon (König et al., 2016).

When talking about the protecting effect of the microbiome we see that many effects depend on compounds produced by the microorganisms. The third definition of the microbiome, where the organism's "theater of activity" is a part of the definition, therefore makes the most sense as this definition includes all the protecting compounds produced by the organisms.

1.3 The Microbiome in Fish

Most fish, with some exceptions like the guppy, are egg-laying animals. This means that fish larvae are isolated from the environment by the outer barrier of the egg until they hatch. They therefore remain sterile until this point. When the fish hatch from their eggs, colonization of their skin by bacteria present in the water and on the surface of the eggs can begin immediately. Some time after hatching the fish will open their mouth for the first time, at which point colonization of their gut also begins. Initial colonization of fish therefore differs from the process in humans and other mammals, where colonization begins during the birth and is later also influenced by the mother's milk, of which there is no equivalent in fish (Yan et al., 2016).

The composition of the microbiota in fish depends on multiple factors. The bacteria present in the water and able to encounter the fish is obviously one factor. Diet, age, gender, and genetics also have an influence, as does the composition of the mucus that covers the skin and gills (Vatsos, 2016).

1.4 Studying the Microbiome Using Germ Free Fish

As mentioned earlier, fish larvae are germ free until they emerge from their eggs and are colonized by bacteria from the environment. This means that by sterilizing the surface of the eggs and keeping them in germ free water, the larvae will also be germ free after hatching. Because the larvae get their nutrition from their yolk-sac, they do not require food until their yolk-sac is drained empty. In the case of Atlantic salmon larvae this process takes about ten weeks. During these weeks, the larvae can be kept germ free by storing them in closed vessels, with filters allowing for diffusion of oxygen and carbon dioxide in and out of the vessel. Water quality can be maintained through regular replacement of old water by sterile fresh water, performed in a sterile environment.

The effects of the microbiome in fish larvae can therefore be studied through experiments on germ free larvae and colonized larvae. When assuming that the presence or absence of the

microbiome is the only variable that distinguish the two groups, any significant difference in the experimental outcome between the groups can be attributed to the microbiome.

Stressmann et al. recently published a study on infection in germ free and colonized zebrafish, using *Flavobacterium columnare* as the invading pathogen. They reported that a collection of ten species of bacteria were able to protect the zebrafish against infection (Stressmann et al., 2020). Another recent study comes from Perez-Pascual et al., who stated germ-free Rainbow trout was found to be "extremely sensitive to infection by *Flavobacterium columnare*", and that a commensal *Flavobacterium* strain provided the fish resistance against infection by the *F. columnare* (Pérez-Pascual et al., 2021).

On the other hand, Situmorang et al. reported that absence or precence of a microbiota had no significant effect on survival when Nile tilapia larvae were challenged with the pathogen Edwardsiella ictaluri (Situmorang et al., 2014).

1.5 Immersion challenges

When attempting to infect an animal with a pathogen it is said that the animal is challenged with that pathogen. When attempting to infect fish the common types of challenge used are immersion (or bath) challenges, cohabitation challenges, and injection challenges. In an immersion challenge, the fish are immersed in water containing the given pathogen. In a cohabitation challenge, healthy fish and fish suffering infection from the given pathogen are placed together in the same water tank. The pathogen might then transmit through direct contact between healthy and sick fish, or they might transmit indirectly through the water. In an injection challenge the pathogens are injected into fish by use of a syringe.

Injection challenges in fish tend to yield reproducible results with high rates of mortality among the fish. A contributing factor to this, if not the only factor, is that syringes bypass the epithelial barrier that protects fish against the outside environment, including outside pathogens (Zhou et al., 2013). This also means that the microbiome on the epithelial surface is bypassed. Because of this, injection challenges are not suited when the goal is to investigate the influence of the microbiome on infection. Between immersion challenges and cohabitation challenges, the latter is more difficult to perform as it requires access to fish that are already suffering an infection, while immersion challenges only require access to cultures of the pathogen.

Several immersion challenges with *Y. ruckeri* (mainly strain NVI-11025) and Atlantic salmon larvae was conducted by Martha Drågen, where the influence of various factors on infectivity were tested (Drågen, 2019, 2020). An experiment with concentrations of *Y. ruckeri* ranging from 10^5 CFUs per mL to $>10^8$ CFUs per mL indicated that a concentration of at least 10^8 might increase the mortality compared to lower concentrations, but the increase in mortality was not replicable, and the untreated control fish also suffered 100 % mortality. The same experiment also compared infection in larvae that were two weeks old with larvae that were six weeks old and found that the older fish were more susceptible to infection.

Another of her experiments tested the impact of stressing the fish with formalin or hydrogen peroxide before the challenge. Both treatments resulted in one duplicate with 100 % mortality among the larvae, and another duplicate with zero mortality. Attempting to increase infectivity of the pathogen by adding homogenized fish into the water at the point of challenge was also tried but resulted in zero deaths among the larvae. As in the previous experiment, untreated control larvae suffered 100 % mortality.

A third experiment on the effect of scarring the larvae before challenge with *Y. ruckeri* resulted in many deaths the scarred larvae but also among the control larvae, so the scarring did not seem to have any effect in infection.

Her last experiment was a challenge where temperature (14°C vs 16°C and the "microbial status" were investigated. The latter refers to whether the fish were conventionally raised (colonized by bacteria present on the eggs), conventionalized (colonized by bacteria from lake water), or germ-free (eggs had been surface sterilized). The pathogen used was *Y. ruckeri* 06059. The results from this experiment indicated that *Y. ruckeri* might be capable of infecting conventionalized and germ-free larvae, but only at 16°C. The mortality percentages in triplicates ranged from 0 % to just 30 %, meaning that the numbers were both low and inconsistent. Among the conventionally raised fish the mortality percentages were higher in the control groups than in the groups of larvae treated with Y. ruckeri.

Along with formalin, hydrogen peroxide, and scarring, another method that might increase mortality among the challenged fish is to culture the pathogen(s) in medium containing bipyridyl before their addition to fish rearing water. Bipyridyl is an iron chelator used to reduce the availability of iron in the bacterial growth medium, making the medium more like the conditions inside a fish, where most iron is bound by proteins and not available for uptake. Pathogens have been found to respond to iron-limitation in the environment by upregulating expression of proteins involved in host invasion. For example, Lages et al. used transcriptome-analysis to show that in the fish pathogen Vibrio anguillarum "virulence-related factors were up-regulated under low iron compared to high iron ... although two exceptions were found" (Lages et al., 2019). Culturing the bacteria with bipyridyl is intended to prime the bacteria for infection by initiating such a change in protein expression before the bacteria are added to the water of the fish. Beck et al. reported that in an experiment involving fingerling channel catfish challenged with two different strains (LV and LSU) of Flavobacterium columnare, the culturing of this bacteria in medium with bipyridyl caused significant increase in mortality in the case of strain LV, but not in the case of LSU (Beck et al., 2016).

1.6 Yersinia ruckeri

Yersinia ruckeri is the bacterium responsible for enteric redmouth disease (ERD, also known as yersiniosis), a disease of salmonids such as Atlantic salmon. It is one of many pathogens of the *Yersinia* genus, with some others being *Y. pestis* (responsible for the plague) and *Y. pseudotuberculosis* (one of the bacteriums responsible for pseudotuberculosis.
Y. ruckeri is a rod-shaped, Gram-negative facultative anaerobe and has a chromosome of 3.7 megabases containing about 3500 genes. The bacterium was first isolated in the 1950s from Rainbow trout in the Hagerman Valley, Idaho, USA. Since then, it has been isolated around the world from many other species of fish as well (Kumar et al., 2015).

Strains of *Y. ruckeri* are divided into four serotypes (where each serotype represents a different set of surface antigens), some of which also have subgroups. The strains are also divided into two biotypes (each biotype represents a group of strains sharing certain genes), with biotype 1 being those capable of motility and lipase secretion, and biotype 2 being those capable of neither. Most outbreaks of ERD are caused by strains classified as biotype 1 and serotype O1a (Kumar et al., 2015). However, biotype 2 appears to be becoming more common. It is assumed that this is because biotype 2 does not have any flagella that vaccinated fish can mount an immune reaction against, which would make vaccines a less effective preventative measure against this biotype (Hjeltnes B, 2019).

Enteric redmouth disease tends to be acute in young fish and chronic in adult fish. Outbreaks of ERD typically have a low mortality rate early on, but over time the loss of fish can become very significant (Kumar et al., 2015).

Y. ruckeri enters the bloodstream of fish through the gills, and spreads with the blood to the intestine and kidneys at first. Later it also spreads to the brain, the liver, the heart, and the spleen. Infected fish are characterized by the color of the mouth and skin becoming darker, and these areas are also prone to bleeding. Bleeding may also occur on the surface of certain internal organs, and the spleen can become larger and darker. A turbid yellow-looking fluid can accumulate in the intestine (Kumar et al., 2015).

Y. ruckeri 06059 is a strain that has previously been used successfully in an immersion challenge, where Atlantic salmon larvae (average weight 0.4 grams) were challenged at temperatures of 12°C and 16°C. The density of larvae was 25 larvae per 5 L fish tank. About 10^7 CFUs of *Y. ruckeri* were added per mL rearing water. The challenge lasted 4 hours, and deaths were recorded for 17 days. Mortalities were 84 % and 64 % in two replicates challenged at 16°C. At 12°C the mortailties were substantially lower. There were no deaths in the control group (Haig et al., 2011).

1.7 Flavobacterium psychrophilum

Flavobacterium psychrophilum is the bacterium responsible for bacterial coldwater disease (BCWD, also known as flavobacteriosis or rainbow trout fry syndrome). This is another salmonid

disease, with Rainbow trout and Coho salmon as particularly susceptible species (Barnes, 2011). The first time *F. psychrophilum* was isolated was in 1960, from a Coho salmon in the USA (NVI, 2021a). It has since then, as with *Y. ruckeri*, been isolated from fish across the world (Barnes, 2011).

In the laboratory, the *F. psychrophilum* is a slow-growing species of bacteria that require a specialized growth medium, such as TYES (Tryptone Yeast Extract Salts). One source reported that fastest growth happens at 15°C (Cain & LaFrentz, 2007) while another source claimed that this happened at 18 to 20°C (Barnes, 2011). When cultured on agar plates it forms deeply yellow colonies, while under a microscope they appear as long rods capable of gliding motility (Barnes, 2011). It is Gram-negative, strictly aerobic, and has a chromosome of 2.9 megabases containing about 2400 genes (Duchaud et al., 2007).

Skin injuries are believed to be the main entry point of *F. psychrophilum* into fish. Presence of ectoparasites on the fish, and/or poor water quality, may be factors that increase the infectiousness of *F. psychrophilum* (Barnes, 2011). Disease in most common in fish reared in water with a temperature between 4 and 10°C, but the disease is most deadly in waters with a temperature of 15°C, closer to the pathogen's optimal growth temperature (Barnes, 2011). Infected salmonid fish are characterized by necrosis in the tail fin and by ulcers on the skin, among other things (NVI, 2021a).

1.8 Aeromonas salmonicida

Aeromonas salmonicida are short rod-shaped bacteria. They do not form spores and are gram-negative facultative anaerobes. *A. salmonicida* is divided into five subspecies, with *A. salmonicida ssp. salmonicida* referred to as the typical subspecies and the other four subspecies referred to as atypical. Most strains of the subspecies *salmonicida* are non-motile and can produce a characteristic brown pigment when cultured on agar medium containing tyrosine or phenylalanine, such as tryptic soy agar (CABI, 2019). The chromosome consists of 4.7 megabases with about 4400 genes (Reith et al., 2008).

The first time *A. salmonicida* was isolated was in the late 1800's, from brown trout in a german hatchery (CABI, 2019), making it the first bacteria to become associated with disease in fish. The bacteria have been isolated all over the world, although strains of the subspecies *salmonicida* have not yet been isolated in South America, Australia, or New Zealand (Boily et al., 2019).

Strains of the typical subspecies are the best studied and are responsible for furunculosis (sometimes referred to as typical or classical furunculosis), a disease among the salmonids. Strains of atypical subspecies can cause various other diseases in many species of fish, both salmonid and non-salmonid (A.W.E., 2020).

Furunculosis can be peracute, acute or chronic, and occurs most frequently when the temperature is above 10°C (NVI, 2021b). The peracute form mainly affect younger fish larvae and fingerlings and is characterized by rapid death. The fish may have bulging eyes and/or darker

color. The acute form affects mainly parrs, smolts, and young adults. It is characterized by bleeding from the fins and the mouth, along with a darkening of the skin. The fish tend to die after a couple days. The chronic form is most common in older adult fish, and can have many symptoms, but the most typical symptom is the development of furuncles, which are described as "dark raised tumefactions (swellings) containing serosanguinous fluid (blood or serum) and necrotic tissue in the musculature" (Boily et al., 2019). Heart damage is a possible cause of death. A study that used bioluminiscence imaging to track the colonization sites of *A. salmonicida* in rainbow trout during an immersion challenge found that initial colonization happens in the gills and in the dorsal and pectoral fin (Bartkova et al., 2017). Further details on how the bacteria spreads through its host appear to be unknown.

Strains of *A. salmonicida* might be either glutinating or non-agglutinating. The agglutinating strains contain membrane proteins referred to as the A-layer (Sakai, 1985), and these strains also agglutinate (clump together) when suspended in a 0.9 % natrium chloride solution (McCarthy, 1983). Virulent strains of *A. salmonicida* are found to be agglutinating and to contain an A-layer, while avirulent strains are non-agglutinating and do not have an A-layer. It is theorized that the A-layer aid the bacteria in attachment to hosts (Sakai, 1985). Expression of the A-layer depends on the culture medium of the bacteria: it was found that culturing in brain heart infusion leads to a high degree of agglutination, whereas culturing in TSB leads to a low degree of agglutination (McCarthy, 1983).

A. salmonicida VI-88/09/03175 is a strain that has been successfully used in an immersion challenge. Atlantic salmon (average weight 20 grams) were challenged for 45 min at a temperature of 12°C, with 10^7 CFUs of A. salmonicida added per mL rearing water. The density of fish was 50 fish per 30 L water tank. Deaths were recorded for three weeks after the challenge. Mortality was at least 75 % is all six challenged groups (Nordmo & Ramstad, 1997).

1.9 Aim of This Project

This project was part of a PhD-thesis where the goal is to determine whether the commensal microbiome in Atlantic salmon increases the salmon's resistance towards bacterial infection, and thereafter to show that pathogen-specific phages are a better treatment option for infected salmons compared to non-specific antibiotic treatments that also damages the commensal microbiomes of the fish.

The main aim was to develop a protocol for reproducible pathogen infection of Atlantic salmon larvae through an immersion challenge, and to compare the extent of infection among groups of germ-free and conventionally raised larvae, to see if the presence of a microbiome made any difference.

In addition, we wanted to see whether qPCR could be used to accurately quantify the number of *Y. ruckeri* cells that were present in homogenized tissue samples from Atlantic salmon larvae reared in water containing *Y. ruckeri*, in order to determine the degree of Y. ruckeri colonization in the tissues.

2 Methods

2.1 Bacterial Strains and their Storage

Yersinia ruckeri 06059 was provided by Tim Wallis at Ridgeway Biologicals Ltd. (United Kingdoms). *Aeromonas salmonicida* ssp. *salmonicida* VI-88/09/03175 and 11540 were provided by Duncan Colquhoun at the Norwegian Veterinary Institute. *Flavobacterium psychrophilum* DSM 3660 was purchased from the German Collection of Microorganisms and Cell Cultures. Flavobacterium psychrophilum NCIMB 13383 was provided by Gunhild Hageskal at SINTEF (Norway). Janthinobacterium sp. MM5 had been isolated in our lab from the skin of Atlantic salmon larvae (kan ikke finne at denne ble isolert i Hanne's master thesis).

