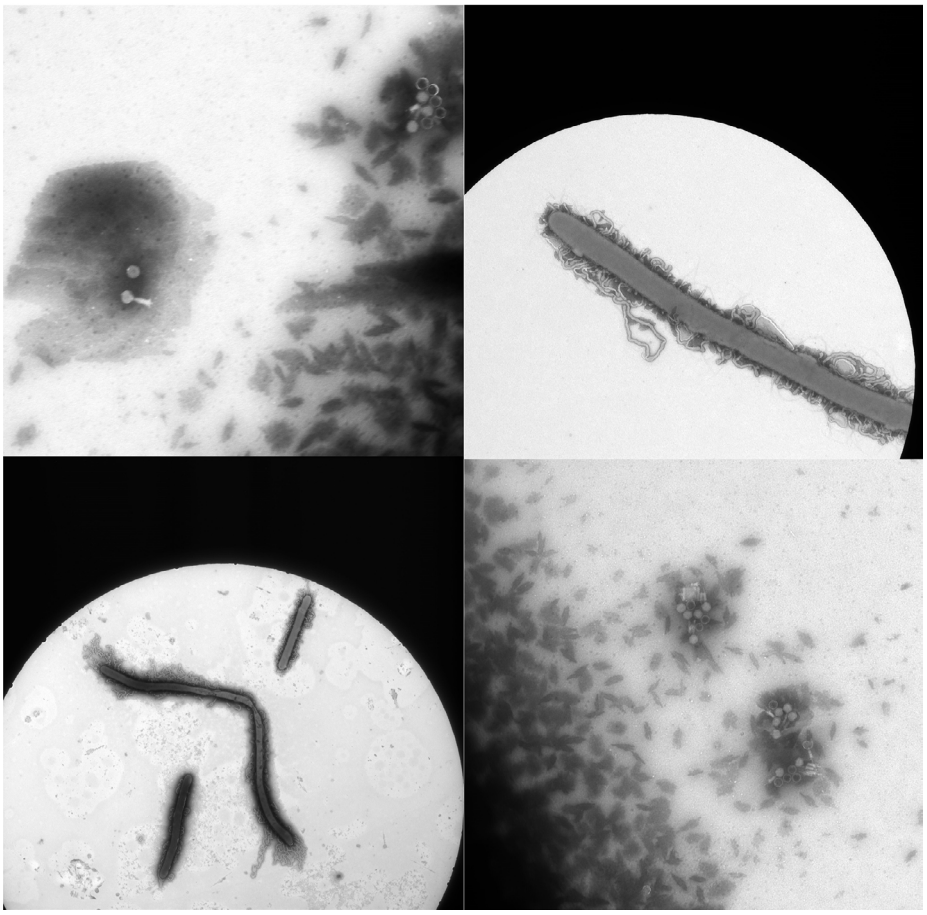
JYU DISSERTATIONS 586

Anniina Runtuvuori-Salmela

The Story of Phage Therapy against *Flavobacterium columnare* Bacterium

Phage-Bacterium Interactions and Utilization of Phage Therapy in Practice



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
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in building Ylistönrinne, auditorium YAA303, on December 16, 2022, at 12 o'clock.

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Cover Figure: Transmission electron microscopy (TEM) images of representative *Flavobacterium columnare* bacterial cells (images with black background) and phages infecting this bacterium (images without black background). Runtuvuori-Salmela, unpublished.

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ABSTRACT

Runtuvuori-Salmela, Saila Maria Anniina
The story of phage therapy against *Flavobacterium columnare* bacterium: Phage-bacterium interactions and utilization of phage therapy in practice
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Diss.

Antibiotic resistance is one of the gravest threats to global health and food production. Resistant strains can cause bacterial infections that were previously treated easily with antibiotics to become lethal. Therefore, there is a need for alternatives to antibiotics, such as bacteriophages, or phages, which are viruses of bacteria. In this thesis, I studied the use of these natural enemies of bacteria against *Flavobacterium columnare*. *F. columnare* is a globally emerging bacterium that causes high mortality among fish and large economic losses at freshwater fish farms during warm-water periods unless treated with antibiotics. At fish farms, the spread of antibiotic-resistant strains and antibiotics in the environment is a risk. Phage therapy has the potential to reduce the use of antibiotics against *F. columnare*. A large collection of new phages that may be used against the pathogen were collected. The most promising phages from this collection were selected so as to study their resistance effects on bacterial strains and to test the different administration routes by which phage therapy can decrease the mortality that columnaris disease causes in rainbow trout. It was seen that *F. columnare* strain adherence and biofilm formation are lower and, consequently, virulence is weaker as a trade-off for phage resistance. It emerged that the optimal means of controlling *F. columnare* infections is to bathe fish in phage solution after the first columnaris symptoms appear. Phage bathing before bacterium infection and the use of immobilised phages on plastic sheets slow the progression of bacterial infections. This thesis reinforces the notion that phage therapy holds considerable promise for combatting bacterial infections, but more studies are needed to understand the interactions between different bacterial strains and these phages.

Keywords: Bacteriophage; columnaris; fish farming; *Flavobacterium columnare*; phage therapy.

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TIIVISTELMÄ

Runtuvuori-Salmela, Saila Maria Anniina
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Diss.

Antibioottiresistenssin on todettu olevan yksi suurimmista uhkista maailmanlaajuiselle terveydelle ja elintarviketuotannolle. Resistentit kannat voivat tehdä hoidettavissa olevista bakteeri-infektioista tappavia. Tästä syystä antibioottihoidoille tarvitaan kipeästi vaihtoehtoja, kuten bakteriofagit eli faagit, jotka ovat bakteerien viruksia. Tässä väitöskirjassa olen tutkinut näitä bakteerien luonnollisia vihollisia *Flavobacerium columnare* -bakteeria vastaan. *F. coumnare* on maailmanlaajuisesti leviävä bakteeri, joka aiheuttaa korkeaa kuolleisuutta kaloilla ja suuria taloudellisia tappioita makean veden kalanviljelylaitoksilla lämpöisinä ajanjaksoina ellei antibioottihoitoja käytetä. Riski antibioottiresistenttien kantojen sekä antibioottien leviämisestä kalanviljelylaitoksilta ympäristöön on olemassa. Tässä tutkimuksessa kerättiin kokoelma *F. columnare* faageja ja lupaavimmat valittiin jatkotutkimuksiin. Havaittiin, että kompromissina faagiresistenssin syntymiselle, *F. columnare* kantojen tarttumis- ja biofilmin muodostuskyky olivat heikompia ja tästä syystä kalojen kuolleisuus oli matalampaa. Erilaisia faagihoitomenetelmiä tutkittaessa havaitiin, että paras tapa hallita *F. columnare* -infektioita oli kylvettää kaloja faagiliuoksessa ensimmäisten columnaris-oireiden puhkeamisen jälkeen. Faagikylvetykset ennen bakteeri-infektioita ja muovikalvoille kiinnitetyt faagit osoittivat myös mahdollisuuksia, sillä ne hidastivat bakteeri-infektioiden puhkeamista. Tämä väitöskirjatyö vahvistaa käsitystä siitä, että faagiterapiaa voidaan käyttää *F. columnare* bakteeri-infektioita vastaan. Lisää tutkimuksia kuitenkin tarvitaan, jotta eri bakteerikantojen ja näiden faagien välisiä vuorovaikutuksia ymmärrettäisiin paremmin.

Avainsanat: Bakteriofagi; columnaris; faagiterapia; *Flavobacterium columnare*; kalanviljely.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–V.

I Runtuvuori-Salmela, A., Kunttu, H.M.T., Laanto, E., Almeida, G.M.F., Mäkelä, K., Middelboe, M. & Sundberg, L.-R. 2022. Prevalence of genetically similar Flavobacterium columnare phages across aquaculture environments reveals a strong potential for pathogen control. *Environmental Microbiology* 24: 2404-2420.

II Runtuvuori-Salmela, A., Sorsa, J., Kunttu, H.M.T., Middelboe, M. & Sundberg, L.-R. Effects of single phage and two phage mixture additions on *Flavobacterium columnare* growth: Implications of phage resistance. Manuscript.

III Kunttu, H.M.T., Runtuvuori-Salmela, A., Sundell, K., Wiklund, T., Middelboe, M., Landor, L., Ashrafi, R., Hoikkala, V. & Sundberg, L.-R. 2021. Bacteriophage Resistance Affects *Flavobacterium columnare* Virulence Partly via Mutations in Genes Related to Gliding Motility and Type IX Secretion System. *Applied and Environmental Microbiology* 87: e00812-21.

IV Donati, V.L., Dalsgaard, I., Runtuvuori-Salmela, A., Kunttu, H., Jørgensen, J., Castillo, D., Sundberg, L.-R., Middelboe, M., & Madsen, L. 2021. Interactions between Rainbow Trout Eyed Eggs and *Flavobacterium* spp. Using a Bath Challenge Model: Preliminary Evaluation of Bacteriophages as Pathogen Control Agents. *Microorganisms* 9: 971.

V Kunttu, H.M.T., Runtuvuori-Salmela, A., Middelboe, M., Clark, J., Sundberg, L.-R. Comparison of Delivery Methods in Phage Therapy against *Flavobacterium columnare* Infections in Rainbow Trout. *Antibiotics* 2021. 10: 914.

Responsibilities of the author:

I Main responsibility for phage isolations and characterizations, transmission electron microscopy, designing the host range study and analyzing the data, and phage genome extraction. Shared responsibilities in study design, water sample collections, virulence experiment, analyzing the sequenced data, and writing.

II Main responsibility in designing and implementing the bacterial phenotypic analyses: proteolytic assay, adhesion and biofilm experiments, bacterial yield and growth rates, analyzing the results, and writing the

manuscript. Shared responsibilities with phage and phage mix exposure and study design.

III Main responsibilities included performing and preparation of the experiment, purifying bacterial isolates from phages, analyzing morphologies, and recording the results. Shared responsibilities in study design and virulence experiment.

IV Main responsibility was to design the methodology and start the experiment in DTU with Valentina Donati, then bring the method for *F. columnare* to JYU and do the *F. columnare* phage treatments and samplings. Shared responsibilities in carrying out experiments, and data curation.

V Main responsibilities were tissue sample preparations from the fish organs from experimental setups, phage samplings from water and tissue samples during the experiments, and designing and executing shelf-life experiments for phages. Shared responsibilities were in study design, virulence experiments, phage production, and maintenance for phage therapy experiments.

ABBREVIATIONS

Abi system	Abortive infection system
ARISA	Automated ribosomal intergenic spacer analysis
Cas system	CRISPR-associated protein system
CFU	Colony forming unit
CRISPR	Clustered regularly interspaced short palindromic repeats
FAO	Food and Agriculture Organization
OMVs	Outer membrane vesicles
PFU	Plaque forming unit
RAS	Recirculating aquaculture system
RFLP	Restriction-fragment length polymorphism
RM system	Restriction-modification system
Sie system	Superinfection exclusive system
TEM	Transmission electron microscope
WHO	World Health Organization

The important thing
is not to stop questioning
Curiosity has its own reason
for existing

Albert Einstein

1 INTRODUCTION

The occurrence of antibiotic-resistant bacterial strains has become a global threat (WHO 2014). The problem arises among humans, animals, and plants, but also in food production. There is a risk that emerging resistant strains can spread resistance from one source to another, causing new problems at new destinations. For example, resistant strains in farm animals can spread to humans, causing problems with the treatment of diseases with antibiotics that are intended for human use. Bacteria can form multiresistant strains, which can no longer be treated with antibiotics.

The practice of having dense populations live under rearing conditions and intensified production increase the risk of farmed animals contracting bacterial diseases. Those diseases must be treated with antibiotics. This increases the risk of the emergence of antibiotic-resistant strains. Aquaculture and fish farming is one of the most rapidly growing sectors of food production (FAO 2020). The nature of fish farming is such that antibiotics and antibiotic-resistant strains can spread directly into the environment and affect environmental microbes (Tamminen *et al.* 2011, Cabello *et al.* 2013). One pathogen that requires frequent antibiotic treatment at freshwater fish farms is *Flavobacterium columnare* (Pulkkinen *et al.* 2010). This environmental pathogen causes high mortality in fish and high economic losses during warm-water periods (Pulkkinen *et al.* 2010). In addition, few approved antibiotics can be used to treat it, which raises concerns about treatments if antibiotic-resistant strains begin to form (Anon 2016).

The threat of antibiotic resistance has caused the search for alternative treatments to intensify. Bacteriophages, also known phages, have been shown to be promising in treating pathogenic bacteria without affecting the normal microbiome (Clokie *et al.* 2011, Loc-Carrillo and Abedon 2011, Chan *et al.* 2013). The viruses of bacteria have been studied within the context of a treatment, phage therapy, which can be used to prevent, to treat, and control bacterial infections. Phages can be found wherever there are bacteria, and their isolation is relatively easy and inexpensive, relative to the discovery of new antibiotics (Kutter *et al.* 2010, Loc-Carrillo and Abedon 2011). In this thesis, I studied the

usability and usefulness of phage therapy against *F. columnare in vitro* and *in vivo* in order to accumulate knowledge about the benefits and possible challenges of phage therapy. I also focused on the optimal administration route for the phages and the manner in which phage resistance affects the bacterium.

2 REVIEW OF THE LITERATURE

2.1 Challenges in fish farming and fish welfare

The world population is expected to reach 8.5 billion in 2030 and 9.7 billion in 2050 (UNDESA 2019). The expansion of the population will inevitably affect demands for food. One sector of food production that is significantly affected by population growth is aquaculture. Fish, molluscs, and crustaceans are increasingly being produced by farming, which takes up space on land and sea. As a fast growing, healthy, and nutritious food source, farmed fish can meet the nutritional challenges of the world. Therefore, fish farming remains one of the most rapidly growing animal production sectors (FAO 2020).

Fish farming can be carried out in ponds, cages, flow-through systems, and recirculating aquaculture systems (RASs; Naylor *et al.* 2021). The pond system can be highly diverse, and fish may be reared in plastic, fibreglass, or ground-based pools, inside or outside, with or without a roof, or even in ditches. Flow-through systems take water from a known source and release it back into the environment, for example into a river. The cage system (with net pens) entails having open water between the fish and the environment. In RASs, water exchange is limited, and wastewater is treated and recycled back into the system (Naylor *et al.* 2021). Almost all of these farming systems can have negative impacts on the environment because water that contains fish-production and treatment-chemical waste may be released from the plant into the surrounding water. Only a RAS limits outlet water, but it has other vulnerabilities.

Intensive farming in aquaculture, which entails high fish densities, monoculture species, optimised feeding, the maintenance of oxygen content, and water circulation, can produce a high amount of nutrition in a delimited capacity (Mansour *et al.* 2021). However, problems cannot be avoided when production becomes more efficient and farming volumes increase.

Monocultures of certain species, high densities, and climate change influence water ecosystems, increasing the risk of fish being exposed to various disease agents (Murray and Peeler 2005). Farmed fish may suffer from structural and reproductive difficulties, but they may also suffer from infectious diseases, which can easily spread between fish under farming conditions (Assefa and Abunna 2018). Some infectious diseases may cause high financial losses at farms (Murray and Peeler 2005, Tavares-Dias *et al.* 2017).

Infectious diseases are among the most critical problems for RASs. Increasing the use of closed water circulation within the RAS has been proposed as a means of avoiding the contamination of the entire system (Naylor *et al.* 2021). It has also been argued that fish can feel pain and stress (Correia *et al.* 2011), which highlights the importance of promoting fish welfare in farming.

A variety of medical treatments must be used to control and avoid disease. Vaccinations, antibiotic treatments, probiotics, and biosecurity measures, such as quarantine and disinfection, can be used to treat and prevent infections and to improve fish health and wellbeing (Murray and Peeler 2005, Assefa and Abunna 2018). However, it remains impossible to control all medical waste in all farming systems.

Parasites, viruses, and bacteria can develop to become more infectious at fish farms (Pulkkinen *et al.* 2010, Mennerat *et al.* 2010, Atkins *et al.* 2013). When released into the environment, they can be detrimental to natural fish stocks. At the same time, changing environmental conditions influence pathogen virulence through complex interactions (Ashrafi *et al.* 2018). Human-induced environmental alterations, like fish farming, can cause phenotypic variation in intensive farming, not necessarily by increasing the virulence of pathogens but through population growth. Consequently, intensive farming can cause increases in mortality (Pulkkinen *et al.* 2022).

Fish are farmed not only for the food industry but also with a view to recovering wild fish stocks and to maintaining the genetic heritage of endangered and threatened species (Bain *et al.* 2007, Trushenski *et al*. 2010). The latter practice is called "enhancement aquaculture". Beyond the fishing industry, diseases at fish farms can hamper the conservation and restoration programs on which the survival of endangered species of fish currently depends. It is therefore exceedingly important that the health and wellbeing of farmed fish be protected.

In this study, I focus on one of the most common bacteria that cause significant problems at fish farms as well as on the treatments that are available now and the ones that may become available in the future.

2.2 *Flavobacterium columnare*

One of the most common pathogens in the fish farming industry is the *Flavobacterium columnare*. *F. columnare* (phylum *Bacteroidetes*, family *Flavobacteriaceae*) causes bacterial infections and epidemics in freshwater fish farms worldwide, especially during warm-water periods (Pulkkinen *et al.* 2010, Declercq *et al.* 2013b). *F. columnare* was previously known as *Bacillus columnaris*, *Chondococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris* (Declercq *et al.* 2013b). This yellow-pigmented, rod-shaped, and Gram-negative bacterium can survive in an unpredictable environment (van der Woude 2006, Sundberg *et al.* 2014). It can survive for extended periods in the water without a host (Declercq *et al.* 2013b). *F. columnare* can infect different wild and farmed fish species (Decostere *et al.* 1999, Morris *et al.* 2006, Faisal *et al.* 2017). One of these species is the rainbow trout (*Oncorhynchus mykiss*), which is therefore used often in columnaris studies (Declercq *et al.* 2013b, FAO 2019), including this thesis.

2.2.1 Bacterial phenotype

When bacterial isolates are isolated from the fish farms, their colony morphology is rhizoid (Fig. 1, a). *F. columnare* can change from rhizoid morphology to soft and rough (Fig. 1). Kunttu *et al.* (2009) discovered that there was a correlation between phenotypes and virulence against fish. In its virulent form, *F. columnare* is rhizoid (Fig. 1a) and forms an organised structure with a thick layer of extracellular filamentous material (Laanto *et al.* 2014). Two less virulent structures, which form rough (Fig. 1b) or soft (Fig. 1c) morphologies, have lost the shape of a sun-like colony and are less organised at the cellular level (soft are less organised than rough, Laanto *et al.* 2014).

F. columnare may encounter various environmental changes that may pose a threat to the bacterium, such as temperature variation, nutrient scarcity, and parasitism (van der Woude 2006, Sundberg *et al.* 2014). One way for *F. columnare* to adjust to these challenges might be connected to structural changes on the cell surface. Phenotypic changes can correspond to environmental pressures (Pulkkinen *et al.* 2022), and structural changes on the cell surface can affect the morphology of a colony (Kunttu *et al.* 2009), influencing the features of the bacterium (Penttinen *et al.* 2018). This transient change from a rhizoid to a less virulent form can serve as a safeguard for the bacterium in the short term; however, it can also impair its virulence (Kunttu *et al.* 2009), which is disadvantageous in the long run.

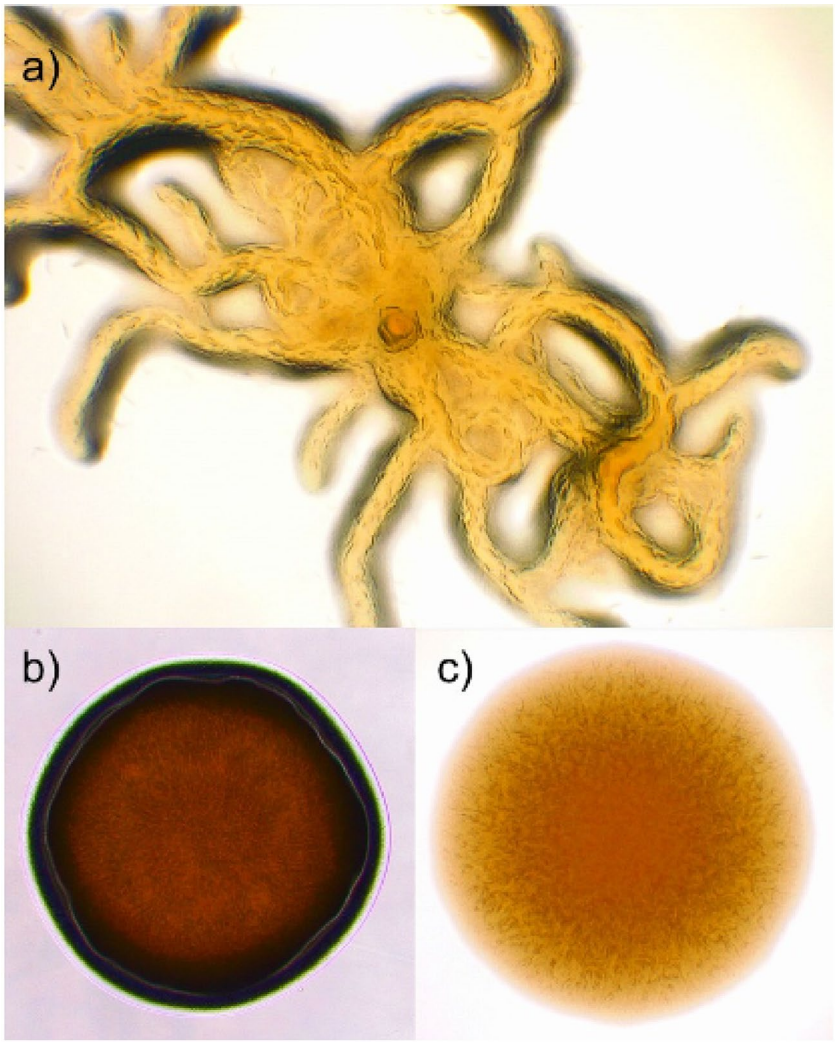


FIGURE 1 *Flavobacterium columnare* different colony morphologies: (a) rhizoid, (b) rough, and (c) soft. Figure from Kunttu *et al.* 2021. CC by 4.0 License.

2.2.2 Bacterial genotype

F. columnare genotypes have been studied with a view to understanding the bacterium and its features. At first, bacterium genetic material needs to be identified and recognised as belonging to a certain bacterial species or subspecies. Ribosomal ribonucleic acid (rRNA) genes are particularly useful because locations in operons, which amount in bacterial genomes, are bacterium dependent (Klappenbach *et al.* 2000, 2001). Among these ribosomal RNA genes (16S-23S-5S, order in operons), 16S is the most widely used target for bacterial identification (Church *et al.* 2020).

Deoxyribonucleic acid (DNA) can be cut into different sizes and fragments by using restriction enzymes (Triyanto and Wakabayashi 1999). This restriction-fragment length polymorphism (RFLP) is used to recognise and categorise fragments (Triyanto and Wakabayashi 1999). *F. columnare* can be

identified in six genomovars (I, II, IIA, IIB, III, and I/II) on the basis of the RFLP of the 16S rRNA gene (16S rDNA).

In Europe, genomovars I and II have been reported to cause infections. Most of the Finnish *F. columnare* strains belong to the common European genomovar I (Michel *et al.* 2002, Suomalainen *et al.* 2006), while Asian-type strains (genomovar II) are probably imported (Michel *et al.* 2002). To study genomovars more closely at the genetic level, non-coding sequence lengths between the 16S and 23S genes (García-Martínez *et al.* 1996), which are called internal transcribed spacers (ITS), can be compared by automated ribosomal intergenic spacer analysis (ARISA) of related bacteria (Fisher and Triplett 1999). Finnish *F. columnare* can be classified into eight different genetic groups: A, B, C, D, E, F, G, and H (Suomalainen *et al.* 2006). Ashrafi *et al.* (2015) showed that Finnish strains can be separated into different sequence types, but no correlation with environmental factors was observed. However, the authors found that stocks that had been isolated from the environment clustered with epidemic stocks that had been isolated at fish farms, indicating that environmental bacteria can be the source of epidemical strains (Ashrafi *et al.* 2015), which Kunttu *et al.* (2012) also predicted. More recently, whole genome sequencing (WGS) has been conducted with the extracted genome, which is cut into previously designed short fragments of predetermined sizes by using enzymes (Gautam *et al.* 2019). *F. columnare* WGS has been made for 105 strains (NCBI datasets, 6.7.2022), and more are needed for a better understanding of the genetic characteristics of this pathogen and the effects of the genes on the life cycle of the bacterium.

2.2.3 Infections at fish farms

It is possible to isolate *F. columnare* from natural water sources (Kunttu *et al.* 2012) and from tropical freshwater aquarium fish (Decostere *et al.* 1998), but fish farms are the most probable source of bacteria, especially during warm-water periods, when the water temperature rises over 18 °C (Pulkkinen *et al.* 2010, Declercq *et al.* 2013b). *F. columnare* causes columnaris diseases in fish. This disease is also known as myxobacterial disease, saddleback, cotton wool disease, mouth fungus, and fin rot (Declercq *et al.* 2013b). These names refer to the parts of the fish where the infection is most visible and pronounced. The most likely cause of fish farm columnaris disease is the water sources (Kunttu *et al.* 2012). When the bacterium enters the fish farm, it may adhere to the surfaces of ponds and to other surfaces as well as to fish gills and mucus. After adherence, the bacterium multiplies and forms a biofilm.

During cell division, the bacterium secretes extracellular enzymes which are involved in the process of the decomposition of the surrounding biological material (Declercq *et al.* 2013b). Columnaris disease causes damage at various levels. It can progress from the mucosal surface to the scales and the skin and then to muscle tissue (Declercq *et al.* 2013b). *F. columnare* can, depending on the bacterial strain and the environmental circumstances, cause acute and chronic infections. In acute infection, external damages are visible, and mortality is

high. In chronic infection, the symptoms are not necessarily noticeable before death. Acute infections normally occur and lead to death more rapidly than chronic infections. In the latter, several symptoms are not particularly clear, which can lead to misdiagnosis (Declercq *et al.* 2013b). Several different individual virulence factors have been proposed. Together, they may interact with virulence (Suomalainen *et al.* 2006, Klesius *et al.* 2008, 2010, Kunttu *et al.* 2011).

It appears that the age of the fish host might affect disease progression. – Young fish become acutely ill through the gills, with major symptoms also present in the body, mouth, and tail, while older fish develop chronic disease with necrotic tissues in the gills and slower lesions on other body parts (Declercq *et al.* 2013b). Because bacteria can quickly cause high mortality and epidemic spread, farms with fish of different ages challenge to detect infections at an early stage. The timely initiation of treatment is extremely important. Accordingly, fish farmers follow temperature fluctuations because the likelihood of *F. columnare* appearing increases with water temperature (Pulkkinen *et al.* 2010, 2022, Ashrafi *et al.* 2018).

It has been suggested that the fish egg surface microbiome has an important role in fish welfare as a first source in the formation of the intestinal microbiome (Hansen and Olafsen 1999, Llewellyn *et al.* 2014). When the eggs hatch, the sterile fry will have their first contacts with microbes from the egg surface and the surrounding water (Llewellyn *et al.*, 2014). The microbiome on the surface of the eggs can be the first developer of the immune response of the fry and thus an important factor for the strength of immune defences for the rest of the life of the fish (Hansen and Olafsen 1999, Llewellyn *et al.* 2014). Due to the influence of the environment, this microbiome may also contain potential bacterial pathogens such as *Pseudomonas* spp., *Vibrio* spp., and *Flavobacterium* spp. (Hansen and Olafsen 1989, 1999). At farms, disinfection protocols for eggs are used often to avoid bacterial infections among fish fry during hatching (de Swaef *et al.* 2016). Disinfection with chemicals may destroy the desirable microbes, which ensure the welfare of the fish. Therefore, different protective methods should be studied. *F. psychrophilum* has been known to survive in fertilised eggs after disinfection (de Swaef *et al.* 2016), meaning the eggs may be one possible route for *F. psychrophilum* infections among fish fries. It has been assumed that *F. columnare* might also survive on fish eggs and cause problems with fish fries. Previously, it has been observed that the virulence of *F. columnare* in fish eggs depends on the strain of the bacteria and the species of the fish (Evenhuis *et al.* 2021). The effects of bacteria on the surfaces of eyed eggs have received little attention. Thus, the effect of *F. columnare* in eyed eggs remains poorly understood.

2.2.4 F. columnare versus antibiotics

Fish farming is exposed to environmental fluctuations, some of which increase the risk of pathogen occurrence and epidemics. To ensure the efficiency of fish production and the wellbeing of fish, it is necessary to treat some bacterial

diseases with antibiotics. Since the use of antibiotics can have both direct and indirect effects on other animals, the environment, farm workers, and those who eat the fish, efforts are being made to control it. The use of antibiotics in the EU and Finland is currently governed by legislation and monitored. In Finland this is done by the Finnish Food Authority as well as by veterinarians. For example, the use of antimicrobial drugs that are intended for the treatment of serious infections in humans on animals is prohibited due to the possible emergence of resistance (Anon 2016).

In fish farming, attention must be paid to the use of antibiotics and the development of antibiotic resistance. Only a few drugs for fish are available. In freshwater fish farms in Finland, *F. psychrophilum* and *F. columnare* are treated primarily with oxytetracycline. If resistance against oxytetracycline treatment is detected, the secondary treatment is florfenicol (Anon. 2016). Both antibiotics are also used in the treatment of bacterial infections in other production animals, such as ruminants and swine. After antibiotic treatment, fish that are close to slaughter size must be kept isolated until the antibiotic residues have been removed completely. Given the nature of *F. columnare*, which is an opportunistic bacterium (Kunttu *et al.* 2012, Declercq *et al.* 2013b), and environmental effects such as longer warm-water periods, antibiotic treatments have to be repeated regularly (Pulkkinen *et al.* 2010, 2022). Increasing the number of treatments elevates the risk of the development of antibiotic resistance. *F. columnare* is not an exception, and it is capable of forming antibiotic resistance against oxytetracycline (Suomalainen *et al.* 2006; Declercq *et al.* 2013a).

The challenges that follow from the use of antibiotics are not limited to fish farms because between 70% and 80% of the antibiotics that are administered may be released into the environment and affect its bacteria (Tamminen *et al.* 2011, Yang *et al.* 2013, Cabello *et al.* 2013, Watts *et al.* 2017). This leakage of antibiotics into the environment may lead to the emergence of antibiotic-resistant strains. It can also affect the balance and expression of microbial communities in the aquatic environments of fish farms (Alanis 2005, Davies and Davies 2010). The risks of antibiotics for fish farms, food production, and the environment are known, but the use of antibiotics is essential for fish welfare and food production in the absence of superior alternatives.

2.3 Bacteriophages

Bacteria, like mammals, face parasitism. Bacteriophages (i.e., phages) are viruses that only infect bacteria and can be found wherever bacteria exist. The name "bacteriophage" is derived from the Greek words' "bacteria" and "phage" ("to eat"). The whole word therefore means "bacteria-eater" (d'Herelle 2007). These viruses are the most abundant biological entities that are known to humankind at present (Clokie *et al.* 2011).

Phages can vary considerably in structure and genetic material. Genetic material, that is, single- or double-stranded nucleic acids, such as DNA or RNA, are protected with a capsid which can be polyhedral, filamentous, or pleomorphic. Genetic material can also be connected to a tail (Ackermann 2007, *Dion et al.* 2020). Phages can have icosahedral, filamentous, or pleomorphic shapes, and some capsids might bind to the tail, which is built with tail proteins. The structure of some phages may also contain lipids, spike proteins, maturation proteins, and tail fibres as well as baseplate, collar, connector, or head-to-tail joining proteins (Ackermann 2007).

Phages can be classified by genome and morphology. The Bacterial and Archaeal Viruses Subcommittee (BAVS) of the International Committee on the Taxonomy of Viruses (ICTV) is responsible for classification, which is based on pre-agreed methods (Adriaenssens *et al.* 2017). In this thesis, I will focus on structural classification in order to keep the volume of the literature that I review tractable and to develop the exposition consistently.

New methods, developments in research, and the discovery of new phages have produced changes in their structural classification in recent years. Previously, phages were classified on the basis of their structure, which was observed by using transmission electron microscopy (TEM). Most isolated phages are tailed and belong to the order of *Caudovirales* (Suttle 2005, Ackermann 2007). *Caudovirales* were divided into three families, which were classified as *Myoviridae* (contractile tail), *Siphoviridae* (long non-contractile tail), and *Podoviridae* (short non-contractile tail). In this older classification, all phages were not sharing similarities and were not grouped into orders (Ackermann 2007).

When phages are isolated, tailed phages still seem to dominate morphologies, probably because of the isolation methods that are in use and the manner in which tailed phages create clear and easily visible plaques. For example, exceptionally large phages may go undetected (Abedon 2008). As methods evolve and knowledge accumulates, new phages will certainly be found. Some phages have not been classified, which, coupled with the intensification of research, has created considerable demand for an updated taxonomy (Adriaenssens *et al.* 2017, Turner *et al.* 2021). The order of *Caudovirales* no longer exists. At present, tailed phages are known as a class of *Caudoviricetes*, with morphologically divided phages, namely myoviruses, siphoviruses, and podoviruses (Turner *et al.* 2021). The new taxonomy relies on phage genome sequencing, which is why it has been found that, prior to the three families, classified phages belong to different orders and share similarities with phages from other families. New families have also been found (Turner *et al.* 2021).

2.3.1 History of phages (and a bit of antibiotic)

Although phages have most likely existed as long as bacteria, their discovery necessitated the development of novel research methods, as well as luck. Two researchers discovered phages by coincidence and almost at the same time, namely Frederick Twort and Félix d´Hérelle (Twort 1915, d'Herelle 2007).

Twort discovered a glassy transformation from "*Micrococcus*" plates (Twort 1915) while d´Hérelle was able to isolate an "anti-microbe" for the first time. One of Twort´s conclusions was that the reason for bacterial colony transformation is a virus-like factor. D´Hérelle described the lysis of *Shigella* cultures, drew conclusions about amplification inside the cell, developed methods that are still in use, and was the founder of phage therapy. It was from d´Hérelle that bacteriophages acquired their name (d'Herelle 2007). The first image of phages was not obtained until the 1940s, long after they had been discovered and described (Ackermann 2007).

Before antibiotics, bacterial infections were severe. Treatments for infections had to be found, and phages provided a promising solution to this problem. D´Hérelle studied the potential of phage therapy, and the commercialisation of phages aroused interest until the start of the antibiotics era (Summers 2001, Abedon *et al.* 2011).

Any study that refers to phages as controlling agents in bacterial growth must mention antibiotics, a requirement that becomes even more pressing when it is the history of phages that is in focus. Antimicrobials are natural compounds which were part of the fight against bacteria as early as the period between 350 CE and 550 CE, although their involvement was not known to science. For example, residues of tetracycline have been found in skeletal remains, which has sparked speculation about a diet that contained a source of tetracycline, namely *Streptomyces* species (Bassett *et al.* 1980, Cook *et al.* 1989, Nelson *et al.* 2010). The conscious use of antibiotics did not begin until much later.

In 1928, Alexander Fleming discovered penicillin (Fleming 1929, Chain *et al.* 2005). His results, together with Howard Florey and Ernest Chain's penicillin purification protocol (Chain *et al.* 2005), enabled the mass production of penicillin to commence in 1945.

The lack of advanced technology and the poor understanding of the nature of phages obstructed research on their therapeutic use before and after the antibiotic era (Abedon *et al.* 2011). Scientists in some countries, such as Poland, the US, the Soviet Union, and Georgia, continued studying phages (Abedon *et al.* 2011). Although the study of phage therapy was interrupted, research on phages continued, and the focus shifted to their molecular biology. Phages have played a key role in the development of methods that are commonplace in contemporary laboratories as well as in the study of replication mechanisms, virus structure, genetic engineering, sequencing, and restriction enzymes (Henry and Debarbieux 2012, Salmond and Fineran 2015). The basics of molecular biology have a strong foundation in the early stages of phage research (Henry and Debarbieux 2012).

Concern about antibiotic resistance arose after the antibiotic era. Phages were rediscovered when the threat of antibiotic resistance was understood and when the search for alternatives began (Williams Smith *et al.* 1987, Theuretzbacher 2013). Due to the pressure of antibiotic resistance, the amount of resources that is allocated to phage research has increased. New technologies have precipitated considerable progress in the study of the nature and biology of phages; new phages are being discovered every day.

2.3.2 How phages infect bacteria

Phages are viruses, meaning that they lack reproductive mechanisms. Therefore, they depend on a suitable host cell for the continuation of their life cycle. Phages have learned to adapt to the living conditions of their host bacteria, which has equipped some with the ability to survive in extreme conditions, such as those in hot springs (Rice *et al.* 2001, Breitbart *et al.* 2004) or Antarctic Sea ice (Luhtanen *et al.* 2018). Although bacteria and viruses are thought of as antagonistic, they interact constantly and affect each other´s evolution. Phages have been said to have a key role in the biology of microbes (Clokie *et al.* 2011). The phage lifecycle is usually described by reference to tailed phages, which account for the majority of phages that have been isolated (Suttle 2005, Ackermann 2007). In this thesis, too, the examples of the proliferation of phages generally concern tailed phages. Moreover, the phages that I studied are tailed.

Phages can go through lytic or lysogenic life cycles. In the beginning, phages and bacteria must be in the same environment. When the phage and the bacterial cell meet, the phage uses its tail or spikes to recognise a surface component, that is, a receptor (Letellier *et al.* 2004). Almost all surface components can serve as receptors for phages. The examples include flagella, pili, capsules, lipopolysaccharides, and proteins (Letellier *et al.* 2004). This adsorption initiates phage infection.

At the beginning of phage infection, phages eject the genomic DNA from the head via the tail tube, through the bacterial cell wall, or walls, and into the cytoplasm (Letellier *et al.* 2004). The empty phage body that is left outside the bacterial cell is sometimes called a phage ghost (Herriott and Barlow 1957). When phage DNA is inside the cell, host-encoded RNA polymerase initiates the transcription of the phage genes. During phage genome replication and viral protein transcription, the packing of new virus particles can commence. The phage head begins to form first. It is followed by connector-complex formation at one point of the prohead. In the phage assembly phase, the terminase translocates the phage genome into the procapsid. Through this connector complex, the genome is packed inside the prohead and released during phage infection. The connector complex also functions as an attachment point for the forming tail, which is connected to the complex after the phage genome is packed (Letellier *et al*. 2004). After this packing and assembly, the formed virus is mature and ready for release. Phages use lysis proteins (e.g., endolysin and holin) to break the host bacterium cell and release mature virions by osmotic pressure (Letellier *et al*. 2004, Fortier and Sekulovic 2013).

The temperate phage is capable of completing the lytic cycle and the lysogenic cycle. During the lysogenic cycle, the phage injects the genome into the bacterium cytoplasm, as in the lytic cycle. Instead of starting the lytic cycle, the phage genome integrates into the bacterial genome (Hampton *et al.* 2020, Mäntynen *et al.* 2021). This genome, which the phage brings to the bacterial cell, might contain genes that code for toxins, improve pathogen survivability, or help in the fight against antibiotics (Gill and Hyman 2010). Integrated phage

DNA (prophage) remains in this stable state in the bacterium genome and can be replicated with the bacteria. The lytic cycle is activated when the circumstances are suitable for the phage to emerge (Mäntynen *et al.* 2021).

2.3.3 Phages versus *F. columnare*

Research on phages and *F. columnare* had begun before 1955, which was when the first known *F. columnare* phages were found after persistent research (Anacker and Ordal 1955). The study of the use of phages against columnaris continued slowly. However, by 1966, the phage genome had been identified as DNA, TEM images had been taken, and researchers were more aware of the manner in which phages infect *F. columnare* bacteria (Kingsbury and Ordal 1966).

Research methods and tools have evolved significantly since 1953, which has enabled the study of phages to become more profound and to develop more rapidly. Laanto *et al*. (2011) isolated and characterised phages that may be used against *F. columnare*, possibly for the first time in Europe. Since the publication of that study, complete genomes have been sequenced and analysed, and treatment potential for *F. columnare* infections has been examined (Laanto *et al.* 2015).

F. columnare phages have the same sources as bacteria, namely freshwater and aquaculture environments. The most straightforward method of isolating phages is to collect water samples at fish farms during an *F. columnare* outbreak (Laanto *et al.* 2011). Water samples are filtered to remove bacteria, and a phage search is performed by presenting potential host bacteria for phages to potentially infect. Phages are detected visually on Agar plates with growing *F. columnare*. Like other phages, *F. columnare* phages create plaques and clear areas on the bacterial lawn, which are indicative of the lysis of the bacterium culture (Fig. 2). Not all phages infect all *F. columnare* bacteria. Furthermore, even if a phage infects a certain bacterial strain, the latter can learn to resist infection.

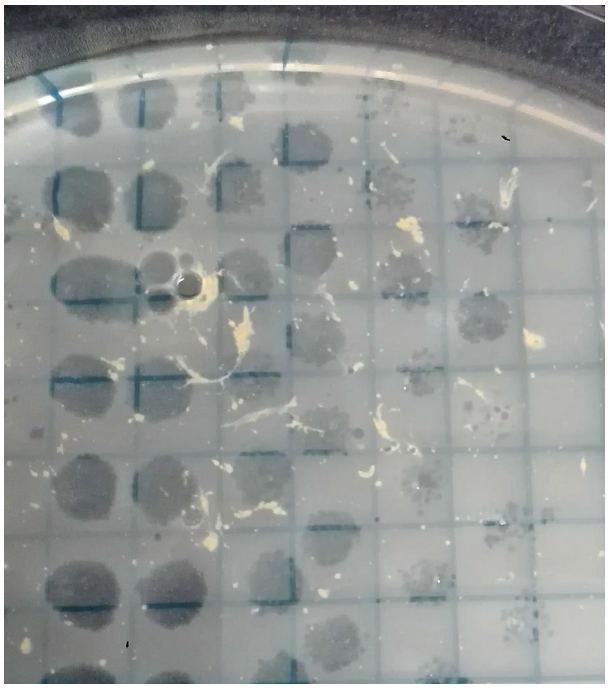


FIGURE 2 Phage plaques on the *F. columnare* bacterial mat. Large clear areas on left are phage drops, while the smaller clear areas, or holes, on the right are individual plaques that indicate one phage unit. Runtuvuori-Salmela, Unpublished.

2.3.3.1.1 *F. columnare* protection against phages and how phages can circumvent these mechanisms

When phages encounter bacterial cells, they adsorb to a specific receptor to start the infection. However, the bacteria do not remain inactive under this pressure. Due to years of competition, they have developed several different defence mechanisms against phages. Bacteria can go through mutations, where the surface proteins that phages use are modified to become unsuitable for them (Labrie *et al.* 2010, Hampton *et al.* 2020). This can entail modifying, altering, masking, and disguising receptors. The bacterium can also form outer membrane vesicles (OMVs) which have phage receptors on their surfaces and reduce the likelihood of a successful phage infection (Hampton *et al.* 2020). However, the resulting phage resistance can affect the fitness of the bacterium and its virulence (Laanto *et al.* 2012, Penttinen *et al.* 2018, Hampton *et al.* 2020).

At the same time, phages and bacteria have coexisted continuously, and phages have learned to compete with bacterial mutations and to overcome resistance. When bacteria modify their receptors in different ways, phages cannot attach to the surface of bacterial cell. Phages can navigate these bacterial changes by evolving or by developing means of bypassing resistance. They can employ mutated receptor-binding proteins to infect the mutated receptor successfully (Hampton *et al.* 2020). Furthermore, they can produce enzymes to degrade the mask from the masked receptors of bacterial surfaces (Labrie *et al.* 2010, Hampton *et al.* 2020). Even though OMVs reduce the likelihood of a

particular cell being infected by phages, they can extend the range of the phage host by transferring phage receptors to another strain or to another bacterial species (Hampton *et al.* 2020).

The other mechanisms that bacteria use to prevent phage infection include superinfection exclusive (Sie) systems, restriction-modification (RM) systems, abortive infection (Abi) systems, and pan-immune systems (Labrie *et al.* 2010, Bernheim and Sorek 2020, Hampton *et al.* 2020). In Sie systems, the proteins that are encoded by phages (prophages) block the entry of other related phage genomes into the host cell (Labrie *et al.* 2010). In RM systems, the phage adsorbs to the bacterial cell and injects a genome into the bacterium. RM systems recognise and degrade the unmethylated phage genome. The bacterium can also methylate the phage genome, which marks new virions. Released phages use this mark during the next infection, whereby other bacterial cells with the same or related RM systems do not recognise the invading genome. The phage can thus circumvent the RM system and infect the cell (Labrie *et al.* 2010, Bernheim and Sorek 2020).

Phages can overcome RM systems by mutating and by reducing or deleting used RM sites. Phages can also modify these sites in the genome so as to be unrecognisable, and they may protect the DNA from the restriction of endonucleases by using their own methylase genes (Labrie *et al.* 2010, Hampton *et al.* 2020).

Abi systems enable the infected cell to either kill itself or to stop its metabolism so that the phages within become incapable of spreading and destroying the surrounding bacterial populations (Labrie *et al.* 2010, Bernheim and Sorek 2020, Hampton *et al.* 2020). Even though these systems are found in different species, the mechanisms that underlie them remain unknown. Phages can overcome Abi systems by escaping from them. It has been found that the recombination of the prophage and the lytic phage can lead to escape. These phages gain or lose genes (Hampton *et al.* 2020).

If phage DNA penetrates the cell, bacteria can also cleave phage DNA by using clustered regularly interspaced short palindromic repeats (CRISPR) through CRISPR-associated protein (Cas) systems. The term "CRISPR-Cas" denotes a region in the bacterium chromosome. There are small palindromic repeats, which are identical, in this region (Borges *et al.* 2017). There are also spacers between these repeats. The spacers originate from foreign DNA, and the bacterium uses them to recognise it in the cell (Borges *et al.* 2017). In order to function as an immune system, the CRISPR system needs *cas* genes. When phage DNA enters the cell, Cas proteins cut it and add a small part of the DNA into the CRISPR array so that it can be stored and used against similar threats in the future (Borges *et al.* 2017). During the next phage infection, the bacterium compares the phage genome to the CRISPR-Cas spacers and activates DNA cleavage. Phages can bypass the CRISPR-Cas system by mutating the site that matches a CRISPR spacer that is stored in the array (Labrie *et al.* 2010, Hampton *et al.* 2020). The mutation is not always unproblematic, in that the viability of the escaped phages might decrease in the course of mutation (Hampton *et al.* 2020). Furthermore, some phages, in order to fight the CRISPR-Cas system,

have anti-CRISPR (Acr) proteins that discharge a protective function. Arc proteins can inactivate the CRISPR system by blocking Cas protein activity in most of the systems (Hampton *et al.* 2020).

There are multiple defence systems that bacteria can use against phages. However, no bacterial strain activates all of these systems during the phage infection, nor is any bacterial strain capable of such activation. It is not cost efficient to activate all systems at the same time. In the pan-immune system, bacterial strains can protect themselves together with different defence systems. The term "pan-genome" refers to the totality of the defence systems that the bacterial population activates, which can be used against phage infection. During the infection, different defence mechanisms are encoded in different strains. This ensures that some of the bacterial strains survive the infection as a part of the population (Bernheim and Sorek 2020). The diversity of populations strengthens the ability of the bacterium to survive.

F. columnare can protect itself from phages by modifying its surface, which also blocks phage receptors (Laanto *et al.* 2012, 2020). *F. columnare* uses the same method to survive starvation, environmental stress, and other unsuitable conditions – the bacterial morphology can change from rhizoid to rough or soft (Kunttu *et al.* 2009, Laanto *et al.* 2012). These modifications prevent phage adsorption to the cell surface. However, phages can sometimes still inject the genomic DNA into the cell. Laanto *et al.* (2020) showed that the phage genome has mutated during the course of evolution and that the mutations in question probably pertain to the tail-encoding genes. This indicates that the tail proteins of the phage have mutated in order to acquire the ability to adapt to the surface of the resistant strain. In the same study, the authors noticed that one phage-resistant strain had activated the CRISPR-Cas system by acquiring a new spacer in the locus.

2.4 Phage therapy

The increased threat of the spread of antibiotic-resistant strains has highlighted the need to find alternative means of treating bacterial infections (Summers 2001, Abedon *et al.* 2011, Theuretzbacher 2013). Phage therapy is one option that enables the uncontrolled spread of bacteria to be combatted naturally (Abedon *et al.* 2011, Chan *et al.* 2013). The development of technology and the rediscovery of phages have highlighted their features and use cases (Williams Smith *et al.* 1987, Abedon *et al.* 2011). Although phage utilisation has considerable potential, the effects of phages on target cells and their habitats need to be understood well before use is extended. How can phages shape the features of a target bacterium? What are their effects on, for example, human and animal welfare or the environment? In this section, I explain the meaning of phage therapy, its current status worldwide, and its development in the context of *F. columnare*.

2.4.1 The basic idea of phage therapy

Phage therapy, that is, the use of phages for the treatment of bacterial infections, can entail preventive treatment, the acute treatment of bacterial infections, combatting chronic infections, and the parallel use of antibiotics. The main goal of all of these approaches is to kill infection-causing pathogenic bacteria, to keep natural microbiota intact, and to limit harm to the surrounding tissue and the environment (Chan *et al.* 2013).

Phages have been used preventively, for example to support the mammalian immune system, when the risk of bacterial infection has been known to be high (Krut and Bekeredjian-Ding 2018). Preventive use in the form of bacterial disinfection can also be implemented on the surfaces of food packages to avoid *Listeria* (Lone *et al.* 2016). The treatment of acute or chronic infections differs with the type of bacterial infection. In the former, a bacterial infection has already started when phage therapy begins. The bacterium quickly causes a strong inflammation of the tissues or organs when the immune system tries to respond. For example, a urinary tract infection that is caused by *Escherichia coli* can cause acute infection (Sanchez *et al.* 2022). In chronic infection, bacteria might appear for a long time, potentially causing continuous damage without the host organism, say a mammalian one, managing to eliminate them independently. The bacterial species that are commonly encountered in chronic infections include *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (Broxmeyer *et al.* 2002, Friman *et al.* 2016). In both forms of infection, phage therapy has been shown to be a potential treatment (Merril *et al.* 1996, Broxmeyer *et al.* 2002). In parallel treatment with antibiotics, the aim is to utilise the accuracy of phages in infecting a certain strain of bacteria as well as to consider the possible resistance that is caused by phages. Phages and antibiotics can be combined to combat bacterial infections. The effect of this combination has been the subject of a broad debate. It seems that combining phage therapy and antibiotic treatment is promising but environment dependent (Abedon 2019). It has been assumed that parallel treatment could prevent phage and antibiotic resistance (Chaudhry *et al.* 2017), but research remains at a nascent stage. In this thesis, I will focus on the use of phages to prevent bacterial infection and to treat acute infection.

When phage therapy is planned, the pathogen that causes the bacterial infection must be known, and a comprehensive and well-studied phage library should be available. The selection of phages for phage therapy is critical. The phages should be sufficiently purified because there may be toxins in the original lysates from the lysed bacterial cells. Phages should be infective to the targeted bacterium, and suitable transfer and storage conditions must be considered (Gill and Hyman 2010, Hyman 2019). First, the phages must be lytic. Temperate phages can mediate bacterial DNA movement between cells, which might increase pathogenicity (Gill and Hyman 2010). This can affect a bacterium and its genomic material through the transfer of, among others, potentially antibiotic-resistant genes (Gill and Hyman 2010, Kutter *et al.* 2010). During the lysogenic cycle, the temperate phage can also cause immunity

against the phage that is used or against related phages. Caution must be exercised when lytic phages are used because a lytic infection can cause toxic shock due to the endotoxins that the decomposing bacteria produce (Drulis-Kawa *et al.* 2012).

It has been suggested that the optimal phage for therapy would be a virus that has as wide a host range as possible (Gill and Hyman 2010, Chan *et al.* 2013). A phage has a broad host range when it can infect many or all of the strains of the target pathogen as well as pathogens that are related to the original host (and produce active virions during lysis). This kind of phage could be used to kill pathogens in a single-phage treatment. Conversely, such a phage might also kill nontargeted bacteria, which may, in the worst-case scenario, be beneficial (Hyman 2019). For this reason, Hyman (2019) wrote that the optimal phage would be one that has a host range which is limited to one species.

Some phages have a narrow host range, and they may even infect only certain strains in a given species. Therefore, phage mixtures, that is, cocktails, have also been considered for phage therapy (Gill and Hyman 2010, Chan *et al.* 2013, Hyman 2019). In such a case, more than one phage is used to infect the target pathogen. Phages with different host ranges are employed to expand aggregate host range and to ensure efficient infection. The number of phages in the mixtures depends on the properties of the phages, their interaction, and the properties of the target bacterium. Wright *et al.* (2021), in a study of *P. aeruginosa*, found that phages in phage cocktails that exhibit high richness or lower richness with functionally diverse combinations affect the efficacy of phage combination positively. Selecting phages that target different receptors might bypass the resistance of mutated strains (Gill and Hyman 2010, Chan *et al.* 2013). Studies of the use of phage cocktails in laboratory conditions mimic the real conditions in which phage therapy should work. Even though bacteria might become resistant to phages, phages can evolve and learn to infect new strains. Friman *et al.* (2016) showed that these evolved phages also exhibit better infectivity to older ancestral strains.

The limitations of phages must be considered in the development of therapies. Phages are viruses, and it is important for their replication that the host does not disappear completely. For this reason, the phage may not be able to eradicate the pathogen altogether. The bacterium can form phage-resistant strains during infection, as mentioned previously. However, it has been shown that resistance entails a trade-off for bacteria, and virulence and antibiotic sensitivity are affected in many cases (Levin and Bull 2004, Laanto *et al.* 2014, Castledine *et al.* 2022). Furthermore, Wright *et al.* (2019), in a study on *P. aeruginosa* phages, showed that the order and timing of phage exposure influence the formation of resistance. This indicates that the design of phage mixtures and their study are important for preventing the emergence of resistance.

Phage therapy is unlikely to be the only treatment for bacterial infections, but research and development have uncovered its potential in the treatment of certain bacterial diseases (Gill and Hyman 2010, Brüssow 2012). A transition from laboratory studies to clinical trials is currently underway. In some

countries, that transition is even complete (Wright *et al.* 2009, Sarker *et al.* 2012). However, in other countries, legislation and the high costs of research are obstructing the shift (Kutateladze and Adamia 2010, Pirnay *et al.* 2011, Brüssow 2012, Chan *et al.* 2013). Phage therapy has attracted interest in the EU, and its benefits have begun to be examined (Pelfrene *et al.* 2016). At the same time, prudence has sharpened awareness of the risks that the introduction of antibiotics revealed. It is important to study phage therapy and to clarify the complex interactions between phage and bacteria before implementation. However, if new information that is relevant to further development is to be obtained, it is also important that well-researched treatments can progress from the laboratory level to clinical trials.

2.4.2 *F. columnare* versus phage therapy

Phage therapy has been studied as a novel method for treating pathogenic bacteria in plants, humans, and animals. It has been also studied in aquaculture with different results, some of which very promising and some less so (Oliveira *et al.* 2012). Promising results have been reported against *Lactococcus garvieae* (Nakai *et al.* 1999), *Pseudomonas plecoglossicida* (Park and Nakai 2003), *F. psychrophilum* (Castillo *et al.* 2012), and *Vibrio anguillarum* (Higuera *et al.* 2013). A product on the Norwegian market, CUSTUS$_{YRS}$ (ACD Pharma), is sold to control *Yersinia ruckeri* outbreaks at fish farms (CUSTUS®YRS), which is indicative of the potential of phage therapy against other bacterial infections.

F. columnare is a challenging bacterium because it can reproduce even without a host in the environment. Although the treatment of the bacterium may be successful once, the pathogen may reappear and resistant strains may develop. With *F. columnare,* it is also possible for phage infection to induce a trade-off whereby the bacteria are forced to choose between phage resistance and death (Levin and Bull 2004, Laanto *et al.* 2012, Bernheim and Sorek 2020, Castledine *et al.* 2022). Natural competition between phage-resistant *F. columnare* and phages can lead to the stable presence of bacteria in the environment, which does not necessarily prevent the emergence or re-emergence of bacterial infection (Merikanto *et al.* 2018). Scholars have highlighted the importance of sufficient infectivity and the speed of phage infection for eradicating bacteria. Phage therapy also offers opportunities to restore the microbial community of the fish and the other microbes surrounding the treatment environment. When the pathogenic and possibly opportunistic bacterial population decreases and the pathogen begins to compete with other microbes, it must choose between infectivity and phage resistance (Laanto *et al.* 2012). In this case, the phages probably have a higher chance of infecting the non-resistant strain, and other microbes can also multiply and, for example, produce antibacterial substances in the area. Diverse microbiota have a role in maintaining a balance so that one species does not dominate (Anttila *et al.* 2013, Merikanto *et al.* 2014).

Phage therapy for *F. columnare* has enjoyed varying degrees of success. The identification of the host range of phages and bacterial resistance formation

patterns has been particularly important. Laanto *et al.* (2015) studied the ability of the FCL-2 phage to reduce the mortality of zebrafish (*Danio rerio*) and rainbow trout. The use of FCL-2 against the Finnish *F. columnare* strain reduced pathogen virulence by 100% for zebra fish and by 50% for rainbow trout. Only a single addition of phages was employed.

Studies of phage therapy and columnaris disease have also been conducted, but they are limited in number, which is particularly true of *in vivo* research. The studies that have been executed are limited to single-phage experiments, and separate phage administration methods have not been tested adequately because phage therapy against *F. columnare* is still relatively new. More research with different bacterial strains and their phages is needed. In this thesis, I have focused on these very challenges and areas for improvement.

3 AIMS OF THE STUDY

The increased risk of the emergence of antibiotic-resistant strains is a reality in the fish industry. Phage therapy is a potential alternative to the antibiotics that are used against the *F. columnare* bacterium. It is also a potential alternative to the antibiotics that are employed to treat the columnaris disease that *F. columnare* causes. Promising decreases in fish mortality have been reported (Laanto *et al.* 2015). However, relatively few *in vivo* studies have been conducted, and there has been no research on different administration routes. Furthermore, phage-mixture experiments are a new approach to columnaris disease. Phage mixtures with narrow host range needs to be considered as one potential form of phage therapy against this pathogen.

The main goal of this thesis was to study the efficacy of different phages against *F. columnare* strains from different genetic groups and to identify the optimal administration routes for phage therapy.

The other goal was to determine whether phage resistance is a problem for phage therapy.

4 SUMMARY OF THE MATERIALS AND METHODS

The materials and methods of this thesis are described in the original publications (I-V). Table 1 displays those methods and the corresponding original publications.

TABLE 1. Materials and methods used in thesis with original publications (where available).

Methods	Publication
Isolation of phages	I
Phage purification	V
Phage genome sequencing	I
TEM imaging	I
Host range of phages infecting *F. columnare*	I, II
Shelf life of *F. columnare* phages	V
Phage therapies with fish	V
Phage sampling from fish organs	V
Phage therapy with fish eggs	IV
Phage mixture treatment	II
Training phages to infect *F. columnare* strains from different genetic clusters *in vitro*	II
Isolation of bacteria	I
Characterising of *F. columnare* genetic clusters	I
Sequencing of *F. columnare*	I, III
Virulence experiments	I, III, V
Phage resistance of *F. columnare*	II, III

5 RESULTS AND DISCUSSION

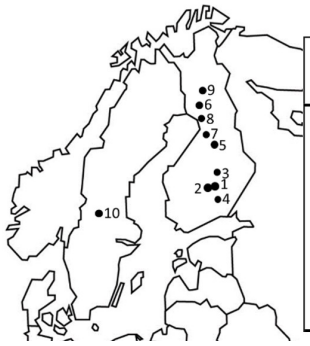
5.1 Phage therapy in action: the potential of phages against *F. columnare* infections in rainbow trout fry

Antibiotics are necessary when columnaris disease occurs at fish farms. At present, fish farmers have no other means of controlling the disease. Climate change and rising water temperatures will likely lead to repeated *F. columnare* infections (Pulkkinen *et al.* 2010). Continuous infections and repeated antibiotic treatments increase the risk of the emergence of antibiotic-resistant strains. Due to the increasing threat of antibiotic-resistant strains in aquaculture, for which the availability of antibiotics in Finland is limited (Anon 2016), phages have been identified as an alternative treatment for *F. columnare* infections. One of the most significant benefits of phages, which antibiotics do not possess, is their ability to reproduce in the presence of a suitable host bacterium. Another useful feature is targeted infection. Phages infect the host without causing damage to other bacteria, which is one of the side effects of antibiotics. Therefore, researching phage therapy for *F. columnare* is interesting. I investigate its potential uses in the treatment of columnaris disease.

Personalised treatment with phage therapy has been considered and studied, for example in human trials (Chan *et al.* 2013). A special motivation of this line of research is that not all treatments and phage mixtures are suitable for everyone. It would be interesting to see whether medication could be tailored similarly in the treatment of columnaris disease. Targeting specific genetic groups demands familiarity with the bacterial strains that occur and knowledge of the phages that infect them.

5.1.1 Compilation of phage collection for *F. columnare*

At the beginning of the research that underlies this thesis, *F. columnare* strains and phages that can be used against this bacterium were isolated from different fish farms in Finland and Sweden (I, Fig. 3). Comprehensive libraries of *F. columnare* strains and phages were created for further studies of phage therapy. It was found that certain genetic groups of *F. columnare* occur together and at the same time at fish farms (Fig. 3). This result is in line with previous studies in which certain bacterial genetic groups were isolated from the same fish farms – certain groups co-occurred (Laanto *et al.* 2011, Ashrafi *et al.* 2015, Sundberg *et al.* 2016). Bacterial strains should be monitored continuously to ensure that these specific groups occur repeatedly at the fish farms in question. The presence of bacteria has not been monitored in real time, either in this work or in the extant literature, due to the laborious nature of the isolation and characterizing of bacteria. The most comprehensive result is from Farm 1, which was sampled most frequently because of its proximity to the laboratory. If the assumption of farm-specific strains is plausible, phage therapy can target the strains that are present at specific sites. At the same time, the per-farm costs of phage therapy may fall because it would not be necessary to search for phages that are effective against all strains.



Farm number	Number of isolated *Flavobacterium columnare* bacteria (*Fc*) and their phages per genetic group													
	A		C		E		G		ND1		ND2		ND3	
	Fc	Phage	*Fc*	Phage	*Fc*	Phage	Fc	Phage	*Fc*	Phage	*Fc*	Phage	*Fc*	Phage
1	1	1	50	23	-	-	-	6	-	-	-	-	-	-
2	-	-	-	1	-	-	8	2	-	-	-	-	-	-
3	-	-	-	5	6	-	-		-	-	-	-	-	-
4	-	-	-	13	-	-	-	-	-	-	-	-	-	-
5	-	-	20	3	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	16	-	-	-	-	-	-	-	-	-
7	-	-	3	7	-	-	-	-	-	-	-	-	-	-
8	7	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	8	2	-	-	2	-	-	-	1	-	1	-	3	-

FIGURE 3 Sampling locations in Finland and Sweden. Left: map of Northern Europe in which each number denotes a farm at which water and fish samples were collected. Right: number of bacterial and bacteriophage isolates from individual fish farms. "A", "C", "E", "G", "ND1", "ND2", and "ND3" indicate the genetic groups of the isolated bacteria and the isolation hosts of the phages. ND = genetic group not determined. Runtuvuori-Salmela *et al.* 2022. CC BY-NC-ND 4.0.

In study I, bacteria that represent four genetic groups, A, C, G, and E, were found at fish farms in Finland, (Fig. 3, Suomalainen *et al.* 2006). The bacteria of all genetic groups caused high mortality in rainbow trout fry. C and E were found to be the most virulent (I). Ashrafi *et al.* (2018) reported similar results with these genetic groups, which supports these results. For phage therapy, it was necessary to discover how virulent those bacterial strains are. Assessing the effectiveness of the phages against highly virulent strains is desirable and important. Highly virulent strains cause the most rapid mortality at fish farms.

If phages can infect these strains, phage therapy might be an effective means of decreasing the mortality that columnaris causes. Even if phages cannot eliminate pathogens at farms completely, a delay in the progression of infection could support the immune defences of the fish and the maintenance of microbial balance.

Furthermore, in study III, bacteria from genetic group C caused mortality more rapidly than bacteria from genetic group G, although the difference was only four hours. In study III, only one strain from these genetic groups was tested, which means that there may be some variations in the virulence of strains from the same genetic groups. All genetic groups were therefore suitable for and relevant to the study of phage therapy.

A large collection of new phages was isolated and well-characterized, and a host range was studied (I). A total of 63 phages, that is, myophages, were isolated at fish farms in Finland and Sweden against *F. columnare* (I). These phages were divided into three clusters, namely A, C, and G, on the basis of their specificity to the genetic groups of the host bacterium (I). The host range of the phages in this thesis and of the previously isolated phages (71 in total) were tested against 227 strains. In total, 16,117 phage-bacterium interactions were examined. Even though the isolated phages were genetically similar to each other and to previously isolated phages (Laanto *et al.* 2011), they differed in their host ranges and had some mutations in their genomes (I). For some phages, the mutations were connected with the ability to infect strains from other genetic groups, which could facilitate the design of phage therapies in the future, where fewer phages could be used to infect more strains. These results also support the prediction that phages coevolve with bacterial strains. Genetic mutations were detected in possible tail fibres (I), which could indicate that these phages have adapted to use different receptors. Similar findings have been obtained with *F. psychrophilum* and *V. anguillarum* where phages with similarities in their genomic levels differed in their host range, which might explain the phenotypic differences and the coevolution of hosts and phages (Castillo and Middelboe 2016, Kalatzis *et al.* 2017).

From the perspective of phage therapy, it is important to identify phages that infect different genetic groups of *F. columnare*. Phages that are effective against pathogenic genetic groups A, C, and G were identified on the same farms at which their hosts had been found (I, Laanto *et al.* 2011). Given that phages that are effective against bacterial strains that could not be isolated during the study were also found, the hosts of these phages likely occur at the farms in question (I). Unfortunately, no phages that infect group E (I) were found. It was for this reason that the bacterial genetic groups A, C, and G were chosen for further phage therapy studies. It might be that the method that was used to isolate phages was not suitable for the E cluster phages. It might also be that the strain that was used for isolation had inherent resistance against phages. For example, it could have had an active CRISPR-Cas or blocked receptors. It is also possible that the isolation host had its own prophage which activates the Sie system that blocks the entry of phage DNA into the cell (Labrie *et al.* 2010, Hampton *et al.* 2020). For these speculations to be verified, the

isolation host genome must be sequenced, and the prophage must be detected. In the future, it will be important to find and describe phages that are effective against the genetic group E in order to develop a phage therapy successfully. This strain has been shown to be highly virulent among rainbow trout fries. Nevertheless, three C cluster phages infected strains from genetic group E successfully (I).

5.1.2 Using phages as a therapy and preventive treatment

Previous phage therapy studies have yielded results that are promising for the fight against columnaris disease (Laanto *et al.* 2015). The G cluster phage protected 100% of zebrafish and 50% of rainbow trout fry from *F. columnare*. The results showed that using only one dose of phages was highly effective. In this thesis, before the phage therapy experiments on fish fry, phage-bacterium interactions were studied *in vitro* with a single phage (II, III) and with two phage mixtures (II). Phages effectively inhibited the growth of bacteria at the same time as the growth of phages was observed. Phage treatments *in vitro* showed a decrease in bacterial adherence and biofilm formation as well as a lower growth rate for phage-induced soft colonies (II, III). These properties were combined into virulence factors. Those key factors are connected with high virulence. As far as *F. columnare* is concerned, it would seem that no single virulence factor causes high mortality and that virulence may be the result of several such factors operating together. The phage treatments did not eradicate the bacteria, and phage resistance appeared relatively quickly (II, III). However, phage resistance did not affect bacterial antibiotic susceptibility, indicating that, despite phage resistance, these strains can be treated with antibiotics if needed. In some cases, phage infection even increased antibiotic sensitivity (III). The lack of connection between phage resistance and antibiotic resistance is promising for cases in which phage therapy must be administered together with antibiotics or ones in which antibiotics must be administered after phage therapy. In such instances, phage resistance would not weaken the effectiveness of antibiotics. Similar results have been observed with *Acinetobacter baumannii* (Altamirano *et al.* 2022) and *Klebsiella pneumoniae* (Eskenazi *et al.* 2022). In these studies, it was found that phage resistance increases bacterial sensitivity to antibiotics and that phage therapy supports antibiotic treatment. Unfortunately, the antibiotic sensitivity assay was not replicated in my thesis. Therefore, the results cannot be considered completely certain (III). In the experiments that are presented in this thesis, the phage infection did not destroy all bacterial cells, and phage-resistant strains formed during the *in vitro* experiments. However, it seems that the ability of phage-resistant bacteria to infect and spread was weaker (II, III). From the perspective of phage therapy, it is important that the phage-resistant strains that form are not more harmful than the original ones and that the resulting resistance does not create antibiotic resistance in the bacteria. I will discuss phage resistance more extensively in the next chapter.

Phage therapy was studied *in vivo* by using rainbow trout fingerlings and fish eggs as well as different strains of *F. columnare* (IV, V). In the past, phage

therapy for *F. columnare* has only been conducted by adding the phage solution into the aquarium that contains the fish and the bacteria directly (Laanto *et al.* 2015). Different options must be evaluated to ascertain the optimal means of administering phages, and the environment in which phage therapy is implemented must be considered. For example, it is known that *F. columnare* creates biofilms at fish farms. Therefore, phage sheets (plastic sheets with immobilised phages) were considered. *F. columnare* is also known to appear on farms during warm-water periods, in which case phage-coated feed could be a preventive treatment that avoids the emergence of columnaris disease. Bathing is employed at fish farms, for example to evict parasites, which is why it could also be a feasible method of delivering phage therapy. The delivery routes that were tested included keeping fish and fish eggs in liquid that is supplemented with phages, direct dosing in aquarium water at different phage concentrations and with different combinations, bathing fish with phages before and after bacterial infection, coating plastic sheets with immobilised phages, and phage-coated fish feed. In the following paragraphs, I overview the advantages and disadvantages of these administration routes.

Previously, it has been suggested that eyed fish eggs are one of the sources of *F. columnare* infections. The bacterium has been found to survive on the surface of chinook salmon (*Oncorhynchus tshawytscha*) eggs (Barnes *et al.* 2005). Barnes *et al.* (2005) showed that the bacterium can attach to and multiply on the surface of the egg and influence the survival of eyed eggs. If *F. columnare* spreads from eyed eggs to hatching fish and then to farms, phage therapy for eggs could potentially prevent spread and increase survival rates among juvenile fish fry. In study IV, phages were added to the eggs of eyed rainbow trout. The bacterium did survive on the surface of the eggs (IV) but did not appear to harm them, probably because *F. columnare* growth depends on water temperature. The suitable temperature for rainbow trout eggs in hatcheries is between 7 °C and 12 °C (Carter 2005). In a previous experiment, *F. columnare* on fish eggs was studied by using a water temperature of 20 °C (Barens *et al*. 2005). The difference between the approach in this thesis and the temperatures that have been employed in past research have to do with the use of different fish species. In this thesis (IV), 20 °C would be too high a temperature for rainbow trout eggs to survive. Conversely, 10 °C would be too low for the bacterium to multiply (Kunttu *et al.* 2011, Declercq *et al.* 2013b). *F. columnare* does not cause significant problems for the rainbow trout eggs, but it can contaminate eggs, and there might be a risk of bacterial infection when the eggs are hatching.

Phages did not emerge to lyse the bacterium during the experiment, and well-purified phages did not cause any harm to the eggs, which suggests that the phages were safe to use (IV). Unfortunately, the experiment did not continue until the juvenile fish phase. It would have been interesting to see whether the bacterium from the egg surface survives to the stage of the life of the fish at which it encounters the optimum replication temperature for *F. columnare* and thus whether phages can protect fish after hatching. This might be possible because bacteria survive in low temperatures and are attached to the surface of eggs, where they may be transferred to hatching fish.

A phage bath indicated the potential of *F. psyschrophilum*, a cold-water bacterium, to prevent the attachment of bacteria to fish eggs during a 24-hour experiment (IV), which also supports the assumption that temperature is important for the functionality of phages. It is possible that conditions that are stressful for *F. columnare* (low temperature) made the bacteria change their colony morphology from spreading rhizoid to less spreading soft or rough (Kunttu *et al.* 2011) and therefore probably to decreased phage sensitivity. Phages have been found to infect rhizoid morphologies, potentially due to the expressed receptors on cell surfaces that might be missing from soft and rough colony morphotypes (Laanto *et al.* 2012). Rough morphotypes can revert to being rhizoid (Laanto *et al.* 2012), which would allow infection to commence when conditions are optimal for the bacterium. Study IV shows that bacterial features have an important role in phage usability.

In study V, different delivery methods were tested with rainbow trout fry. One option is to add phages directly to the aquarium of the fish that are infected with *F. columnare*. At first, phages were added directly to the closed water system of an aquarium that contained rainbow trout fry (the water inside the aquarium did not change during the experiment). In the closed system, bacterium and phage interacted until the end of the experiment. The challenges of this method are that it may not be suitable for larger fish (or may require a larger aquarium due to the size of the fish) and that the oxygen level starts to decrease because of the stressful and warm conditions in which fish consume more oxygen. This experiment does not necessarily yield a realistic representation of the functionality of phage therapy at fish farms where flow-through systems are used. In a flow-through system, the water flows in and out of the fish tanks in a controlled manner. However, adding phages to a closed water system that contains fish and bacteria confirmed that phages do not harm fish fry (V), a promising indication of the safety of phage therapy.

The interaction between different bacterial genetic groups and the phages varied considerably in the study on phage therapy and rainbow trout fry (V). The strain from genetic group A did not exhibit sensitivity to its phage, the strain from genetic group G was somewhat sensitive, and the strain from genetic group C was the most sensitive. Even though an A cluster phage for bacteria from genetic group A was isolated (I), it is possible that this bacterium is not an optimal host for the phage. This could explain the high fish mortality (V). Furthermore, bacteria were added at the same time as phages during the experiment, which might have resulted in phage resistance. In studies of *E. coli*, it has been observed that the strain that is introduced to the murine intestine can differentiate into two populations, one of phage-sensitive rod-shaped *E. coli,* which is observed in laboratory conditions, and another of phage-resistant coccoid-type cells (Chibani-Chennoufi *et al.* 2004). It has been assumed that the mucosal layer might protect *E. coli* from phage infection. In study V, phage titers increased in the water, which is indicative of some successful phage replication and bacterial lysis. However, bacterial colony morphology and phage sensitivity were not tested from fish skin mucus. It would have been interesting to see whether there the morphology of bacteria that are isolated

from skin mucus changes and if the A cluster phage can infect bacteria in skin mucus.

It is possible to administer preventive phage treatments for *F. columnare* infections to fish fry. Bathing fish is a normal procedure in fish farms, for example when new fish populations are conducted from hatcheries. The fish are transferred to a separate pool that contains water, with or without health-supporting ingredients as disinfectants. In this thesis, the use of a phage bath before bacterial infection (preventive treatment) decreased fish mortality, including from the bacterium from genetic group A (V). This could support the argument that if the bacterium is present on the mucin of the skin first, it can protect itself from the phage. Conversely, if the phage is present on the mucin first, it can lyse the bacterium. However, this argument is speculative. More detailed studies are required.

Since phage treatment for bacteria from genetic group A was not unequivocally successful, its operation was not studied in a flow-through system. The properties of this genetic group must be studied further and separately in the future.