Y. ruckeri 06059 was originally isolated in the United Kingdoms in 2006 from Atlantic salmon and has previously been used successfully in an immersion challenge with Atlantic salmon larvae (Haig et al., 2011). *A. salmonicida* VI-88/09/03175 was originally isolated in Norway from Atlantic salmon suffering from furunculosis and has also previously been used successfully in an immersion challenge with Atlantic salmon (Nordmo & Ramstad, 1997). *A. salmonicida* 11540 was originally isolated from a wild, sick fish in Trøndelag, Norway in 2017 (Ref: Duncan Colquhoun 2020, personal communication) and has not yet been used in an immersion challenge.

F. psychrophilum NCIMB 13383 was originally isolated in Denmark from a sick Rainbow trout suffering from bacterial coldwater disease (Lorenzen, 1997). It was used in one immersion challenge that resulted in no mortalities (Lorenzen et al., 2010). However, the aim of this particular immersion challenge was not to make the fish so sick they would die, but rather to try to make them immune against the pathogen, and then perform an injection challenge later on to test for immunity. Challenge condition might therefore not have been optimal in regard to inducing serere disease. F. psychrophilum DSM 3660 was originally isolated in USA in 1948 from a sick Coho salmon and has been used successfully in an immersion challenge (Aoki et al., 2005).

All strains were stored as cryo cultures at -80 °C in a glycerol solution (25% glycerol in the case of F. psychrophilum, 50% glycerol for all other strains). Bacteria from cryo cultures were streaked out on agar plates before use.

To verify the identity of our strains, the v3-v4-region of their 16S rRNA gene was Sanger sequenced (section 2.3), with universal primers 338F and 805R being used in the DNA amplification step. Once the sequencing data was returned, the sequencing quality was checked on the chromatograms, and the genus of each bacterium was determined by using RPD Classifier v11 (Wang et al., 2007) (sequence data given in Appendix X).

2.2 Culturing of Bacteria

Strains of *Y. ruckeri*, *A. salmonicida*, and *J.* sp. MM5 were cultured aerobically in TSB (3 mL) at 20°C and 120 RPM, and on TSA plates at 20°C. Strains of *A. salmonicida* were also cultured aerobically in BHI (3 mL) at 20°C and 120 RPM. Strains of *F. psychrophilum* were cultured aerobically in TYES broth (3 mL) and on TYES agar plates at 15°C and 140 RPM. The recipes for

TSB, TYES broth and BHI are given in Table 1. TSA and TYES agar were made by adding agar (15 g/L, VWR) to TSB and TYES broth.

In some experiments, *Y. ruckeri* and *A. salmonicida* were also cultured in TSB w/ bipyridyl. *A. salmonicida* was additionall cultured in BHI w/ bipyridyl. *F. psychrophilum* was cultures in TYES broth w/ bipyridyl. Culture conditions were the same as in the previous paragraph.

Liquid cultures were routinely subcultured by transferring 5 % (v/v) of an outgrown culture into fresh sterile medium. Agar plate cultures were routinely subcultured by picking a single colony from a plate and streaking onto a fresh agar plate.

Table 1: Recipes for bacterial growth media. All three media were made in a variant with bipyridyl and a variant without. Media were made by dissolving the ingredients in Milli-Q water, followed by autoclaving (20 min at 121 °C, Astell AMA270BT65) before use.

Medium	Ingredient	Weight (g/L)	Manufacturer
TSB	Tryptic Soy Broth	30	Sigma-Aldrich
(w/ bipyridyl)	(2,2'-bipyridyl)	(0.0156)	(Sigma-Aldrich)
TYES broth (w/ bipyridyl)	Magnesium Sulfate Heptahydrate	0.5	Sigma-Aldrich
	Calcium Chloride Dihydrate	0.5	Sigma-Aldrich
	Yeast Extract	0.4	Oxoid
	Tryptone	4	VWR
	(2,2'-bipyridyl)	(0.0078)	(Sigma-Aldrich)
BHI	Brain Heart Infusion	37	Merck
(w/ bipyridyl)	(2,2'-bipyridyl)	(0.0156)	(Sigma-Aldrich)

2.3 Sanger Sequencing

To sequence DNA from bacteria, we first filled microcentrifuge tubes with 1 mL bacterial culture and centrifuged the tubes (13 000 g, 1 min). The supernatants were discarded, and the bacterial pellets were resuspended in powerbead tubes from the Qiagen DNeasy PowerSoil kit. The DNA was extracted according to Qiagen's kit instructions. A sequence of the DNA extract was amplified with PCR (section 2.3). To confirm successful amplification and purity of the PCR product, the product was run through an agarose gel (section 2.4).

PCR products were purified using the Qiagen QIAquick PCR Purification Kit, according to Qiagen's kit instructions. 30 µL PCR grade water was used in the elution step. For sequencing, 5 µL of each purified product was mixed with 5 µL of a 5 µM primer in a PCR tube (primer 338F or 1492F depending on which we used in the amplification), and the tubes were sent to Eurofins Genomics for Sanger sequencing.

2.4 PCR-Amplification of the 16S rRNA Gene

DNA was PCR-amplified by preparing reactions as in Table 2 and running the reactions in a thermal cycler (Bio-Rad T100) with the program specified in Table 3.

Table 2: Ingredients for PCR. Total reaction volume was 25 µL. The exact forward and reverse primer used is specified in other sections, as different primers were used in different amplifications.

Ingredient	Volume (µL) per reaction
PCR grade water (VWR Life Science)	16.82
5x Phusion HF-buffer (Thermo Scientific)	5
Forward primer EUB8F or 338F (10 µM)	0.75
Reverse primer 805R or 1492R (10 µM)	0.75
dNTP mix (10 mM of each dNTP, VWR Life Science)	0.5
Phusion HotStart II DNA polymerase (Thermo Scientific)	0.18
Sample DNA	1

Table 3: PCR program for amplification of bacterial 16S rRNA gene.

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	60	
Denaturation	98	15	
Annealing	55	20	36
Elongation	72	20	
Final elongation	72	300	
Cooling	10	Hold	

2.5 Agarose Gel Electrophoresis

Gel electrophoresis was used to check whether amplification of DNA by PCR had been successful. 4 µL PCR product was mixed with 1 µL 6x DNA Loading Dye (Thermo Scientific). The mix was loaded on an agarose gel (1 % w/v agarose dissolved in TAE buffer by microwave-boiling, stored at 60°C) covered in TAE buffer. The gel contained GelRed nucleic acid stain (10 000x, Biotium, 5 µL GelRed per 100 mL gel). A DNA ladder (GeneRuler 1 kb Plus, Thermo Scientific) was also loaded on the gel. The loaded PCR products were run through the gel using a voltage of 110 V for about an hour and were then visualized with an UV imaging device (Syngene G:BOX).

TAE buffer was prepared as a 50x stock solution by dissolving 242 grams of Tris-base (VWR) in 700 mL distilled water, then adding 57.1 mL glacial acetic acid (VWR) and 100 mL 0.5 M EDTA

(VWR). Distilled water was added to make the final volume 1 L, and the solution was autoclaved (20 min at 121 °C, Astell AMA270BT65). The 50x stock solution was diluted 1:50 with Milli-Q water before use.

2.6 Bacterial Growth Curves

To determine how fast our pathogenic strains would reach their maximum density in liquid culture we plotted growth curves for each strain.

Two different methods were used in creation of the curves. With the first method, 100 mL conical flasks were filled with 40 mL fresh sterile medium. Outgrown culture was transferred to a flask (5 % v/v). The OD_{600} of samples (750 µL) from the flask were measured in plastic cuvettes at intervals using a spectrophotometer (Hitachi U-5100) until the bacteria reached the stationary phase. Fresh sterile medium was used as blank reference.

With the second method, 15 mL glass culture tubes were filled with 3 mL fresh sterile medium. Outgrown culture was transferred to a tube (5 % v/v). The OD_{600} was measured in the tube by placing the tube directly into the spectrophotometer. This was done at intervals until the bacteria reached the stationary phase. Fresh sterile medium in a glass tube was used as blank reference.

The OD_{600} measured in cuvettes was expected to differ from the OD_{600} measured in glass tubes, because the two have different sizes, shapes, and materials. Therefore, an experiment was conducted to compare the results generated by the first and second method.

The OD_{600} was measured in a 15 mL glass tube containing 3 mL culture. 750 µL of the culture was then transferred to a cuvette, and the OD_{600} of the cuvette was measured. The ratio of OD_{600} between the glass tube and the cuvette was calculated. Then, 750 µL fresh medium was added to the glass tube, resulting in a dilution of the bacterial density.

The whole process of comparing the OD_{600} and diluting the culture was now repeated nine times, so that we ended up with ten OD_{600} ratios, each taken with a different bacterial density.

2.7 Determining the Relationship between OD and CFUs in Bacterial Cultures

Cultures of *A. salmonicida* and *F. psychrophilum* (cultured without bipyridyl) were diluted to an OD_{600} of approximately 1 using a spectrophotometer (Hitachi U-5100). The OD_{600} values were recorded.

An aliquot from each culture was serially diluted 1:10 seven times. From the 10^{-5}, 10^{-6} and 10^{-7} dilutions triplicate aliquots (70 µL) were pipetted onto agar plates (TSA for *A. salmonicida*, TYES agar for *F. psychrophilum*) topped with roughly twenty sterile glass beads (ø = 4 mm). The plates were shaken horizontally for at least ten seconds, ensuring the bacteria were evenly distributed over the plates. The beads were collected in a beaker and were later autoclaved and washed for reuse. The TSA plates were incubated at RT (room temperature), while the TYES agar plates were incubated at 15 °C.

To increase the amount of data, the cultures of OD 1 were diluted to approximately OD 0.75, 0.50, and 0.25, and the procedure in the last paragraph was repeated for each dilution. It was assumed that OD and cell density has a linear relationship for OD values in the range 0.1 to 1.0.

After a few days, when clearly visible colonies had appeared on the plates, the colonies were counted. Plates where individual colonies were hard to distinguish from each other (meaning plates with roughly >300 colonies) were discarded. From each CFU count, the theoretic number of bacteria per 1 mL at OD 1.0 was calculated according to Equation 1. The mean of these numbers was taken as our best estimate of the actual number of bacteria per 1 mL at OD_{600} = 1.0.

$$Bacteria\ per\ 1\ mL\ (OD600 = 1.0) = \frac{Colony\ count * 1000\ \mu L}{Dilution\ factor * Recorded\ OD600 * 70\ \mu L} \quad (1)$$

2.8 General Handling of Atlantic Salmon Eggs and Larvae

Atlantic Salmon eggs to be used in the experiments were ordered from AquaGen Hemne AS. For each experiment we ordered a larger number of eggs than what was needed, to account for damaged eggs and premature death of fish larvae. The temperature in the fish lab was 6.0 ± 0.4 °C. To minimize stress on the fish they were kept in the dark, except when they were being worked with.

Generally, the protocol established by Gomez et al. (manuscript in preparation) was followed for the handling and derivation of the Atlantic salmon eggs and larvae.

The eggs were delivered a week before their expected hatching date. Once received, the damaged or prematurely hatched eggs were discarded, and the intact eggs were distributed into petri dishes (ø = 140 mm) using sterile forceps, with up to 100 eggs per dish. The eggs were covered with SGM (Salmon Gnotobiotic Medium, Appendix A) for acclimation. The dishes were swirled to wash the eggs with the medium, to homogenize the bacteria on the eggs.

From this point onwards, all work with the eggs (and later, with the fish larvae) was performed in a type II biological safety cabinet, to prevent the eggs (and fish larvae) from being contaminated by bacteria from the air. Non-biological items, such as pipettes, were cleaned with ethanol (70 %) before being placed in the cabinet and were then treated with UV light (30 min) before use. Afterwards the biological items, such as fish flasks, were also cleaned with ethanol before being placed into the cabinet. Each time work in the cabinet was done, the cabinet was cleaned with ethanol and treated with UV-light before the next use.

After 24 hours, tissue culture flasks (250 mL, non-treated, sterilized, with vent caps, VWR) were filled with 100 mL SGM. The eggs were transferred into the flasks using sterile forceps, with 17 eggs per flask (except the last flask, which got any number of remaining eggs).

Every Monday, Wednesday, and Friday, 60 mL SGM was removed from each flask by using sterile 50 mL (max 60 mL) pipettes. Fresh sterile SGM was poured into the flasks until the 100 mL mark was reached. The removed SGM was collected in a large plastic beaker (5 L). At the end of the SGM replacement, 150 mL concentrated chlorine was added to the removed SGM, and spring water was added to bring the volume up to 4 L. The chlorine solution was left in the beaker for at least an hour for complete disinfection before it was discarded into the drain.

During the routine SGM replacement, any dead fish were also removed by using sterile 10 mL pipettes to grab onto their yolk sacs. The number of dead fish in the flasks was recorded in a log, along with the date of the deaths.

When the majority of all the eggs had hatched, the date was recorded as the “hatching day”. Any eggs that had not hatched some days after this date were assumed dead and were removed.

Five weeks after hatching, the number of fish in each flask was adjusted to fifteen by transfering from flasks with more fish to flasks with less. Transfers were done by grabbing fish at their yolk sac with a 10 mL pipette.

Immersion challenge experiments started the day when the fish larvae had reached six weeks old. On this day, pre-challenge CFU counts was prepared using water samples from all flasks except those supposed to be sterile (section 2.10). Bacterial doses were prepared (section 2.11) and were then added to the flasks. Afterwards, post-challenge CFU counts were prepared for all flasks except those supposed to be sterile. The CFU counts were used to check how many bacteria had been added to the flasks.

All challenge experiments lasted three weeks. During this time, the fish larvae were checked twice a day: once in the morning and once in the evening. If any larvae were dead, they were removed and the deaths were logged, as done before infection. At the end of every challenge the remaining live fish was euthanized by immersing them for over a minute in tricaine solution (see Appendix E).

2.9 Derivation of Atlantic Salmon Eggs

In two of the three immersion challenge experiments, a special procedure was performed to sterilize the fish eggs of any microorganism found on their surface. This was done by submerging the eggs in an antibiotic solution for 24 hours, then submerging them in a 1:200 diluted buffodine solution for 30 minutes. This egg surface sterilization, referred to as “derivation”, happened after the 24 hours of acclimatization to SGM in the petri dishes and before the distribution of eggs into flasks (see previous section).

The SGM in the dishes was removed by pipetting, and the eggs were covered with antibiotic SGM (Appendix B). The dishes were swirled to wash the eggs with the antibiotics.

After 24 hours of antibiotic treatment the dishes were placed into a type II biological safety cabinet. 50 mL conical tubes were filled with 49.75 mL SGM and 250 µL buffodine solution

(FishTech AS, 1.11 % (w/v) iodine), and the solutions were mixed by inverting the tubes, resulting in a 1:200 diluted buffodine solution in each tube.

Seventeen eggs were transferred into an empty conical tube using a sterile forceps and 50 mL diluted buffodine was poured onto the eggs for thirty minutes. The tubes were gently moved back and forth every three minutes, to wash the surface of the eggs with buffodine. After thirty minutes the buffodine was discarded and the eggs were washed with SGM four times. They were then poured into a flask containing 100 mL SGM.

2.10 CFU Counts of Rearing Water

CFU counts were performed with samples of fish rearing water to determine the number of bacteria that were added in the immersion challenges. A sample from each fish flask was serially diluted in steps of 1:10 up to seven times for a final dilution of 10^{-7}. 70 µL of dilutions were pipetted onto agar plates (TYES agar if *F. psychrophilum* was to be added to the given flask, TSA if *A. Salmonicida*, *Y. ruckeri* or *J. sp.* MM5 was to be added) topped with roughly twenty glass beads (ø = 4 mm). The plates were shaken horizontally for at least ten seconds, ensuring the bacteria were evenly distributed over the plates. The beads were collected, autoclaved, and washed for reuse. The TYES agar plates were incubated at 16 °C, while the TSA plates were incubated at RT.

2.11 Preparation of Bacterial Doses for the Immersion Challenges

Late exponential phase bacterial cultures grown with bipyridyl were (if necessary) diluted to an OD_{600} of 1.0, which for *A. salmonicida* corresponded to approximately 10^8 CFUs per mL, and for *F. psychrophilum* and *Y. ruckeri* corresponded to approximately 10^9 CFUs per mL (Drågen, 2020). 1 mL of cultures were transferred into 1.5 mL microcentrifuge tubes and centrifuged at max speed for 5 min. The culture media were discarded, and the bacterial pellets were resuspended in 1 mL SGM to wash away remains of the medium. The bacteria were again centrifuged, the SGM discarded, and the pellets were again resuspended in SGM, now ready for transfer into the flasks.