In study V, the phage bath was performed by using two phage mixtures against two bacterial strains. The phage mixture decreased the mortality that these two bacterial strains cause, suggesting that the phage mixtures have the potential to be used against columnaris disease or at least against the strains under examination here. The most effective method for decreasing pathogen-induced mortality was to bathe fish in a phage solution when the first columnaris symptoms appeared (V, Fig. 4). The multiplicity of infection (MOI) of the phage solution was 1 with the bacterial solution before bacteria and phage-mixtures were added to the aquarium. It is particularly interesting that the experiment was conducted in a flow-through system and that the infections involved the simultaneous use of two bacterial strains (co-infection). This resembles a realistic situation at fish farms, where fish in flow-through systems are kept in high densities. It is likely that different strains co-occur during bacterial infections at fish farms. Interestingly, for fish, a phage bath is not as effective against *Aeromonas hydrophila* as an intraperitoneal injection (Gordola *et al.* 2020). This is probably due to the nature of the *A. hydrophila* infection, which spreads in the bloodstream. *F. columnare* adheres primarily to the surface of the fish. Phage bathing is therefore not a universal solution for the treatment of all bacterial infections in fish. When planning phage therapy, the nature of the target bacterium and the route of infection spread must be considered so as to target the administration of phages accurately.

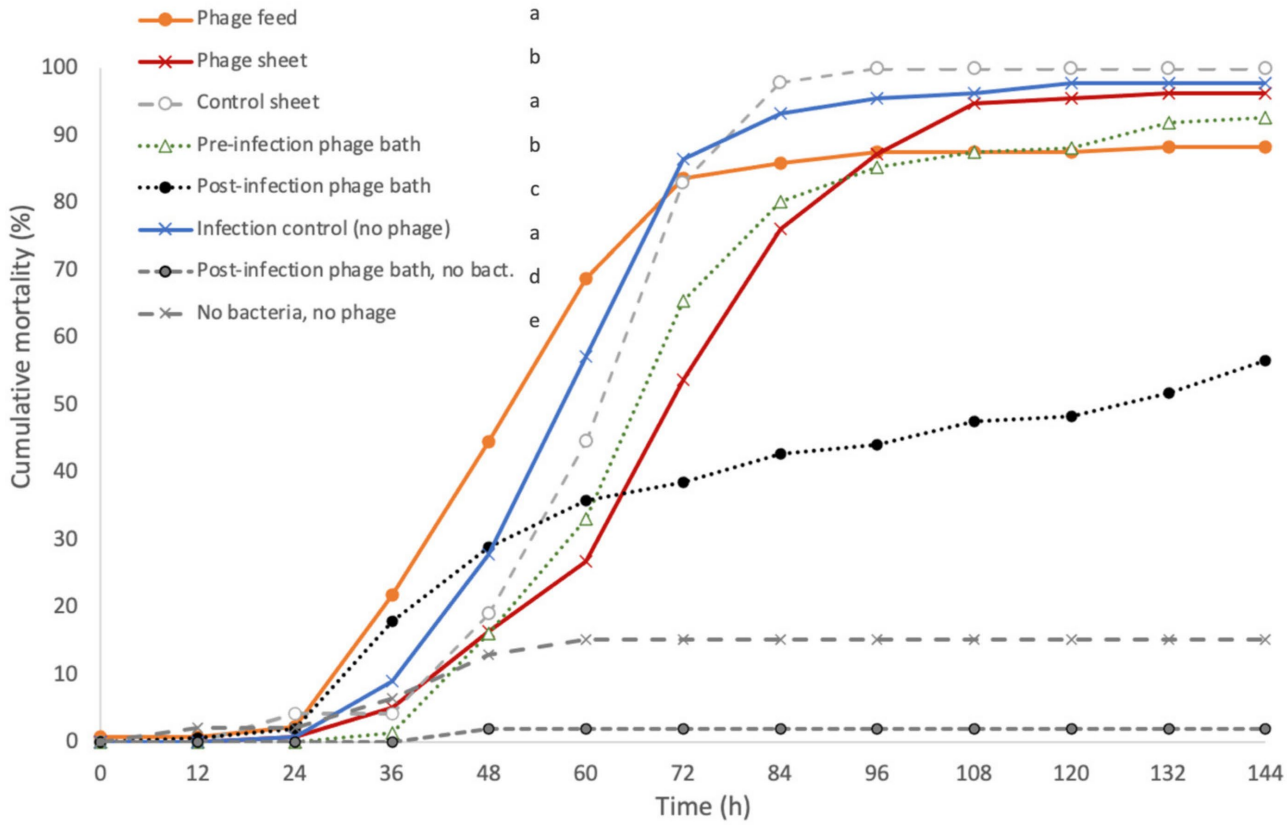


FIGURE 4 Cumulative mortalities (%) of rainbow trout fry that were infected with a mix of *Flavobacterium columnare* isolates. The fish were fed with phage mix-coated feed (phage feed) for 7 days before the bacterial infection and kept with a phage mix-coated sheet (phage sheet) or a control sheet without phage mix (control sheet) during the whole experiment, starting from 7 days before bacterial infection. Alternatively, they were treated with a 2 h phage mix bath one day before bacterial infection (pre-infection phage bath) and immediately after the first symptoms of columnaris disease appeared after the bacterial infection (post-infection phage bath). The negative controls for pre-infection phage bath and phage feed did not cause any mortality and are not shown in the graph. Kunttu *et al*. 2021. CC BY 4.0.

The success of preventive phage inoculation can be partially due to the surface of the fish. It has been shown that *F. columnare* phages can bind to the primary mucosa of rainbow trout and remain there for up to 7 days (Almeida *et al.* 2019). In a flow-through study (V), C cluster phages were not detected on the skin mucus during preventive or post-infection treatment. After the experiment, a C cluster phage was detected in the water. A G cluster phage was detected in the water only during the post-infection treatment. Turning to the analysis of the efficiency of phage treatment, of mortality, and of phage appearance in the water or in fish organs, the use of phages decreased mortality, indicating that the treatment succeeded, even though they were not always detectable in the mucosa or the water. New phages that are released during lysis may have been removed from the aquarium by a flow-through system. Phages have previously been found on the skin mucus of rainbow trout (Almeida *et al.* 2019). If this thesis is compared to previous study, the difference in phage detection is likely attributable to the higher concentration (10^8 PFU/mL) that is used in the latter (Almeida *et al.* 2019). The theoretical MOI in the present thesis is 1, with a total phage concentration of 10^6 PFU/mL. Still, the phage mixture decreased the mortality that *F. columnare* causes, indicating that a lower concentration might be sufficient to treat fish. This lower concentration might be more convenient for the fish farmers who must store phages. There is no need for large, highly concentrated and space-consuming stocks. Producing highly concentrated phage stocks in small volumes for use in large fish farming tanks remains challenging.

Different phage-to-bacterium ratios affected fish mortality rates. A MOI of 0.1 was too low to decrease mortality. A MOI of 10 had the strongest effect (V). The most probable explanation is that significantly more phages than bacteria were present, which resulted in more interactions between phages and bacteria. Similar results have been observed in the use of phages against *Clostridium difficile*, where increasing MOI from 7 to 10 resulted in no viable *C. difficile* cells being detected (Meader *et al.* 2010). However, in the case of *E. coli*, increasing MOI from 1 to 1,000 does not significantly affect bacterial cell survival (Silva *et al.* 2014). MOI must therefore be tested for each bacterial strain separately in order to find the concentration of phages that is optimal for controlling bacterial growth. Even if a MOI of 10 seems to result in an effective treatment for *F. columnare* (V), it is still necessary to consider whether the use of such a concentrated phage solution is profitable and practical in farming conditions. The stronger the phage solution, the more expensive it is to produce. The implementation of the bathing method in fish farming would be possible, even though the volume of the phage solutions would need to be very large, because it does not require phage stocks that are as concentrated as those that are needed if phages are added directly to the tanks or if a higher MOI is employed. Still, storing phages in liquid might take up much space at farms or at the premises of companies that sell phage products. Given that *F. columnare* phages withstand temperature changes and dryness well in sterile lake water and bacterial growth media (V), the optimisation of storage methods could provide a solution to the problem of large liquid stocks.

5.1.3 Usability of immobilised phages

One option for administrating phages is to use phage-coated plastic sheets. This approach, when used for prevention and control, could resolve the *F. columnare* biofilm problem. For example, food packages can be covered with phages to prevent *Listeria* (Lone *et al.* 2016), and phages in wound dressing can prevent *P. aeruginosa* (Nogueira *et al.* 2017). In study V, plastic sheets were covered with C and G cluster phages. The phage immobilisation solution was manufactured and supplied by Fixed Phage Ltd (Glasgow, Scotland, UK), which uses proprietary and patented technology. The A cluster phage was not used because it did not show promise when applied against its host in *in vivo* experiments. These sheets were rolled so that the edges did not overlap and placed into the aquaria with the fish before bacterial infection. The use of sheets was not as efficient as the other methods but delayed fish mortality (V, Fig. 4). The problem might be that the phages are immobilised and the bacteria are in the water. Phage infection and the production of new virions in the water only occur when the bacterium is within the reach of the phage. At the same time, if the bacteria arrive at the fish farm from inlet water, as has been argued in the past (Kunttu *et al.* 2012), a phage-coated inlet pipe or filter would increase the probability of encounters between phages and bacteria. Immobilised phages would infect incoming bacteria, and bacterial lysis would release new phages into the water to prevent columnaris disease.

F. columnare can cause high mortality quickly (I, Declercq *et al*. 2013b), which means that the alternative treatment ought to be efficient. The replication of *F. columnare* phages begins relatively rapidly, and new virions are produced after 2–8 hours of infection (II). Using the correct phages in phage-coated filters for incoming water could increase the possibility of encounters between phages and bacteria and prevent the transfer of pathogenic bacteria from the water source to the fish farm. Indeed, Almeida *et al.* (2019) showed that a single dose of phages can remain viable in a RAS for up to three weeks. A RAS is a highly sensitive farming system which can be paralysed by bacterial infections, and chemicals and antibiotics disrupt the microbiome of the biofilters. Phage-coated filters could be one solution for RASs. However, its adoption would require significant optimisation and a comprehensive selection of sufficiently new phages that have been proven to infect those strains which occur on a given farm as well as testing before implementation.

Another means of administering phages to fish that this thesis examines entails immobilising phages on fish feed (Fixed Phage Ltd). Previously, it has been shown that phage-coated feed protects fish from *P. plecoglossicida* (Park and Nakai 2003) and *Lactococcus garvieae* (Nakai *et al.* 1999). In study V, phage-covered feed did not protect fish from *F. columnare* infection (V, Fig. 4). Once more, this is likely due to the nature of the bacteria that use water to transmit to the fish and to create biofilm, mainly on the fish surface. Phages for *A. hydrophila* were highly efficient when phage feed was used, but it was observed that bacterial infection decreased the appetite of the fish, which reduced the dosage of phages (Gordola *et al.* 2020). Furthermore, the digestive system could

reduce the potency of phages in food before they enter the bloodstream. In study V, even though phages were administered through feed, they were found in the skin mucus before bacterial infection. This should have created a protective effect, but the phages did not prevent mortality, and no phages were found after the experiment. A similar phage-coating technique was used for *F. psychrophilum*, where the immobilised phages also did not protect the fish from bacterial infections (Donati *et al.* 2021). This indicates that phage-coated feed might not be a suitable administration route for these bacterial pathogens.

Although bathing fish in phage solution emerged to be the optimal method for phage delivery in this thesis, it must be noted that different bacterial strains react to the presence of phages in different ways. Phage therapy must be planned individually for specific *F. columnare* genetic groups. In study II, study III, and study V, the genetic groups reacted differently to phage infection. Even though the phages infected the bacterial strains in the *in vitro* host range experiments (I), A and G cluster phages were less effective in decreasing mortality than C cluster phages *in vivo* (V). Moreover, the phage mixture decreased the mortality of the bacterium from genetic group C to a larger extent than the other cluster phages that were used to infect their hosts (V). These results indicate that *F. columnare* strains behave differently *in vivo* and *in vitro*. More phage studies on fish populations ought to be conducted with different strains and phages in order to arrive at a more comprehensible representation of phage therapy in action. The combined effect of phage and bacterial mixtures should also be considered in future experiments. This thesis highlights the importance of the design of phage therapy design by using *in vivo* experiments to generate information about the ability of the phage to function in farming environments.

5.2 The most difficult challenge for phage therapy: phage resistance?

One of the most significant challenges for phage therapy is the rapid emergence of phage resistance. The critical concern is that phages that are administered may subsequently become unusable. Phage resistance might increase bacterium virulence and thus precipitate an increase in mortality (Waldor and Mekalanos, 1996, Gill and Hyman 2010, Loc-Carrillo and Abedon 2011). Using temperate or poorly characterised phages might result in the transfer of unwanted features (for example, antibiotic resistance) to bacterial cells, which can cause changes in bacterial virulence (Waldor and Mekalanos, 1996, Gill and Hyman 2010). In general, phage resistance decreases bacterial virulence (León and Bastías, 2005). If the errors that plagued the implementation of antibiotics are to be avoided, it is important to study the effects of phages on the targeted bacterium and the manner in which phage infection changes bacterial virulence. It has also been suggested that using a phage mixture could prevent phage resistance (Gill and

Hyman 2010, Chan *et al.* 2013, Hyman 2019). Studying phage resistance in *F. columnare* is important and necessary because several fish farms use flow-through systems, which might spread resistant bacteria into the environment. I studied the emergence of phage resistance (II, III), the genetic mechanisms that may be involved (III), and the manner in which phage resistance affects *F. columnare* virulence in rainbow trout (III).

5.2.1 Emergence of phage resistance

In study II and study III, phage-resistant mutants emerged rapidly, that is, approximately 48 hours after infection. After phage lysis, the bacterial concentration fell but then reverted or almost reverted to its original density. In study II, the use of single phages or two-phage mixtures made no difference to the speed with which phage resistance emerged. Although it has been speculated that a phage mixture could obstruct the generation of phage-resistant strains (Gill and Hyman 2010, Chan *et al.* 2013, Nobrega *et al.* 2015), I could not confirm this proposition with the two-phage mixtures that I used for *F. columnare* (II). Furthermore, in studies of *F. psychrophilum,* it has been shown that the phage mixture decreases bacterial cell re-growth to a larger extent than a single-phage treatment (Christiansen *et al.* 2016). However, phage resistance did not develop during an experiment that saw a three-phage mixture used against *Vibrio cholera* in mice (Yen *et al.* 2017). Notably, it emerged that the timing of phage treatment is important for the emergence of resistance in *V. cholerae*. If phages are administered too early, their dilution may cause phage resistance (Yen *et al.* 2017). A mixture of two phages does not seem to be more effective against *F. columnare* than the one-phage treatment, nor does it cause larger changes in the bacteria. Interestingly, however, a mix of two phages prevents mortality in rainbow trout effectively when the infections are caused by two bacteria (II, V). In light of these findings, it remains unclear whether the result should be attributed to the number of phages, which probably share the same target receptor, to the number of bacteria, or to the competitive situation of the strains that are affected by phage infection. Both *in vivo* and *in vitro* studies are needed to develop the study of phage therapy.

In this thesis, a two-phage mixture (total phage MOI of 1) caused resistance to emerge *in vitro* (II). If I had used a higher MOI and more phages that attach to different receptors or cell-surface particles, the results might have been different. As far as *F. psychrophilum* is concerned, a higher initial MOI was connected to a lower number of resistant populations and to the ability to compete with ancestral strains (Christiansen *et al.* 2016). A MOI of 10 was used against *F. columnare* in the rainbow trout experiment, in which it emerged to be more efficient than a MOI of 1, but the emergence of resistance was not tested (V). The practicality of a high MOI must also be considered. Adding too many phages to mixtures increases the costs of production and is liable to affect nontargeted bacteria adversely (Chan *et al.* 2013). It is difficult to estimate the concentrations of the bacteria that arrive into fish farms or the bacterial density that occurs during columnaris disease without culturing or employing PCR-

based methods. Therefore, it is important to discover an MOI that is sufficient to control bacterium infection as well as a delivery method that is appropriate for everyday use at farms. The optimisation of phage amounts and the aforementioned options could be the next stage of experimental research on phage mixtures for *F. columnare*.

It should be noticed that responses to phage infections differ across genetic groups (II, III). Bacteria from genetic group C, which is highly virulent (I), are lysed more slowly and more efficiently (bacterium concentration decreases effectively). In the less virulent genetic group G (I), bacterial lysis begins earlier, takes longer, and is less efficient in decreasing bacterium concentration (II, III). This may be due to the different growth characteristics of the strains. Suomalainen *et al.* (2006) showed that Finnish genetic groups respond differently in various circumstances. However, there were no significant disparities between groups C and G. That virulence varies (I) means that the same is also likely true of responses to phage infections and the formation of phage-resistant mutants. It may be that adhesion and biofilm formation (II, III) are related to phage susceptibility and interact to produce various structural and genetic changes within the bacterium. These results and the results from the phage therapy experiments (V) ought to encourage further research on the interactions between different *F. columnare* genetic groups and phages.

5.2.2 Effects of phage resistance on the *F. columnare* phenotype

It emerged from this thesis that variations in phage-induced colony type exert a stronger influence on the differences between phage-resistant strains than the number of phages (II). Both a single phage and a phage mixture induced rhizoid, rough, and soft colonies (II, III). Soft and rough colonies were resistant to ancestral phages. The change in morphology can be an energy-consuming process during the bacterial life cycle. Therefore, the bacterium reacts to the threat with morphological change when it benefits from it (Young 2007, Yang *et al.* 2016). Young (2007) presented the three main environmental pressures that affect bacterial survival: nutrient acquisition, cell division, and predation. The other mechanisms which are used by the bacterium to address the three factors are adherence, passive dispersal, active motility, and internal or external differentiation. These factors might also affect the features of *F. columnare* and phage resistance, which are adapted to environmental conditions, such as phage infections, to ensure the survival of the bacterium. As far as *F. columnare* is concerned, the bacterium uses a modification of surface molecules to survive predation, phages, and morphological changes, with different strains affecting adherence, biofilm formation, caseinase activity, growth rates, phage resistance, and virulence (II, III). It seems that soft colonies that are phage induced and phage resistant are less likely to adhere and form biofilm than rhizoid colonies (II). The growth rates of phage-induced soft colonies were lower than those of rough or rhizoid ones, but the maximum yield was not affected. Furthermore, Laanto *et al.* (2014) showed that these three colony morphotypes differ in cell

organisation, the profile of the secreted protein, and OMVs. They assumed that these factors might be associated with bacterial virulence.

Kunttu *et al.* (2011) showed that colony type influences virulence in *F. columnare*. Study III also showed that phage-resistant soft and rough colonies are less virulent than rhizoid colonies on rainbow trout (III). This decrease in virulence is likely caused by selection and the need to survive phage infection as well as by a combination of phage-induced effects, such as weaker adhesion, lower biofilm formation, and fewer motile and morphological changes from rhizoid to rough or soft colonies (II, III). It is possible that the surviving bacterial cells have evolved resistance against the phages that are used or that resistant cells that were originally in the population of strains gained more living space and a competitive advantage when phages caused the sensitive bacterial population to diminish. Interestingly, one isolate maintained its rhizoid morphology after phage exposure and was found to be virulent in rainbow trout. It also partly maintained its sensitivity to phages. Even though phage therapy induces the emergence of resistant strains, they are less harmful to the fish. If virulent rhizoid strains remain, they are sensitive to the phages that have been used, probably because of the available receptors.

5.2.3 Genetic factors behind the phage resistance of *F. columnare*

The evolution of bacteria is not always visible from the structure of the bacterium – changes also occur inside of it. The internal differentiations which might influence the phage resistance of *F. columnare* strains include CRISPR-Cas and secretion IX systems (Li *et al.* 2017, Laanto *et al.* 2017, Hoikkala *et al.* 2021). In study III, the virulence of all phage-resistant strains was reduced. Three strains retained their original rhizoid morphology and high virulence in rainbow trout. It is possible that CRISPR immunity was involved because the phage sensitivity of these strains was weakened (III). *F. columnare* has two CRISPR systems, which protect the bacterium from phages (Laanto *et al.* 2017). Hoikkala *et al.* (2021) showed that *F. columnare* acquires CRISPR spacers when the bacterium is cocultured with phage in a low-nutrient medium, which is followed by enrichment in a high-nutrient medium. This change in nutrient content might also occur also when the bacterium is transferred from natural waters to a fish farming environment. However, no new CRISPR spacers were detected during WGS in study III, which is why CRISPR is probably not the reason for the emergence of partial phage resistance. It would be interesting to discover whether the CRISPR system activates during a columnaris outbreak because of the environment of fish farms. It might be that phage resistance does not necessarily manifest in the activation of the CRISPR system, as it does, for example, in natural waters. Indeed, Laanto *et al.* (2017) observed that, for some *F. columnare* strains, there are fewer phage-targeting spacers in strains that are isolated during a columnaris outbreak than there are strains from natural water sources. In study III, the three virulent strains which were connected with phages were partly phage sensitive, which indicated that some spontaneous mutations might have occurred during the phage infection. Similar results have

been reported for strains of *F. psychrophilum* with phage-induced resistance (Castillo *et al.* 2015). The phage-resistant strains had insertions, deletions, or point mutations, and some of these mutations were connected with surface properties and gliding motility, which might be the reason for phage resistance (Castillo *et al.* 2015).

In study III, phage resistance and phage-induced decreases in virulence were connected, in most of the cases, with the mutations of the gliding motility protein which are a part of the type IX secretion system. Most of the isolates which exhibited changes in motility, adhesion, protein secretion, and virulence had mutations in these genes (III). Many members of *Bacteroidetes* (which also includes *F. columnare*) have a type IX secretion system, which is needed to transport and attach proteins to the cell surface through the outer membrane. This system is also part of the larger systems that are used for gliding motility (Gorasia *et al.* 2020). Mutations were not found in strains that had not been exposed to phages (III). Laanto *et al.* (2012), in a study of zebra fish, showed that decreased virulence is related to decreased motility, which also emerged from study III. It has been shown that deleting a gene from the *F. columnare* type IX secretion system affects gliding motility and decreases the mortality of zebrafish, channel catfish, and rainbow trout (Li *et al.* 2017). Similar results were obtained with *F. psychrophilum* (Barbier *et al.* 2020). The mutations that phages induce might force resistant bacteria to choose exposure to phages or phage resistance. These phage-resistant mutants might lose some surface protein, gliding motility, and, probably as a result of these mutations, virulence.

5.2.4 Effect of treatment environment on phage resistance

The emergence of phage resistance does not necessarily mean that the formed bacteria are completely inaccessible to phages. Castillo *et al.* (2014), in a study of *F. psychrophilum*, showed that when phage resistance emerges, it covers several phages. This is an alarming finding for phage therapy, especially if a regimen that has been used once induces widespread resistance against different phages. However, the authors suspected that the resistance was mainly due to the similarity between the tested phages, which probably use the same receptor when infecting a bacterial cell (Castillo *et al.* 2014). Interestingly, they also found that the development of phage resistance makes some bacteria sensitive to other phages that cannot infect the ancestral strain. They suspected that this sensitivity is due to the coexistence of bacteria and phages in an environment in which they exert mutual influences on their development (Castillo *et al.* 2014). Furthermore, phage-induced decreases in adherence, biofilm formation, and virulence (II, III) might put the phage-resistant strain at a disadvantage when other bacteria are present in the environment. The other bacteria may enter a more balanced state, and *F. columnare* would not cause an infection (Merikanto *et al.* 2018).

Interestingly, mucin was found to increase biofilm formation in some colony types. Phage infections and colony morphologies did not necessarily influence this process (II). Mucin increased significantly in ancestral-strain

biofilm formation, which might mimic the conditions of the bacteria on fish skin most closely. The fish skin mucosa is mainly formed by mucins, which protect the fish from physical stress and environmental pathogens. It also serves as an attachment surface for *F. columnare* (Almeida *et al.* 2019). Almeida *et al.* (2019) noticed that, if cultured in mucin, *F. columnare* bacterial virulence increases. However, there is a trade-off, in that bacteria that are grown in mucin are also more susceptible to phage infections. These results suggest that the cell mucus plays an important role in the pathogenicity of the bacterium as well as in its interaction with the phage. It would be interesting to study the sensitivity of the phage-resistant strains that formed in study II in the presence of mucin to determine whether mucin also affects the virulence, morphology, and motility of phage-resistant strains in rainbow trout.

F. columnare is an environmental bacterium which coevolves with its phages in natural waters and is engaged in a constant arms race with them (Laanto *et al.* 2017). When the water temperature rises above a critical point, the bacteria start to multiply, and a larger bacterial load begins to arrive at fish farms. *F. columnare* strains have been collected over long periods of time in order to study the features of the pathogen (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2010, Laanto *et al.* 2011, Ashrafi *et al.* 2015). In study I, phage host range was tested with *F. columnare* strains that had been collected in the past and during the experiment. The newly isolated phages infected more strains than previously isolated ones. These new phages infected both previously and newly isolated strains, while older phages mostly infected older strains (I). This might be due to the ability of bacteria to evolve to resist the phages that they encounter at fish farms or in water sources. Laanto *et al.* (2017) showed that *F. columnare* and its phages undergo coevolution and genetic change to survive in challenging environments. This constant interaction provides phage researchers with an inexhaustible source of new phages that can be used in therapy. Therefore, it is useful for the isolation and maintenance of a comprehensive phage library. However, isolation from the environment and characterisation may be time consuming and expensive, especially if phages that have already been isolated can be trained and developed in a controlled manner and under laboratory conditions (Castillo *et al.* 2022).

5.2.5 Phage evolution as a solution to phage resistance

Phage resistance has been described as an important problem for phage therapy, which is why it is important to follow and detect phage-bacterium interactions (León and Bastías, 2005). In nature, phages and bacteria mix every day without suffering a competitive disadvantage. Phages can adapt and learn to infect phage-resistant strains. In study II, it was found that the rhizoid bacterium from genetic group C was resistant to the ancestral phages. However, it was also shown that *F. columnare* phages can evolve in the long term. These evolved phages were able to infect the strains that were resistant to the ancestral phages. Unfortunately, the sequencing was not successful, and phages also lost infectivity during storage (II). Laanto *et al.* (2020) showed that the

evolved *F. columnare* phage has mutations in the tail proteins, which may affect its ability to adhere to the resistant bacterial surface. Furthermore, Friman *et al.* (2016), who worked with *P. aeruginosa*, showed that evolved phages are more efficient in decreasing bacterial concentration than ancestral phages. They found that the ancestral bacteria were not able to generate resistance against the evolved phages. One way to circumvent or address this problem in phage therapy is to develop a means of refining phages and causing them to evolve in the desired direction. In a study on *F. psychrophilum*, Castillo *et al.* (2022) showed that, as far as phage-resistant strains are concerned, there is an alternative to isolating phages from the environment. They used a similar method to that which I employed in study II on a larger scale, as well as several additional phages. They found that forced evolution is a relatively rapid method for selecting new phages for use against the targeted pathogen despite phage resistance. For *F. columnare,* the creation of one (Laanto *et al.* 2020) or three (II) evolved phages took between one and three weeks. In these studies, only one dose of phages was added to the bacterial cultures. Castillo *et al.* (2022) isolated 12 evolved phages in the first or second week by using *F. psychrophilum* strains that were already resistant as well as single and multiple phage stocks with serial passages. A similar experiment could be performed with *F. columnare,* and it would be interesting to see whether it results in more evolved phages than a single-dose treatment. Similarly to bacteria, phage resistance entails a trade-off between survival and the loss of some features. The evolved *F. columnare* phage was found to suffer from reduced adsorption when used against other strains (Laanto *et al.* 2020). This might explain the instability of the evolved phages during storage and the failure of the sequencing attempts in study II. Having become infective to a resistant strain, the phage might have been weakened by a mutation that influences survival. It is possible that more evolved and stable phages would have formed if the experiment had continued for a longer time under the same conditions or if it had been conducted with serial passages.

In study V, the attachment of phages to plastic film somewhat decelerated fish mortality. It may reduce the amount of pathogens in incoming water and prevent biofilm formation. However, the risk of the inlet tube that is coated with phages resulting in resistant incoming bacteria must be considered. It is possible that the functionality of the phage bathing method would decrease as a result. In this scenario, it would be important for the phage-coated tubes or filters to be up to date and to contain phages that are capable of infecting contemporary strains. The advantage of phages is that they can infect and lyse bacteria rapidly and that they produce a larger number of virions than that which is used during the infection. The daughter phages are released into the water, where the infection may continue if free bacteria are available. The assumption is that when *F. columnare* enters a fish farm, it becomes pathogenic in the nutrient-rich conditions of the facility (Pulkkinen *et al.* 2022). If immobilised phages can eradicate the bacterial cells from the incoming water, the formation of bacterial pathogenicity may be prevented.

Although *F. columnare* phages are very host specific, some seem to have the ability to learn how to infect other strains (II). In study I, the FCOV-F13 phage was isolated against bacteria from genetic group G, and it exhibited infectivity to strains from this group. In study II, FCOV-F13 showed an ability to learn how to infect bacterial strains from genetic group C. This ability might be the result of coevolution in nature, where the FCOV-F13 phage has encountered representatives of both strains (I). This development is promising for phage therapy because a solution to narrowing host ranges or the emergence of phage resistance can also be found in phages that have already been isolated. In the future, the FCOV-F13 phage could be tested in an evolution experiment that mimics the experiment that Castillo *et al.* (2022) conducted. The FCOV-F13 phage is exactly like two other G cluster phages (I), which would suggest that these two phages may infect genetic group C. A future experiment of this kind would be interesting.

5.3 The future of phage therapy for *F. columnare*

It is important to avoid the mistakes that were made with antibiotics when studying and developing phage therapy. A blue-eyed and unnecessarily optimistic attitude towards the possible dangers can lead to a similar outcome. It was hoped that the development of resistance to antibiotics would not be a problem (Rollo *et al.* 1952). Unfortunately, however, this hope transpired to be misplaced. Similar mistakes can be made with phages, especially when devices evolve and when the amount of information and its availability increase.

Well-planned and multifactorial phage therapy is a potential alternative to antibiotics in the treatment of bacterial infections (Górski *et al.* 2020). One of the most obvious benefits of phage therapy is that bacterial cells can be targeted for infection precisely. In phage therapy, the harmful bacterium must be identified, and well-studied phages that are effective against it must be available. Using phages reduces harm to other microbiota (Drulis-Kawa *et al.* 2012), a significant advantage over antibiotic treatments. Since antibiotics may kill a broad spectrum of bacteria (Dethlefsen and Relman 2011), it is possible for beneficial ones to die. The regeneration of the microbiome may take a long time. If some cells have survived due to insufficient dosage, the pathogen may reconquer the area and reinitiate infection during that period. This is also a possibility with *F. columnare*, which has been shown to cause repeated outbreaks despite antibiotic treatment (Pulkkinen *et al.* 2010), for example due to natural sources (Kunttu *et al.* 2012) or biofilm in farming systems (Cai *et al.* 2013). In Finland, few antibiotics are approved for use against *F. columnare* in fish farms (Anon 2016), which increases demand for alternative treatments.

As far as phage therapy is concerned, it is also desirable to consider the results of interactions between phages and bacteria as well as their possible effects on humans, animals, and the environment. The epidemiological

dynamics of pathogens that grow in the environment may serve as an example (Merikanto *et al.* 2018). It is known that agriculture plays a role in the emergence of resistance to antibiotics. The resistant strains can cause problems in humans (Meaden and Koskella 2013). If similar developments are to be avoided in phage therapy, it must be studied and implemented in a controlled manner. Since the study of phage therapy in agriculture and aquaculture is at an early stage of its development, the effect of concentrated phage treatments on the environment and its microbiome is unknown. The effect, if any, of the transfer of phages from agriculture or aquaculture to humans has to be ascertained. Nguyen *et al.* (2017) found that the *E. coli* T4 phage can cross the epithelial cell layers of humans. They noted that this is normal for phages because some of them live in constant interaction with humans and human bacteria and travel between organs. Lehti *et al.* (2017), working on the *E. coli* PK1A2 phage, noticed that it can attach to the structure of the eukaryotic neuroblastoma cell surface. This polysialic acid is related to the receptor that phages use to infect *E. coli*. The phage does not cause harm to the cell, which degraded it during the experiment (Lehti *et al.* 2017). These two studies show that much must be discovered about phages and their features before their effects can be understood fully. In this thesis, it was shown that *F. columnare* and its phages does not harm the eyed eggs of rainbow trout. *F. columnare*, its phages and phage-resistant strains does not harm the welfare of fish fry (IV, V), and phage-resistance does not seem to increase antibiotic resistance (III). Water from fish farms is released into the environment, for example into rivers. If phages must be removed from outlet waters, the amount of *F. columnare* phages can be decreased through NaCl processing (V). This thesis indicates that phage therapy for columnaris disease is safe to use.

F. columnare bacteria occur in freshwater around the world. However, strains can vary between continents and even between countries (I, Michel *et al.* 2002, Suomalainen *et al.* 2006). The same appears to be true of phages. It may be that the phages that have been isolated in Finland and which are suitable for effective phage therapy do not work at all in, say, the US (I). During the host range experiment from study I, Finnish phages seemed not to infect US or French strains of *F. columnare*. Of course, in this study, there were no phages from these countries, which is why it is impossible to say whether US or French phages can infect Finnish bacterial strains. This would be an interesting avenue for future research. Similar geographical differences were observed with Finnish and Swedish samples (I). However, the samples of isolates from outside of Finland were small, which is why conclusions that are too direct should be avoided. The results must be investigated more broadly to arrive at a more comprehensive theory. Similar results have been reported for *F. psychrophilum* – phages from Chile infect fewer tested strains than Danish phages (Castillo *et al.* 2014). Danish phages form clear zones with Danish strains most frequently, which is indicative of lysis during tests, when the turbid zone might indicate phage resistance or inhibition. Castillo *et al.* (2014) wrote that this difference might not be the only reason for the observed geographical distribution. The fish from which the bacterial strains were isolated may also serve as an

explanation. At the same time, Castillo *et al.*'s (2014) results support the notion that geographical separation may have supported the local diversification of the phages. It seems that, for both *F. columnare* and *F. psychrophilum*, local bacterial strains have affected the evolution of the phage population and that the phage population has shaped the development of phages. Awareness of this relationship is essential for the planning of phage therapy.

If fish fry production is concentrated in the hands of a few producers or if fish are moved from one country to another, the bacterial strains which are now emerging at fish farms might be affected. Foreign strains might spread from farms to the environment. For example, it is possible that suppliers of offspring and eyed eggs in Denmark have contributed to the spread of *F. psychrophilum* (Castillo *et al.* 2014). The native *F. columnare* may be displaced by new strains, which may be more harmful. This poses challenges for both antibiotic treatments and the planning of phage therapy because region-specific phages are not necessarily effective against new strains. A new strain can also merge with an old one. If transnational disease control fails, Nature´s own way of overcoming these strains is the only hope. Phages have evolved together with bacteria. Accordingly, it is believed that they could also circumvent the potential resistances of new strains. If new *F. columnare* strains appear, the supervisory authorities must monitor the development of bacterial strains, the effectiveness of antibiotics, and, if phage therapy is developed, the infectivity of phages in the phage library to strains that have been isolated at fish farms.

F. columnare can cause acute and chronic infections, which makes treatment challenging (Declercq *et al.* 2013b). It could be possible to use phage therapy and prophylaxis in the treatment of acute or chronic infections. The bacterium is an environmental and temperature-dependent pathogen, and different strains might appear at different fish farms (I). Using a phage cocktail preventively, for example with immobilising phages in farming systems, could be one way of avoiding columnaris disease. When farming waters are warming, preventive treatment could decrease the incidence of *F. columnare*, slow the multiplication of bacterial strains, and reduce financial losses and demand for antibiotics. The useful life of antibiotics could be prolonged. When their use decreases, so does the probability of the emergence of antibiotic-resistant strains. At the same time, the use of antibiotics could be focused on acute infections because not every warm-water period would cause columnaris disease.

The development of phage therapy for *F. columnare* calls for further *in vitro* and *in vivo* studies that would ensure that it is a suitable alternative to antibiotics and that it is safe to use on a larger scale. In the US, the Food and Drug Administration has approved the sale of phages that are used to treat food products before they arrive on the market (FDA 2013). The European Parliament has proposed a motion on phage therapy (European Parliament 2014), and there have been discussions and plans to provide support for phage therapy in the fight against antibiotic resistance. The regulation of biological medicinal products affects studies on phage therapy and its use in the EU (Pelfrene *et al.* 2016). These regulations also concern phage therapy against *F.*

columnare. Phage therapy already meets some of the regulatory requirements. If certain difficulties, such as resistant strains, are to be overcome, license conditions must be updated, which can be time consuming and expensive (Pelfrene *et al.* 2016). Furthermore, if a new phage is added to a mixture, the resultant cocktail is deemed to be a novel drug rather than a variation of an old one. The main goal of this legislation is to protect individuals from harmful drugs and their side effects. However, it is obstructing progress in the study of phage therapy. At the same time, clinical treatments that would enable effectiveness and safety to be studied, the output of which would enable the regulations to be supplemented, are lacking. The matter is being discussed in the European Commission (European Parliament 2017, 2021), which is a step forward for phage therapy in Europe.

5.4 Conclusions

The success of phage therapy does not depend solely on the properties of the phage or its host bacteria. It is also tied to multiple complex interactions between the two, the target bacterial host, and the environment. This study confirmed that phage therapy is a potential alternative to antibiotics in the fight against *F. columnare*. Phages were relatively easy to isolate, and the isolated phages exhibited satisfactory infectivity to targeted bacterial strains (I). Isolating phages from the fish farms also showed that new phages had evolved to infect new bacterial strains when previously isolated phages were infecting them less successfully (I). The optimal method of administering phage therapy is phage bathing after the appearance of the first columnaris symptoms (V). Other routes were also tested. Some of those methods, such as immobilising phages on plastic sheets, could, if refined, be used to control bacteria before the outbreak of disease. Phage resistance did not seem to be problematic for phage therapy because the resistant strains exhibited decreases in virulence, adherence, and spread (II, III).