2.12 Sterility Checks of Rearing Water

Water samples (150 µL) from the fish flasks were pipetted onto TSA plates and into four different liquid media (3 mL of each) (recipes in Table 4). The plate and the tubes were incubated at room temperature for two weeks. Through these weeks the tubes and plates were checked multiple times, and if colonies or turbidity was noticed, the source flask was considered contaminated.

Table 4: Recipes for media used in sterility checks. Media were made by dissolving the ingredients in Milli-Q water, followed by autoclaving (20 min at 121 °C, Astell AMA270BT65) before use.

Medium	Ingredient	Weight (g/L)	Manufacturer
BHI	Brain Heart Infusion	37	Merck
GYE broth	D-(+)-Glucose	10	Sigma-Aldrich
	Yeast Extract	2.5	Oxoid
SD broth	Sabourad 2 % Dextrose Broth	30	Merck
NB	Nutrient Broth	8	VWR

2.13 First Immersion Challenge - Attempting to Infect Atlantic Salmon Larvae with *A. salmonicida* and *F. psychrophilum*

In the first immersion challenge experiment four different pathogenic strains (Table 5) were tested for ability to infect CR (conventionally raised) Atlantic salmon larvae. For the *A. salmonicida* strains we wanted to test if ability to infect the fish was influenced by the growth medium used to culture the bacteria before challenge. These two strains were therefore cultured in two different media (TSB and BHI). In addition, we also had one control group with a non-pathogenic strain (*Janthinobacterium sp.* MM5) and one control group with no bacteria added. All strains were cultured in medium with bipyridyl, but *A. salmonicida* VI-88/09/03175 did not grow in TSB w/ bipyridyl on the day of challenge. A culture from TSB without bipyridyl was therefore used as replacement.

Table 5: The eight different groups used in the first immersion challenge experiment.

Treatment Name	Microbial Status of Fish	Bacterial Strain Added	Bacterial Culture Medium
CR_ASAL_03175_TSB	CR	*Aeromonas salmonicida* ssp. *salmonicida* VI-88/09/03175	TSB
CR_ASAL_03175_BHI_BP	CR	As above	BHI w/ bipyridyl (100 µM)
CR_ASAL_11540_TSB_BP	CR	*Aeromonas salmonicida* ssp. *salmonicida* 11540	TSB w/ bipyridyl (100 µM)
CR_ASAL_11540_BHI_BP	CR	As above	BHI w/ bipyridyl (100 µM)
CR_FPSY_3660_BP	CR	*Flavobacterium psychrophilum* DSM 3660	TYES w/ bipyridyl (50 µM)
CR_FPSY_13383_BP	CR	*Flavobacterium psychrophilum* NCIMB 13383	TYES w/ bipyridyl (50 µM)
CR_JMM5_BP	CR	*Janthinobacterium sp.* MM5	TSB w/ bipyridyl (100 µM)
CR_NO_BAC_ADDED	CR	None	---

680 eggs were ordered. When the eggs arrived, they were distributed into fourty flasks and handled as described in section 2.8 until the start of the immersion challenge (six weeks past hatching).

At 11 DBC (days before challenge), all strains used in the challenge were transferred from cryo stocks to agar plates (TYES agar for strains of *F. psychrophilum*, TSA for *Janthinobacterium sp.* MM5 and strains of *A. Salmonicida*).

At 7 DBC, bacterial colonies were transferred from agar plates into liquid media without bipyridyl (TYES broth for *F. psychrophilum*, TSB for *A. salmonicida* and *Janthinobacterium sp.* MM5, with *A. salmonicida* also transferred into BHI). The temperature in the fish lab was increased from 6.0°C to 8.0°C. The fish flasks were changed from a standing to a lying position to increase the surface area of the water, increasing the rate of oxygen diffusion into the water. Each of the two following days the temperature was increased by +2°C, giving a challenge temperature of 11.8 ± 0.3°C.

At 3 DBC and 2 DBC, the two strains of *F. psychrophilum* were transferred from TYES broth into TYES broth w/ bipyridyl (50 µM). At 2 DBC and 1 DBC, *Janthinobacterium sp.* MM5 and the two

strains of *A. salmonicida* were transferred from TSB into TSB w/ bipyridyl (100 µM). The two strains of *A. salmonicida* were also transferred from BHI into BHI w/ bipyridyl (100 µM). Transfer of the bacteria at multiple time points was done to make sure at least one culture would be in the late exponential growth phase upon addition to the fish flasks.

24 flasks were used for the experiment, three flasks for each of the eight groups. Two other flasks were used to measure the length and weight of the fish. This was done after euthanizing the fish by keeping them in tricaine solution for a minute.

Before the challenge bacteria were added to the fish flasks, the water in each flask was transferred into an empty flask. The fish were left in the original flask without water for five minutes, after which the water in the second flask was poured back into the original flask and the doses of bacteria, prepared as in section 2.11, were added to the flasks. For flasks receiving *F. psychrophilum* approximately 10^7 CFUs were added per mL rearing water, while for flasks receiving A. salmonicida approximately 10^6 CFUs were added per mL rearing water. It was believed that leaving the fish out of the water for some time might cause stress that increase their susceptibility to disease.

2.14 Second Infection Challenge - Attempting to Infect Atlantic Salmon Larvae with *Y. Ruckeri*

In the second immersion challenge experiment, *Y. ruckeri* 06059's ability to infect GF (germ-free) and CVZ (conventionalized) Atlantic salmon larvae was tested. Whether the pathogenicity of *Y. ruckeri* was influenced by the presence of bipyridyl in its pre-challenge growth medium was also tested. In addition, we also had GF and CVZ control groups with a non-pathogenic strain (*Janthinobacterium sp.* MM5) and with no bacteria added (Table 6).

Table 6: The eight different groups used in the second immersion challenge experiment.

Treatment Name	Microbial Status of Fish	Bacterial Strain Added	Bacterial Culture Medium
GF_YRUC_BP	GF	*Y. ruckeri* 06059	TSB w/ bipyridyl (100 µM)
CVZ_YRUC_BP	CVZ	*Y. ruckeri* 06059	TSB w/ bipyridyl (100 µM)
GF_YRUC	GF	*Y. ruckeri* 06059	TSB
CVZ_YRUC	CVZ	*Y. ruckeri* 06059	TSB
GF_JMM5	GF	*Janthinobacterium sp.* MM5	TSB
CVZ_JMM5	CVZ	*Janthinobacterium sp.* MM5	TSB
GF_NO_BAC_ADDED	GF	None	---
CVZ_NO_BAC_ADDED	CVZ	None	---

470 eggs were ordered. When the eggs arrived, they were handled as described in section 2.8. All the eggs were derivated (section 2.9). As in the previous challenge, this challenge started six weeks past hatching of the eggs.

On the hatching day, a sterility check was performed on water samples from every flask (section 2.12). One week after the eggs had hatched, twelve flasks that tested non-sterile were conventionalized. Conventionalization was performed during a SGM replacement, after 60 mL SGM had been removed from the flasks. Instead of adding 60 mL fresh sterile SGM to each flask as in a typical SGM replacement, we added 60 mL of untreated water from lake Jonsvatnet. Additional sterility checks were performed on GF-flasks four weeks past hatching and at one DBC. Contaminated flasks were removed.

At 11 DBC (days before challenge), *Y. ruckeri* 06059 and *Janthinobacterium sp.* MM5 were transferred from cryo stocks to agar plates (TSA). At 7 DBC, bacterial colonies were transferred from agar plates into liquid media (TSB). The fish flasks were incubated at 8.0°C in a lying position. Each of the four following days the temperature was increased by +2°C, so that the temperature during the challenge would be 15.8°C.

At 2 DBC and 1 DBC, *Y. ruckeri* 06059 was transferred from TSB into fresh TSB and into TSB w/ bipyridyl (100 µM). *Janthinobacterium sp.* MM5 was transferred from TSB into fresh TSB.

At 1 DBC, two flasks with germ-free fish were used to measure the length and weight of the fish. This was done after euthanizing the fish by keeping them in tricaine solution for a minute.

On challenge day, bacterial doses were prepared (section 2.11) and added to the flasks. The amount was approximately 10^7 CFUs per mL rearing water.

2.15 Third Infection Challenge - Attempting to Infect Atlantic Salmon Larvae with *Y. Ruckeri* (Again)

The third infection experiment was a retry of the second experiment, albeith slightly simplified.

The two groups in the second challenge where *Y. ruckeri* had been cultured in medium without bipyridyl were dropped, and *J. sp.* MM5 was cultured in medium with bipyridyl. Apart from this the groups were the same as in the second challenge (Table 7). Each GF group had five replicate flasks. The groups CVZ_YRUC_BP_RETRY and CVZ_NO_ADDED_RETRY had four replicates, while CVZ_JMM5_BP_RETRY had three.

Table 7: The six different groups used in the third immersion challenge experiment.

Treatment Name	Microbial Status of Fish	Bacterial Strain Added	Bacterial Culture Medium
GF_YRUC_BP_RETRY	GF	*Y. ruckeri* 06059	TSB w/ bipyridyl (100 µM)
CVZ_YRUC_BP_RETRY	CVZ	*Y. ruckeri* 06059	As above
GF_JMM5_BP_RETRY	GF	*Janthinobacterium sp.* MM5	As above
CVZ_JMM5_BP_RETRY	CVZ	*Janthinobacterium sp.* MM5	As above
GF_NO_ADDED_RETRY	GF	None	---
CVZ_NO_ADDED_RETRY	CVZ	None	---

750 eggs were ordered. When the eggs arrived, they were handled as described in section 2.8. All the eggs were derivated (section 2.9). The challenge was set to start when the larvae reached six weeks of age, as in the previous experiments. The fish were again challenged at six weeks past hatching.

On the hatching day, a sterility check was performed on water samples from every flask (section 2.12). Additional sterility checks were also performed one week after hatching, four weeks after hatching, and one DBC. All non-sterile flasks were removed. One week after the eggs had hatched, eleven flasks were conventionalized. Conventionalization was performed as described previously in section 2.14.

At 11 DBC, *Y. ruckeri* 06059 and *Janthinobacterium sp.* MM5 were transferred from cryo stocks to agar plates (TSA). At 7 DBC, bacterial colonies were transferred from agar plates into liquid media (TSB without bipyridyl). The temperature in the room was increased to 8.0°C. The flasks were placed in a lying position. Each of the four following days the temperature was increased by +2°C, so that the temperature during the challenge would be 15.5 ± 0.3°C.

At 2 DBC and 1 DBC, *Y. ruckeri* 06059 and *Janthinobacterium sp.* MM5 were transferred from TSB into TSB w/ bipyridyl (100 µM).

On challenge day, bacterial doses were prepared (2.11) and added to the flasks. The amount was approximately 10^7 CFUs per mL rearing water.

At 18 days past start of challenge, a sensor for dissolved oxygen (Oxi 3315, WTW) was used to measure the amount of oxygen in the water in nine different CVZ fish flasks.

At the end of the three weeks of the third infection experiment, a last sterility check was performed on the water in every flask in the GF_NO_ADDED_RETRY treatment. An end-of-

challenge CFU count was prepared using water samples from the flasks in the other five treatments.

2.16 The Contaminating Microorganism in the Third Infection Challenge

Early into the third infection experiment a sterility check showed that 18 of the supposed sterile fish flasks were contaminated with a microorganism, which grew on TSA plates and in TSB and GYE broth at room temperature in aerobic conditions.

The 16S rRNA gene in bacterial samples was Sanger sequenced (section 2.3), with universal primers EUB8F and 1492R being used in the PCR (primer sequences are given in Appendix C).

To test whether the contaminating microorganism could survive the autoclave, 3 mL of outgrown culture was poured into a bottle of 1 L SGM. The bottle was placed in an autoclave (Astell AMA270BT65) that was already run warm (approximately 80°C), so that the autoclave itself would reach 121°C more quickly than the SGM bottle. This would result in the SGM spending less time at the maximum teperature, which would give the microorganism a higher chance of surviving. An additional eight SGM bottles (1 L) were placed around the first bottle, to act as a heat buffer. The bottles were then autoclaved (20 min at 121 °C). After the autoclave had finished, 150 µL samples of SGM with (possibly still alive) bacteria were transferred into 3 mL GYE broth and incubated at room temperature.

We also wanted to test if the contamination might have been present on the fish eggs upon reception and had been able to survive the egg surface treatments with antibiotics and buffodine. To do this, we first transferred 1 mL culture of the contaminating organism to 1.5 mL microcentrifuge tubes and centrifuged at 13 000 g for 1 min. The supernatants were discarded. Some of the pellets were resuspended in 1 mL antibiotic SGM (see Appendix B), while others were resuspended in 1 mL of a 1:200 buffodine solution (diluted in SGM). The tubes were incubated at RT. Note that the antibiotic SGM used in this test did not contain any Amphotericin B, as had been used in the derivation (Amphotericin B is an antifungal and its absence is not expected to matter since we were dealing with a bacterial contamination).

After 30 minutes the tubes with buffodine-SGM-solution were centrifuged at 13 000 g for 1 min and the supernatants were discarded. The bacterial pellets were resuspended in GYE broth and vortexed at medium speed for 3-4 seconds. The centrifuging, media discarding, resuspending and vortexing was repeated an additional three times to wash out the buffodine. 150 µL culture was then transferred from each microcentrifuge tube into larger culture tubes containing 3 mL GYE broth that were incubated at room temperature.

After 24 hours the tubes with antibiotic SGM were centrifuged at 13 000 g for 1 min and the supernatants were discarded. The bacterial pellets were resuspended in GYE broth and vortexed at medium speed for 3-4 seconds. 150 µL culture was then transferred from each microcentrifuge tube into larger culture tubes containing 3 mL GYE broth that were incubated at room temperature.

To check if the contaminating microorganism could grow in medium containing antibiotics, 150 µL culture samples of the microorganism (cultured in TSB) were transferred into tubes containing 3 mL antibiotic TSB (see Appendix B). The tubes were incubated at room temperature and were regularly checked for sign of microbial growth over a period of eleven days.

2.17 Determining the Amount of *Y. ruckeri* in Atlantic Salmon Larvae Tissue by Using qPCR

To determine how efficiently *Y. ruckeri* can colonize skin and gut of Atlantic salmon larvae, multiple qPCRs were performed on DNA extracted from homogenized larvae skin and gut and from the rearing water of the larvae. One group of larvae had been reared in water containing *Y. ruckeri*. The two other groups had been reared in water containing either *Janthinobacterium sp.* MM5 or no bacteria.

The tissue homogenization and DNA extraction had been performed earlier in our lab. DNA had been extracted using a KingFisher Flex (Thermo Fisher) and the ZymoBIOMICS^{TM} 96 MagBead DNA Kit (Zymo) and the extracts had been stored at -80°C.

After thawing, each DNA extract was diluted 1:10 with PCR grade water, and triplicate qPCR reactions were prepared from each diluted DNA extract. The reactions were prepared by mixing the diluted DNA with MasterMix, PCR grade water, and primers targeting a region of *Y.ruckeri*'s hom7 gene (Table 8). This was done in 96-well plates. Each plate also contained triplicates of five DNA concentration standards (DNA concentrations ranging from 10^{-3} to 10^{-7} ng/µL), and triplicate non-template control samples that contained PCR grade water in place of DNA. The target DNA sequence was amplified using a QuantStudio 5 Real-Time PCR System (ThermoFisher) with the program specified in Table 9.

The hom7 gene is used in synthesis of Holomycin and is assumed to be present in only one copy per genome, so that the number of gene sequences is equal to the number of *Y. ruckeri* cells in the original sample.

Table 8: qPCR ingredients for amplification of *Y. ruckeri's* hom7 gene. Primer sequences are given in Appendix X.