Despite the promising results, it emerged that more research must be conducted. Different *F. columnare* strains appear to require different phage therapeutic approaches. Similar doses, schedules, and administration routes did not seem to affect all strains in the same way (V). There is a difference between *in vitro* and *in vivo* research on phage therapy. Both must intensify for the general effects and efficiency of phage therapy to become apparent. At present, it seems that phage therapy for *F. columnare* will not be developed in the next few years. However, studies show that as research progresses and new methods are devised, phage therapy may become a treatment for columnaris disease.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kertomus faagiterapiasta *Flavobacterium columnare* bakteeria vastaan: faagin ja bakteerin välinen vuorovaikutus ja faagiterapian hyödyntäminen käytännössä

Kun antibiootit löydettiin, näistä tuli korvaamaton apu erilaisten bakteeri-infektioiden hoidossa. Ennen jopa tappavat taudit olivat antibioottien ansiosta helposti hoidettavissa. Antibioottien huoleton käyttö sekä terveydenhuollossa että ruoantuotannossa on kuitenkin synnyttänyt uuden ongelman. Bakteerit voivat muodostaa vastustuskyvyn eli resistenssin antibiootteja vastaan ja tällaiset antibioottiresistentit kannat voivat levitä alkulähteestä ympäristöön ja levittää resistenssiä esimerkiksi maataloudesta ihmisiin. Antibioottiresistenssin takia helposti hoidettavasta taudista voi muodostua kohtalokas tai se voi vaarantaa ruoantuotannon. Uusien antibioottien etsiminen ei pysy resistenssien kantojen muodostumisen perässä ja uusia vaihtoehtoja antibiooteille on löydettävä.

Kalankasvatus on yksi nopeimmin kehittyvistä ruoantuotannon sektoreista, joka tarjoaa ravintoa useille ihmisille ympäri maailmaa. Kalojen kasvatus suurissa tiheyksissä tehostaa tuotantoa, mutta altistaa samalla kaloja jatkuvalle stressille ja erilaisille sairauksille. Yksi näistä on *Flavobacterium columnare* -bakteerin aiheuttama columnaris-tauti, jota esiintyy makean veden kalanviljelylaitoksilla lämpiminä ajanjaksoina. Tämä bakteeri voi aiheuttaa kalanviljelyssä tartuntoja, korkeaa kuolleisuutta ja taloudellisia tappioita ellei sitä hoideta antibioottien avulla. *F. columnare* -infektiot vaativat kuitenkin toistuvia antibioottikuureja, sillä ne uusiutuvat helposti. Nämä toistuvat kuurit lisäävät resistenttien kantojen ilmenemisen riskiä. Tällä hetkellä Suomessa on käytössä kaksi antibioottia (oxytetrasykliiniä ja florfenikoli) *F. columnare* -bakteerin hoitoon, mikä asettaa haasteita kalantuotannolle, jos resistenssi ilmenee molempia antibiootteja vastaan. Kalanviljelyssä antibioottijäämät ja antibioottiresistentit kannat saattavat levitä läpivirtaavan veden mukana ympäristöön, jossa ne voivat vaikuttaa luonnon mikrobien elinkulkuun.

Bakteriofagit, eli faagit, ovat viruksia, jotka infektoivat spesifisesti omia isäntäbakteereja. Faagien uskotaan tarjoavan vaihtoehdon bakteeri-infektioiden hoitoon, sillä infektoidessaan kohdebakteeria, ne jättävät muut bakteerit rauhaan. Faagihoito eli faagiterapia ei aiheuta haittaa mahdollisesti hyödyllisille bakteereille. Faagi-infektion aikana faagi lisääntyy bakteerin sisällä ja uusien viruspartikkelien vapautuessa bakteerisolu kuolee. Uudet faagit ovat valmiita jatkamaan infektiota.

Tässä väitöskirjassa olen tutkinut faagiterapian käyttöä *F. columnare* -bakteerin aiheuttamaa columnaris-tautia vastaan. Tutkimus aloitettiin eristämällä ja kartoittamalla uusia faageja ja bakteereja kalanviljelylaitoksilta (I). Havaittiin, että kaikki eristetyt bakteerikannat olivat hyvin virulentteja kirjolohen (*Oncorhynchus mykiss*) poikasilla tehdyssä kokeessa. Kun faagien ominaisuuksia tutkittiin, huomattiin, että uusien faagien isäntäkirjo *F. columnare* bakteerilajin sisällä

oli laajempi kuin aikaisemmin eristettyjen faagien. Tämä viittaisi siihen, että eristetyt faagit olivat oppineet luonnossa infektoimaan uusia bakteerikantoja, joita aikaisemmin eristetyt faagit eivät olleet aikaisemmin tavanneet. Tästä bakteeri ja faagi kokoelmasta pystyttiin valitsemaan kaikista tautia aiheuttavimmat bakteerikannat ja näitä infektoivat faagit tulevia tutkimuksia varten.

Bakteerit ja faagit elävät luonnossa jatkuvassa vuorovaikutuksessa keskenään, minkä vuoksi bakteereilla on kyky muodostaa resistenssi faageja vastaan. Faagiresistenssin on ajateltu olevan yksi isoimmista haasteista faagiterapian käytön kannalta, jos resistenttien kantojen sairauden aiheuttamiskyky on säilynyt tai jopa lisääntynyt eikä näitä kantoja voida hoitaa esimerkiksi antibiooteilla tai toisilla faageilla. Osatutkimuksissa II ja III tutkin sekä yhden että kahden erilaisen faagin aiheuttaman resistenssin mahdollisia vaikutuksia *F. columnare* kantojen ominaisuuksiin. Havaittiin, että faagilla tai niiden lukumäärällä ei ole merkitystä havaituissa eroavaisuuksissa vaan suurin merkitys on faagin aiheuttamalla bakteerin pesäketyypin muutoksella. Faagien aikaansaamat muutokset pesäketyypissä ja tätä kautta bakteerisolujen käyttäytymisessä heikensi bakteerisolujen tarttumiskykyä ja biofilmin muodostusta sekä vähensi bakteerin aiheuttamaa kuolleisuutta kirjolohen poikasilla. Faagien aiheuttama resistenssi vaikutti myös säilyttävän bakteerikantojen antibioottiherkkyyden. Nämä menetetyt ominaisuudet mahdollisesti myös heikentävät bakteerin kilpailukykyä kalanviljelylaitoksilla ja luonnossa, jolloin ne eivät pääse muodostamaan suurintaosaa ilmenevästä bakteeripopulaatiosta ja kehittymään tautia aiheuttaviksi. Näiden osatutkimusten tulosten perusteella faagiterapian aikaansaama faagiresistenssi ei vaikuta olevan ongelma.

Faagiterapiaa tutkittaessa on mietittävä miten faagiterapian antaminen toteutetaan. Yksi mahdollinen *F. columnare* -bakteerin lähde on mätimunat, joista kuoriutuu viljelykaloja laitoksille. Osatutkimuksessa IV tutkittiin ensimmäistä kertaa *F. columnare* ja tämän sukulaisbakteerin *F. psychrophilum* -bakteerien tarttumista ja selviytymistä hedelmöitetyillä kirjolohen mädeillä. Samassa tutkimuksessa selvitettiin faagiterapian käyttömahdollisuutta ennaltaehkäisevänä keinona kontrolloida bakteerien esiintymistä mätimunissa. Kirjolohen poikasten mätimunia pidettiin 10 °C, sillä liian lämpimissä olosuhteissa munat eivät selviytyneet hengissä. Havaittiin, että tämä lämpötila on liian matala *F. columnare* -bakteerin lisääntymiselle mutta bakteeri sekä sen faagit säilyivät mätimunien kanssa. *F. psychrophilum* -bakteeri puolestaan tarvitsee matalamman lämpötilan lisääntyäkseen ja tutkimuksen aikana havaittiin, että faagiterapialla oli potentiaalia kontrolloida bakteerin esiintymistä mätimunissa.

Faagien antamista kirjolohen poikasille tutkittiin eri menetelmiä hyödyntäen (V). Faagien lisääminen yhdessä bakteerin kanssa yhden kalan koealtaisiin oli todettu jo aikaisemmin toimivaksi menetelmäksi ja osatutkimuksessa V vahvistettiin, että tällä menetelmällä pystyttiin vähentämään kalojen kuolleisuutta. Kalanviljelijät eivät kuitenkaan tiedä bakteeri-infektioista yleensä vasta kun oireita alkaa ilmaantua, minkä vuoksi myös erilaisien annostusaikataulujen ja kalojen faagiseoksessa kylvettämisen vaikutuksia selvitettiin. Havaittiin, että faageilla päällystetty muovikalvo tarjosi myös suojaa kaloille bakteeri-infektioita

vastaan, vaikkakaan ei yhtä tehokkaasti kuin vapaat faagit vedessä. Koska suljetut yhden kalan koealtaat eivät ole verrattavissa kalanviljelylaitosten olosuhteisiin, tehtiin isompi koe kalapopulaatioiden ja läpivirtausaltaiden avulla. Kaikista tutkituista menetelmistä kahden bakteerikannan aikaansaamalta columnaris-taudilta suojasi parhaiten kalojen kylvetys kahden faagin seoksessa. Kylvetys toteutettiin, kun kalat alkoivat ilmentää ensimmäisiä columnaris-taudin oireita. Kahdella faagilla päällystetty kalvo ja 2 tuntia ennen bakteeri-infektion aloitusta annettu faagikylvetys hidastivat columnaris-taudin puhkeamista, mikä voisi kalanviljelylaitoksen olosuhteissa suojata kaloja varsinaisen taudin ilmenemiseltä tai antaa viljelijöille lisäaikaa reagoida ja hoitaa mahdollisen taudin puhkeaminen.

Tässä väitöskirjassa selvitettiin, että faagiterapialla on mahdollisuuksia taistelussa *F. columnare* -bakteerin aiheuttamaa columnaris-tautia vastaan. Potentiaalisista tuloksista huolimatta on kuitenkin huomioitava, että erilaiset bakteerikannat ja näiden faagien infektiot vaihtelivat keskenään ja vaikuttaisi siltä, että yksi menetelmä tai tapa käyttää faageja ei ole sopiva kaikille *F. columnare* kannoille. Tästä syystä lisää erilaisia menetelmiä on tutkittava sekä *in vitro* ja *in vivo*, jotta saadaan lisää tietoa faagien ja bakteerien välisestä vuorovaikutuksesta sekä laboratorioissa että viljelyolosuhteissa.

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ORIGINAL PAPERS

I

PREVALENCE OF GENETICALLY SIMILAR FLAVOBACTERIUM COLUMNARE PHAGES ACROSS AQUACULTURE ENVIRONMENTS REVEALS A STRONG POTENTIAL FOR PATHOGEN CONTROL

by

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Prevalence of genetically similar *Flavobacterium columnare* phages across aquaculture environments reveals a strong potential for pathogen control

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Summary

Intensive aquaculture conditions expose fish to bacterial infections, leading to significant financial losses, extensive antibiotic use and risk of antibiotic resistance in target bacteria. *Flavobacterium columnare* causes columnaris disease in aquaculture worldwide. To develop a bacteriophage-based control of columnaris disease, we isolated and characterized 126 *F. columnare* strains and 63 phages against *F. columnare* from Finland and Sweden in 2017. Bacterial isolates were virulent on rainbow trout (*Oncorhynchus mykiss*) and fell into four previously described genetic groups A, C, E and G, with genetic groups C and E being the most virulent. Phage host range studied against a collection of 227 bacterial isolates (from 2013 to 2017) demonstrated modular infection patterns based on host genetic group. Phages infected contemporary and previously isolated bacterial hosts, but bacteria isolated most recently were generally resistant to previously isolated phages. Despite large differences in geographical origin, isolation year or host range of the phages, whole-genome sequencing of 56 phages showed high level of genetic similarity to previously isolated *F. columnare* phages (*Ficleduovirus*, *Myoviridae*). Altogether, this phage collection demonstrates a potential for use in phage therapy.

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Introduction

During the past 20 years, aquaculture has been the fastest-growing food production sector (FAO, 2014), providing an important source of protein for human consumption. Intensive aquaculture production is based on monocultures of certain species, which are reared in high population densities, resulting in increased transmission of infections (Pulkkinen *et al*., 2010; Oidtmann *et al*., 2011) and antibiotic use (FAO, 2014). Approximately 70%–80% of antibiotics in aquaculture may end up in the environment (Cabello *et al*., 2013; Watts *et al*., 2017), where they select for antibiotic resistance also in the environmental bacteria (Tamminen *et al*., 2011; Yang *et al*., 2013). The World Health Organization (WHO) has declared antibiotic resistance as a major risk for global health and food security, and means to control diseases without antibiotics are therefore urgently needed.

Due to increased issues with antibiotic resistance and lack of efficient vaccines, application of lytic bacteriophages (phages) has been suggested as an alternative for controlling pathogenic bacteria (reviewed by e.g. Watts, 2017; Kortright *et al*., 2019). Their host specificity makes phages strong candidates as tools for targeted eradication of pathogenic bacteria. Indeed, the use of phage as therapeutics has a long history in medicine (Almeida and Sundberg, 2020), and recently interest towards other types of applications has increased. Phages can be used, for example to extend the food shelf life (Moye *et al*., 2018), and are already in use against *Listeria* in salad, salmon and meat packages (Sulakvelidze, 2013; Lone *et al*., 2016). Interest towards using phages in aquaculture has been steadily increasing the past decade, including studies on phage–bacterium

interactions for important fish pathogens such as Flavobacteria, Vibrio and Aeromonas (Castillo *et al*., 2012; Silva *et al*., 2014; Tan *et al*., 2014).

Flavobacterium columnare (Bacteroidetes) is a Gram-negative bacterium (Bernardet *et al*., 1996) causing columnaris epidemics at freshwater fish farms worldwide (Declercq *et al*., 2013). Conditions such as water temperature over 18°C and high fish density promote columnaris epidemics, which spread rapidly in the rearing units, and may lead to high mortality if not treated by antibiotics (Suomalainen *et al*., 2005; Karvonen *et al*., 2010; Pulkkinen *et al*., 2010). Moreover, the intensive production of rainbow trout (*Oncorhynchus mykiss*) has been shown to select for highly virulent *F. columnare* strains in the aquaculture environment (Sundberg *et al*., 2016), with different genetic groups (genotypes hereafter) being connected to different host species at the global scale (LaFrentz *et al*., 2018).

However, how the pathogen population structure in aquaculture systems influences the genetic and phenotypic (especially host range) patterns in phages is not understood. Phages infecting *F. columnare* described so far have been relatively host specific with a narrow host range (Laanto *et al*., 2011), and phage addition during experimental columnaris infection has shown promising results on the survival of rainbow trout fry (Laanto *et al*., 2015; Almeida *et al*., 2019). However, for development of successful phage therapy approach, it is essential to obtain an overview of the diversity and spatial distribution of both phage and bacterial populations, and establish a phage collection that covers this diversity. This requires isolation, and subsequent genetic and functional characterization of phage and host communities, and description of the phage–host interactions.

In this study, we isolated new *F. columnare* strains and their phages from 10 different aquaculture locations in Finland and Sweden during summer 2017. Highly virulent bacterial strains occurred at several farms. Phage infection patterns were studied on 227 different bacterial strains isolated during 2013–2017. Bacterial isolates were genetically characterized and their virulence was determined on rainbow trout. Morphology of the phages was confirmed with transmission electron microscopy (TEM), and whole-genome sequencing was performed for 56 of the isolated phages. We show that geographically distant *F. columnare* phages have very similar genomic composition and cluster according to the genetic groups of the host bacteria. The phages were able to infect bacteria isolated from different fish farms; however, the impaired capacity of phages isolated earlier to infect bacteria in a later time point suggests that bacteria evolve resistance against phage in the aquaculture conditions.

Results

Isolation and genetic characterization of F. columnare *strains*

We isolated 111 *F. columnare* strains from water samples from 10 different locations in Finland and Sweden (Supplementary Table S1). In addition, 15 Swedish *F. columnare* strains were obtained from National Veterinary Institute, Sweden. Globally, *F. columnare* strains can be classified into six genomovars (I, I/II, II, II-A, II-B and III) by restriction fragment length polymorphism (RFLP) of 16S rDNA (Hadisaputro and Wakabayashi, 1999; LaFrentz *et al*., 2014; LaFrentz *et al*., 2017; García *et al*., 2018). In this study, all isolated bacterial strains were classified as genomovar I strains, except for the previously isolated Swedish strains F310, F383 and F514, which were classified as genomovar I/II (Table 1, Supplementary Table S1).

From altogether 126 bacterial isolates, 121 Finnish and Swedish strains could be assigned into previously defined genetic groups (A, C, G, E, Suomalainen *et al*., 2006) using RFLP analysis of the internal transcribed spacer (ITS) region between 16S rRNA and 23S rRNA genes. Most of the strains fell into genetic group C (73 strains) or E (24 strains) (Fig. 1; Supplementary Table S1), whereas eight strains belonged to group G and 16 to group A. Bacterial strains isolated from Sweden fell into two genetic groups (A and E), but during 2017, only genetic group A strains were isolated (Supplementary Table S1). Five Swedish strains (Supplementary Table S1) could not be assigned to any of the previously defined genetic groups and were designated as ND1 (F397), ND2 (F512) and ND3 (F310, F383 and F514).

In general, the isolates from each fish farm represented a specific genomic group (Fig. 1) suggesting that specific genomic groups are dominating the *F. columnare* communities at the individual fish farm. Only from the most frequently sampled farm (Farm 1), we isolated bacteria belonging to two genetic groups (A and C) (Fig. 1, Supplementary Table S1). *Flavobacterium columnare* was not isolated from farms 4 and 9.

Virulence experiment

Virulence of 34 selected *F. columnare* isolates representing different genetic groups was studied on rainbow trout fry. Of the isolates, 17 were categorized as high virulence (estimated survival time <15 h), 16 as medium virulence (estimated survival time >15 h) and one as low virulence (no difference to uninfected control: $p = 0.662$, Kaplan–Meier survival analysis) isolate. The virulence observed among isolates belonging to genetic groups E and C (Table 1) were significantly higher than for groups A and G isolates ($p < 0.001$) (Fig. 2), with

Table 1. Virulence of 34 *Flavobacterium columnare* isolates in rainbow trout fry (*Oncorhynchus mykiss*) in a 24-h experiment.

Isolate	Isolation year	Country	Farm n:o	Genomovar	Genetic group	Mortality (%)	Mean estimated survival time (h)	Virulence
FCO-F16	2017	Finland	3	I	E	100.0	12.87	High
FCO-F81	2017	Finland	6	I	E	100.0	13.00	High
FCO-F88	2017	Finland	6	I	E	100.0	13.07	High
FCO-F2	2017	Finland	1	I	C	100.0	13.13	High
FCO-F13	2017	Finland	3	I	E	100.0	13.53	High
FCO-F118	2017	Finland	7	I	C	100.0	13.53	High
FCO-F11	2017	Finland	3	I	E	100.0	13.60	High
FCO-F22	2017	Finland	1	I	C	100.0	13.60	High
FCO-F33	2017	Finland	1	I	C	100.0	13.67	High
FCO-F58	2017	Finland	1	I	C	100.0	13.73	High
FCO-F98	2017	Finland	5	I	C	100.0	13.80	High
FCO-F50	2017	Finland	1	I	C	100.0	13.93	High
FCO-F86	2017	Finland	6	I	E	100.0	14.00	High
FCO-F30	2017	Finland	1	I	C	100.0	14.13	High
FCO-F78	2017	Finland	5	I	C	100.0	14.20	High
FCO-F41	2017	Finland	1	I	C	100.0	14.33	High
F310	2000	Sweden	10	I/II	ND3	100.0	14.93	High
F387	2002	Sweden	10	I	E	93.3	15.33	Medium
F383	2002	Sweden	10	I/II	ND3	100.0	15.40	Medium
FCO-F45	2017	Finland	8	I	A	100.0	15.53	Medium
F514	2013	Sweden	10	I/II	ND3	100.0	15.87	Medium
FCO-F42	2017	Finland	8	I	A	100.0	15.87	Medium
FCO-S1	2017	Sweden	10	I	A	100.0	16.00	Medium
FCO-F32	2017	Finland	1	I	A	100.0	16.21	Medium
FCO-F40	2017	Finland	1	I	C	100.0	16.27	Medium
FCO-F47	2017	Finland	8	I	A	100.0	16.33	Medium
3/3449	2017	Sweden	10	I	A	100.0	16.40	Medium
FCO-F5	2017	Finland	2	I	G	100.0	16.53	Medium
FCO-F3	2017	Finland	2	I	G	100.0	16.60	Medium
FCO-F9	2017	Finland	2	I	G	100.0	17.00	Medium
FCO-F8	2017	Finland	2	I	G	100.0	17.13	Medium
F524	2014	Sweden	10	I	A	100.0	19.87	Medium
F512	2013	Sweden	10	I	ND2	100.0	20.67	Medium
F397	2002	Sweden	10	I	ND1	20.0	22.33	Low
Neg. control						30.0	23.90	

Virulence levels (high, medium or low) were based on estimated survival time, Kaplan–Meier survival analysis.

significantly faster cumulative mortality caused by group E than group C isolates ($p < 0.001$). Mortality caused by genetic groups A and G, on the other hand, did not differ from each other ($p = 0.865$).

In addition to the genetic group, initial isolation source of the bacterium (fish farm water or directly from fish, Supplementary Table S1) had a significant effect on bacterial virulence, with isolates from tank water being more virulent than bacteria isolated from fish ($p < 0.001$). There were also differences in mortalities caused by bacteria isolated from different fish species/rearing tanks of different species ($p < 0.001$), the isolates from salmon (*Salmo salar*) being the most and the isolates from trout (*S. trutta*) the least virulent.

Characterization of bacteriophages

Sixty-three bacteriophages were isolated from the water samples originating from seven different fish farms (Fig. 1; Table 2). The majority (52 out of 63) of the phages were isolated against hosts from genetic group C, while the rest of the phages were isolated against genetic groups A or G. No phages were isolated from mixed bacterial enrichment cultures or with genetic group E bacteria. Phages infecting hosts from different genetic groups [FCOV-S1 (A), FCOV-F13 (G) and FCOV-F27 (C), Supplementary Fig. S1] were morphologically similar under TEM and displayed typical characteristics of myoviruses, icosahedral capsid with a rigid, relatively thick tail (Supplementary Fig. S1). Interestingly, phages against genetic group C were isolated from fish farm 4, even though no *F. columnare* was detected from the farm during the sampling.

Host range of 71 *F. columnare* phages isolated in this study and previously isolated phages (Table 2) was tested in total with 227 different bacterial strains (Supplementary Table S4). Of these, 51 Finnish and eight Swedish bacterial strains were susceptible to one or more of the phages (Supplementary Table S4). In cases where clear infections and plaques were not

Farm number	Number of isolated *Flavobacterium columnare* bacteria (*Fc*) and their phages per genetic group													
	A		C		E		G		ND1		ND2		ND3	
	Fc	Phage	*Fc*	Phage	*Fc*	Phage	Fc	Phage	*Fc*	Phage	*Fc*	Phage	*Fc*	Phage
1	1	1	50	23	-	-	-	6	-	-	-	-	-	-
2	-	-	-	1	-	-	8	2	-	-	-	-	-	-
3	-	-	-	5	6	-	-		-	-	-	-	-	-
4	-	-	-	13	-	-	-	-	-	-	-	-	-	-
5	-	-	20	3	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	16	-	-	-	-	-	-	-	-	-
7	-	-	3	7	-	-	-	-	-	-	-	-	-	-
8	7	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	8	2	-	-	2	-	-	-	1	-	1	-	3	-

Fig. 1. Sampling locations of fish farms in Finland and Sweden. On the left: Map of Northern Europe, where each number indicates a farm, where the water and fish samples were collected. Exact number and locations of Swedish farms are not known, and they are jointly marked as farm 10. On the right: Number of bacterial and bacteriophage isolates from individual fish farms. A, C, E, G, ND1, ND2 and ND3 indicate the different genetic groups of the isolated bacteria and the isolation hosts of the phage. ND = genetic group not determined.

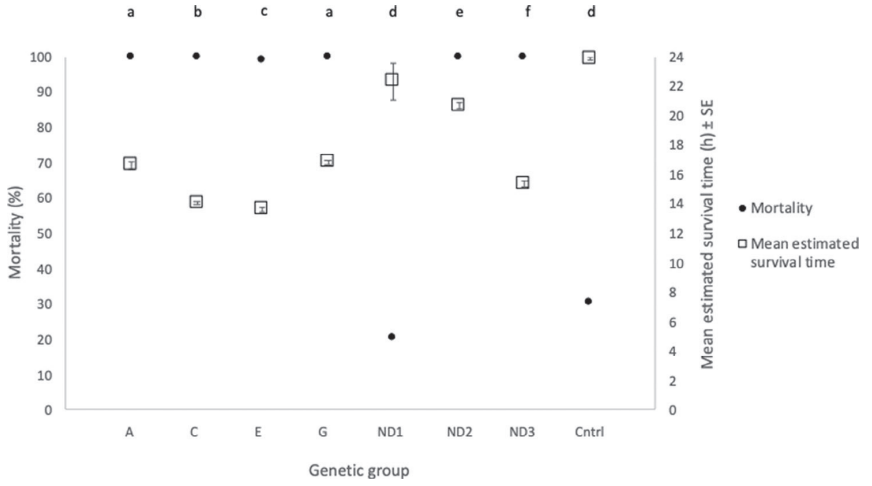

Fig. 2. Mortality percent and mean estimated survival time (±SE) of rainbow trout (*Oncorhynchus mykiss*) during 24-h experimental infection with *Flavobacterium columnare* isolates representing genetic groups A, C, E and G. ND = genetic group not determined, Cntrl = control with no bacterial infection. Different lowercase letters indicate statistical difference in cumulative mortality (Kaplan–Meier Survival Analysis) between the genetic groups.

observed, majority of the phages caused growth inhibition on bacterial lawn (Supplementary Table S4).

Bacteria isolated in 2017 were generally resistant to phages isolated earlier (Fig. 3, Supplementary Table S4). The phages characterized in this study did not, in general, infect bacteria isolated in the USA or other tested Flavobacterium species.

The phages isolated in 2017 infected both contemporary and previously isolated *F. columnare* strains within the genetic cluster, regardless of the isolation origin of the bacteria. Network analysis of the phage infection patterns revealed clustered interactions defined by the host genetic group (Fig. 3, see also Supplementary Table S4). In other words, phages isolated against a host from a specific genetic group infected generally bacteria from the same genetic group. For example FCOV-S1 isolated against host from genetic group A, infected generally bacteria from genetic group A.

Few phages deviated from these cluster patterns, showing cross-infection to hosts from a different genetic group. C-genetic group infecting phages FCOV-F25, F26, and F27 were able to infect bacterial isolates from genetic groups E (isolates FCO-F13 and FCO-F14) and G (isolate B442). FCOV-F28 was able to infect bacteria from genetic group G (isolate B442) in addition to group C bacteria (Supplementary Table S4). The pattern differentiated also when isolation year was regarded, as the phages isolated earlier (V183 and V184, V188, V189 isolated in 2015 from farm 1) were able to infect only earlier isolated bacterial strains (B245 in 2009 and B526 in 2012).

Four phages isolated with G host B185 (FCOV-F13, F14, F15 and F16) were able to also infect genetic group

Table 2. Bacteriophages isolated in this study.

Phage	Fish farm n:o	Isolation year/date	Source	Isolation host/ genetic group	Number of predicted ORFs	Genome length (kbp)	TEM	Accession number	Genetically identical isolates
FCL-1*	2	2007		B076/A					
FCL-2*	2	2008	–	B185/G		47.1	x	NC_027125	c
FCV-1*	1	2009	–	C1/C		46.5	x	NC_041845	h
V182*	1	2014	–	B245/C		49.1		KY979242	i
V183	1	2015	–	B245/C	76	49.1		MT585311	p
V184	1	2015	–	B245/C	76	49.1		MT585312	p
V186	1	2015	–	B067/A	74	46.5		MT585313	b
V188	1	2015	–	B245/C	76	49.1		MT585314	p
V189	1	2015	–	B245/C	76	49.1		MT585315	p
FCOV-S1	10	3.8.2017	Tank water	B534/A	74	46.5	x	MK756094	a
FCOV-S2	10	3.8.2017	Tank water	B067/A	74	46.4	x	MK756095	a
FCOV-F1	1	6.7.2017	Tank water	B537/C	76	49.1		MT585273	j
FCOV-F2	1	6.7.2017	Tank water	B537/C	76	49.1	x	MK756083	j
FCOV-F3	1	6.7.2017	Tank water	B537/C	76	49.1	x	MT585274	j
FCOV-F4	1	6.7.2017	Tank water	B537/C	76	49.1		MT585275	j
FCOV-F5	3	24.7.2017	Tank water	B537/C			x		
FCOV-F6	3	24.7.2017	Tank water	B537/C	76	49.1		MK756084	j
FCOV-F7	3	24.7.2017	Tank water	B537/C	76	49.1		MT585276	j
FCOV-F8	1	7.8.2017	Farm outlet water	B537/C	76	49.1		MT585277	k
FCOV-F9	1	7.8.2017	Tank water	B537/C	76	49.1	x	MK756085	k
FCOV-F10	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585278	j
FCOV-F11	1	7.8.2017	Tank water	FCO-F2/C	76	49.1	x	MT585279	j
FCOV-F12	4	8.8.2017	Farm outlet water	FCO-F2/C	76	49.1		MT585280	n
FCOV-F13	1	7.8.2017	Tank water	B185/G	74	47.2	x	MK756086	d
FCOV-F14	1	7.8.2017	Tank water	B185/G	74	47.2		MT585281	e
FCOV-F15	1	7.8.2017	Tank water	B185/G	74	47.2		MT585282	d
FCOV-F16	1	7.8.2017	Tank water	B185/G	74	47.2	x	MK756087	e
FCOV-F17	1	7.8.2017	Tank water	B537/C	76	49.1		MT585283	k
FCOV-F18	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585284	k
FCOV-F19	7	23.8.2017	Tank water	B537/C	76	49.1		MT585285	o
FCOV-F20	5	18.8.2017	Tank water	B537/C	76	49.1		MT585286	o
FCOV-F21	5	18.8.2017	Tank water	B537/C	76	49.1		MT585287	o
FCOV-F22	7	23.8.2017	Farm outlet water	FCO-F2/C	76	49.1		MT585288	o
FCOV-F23	1	7.8.2017	Tank water	B537/C	76	49.1		MT585289	k
FCOV-F24	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585290	j
FCOV-F25	1	7.8.2017	Tank water	B537/C	76	49.1	x	MK756088	j
FCOV-F26	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585291	j
FCOV-F27	1	7.8.2017	Tank water	FCO-F2/C	76	49.2		MK756089	j
FCOV-F28	1	7.8.2017	Tank water	B537/C	76	49.1		MT585292	m
FCOV-F29	1	7.8.2017	Tank water	B537/C	76	49.1		MT585293	l
FCOV-F30	1	7.8.2017	Tank water	B537/C	76	49.1		MT585294	k
FCOV-F31	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585295	l
FCOV-F32	1	7.8.2017	Tank water	FCO-F2/C	76	49.1		MT585296	j
FCOV-F33	4	8.8.2017	Tank water	B537/C	76	49.1		MT585297	n
FCOV-F34	4	8.8.2017	Tank water	B537/C	76	49.1		MT585298	n
FCOV-F35	4	8.8.2017	Tank water	B537/C	76	49.1		MT585299	n
FCOV-F36	4	8.8.2017	Tank water	B537/C	76	49.1		MT585300	n
FCOV-F37	4	8.8.2017	Tank water	B537/C	76	49.1		MK756090	n
FCOV-F38	4	8.8.2017	Tank water	B537/C	76	49.1		MT585301	n
FCOV-F39	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585302	n
FCOV-F40	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585303	n
FCOV-F41	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585304	n
FCOV-F42	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585305	n
FCOV-F43	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585306	n
FCOV-F44	4	8.8.2017	Tank water	FCO-F2/C	76	49.1		MT585307	n
FCOV-F45	2	15.8.2017	Tank water	B185/G	74	47.2		MK756091	f
FCOV-F46§	2	28.8.2017	Tank water	B185/G	74	47.2		MK756092	g
	5	18.8.2017	Tank water	B537/C	76	49.1		MT585308	o

(Continues)

Table 2. Continued

Phage	Fish farm n:o	Isolation year/date	Source	Isolation host/ genetic group	Number of predicted ORFs	Genome length (kbp)	TEM	Accession number	Genetically identical isolates
FCOV-F47§									
FCOV-F48§	2	15.8.2017	Tank water	B537/C	76	49.1		MT585309	p
FCOV-F49§	7	23.8.2017	Tank water	B537/C					
FCOV-F50§	7	23.8.2017	Tank water	B537/C					
FCOV-F51§	3	24.7.2017	Tank water	B537/C					
FCOV-F52§	7	7.8.2017	Tank water	B537/C					
FCOV-F53§	7	7.8.2017	Tank water	B537/C					
FCOV-F54§	1	3.9.2017	Tank water	B185/G	74	47.2		MK756093	d
FCOV-F55§	1	3.9.2017	Tank water	B185/G					
FCOV-F56§	1	3.9.2017	Tank water	B067A	74	46.5		MT585310	b
FCOV-F58§	7	7.8.2017	Tank water	B537/C					
FCOV-F59§	1	7.8.2017	Tank water	B537/C					
FCOV-F60§	1	7.8.2017	Tank water	B537/C					
FCOV-F61§	1	7.8.2017	Tank water	B537/C					
FCOV-F62§	3	24.7.2017	Tank water	B537/C					

Phages marked with asterisks have been isolated and characterized in previous studies. Small letters in the last column indicate genetically identical genomes (same letter = identical genome). ORFs were predicted with GenemarkS. For details of isolation hosts, see Supplementary Table S2.

§ phages isolated with mucin supplement (Almeida *et al*., 2019).

* Phages characterized in Laanto *et al*. (2011) and Laanto *et al*. (2017).

A (3/3449, 5/3451, isolated from Sweden in 2017) bacteria (Fig. 5, Supplementary Table S4). In addition, FCOV-F16 was able to infect also ND1 (isolate F397) and ND3 (isolate F310) strains from Sweden.

Interestingly, these extended host ranges were associated with minimal or no genetic differences in phage genomes (see below).

Genetic characterization of bacteriophages

Sequencing of the phage genomes revealed highly similar genomes despite the different host range of the phages (Fig. 4). Comparison of phage genomes across infection clusters (i.e. across host genomic groups, VIRIDIC) showed a nucleotide level identity between representative phage genomes of C- and G-phages of 84.4%, whereas A and G phages shared 93.41% identity, and A and C phages 88.3% (Fig. 4, Supplementary Fig. S2).

Length of the linear phage genomes varied from 46 kbp with 74 open reading frames (ORFs) (Cluster A phages) and 47 kbp with 74 ORFs (Cluster G) up to 49 kbp with 76 ORFs (Cluster C) (Supplementary Table S2). The ends of the linear DNA were Sanger sequenced here for two A cluster phages and the sequences were identical to the previously determined genome ends. The ends of these linear phage genomes have a 13 nt long 5′ cohesive ends with base-pairing complementary. This has previously been experimentally verified by ligation following sequencing of the overlap in the circularized genomes (data not shown). Most of the differences between phages infecting A-, C- and G -hosts were located at the left side of the genome, whereas approximately the first 29 kbps of the genomes were more conserved (Fig. 4, Supplementary Table S5). The conserved area consists of the predicted, e.g. packaging and structural genes. None of the phages had ORFs that could be directly associated with lysogeny, virulence, horizontal gene transfer or antibiotic resistance.

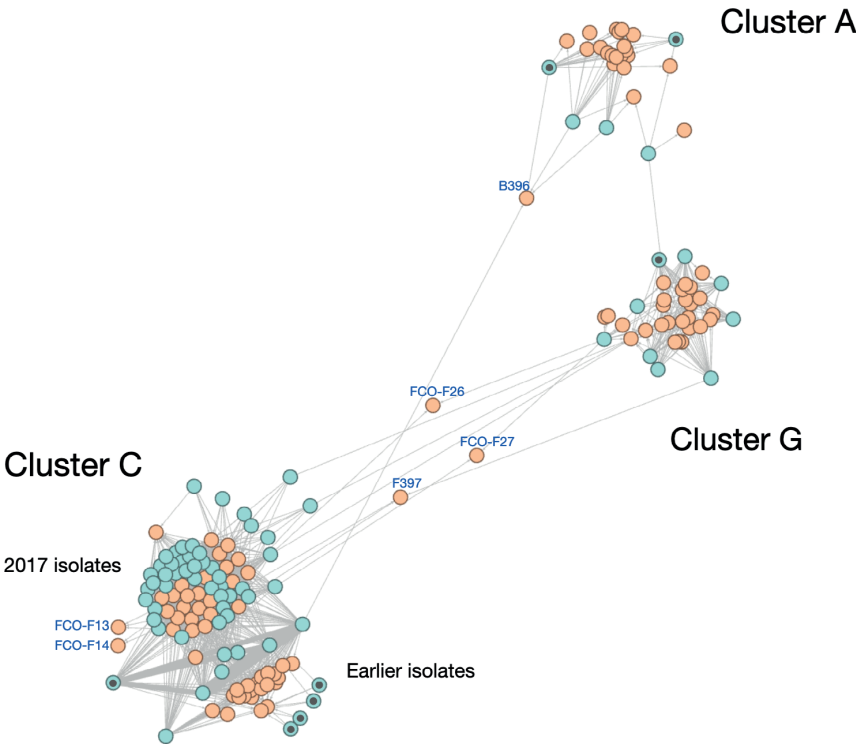

Fig. 3. Phage–bacterium interaction network. Infection patterns of phages (light blue) in bacterial hosts (orange) are clustered based on the genetic group of the bacterial host (A, C and G). Dark dot within phage circles indicates phages isolated earlier. Some key bacterial species infected by two different phage groups (FCO-F26, FCO-F27, F397, B396) or belonging to genetic group E (FCO-F13 and FCO-14) are indicated. Network was visualized using Force Atlas 2 algorithm in Gephi, where modularity of the community is interpreted by comparing the nodes with each other.

Phylogenetic tree based on complete phage genomes indicated similar clustering as the phage infection patterns (Fig. 5). Phylogenomic Genome-BLAST Distance Phylogeny (GBDP) tree inferred using the formula D6 yielded average support of 70%. The OPTSIL clustering yielded two species clusters (C-phages and G + A phages), and one genus level. All the phages characterized here can be assigned to the unclassified Ficleduoviruses (*Myoviridae*), but represent two different species.

Within the individual infection and species clusters, the phages had very similar genomes. The nucleotide level identities between phage genomes within infection clusters A, C and G (Fig. 3) were high: 99.7%–100% between A-phages, 98.2%–100% between G phages (including FCL-2) and 94.9%–100% between C-phages (Supplementary Fig. S2). It should also be noted that genetically identical phages were isolated from different fish farms, e.g. cluster C phages FCOV-F4 and FCOV-F6 (from Farms 1 and 3 respectively) and FCOV-F20 and FCOV-F22 (from Farms 5 and 7), and Cluster G phages FCOV-F45 and FCOV-F54 (Farms 2 and 1) (Table 1).

In general, the nucleotide level differences leading to amino acid level changes were detected in the putative structural proteins, in addition to several ORFs without putative annotated function (hypothetical proteins). Detailed list of differing ORFs is provided in Supplementary Table S5. In cluster A phages, changes were seen also in the putative 3′-phosphoadenosine-5′-phosphosulfate (PAPS), putative DNA methylase (ORF 47), and in the putative replication proteins (ORFs 50 and 51). In cluster G phages, the additional ORFs with amino acid changes were the putative peptidase (ORF 16) and ssDNA-binding protein (ORF 63).

Notably, Cluster A-phages originating from different countries (Fig. 6A) shared surprisingly high level of genetic identity. Within each phage cluster the hot spots for genetical variability were found in different parts of the genomes (compared with the consensus, Fig. 6). In Cluster A-phages the hot spot was located in the area coding for putative replication proteins (after 35 kbp), while in C-phages it was in the area encoding putative tail proteins. In addition, the area around 25 kbp (from the genome start), which has been speculated to code for tail fibre proteins (e.g. in FCOV-F25 ORFs 35 and 36) (Laanto *et al*., 2017), was also characterized by variability among phages.

Within Cluster G phages, genetically identical FCOV-F45 and FCOV-F46 differed from the other Cluster G phages isolated in 2017, although only two nucleotide-level differences were detected. One change was located in non-coding area (11 837 bp) and the other one was a

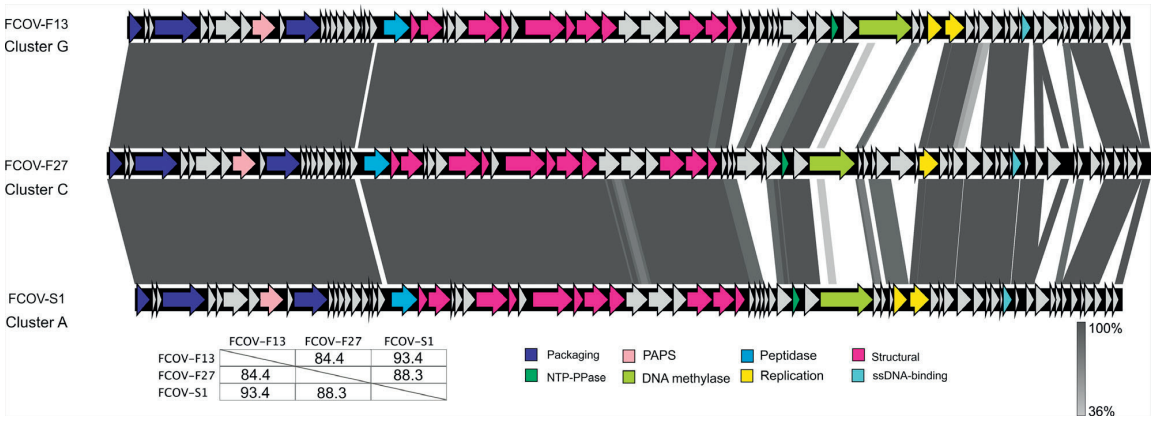

Fig. 4. Genomic comparison of representative phages of the Clusters A (FCOV-S1), C (FCOV-F27) and G (FCOV-F13). Arrows in the image indicate locations and orientations of ORFs. ORFs with unknown function are marked with grey, ORFs with putative function are marked with colours indicating the putative function as marked in the bottom. PAPS stands for 3′-phosphoadenosine-5′-phosphosulfate. The nucleotide level identity between the genomes is indicated in the box bottom left. Grey regions between the genomes indicate the level of identity (see the legend).

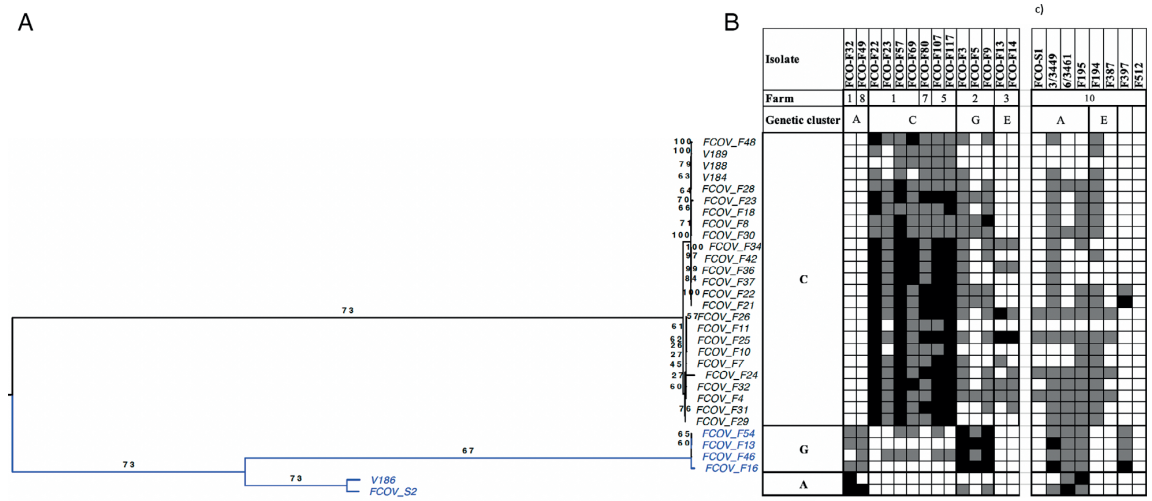

Fig. 5. Genetic distance versus host range of sequenced phages.
A. Genome BLAST Distance Phylogeny (GBDP) tree. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications. Host range of sequenced bacteriophages against representative bacterial strains isolated in (B) Finland, and (C) in Sweden. Black square indicates phage infection, grey square growth inhibition and white square bacterial resistance. Complete host range is provided in Supplementary materials.

non-synonymous change in a putative tail protein (16 932 bp). However, these phages (FCOV-F45 and FCOV-F46) differ from each other in their host range, with FCOV-F46 able to infect also eight bacterial isolates in the genetic group C. The previously isolated phage FCL-2 (from 2008) differs from other Cluster G phages, especially in the putative tail tape measure protein, although it has been isolated from the same fish farm than FCOV-F45 and FCOV-F46 (Fig. 6B). In this comparison genetic changes were also seen in the ORF coding for speculated tail fibre proteins (hypothetical proteins around 25 kbp, similarly to Cluster C phages, see below).

Cluster C-phages were isolated most frequently, allowing higher molecular resolution of phage genomes in this group. Although the genomes were highly identical (Fig. 5; Table 1), comparisons revealed seven genetically variable areas, forming five genetic variants (GV 1–5)

where nucleotide differences leading to amino acid change occurred (Fig. 6C). The variation and similarity were not dependent on bacterial strain used in phage isolation (Table 1).

Most of the genetic variance occurred in the hypothetical proteins, but in GV2 a putative tail protein was included (ORF27). In addition, GV4 includes the speculated tail fibre proteins in the 25 kbp area. Here, amino acid-level differences in ORFs 35 and 36 between FCOV-F25 and FCOV-F28 might explain differences in host range (Fig. 6D, Supplementary Table S4).

Discussion

In this study, we examined phenotypic and genetic characteristics of 126 *F. columnare* isolates and 63 phages from fish farms in Finland and Sweden. Bacterial isolates represented two previously characterized genomovars (I, I/II, based on 16S rDNA, LaFrentz *et al*., 2014) and four genetic groups (A, C, G, E, Suomalainen *et al*., 2006). Bacteria belonging to genetic groups C and E had the highest virulence in rainbow trout, but also other genetic groups caused high mortality. The isolated phages were all tailed *Myoviridae* dsDNA phages, and were genetically similar compared to previously described *F. columnare* phages (Laanto *et al*., 2017). The phages clustered into specific units of infection based on the bacterial genomic groups, with a few exceptions of phages able to cross infect to other bacterial groups (see below). Importantly, the isolated phages were able to impair the growth of the virulent bacteria, suggesting potential to be used in phage therapy against columnaris disease.

Previous studies have shown that genetically different *F. columnare* strains co-occur at fish farms (Ashrafi *et al*., 2015; Sundberg *et al*., 2016). Our results confirm the presence of previously isolated genetic groups in Finnish fish farms, suggesting that these bacterial populations have been circulating at farms during the last decades (Suomalainen *et al*., 2006; Ashrafi *et al*., 2015). Furthermore, the intensive aquaculture production in salmonid fish species in the Nordic countries may also select for certain host-associated *F. columnare* strains (LaFrentz *et al*., 2018). Due to the convenient distance from the laboratory, Farm 1 was sampled the most frequently, and thus the majority of the isolated bacteria (51) and phages (30) were obtained from this farm, likely explaining the higher diversity obtained here. Nevertheless, the sampling showed presence of virulent *F. columnare* genetic groups (C and E) at almost all farms in Finland (Fig. 1), and also in Sweden (exact farm locations for Swedish farms are not known). However, the Swedish *F. columnare* population was genetically more diverse with more genetic group isolated, some of which have not been found in Finland. Yet, the virulence of Swedish strains was lower than Finnish ones. To our knowledge, *F. columnare* isolates from Sweden have not been characterized previously.

We isolated phages infecting *F. columnare* from six farms in Finland and from one sample collected from Sweden. Interestingly, phages were also isolated when their bacterial hosts were not, suggesting that phages can be useful indicators of pathogen diversity during epidemics. Phage occurrence as a proxy for pathogen presence has been used also elsewhere in aquatic environments (Jofre *et al*., 2016; McMinn *et al*., 2017; Farkas *et al*., 2019).

Phage infection patterns clustered according to *F. columnare* genetic groups, although there are a few exceptions. For example, no phages were isolated using group E bacteria as isolation hosts. However, a few phages (FCOV-F25 - F27) had the ability to infect group E bacteria (FCO-F13 and FCO-14) in addition to their isolation host (C). Despite this, these phages did not genetically differ from some of the other C-phages (indicated by letter b in Table 1, e.g. FCOV-F1 – F7), which could only infect C type bacteria. Similarly, phage FCOV-F46 clustering to group G had the ability to infect a few group C bacteria, although sharing high genetical similarity with FCOV-F45 infecting only G bacteria. A previous study with Bacteroidetes phages showed similar results, as serial passage of phages in bacterial hosts can result in changes in host range, even without detectable genetic changes (Holmfeldt *et al*., 2016). It is also possible that epigenetic modifications play a role in host range (Hattman, 2009) but that remains to be verified in these phages. However, some differences in host range were associated with clear genetic changes. FCOV-F28 was able to infect genetic groups C and G, and comparison to FCOV-F25 revealed several non-synonymous nucleotide changes in the previously speculated tail fibre genes, ORFs35 and 36 (Laanto *et al*., 2017). In our previous study these same ORFs accumulated several mutations during a co-culture of *F. columnare* and phage FCV-1, leading to change in host range (Laanto *et al*., 2020). Previous data with other species also suggest point mutations in tail fibre proteins increase phage infectivity (Uchiyama *et al*., 2011; Boon *et al*., 2020).

Although the increased accumulation of phage genomic and metagenomics data has revealed their enormous genetic diversity both on global and local scales (Salmond and Fineran, 2015), specific phages infecting specific hosts have been isolated across large geographical areas (Kellogg *et al*., 1995; Wolf *et al*., 2003; Sonnenschein *et al*., 2017), suggesting that some groups of closely related phages may have a worldwide distribution. An important finding in this study is that similar phages exist at different fish farms and countries, although some small genetic differences occur. In

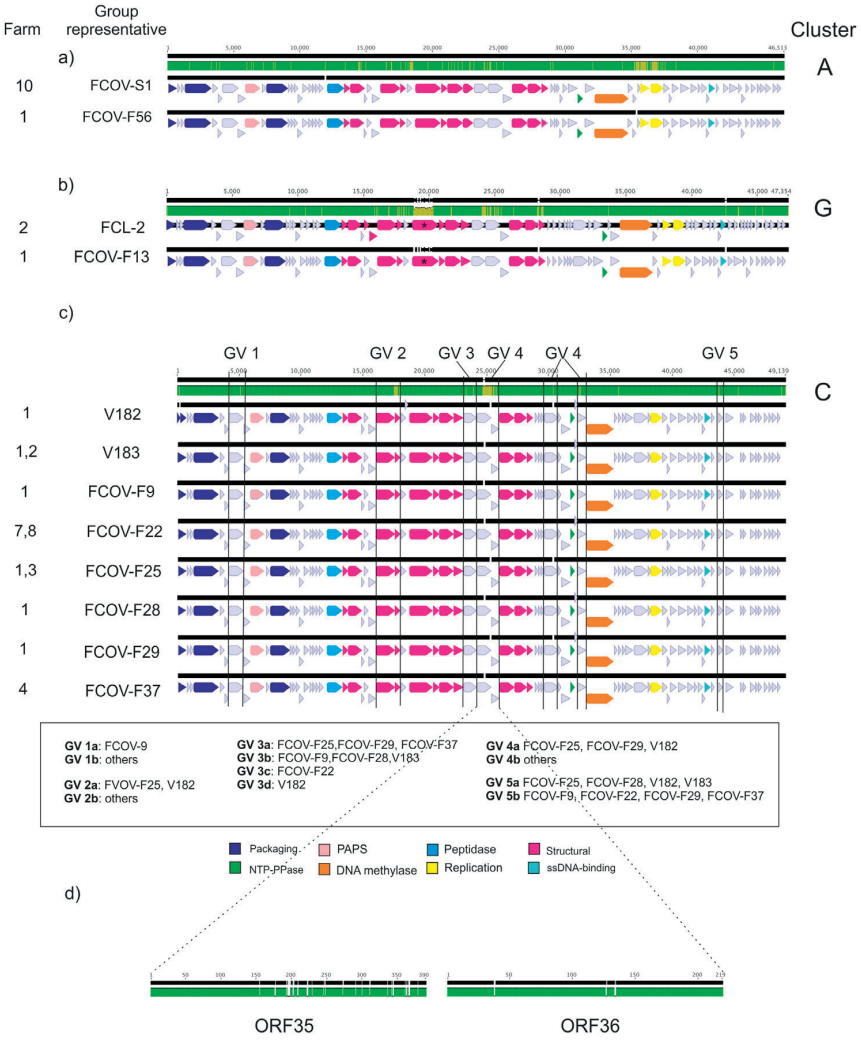

Fig. 6. Nucleotide alignments of genetically different phages infecting *Flavobacterium columnare*.
A. Cluster A (FVOV-S1 from Sweden and FCOV-F56 from Finland), (B) Cluster G (FCL-2 isolated in 2008), and (C) Cluster C phages (previously analysed V182 as a reference), and (D) consensus of the amino acid alignments of ORF 35 and ORF 36 (speculated tail fibre proteins) between FCOV-F25 and FCOV-F28, which may be linked with differences in host range. In the genome consensus (on top of each alignment) green colour indicates 100% identical sequence and yellow >30%. GV indicates genetically variable area (GV 1–5) where the differences localize. ORFs with unknown function are marked with grey, ORFs with putative function are marked with colours indicating the putative function as marked in the bottom. Letters after GV (a, b, c or d) indicate phages, which are similar in certain GV area but differ in other areas. PAPS = 3′-phosphoadenosine-5′-phosphosulfate. Asterisk marks the tape tail measure protein in case of cluster G phages. Phage V182 isolated in 2014 was included in alignments as a reference to the last time point of the phage genome evolution dataset published earlier (Laanto *et al*., 2017).

addition, the isolated phage genomes were highly similar to the previously described phages in our dataset, as also reported earlier with phages infecting the genetic group C bacteria (Laanto *et al*., 2017). Comparable findings have been reported in aquaculture-related phage-bacterium systems, for example with pathogens *F. psychrophilum* and *Vibrio anguillarum* (Castillo and Middelboe, 2016; Kalatzis *et al*., 2017), where genetically similar phages were widely distributed across large temporal and spatial scales. Furthermore, genetically similar phages have been isolated also in other types of phage-bacterium systems, such as *Salmonella*, *Synechococcus* and *Escherichia* (Gregory *et al*., 2016; Michniewski *et al*., 2019; Thanki *et al*., 2019).

Phages infecting flavobacterial species are known to regulate the genetic and phenotypic diversity of their bacterial hosts. As a co-evolutionary response, this should select for diversity also in the phage population. Yet, *F. columnare* phages with 100% nucleotide identity were isolated from different fish farms and using enrichment hosts isolated in different years (Table 1). For example, Cluster C phages FCOV-F29 (isolated using B537 host from 2013) and FCOV-F31 (isolated with FCO-F2 from 2017) are identical, as are also e.g. V183 (B245 from 2009) and FCOV-F48 (B537), and A-phages FCOV-S1 and FCOV-S2 (isolated using B534 from 2013 and B067 from 2007 respectively). A possible explanation for the low genetic diversity among *F. columnare* phages includes a potential for transfer of phages and bacteria between farms with the fish stocks or via water sources. This could explain the 100% similarity of phages from Farms 5 and 7, which are located close to each

other and share the same source of water. Similarly, the shared water source and transfer of fish fry from Farm 1 to Farm 3 may contribute to the isolation of identical phages at these farms. On the other hand, A-phages originating from Finland and Sweden shared 99.7% identity despite the geographical distance and different isolation hosts. Therefore, the low genetic diversity and the high costs of phage resistance (Laanto *et al*., 2012; Laanto *et al*., 2014) in *F. columnare* may select for a low genetic diversity of the phages. Also, the use of antibiotics may play a central role in maintaining the low genetic diversity of the host bacteria in aquaculture. Application of antibiotic treatment to control disease epidemics rapidly impact the bacterial population size, leaving less possibilities for phage–bacterium interaction at short (within-season) time span.

When looking at the temporal differences of phage isolates our results are in accordance with previous findings: the phages isolated in most recently (2017) had broader host range than the previously isolated phages [FCV-1 (2008), V183-V189 (2015), Cluster C, Supplementary Table S4] from the same fish farm, which were, mostly able to infect isolates from earlier time points. This indicates a coevolutionary response to evolution of bacterial resistance towards previously isolated phages. At the same time, the bacterial isolates were susceptible to contemporary phages, which may have evolved to overcome the resistance mechanisms of the hosts. Similar results from environmental data have been derived also in other phage–bacterium systems (Koskella and Parr, 2015). However, the low diversity both in the phage and host *F. columnare* populations seems to restrict the genetic changes to small areas in the phage genomes. Our genetic data indicate changes at the end of the genome which might explain why host range between 2015 and 2017 phages differs in Cluster C phages. Phage V182 (isolated in 2014, Farm 1) used as a reference in genome alignment (Fig. 6C) was distinct to 2015 phages from the same farm. V182 was the most recent phage isolate used in our previous study on phage–bacterium coevolution during 2007–2014 at Farm 1 (Laanto *et al*., 2017). The genetic comparisons thus suggest that bacterial resistance mechanisms cause directional selection in the phage genome over long time spans. However, this phenomenon was not detectable in isolates obtained within one outbreak season at Farm 1, as phages isolated during three time points were identical (Table 1). This might have been caused by other factors at farms (e.g. the use of antibiotics) or in the natural waters. Similar results can be derived from comparison of Cluster G phages isolated from farm 2. FCL-2 (isolated in 2008) was genetically different to phages isolated in 2017, which can also be seen in differences in the host range.

In relation to the genetic similarity, another main finding of our data is that phages isolated from certain farms were able to infect bacterial hosts from other farms. This indicates that the aquatic farming environment (probably together with fish transfers and other reasons mentioned above) does not form isolation barriers between geographic locations, which would lead to locally adapting phage populations. At the nucleotide level, some farm-specific differences between the phages were observed, but this did not impact their host range. In a broader perspective, none of the phages was able to infect *F. columnare* strains isolated from Central Europe, and only one infected isolate from the USA. Also in Swedish hosts the infectivity was limited to few strains. It is therefore likely that phage–bacterium coevolution has different trajectories in different geographic areas. This remains to be demonstrated until phage–*F. columnare* interactions have been characterized outside Nordic countries. In addition, further genomic analysis of the host bacteria could reveal the resistance mechanisms in bacteria, explaining the phage host range between farms and in time.

Because of specific fatal effect against bacteria, lytic phages have been considered and studied as antimicrobial agents against bacterial infections instead of antibiotics (Lin *et al*., 2017; Ooi *et al*., 2019). Here we characterized phage diversity against *F. columnare* and its four different genetic groups. In this study, we increased the collection of the isolated and characterized *F. columnare* strains and phages from different fish farms. Our findings suggest phages capable of infecting virulent *F. columnare* strains are present at fish farms and these phages could be used as potential antimicrobial agents in future applications.

Experimental procedures

Isolation of bacteria

Samples from >10 fish farms were collected between June and August 2017 during columnaris disease outbreaks (Fig. 1 and Supplementary Table S1; exact number and locations of the Swedish fish farms are confidential and not known by the authors, so they are jointly marked as Farm 10). *Flavobacterium columnare* was isolated from water samples and directly from infected fish, using standard culture methods on Shieh agar plates supplemented with tobramycin (Song *et al*., 1988; Decostere *et al*., 1997). Water samples (1000 ml) were collected from earthen ponds, fibreglass and plastic tanks, and from the outlet water of the farms. The obtained isolates were pure cultured and stored at −80°C with 10% glycerol and 10% fetal calf serum.

Genetic characterization of bacterial strains

Bacterial genomic DNA was extracted from overnight bacterial liquid cultures with DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. For some bacterial isolates, a template for PCR reaction for genomovar and genetic group classification (see below) was obtained by picking one bacterial colony into 100 μl of sterile distilled water.

Flavobacterium columnare isolates were classified to genomovars with RFLP analysis of 16S rRNA gene according to LaFrentz *et al*. (2014) with some modifications. 16S rDNA was amplified by PCR (10 min at 95°C; 40 cycles of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C; 10 min at 72°C) using universal primers fD1 (Weisburg *et al*., 1991) and 1500R (Hadisaputro and Wakabayashi, 1999) with 1 μM of each primer, 1× DreamTaq Green buffer, 0.2 mM dNTP mix, 0.5 U Dream Taq DNA polymerase. PCR products (10 μl) were digested overnight at 37°C with 3.3 U of HaeIII. Restriction fragments were run in 12% acrylamide gels, which were stained with ethidium bromide and visualized under UV light.

The bacteria were further classified into genetic groups by RFLP of 16S – 23S ITS region correlating with the ARISA analysis designed for *F. columnare* (Suomalainen *et al*., 2006). ITS region was amplified by PCR (2 min at 95°C; 35 cycles of 30 s at 94°C, 45 s at 52°C and 3 min at 72°C; 15 min at 72°C) using primer pair rD1f (Weisburg *et al*., 1991) and 23Sr (Borneman and Triplett, 1997) as above. PCR products (10 μl) were double-digested overnight at 37°C with 3.3 U of both HaeIII and HinfI. Restriction fragments were run and visualized as described above.

Virulence experiment

Thirty-four bacterial isolates representing all the genetic groups were selected for virulence testing on rainbow trout fry (Supplementary Table S1). Bacteria were revived from −80°C by inoculation to 5 ml of Shieh medium and cultured overnight at 25°C under constant agitation (120 rpm). Bacteria were enriched by subculturing (1:10) and incubating for 24 h. Bacterial cell density was measured as an optical density (OD, 595 nm; Multiskan FC Thermo Scientific) and colony-forming units per ml (CFU ml^{-1}) estimated based on our previously determined OD–CFU relationship.

A total of 527 rainbow trout fry (*Oncorhynchus mykiss*), average weight 1.25 g, were randomly selected and placed individually into experimental aquaria with 500 ml of pre-aerated water (25°C). For each bacterial isolate, 14–15 individual fish (20 for negative control) were infected by pipetting into each aquarium 500 μl of bacterial solution giving a final dose of 5×10^3 CFU ml^{-1}. Shieh medium was used for negative controls. Fish morbidity and symptoms were observed at 1-h intervals for 24 h. Symptomatic fish not responding to stimuli were removed from the experiment and measured. To confirm the presence/absence of the bacterium, cultivations from gills of the dead fish were made on Shieh agar supplemented with tobramycin (Decostere *et al*., 1997). At the end of the experiment, surviving fish were euthanatized with overdose of benzocaine. Mortality data were analyzed using Kaplan–Meier survival analysis in IBM-SPSS statistics 24 SPSS. High and medium virulence of individual isolates were defined by an estimated survival time of <15 h and >15 h respectively, and low virulence was when no significant difference was detected compared to the control group.

Virulence test was performed according to the Finnish Act on Use of Animal for Experimental Purpose, under permission ESAVI/3940/04.10.07/2015 granted for Lotta-Riina Sundberg by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Isolation and characterization of bacteriophages

For bacteriophage isolation, 500 ml of water sample was filtered using rapid flow filters (PES membrane, pore size 0.45 μm, Nalgene®). 5× Shieh medium was diluted to 1× using filtered water sample. 1 ml of overnight-grown bacterial host (or mixture of hosts) was added to the diluted Shieh (total volume 21 ml). In some isolations, Shieh was diluted to 0.5× Shieh supplemented with 0.1% mucin (Almeida *et al*., 2019).

Four previously isolated and characterized *F. columnare* strains (genetic group in parenthesis) were used as enrichment hosts; B185 (G), B480 (E), B534 (A) and B537 (C). In addition, strains F514 (ND3) isolated from Sweden and FCO-F2 (C) isolated from Finland were used in some of the enrichments (see Supplementary Table S1). All the strains were used both individually and as a mixture. In mixed cultures, the total bacterial cell density (at OD 595 nm) was adjusted to the same OD level as the bacterium with the lowest OD in the individual enrichments.

After incubating for 24 h at 25°C (120 rpm), 0.5 ml samples were taken from enrichment cultures, centrifuged (3 min, 8000*g*), and supernatants were collected. Presence of phages was detected using double-layer agar method. 300 μl of fresh indicator bacterial culture and 300 μl of supernatant from the enrichment culture were mixed with 3 ml of soft Shieh agar (0.5%) tempered to 46°C and poured on Shieh agar plates. When mucin was used in the isolation procedure, also the soft agar contained 0.1% mucin. After 1–2 days of incubation at

25°C, individual plaques were transferred to a 500 µl Shieh medium and subjected to three rounds of plaque purification.

Phage stocks were prepared by adding 5 ml Shieh medium on confluent (lysis of all or almost all bacterial cells) and semi-confluent (lysis of approximately half of the bacterial cells) plates. Plates were incubated in a constant shaking (90 rpm) in cold room (7°C) for approximately 12 h. The lysate was collected with a syringe, sterile-filtered (Acrodisc® Syringe Filters with Supor® Membrane, pore size 0.45 µm), and stored at 7°C for further use.

Transmission electron microscopy

Three representative phages infecting different hosts and originating from different locations were selected for TEM imaging. TEM samples were prepared from phage lysates on Cu-grids. A drop of lysate was added to the grid and after 15–30 s the grids were dried with moist filter paper (Whatman). Dried samples were incubated with phospho-tungstic acid (1% PTA, pH 7.5) for 30–60 s and dried as above. Grids were air-dried overnight and protected from light. Imaging was done with JEOL JEM-1400 with 80 kVA.

Phage host range

Host range of 71 phages (Table 2) was tested on 227 different bacterial hosts (Supplementary Tables S1, S2 and S3) using the double-layer agar method. Two microliters of each high-titre phage lysate and their 10- and 100-fold dilutions were spotted on bacterial lawns. Results were recorded after 2 days of incubation at room temperature. Phages were considered to infect the bacterium if all phage dilutions had clear spots or if individual plaques were observed. When the drop area was not clearly lysed, but bacterial growth was impaired, phages were considered to cause growth inhibition. Bacteria were considered resistant if the phage had no effect on the growth.

Interaction map of the phage–bacterium infection patterns was done with Gephi 0.9.2 (Bastian *et al*., 2009) using the Force Atlas 2 network visualization algorithm (Jacomy *et al*., 2014).

Genetic characterization of bacteriophages

High-titre phage lysates (from 10^{10} to 10^{12} PFU ml^{-1}) were used for phage DNA extraction using the zinc chloride method (Santos, 1991) with small modifications. DNase and RNase (final concentrations 1 and 10 µg ml^{-1} respectively) were added to filtered phage lysates and incubated at 37°C for 30 min. Particles were concentrated with 0.2 M filtered $ZnCl_2$ and treated with 0.8 mg ml^{-1} Proteinase K (Thermo Scientific). Finally, DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) (Sambrook and Russell, 2001) and precipitated with isopropanol. DNA pellets were dissolved to 10–30 µl of dH_2O. Absence of bacterial host DNA was confirmed with PCR using universal bacterial 16S rDNA primers fD1 and rD1 (Weisburg *et al*., 1991). Genome restriction analysis with EcoRI was performed to confirm phage cluster type (data not shown).

Phage genome sequencing was performed by the Institute for Molecular Medicine Finland (FIMM). Fifty six phages were selected for sequencing based on host genetic group, isolation farm and isolation method (with and without added mucin). Phage genomes were sequenced with Illumina HiSeq2500, and PE100 sequencing produced on average 800× coverage of the genomes.

Phage genome assemblies were produced by mapping the reads to reference sequence (KY979242 for genetic group C-infecting phages, and NC_027125 for genetic group G-infecting phages) using Geneious mapper (Geneious 7.1.4 and later versions, Biomatters) with the option to find structural variants, short insertions and deletions of any size (up to 1000 bp). Minimum support for structural variant discovery was two reads, and the option to detect insertion in structural variants was included. Gaps were also allowed.

Genetic group A-infecting phages were *de novo* assembled with Velvet 1.2.10 (in Geneious). Genome ends were checked from V186 and FCOV-S1 with Sanger sequencing using primers designed for *F. columnare* phage FCV-1 genome end verification (Laanto *et al*., 2017). Briefly, primer walking was done towards the end of the genome and detection of a peak in the electropherogram (artificial base added by the sequencing polymerase) and the end of sequence suggested the physical ends of the genome.

ORFs were predicted using GenemarkS (Besemer *et al*., 2001) and Glimmer (Kelley *et al*., 2012). BLASTP (Altschul *et al*., 1990) and HHPred (Söding *et al*., 2005) were employed for annotating the putative function of the ORFs. Genomes were aligned using MUSCLE (Edgar, 2004) using default settings suggested by Geneious 7.1.4 (Biomatters). Phage V182 isolate from farm 1 in 2014 was included in alignments as a reference to the last time point of the phage genome evolution dataset published earlier (Laanto *et al*., 2017). Genome comparison of the three phages (FCOV-S1, FCOV-F13 and FCOV-F27) infecting different host genetic groups was created with Easyfig (Sullivan *et al*., 2011) employing BLASTX. All versus all comparisons for genomes were done using VIRIDIC (Moraru *et al*., 2020). Putative transposases, virulence factors and antibiotic-resistant genes were manually checked using BLASTP.

Phage genetic distances were analyzed with Victor (Meier-Kolthoff and Göker, 2017). All pairwise comparisons of the nucleotide sequences were conducted using the GBDP method (Meier-Kolthoff *et al*., 2013) under settings recommended for prokaryotic viruses (Meier-Kolthoff and Göker, 2017).

The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME including SPR postprocessing (Lefort *et al*., 2015) for each of the formulas D0, D4 and D6 respectively. Branch support was inferred from 100 pseudo-bootstrap replicates each. Trees were rooted at the midpoint (Farris, 1972) and visualized with FigTree (Rambaut, 2016). Taxon boundaries at the species, genus and family level were estimated with the OPTSIL program (Göker *et al*., 2009), the recommended clustering thresholds (Meier-Kolthoff and Göker, 2017) and an *F* value (fraction of links required for cluster fusion) of 0.5 (Meier-Kolthoff *et al*., 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information.

Appendix S2: Supporting Information.

Supplementary Fig. S1. Transmission electron microscopy (TEM) images of representative F. columnare phages infecting different genetic groups of the host (A, C and G). A). FCOV-S1, B). FCOV-F13 and C). FCOV-F27. Scale bar in A) and C) is 200 nm and B) 500 nm.

Supplementary Table S1. Bacterial strains isolated and/or characterized in this study. Swedish fish farms are confidential and not known by the authors, so they are jointly marked as Farm 10.

Supplementary Table S2. Previously isolated *F. columnare* bacterial strains used in host range studies. *F. columnare* strains from H to B533 have been previously characterized (Ashrafi *et al*., 2015). Strains marked with asterisks were used in phage isolation. Strains collected from farms 5-9 were isolated and kindly donated by Dr. Päivi Rintamäki.

Supplementary Table S3. Previously isolated *F. columnare* strains from USA and France, and other bacterial species used in host range studies.

Supplementary Table S4. Host range of phages in *F. columnare* strains isolated in this study, in previously isolated *F. columnare* strains, and in other flavobacterial species. Black square indicates infection (clear lysis in three consequent phage dilutions), grey square indicates growth inhibition, and white square no effect (i.e. bacterial resistance). Each column represents a phage isolate and each row represents a host isolate.

Supplementary Table S5. List of open reading frames (ORFs) that displayed differences between phage genomes in phages infecting genetic group C, A and G hosts.

Supplementary Fig. S2. A heatmap of all *Flavobacterium columnare* phage genomes in this study (including also previously described phages FCL-2 and V182) generated with VIRIDIC incorporating intergenomic similarity values (right) and alignment indicators (left). Numbers on the right half represent the similarity values for each genome pair and in the left three values represent (from top to bottom): aligned fraction for the genome that is found in this row (top), genome length ratio for the genomes found in this pair (middle) and aligned fraction for the genome found in this column (bottom). On the right, the darker the colours the more closely-related the genomes. On the left the darker colours indicate low values and white colours indicate genome pairs with higher similarity values.

Supplementary materials

Prevalence of genetically similar *Flavobacterium columnare* phages across aquaculture environments reveals a strong potential for pathogen control

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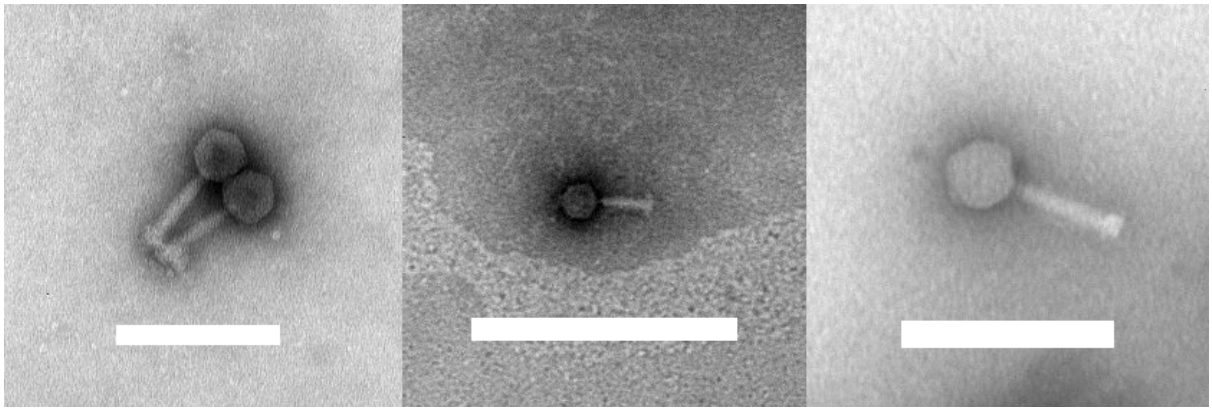
Supplementary Figures



Supplementary Figure S1. Transmission electron microscopy (TEM) images of representative *F. columnare* phages infecting different genetic groups of the host (A, C and G). A). FCOV-S1, B). FCOV-F13 and C). FCOV-F27. Scale bar in A) and C) is 200 nm and B) 500 nm.

Supplementary Figure S2 (Separate pdf file). A heatmap of all *Flavobacterium columnare* phage genomes in this study (including also previously described phages FCL-2 and V182) generated with VIRIDIC incorporating intergenomic similarity values (right) and alignment indicators (left). Numbers on the right half represent the similarity values for each genome pair and in the left three values represent (from top to bottom): aligned fraction for the genome that is found in this row (top), genome length ratio for the genomes found in this pair (middle) and aligned fraction for the genome found in this column (bottom). On the right, the darker the colors the more closely-related the genomes. On the left the darker colors indicate low values and white colors indicate genome pairs with higher similarity values.