Ingredient	Volume (µL) per reaction
Power SYBR Green PCR Master Mix (2x concentration, ThermoFisher)	10
PCR grade water	3
Forward primer 685F (10 µM)	1
Reverse primer 685R (10 µM)	1
DNA solution	5

Table 9: qPCR program. The temperature changes at a rate of 1.6 °C s^{-1} between all steps except between steps Melt Curve #2 and #3, where it changes at a rate of 0.15 °C s^{-1}.

Step	Temperature (°C)	Time (sec)	Cycles
Initial heating #1	50	120	
Initial heating #2	95	600	
Denaturation	95	15	40
Annealing & Elongation	60	60	
Melt Curve #1	95	15	
Melt Curve #2	60	60	
Melt Curve #3	95	1	

For each qPCR run, the Ct values of the five DNA concentration standards were plotted against the $\log_{10}$ DNA copy numbers of the standards. The DNA copy numbers of the standards were calculated by Equation 2:

$$\frac{DNA\ concentration * DNA\ volume}{weight\ per\ target\ DNA\ (including\ the\ complementary\ strand)} \quad (2)$$

where the DNA density ranged from 10^{-3} to 10^{-7} depending on the DNA standard, the volume was 5 µL, and the molecular weight of one DNA molecule was determined to be 6.547211e-11 ng (39 428.20 Da) based on its sequence: CCTCGGCGTCTATACGGAAGTGCTGCGTCAATGGTCCCGCCGTCAGGATTTCACGCTCACCCTG.

Using linear regression on the plots, we obtained standard curves with equation of the form (Equation 3), where a and b are the slope and intersect of a particular standard curve. The equation can be rewritten as (Equation 4), which gives the copy number as a function of the Ct-value. It was assumed that the copy number in a sample volume corresponded to the number of *Y. ruckeri* in that volume.

$$Ct = a(log_{10} copy\ number) + b \quad (3)$$

$$copy\ number = 10^{\frac{(Ct-b)}{a}} \quad (4)$$

After each qPCR run the data quality was checked. Samples with a Ct-value higher than 36 were discarded. Samples with one or more melting curve peaks that did not align at the same temperature as the average melting peak were also discarded. Samples with an amplification curve that did not match the other amplification curves in the same triplicate were discarded. If a triplicate now only had one remaining sample, this sample was also discarded. For triplicates where two or three samples remained, the Ct-values of these samples were converted into DNA copy numbers. The means and standard deviations for these samples were calculated.

2.18 Preparing the qPCR Standard Curve

A standard curve was needed to relate the Ct-values obtained in the qPCR to the number of *Y. ruckeri* present in a sample. The curve was based on five different DNA concentration (10^{-3} to 10^{-7} ng/µL). To make these DNA concentrations we first centrifuged 1 mL outgrown culture of *Y. ruckeri* 06059 at 13 000 g for 1 min. The supernatant was discarded, and the bacterial pellet was resuspended in a powerbead tube from the Qiagen DNeasy PowerSoil kit. The DNA was extracted according to the kit instructions provided by Qiagen.

The amount and purity of the extract was determined using a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). Forward and reverse primers targeting a region of the hom7-gene were mixed with extracted DNA and other PCR ingredients (Table 10) (both undiluted and 1:10 diluted DNA extract was used, in two separate reaction mixes). The target sequence was amplified using a thermal cycler (Bio-Rad T100) with the program specified in Table 11. To assess the amount and purity of the PCR product, the product was run through an agarose gel (section 2.5).

Table 10: PCR ingredients for amplification of *Y. ruckeri's* hom7-gene. Primer sequences are given in Appendix X.

Ingredient	Volume (µL) per reaction
PCR grade water (VWR)	33.64
5x Phusion HF-buffer (Thermo Scientific)	10
Forward primer 685F (10 µM)	1.5
Reverse primer 685R (10 µM)	1.5
dNTP mix (10 mM of each dNTP, VWR Life Science)	1
Phusion HotStart II DNA polymerase (Thermo Scientific)	0.36
Sample DNA	2

Table 11: PCR program for amplification of the hom7 gene.

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	60	
Denaturation	98	15	38
Annealing	55	20	
Elongation	72	20	
Final elongation	72	300	
Cooling	10	Hold	

After amplification, the PCR products were purified using QIAquick PCR Purification Kit, according to kit instructions provided by the manufacturer (30 µL PCR grade water was used during the elution step). Because the brightness of the gel bands for the undiluted and 1:10 diluted PCR products had indicated that they had a similar DNA concentration, and because the volume of each individual PCR product was low, the two PCR products were added together in one tube.

The concentration of the DNA product was determined with a Qubit 3 Fluorometer (ThermoFisher). An aliquot of DNA was used to make a 10-fold dilution series starting at 10^{-3} ng/µL of DNA and ending at 10^{-7} ng/µL. These five dilutions were aliquoted and stored at -20°C until use.

3. Results

3.1 Relationship Between OD and CFU in Bacterial Cultures

For *F. psychrophilum* DSM 3660 we calculated a mean of $1.3x10^9$ CFUs per mL at OD_{600} = 1.0, with a standard deviation of $1.5x10^9$, based on fifteen plate counts. For *A. salmonicida* ssp. *salmonicida* VI-88/09/03175 the mean at OD_{600} = 1.0 was $1.7x10^8$, with a standard deviation of $2.2x10^8$, based on 36 plate counts.

As the standard deviations of the means are very large for both *F. psychrophilum* and *A. salmonicida*, the means are poor estimates for the real number of bacterial cells in a sample with OD_{600} = 1.0.

3.2 Growth curves

We wanted the pathogens used in the immersion challenges to be in the late exponential phase at the point of addition. Growth curves for our strains were therefore made. A comparison between measurements taken in plastic cuvettes and in glass tubes were also performed, to see if glass tubes could be used in the place of cuvettes (Table 12). The comparison indicates that if the OD_{600} measured in a glass tube is at least 0.380, then the measured value can be divided by 1.2 to get a good approximation of what the value would have been if measured in a cyvette. The spectrophotometer used for measurements was designed to calculate OD for samples in cuvettes. Therefore, when cultures in glass tubes were used to create growth curves, the measurements were divided by 1.2 before the curves were plotted.

Table 12: Difference in OD_{600} measurements of a bacterial culture in glass tubes and in cuvettes. The top table entry is a sample of an undiluted culture, the other rows are incremental dilutions of this culture.

OD_{600}, culture in glass tube	**OD_{600}, culture in cuvette**	**Ratio (tube to cuvette)**
2.207	1.757	1.256
1.896	1.607	1.180
1.520	1.239	1.227
0.864	0.694	1.245
0.617	0.532	1.160
0.380	0.315	1.206
0.190	0.252	0.754
0.201	0.210	0.957
0.077	0.133	0.579
0.301	0.104	2.890

Cultures of *Y. ruckeri* 06059 reaches the stationary growth phase after about 12 to 15 hours, regardless of presence or absence of bipyridyl. The maximum bacterial density is also unaffected by the bipyridyl (Figure 2).

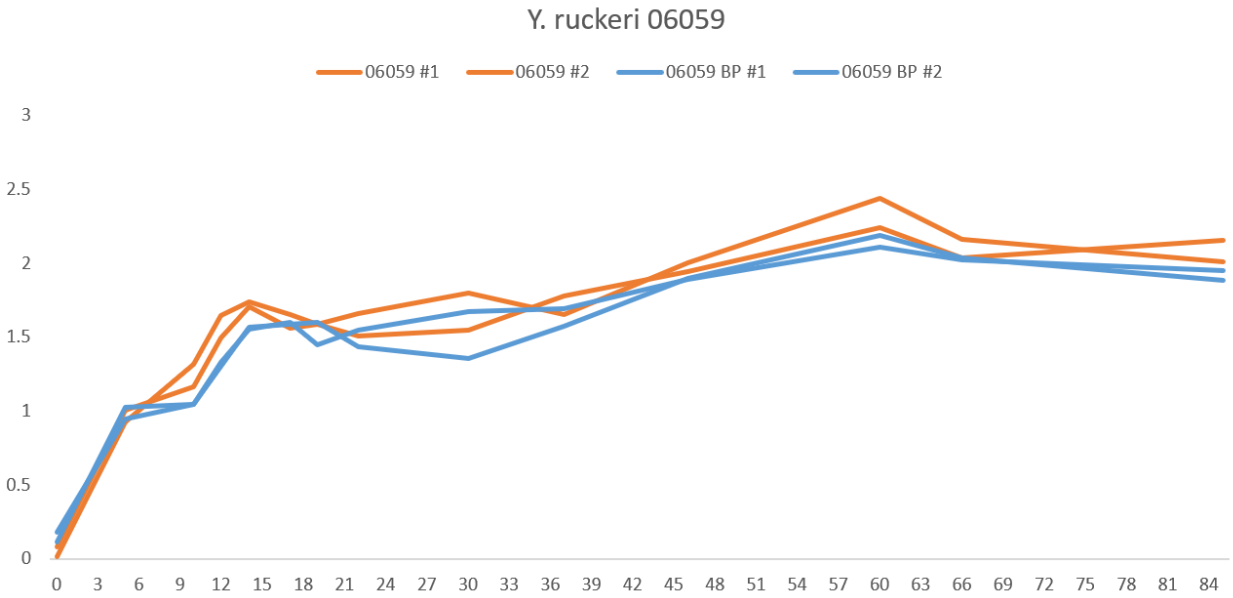

Figure 2: Growth curves for duplicate cultures of *Y. ruckeri* 06059, cultured with and without bipyridyl (BP). The X-axis shows hours since the starting of the culture, while the Y-axis shows the OD_{600}.

Cultures of *A. salmonicida* strains seem to reach the stationary growth phase after 12 to 15 hours. The cultures with bipyridyl have a slightly lower OD compared to the cultures without bipyridyl (Figure 3).

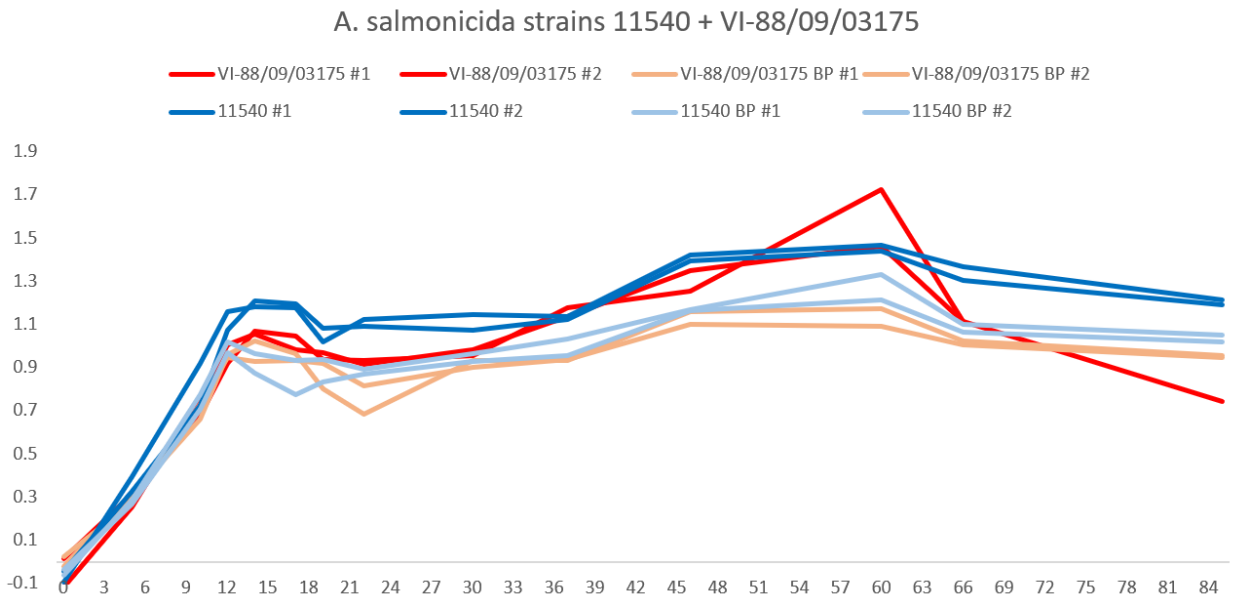

Figure 3: Growth curves for duplicate cultures of *A. salmonicida* ssp. *salmonicida* VI-88/09/03175 and *A. salmonicida* ssp. *salmonicida* 11540, cultured with and without bipyridyl (BP). The X-axis shows hours since the starting of the culture, while the Y-axis shows the OD_{600}.

Both strains of F. psychrophilum strains grow to a higher maximum density when cultured without bipyridyl. When cultured with bipyridyl, the max density is reached after about 70 hours for both strains, whereas when cultured without bipyridyl this takes only about 45 hours (Figure 4).

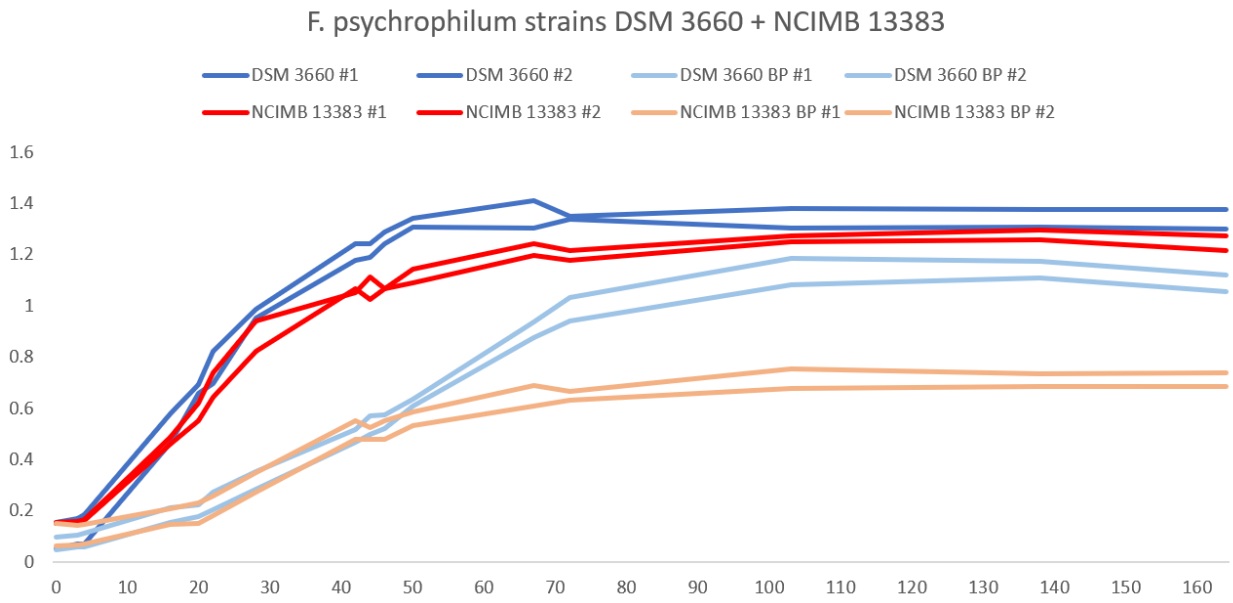

Figure 4: Growth curves for duplicate cultures of *F. psychrophilum* DSM 3660 and *F. psychrophilum* NCIMB 13383, cultured in TYES broth with and without 50 µM bipyridyl (BP). The X-axis shows hours since the starting of the culture, while the Y-axis shows the OD_{600}.

3.3 Results of the First Immersion Challenge

The goal of this experiment was to identify a pathogenic strain capable of infecting conventionally raised Atlantic salmon larvae through an immersion challenge. We tested two strains each of the major salmonid pathogens *A. salmonicida* and *F. psychrophilum*. Each strain of *A. salmonicida* was tested with two different growth media. We therefore had six different challenge groups, in addition to a control group with *Janthinobacterium sp.* MM5 and a control group with no bacteria added. The reason for having the '*Janthinobacterium sp.* MM5' group on top of the 'no bacteria added' group was that we wanted to check if the addition of non-pathogenic bacteria could influence the death rate of the fish. The groups will be referenced to with the treatment names given in Table 5.

We initially had 40 flasks, but in 14 of these all fish died before the challenge started. This left 26 flasks, where 24 were used in the challenge. The last two were used to measure the length and weight of the fish on 1 DBC. The weight was 0.121 ± 0.01 grams, and the length was 2.6 ± 0.09 cm, based on twenty measured fish.

In the week prior to the challenge, when the temperature of the fish lab was increased towards 12°C, larvae started to die in three of the 24 remaining flasks. We also noticed a biofilm growing on the inside of these three flasks. After only 1 DPC (day past challenge) we noticed the same in a fourth flask. All four flasks with biofilm on the inside were removed from the experiment. Because four flasks were removed from the experiment, four of the eight treatment groups

(CR_ASAL_11540_TSB_BP, CR_ASAL_11540_BHI_BP, CR_FPSY_3660_BP, CR_NO_BAC_ADDED) ended up with duplicate flasks instead of triplicates. Three other flasks developed biofilm at later points in the challenge, but these flasks were not removed.

DNA from bacteria in the biofilm was extracted, amplified with primers EUB8F and 1492R, purified, and sent to Sanger sequencing (section 2.3). When sequence data was returned, the sequence was determined to belong to a bacterium of the Pseudomonadaceae family by using RPD Classifier v11 (Wang et al., 2007).

To estimate the number of bacteria added we performed pre-challenge and post-challenge CFU counts on water samples from every flask. The challenge bacteria we added should give the flasks 10^7 CFUs per mL in the post-challenge count, but most flasks already had more than 10^7 CFUs per mL according to the pre-challenge count. We could therefore not verify that the correct number of bacteria had been added.

To determine whether any of the pathogens had successfully infected the fish, the survival of fish throughout the challenge was plotted for every flask (Figure 5). None of the fish died on the challenge day during the five minutes they were without rearing water. Both control groups (CR_JMM5_BP and CR_NO_BAC_ADDED) had one flask with a low survival percentage (<30 %), while the other three flasks in the control groups had no or very few deaths. The flasks with low survival percentages had maintained high survival percentages until 18 DPC, from which point many fish died in rapid succession over the next two days. For the flask in the CR_JMM5_BP treatment the time of deaths correlated with the rearing water becoming turbid, indicating high bacterial growth in the rearing water.

Flasks challenged with F. psychrophilum (CR_FPSY_13383_BP and CR_FPSY_3660_BP) had zero deaths, with exception of a flask in the CR_FPSY_3660_BP group where all fish died in rapid succession about 7 DPC.

The groups challenged with A. salmonicida cultured in BHI had more deaths than any other group, but the survival percentages varied wildly between replicates. One flask had 100 % survival, two flasks had 0 % survival, and the last two had 60 % and 40 % survival. In the CR_ASAL_03175_BHI_BP group there is one flask that stands out from every other flask in the experiment. This flask had 0 % survival, with deaths almost evenly distributed from 10 DPC to 19 DPC. This might be a successful infection, but the result was not replicated in the other two flasks in the group, where survival was 60 % and 100 %. Flasks challenged with A. salmonicida cultured in TSB (CR_ASAL_03175_TSB and CR_ASAL_11540_TSB_BP) all had over 70 % survival, except one flask with less than 30 % survival. The deaths mainly occured in the last few days of the experiment. None of the *A. salmonicida* flasks seemed to have had a successful infection.

In general, when a flask has low survival (<40 %) the fish tend to die in rapid succession over a period of two to three days. The only noticable pattern to these rapid deaths is that the flasks develop poor water quality at the same time as the deaths take place. Poor water quality means that the water was turbid, or yellow, or had white clumps of bacteria floating around (Figures 6,

7, 8). It is undetermined whether reduction of water quality happens before the onset of the rapid deaths and therefore could be the cause, or if it is just a consequence of the deaths.

Figure 5: Survival of fish in the eight groups of the first immersion challenge experiment. Each graph represents a different fish flask. The X-axis shows time measured in days past challenge, and the Y-axis shows the number of live fish at the given time.

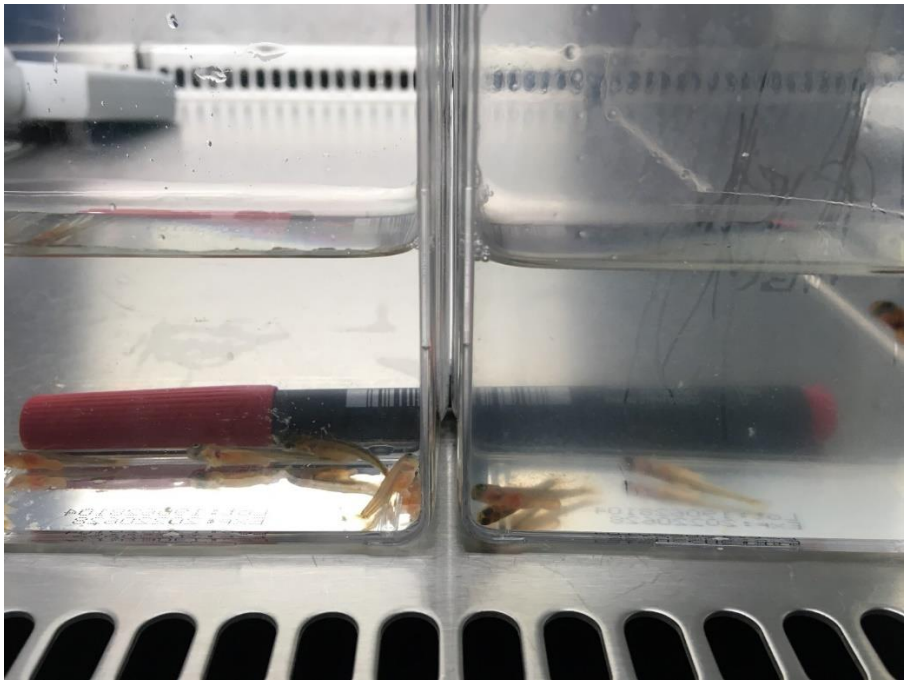

Figure 6: The water quality in some flasks in the first experiment was relatively poor, as seen here with much more turbid water in the flask on the right side. Note that the flask with turbid water also has a biofilm on the inside of the wall.

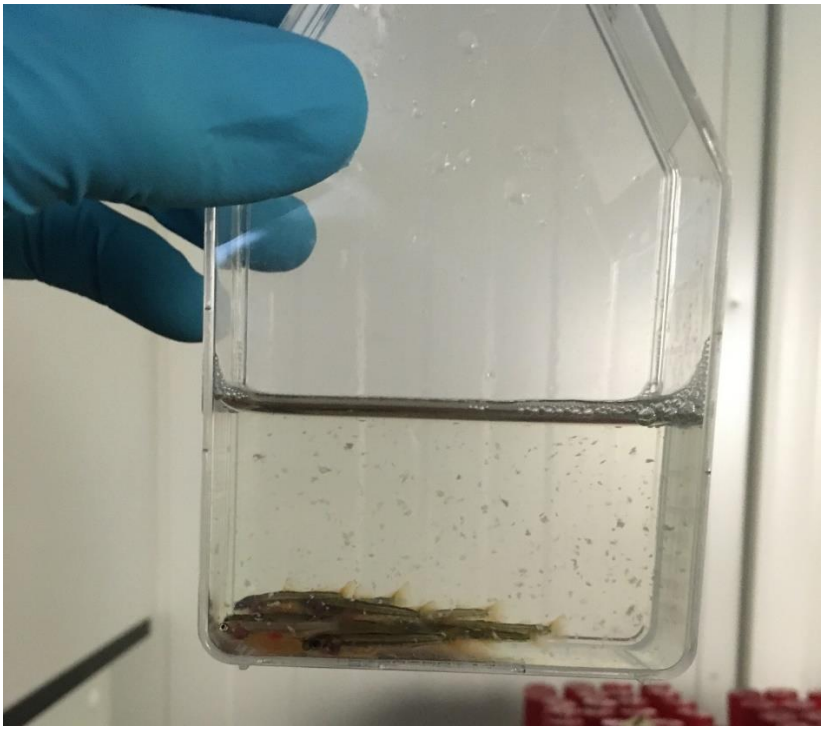

Figure 7: Another example of poor water quality. Some flasks had their water full of white clumps, assumed to be aggregates of bacteria. This might indicate fast growth of a particular bacteria, possibly using calcium crystals present in the SGM as start points for the development of clumps.

Figure 8: A last example of issues with the water quality. Notice how the leftmost flask has a yellow tint in the water. This could be caused by high density of a yellow-colored bacteria.

3.4 Results of the Second Immersion Challenge

The purpose of the second immersion challenge experiment was to determine whether germ-free (GF) Atlantic salmon larvae are more susceptible to infection of *Y. ruckeri* 06059 compared to conventionalized (CVZ) larvae. In other words, to test whether an established microbiome can influence the extent of infection in the larvae. We also wanted to see if bacteria were more infectious when cultured in medium with an iron chelator (bipyridyl). We therefore challenged both GF and CVZ fish with *Y. ruckeri* cultured in TSB with bipyridyl and in TSB without bipyridyl. In addition, we had a control group with *Janthinobacterium sp.* MM5 and another control group with no bacteria added. The treatments will be referenced with the treatment names given in Table 6.

For this experiment we needed 24 flasks, half of them GF and the other half CVZ. After having received and distributed our eggs into the flasks, we initially had 30 flasks. After derivation and hatching of the eggs, a sterility check indicated twelve of the flasks were not sterile. These flasks were conventionalized, while the eighteen flasks that tested sterile became GF-flasks.

Five of the eighteen GF-flasks became contaminated before the challenge started and were removed. Two other GF-flasks were used to measure weight and length of the larvae. Relocation of larvae in GF-flasks yielded one additional GF-flask and gave us exactly twelve GF-flasks for the immersion challenge. However, during the challenge two of the GF-flasks in the GF_NO_BAC_ADDED group became contaminated and were removed.

The weight and length of the GF-fish were measured at 1 DBC. The weight was 0.145 ± 0.02 grams, and the length was 2.6 ± 0.16 cm, based on 24 measurements. Compared to the CVR-fish of the first experiment, the GF-fish were a little heavier (0.145 grams vs 0.121 grams) but had the same mean length.

To estimate the number of bacteria added we performed pre- and post-challenge CFU counts. From the pre-challenge CFU counts we found that CVZ flasks had <= 10^6 CFUs per mL. The post-challenge CFU counts from both GF-flasks and CVZ-flasks also showed <= 10^6 CFUs per mL, indicated that we had added less bacteria than planned (the plan being 10^7 CFUs per mL). The dilutions used in these CFU counts had been too high for accurate CFU-determination, and so another CFU-count was performed at 3 DPC. This count resulted in between 10^4 and 10^5 CFUs per mL in the different flasks, affirming that we had added too few bacteria. Yet another CFU count, performed 14 DPC using only samples from GF-flasks, indicated that both *Y. ruckeri* and *Janthinobacterium sp.* MM5 had grown to numbers above 10^6 CFUs per mL in these flasks.

As in the first challenge experiment, survival of the larvae through the three weeks of this challenge was plotted for each group to determine whether a successful infection had taken place (Figure 9). The two GF control groups (GF_JMM5 and GF_NO_BAC_ADDED) both maintained very high survival percentages throughout the experiment, although the latter group only has a single flask as the two others had been contaminated and removed.

The GF_YRUC_BP group seems to have had a successful infection, with all three flasks having less than 40 % survival. In each flask the deaths are spread out over the last two weeks of the experiment. The GF_YRUC group had two flasks with no surviving fish at the end of the experiment, but the last flask had 87 % survival. In this group most of the fish also died in rapid succession over two to three days, in contrast to the fish in the GF_YRUC_BP treatment.

The CVZ control groups (CVZ_JMM5 and CVZ_NO_BAC_ADDED) generally have very poor survival. Particularly, in CVZ_NO_BAC_ADDED every fish in every replicate flask die within 13 days. In some of the flasks the deaths are spread out over a period of a week and a half, whereas in other flasks the deaths all occurred rapidly in a period of two days.

In the CVZ pathogen groups (CVZ_YRUC and CVZ_YRUC_BP) survival is below 40 % in every flask. Both treatments have at least one flask with zero survival and another flask with a low survival percentage, which we also see in the CVZ_JMM5 treatment. When compared to the control group CVZ_NO_BAC_ADDED, the two CVZ pathogen groups actually have higher rates of fish survival. And most of the fish in these two groups died in rapid succession. Based on this, the deaths in CVZ_YRUC and CVZ_YRUC_BP cannot be attributed to infection from *Y. ruckeri*.

By comparing treatments with germ-free fish to treatments with conventionalized fish, we see that there is generally less deaths among the germ-free fish, and this is especially true for the GF control treatments compared to the CVZ control treatments.

Poor water quality was noted for all the CVZ flasks and multiple GF flasks, and these issues developed at the same time as when fish in each flask started dying. Only six flasks maintained good water quality throughout the experiment. These included all three flasks in the GF control treatments (GF_JMM5 and GF_NO_BAC_ADDED), one flask with high survival in the GF_YRUC treatment and two flasks with low survival in the GF_YRUC_BP treatment. That fish in the two latter flasks were dying despite good water quality supports the assumption that we achieved a successful infection in this treatment group.

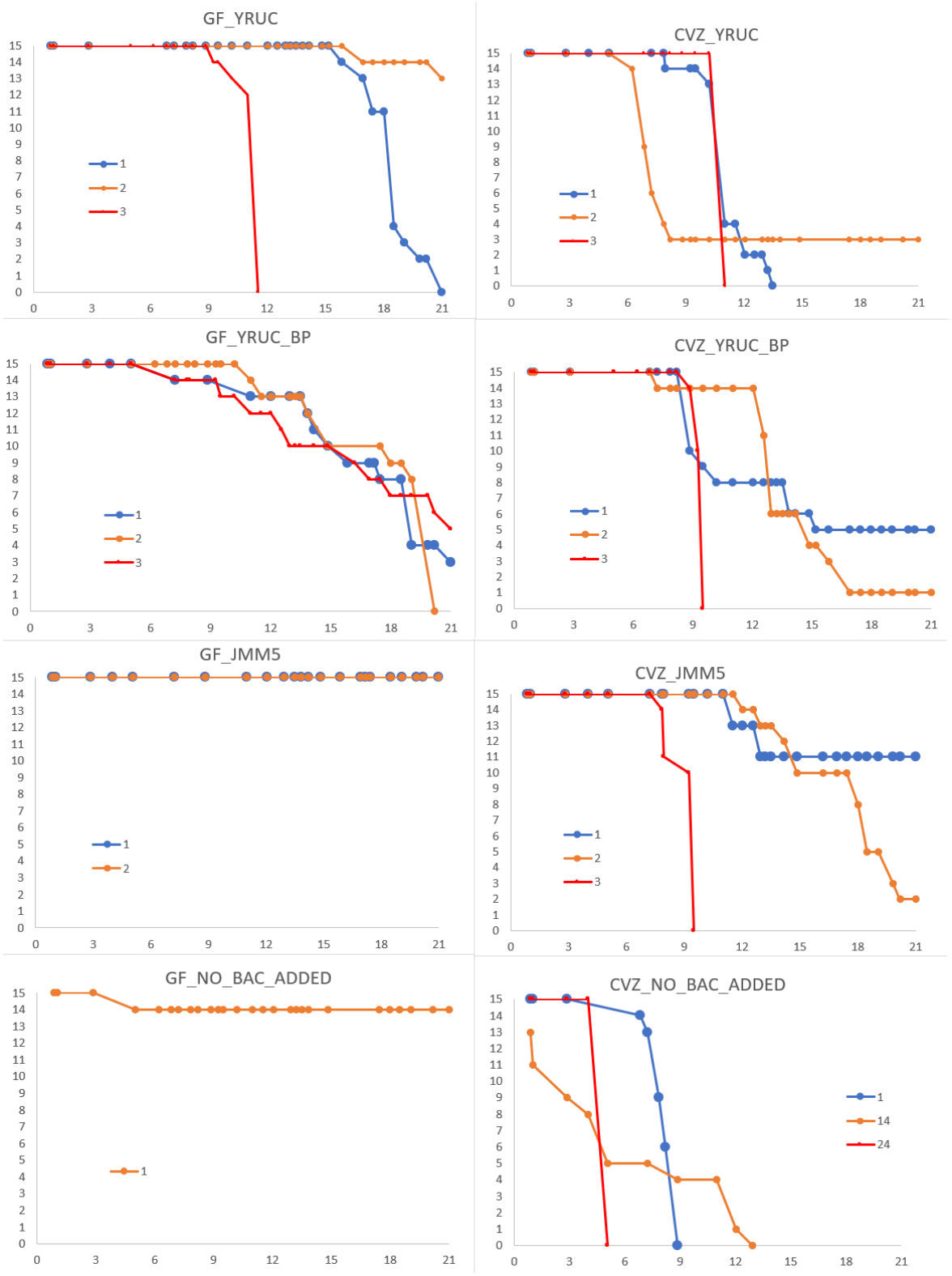

Figure 9: Survival of fish in the eight groups of the second immersion challenge experiment. Each graph represents a different fish flask. The X-axis shows time measured in days past challenge, and the Y-axis shows the number of live fish at the given time.