Supplementary Table S1. Bacterial strains isolated and/or characterized in this study. Swedish fish farms are confidential and not known by the authors, so they are jointly marked as Farm 10

Isolate	Isolation year	Fish farm n:o	Fish species	Source	Genomovar	Genetic group	Virulence tested
F194	1997	Sweden/10	Brown trout	Kidney	I	E	
F195	1997	Sweden/10	Brown trout	Skin	I	A	
F310	2000	Sweden/10	Brown trout	Skin	I/II	ND3	yes
F383	2002	Sweden/10	Salmon	Skin	I/II	ND3	yes
F387	2002	Sweden/10	Brown trout	Skin	I	E	yes
F397	2002	Sweden/10	Brown trout	Kidney	I	ND1	yes
F512	2013	Sweden/10	Brown trout	Kidney	I	ND2	yes
F514	2013	Sweden/10	Brown trout	Kidney	I/II	ND3	yes
F524	2014	Sweden/10	Rainbow trout	Kidney	I	A	yes
3/3449	2017	Sweden/10	Rainbow trout	Skin ulcer	I	A	yes
4/3450	2017	Sweden/10	Rainbow trout	Skin ulcer	I	A	
5/3451	2017	Sweden/10	Rainbow trout	Skin ulcer	I	A	
5/3460	2017	Sweden/10	Rainbow trout	Kidney and gills	I	A	
6/3461	2017	Sweden/10	Rainbow trout	Kidney and gills	I	A	
FCO-S1	2017	Sweden/10	Rainbow trout	Fish tissue	I	A	yes
FCO-F1	2017	1	Salmon	Tank water	I	C	
FCO-F2	2017	1	Salmon	Tank water	I	C	yes
FCO-F3	2017	2	Lake trout	Dorsal fin	I	G	yes
FCO-F4	2017	2	Sea trout	Gill	I	G	
FCO-F5	2017	2	Lake trout	Gill	I	G	yes
FCO-F6	2017	2	Lake trout	Gill	I	G	
FCO-F7	2017	2	Lake trout	Gill	I	G	
FCO-F8	2017	2	Lake trout	Tank water	I	G	yes
FCO-F9	2017	2	Sea trout	Tank water	I	G	yes
FCO-F10	2017	2	Sea trout	Tank water	I	G	
FCO-F11	2017	3	Rainbow trout	Gill	I	E	yes
FCO-F12	2017	3	Rainbow trout	Gill	I	E	
FCO-F13	2017	3	Rainbow trout	Tank water	I	E	yes
FCO-F14	2017	3	Rainbow trout	Tank water	I	E	
FCO-F15	2017	3	Rainbow trout	Tank water	I	E	
FCO-F16	2017	3		Outgoing water	I	E	yes
FCO-F19	2017	1	Rainbow trout	Gill	I	C	
FCO-F20	2017	1	Rainbow trout	Pectoral fin	I	C	
FCO-F21	2017	1	Rainbow trout	Gill	I	C	
FCO-F22	2017	1	Rainbow trout	Gill	I	C	yes
FCO-F23	2017	1	Rainbow trout	Gill	I	C	
FCO-F24	2017	1	Rainbow trout	Gill	I	C	
FCO-F25	2017	1	Rainbow trout	Gill	I	C	
FCO-F26	2017	1	Rainbow trout	Tank water	I	C	
FCO-F27	2017	1	Rainbow trout	Tank water	I	C	
FCO-F28	2017	1	Rainbow trout	Tank water	I	C	
FCO-F29	2017	1	Rainbow trout	Tank water	I	C	

FCO-F30	2017	1	Rainbow trout	Tank water	I	C	yes
FCO-F31	2017	1	Rainbow trout	Tank water	I	C	
FCO-F32	2017	1	Rainbow trout	Tank water	I	A	yes
FCO-F33	2017	1	Rainbow trout	Tank water	I	C	yes
FCO-F35	2017	1		Outgoing water	I	C	
FCO-F36	2017	1		Outgoing water	I	C	
FCO-F37	2017	1		Outgoing water	I	C	
FCO-F40	2017	1		Outgoing water	I	C	yes
FCO-F41	2017	1		Outgoing water	I	C	yes
FCO-F42	2017	8	Lake trout	Kidney	I	A	yes
FCO-F43	2017	8	Lake trout	Gill	I	A	
FCO-F44	2017	8	Lake trout	Kidney	I	A	
FCO-F45	2017	8	Lake trout	Tank water	I	A	yes
FCO-F46	2017	8	Lake trout	Tank water	I	A	
FCO-F47	2017	8		Outgoing water	I	A	yes
FCO-F49	2017	8		Outgoing water	I	A	
FCO-F50	2017	1	Salmon	Gill	I	C	yes
FCO-F51	2017	1	Salmon	Gill	I	C	
FCO-F52	2017	1	Salmon	Gill	I	C	
FCO-F53	2017	1	Salmon	Gill	I	C	
FCO-F54	2017	1	Salmon	Gill	I	C	
FCO-F55	2017	1	Salmon	Gill	I	C	
FCO-F56	2017	1	Salmon	Gill	I	C	
FCO-F57	2017	1	Salmon	Gill	I	C	
FCO-F58	2017	1	Salmon	Tank water	I	C	yes
FCO-F59	2017	1	Salmon	Tank water	I	C	
FCO-F60	2017	1	Salmon	Tank water	I	C	
FCO-F61	2017	1	Salmon	Tank water	I	C	
FCO-F62	2017	1	Salmon	Tank water	I	C	
FCO-F63	2017	1	Salmon	Tank water	I	C	
FCO-F64	2017	1	Salmon	Tank water	I	C	
FCO-F65	2017	1	Salmon	Tank water	I	C	
FCO-F66	2017	1	Salmon	Tank water	I	C	
FCO-F67	2017	1	Salmon	Tank water	I	C	
FCO-F68	2017	1	Salmon	Tank water	I	C	
FCO-F69	2017	1	Salmon	Tank water	I	C	
FCO-F70	2017	1	Salmon	Tank water	I	C	
FCO-F71	2017	1	Salmon	Tank water	I	C	
FCO-F72	2017	1	Salmon	Tank water	I	C	
FCO-F73	2017	1	Salmon	Tank water	I	C	
FCO-F74	2017	1	Salmon	Tank water	I	C	
FCO-F75	2017	1	Salmon	Tank water	I	C	
FCO-F76	2017	1	Salmon	Tank water	I	C	
FCO-F77	2017	1	Whitefish	Gill	I	C	
FCO-F78	2017	7	Lake trout	Kidney	I	C	yes
FCO-F80	2017	7	Lake trout	Pectoral fin	I	C	
FCO-F81	2017	6	Salmon	Gill	I	E	yes

FCO-F82	2017	6	Salmon	Kidney	I	E	
FCO-F83	2017	6	Salmon	Kidney	I	E	
FCO-F84	2017	6	Salmon	Kidney	I	E	
FCO-F85	2017	6	Salmon	Kidney	I	E	
FCO-F86	2017	6	Salmon	Gill	I	E	yes
FCO-F87	2017	1		Outgoing water	I	C	
FCO-F88	2017	6	Salmon	Tank water	I	E	yes
FCO-F89	2017	6	Salmon	Tank water	I	E	
FCO-F90	2017	6	Salmon	Tank water	I	E	
FCO-F91	2017	6	Salmon	Tank water	I	E	
FCO-F92	2017	6	Salmon	Tank water	I	E	
FCO-F93	2017	6	Salmon	Tank water	I	E	
FCO-F94	2017	6	Salmon	Tank water	I	E	
FCO-F95	2017	6	Salmon	Tank water	I	E	
FCO-F96	2017	6	Salmon	Tank water	I	E	
FCO-F97	2017	6	Salmon	Tank water	I	E	
FCO-F98	2017	5	Sea trout	Skin/skin lesion	I	C	yes
FCO-F99	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F100	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F101	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F102	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F103	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F104	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F105	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F106	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F107	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F108	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F109	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F110	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F111	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F112	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F113	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F114	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F115	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F116	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F117	2017	5	Sea trout	Skin/skin lesion	I	C	
FCO-F118	2017	7	Salmon	Kidney	I	C	yes

Supplementary Table S2. Previously isolated *F. columnare* bacterial strains used in host range studies. *F. columnare* strains from H to B533 have been previously characterized (Ashrafi *et al.*, 2015). Strains marked with asterisks were used in phage isolation. Strains collected from farms 5-9 were isolated and kindly donated by Dr. Päivi Rintamäki.

Bacterial strain	Genomovar	Genetic group	Isolation year	Isolation farm	Isolation source
H	I	H	2003	1	rainbow trout (*Oncorhynchus mykiss*)
Tulo2	I	A	2010	1	tank water
C1*	I	C		unknown	
B067*	I	A	2007	2	Atlantic salmon (*Salmo salar*)
B185*	I	G	2008	2	water
B230	I	C	2009	1	tank water
B245	I	C	2009	1	tank water
B259	I	C	2009	1	tank water
B261	I	C	2009	1	tank water
B269	I	A	2009	1	tank water
B270	I	C	2009	1	tank water
B357	I	C	2010	1	earthen pond water
B366	I	C	2010	1	farm oulet water
B367	I	E	2010	1	tank water
B369	I	E	2010	1	brown trout (*Salmo trutta*)
B376	I	E	2010	1	tank water
B377	I	E	2010	1	earthen pond water
B379	I	E	2010	1	farm oulet water
B393	I	G	2010	nature/Hankasalmi	bream (*Abramis brama*)
B396	I	A	2010	1	inlet biofilm
B398	I	A	2010	1	inlet water
B399	I	G	2010	1	tank water
B402	I	C	2010	1	European whitefish(*Coregonus lavaretus*)
B405	I	C	2010	nature/Lake Jyvasjarvi	water
B407	I	C	2010	nature/Hankasalmi	water
B408	I	C	2010	nature/Hankasalmi	water
B409	I	C	2010	7	sea trout (*Salmo trutta trutta*)
B416	I	C	2008	7	brown trout (*Salmo trutta)*
B417	I	C	2008	7	Atlantic salmon (*Salmo salar*)
B418	I	C	2009	7	brown trout (*Salmo trutta*)
B419	I	C	2009	8	Atlantic salmon (*Salmo salar*)
B420	I	G	2009	8	Atlantic salmon (*Salmo salar*)
B421	I	C	2008	8	Atlantic salmon (*Salmo salar*)
B422	I	A	2009	9	sea trout (*Salmo trutta trutta*)
B423	I	A	2009	9	lake trout (*Salmo trutta lacustris*)

B424	I	C	2007	7	brown trout (*Salmo trutta*)
B426	I	C	2006	8	Atlantic salmon (*Salmo salar)*
B427	I	A	2006	9	brown trout (*Salmo trutta*)
B429	I	H	2003	2	Pike perch (*Stizostedion lucioperca*)
B430	I	E	2003	2	Pike perch (*Stizostedion lucioperca*)
B431	I	A	2003	2	grayling *Thymallus thymallus*
B434	I	G	2005	6	Atlantic salmon (*Salmo salar*)
B435	I	G	2005	6	Atlantic salmon (*Salmo salar*)
B436	I	G	2006	6	Atlantic salmon (*Salmo salar*)
B437	I	C	2006	8	brown trout (*Salmo trutta*)
B438	I	A	2006	9	rainbow trout (*Oncorhynchus mykiss*)
B439	I	C	2006	3	rainbow trout (*Oncorhynchus mykiss*)
B440	I	G	2007	8	Atlantic salmon (*Salmo salar*)
B441	I	C	2006	8	rainbow trout (*Oncorhynchus mykiss*)
B442	I	G	2007	6	Atlantic salmon (*Salmo salar*)
B444	I	G	2007	6	Atlantic salmon (*Salmo salar*)
B445	I	E	2007	nature/Hankasalmi	water
B446	I	E	2007	nature/Hankasalmi	water
B447	I	C	2007	nature/Hankasalmi	water
B448	I	C	2007	nature/Lake Jyvasjarvi	water
B449	I	C	2007	nature/Lake Jyvasjarvi	water
B450	I	G	2007	2	Pike perch (*Stizostedion lucioperca*)
B451	I	G	2007	2	Pike perch (*Stizostedion lucioperca*)
B452	I	A	2007	2	Pike perch (*Stizostedion lucioperca*)
B453	I	E	2008	1	rainbow trout (*Oncorhynchus mykiss*)
B454	I	E	2008	1	rainbow trout (*Oncorhynchus mykiss*)
B458	I	C	2009	8	brown trout (*Salmo trutta*)
B463	I	C	2011	3	rainbow trout (*Oncorhynchus mykiss*)
B480*	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B491	I	E	2012	1	tank water
B496	I	E	2012	1	tank water
B503	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B504	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B508	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B510	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B511	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)

B513	I	E	2012	1	rainbow trout (*Oncorhynchus mykiss*)
B517	I	E	2011	nature/Hankasalmi	water
B518	I	C	2006	8	Atlantic salmon (*Salmo salar*)
B519	I	C	2006	7	brown trout (*Salmo trutta*)
B520	I	C	2006	7	Atlantic salmon (*Salmo salar*)
B521	I	C	2006	7	Atlantic salmon (*Salmo salar*)
B523	I	A	2012	9	brown trout (*Salmo trutta*)
B526	I	C	2012	6	Atlantic salmon (*Salmo salar)*
B529	I	C	2012	6	Atlantic salmon (*Salmo salar)*
B531	I	C	2012	7	brown trout (*Salmo trutta*)
B532	I	C	2012	7	brown trout (*Salmo trutta*)
B533	I	H	2012	8	Atlantic salmon (*Salmo salar)*
B534*	I	A	2013	1	rainbow trout (*Oncorhynchus mykiss*)
B537*	I	C	2013	1	rainbow trout (*Oncorhynchus mykiss*)

Supplementary Table S3. Previously isolated *F. columnare* strains from USA and France, and other bacterial species used in host range studies.

Strain/species	Genomovar	Isolation year	Provided by	Reference (if available)
ATCC49513	I	1987	Jean-Francois Bernardet, INRA, France	(Bernardet, 1989)
ATCC49512	I	1987	”	(Bernardet, 1989)
LDA 39	I		”	
NCIMB2248T	I			(Bernardet and Grimont, 1989)
CSF258-10 (USA)	I		Prof Mark McBride, University of Wisconsin Milwaukee, USA	(Evenhuis *et al.*, 2016, 2017)
MSFC4 (USA)	I		”	(Evenhuis *et al.*, 2014; Bartelme *et al.*, 2018)
90-106 (USA)	III	1990	Dr Attila Karsi	(Soto *et al.*, 2008)
L90-629 (USA)	III	1990	”	(Soto *et al.*, 2008)
S03-579 (USA)	II/B	2005	”	
S09-108 (USA)	II	2009	”	
S09-157 (USA)	III	2009	”	
S10-025 (USA)	III	2010	”	
S10-239 (USA)	II	2010	”	
C-069 (USA)	II	2010	”	
C-074 (USA)	II	2010	”	
CB10-151 (USA)	I	2010	”	
Flavobacterium sp.B330				(Laanto, Mäntynen, *et al.*, 2017)
Flavobacterium sp.B183				(Laanto, Ravantti, *et al.*, 2017)
F. johnsoniae UW101				(McBride and Braun, 2004)
F.psychrophilum 950106-1/1				(Stenholm *et al.*, 2008)
F.psychrophilum MH1				(Castillo *et al.*, 2012)

Supplementary Table S4. Host range of phages in *F. columnare* strains isolated in this study, in previously isolated *F. columnare* strains, and in other flavobacterial species. Black square indicates infection (clear lysis in three consequent phage dilutions), grey square indicates growth inhibition, and white square no effect (i.e. bacterial resistance). Each column represents a phage isolate and each row represents a host isolate.

(SEPARATE EXCEL FILE)

Supplementary Table S5. List of open reading frames (ORFs) that displayed differences between phage genomes in phages infecting genetic group C, A and G hosts.

(SEPARATE EXCEL FILE)

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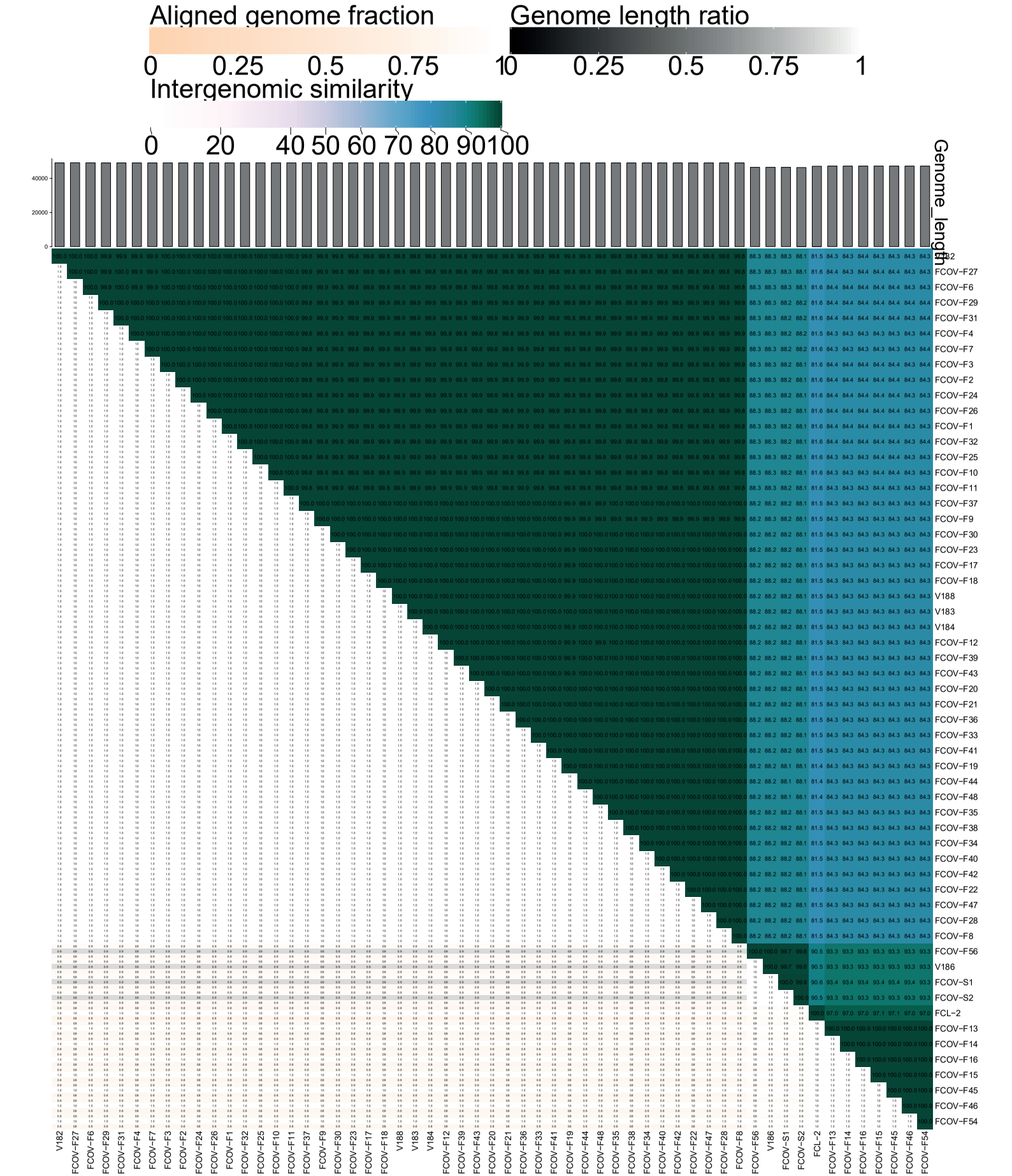
Aligned genome fraction
0
0.25
0.5
0.75
1
Genome length ratio
0
0.25
0.5
0.75
1
Intergenomic similarity
0
20
40
50
60
70
80
90
100
Genome_length
40000
20000
0
V182
FCOV-F27
FCOV-F6
FCOV-F29
FCOV-F31
FCOV-F4
FCOV-F7
FCOV-F3
FCOV-F2
FCOV-F24
FCOV-F26
FCOV-F1
FCOV-F32
FCOV-F25
FCOV-F10
FCOV-F11
FCOV-F37
FCOV-F9
FCOV-F30
FCOV-F23
FCOV-F17
FCOV-F18
V188
V183
V184
FCOV-F12
FCOV-F39
FCOV-F43
FCOV-F20
FCOV-F21
FCOV-F36
FCOV-F33
FCOV-F41
FCOV-F19
FCOV-F44
FCOV-F48
FCOV-F35
FCOV-F38
FCOV-F34
FCOV-F40
FCOV-F42
FCOV-F22
FCOV-F47
FCOV-F28
FCOV-F8
FCOV-F56
V186
FCOV-S1
FCOV-S2
FCL-2
FCOV-F13
FCOV-F14
FCOV-F16
FCOV-F15
FCOV-F45
FCOV-F46
FCOV-F54

II

EFFECTS OF SINGLE PHAGE AND TWO PHAGE MIXTURE ADDITIONS ON *FLAVOBACTERIUM COLUMNARE* GROWTH: IMPLICATIONS OF PHAGE RESISTANCE

by

Anniina Runtuvuori-Salmela, Joanda Sorsa, Heidi Kunttu, Mathias Middelboe & Lotta-Riina Sundberg 2022

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III

BACTERIOPHAGE RESISTANCE AFFECTS *FLAVOBACTERIUM COLUMNARE* VIRULENCE PARTLY VIA MUTATIONS IN GENES RELATED TO GLIDING MOTILITY AND TYPE IX SECRETION SYSTEM

by

Heidi Kunttu, Anniina Runtuvuori-Salmela, Krister Sundell, Tom Wiklund, Mathias Middelboe, Lotta Landor, Roghaieh Ashrafi, Ville Hoikkala & Lotta-Riina Sundberg 2021

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ENVIRONMENTAL MICROBIOLOGY

Bacteriophage Resistance Affects *Flavobacterium columnare* Virulence Partly via Mutations in Genes Related to Gliding Motility and the Type IX Secretion System

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ABSTRACT Increasing problems with antibiotic resistance have directed interest toward phage therapy in the aquaculture industry. However, phage resistance evolving in target bacteria is considered a challenge. To investigate how phage resistance influences the fish pathogen *Flavobacterium columnare*, two wild-type bacterial isolates, FCO-F2 and FCO-F9, were exposed to phages (FCO-F2 to FCOV-F2, FCOV-F5, and FCOV-F25, and FCO-F9 to FCL-2, FCOV-F13, and FCOV-F45), and resulting phenotypic and genetic changes in bacteria were analyzed. Bacterial viability first decreased in the exposure cultures but started to increase after 1 to 2 days, along with a change in colony morphology from original rhizoid to rough, leading to 98% prevalence of the rough morphotype. Twenty-four isolates (including four isolates from no-phage treatments) were further characterized for phage resistance, antibiotic susceptibility, motility, adhesion, and biofilm formation, protease activity, whole-genome sequencing, and virulence in rainbow trout fry. The rough isolates arising in phage exposure were phage resistant with low virulence, whereas rhizoid isolates maintained phage susceptibility and high virulence. Gliding motility and protease activity were also related to the phage susceptibility. Observed mutations in phage-resistant isolates were mostly located in genes encoding the type IX secretion system, a component of the *Bacteroidetes* gliding motility machinery. However, not all phage-resistant isolates had mutations, indicating that phage resistance in *F. columnare* is a multifactorial process, including both genetic mutations and changes in gene expression. Phage resistance may not, however, be a challenge for development of phage therapy against *F. columnare* infections since phage resistance is associated with decreases in bacterial virulence.

IMPORTANCE Phage resistance of infectious bacteria is a common phenomenon posing challenges for the development of phage therapy. Along with a growing world population and the need for increased food production, constantly intensifying animal farming has to face increasing problems of infectious diseases. Columnaris disease, caused by *Flavobacterium columnare*, is a worldwide threat for salmonid fry and juvenile farming. Without antibiotic treatments, infections can lead to 100% mortality in a fish stock. Phage therapy of columnaris disease would reduce the development of antibiotic-resistant bacteria and antibiotic loads by the aquaculture industry, but phage-resistant bacterial isolates may become a risk. However, phenotypic and genetic characterization of phage-resistant *F. columnare* isolates in this study revealed that they are less virulent than phage-susceptible isolates and thus not a challenge for phage therapy against columnaris disease. This is valuable information for the fish farming industry globally when considering phage-based prevention and curing methods for *F. columnare* infections.

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Aquaculture has a central role in supporting the increasing demand for high quality protein and healthy food. However, the use of chemotherapy in disease treatment in the industry has led to increased resistance of disease-causing agents to commonly used antibiotics (1, 2). Further, in the face of climate warming, the production of protein with a smaller carbon footprint is of increasing importance. This has put pressure on the aquaculture industry to increase efficiency in food production, which also means developing more effective ways to fight infectious diseases in intensive farming, including reducing the use of antibiotics. Although vaccines against many microbial diseases are in use globally in aquaculture, there are still many diseases with no potent immunization method available (3). This applies especially to infections of fish fry, where efficiency of vaccination is poor due to lack of development of fish secondary immunity at the early life stage.

One of these diseases affecting fry is caused by the fish pathogenic bacterium *Flavobacterium columnare*, the infectious agent of columnaris disease. Columnaris infections cause extensive losses in farmed salmonid fry and juveniles, populations of different catfish species, and ayu (*Plecoglossus altivelis*) around the world in water temperatures above 18°C. The only effective treatment method is antibiotic treatment. However, infections often occur repeatedly and may cause up to 100% mortality in rainbow trout fry populations if not treated, thus causing major economic losses to the industry (4, 5). In addition, elevated water temperatures due to warmer summers in the recent years are suggested to enhance virulence development in *F. columnare* (5). Although antibiotic resistance in this bacterium is not yet as severe a problem as in related pathogens (e.g., *Flavobacterium psychrophilum* [6, 7] or *Vibrio* species [8, 9]), strains that have acquired resistance toward commonly used antibiotics already exist (10).

Bacteriophages (phages) are viruses that specifically infect their host bacteria without harming the surrounding microbial community (reviewed in reference 11). Among the alternatives to traditional antibiotics, phage therapy (i.e., the use of phages against bacterial infections) has demonstrated a strong potential for controlling disease outbreaks in aquaculture (12–14). Promising results have been gained also in phage therapy trials of flavobacterial infections. In a study by Castillo et al. (15), phage treatment reduced the mortality of *F. psychrophilum*-infected Atlantic salmon (*Salmo salar*) by 60% and rainbow trout (*Oncorhynchus mykiss*) by 67%. In studies with columnaris infections, mortality of zebra fish (*Danio rerio*) and rainbow trout were reduced by 100% and nearly 42%, respectively, in the presence of phages (16). In addition, precolonization of fish with phage significantly slowed down the infection and reduced the mortality of rainbow trout (17).

One of the biggest challenges for phage therapy is the imposed selection for phage resistance among phage-exposed bacteria. Bacteria have developed a variety of phage defense strategies, including surface modification and cell aggregation, inactivation of intruding phage DNA by restriction modification and CRISPR-Cas systems, proteolytic digestion of phage particles, and quorum sensing regulation of phage receptor expression (18–20). Prevalence and control of these resistance mechanisms depend specifically on the phage-bacterium interaction, on the type and function of the receptor, and the costs of engaging the different mechanisms under various environmental conditions. In many pathogenic bacteria, the cell surface molecules are functioning as virulence factors, and phage-driven changes in these structures leading to phage resistance often lead to simultaneous reduction in virulence (21). This trade-off has been detected also among several bacterial fish pathogens (e.g., in *Pseudomonas plecoglossicida* [22], *F. psychrophilum* [23], and *Vibrio anguillarum* [24]).

Exposing *F. columnare* to phages has been observed to cause a change in colony morpohotype from the ancestral rhizoid form to rough form, which is associated with loss of gliding motility and virulence (25–27). Since a change in colony morphology

and loss of virulence have been observed previously by deletion of genes in the type IX secretion system involved in gliding motility of *F. columnare* (28), it is likely that mutations in this secretion system are also linked with phage resistance in *F. columnare* (29). Yet, the exact mechanisms by which phages select for colony morphology change in *F. columnare*, and the functional implications for the bacteria have not been previously explored.

Understanding the mechanisms and consequences of phage resistance in the target bacteria is central for development of successful phage therapy. Thus, in this study, we exposed two *F. columnare* isolates (FCO-F2 and FCO-F9) separately to three different phages and studied infection dynamics, bacterial viability, and colony morphology and isolated phage-resistant bacteria. Twenty-four phage-exposed and no-phage control isolates were further characterized for their phage resistance, antibiotic susceptibility, motility, adhesion, and biofilm formation on a polystyrene surface, protease (elastinase, gelatinase, and caseinase) activity, virulence on rainbow trout fry, and whole-genome sequence. Our results show that if phage resistance in *F. columnare* is gained via surface modification leading to morphotype change, virulence decreases. However, if the colony morphology remains rhizoid, the isolates remain highly virulent with reduced susceptibility to phage compared to the ancestral wild-type strain.

RESULTS

Isolates from phage exposures: growth, colony morphology, and phage resistance. In all phage exposure cultures of FCO-F2, there was a strong initial phage control of the host population during the first day in all the phage-exposed cultures compared with control culture without phages (Fig. 1a). After this, the bacterial density started to recover. The phage-free cultures grew exponentially during the first day, after which they reached a plateau phase. Along with the population decline on day 1, bacterial colony morphotype changed from ancestral rhizoid to rough (Fig. 2). From day 1 onwards, more than 88% of the colonies formed by phage-exposed bacterial isolates were rough, the amount reaching at least 98% at the end of the experiment (Fig. 1c). In addition, in FCOV-F25 exposure, few soft colonies were observed on day 2 (Fig. 2), and in no-phage control cultures, some rough colonies appeared among the prevailing rhizoid colonies.

FCO-F9 showed slightly different growth dynamics. The bacterial population size increased exponentially during the first day in all cultures (Fig. 1b) but decreased drastically on day 2 in response to phage exposure and then reached exponential growth again. The phage-free cultures reached a plateau phase on day 2, after which the amount of culturable bacteria decreased. From the day 2 population crash and onwards, more than 85% of the colonies formed by phage-exposed bacteria had rough morphology (Fig. 1d). At the end of the experiment, more than 98% of the colonies were rough. In FCOV-F13 exposure, a few rough colonies were observed already on day 1 and some soft colonies on days 2 and 3. In no-phage control cultures, some (4%) rough colonies appeared among the rhizoid colonies on day 3.

Out of 189 colonies collected from phage exposures, 20 phage-exposed and 4 no-phage control isolates were characterized further (Table 1). Of these isolates, the no-phage control isolates all formed rhizoid colonies similar to their wild-type parent phage-susceptible isolates FCO-F2 and FCO-F9. Most of the phage-exposed isolates were of rough colony morphology, but F2R58, F2R66, and F9R56 had a rhizoid morphology and F9R69 had a soft colony morphology. It should be noted, however, that rough colonies also appeared spontaneously in the no-phage control treatments (Fig. 1).

All the phage-exposed rough isolates were resistant to all the phages used to infect the ancestor wild-type bacteria (Table 1). In addition, in some cases, phage caused visible inhibition of bacterial growth in the double layer agar assay, but colonies were considered phage resistant because no clear plaques due to phage infection were detected. The rhizoid phage-exposed isolates turned out to be partly phage resistant,

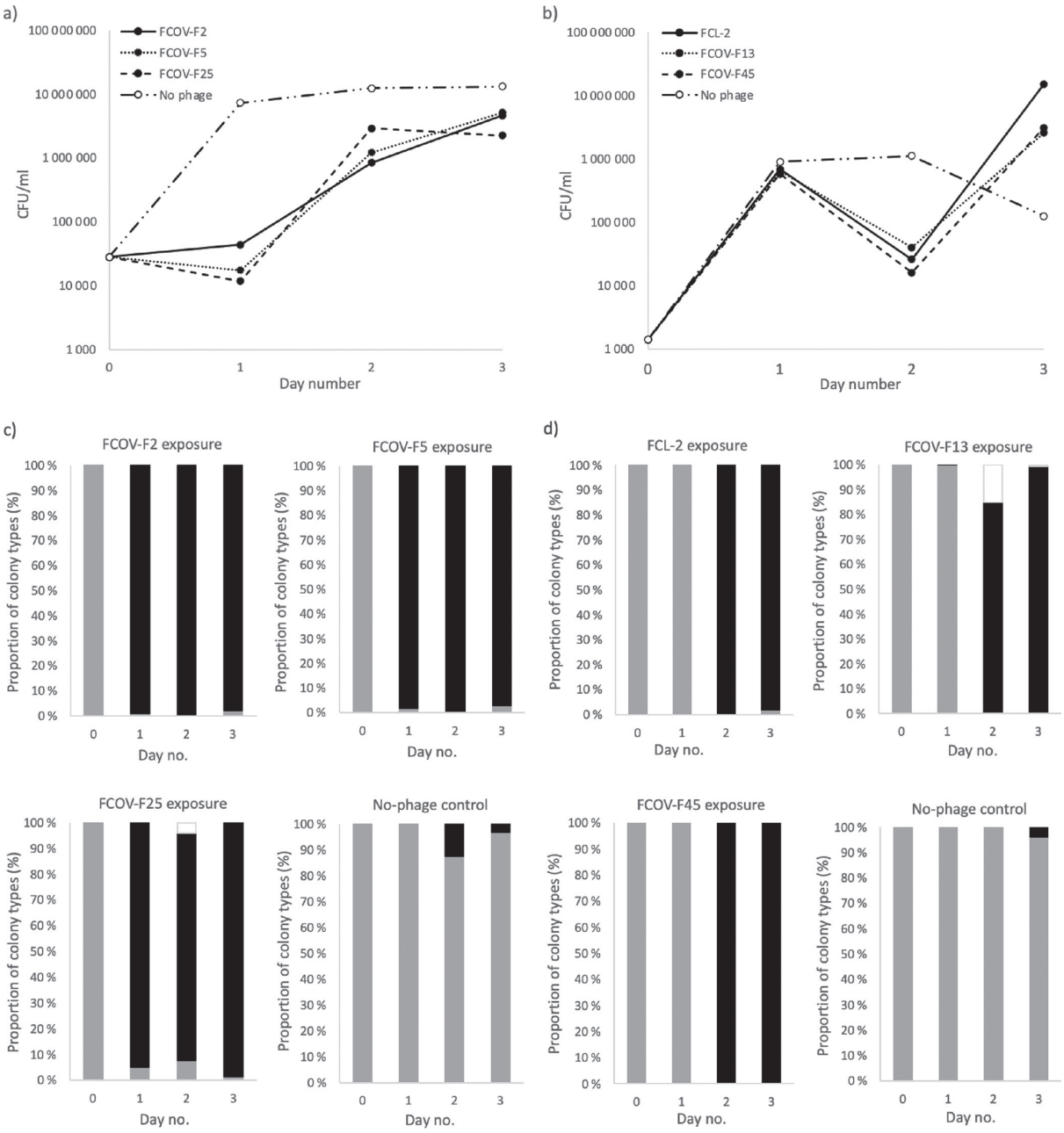

FIG 1 (a, b) Bacterial growth determined from three replicate phage exposure and no-phage control cultures represented as CFU ml^{-1} (± standard error [SE]). (c, d) Proportion (%) of different colony types of *Flavobacterium columnare* isolates FCO-F2 (c) and FCO-F9 (d) during the 3-day exposure to phages FCOV-F2, FCOV-F5, FCOV-F25, FCL-2, FCOV-F13, and FCOV-F45. The dark gray bar indicates the proportion of isolates forming a rhizoid colony morphology, the black bar indicates the proportion of isolates forming a rough colony morphology, and the white bar indicates the proportion of isolates forming a soft colony morphology.

with a 5.5×10^5- to 11×10^5-fold reduction in phage susceptibility (efficiency of plating) compared to the wild-type isolates, depending on the specific phage (results not shown). Throughout this paper, these isolates with decreased phage susceptibility are grouped together with the phage-susceptible isolates.

Antibiotic susceptibility. In general, phage-resistant isolates showed antibiotic susceptibility patterns similar to the parent wild-type isolates; however, some differences

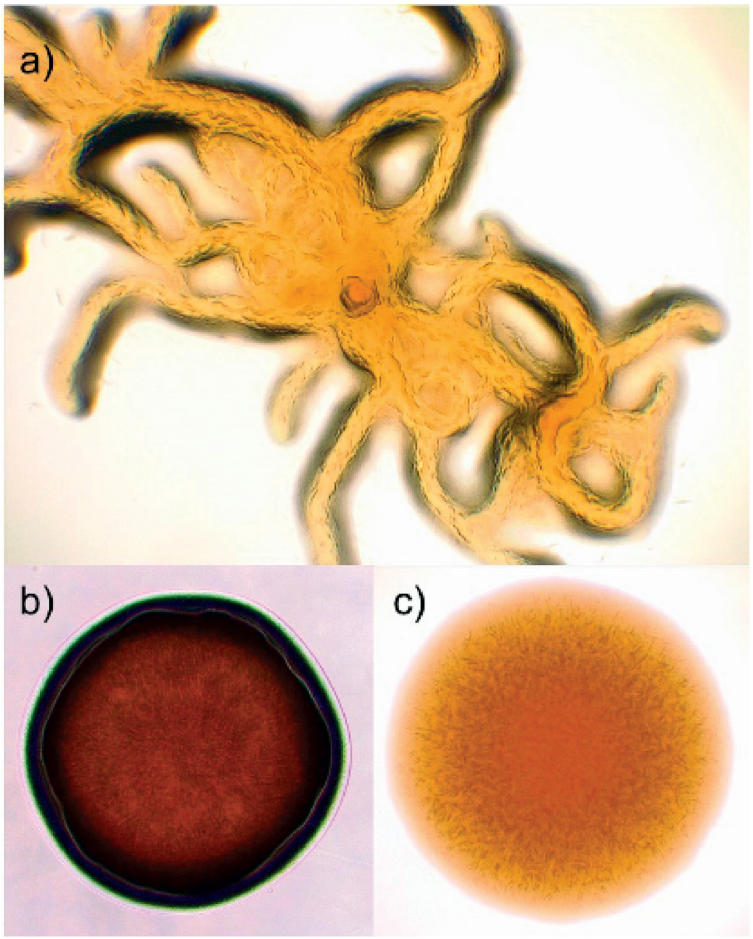

FIG 2 Different colony morphologies formed by *Flavobacterium columnare* on Shieh agar plates after phage exposure: (a) rhizoid, (b) rough, and (c) soft. For the approximate size of each colony morphology, see Fig. 3.

were also observed (Fig. S1 and Table S1 in the supplemental material). For example, in most cases, the inhibition zone of tetracycline (used against columnaris) increased in the phage-resistant strains compared to the ancestral type, which may indicate increased susceptibility. However, the assay was not replicated, so exact conclusions cannot be drawn.

Motility, adhesion, and biofilm formation. Phage-susceptible bacteria forming rhizoid colonies were significantly more motile (determined as colony spreading) than phage-resistant rough or soft morphotypes, irrespective of isolation history (F2 isolates, $P < 0.001$, one-way analysis of variance [ANOVA], $\log_{10}$ transformation; F9 isolates, $P \leq 0.004$, Mann-Whitney test) (Fig. 3).

Compared to the parent wild-type FCO-F2 isolate, there was a large variability in the adhesion capacity of individual phage-resistant F2 isolates (Fig. 4a). Phage susceptibility (rhizoid versus rough colony type) or phage used in the coculture experiment did not influence bacterial adhesion capacity ($P = 0.3$, Mann-Whitney test, and $P = 0.564$, Kruskal-Wallis test, respectively).