3.5 Results of the Third Immersion Challenge

The purpose of the third immersion challenge experiment was to try to confirm the findings of the second immersion challenge: that *Y. ruckeri* 06059 is capable of infecting Atlantic salmon larvae if the *Y. ruckeri* is cultured in TSB containing bipyridyl. This third experiment was identical to the second experiment, except this time the groups challenges with Y. ruckeri cultured in TSB without bipyridyl were dropped. The treatments will be referenced with the treatment names given in Table 7.

After having received, derivated and distributed our eggs into the flasks, we initially had 49 flasks. After hatching, a sterility check indicated that eighteen of the flasks were contaminated. These flasks were removed, and 31 flasks remained. Eleven of the remaining flasks were conventionalized, meaning that twenty flasks remained germ-free.

Another sterility check, performed when the fish were four weeks old, indicated that three of the twenty GF flasks were contaminated. These three flasks were also removed. Relocation of fish before the challenge yielded an additional GF flask, resulting in a total of 18 GF flasks. Three of these were given to someone else for use in other experiments (Thesis Mølmen 2021), meaning that there were fifteen GF flasks for this immersion challenge. Each GF group therefore got five flasks instead of three, but one GF-flask in GF_NO_ADDED_RETRY became contaminated during the experimen and was removed.

With eleven conventionalized flasks, the CVZ_YRUC_BP_RETRY and CVZ_NO_ADDED_RETRY treatments got four flasks instead of the initially planned three. Four of the eleven CVZ flasks had to start with fourteen fish instead of fifteen, because fish died on the day before the experiment began.

Pre-challenge and post-challenge CFU counts indicated that the CVZ flasks contained between 10^6 and 10^7 CFUs per mL both before and after the addition of bacteria, while GF flasks contained about 10^6 CFUs per mL after addition of bacteria. This means that as in the previous experiment, the planned amount of 10^7 CFUs per mL was not achieved.

Survival of fish throughout the challenge was plotted to check whether this challenge yielded similar results as the previous challenge (Figure 10). We can immediately see that this was not the case, and that the third challenge experiment had failed to reproduce the results of the second challenge experiment. Among the three treatments with GF fish there was no difference between the pathogen group (GF_YRUC_BP_RETRY) and the two control groups (GF_JMM5_BP_RETRY and GF_NO_ADDED_RETRY). In all three GF groups, the survival percentage in the flasks ranged from a perfect 100 % and down to about 50 %. The deaths in every flask were spaced out over time, with most deaths occuring in the second half of the challenge.

This in strong contrast to the treatments with germ-free fish in the second challenge, where the pathogen group (GF_YRUC_TSB_BP) had at least less than 40 % survival in every flask, while the

control groups (GF_JMM5_TSB and GF_NO_BAC_ADDED) had near-perfect survival with only one dead fish.

The results from the three CVZ groups show more similarity to their equivalent groups in the second immersion challenge. Among the control groups (CVZ_JMM5_BP_RETRY and CVZ_NO_ADDED_RETRY) all but one flask had less than 40 % survival, which also happened in the previous challenge. In some flasks the deaths occur rapidly over one to two days, while in other flasks the deaths are spread out over a longer period. In the pathogen group (CVZ_YRUC_BP_RETRY) we see one flask maintaining a higher survival rate than any of the flasks in the two control groups. The other three flasks in the pathogen group have survival ranging from 27 % to 0 %, which also resemble flasks in the control groups. The deaths in the pathogen group therefore cannot be attributed to infection.

Water quality was generally better in GF flasks than in CVZ flasks. Among the GF groups, five flasks (one in GF_YRUC_BP_RETRY, two in GF_JMM5_BP_RETRY, and two in GF_NO_ADDED_RETRY) were noted to have had been turbid at one or more points during the challenge experiment. In comparison, all but one of the eleven CVZ flasks were noted to have been turbid at some point, with two turbid flasks also having yellow water and another having what we assumed to be clumps of bacteria in the water.

At 17 DPC, right before a water replacement, we tested the dissolved oxygen (DO) of water samples from six flasks with conventionalized fish. The six contained varying numbers of live fish larvae (between one and nine) Three flasks where all the fish had died at an earlier date were also tested. The water in these flasks was very turbid. A sample of SGM was tested for reference (Table 13).

Table 13: The number of live fish and dissolved oxygen in different CVZ flasks, with SGM as a reference. The three removed flasks were flasks where all the fish had died before the measurement was done.

Water source	Live fish in flask (#)	Dissolved Oxygen (%)
CVZ_NO_ADDED_RETRY #1	7	64
CVZ_NO_ADDED_RETRY #2	9	56
CVZ_JSP_BP_RETRY #1	8	66
CVZ_JSP_BP_RETRY #2	1	80
CVZ_YRUC_BP_RETRY #1	2	78
CVZ_YRUC_BP_RETRY #2	4	77
Removed #1	0	31
Removed #2	0	20
Removed #3	0	16
SGM	0	101

From the six flasks with conventionalized fish, we see that a higher number of fish in a flask correlate with a smaller amount of DO. The flask that has the most fish (CVZ_NO_ADDED_RETRY #2) has the lowest amount of DO (56 %), while the flask with the smallest number of fish (CVZ_JMM5_BP_RETRY #2) has the highest amount of DO (80 %). In the three flasks that had been removed from the experiment we see DO vary from 16 to 31 %. Although these flasks had no live fish, they still contained several dead fish, and they were no longer subject to the routine water replacement. This allowed the bacteria in the flasks to reach high densities, which increased oxygen consumption. The SGM, which contained no fish or bacteria, shows a DO of approximately 100 % as expected.

From a last CFU count performed at 21 DPC we found that GF flasks contained about 10^6 CFUs per mL and CVZ flasks contained about 10^7 CFUs per mL. The results were similar to the post-infection counts, which showed that the bacterial densities over the three weeks of the experiment had not changed with more than a magnitude.

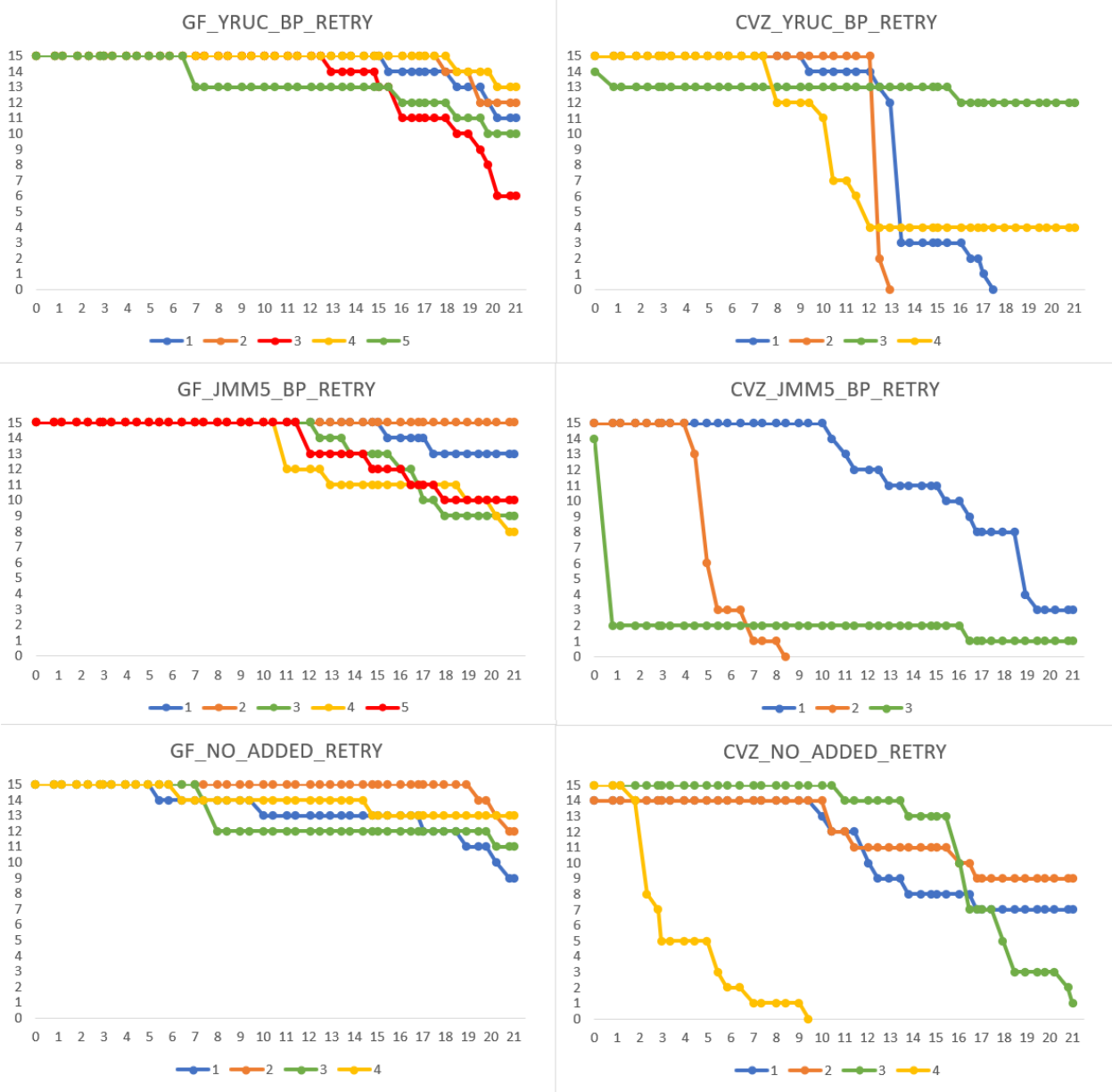

Figure 10: Survival of fish in the six groups of the third immersion challenge experiment. Each graph represents a different fish flask. The X-axis shows time measured in days past challenge, and the Y-axis shows the number of live fish at the given time.

3.6 Third Immersion Challenge – The Contaminating Microorganism

In the third immersion challenge, the first sterility check performed on the fish flasks showed that 18 out of 49 were contaminated. When growing on TSA, the contaminating bacteria from all eighteen flasks developed yellow colonies (Figure 11). Microscopy of bacterial samples revealed

that the bacteria grew in long filaments (Figure 12), regardless of what flask they originated from. These two results suggested that all eighteen flasks were contaminated by the same bacteria.

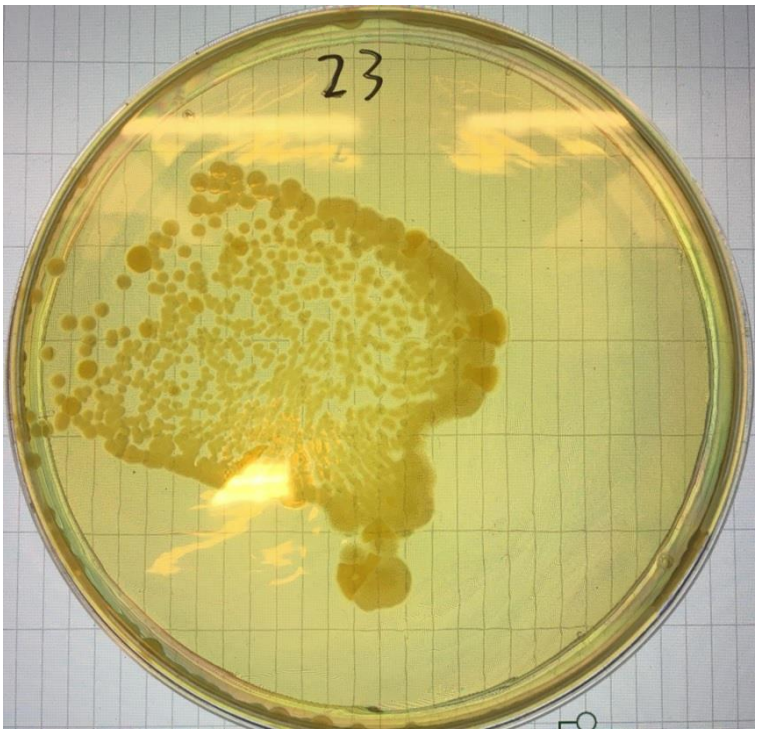

Figure 11: Bacteria originating from a contaminated fish flask in the third immersion experiment, here cultured on a TSA plate.

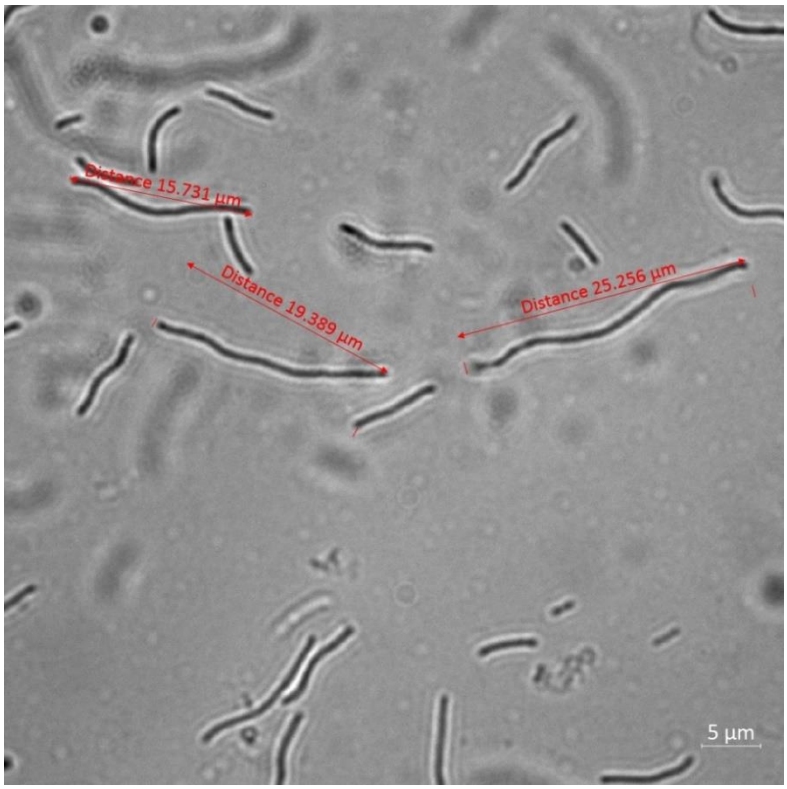

Figure 12: Bacteria originating from a contaminated fish flask in the third immersion challenge experiment, as seen in a microscope. The bacteria grow in filaments up to 25 µm long.

We tested if the contaminating bacteria might have entered the flasks by surviving the autoclaving of the SGM. Bacterial culture was added to a bottle containing 1 L SGM and the bottle was autoclaved at 121°C for 20 min. 150 µL SGM samples were transferred into GYE broth tubes and onto TSA plates and incubated at RT. None of the tubes or plates showed any sign of bacterial growth, meaning that SGM was probably not the source of the contamination.

We also tested if the bacteria could survive 30 minutes of treatment with 1:200 buffodine solution or 24 hours of treatment with antibiotic SGM. After the specified lenght of time the bacteria were washed and incubated in tubes containing GYE broth at RT. Growth was observed after a few days, both in tubes with buffodine-treated bacteria and in tubes with antibiotic-treated bacteria. This means that the contaminating bacteria probably got into the flasks by being present on the surface of the eggs when we received them, and and that they had survived the derivation procedure.

As an additional test we had attempted to culture the contaminating bacteria in tubes containing antibiotic TSB, incubated at RT. After eleven days there was no sign of growth in any tube, which meant that the bacteria could survive, but not grow, in the presence of the antibiotics.

DNA from the contaminating bacteria was extracted, the 16S rRNA gene was amplified with PCR using primers EUB8F and 1492R, and the PCR product was purified and Sanger-sequenced (section 2.3). The returned sequencing data was as follows:

GAAGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGTACGA
GAAGAAACACTCCTATGTATAGGAGCTTGACGGTATCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAATAAGTCAGT
GGTGAAAGCCCATCGCTCAACGGTGGAACGGCCATTGATACTGTTAAACTTGAATTATTAGGAAGTAACTAGAAT
ATGTAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATACCAATTGCGAAGGCAGGTTACTACTAATGGATTG
ACGCTGATGGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCGGGGTAGTCAA.

By using RPD Classifier v11 (Wang et al., 2007), the sequence was determined to come from an unidentified species of *Flavobacterium*.

3.7 qPCR – Degree of *Y. ruckeri* Colonization in Atlantic Salmon Larvae Tissue

We had wanted to test if we could quantify the amount of *Y. ruckeri* in skin and gut of Atlantic salmon larvae, and in the rearing water of the larvae, by using qPCR on DNA extracted from fish skin and gut and from the water. Some of the fish were CR while others were CVZ. qPCR might enable a more accurate quantification of *Y. ruckeri* compared to CFU counts. To quantify *Y. ruckeri* with qPCR we used forward and reverse primers targeting a region of the hom7-gene, one of the genes in the holomycin synthetic pathway (Qin et al., 2013), which we assume is present in a single copy in *Y. ruckeri* but not in other species of bacteria.

Seven qPCRs were run. The Ct values were converted into DNA copy numbers, and these were presented as means of sample duplicates or triplicates, with associated standard deviations (Table 14). We see that none of the control samples (samples with *Janthinobacterium sp.* MM5 or no bacteria added) have any amplification, while most of the infected samples (with *Y. ruckeri*) show amplification. This proves that our amplification primers bind specifically to DNA from *Y. ruckeri* and do not give false positives.

When there is amplification, the standard deviations are in some cases quite large. For example, in the fourth run of sample #6, the standard deviation is almost as large as the mean (21 056 vs 26 766). Sample #9, #18, #23, and #47 also have runs where the deviation is larger than half of the mean.

The difference between multiple runs of the same sample can also be large, both in relative and absolute terms. For example, for sample #5 the second run resulted in a mean almost four times larger than the mean from the fourth run (157 937 vs 32 499). Sample #6 and #29 also have means with similarly large relative difference. For sample #43, the second run gave a mean that was 85 million than the mean from the fourth run (145×10^6 vs 60×10^6). An absolute difference in the tens of millions was also seen between the means from sample #8.

We also see that sample #7, #21 and #23 had some runs that resulted in no amplification, and other runs that resulted in amplification. These amplifications were all low and indicated less than 10 000 cells of *Y. ruckeri* being present. It is therefore possible to get false negatives, at least when *Y. ruckeri* is only present in relatively small numbers.

Table 14: Shows the copy number of *Y. ruckeri* 06059 in samples of fish skin, fish gut and fish rearing water. Each entry is the mean of a duplicate or triplicate measurement, with the numbers in parentheses being the standard deviations of the means. The column "Bacteria" shows which bacteria had been added to the rearing water. The rows that share the same source are samples taken from different fish, or in the case of water samples, taken from different fish flasks.

#	Bacteria	CR/CVZ	Source	First qPCR	Second qPCR	Third qPCR	Fourth qPCR	Fifth qPCR	Sixth qPCR	Seventh qPCR
1	*Y. ruckeri* 06059	CR	Skin		0		0	0		
2	*Y. ruckeri* 06059	CR	Skin		0		0			
3	*Y. ruckeri* 06059	CR	Skin	26 407 (10 783)	13 286 (5 226)		23 050 (8 372)		34 267 (9 101)	
4	*Y. ruckeri* 06059	CR	Skin				0			
5	*Y. ruckeri* 06059	CR	Skin		157 937 (50 780)		32 499 (1 238)			
6	*Y. ruckeri* 06059	CR	Skin		47 540 (4 858)		26 766 (21 056)	12 065 (2 549)	21 953 (467)	36 755 (14 738)
7	*Y. ruckeri* 06059	CR	Skin		8 956 (2 656)		0	0		
8	*Y. ruckeri* 06059	CR	Skin		43e6 (9e6)		43e6 (3e6)	34e6 (4e6)	50e6 (4e6)	62e6 (1e6)
9	*Y. ruckeri* 06059	CVZ	Skin	31 614 (4 537)		34 744 (18 057)				
10	*Y. ruckeri* 06059	CVZ	Skin					22 509 (9 289)		
11	*Y. ruckeri* 06059	CVZ	Skin			5 941 (389)				
12	*Y. ruckeri* 06059	CVZ	Skin			232 782 (2 156)				
13	*Y. ruckeri* 06059	CVZ	Skin					3 776 (251)		
14	*Y. ruckeri* 06059	CVZ	Skin			31 981 (6 515)				
15	*Y. ruckeri* 06059	CVZ	Skin			12 829 (3 594)				
16	*Y. ruckeri* 06059	CVZ	Skin			13 619 (4 944)				
17	*Y. ruckeri* 06059	CVZ	Skin			0			0	
18	*Y. ruckeri* 06059	CR	Gut		6 163 (1 113)		10 852 (7 432)		22 404 (6 940)	
19	*Y. ruckeri* 06059	CR	Gut		0		0			
20	*Y. ruckeri* 06059	CR	Gut		0		0	0		
21	*Y. ruckeri* 06059	CR	Gut		8 307 (1 543)		0	0	12 814 (5 828)	
22	*Y. ruckeri* 06059	CR	Gut				0	0		
23	*Y. ruckeri* 06059	CR	Gut		6 448 (3 582)		0	0		
24	*Y. ruckeri* 06059	CR	Gut				0	0		
25	*Y. ruckeri* 06059	CVZ	Gut			0				
26	*Y. ruckeri* 06059	CVZ	Gut	1e6 (147 870)		2e6 (58 539)				
27	*Y. ruckeri* 06059	CVZ	Gut			464 659 (26 725)				
28	*Y. ruckeri* 06059	CVZ	Gut					2e6 (899 834)		
29	*Y. ruckeri* 06059	CVZ	Gut			31 442 (8 040)		37 893 (11 322)	117 093 (32 921)	
30	*Y. ruckeri* 06059	CVZ	Gut			5e6 (2e6)				
31	*J. sp.* MM5	CR	Skin		0		0			
32	*J. sp.* MM5	CR	Skin		0		0			
33	*J. sp.* MM5	CVZ	Skin			0				
34	*J. sp.* MM5	CR	Gut		0		0			
35	*J. sp.* MM5	CVZ	Gut			0				
36	None	CR	Skin		0		0			
37	None	CR	Skin		0		0			
38	None	CVZ	Skin			0				
39	None	CR	Gut		0		0			
40	None	CR	Gut						0	0
41	None	CVZ	Gut			0		0	0	0
42	*Y. ruckeri* 06059	CR	Water		26e6 (8e6)		27e6 (3e6)			
43	*Y. ruckeri* 06059	CR	Water		145e6 (22e6)		60e6 (2e6)			
44	*Y. ruckeri* 06059	CR	Water		5e6 (86 350)		6e6 (154 214)			
45	*Y. ruckeri* 06059	CVZ	Water	2e6 (58 682)		2e6 (231 257)				
46	*Y. ruckeri* 06059	CVZ	Water			2e6 (59 877)				
47	*Y. ruckeri* 06059	CVZ	Water			1e6 (597 081)				
48	*J. sp.* MM5	CR	Water				0			

4 Discussion

The microbiome has been shown to aid its host in defense against pathogen infection. For example, commensal microorganisms have been found to provide both Rainbow trout and zebrafish with increased resistance against infection by the pathogen *F. columnare*. However, no study has yet been performed on the protective effect of the microbiome in Atlantic salmon.

Therefore, three immersion challenge experiments with Atlantic salmon larvae were conducted, the first one with strains of the pathogens *A. salmonicida* and *F. psychrophilum*, and two other ones with the pathogen *Y. ruckeri* 06059. The purpose of the first challenge was to identify a strain capable of infecting the Atlantic salmon larvae. Only conventionally raised larvae were used in this first challenge. The criterion for successful infection was that at least 60 % of fish in a challenged population should become infected, i.e., they should die, while the control populations should have minimal deaths. This criterion had been used in a previous immersion challenge (Long et al., 2014). If a pathogen capable of causing such degree of infection was found, the intention was to use it later in immersion challenges with germ-free larvae.

The purpose of the other two challenges was to try to confirm the findings from an older experiment: that *Y. ruckeri* might be able to infect germ-free larvae, but not conventionalized larvae. In all three immersion challenges we included two different control groups: one where the larvae were mock challenged with the commensal strain *Janthinobacterium sp.* MM5, and one where the larvae were not challenged with any bacteria. This strain had been isolated from the skin of Atlantic salmon (Mallasvik, 2019) and had also been found as a common member of the rearing water of Atlantic salmon larvae in earlier experiments (Fiedler et al., manuscript in preparation).

Already in the first immersion challenge there was very large variation in mortality between the replicates, both in the control groups and in the challenged groups, with some replicates suffering many mortalities and other replicates suffering none. Among the flasks with many mortalities were two of the five control flasks, where no deaths were supposed to occur. This indicated that there was a problem with the experimental conditions.

In some of the challenged groups the result was zero to low mortality across all replicates the given group, meaning there was no sign of successful infection. In other challenged groups there was large variation in the mortality percentage between replicates, with some having high mortality and some having little, like in the control groups. One challenged group, CR_ASAL_11540_BHI_BP, had at least 60 % mortality in both flasks. In each of these two flasks the mortalities all took place in a period of about 24 hours. This is similar to how rapidly the fish died in the two control flasks, which means that whatever killed the fish in the control flasks might be the cause of death in this challenged flask, too.

Based on similarity between these challenged groups and the control groups, the high mortality percentage in some of the challenge group replicates cannot be assumed to be a sign of infection. It must rather be taken as a sign of poor experimental conditions, such as bad water quality.

One flask with a high mortality percentage stood out in that the first and last death were spaced thirteen days apart, with deaths occuring regularly throughout that period (see the gentle downslope on the graph of the second flask in treatment CR_ASAL_03175_BHI_BP, Figure 5). This could indicate that the larvae in this flask died for another reason than larvae in other flasks. However, if we assume that the reason was a successful infection, then the infection could not be replicated in the other two replicates in the same group.

The second immersion challenge was performed in an attempt to confirm the findings of Martha Drågen's experiment with germ-free larvae: that *Y. ruckeri* 06059 might be able to infect conventionalized and germ-free Atlantic salmon larvae at 16°C. The mortalities in the challenged replicates had been up to 30 %, while the controls suffered no mortalities at all. While not an ideal result, as 30 % is far from the desired >60 % mortality, it could have marked a step in the right direction if a way to enhance the mortality was found.

The results from Drågen's previous experiment were not fully replicated in our second challenge. Among the germ-free larvae the controls had near-perfect survival percentages, as they had had in the previous experiment. But the larvae challenged with *Y. ruckeri* ended up with 100 % mortality in two of the replicates, a much higher percentage than found previously. Most of the deaths in these two replicates happened rapidly, in periods of about 48 hours. A new GF treatment group added to this experiment, where *Y. ruckeri* had been cultured in medium with bipyridyl, had over 60 % mortality in every replicate. Over half of these deaths occured in the third week. In our experiment we recorded deaths for three weeks after the start of the challenge, whereas Drågen had recorded for two weeks. The deaths in the last week constituted a significant share of the mortality percentages obtained at the end. A direct comparison between our and Marthas results is therefore not appropriate. If we look away from deaths in the third week, the results from the germ-free groups in our and Drågen's experiments would be more similar.

Among the conventionalized larvae in our second immersion challenge there were many deaths in the controls, with multiple replicates having 100 % mortality. This holds true even if the deaths recorded in the third week of the challenge are ignored. The mortality percentages in replicates of challenged fish were similarly high. This result is completely different from the one obtained in Drågen's experiment, and as in our first experiment, there seemed to be something wrong with our experimental conditions.

But despite of the unexpected deaths in the CVZ groups, the results from the GF larvae were very promising, especially in the group of larvae challenged with *Y. ruckeri* cultured in bipyridyl. We therefore wanted to do one third challenge experiment, which would simply be a rerun of the second, to see if these promising results could be replicated.

But the mortality percentages obtained at the end of the third challenge were disappointing. Among the germ-free larvae, both the challenged group and the control groups had replicate average mortalities of about 25 %, with a spread of about ± 25 % for the individual flasks. This

indicated that none of the flasks had become infected. Among the conventionalized larvae there were again a large degree of variation between replicates, and the control groups contained multiple flasks with over 90 % mortality.

Across all three immersion challenges there are a few things that stand out. The variation in mortality percentage between replicates is usually large, and the mortality percentages obtained in some of the controls (especially CVZ and CR controls) are too high. These were also common issues in Drågen's experiments. In some flasks with high mortalities the fish are dying one or a few per day, while in other flasks all (or almost all) fish die within a period of one to three days. This difference between flasks might be related to the cause of death among the fish.

A severe drop in water quality, such as a drop in the level of DO (dissolved oxygen), might cause many fish to die from suffocation at about the same time, with the exact time of suffocation depending on how well the individual fish is able to handle the lack of oxygen.

From our measurement of DO in the third experiment we saw that the level of DO was inversely correlated with the density of live larvae. If the main cause of death in a flask was a low level of dissolved oxygen, then it makes sense that after several larvae die and are removed from the flask, OD should increase, and the number of deaths will stabilize. However, death of larvae correlates with increase in bacterial growth in the water (as indicated by increased turbulence), and the DO might therefore not improve until the water has been replaced. It would have been interesting to have the dates of water replacement marked in the survival graphs from the three challenges, to see if the water replacements cause the graphs to flatten out.

There is a possibility of using continous water flow systems or aeration pumps to maintain oxygen in the water, the former method also being able to monitor and control factors such as pH, salinity, and oxygen content (Nordmo & Ramstad, 1997). But these methods may not be possible to use when the larvae are supposed to remain germ-free. Alternatives are to reduce the density of larvae per flask (and have more replicate flasks), or to replace the water more frequently or to replace a greater proportion of water (more than 60 %) each time. Larger volumes of water per flask is perhaps also a possibility but would require bigger flasks. This is because an increased volume of water will not increase the area of the water surface, so gas diffusion between air and water would be the same unless the dimentions of the flask were increased. Perhaps most ideal would be big and flat flasks which allow the use of greater water volumes and a greater water surface area, but such flasks may not exist. Concentration of bacteria in the rearing waters could be monitored by using flow cytometry on water samples.

As for the contaminating bacteria in the third immersion challenge, our experiments showed that it could survive the 24 hours of antibiotic treatment but could not survive the autoclave. This indicates the bacteria was probably present on the surface of the eggs and survived derivation. It is unlikely that the bacteria was present in any of the salt stocks used to make SGM, as these were used throughout the experiment and would therefore have resulted in larger numbers of contaminated flasks in the later sterility checks, not just the first one. If contamination of flasks

becomes a big issue in yet an experiment, the derivation should perhaps be modified with use of additional antibiotics or increased antibiotic concentrations (the latter probably being more reasonable since we already use six different types of antibiotics, unless a higher concentration could damage the fish eggs).

5 Conclusion

In this thesis the aim was to develop a protocol for reproducible pathogen infection of Atlantic salmon larvae through an immersion challenge, a goal that was not achieved. Our first immersion challenge with strains of A. salmonicida and F. psychrophilum had issues both with reproduction of results between replicates, and further issues with many deaths in the control groups. It did not seem like any of the strains could cause a severe infection in our fish, given the experimental conditions.