Most of the individual phage-exposed and no-phage control F2 isolates had significantly lower biofilm-forming capacity than parent wild-type FCO-F2 ($P \leq 0.017$, one-way ANOVA, least significant difference [LSD] multiple comparisons, square root

TABLE 1 Experimental setup of phage exposure of two phage-susceptible wild-type *Flavobacterium columnare* isolates FCO-F2 (high-virulence, genotype C; exposed for phages FCOV-F2, FCOV-F5, and FCOV-F25) and FCO-F9 (medium-virulence, genotype G; exposed for phages FCL-2, FCOV-F13, and FCOV-F45), and colony morphologies and phage susceptibilities of the 20 phage-exposed (F2R- and F9R-) and 4 no-phage control (F2S- and F9S-) isolates obtained from the exposure cultures

Wild-type isolate	Phage	Phage-exposed isolate[a]	No-phage control isolate[a]	Colony morphology of the isolate	Phage susceptibility of the isolate[b]					
					FCOV-F2	FCOV-F5	FCOV-F25	FCL-2	FCOV-F13	FCO-F45
FCO-F2				Rhizoid	+	+	+	−	−	−
	FCOV-F2	F2R58		Rhizoid	±	±	±	−	−	−
		F2R60		Rough	−	−	−	−	−	−
		F2R62		Rough	−	−	−	−	−	−
	FCOV-F5	F2R64		Rough	−	−	−	−	−	−
		F2R65		Rough	−	−	−	−	−	−
		F2R66		Rhizoid	±	±	±	−	−	−
		F2R67		Rough	−	−	−	−	−	−
		F2R68		Rough	−	−	−	−	−	−
	FCOV-F25	F2R70		Rough	i	i	i	−	−	−
		F2R72		Rough	−	−	−	−	−	−
		F2R74		Rough	−	−	−	−	−	−
	No phage		F2S4	Rhizoid	+	+	+	−	−	−
			F2S17	Rhizoid	+	+	+	−	−	−
FCO-F9				Rhizoid	−	−	−	+	+	+
	FCL-2	F9R56		Rhizoid	−	−	−	±	±	±
		F9R58		Rough	−	−	−	i	i	i
		F9R61		Rough	−	−	−	i	i	i
	FCOV-F13	F9R64		Rough	−	−	−	−	−	−
		F9R66		Rough	−	−	−	i	i	i
		F9R69		Soft	−	−	−	i	i	i
	FCOV-F45	F9R72		Rough	−	−	−	i	i	i
		F9R75		Rough	−	−	−	i	i	−
		F9R78		Rough	−	−	−	−	−	−
	No phage		F9S15	Rhizoid	−	−	−	+	+	+
			F9S17	Rhizoid	−	−	−	+	+	+

[a]Isolates are shown according to the phage to which they were exposed.
[b]The following indicate the susceptibility of the isolates to phages used in exposures: +, susceptible; –, resistant; ±, susceptibility decreased compared to the parent wild-type isolate; i, inhibition of bacterial growth, considered phage resistance.

transformation) (Fig. 4c). Still, there was no statistical difference in biofilm formation between phage-susceptible rhizoid and phage-resistant rough morphology F2 isolates ($P = 0.062$, one-way ANOVA).

Again, the bacterial strain F9 behaved differently compared to F2. In contrast to the phage-resistant F2 isolates, the phage-resistant rough and soft morphology F9 isolates had significantly lower adherence than susceptible rhizoid isolates ($P < 0.001$, one-way ANOVA, LSD multiple comparisons, square root transformation) (Fig. 4b). In addition, isolates exposed to phages isolated in 2017, FCOV-F13 and FCOV-F45 (F9R64, F9R66, and F9R69, and F9R72, F9R75, and F9R78, respectively), had significantly lower adhesion capacity than isolates exposed to FCL-2 (F9R56, F9R58, and F9R61) isolated in 2008 ($P < 0.001$, Mann-Whitney test). This may indicate that phage FCL-2 uses a different phage receptor (see below).

In contrast to adhesion ability, biofilm-forming capacity of most of the individual phage-exposed and no-phage control F9 isolates was significantly higher than that in the wild-type parent isolate ($P \leq 0.004$, one-way ANOVA, LSD multiple comparisons) (Fig. 4d). F9R69 with soft colony morphology did not form any biofilm and was thus excluded from the multiple comparisons. Phage-resistant rough F9 isolates had significantly higher biofilm-forming capacity than the phage-susceptible rhizoid morphotypes ($P < 0.001$, one-way ANOVA, square root transformation).

Protease activity: elastinase, gelatinase, and caseinase. Elastinase activity was detected in the wild-type and all the phage-susceptible rhizoid FCO-F2 isolates and one resistant, rough F2 isolate (clear zone ratio of >1), whereas all remaining resistant, rough morphology isolates had completely lost the ability to degrade elastin (Fig. 5a).

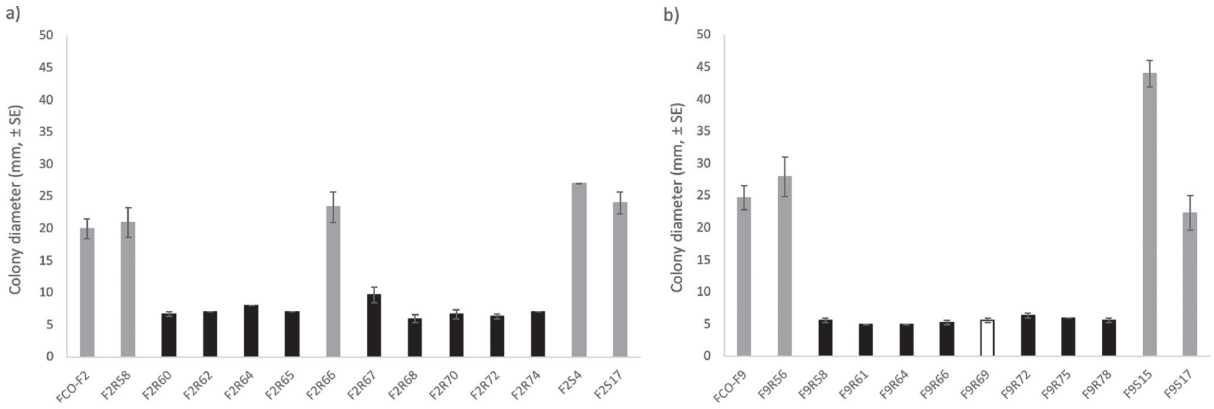

FIG 3 Colony spreading of *Flavobacterium columnare* wild-type FCO-F2 (a) and FCO-F9 (b) isolates and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates expressed as colony diameter (mm, ±SE) on TYES agar. All the phage-susceptible rhizoid colony-forming isolates (dark gray bars) produced significantly more spreading than phage-resistant rough (black bar) or soft (white bar) morphology isolates (F2 isolates, $P < 0.001$, one-way ANOVA, $\log_{10}$ transformation; F9 isolates, $P \leq 0.004$, Mann-Whitney test).

There were no differences in elastinase activity between the elastinase-positive isolates ($P = 0.843$, one-way ANOVA). Elastinase activity was not detected in any of the F9 isolates (clear zone ratio of 1) (Fig. 5b).

There were variations in gelatinase activity between individual F2 and F9 isolates (one-way ANOVA, LSD multiple comparisons) (Fig. 5c and d). However, among both F2 and F9 isolates, gelatinase activity of phage-resistant rough morphotypes was lower than that of susceptible rhizoid morphotypes (F2 isolates, $P = 0.018$, one-way ANOVA, exponential transformation; F9 isolates, $P < 0.001$, one-way ANOVA). Two of the phage-exposed F9 isolates (F9R69 and F9R78) did not have any gelatinase activity and were thus excluded from the multiple comparisons.

Less variation in caseinase activity between individual isolates was observed (one-way ANOVA, LSD multiple comparisons) (Fig. 5e and f), and phage-susceptible rhizoid and resistant rough F2 isolates did not differ from each other ($P = 0.058$, one-way ANOVA). On the other hand, caseinase activity of phage-resistant rough and soft F9 isolates was lower than that of susceptible rhizoid isolates ($P = 0.007$, one-way ANOVA).

Virulence. Rainbow trout fry were exposed to wild-type, phage-exposed, and no-phage control isolates, and all of them caused mortality during 24 h (Fig. 6). The phage-susceptible rhizoid morphotypes were most virulent, causing 100% mortality, whereas resistant rough and soft morphotypes were less virulent, causing 46.7% mortality at the highest (except for phage-resistant rough morphotype F2R70, which caused 100% mortality). Mortality of control fish was 15%, but no bacterial growth was observed from these fish. This mortality is most likely caused by the high water temperature used in the experiment (+25°C). However, *F. columnare* growth was observed from all fish exposed to bacteria. Colony morphotype of the bacterial isolates did not change during the infection.

When comparing the data according to phage susceptibility and thus colony morphology, cumulative mortality of fish infected with phage-susceptible rhizoid morphotypes, irrespective of if they were wild-type, phage-exposed, or no-phage control isolates, was significantly higher than mortality caused by phage-resistant rough or soft morphotypes among both F2 and F9 isolates ($P < 0.001$, Kaplan-Meier survival analysis). Also, the estimated survival time (Kaplan-Meier survival analysis) was shortest in fish infected with susceptible rhizoid isolates (Fig. 6). In the case of F2 isolates, mortality caused by phage-resistant rough isolates was also significantly higher than mortality of control fish, but mortality caused by resistant rough and soft F9 isolates did not differ from each other or from the control fish mortality. Mortality caused by rhizoid phage-susceptible F2 isolates started to peak at 12 h postinfection (h p.i.) and in F9 at 16 h p.i. ($P < 0.001$, Kaplan-Meier survival analysis), but between rough phage-resistant F2 and F9

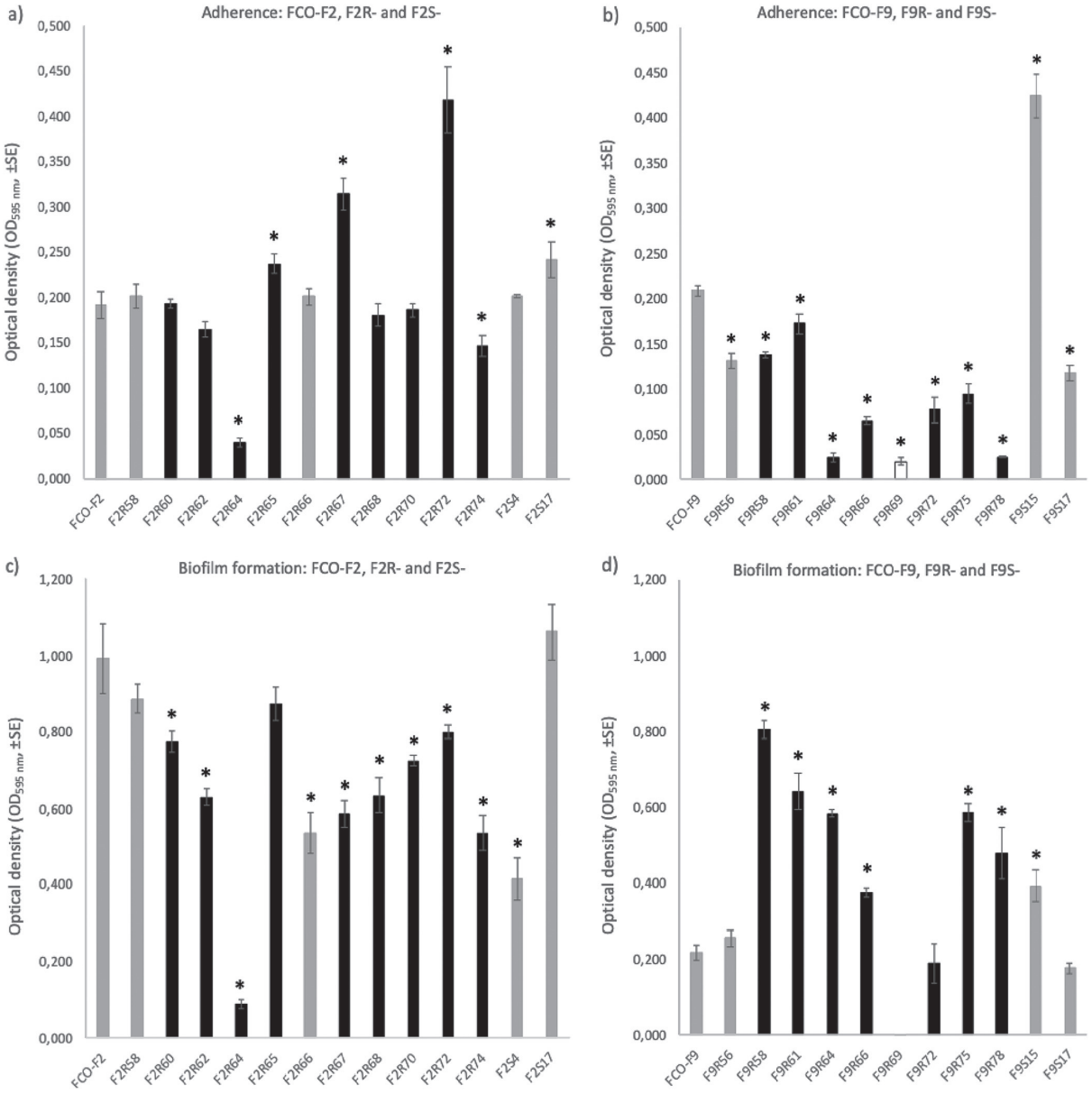

FIG 4 Adherence (a, b) and biofilm-forming capacity (c, d) of *Flavobacterium columnare* wild-type FCO-F2 (a, c) and FCO-F9 (b, d) isolates and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates on a polystyrene surface measured as optical density (OD_{595}, ±SE). Asterisks indicate a statistically significant difference ($P < 0.05$) compared to the parent wild-type isolate. F9R69 did not form any biofilm and was thus excluded from the statistical analyses. Dark gray bars, phage-susceptible isolates forming a rhizoid colony morphology; black bars, phage-resistant isolates forming a rough morphology; white bar, phage-resistant isolate forming a soft morphology.

isolates, the mortality patterns were more similar, starting to increase slowly at 2 to 3 h p.i. ($P = 0.217$, Kaplan-Meier survival analysis). However, there were differences in cumulative mortalities caused by individual isolates in each morphology group (Data Set S1).

Whole-genome sequencing. The aim of the genomic analysis was to identify the mutations selected by phage exposure; therefore, the spontaneously formed rough colonies were not sequenced. Genomic data of wild-type *F. columnare* isolates FCO-F2 and FCO-F9 are presented in Table 2.

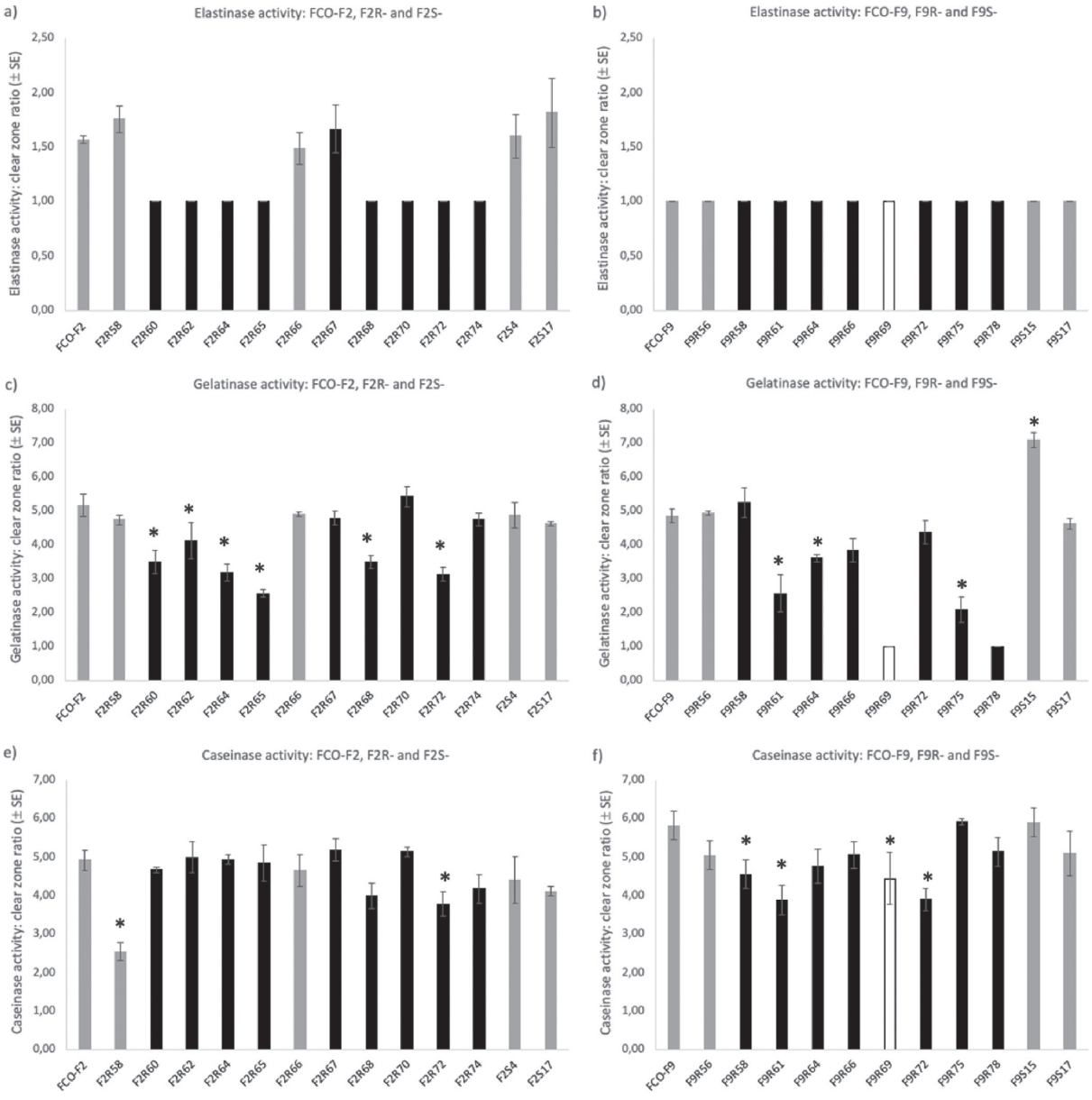

FIG 5 Elastinase (a, b), gelatinase (c, d), and caseinase (e, f) activity of the *Flavobacterium columnare* FCO-F2 (a, c, e) and FCO-F9 (b, d, f) isolates and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. Activity was measured as the clear zone ratio (clear zone diameter/colony diameter, ±SE) on TYES agar supplemented with elastin, gelatin, and skim milk (caseinase). The asterisk indicates a significant reduction in protease activity ($P < 0.05$) compared to the parent wild-type isolate. A clear zone ratio of 1 indicates no protease activity. Isolates with no activity were excluded from the statistical analyses. Dark gray bars, phage-susceptible isolates forming a rhizoid colony morphology; black bars, phage-resistant isolates forming a rough morphology; white bar, phage-resistant isolate forming a soft morphology.

Genomic comparisons between F2 wild-type and phage-exposed isolates revealed a limited number of genomic changes. In 7 out of 11 isolates, single mutation leading to the formation of wrong or truncated proteins was observed in the phage-resistant mutants (Table 3). Notably, the majority of the mutations were located in genes encoding gliding motility proteins *gldB* (F2R67), *gldN* (F2R72), and *sprA* (F2R60, F2R64, F2R65, and F2R74), of which *gldN* and *sprA* are also parts of the type IX secretion system (29).

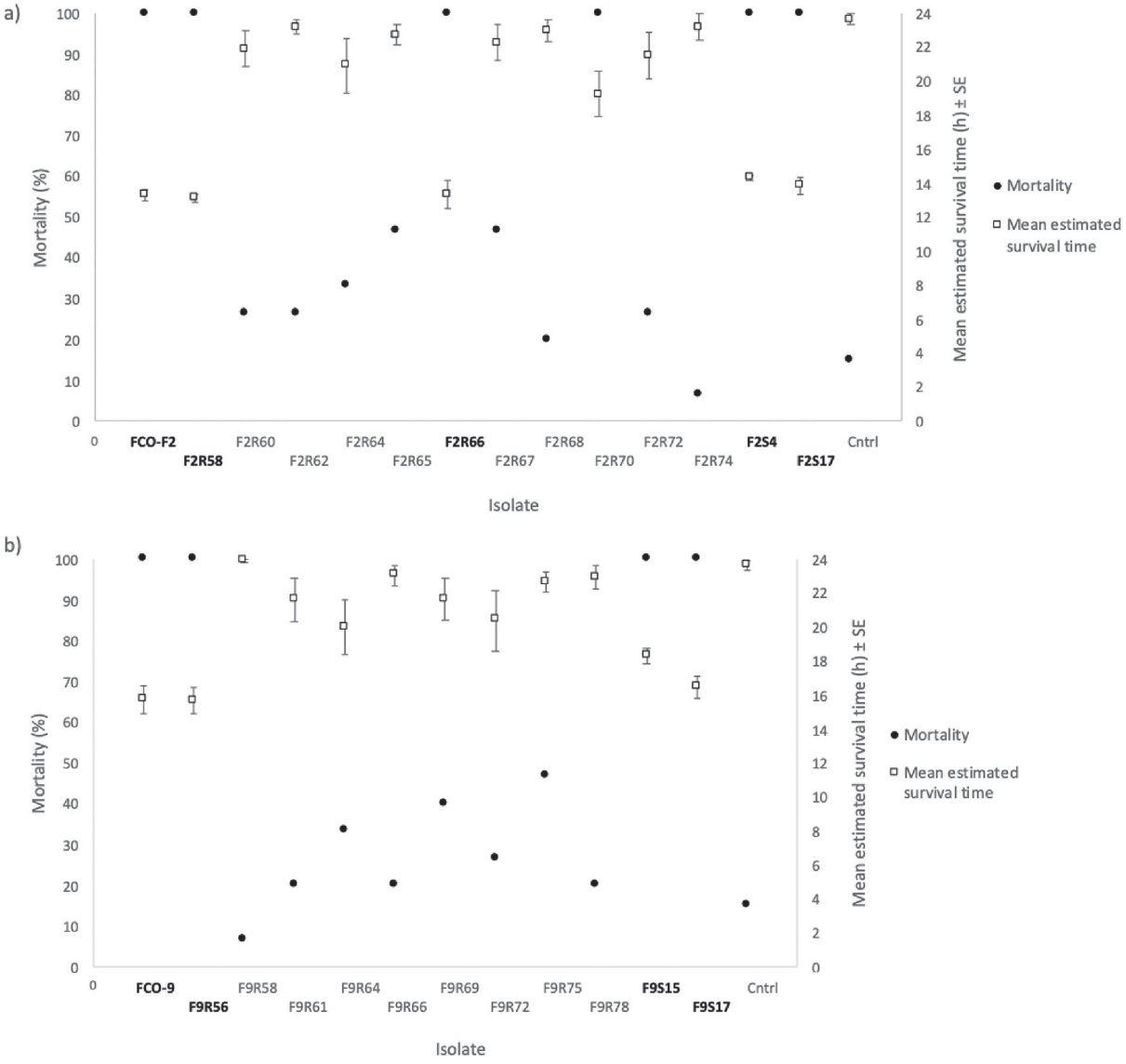

FIG 6 Mortality percent and estimated survival time (±SE) of rainbow trout (*Oncorhynchus mykiss*) during a 24-h experimental infection with wild-type *Flavobacterium columnare* FCO-F2 (a) and FCO-F9 (b) and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. Phage-susceptible rhizoid colony-forming isolates are written in bold. Cntrl, control with no bacterial infection.

Isolate F2R70, the only virulent rough isolate, had one nucleotide insertion in an outer membrane protein H (OmpH) family protein-coding gene. Three isolates (F2R62, F2R66, and F2R68) did not show any genomic changes relative to the wild type. In isolate F2R58 with decreased phage susceptibility, one nucleotide change in the *rlmF* gene (encoding rRNA large subunit methyltransferase F) did not lead to an amino acid change. No mutations were observed in the no-phage control isolates. At certain points of rRNA operons in all phage-exposed and no-phage control isolates, and also in a 736,221-bp sequence (hypothetical protein coding sequence in the wild-type FCO-F2 genome used as a reference) in phage-exposed isolates F2R66 and F2R68, there was poor coverage of reads leading to unclear sequences, which prevented detection of possible mutations in this region.

TABLE 2 Data on genomes of wild-type *Flavobacterium columnare* strains FCO-F2 and FCO-F9

Wild-type isolate	Genetic group	Genome size (bases)	No. of ORFs[a]	GC %
FCO-F2	C	3,221,312	3,280	31.7
FCO-F9	G	3,261,403	3,374	31.7

[a]ORFs, open reading frames.

In F9 phage-exposed isolates, excluding F9R58, one or two mutations per isolate were observed (Table 4). Mutations in isolates exposed to FCOV-F45 had insertions, whereas FCOV-F13-exposed isolates had deletions or single nucleotide changes in genes encoding gliding motility proteins *gldG* (F9R72), *gldM* (F9R64, F9R69, and F9R78), and *gldN* (F9R69 and F9R75), leading to formation of wrong or truncated proteins. Interestingly, in the isolate F9R69 (exposed to FCOV-F13) with a soft colony type, deletion of a genomic region of 4,701 bp was observed, spanning over gliding motility genes *gldM* and *gldN* and sequences encoding flavin adenine dinucleotide (FAD)-binding oxidoreductase, DUF3492 domain-containing protein, and a hypothetical protein (Fig. 7).

On the contrary, no mutations in gliding motility genes were observed in the sequenced F9 isolates exposed to FCL-2; but, instead, two of these isolates had one nucleotide change in genes encoding DegT/DnrJ/EryC1/StrS family aminotransferase, DUF255-domain containing protein (F9R56), and cystathionine gamma-synthase (F9R61), leading to either one amino acid change or truncated protein. No mutations were observed in no-phage control isolates. Around 2,000,620 bp (hypothetical protein coding sequence in the B185 genome used as a reference), there was poor coverage of reads, leading to unclear sequence in both wild-type FCO-F9, phage-exposed, and no-phage control isolates, which prevented detection of possible mutations in this region.

DISCUSSION

Phage therapy is seen as an attractive option to treat and prevent bacterial diseases, but the development of phage resistance in target bacteria is considered one of the main problems related to the use of phages. Our results describe the selection for phage resistance in two different *F. columnare* isolates upon exposure to six specific phages. We show that phage resistance is associated with reduction in virulence and virulence-related phenotypic changes in the bacterium. Our genetic data indicate that, in most cases, phage resistance is linked to surface modifications, often related to the type IX secretion system connected to *Bacteroidetes* gliding motility machinery. Mutations in the genes encoding an outer membrane protein or genes related to gliding motility seem to be phage specific and likely prevent phage attachment, possibly in a phage-specific manner, selecting morphology change and loss of virulence.

In the present study, phage exposure caused significant changes in bacterial phenotypic characteristics (motility, adhesion, protein secretion, and virulence; see details below), leading to phage resistance. In most isolates, these changes could be linked to changes in gliding motility-related genes. Flavobacteria show gliding motility on surfaces (29), and mutations in any of the genes encoding gliding motility machinery proteins have been shown to lead to loss of motility (30, 31). Gliding is also connected to virulence, since part of the gliding motility machinery (GldK, GldL, GldM, GldN, PorV, SprA, SprE, SprF, and SprT) is used as a type IX secretion system found in *Bacteroidetes* (28, 32). Indeed, phage resistance due to loss of motility has been linked to decreased virulence in *F. columnare* previously (27), and *F. columnare gldN* mutants have been shown to exhibit both decreased proteolytic and chondroitinase activity and virulence on rainbow trout (28). Similarly, phage resistance was associated with loss of motility and mutations in genes related to cell surface properties and gliding motility in *F. psychrophilum* (23) and in *F. johnsoniae* (31, 33). It has been suggested that surface proteins secreted by the type IX secretion system in *F. johnsoniae* (such as SprB and RemA)

TABLE 3 Mutations revealed by whole-genome sequencing (Illumina) in F2 phage-exposed *Flavobacterium columnare* isolates compared to their wild-type (wt) isolate FCO-F2

(Phage) phage-exposed isolate[a]	Colony morphology	Gene/CDS[b]	Mutation	Location (base no.) in wt genome	Outcome
(FCOV-F2)					
F2R58	Rhizoid	*rlmF* (rRNA large subunit methyltransferase F)	T → A	21,350	No aa change
F2R60	Rough	*sprA* (component of type IX SS)	Ins GT	1,314,323–1,314,324	Change in reading frame → stop codon → two truncated proteins
F2R62	Rough				
(FCOV-F5)					
F2R64	Rough	*sprA* (component of type IX SS)	Ins G	1,317,523	Change in reading frame → stop codon → two truncated proteins
F2R65	Rough	*sprA* (component of type IX SS)	Ins G	1,317,524	Change in reading frame → stop codon → two truncated proteins
F2R66	Rhizoid				
F2R67	Rough	*gldB* (gliding motility machinery protein)	Del T	1,122,801	Truncated/wrong protein
F2R68	Rough				
(FCOV-F25)					
F2R70	Rough	OmpH family outer membrane protein	Ins G	1,275,242	Change in reading frame → wrong protein
F2R72	Rough	*gldN* (component of type IX SS)	Ins TCTAC	1,013,274–1,013,278	Change in reading frame → stop codon → two truncated proteins
F2R74	Rough	*sprA* (component of type IX SS)	Del A	1,313,911	Change in reading frame → stop codon → two truncated proteins

[a]Isolates are shown according to the phage to which they were exposed.
[b]→, change to; aa, amino acid; CDS, coding sequence; Del, deletion; Ins, insertion; SS, secretion system.

may function as phage receptors (31, 34). Mutations in either gliding (*gldB, gldG, gldL, gldM, gldN*) or type IX secretion system (*sprA, gldL, gldM, gldN*) genes in *F. johnsoniae* will disrupt SprB/RemA secretion, resulting in phage resistance due to the lack of receptors on the host cell surface. Together, the results suggest that the type IX secretion system is a key target for infection by a wide range of phages and across the *Flavobacterium* genus, and that phage exposure selects for mutations linked with morphology changes and loss of motility in this bacterial group.

Exposure to a specific phage selected for different gliding motility mutations in different *F. columnare* isolates, as has also been seen in phage-resistant *F. psychrophilum* (23), indicating that several genes are involved in phage attachment and infection of *F. columnare* phages. Furthermore, genomic analysis of one soft colony isolate revealed a large deletion (4,701 bp) spanning over two gliding motility genes. It is possible that this region is important for adhesion and biofilm formation. However, although all rough colony-forming isolates were phage resistant, not all of these isolates (F2R62, F2R68, and F9R58) had mutations in genes encoding proteins related to gliding motility or elsewhere in their genomes. This may indicate that development of phage resistance and colony morphology change are also influenced by gene expression or epigenetic modifications, leading to variation in colony morphology, as suggested previously (35). For example, in *Bordetella* spp., phage resistance is regulated via phase variation in virulence-related factors, such as some adhesins, toxins, and the type III secretion system (reviewed in reference 36). Furthermore, our data indicate that phage exposure can also select for mutations encoding other outer membrane proteins versus those related to gliding motility, such as *ompH* in the case of F2R70. Although it had a rough colony morphotype, this isolate was virulent in rainbow trout. Interestingly, isolates exposed to FCL-2 did not have mutations in gliding motility-

TABLE 4 Mutations revealed by whole-genome sequencing (Illumina) in F9 phage-exposed *Flavobacterium columnare* isolates compared to their wild-type (wt) isolate FCO-F9

(Phage) phage-exposed isolate[a]	Colony morphology	Gene/CDS[b]	Mutation	Location (base no.) in wt genome	Outcome
(FCL-2)					
F9R56	Rhizoid	DegT/DnrJ/EryC1/StrS family aminotransferase	C → T	657,725	Cys → Tyr
		DUF255 domain-containing protein	C → T	2,542,435	Stop codon → truncated protein
F9R58	Rough				
F9R61	Rough	Cystathionine gamma-synthase	G → A	1,720,857	His → Tyr
(FCOV-F13)					
F9R64	Rough	*gldM* (component of type IX SS)	Del CAA	2,732,551	Del Thr
F9R66	Rough	Gliding motility protein	G → A	1,849,668	Stop codon → truncated protein
F9R69	Soft	*gldM* (component of type IX SS)	Del 255 3′ nt	2,732,457–	No/truncated protein
		gldN (component of type IX SS)	Del CDS		No protein
		FAD-binding oxidoreductase	Del CDS		No protein
		DUF3492 domain containing protein	Del CDS		No protein
		Hypothetical protein	Del 454 5′ nt	–2,737,157	No/truncated protein
(FCOV-F45)					
F9R72	Rough	*gldG* (gliding motility machinery protein)	Ins T	3,023,647	Change in reading frame → wrong protein
F9R75	Rough	*gldN* (component of type IX SS)	Ins G	2,733,099	Start and stop codon → two truncated proteins
F9R78	Rough	*gldM* component of type IX SS)	Ins A	2,731,567	Change in reading frame → stop codon → truncated protein

[a]Isolates are shown according to the phage to which they were exposed.
[b]→, change to; CDS, coding sequence; Del, deletion; Ins, insertion; nt, nucleotide; SS, secretion system.

related genes. To uncover if FCL-2 uses different receptors for infection of *F. columnare* than the other phages in this study, more phage-resistant isolates should be sequenced. However, FCL-2 differs genetically from other phages infecting genetic group G bacteria (this article was submitted to an online preprint archive [37]), supporting this suggestion.

Generally, point mutations and changes in receptor expression enable a rapid and efficient response of bacterial populations to phage exposure. However, the large phenotypic costs of mutation-derived phage resistance observed in *F. columnare* in this study suggest that these mutations may be dynamic and likely also rapidly revert back to the susceptible form in *F. columnare*. Indeed, reversion of both phage-driven and

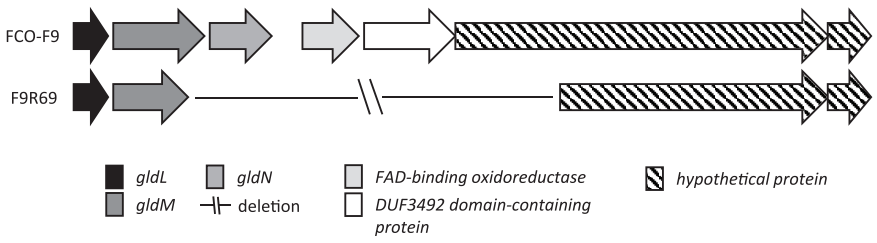

FIG 7 Deletion of genomic region covering 4,701 bp in FCOV-F13-exposed soft colony-forming phage-resistant *Flavobacterium columnare* isolate F9R69.

spontaneously formed rough colony types back to rhizoid has been observed to happen in *F. columnare* subcultures, similar to the occasional appearance of spontaneous rough colonies (27) as also observed in this study. Various mechanisms to regain phage resistance have also been found in fish pathogenic *F. psychrophilum* (23) and *V. anguillarum* (24), in which a rapid reversion back to a phage-susceptible phenotype has been shown to occur. These sorts of dynamics in phage resistance have also been observed in a human symbiont *Bacteroides thetaiotaomicron* (38), suggesting that the phenomenon may be common among a wide variety of bacteria.

Phage-exposed *F. columnare* isolates F2R58, F2R66, and F9R56 did not respond to phage infection with colony morphotype change but maintained their original rhizoid colony morphotype and high virulence. These rhizoid isolates were not completely resistant to phage, although phage infection efficiency dropped markedly (up to a one-million-fold decrease on efficiency of plating), suggesting some other mechanism for reducing infection efficiency. *F. columnare* has two functional CRISPR systems that have been shown to adapt under phage exposure at fish farms (39). However, we did not observe additional CRISPR spacers in whole-genome sequencing. The same was observed in phage-exposed *F. psychrophilum* isolates in which no differences to the wild-type strain's CRISPR composition were found (23). In our experience, CRISPR adaptation in *F. columnare* requires a different experimental setup with longer coculture time in low-nutrient medium, followed by enrichment in high-nutrient medium (40). Thus, the decreased phage susceptibility of rhizoid phage-exposed isolates is likely a consequence of as of yet unknown functions that need to be studied in the future.

In addition to the type IX secretion system, type I and VI secretion systems are also known to function in *F. columnare* (41). Possible secretion of virulence-related factors through type I and VI secretion systems in *F. columnare* could be one of the reasons why rough phage-resistant isolates caused some mortality in fish and explains their gelatinase and caseinase activity despite morphology change. It has also been shown recently that virulence of *F. columnare* increases in the mucus and with increasing mucin concentration (17). As the mucus-covered fish surface is the main infection route of *F. columnare*, it is probable that some *F. columnare* virulence factors, such as proteinase activity, are expressed differently in growth media compared to in an *in vivo* infection situation. This possible differential expression could also explain the mortality caused by phage-resistant rough isolates.