In the second immersion challenge, this time using Y. ruckeri 06059, it seemed like we managed to successfully infect the GF fish group challenged with Y. ruckeri cultured in bipyridyl. In this group there was more than 60 % mortality in all three replicates. The GF control groups had minimal deaths in comparison. A third immersion challenge was performed in an attemt at reproducing this result. The result was small to intermediate mortality in all GF groups, both the one challenged and the two controls. Reproduction of the previous result was therefore not achieved.

Both in the second and third challenge experiments we had problems with the CVZ fish, with many deaths in some of the control flasks and big variation in mortality between replicate flasks, as in the first challenge experiment. We believe that the water quality might be responsible for the issues. In particular,we believe that rapid growth of aerobic bacteria can reduce the amount of dissolved oxygen in the water and cause the fish to suffocate.

Previous immersion challenge experiments have used continous flow systems or aeration pumps to maintain good water quality and have also used devices for automatic monitoring of factors influencing the water quality, such as pH and salinity. These were unfortunately not options for us, as our setup needed to be compatible with GF fish. One possible alternative would be to use more frequent replacements of the fish rearing water or replace a greater proportion of the rearing water each time.

qPCR does not seem like an ideal method for quantifying the number of *Y. ruckeri* cells in tissue samples. In individual qPCRs the sample replicates sometimes had large standard deviations. Means of standard deviation could also have large variation between different qPCRs of the same samples. Results must be used with caution.

References

A.W.E., D. (2020). Infection with Aeromonas salmonicida - atypical strains. In: Australian Government - Department of Agriculture, Water and the Environment.

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish Shellfish Immunol*, *90*, 210-214. https://doi.org/10.1016/j.fsi.2019.04.066

Aoki, M., Kondo, M., Kawai, K., & Oshima, S. (2005). Experimental bath infection with Flavobacterium psychrophilum, inducing typical signs of rainbow trout Oncorhynchus mykiss fry syndrome. *Diseases of Aquatic Organisms*, *67*, 73-79.

Barnes, M. E. (2011). A Review of Flavobacterium Psychrophilum Biology, Clinical Signs, and Bacterial Cold Water Disease Prevention and Treat. *The Open Fish Science Journal*, *4*(1), 40-48. https://doi.org/10.2174/1874401x01104010040

Bartkova, S., Kokotovic, B., & Dalsgaard, I. (2017). Infection routes ofAeromonas salmonicidain rainbow trout monitoredin vivoby real-time bioluminescence imaging. *Journal of Fish Diseases*, *40*(1), 73-82. https://doi.org/10.1111/jfd.12491

Beck, B. H., Li, C., Farmer, B. D., Barnett, L. M., Lange, M. D., & Peatman, E. (2016). A comparison of high- and low-virulence Flavobacterium columnare strains reveals differences in iron acquisition components and responses to iron restriction. *Journal of Fish Diseases*, *39*(3), 259-268. https://doi.org/10.1111/jfd.12343

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., Mitter, B., Ryan, M., Sarand, I., Smidt, H., Schelkle, B., Roume, H., Kiran, G. S., Selvin, J., Souza, R. S. C. d., van Overbeek, L., Singh, B. K., Wagner, M., Walsh, A., Sessitsch, A., & Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, *8*(1). https://doi.org/10.1186/s40168-020-00875-0

Boily, F., Malcolm, G., & Johnson, S. C. (2019). Characterization of Aeromonas salmonicida and furunculosis to inform pathogen transfer risk assessments in British Columbia. In: Fisheries and Oceans Canada.

CABI. (2019). *Furunculosis in Fish*. https://www.cabi.org/isc/datasheet/81888

Cain, K. D., & LaFrentz, B. R. (2007). Laboratory Maintenance of Flavobacterium psychrophilum and Flavobacterium columnare. *Current Protocols in Microbiology*, *6*(1). https://doi.org/10.1002/9780471729259.mc13b01s6

Chiu, L., Bazin, T., Truchetet, M.-E., Schaeverbeke, T., Delhaes, L., & Pradeu, T. (2017). Protective Microbiota: From Localized to Long-Reaching Co-Immunity. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01678

Davidsen, T. (2018). *Salmon Farming: Sustaining Communities and Feeding the World*.

Drågen, M. (2019). Establishing a protocol for challenging Atlantic salmon yolk sac fry with Yersinia ruckeri. In *TBT4500: Biotechnology, Specialization Project*: NTNU.

Drågen, M. (2020). *Immersion Challenge of Atlantic Salmon (Salmo salar) yolk sac fry with Yersinia ruckeri*. Master's thesis, NTNU.

Duchaud, E., Boussaha, M., Loux, V., Bernardet, J.-F., Michel, C., Kerouault, B., Mondot, S., Nicolas, P., Bossy, R., Caron, C., Bessières, P., Gibrat, J.-F., Claverol, S., Dumetz, F., Hénaff, M. L., & Benmansour, A. (2007). Complete genome sequence of the fish pathogen Flavobacterium psychrophilum. *Nature Biotechnology*, *25*(7), 763-769. https://doi.org/10.1038/nbt1313

FAO. (2020). *The State of World Fisheries and Aquaculture 2020*. https://doi.org/10.4060/ca9229en

Haig, S. J., Davies, R. L., Welch, T. J., Reese, R. A., & Verner-Jeffreys, D. W. (2011). Comparative susceptibility of Atlantic salmon and rainbow trout to Yersinia ruckeri: Relationship to O antigen

serotype and resistance to serum killing. *Veterinary Microbiology*, *147*(1-2), 155-161. https://doi.org/10.1016/j.vetmic.2010.06.022

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS Microbiology Letters*, *366*(10). https://doi.org/10.1093/femsle/fnz117

Hjeltnes B, B.-J. B., Bornø G, Haukaas A, Walde C S (Eds). (2019). *The Health Situation in Norwegian Aquaculture 2018*.

König, J., Wells, J., Cani, P. D., García-Ródenas, C. L., MacDonald, T., Mercenier, A., Whyte, J., Troost, F., & Brummer, R.-J. (2016). Human Intestinal Barrier Function in Health and Disease. *Clinical and Translational Gastroenterology*, *7*(10). https://doi.org/10.1038/ctg.2016.54

Kumar, G., Menanteau-Ledouble, S., Saleh, M., & El-Matbouli, M. (2015). Yersinia ruckeri, the causative agent of enteric redmouth disease in fish. *Veterinary Research*, *46*(1). https://doi.org/10.1186/s13567-015-0238-4

Lages, M. A., Balado, M., & Lemos, M. L. (2019). The Expression of Virulence Factors in Vibrio anguillarum Is Dually Regulated by Iron Levels and Temperature. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02335

Long, A., Fehringer, T. R., LaFrentz, B. R., Call, D. R., & Cain, K. D. (2014). Development of a waterborne challenge model forFlavobacterium psychrophilum. *FEMS Microbiology Letters*, *359*(2), 154-160. https://doi.org/10.1111/1574-6968.12563

Lorenzen, E., Brudeseth, B. E., Wiklund, T., & Lorenzen, N. (2010). Immersion exposure of rainbow trout (Oncorhynchus mykiss) fry to wildtype Flavobacterium psychrophilum induces no mortality, but protects against later intraperitoneal challenge. *Fish & Shellfish Immunology*, *28*(3), 440-444. https://doi.org/10.1016/j.fsi.2009.11.025

Lorenzen, E., Dalsgaard, I., Bernardet, J. F. (1997). Characterization of isolates of Flavobacterium psychrophilum associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies. *Diseases of Aquatic Organisms*, *31*, 197-208.

Mallasvik, H. (2019). *Initial bacterial colonization of Atlantic salmon (Salmo salar) yolk-sac fry using a gnotobiotic model system*. Master's thesis, NTNU.

McCarthy, D. H. (1983). An experimental model for fish furunculosis caused by Aeromonas salmonicida. *Journal of Fish Diseases*, *6*, 231-237.

Nordmo, R., & Ramstad, A. (1997). Comparison of different challenge methods to evaluate the efficacy of furunculosis vaccines in Atlantic salmon, Salmo salar L. *Journal of Fish Diseases*, *20*, 119-126.

NVI. (2021a). *Flavobacterium psychrophilum*. Norwegian Veterinary Institute. Retrieved 28/05/21 from https://www.vetinst.no/sykdom-og-agens/flavobacterium-psychrophilum

NVI. (2021b). *Furunkulose*. Norwegian Veterinary Institute. Retrieved 28/5/21 from https://www.vetinst.no/sykdom-og-agens/furunkulose

Pérez-Pascual, D., Vendrell-Fernández, S., Audrain, B., Bernal-Bayard, J., Patiño-Navarrete, R., Petit, V., Rigaudeau, D., & Ghigo, J.-M. (2021). Gnotobiotic rainbow trout (Oncorhynchus mykiss) model reveals endogenous bacteria that protect against Flavobacterium columnare infection. *PLOS Pathogens*, *17*(1). https://doi.org/10.1371/journal.ppat.1009302

Qin, Z., Baker, A. T., Raab, A., Huang, S., Wang, T., Yu, Y., Jaspars, M., Secombes, C. J., & Deng, H. (2013). The Fish Pathogen Yersinia ruckeri Produces Holomycin and Uses an RNA Methyltransferase for Self-resistance. *Journal of Biological Chemistry*, *288*(21), 14688-14697. https://doi.org/10.1074/jbc.M112.448415

Reith, M. E., Singh, R. K., Curtis, B., Boyd, J. M., Bouevitch, A., Kimball, J., Munholland, J., Murphy, C., Sarty, D., Williams, J., Nash, J. H. E., Johnson, S. C., & Brown, L. L. (2008). The genome of Aeromonas salmonicida subsp. salmonicida A449: insights into the evolution of a fish pathogen. *BMC Genomics*, *9*(1). https://doi.org/10.1186/1471-2164-9-427

Sakai, D. K., Kimura, T. (1985). Relationship between Agglutinative Properties of Aeromonas salmonicida Strains Isolated from Fish in Japan and Their Resistance to Mechanisms of Host Defense. *Fish Pathology*.

Situmorang, M. L., Dierckens, K., Mlingi, F. T., Van Delsen, B., & Bossier, P. (2014). Development of a bacterial challenge test for gnotobiotic Nile tilapia Oreochromis niloticus larvae. *Diseases of Aquatic Organisms*, *109*(1), 23-33. https://doi.org/10.3354/dao02721

Stressmann, F. A., Bernal-Bayard, J., Perez-Pascual, D., Audrain, B., Rendueles, O., Briolat, V., Bruchmann, S., Volant, S., Ghozlane, A., Häussler, S., Duchaud, E., Levraud, J.-P., & Ghigo, J.-M. (2020). Mining zebrafish microbiota reveals key community-level resistance against fish pathogen infection. *The ISME Journal*, *15*(3), 702-719. https://doi.org/10.1038/s41396-020-00807-8

Vatsos, I. N. (2016). Standardizing the microbiota of fish used in research. *Laboratory Animals*, *51*(4), 353-364. https://doi.org/10.1177/0023677216678825

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261-5267. https://doi.org/10.1128/aem.00062-07

Yan, Q., Li, J., Yu, Y., Wang, J., He, Z., Van Nostrand, J. D., Kempher, M. L., Wu, L., Wang, Y., Liao, L., Li, X., Wu, S., Ni, J., Wang, C., & Zhou, J. (2016). Environmental filtering decreases with fish development for the assembly of gut microbiota. *Environmental Microbiology*, *18*(12), 4739-4754. https://doi.org/10.1111/1462-2920.13365

Zhou, D., Henriksen, M. M. M., Madsen, L., & Dalsgaard, I. (2013). Effect of Hydrogen Peroxide on Immersion Challenge of Rainbow Trout Fry with Flavobacterium psychrophilum. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0062590

Appendix A: SGM (Salmon Gnotobiotic Medium)

SGM was prepared from pre-made salt solutions (see Table A1) and was autoclaved before use (20 min at 121 °C, Astell AMA270BT65).

Table A1: How to make 1 L SGM (Salmon Gnotobiotic Medium) from pre-made salt solutions. All salt solutions were made by dissolving the salt in Milli-Q water. Natrium Bicarbonate solution was filter sterilized before use, using 0.2 µm PES filter units (VWR). The other three solutions were autoclaved (121 °C, 20 min, Astell AMA270BT65) before use.

Salt solution / Ingredient	Volume added to 1 L bottle (mL)
Magnesium Sulphate heptahydrate solution (12.3 g/L)	10
Kalium Chloride solution (0.4 g/L)	10
Natrium Bicarbonate solution (9.6 g/L)	10
Calcium Sulphate dihydrate solution (0.3 g/L)	200
Milli-Q water	770

Appendix B: Antibiotic SGM and Antibiotic TSB

To make antibiotic SGM and antibiotic TSB, we first prepared solutions of individual antibiotics. Volumes of these antibiotic solutions were then added to 1 L SGM or 1 L TSB (Table B1).

Table B1: How solutions of individual antibiotics were prepared. Amphotericin B was not added to TSB, only to SGM. All antibiotics were ordered from VWR. Amphotericin B was bought as a premade solution.

Antibiotic	Stock Concentration (mg/mL)	Stock Solvent	Volume added to SGM or TSB (µL)	Working concentration (mg/mL)
Rifampicin	50	Dimethyl Sulfoxide	200	10
Kanamycin	50	Water	200	10
Penicillin G	100	Water	1500	150
Ampicillin	100	Water	1000	100
Oxolinic Acid	12.5	0.05 M NaOH	6000	75
Amphotericin B	0.25	(premade solution)	1000	0.25
Erythromycin	50	90 % ethanol	200	10

Appendix C: Primer Sequences

Table C1: The sequence and target gene for all six primers used in the experiments.

Primer Name	Sequence	Target Gene
338F	5’-CCTACGGGWGGCAGCAG-3’	16S rRNA (v3-v4-region only)
805R	5’-GACTACNVGGGTATCTAAKCC-3’	16S rRNA (v3-v4-region only)
EUB8F	5’-AGAGTTTGATCMTGGCTCAG-3’	16S rRNA (whole gene)
1492R	5’-GGTTACCTTGTTACGACTT-3’	16S rRNA (whole gene)
685F	5’-CCTCGGCGTCTATACGGAAGT-3’	hom7 (small region only)
685R	5’-CAGGGTGAGCGTGAAATCCT-3’	hom7 (small region only)

Appendix D: Sequences of the v3-v4-Region of the 16S rRNA Gene

Sequencing results used to verify the identity of our strains. All three strains were classified to the expected genus using RPD Classifier v11 (Wang et al., 2007).

>FLAVOBACTERIUM_PSYCHROPHILUM_DSM_3660

GAACGCGGCTTACCATGCAAGTCGAGGGGTAGAATAGCAATATTTGAGACCGGCGCACGGGTGCGTAA
CGCGTATGCAATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGTG
AGTTGGCATCAACACACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAGCTAGTTGGT
AAGGTAACGGCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTG
AGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGCAAGCCTGAACC
AGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGCACAGGAAGAAACACTACC
TCGTGAGGTAGCTTGACGGTACTGTGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGGCAGATAAGTCAGT
GGTGAAAGCCCATCGCTCAACGATGGAACGGCCATTGATACTGTTTGGCTTGAATTATTTGGAAGAACC

>AEROMONAS_SALMONICIDA_VI-88/09/03175

AGCTAACACATGCAAGTCGAGCGGCAGCGGGAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGG
GTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCAT
ACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGC
TAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAAC
CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAA
AGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
TGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTC
TTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAG
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAGAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATG
TCTGGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAATCAGACACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC

>AEROMONAS_SALMONICIDA_11540

GGCCTAACACATGCAAGTCGAGCGGCAGCGGGAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACG
GGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCA
TACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAG
CTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAA
ACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGG

AAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC
GGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT
GGCGAAGGCGGCGC

Appendix E: Tricaine Solution

Tricaine solution was prepared by adding 27.3 mL Tris base (1 M, pH = 9) to 972.7 mL SGM, then dissolving 5.2 grams of tricaine into this solution.

NTNU

Norwegian University of
Science and Technology