The ability to adhere and form biofilms has a major role in bacterial infections and in colonizing niches (42). In *F. columnare*, adhesion and biofilm-forming capacity may have a central role in their persistence in the farming environment (e.g., tanks and water systems) (43) but also in establishing the first steps of infection on the fish surfaces (44). Our results indicate that *F. columnare* strains differ in their adherence and biofilm-forming characteristics, and the effect of phage resistance on bacterial phenotype is not straightforward. This is probably associated with different mutations in different isolates and general biological variability. Whereas phage exposure had no clear effect on the adhesion capacity of the F2 isolates, phage resistance led to a decrease in biofilm-forming capacity in most of the individual phage-resistant F2 isolates. This is in agreement with the systematic reduction in biofilm-forming properties of phage-resistant *F. psychrophilum* relative to the wild type (23). Adhesion capacity of F9 phage-resistant isolates, on the other hand, was significantly lower than in the wild-type parent isolate, but rough phage-resistant F9 isolates had a significantly higher biofilm-forming capacity than rhizoid susceptible isolates. These results partly differ from what we have found earlier (25, 26), most likely because in the previous studies, the rough colonies were formed spontaneously without phage exposure. Indeed, morphology of spontaneously formed rough colonies and these morphotypes' ability to move when cultured in low-nutrient media differ from rough morphotypes formed under phage exposure (27). Furthermore, the high variability in the results of biofilm and adhesion assays may reflect the biology of the bacterium, which may have natural variance in its phenotype. As the bacterium has the capacity to survive long periods in the environment outside

TABLE 5 *Flavobacterium columnare* isolates and phages used in this study

Bacterium isolate[a]	Genetic group of the bacterium	Phage isolate	Genetic group of the phage isolation host	Farm no.	Isolation yr
FCO-F2	C			1	2017
FCO-F9	G			2	2017
		FCOV-F2	C	1	2017
		FCOV-F5	C	3	2017
		FCOV-F25	C	1	2017
		FCL-2	G	2	2008
		FCOV-F13	G	1	2017
		FCOV-F45	G	2	2017

[a]Bacteria and phages were isolated from Finnish fish farms. *F. columnare* isolates have previously been categorized into genetic groups by restriction fragment length polymorphism analysis of internal transcribed spacer region between 16S and 23S rRNA genes (36).

of the host, different strategies can improve survival. Furthermore, although adhesion is a prerequisite for biofilm formation, these are different steps of the infection process. It is possible that once the surface adhesion has been established, bacterial gene expression changes to initiate biofilm formation. Nevertheless, together, our results indicate that since *F. columnare* phages are genetically group specific, they might be using different receptors, which, in turn, causes differences in bacterial resistance mechanisms and phenotypic characteristics between genetic groups.

Phage resistance can influence bacterial susceptibility to antibiotics (45–47). In this study, phage resistance did not affect the antibiotic susceptibility of any of the isolates studied. Lack of association between development of antibiotic resistance and bacteriophage resistance has previously been shown (e.g., in *Escherichia coli* [48]). Based on our results, phage resistance may not increase a risk of antibiotic resistance development in *F. columnare*. Thus, phage therapy given at fish farms is not likely to hamper possible concomitant use of antibiotics as therapeutic agents against columnaris infections. Indeed, it has been shown with *Pseudomonas fluorescens* that applying phages together with antibiotic treatments may inhibit the evolution of antibiotic resistance in pathogenic bacteria (49). However, since some changes in inhibition zone tests were detected in our study with *F. columnare*, more thorough analysis of antibiotic resistance would be beneficial to fully describe the association with phage resistance.

To summarize, our results show that even though *F. columnare* rapidly develops phage resistance under phage exposure, the emergence of phage resistance does not pose a high risk in the development of phage therapy against columnaris infections in rainbow trout. This is because, in most cases, phage resistance selects for decreases in bacterial virulence, adherence to surfaces, and protease secretion. Based on our results with experiments with two genetically different wild-type bacterial isolates, development and regulation of phage resistance in *F. columnare* is a multifactorial process, partly affected by the formation of mutations mainly in gliding motility- and type IX secretion system-related genes and partly by other defense mechanisms against phages, the functions of which need to be studied in the future.

MATERIALS AND METHODS

Bacterial and phage isolates used in this study. Bacteria and phages used in this study were isolated from water samples collected from fish farms during columnaris outbreaks (37) (Table 5). The bacteria were confirmed as *F. columnare* by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene and classified into genetic groups by RFLP of the 16S to 23S internal transcribed spacer (ITS) region (37). The six phages used in the experiments have been isolated from different fish farms in Finland (37, 50). Phages belong to the Myoviridae family and have been characterized with respect to host range and genomic composition previously (37). Briefly, *F. columnare* phages infect their host in a genotype-specific manner; phages FCOV-F2, FCOV-F5, and FCOV-F25 infect bacterial strains belonging to genetic group C (here FCO-F2), and phages FCL-2, FCOV-F13, and FCOV-F45 infect bacteria in genetic group G (here FCO-F9).

Bacterial cultures and phage lysates. For phage exposure and virulence testing, *F. columnare* isolates were inoculated from cryopreserved (–80°C) stocks in modified Shieh medium (51) and grown for 48 h at 25°C with 120 rpm agitation. After this, subcultures were made in modified Shieh medium and grown for 24 h at 25°C with 120 rpm agitation. The optical density (OD) of the bacterial broth suspensions was measured spectrophotometrically at 595 nm and adjusted to 5×10^5 CFU ml^{-1} for phage exposures and 5×10^6 CFU ml^{-1} for virulence experiments (based on previously determined OD/CFU relationships). For other tests, *F. columnare* isolates were cultured in tryptone yeast extract salts (TYES) broth (52) and washed in TYES broth by centrifugation at 5,310 $\times$ *g* for 15 min at 4°C. Cultures were then spectrophotometrically adjusted to an OD of 0.6 at 520 nm (approximately 10^8 CFU ml^{-1}).

Phage lysates were produced using the "double layer agar" method (53) as follows: 3 ml of melted (47°C) top agar (0.5%), including 300 μl of a 24-h subculture of the host bacterium and 100 μl of phage (10-fold dilutions in Shieh medium), was poured onto Shieh agar and grown for 48 h at 25°C. Five milliliters of Shieh medium was added on top of Shieh agar plates with confluent lysis and incubated at 7°C for 12 to 18 h with constant agitation (90 rpm). Lysates were collected, filtered (polyethersulfone [PES] membrane, pore size 0.45 μm; Nalgene), and stored at +7°C or at −80°C with 20% glycerol. For phage exposure, phage lysates were diluted with Shieh medium to 5×10^5 PFU ml^{-1}.

Phage exposure experiments and isolation of colonies. Two phage-susceptible wild-type *F. columnare* isolates, the high-virulence FCO-F2 isolate (genetic group C) and the medium-virulence FCO-F9 isolate (genetic group G) (37), were each exposed to three phages in separate experiments with individual phages. Isolate FCO-F2 was exposed to phages FCOV-F2, FCOV-F5, and FCOV-F25, and isolate FCO-F9 was exposed to phages FCL-2, FCOV-F13, and FCOV-F45, in accordance with the host range of the phages. Cultures with only bacteria served as no-phage controls. The exposures were carried out in 20 ml of autoclaved fresh water (Lake Jyväsjärvi) in triplicate cultures under constant agitation (120 rpm) at 25°C for 3 days at a multiplicity of infection (MOI) at inoculation of 1 (1×10^4 CFU and PFU ml^{-1}). The cultures were sampled every 24 h for 3 days by making a serial 10-fold dilution of samples and spreading on Shieh agar plates. After up to 4 days of incubation at room temperature, CFU and colony morphologies were determined from the plate cultures. Two to three colonies from each triplicate culture at each sampling point were picked and pure cultured directly on Shieh agar plates three times to get rid of any phage contamination. Colonies were then checked for phage resistance by spot assay on agar plates; bacterial lawns on top agar were prepared as described above, and 10 μl of 10-fold-diluted original phage lysates (used in initial exposures) was spotted on agar. After a 48-h incubation at 25°C, bacterial plates with no observed plaques or confluent lysis were considered phage resistant. Altogether, 189 colonies from phage-exposed and no-phage control exposures were isolated from plate cultures. From this collection, 20 phage-exposed and 4 no-phage control isolates were randomly selected for further analysis (Table 1).

The phage-exposed and no-phage control isolates were named according to the latter part of the wild-type bacterial host, the letter R for phage-exposed and the letter S for no-phage control isolates plus a running number for the isolated colony. For example, F2R2 is the second selected phage-exposed colony of the *F. columnare* wild-type isolate FCO-F2. Correspondingly, the second *F. columnare* isolate from no-phage control cultures was marked as F2S2. For simplicity, wild-type FCO-F2 and all its subsequent isolates from the phage and control exposures are commonly called F2 isolates in this paper. Correspondingly, wild-type FCO-F9 and its subsequent isolates are called F9 isolates.

Antibiotic sensitivity. Changes in susceptibility of phage-exposed *F. columnare* isolates toward antibiotics was tested using the Kirby-Bauer disc diffusion method (54) on diluted Mueller-Hinton (55) agar medium supplemented with 5% wt/vol fetal calf serum. A 40-μl volume of each isolate suspension (10^9 CFU ml^{-1}) was added to 5 ml of phosphate-buffered saline and poured onto the Mueller-Hinton agar plates. After removing excess bacterial suspension by pipetting, the antibiotic discs (oxolinic acid [2 μg], florfenicol [30 μg], sulfamethoxasol/trimethoprim [25 μg], and tetracycline [30 μg]) were placed on the plates. The plates were then incubated for 3 days at 25°C. After incubation, the inhibition zone around the antibiotic discs was measured. The susceptibility tests did not include replicates. The susceptibility patterns of the selected phage-exposed and no-phage control *F. columnare* isolates to the antibiotics were compared to that of the parent wild-type isolates.

Motility/colony spreading. The effect of phage exposure on bacterial motility was tested by comparing the colony-spreading ability of phage-exposed and no-phage control isolates with that of their parent wild-type isolates. After spotting 5 μl of bacterial suspension (10^9 CFU ml^{-1}) on TYES agar (0.5% agar) plates supplemented with 0.1% baker's yeast and incubating for 3 days at 25°C, the colony diameter of each isolate was measured. Each isolate was tested in three replicates.

Adhesion and biofilm formation. Changes in adherence or biofilm formation capacities between wild-type, phage-exposed, and no-phage control *F. columnare* isolates were studied in flat-bottomed 96-well microtiter plates (Nunclon Delta surface, Nunc) (56). *F. columnare* cells grown on TYES agar were suspended in autoclaved fresh water (Lake Littoistenjärvi) to a concentration of 10^8 CFU ml^{-1} (OD_{520} = 0.6). For testing of bacterial adherence, a 100-μl volume of the prepared bacterial suspensions was added in triplicate into wells of replicate microtiter plates and incubated statically for 1 h at 25°C. For testing of biofilm formation, a 100-μl volume of TYES broth was added to wells containing 100 μl of the prepared bacterial suspensions and allowed to incubate for 3 days. Autoclaved fresh water was used as a negative control. After incubation, the contents were discarded, and the wells were washed three times with sterile 0.5% NaCl to remove nonadherent cells and air dried. The wells were then stained with 0.1% crystal violet solution for 45 min and washed three times by submersion in a container of tap water and air dried. The crystal violet was solubilized with 96% ethanol for 15 min before measuring the absorbance (1 s) at 595 nm (Victor2, Wallac).

TABLE 6 Accession numbers of whole-genome sequences of wild-type *Flavobacterium columnare* isolates FCO-F2 and FCO-F9 and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates submitted to GenBank

Isolate	Accession no.
FCO-F2	CP051861
F2R58	CP054506
F2R60	CP054505
F2R62	CP054504
F2R64	CP054503
F2R65	CP054502
F2R66	CP054501
F2R67	CP054500
F2R68	CP054499
F2R70	CP054498
F2R72	CP054497
F2R74	CP054496
F2S4	CP054495
F2S17	CP054494
FCO-F9	CP054518
F9R56	CP054517
F9R58	CP054516
F9R61	CP054515
F9R64	CP054514
F9R66	CP054513
F9R69	CP054512
F9R72	CP054511
F9R75	CP054510
F9R78	CP054509
F9S15	CP054508
F9S17	CP054507

Protease activity. Changes in protease activity were examined by spotting 1 μl of bacterial TYES broth suspension (10^8 CFU ml^{-1}) of the wild-type isolates and each phage-exposed and no-phage control isolate on TYES agar (1.5% agar) supplemented with elastin (0.1%, wt/vol), gelatin (3%), and skim milk (5%) (caseinase production). The proteolytic activity of each isolate was observed by the presence of a clear zone surrounding the colony after incubation and assessed by measuring the clear zone ratio (diameter of clear zone/diameter of the colony) of three replicate samples. In the absence of a clearing zone outside the colony, the clear zone ratio was defined as 1. The measurements were made after 5 (caseinase and gelatinase) or 10 (elastinase) days of incubation at 25°C.

Virulence. Virulence of phage-exposed and no-phage control *F. columnare* isolates was tested on 1.94-g (average weight) rainbow trout fry and compared to the virulence of wild-type isolates. Fifteen fish per treatment, 20 in the control treatment with no bacteria, were exposed individually in 500 ml of bore hole water (25°C) to cells of single bacterial isolates by constant immersion (5.0×10^3 CFU ml^{-1}). Survival of the fish was monitored hourly for 24 h. Morbid fish that did not respond to stimuli were considered dead, removed from the experiment, and euthanized by decapitation. At the end of the experiment, the fish that survived infection were euthanized using 0.008% benzocaine. Bacterial cultivations from gills of all the dead fish were made on Shieh agar supplemented with tobramycin (57) to confirm the presence/absence of the bacterium. Cumulative percent mortality and estimated survival time (Kaplan-Meier survival analysis) based on observed average survival time of fish after exposure to each isolate were used as measures of virulence, with more virulent isolates having a shorter estimated survival time.

Fish experiments were conducted according to the Finnish Act of Use of Animals for Experimental Purposes under permission ESAVI/8187/2018 granted for Lotta-Riina Sundberg by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Whole-genome sequencing. Genomes of the wild-type FCO-F2 and FCO-F9 *F. columnare* and selected 20 phage-exposed and four no-phage control isolates (Table 1) were sequenced using an Illumina HiSeq platform (Institute of Molecular Medicine Finland). The Illumina data reads of FCO-F9 and its phage-exposed and no-phage control isolates were mapped to a reference genome of *F. columnare* isolate B185 (58) using Geneious software version 11.1.5 (Biomatters Ltd.). The genome of the wild-type FCO-F2 isolate was sequenced using PacBio (BGI, China). PacBio data of FCO-F2 were assembled using >8-kbp reads with Flye (version 2.7, four iterations) and >6-kbp reads with Canu (version 1.9). These multicontig assemblies were then combined using Quickmerge (version 0.3) to produce one 3,221,312-bp contig. This contig was polished with Illumina HiSeq reads using Pilon (version 1.23), with preprocessing done using Trimmomatic (version 0.39), bowtie2 (version 2.3.5.1), and Samtools (version 1.9). The quality of the polished contig was quantified using Busco (version 4.0.2), which reported 100% completeness of the genome against the bacteria_odb10 reference set. The genome was annotated

using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (59, 60) and used as reference genome for mapping of F2 phage-exposed and no-phage control isolates using Geneious software version 11.1.5 (Biomatters Ltd.). The mutations were considered true when the number of reads with nucleotide changes/mutations was bigger than the number of reads with a wild-type sequence.

Statistical analyses. IBM SPSS statistics version 24 was used for statistical analysis of the data. A one-way analysis of variance (ANOVA) was used to compare means from phenotypic analyses between experimental groups (phage-exposed isolates and no-phage control isolates) and parent wild-type isolates. If needed, $\log_{10}$ exponential or square root transformations were made for the data to fulfill the homogeneity of variances assumption. If the homogeneity of variances could not be met by transformations, the data were analyzed using nonparametric Kruskal-Wallis and Mann-Whitney tests. In cases of elastinase and casienase activity and biofilm formation, the isolates with no activity/biofilm-forming capacity were excluded from the ANOVA LSD multiple comparison analyses. Kaplan-Meier survival analysis was used for the analysis of virulence data.

Data availability. The whole-genome sequences of all isolates were submitted to GenBank under accession numbers presented in Table 6.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.03 MB.

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Bacteriophage Resistance Affects *Flavobacterium columnare* Virulence Partly via Mutations in Genes Related to Gliding Motility and Type IX Secretion System

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Supplemental material

The phage-exposed and no-phage control isolates showed antibiotic susceptibility patterns similar to the parent wild-type isolates (Figure S1). Most showed decreased inhibition zone diameter in trimethoprim/sulfamethoxazole test, but including wild-type isolates, this inhibition zone was weak and not totally clear from bacterial growth. In case of other antibiotics, decrease in inhibition zone diameter was within a range of measurement error (1-2 mm) (Table S1). However, these results are based on only one repeat so statistical analyses could not be conducted and no estimates on significance of the results can be made.

In addition to statistical differences between the virulence of phage-sensitive rhizoid and phage-resistant rough and soft isolates, there were also differences between the virulence of individual isolates (Data set S1).

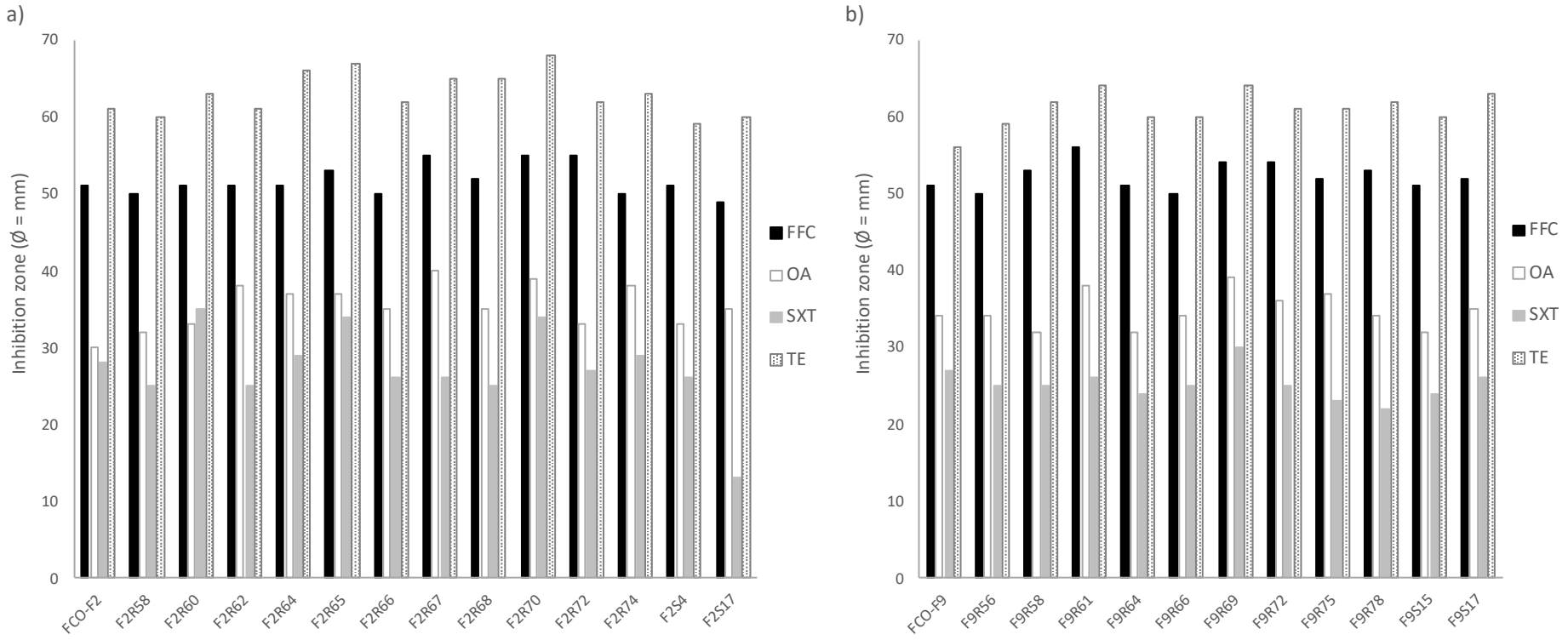

Figure S1. Antibiotic susceptibility of the wild-type *Flavobacterium columnare* a) FCO-F2 and b) FCO-F9 isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates against florfenicol (FFC), oxolinic acid (OA), sulfamethoxasol/trimethoprim (SXT) and tetracycline (TE) measured as the inhibition zone diameter (mm) with the Kirby-Bauer disc diffusion method.

Table S1. Antibiotic susceptibility of the wild-type *Flavobacterium columnare* FCO-F2 and FCO-F9 isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates against florfenicol (FFC), oxolinic acid (OA), sulfamethoxasol/trimethoprim (SXT) and tetracycline (TE) measured as the inhibition zone diameter (mm) with the Kirby-Bauer disc diffusion method. Values of the wild-type isolates are underlined, and values with bold indicate decreased inhibition zone diameter compared to the parent wild-type isolate.

Isolate	Inhibition zone diameter (mm)			
	FFC	OA	SXT	TE
FCO-F2	51	30	28	61
F2R58	**50**	32	**25**	**60**
F2R60	51	33	35	63
F2R62	51	38	**25**	61
F2R64	51	37	29	66
F2R65	53	37	34	67
F2R66	**50**	35	**26**	62
F2R67	55	40	**26**	65
F2R68	52	35	**25**	65
F2R70	55	39	34	68
F2R72	55	33	**27**	62
F2R74	**50**	38	29	63
F2S4	51	33	**26**	**59**
F2S17	**49**	35	**13**	**60**
FCO-F9	51	34	27	56
F9R56	**50**	34	**25**	59
F9R58	53	**32**	**25**	62
F9R61	56	38	**26**	64
F9R64	51	**32**	**24**	60
F9R66	**50**	34	**25**	60
F9R69	54	39	30	64
F9R72	54	36	**25**	61
F9R75	52	37	**23**	61
F9R78	53	34	**22**	62
F9S15	51	**32**	**24**	60
F9S17	52	35	**26**	63

Data set S1. Statistical differences (Kaplan-Meier Survival Analysis, pairwise comparisons; Log-Rank, Mantel Cox) between cumulative mortalities of rainbow trout caused by wild-type *Flavobacterium columnare* FCO-F2 and FCO-F9 isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates.

(SEPARATE EXCEL FILE)

IV

INTERACTIONS BETWEEN RAINBOW TROUT EYED EGGS AND FLAVOBACTERIUM SPP. USING A BATH CHALLENGE MODEL: PRELIMINARY EVALUATION OF BACTERIOPHAGES AS PATHOGEN CONTROL AGENTS

by

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Article

Interactions between Rainbow Trout Eyed Eggs and *Flavobacterium* spp. Using a Bath Challenge Model: Preliminary Evaluation of Bacteriophages as Pathogen Control Agents

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Abstract: The microbial community surrounding fish eyed eggs can harbor pathogenic bacteria. In this study we focused on rainbow trout (*Oncorhynchus mykiss*) eyed eggs and the potential of bacteriophages against the pathogenic bacteria *Flavobacterium psychrophilum* and *F. columnare*. An infection bath method was first established, and the effects of singular phages on fish eggs was assessed (survival of eyed eggs, interaction of phages with eyed eggs). Subsequently, bacteria-challenged eyed eggs were exposed to phages to evaluate their effects in controlling the bacterial population. Culture-based methods were used to enumerate the number of bacteria and/or phages associated with eyed eggs and in the surrounding environment. The results of the study showed that, with our infection model, it was possible to re-isolate *F. psychrophilum* associated with eyed eggs after the infection procedure, without affecting the survival of the eggs in the short term. However, this was not possible for *F. columnare*, as this bacterium grows at higher temperatures than the ones recommended for incubation of rainbow trout eyed eggs. Bacteriophages do not appear to negatively affect the survival of rainbow trout eyed eggs and they do not seem to strongly adhere to the surface of eyed eggs either. Finally, the results demonstrated a strong potential for short term (24 h) phage control of *F. psychrophilum*. However, further studies are needed to explore if phage control can be maintained for a longer period and to further elucidate the mechanisms of interactions between Flavobacteria and their phages in association with fish eggs.

Keywords: *Flavobacterium psychrophilum*; *Flavobacterium columnare*; rainbow trout; eyed eggs; phage-mediated control; bacteriophages

1. Introduction

The physical barrier of the thin chorion (*zona pellucida*) and the thicker inner membrane (*zona radiata*) of teleost eggs varies in structure and thickness among species [1], and represents the first line of defense against bacterial and viral infections. The wide range of the bacteria that surrounds the eggs will contribute to the early establishment of the fish microbiome [2,3]. Within these microbial communities, pathogenic bacteria such as *Cytophaga* spp., *Flavobacterium* spp., *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. also exist, and may represent threats for the development and survival of the fish [3–6]. In

aquaculture facilities, egg disinfection protocols are used to decrease the risk of mortality and pathogen transmission [7].

The transmission of the freshwater pathogen *Flavobacterium psychrophilum* [8,9], an etiological agent of rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (BCWD), among fish populations is not fully understood. Both the vertical and the horizontal routes have been suggested to play a role [10–12], and *F. psychrophilum* has been isolated from milt, ovarian fluids, and in close connection with eggs [12–14], as well as from the surrounding environment of diseased fish [12,15]. Similarly to *F. psychrophilum*, the freshwater pathogen *F. columnare*, which causes mortality in wild and culture freshwater fish, characterize the microbial communities of fish, eggs, and the rearing waters (reviewed by [16]). Persistent colonization of eggs by Flavobacteria thus likely increase the probability of bacterial transmission to fish in all production stages, which can lead to important economic losses and the increased use of antibiotics [16]. Both *F. psychrophilum* and *F. columnare* cause high mortalities in rainbow trout fry populations (up to 80–90%), depending on the size of the fish [17–19]. Good husbandry management and egg disinfection have been highlighted as methods to reduce the development of infections among fish in hatcheries [12].

The utilization of virulent bacteriophages (also called phages) [20] to reduce mortality and prevent the spread of bacterial populations among fish and crustaceans at different stages has gained increased attention (reviewed by [21,22]). Phage therapy is considered a potential alternative to antibiotics, aiming to reduce the issues related to the use of antibiotics, and as a preventive measure against the spread of bacterial infections (reviewed by [23]).

Previous studies on phage control of *Flavobacterial* pathogens in rainbow trout have focused on fry and juvenile stages [24–28]. Here, we report for the first time the use of bacteriophages for reducing these pathogens in connection with rainbow trout eyed eggs. In this work, we explored the potential of using virulent bacteriophages targeting *F. psychrophilum* and *F. columnare* as bacterial control agents in rainbow trout eyed eggs. At first, we established a bacterial challenge bath method (Section A), and secondly, we evaluated the effects of phage addition on eyed eggs (Section B). Subsequently, we exposed rainbow trout eyed eggs to phages to assess their efficiency in eliminating the target bacterium (Section C).

2. Materials and Methods

2.1. Bacteria

Flavobacterium psychrophilum 950106-1/1 and 160401-1/5N, Danish strains isolated from rainbow trout, were selected for the experiments. *F. psychrophilum* 950106-1/1 is a well-characterized strain isolated in 1995 (serotype Fd, virulent) [10,29–31], while *F. psychrophilum* 160401-1/5N was isolated in 2016 and recently characterized (serotype Th, virulent) [31]. An additional strain, *F. psychrophilum* FPS-S6 (serotype Th, virulent, isolated in 2017 in Sweden), was used for the production of high titer phage FPSV-D22 solutions since it was the most efficient host for phage proliferation [27,31]. The strains were stored at −80 °C in tryptone yeast extract salts (TYES) medium [32] and glycerol (15–20%). For phage analysis, *F. psychrophilum* 950106-1/1 (and *F. psychrophilum* 160401-1/5N for Exp. I section C) was inoculated in TYES broth (5 mL, referred as TYES-B) from a −80 °C stock, incubated for 48–72 h (15 °C; 100 rpm) and then streaked onto TYES agar (TYES-B with 1.1% agar, referred as TYES-A). Single colonies were then picked (3–4 days of incubation) and inoculated in TYES-B for 48 h [27]. For bath challenge experiments, the selected bacteria were prepared according to [30]. Specifically, 0.5 mL of a 72 h bacterial culture (5 mL) was transferred into 100 mL TYES-B and incubated at 15 °C. After 48 h of incubation, appropriate dilutions depending on the selected dose of infection were performed prior to the bath. CFU were counted before and after the infection procedure in duplicates.

Two virulent isolates were used in the studies with *F. columnare*: B480 and B185. Originally, both strains were isolated from fish farms during columnaris disease outbreaks

in Finland. Strain B480 was isolated from rainbow trout in 2012, and belongs to the genetic group E [33]. B185 was isolated from rearing tank water in 2008 [34]. Bacterial cultures were stored at −80 °C with 10% glycerol and 10% fetal calf serum. For the experiments, bacteria were revived from −80 °C by inoculation into 5 mL of Shieh medium [35] and cultured overnight at 25 °C under constant agitation (120 RPM). Bacteria were enriched by subculturing (1:10) and incubating for 24 h. Bacterial cell density was measured as an optical density (OD, 595 nm; Multiscan FC Thermo Scientific, Ratastie, Finland) and colony forming units per ml (CFU mL^{-1}) estimated based on our previously determined OD-CFU relationship (unpublished).

2.2. Bacteriophages

F. psychrophilum-targeting lytic bacteriophages FpV4 (isolated in 2005 in Denmark from water with feces samples, Podoviridae family) [36,37] and FPSV-D22 (isolated in 2017 in Denmark from fish tissue samples, Siphoviridae family) [27,31] were selected for the studies. Solutions of FpV4 and FPSV-D22 were purified (0.2 µm pore size sterile filter) and stored in SM buffer (8 mM $MgSO_4$, 50 mM Tris-Cl [pH 7.5], 99 mM NaCl, 0.01% gelatin) and glycerol (15%) at −80 °C [31,36]. For the experiments in section B, phage high titer solutions were prepared from crude lysates following the infection of the strain 950105-1/1 (for FpV4 propagation) and of the strain FPS-S6 (for FPSV-D22 propagation) in TYES-B (MOI = 1). After incubation for 48–72 h, the lysed cultures were then centrifuged (5000× *g*, 10 min, 4 °C) and filtered with a 0.2 µm pore size sterile filter (Sterivex, Millipore; Merck KGaA, Darmstadt, Germany). For the experiments in section B and C, FpV4 and FPSV-D22 crude lysates were further purified and concentrated by PEG-precipitation (24 h-incubation at 4 °C with poly-ethylene glycol 8000 (PEG-8000) and sodium chloride at a final concentration of 10% *w/v* and 1 M, respectively) and subsequent 0.2 µm filtration, centrifugation (10,000× *g*, 30 min, 4 °C), and re-suspension in either sterile TYES-B or sterile SM buffer, as described by [27,38].

F. columnare-infecting lytic Myoviridae phages FCL-2 (isolated in 2008 in Finland, infection of hosts in genetic group G) [34] and FCOV-F27 (isolated in 2017 in Finland, infection of hosts in genetic group C) [33] were used in the experiments (phages were previously isolated from tank water in fish farms during columnaris outbreaks). FCL-2 has previously been shown effective against columnaris infections in rainbow trout [28]. To test the interaction with rainbow trout eggs (section B, experiment III), crude lysates of each phage were produced, as described earlier [28]. To test the efficiency of phages in preventing *F. columnare* replication on eggs, the phage FCL-2 was produced and purified by tangential flow filtration with diafiltration by PhageCosultants Ltd. Briefly, 300 mL of the crude lysate was loaded on the Millipore Labscale Tangential Flow Filtration (TFF) System with Pellicon® XL Ultrafiltration Module Biomax® 100 kDa, 0.005 m^2. The lysate was diafiltrated by using ultrafiltration membranes (PES, 100 kDa pass) to completely remove or lower the concentration of salt, solvent, and metabolites by exchanging the volume of the lysate to 0,9% NaCl three times.

2.3. Rainbow Trout Eyed Eggs

Rainbow trout (*Oncorhynchus mykiss*) eyed eggs (>200 day degrees, dd) used for the experiments concerning *F. psychrophilum* and its phages were purchased from Troutex ApS (Egtved, Denmark). A few hours after arrival at the laboratory (Denmark), the experiments were performed. The status of the eyed eggs was inspected to reveal if any mortality had occurred during the transportation, whereafter the eggs were disinfected according to standard procedures performed at Danish rainbow trout hatcheries (10–15 min treatment in a iodine-based disinfectant for aquaculture) (100 ppm active iodine; 1% Actomar K30 (Desag AF, Uster, Switzerland)) [15] (Figure 1A). After disinfection, the eyed eggs were rinsed with sterile water before the bacteria and phage exposure experiments.

Figure 1. Illustrated overview of the experimental procedure followed in the various experiments concerning *F. psychrophilum* and its phages. (**A**) Disinfection of eyed eggs following standard procedures used in hatchery facilities (iodine-based solution) performed at the start of each experiment; (**B**) eyed eggs during the two-hour bacterial bath challenge with *F. psychrophilum* and incubation at 10 °C (experiments section A and C); (**C**) eyed eggs during the phage bath and incubation at 10 °C (experiments section B and C); (**D**) eyed eggs for phage bath placed in 250 mL sterile glass beakers (experiments section B and C); (**E**) eyed eggs during incubation in 24-well plates (experiments section A, B, and C) (photos by V.L. Donati).

For the experiments concerning *F. columnare* and its phages, rainbow trout eyed eggs (>200 day degrees, dd) were received from a fish farm within a one-hour drive from the laboratory (Finland). The eggs were disinfected with the iodine-based disinfectant Buffodine® (Evans Vanodine International plc, Lancashire, UK) at the farm according to the manufacturer's instructions (10 min treatment), cold-transported to the lab, and used immediately in the experiments. Before the start of an experiment, six eggs were sampled for the presence of *F. columnare* and its phages and found negative.

2.4. Establishment of a Bath Bacterial Challenge Method (Section A)

A series of experiments was initially performed to establish a reproducible method to study the interactions of *F. psychrophilum* and rainbow trout eyed eggs at a small scale. These experiments were performed with the aim of (1) isolating the bacterium in connection with the eggs and (2) recording the effects of the bacterial challenge on the eggs' survival during 24 h incubations. An additional experiment, focused on *F. psychrophilum* growth in different media, was performed. Furthermore, experiments targeting *F. columnare* were set up with the aim of evaluating the effects of temperature and medium on the eggs' survival.

F. psychrophilum 950106-1/1 was chosen for the preliminary experiments. Disinfected eyed eggs were placed in 500 mL sterile glass beakers containing 200 mL of either bacterial solution (Exp. no. 1: 8.7×10^4 CFU mL^{-1}; Exp. no. 2: 1.5×10^7 CFU mL^{-1}; Exp. no. 3: 1.6×10^5 CFU mL^{-1}) or sterile TYES-B (control for the infection) and incubated for 2 h at 10 °C at 80–90 RPM (Figure 1B). After the bath challenge procedure, the eyed eggs

were moved to sterile 24-well plates (one egg per well) (CELLSTAR®, Greiner Bio-One GmbH, Frickenhausen, Germany) containing 2 mL of sterile TYES-B (Exp. no. 1), sterile Milli-Q water (Exp. no. 2), or sterile SM buffer diluted 10 times in Milli-Q water (Exp. no. 3). Eyed eggs were transferred using sterile 10 µL inoculation loops (Figure 1E). The plates were covered with lids and incubated at 10 °C (at 80–90 RPM) for 24 h. In Exp. no. 1, three eggs were sampled at 1, 3, 21, and 25 h after the incubation in 24-well plates. In Exp. no. 2 and no. 3, three eggs were sampled right after the end of the bacterial bath (before the transfer to 24-well plates) and after 24 h of incubation. Exp. no. 1 was performed in December 2018, Exp. no. 2 in May 2019, and Exp. no. 3 in June 2019. Furthermore, to evaluate the growth of *F. psychrophilum* 950106-1/1 in Milli-Q water and 0.2 µm filtered tank water collected in our fish experimental facilities and compare to the growth in TYES medium, a growth experiment was performed as follows: 0.5 mL of a 72 h bacterial culture (5 mL) was transferred into either 100 mL of Milli-Q water, 100 mL of water from fish experimental facilities (fish tanks), or sterile TYES-B, and incubated at 15 °C. The experiment was performed in duplicates and the CFU count was performed at various time points.

In the case of *F. columnare*, various temperatures (5 °C (moved to 10 °C after 72 h), 15 °C, and 20 °C), in combination with different media (pre-aerated with pressurized air until 100% oxygen saturation, non-aerated sterile distilled water, or pre-aerated or non-aerated sterile Shieh medium) were tested (February 2019). For each group, 12 eyed eggs were placed in sterile 24-well plates (Nunc™, Thermo Fisher Scientific, Rochester, USA) containing 2 mL of the selected medium and incubated at the settled temperature without any shaking (similarly as for *F. psychrophilum* in Figure 1E). Four of the 12 eyed eggs in each group were exposed to the *F. columnare* strain B480 by adding 10 µL of overnight culture (1.0×10^8 CFU mL^{-1}) directly to the wells, giving a final density of 5.0×10^5 CFU mL^{-1}. Survival of the eggs (embryo movement and blood flow observed under a light microscope) was followed in 24 h intervals for 144 h, except for the experiments performed at 20 °C, which were carried out until 72 h. In the case of bacterial exposure, samples from the media surrounding the eggs were collected from at least two wells per treatment at 24, 48, and 96 h.

2.5. Interactions of Phages with Rainbow Trout Eyed Eggs (Section B)

In this section, the effects of phages on rainbow trout eyed eggs' survival in the absence of pathogens were evaluated. The experiments were also aimed at evaluating if phages could interact with the surface of the eggs. The effects of two selected *F. psychrophilum* bacteriophages (FpV4 and FPSV-D22; singularly) were tested by constant (Exp. I, section B) and by short-term bath exposure (Exp. II, section B). Similarly, the effects of two selected *F. columnare* bacteriophages (FCL-2 and FCOV-F27, singularly; Exp. III, section B) were tested. An overview of the experiments performed in this section is presented in Table 1.

2.5.1. Constant Exposure of Eyed Eggs to *F. psychrophilum* Phages (Exp. I, Section B)

Eyed eggs were constantly exposed to phages FpV4 (3.0×10^5 PFU mL^{-1} crude lysate and 1.0×10^6 PFU mL^{-1} PEG-purified in TYES-B) and FPSV-D22 (1.2×10^7 PFU mL^{-1} PEG-purified in TYES-B) for 144 h (the experiment was performed in April 2019). A control group without phage exposure was included (eggs were placed in sterile TYES-B). After disinfection, seventy-five eyed eggs were placed in 24-well plates using sterile 10 µL inoculation loops (all groups contained 23 eggs except the group where eggs were exposed to FpV4 in crude lysate where 16 eggs were incubated) with 2 mL of phage solution (sterile TYES broth for the control) (Figure 1E). Covered with lids, the plates were incubated at 10 °C at 80–90 RPM. After 2, 27, 49, and 71 h of incubation, three eggs and their correspondent well content per group were collected. At 144 h after the start of the experiment, the status of three eggs (alive/dead; hatched/not hatched) was characterized, and only the well content was collected for phage analysis.

Table 1. Overview of studies focused on exploring the interactions between rainbow trout eyed eggs and *Flavobacterium* spp. bacteriophages (section B and C).

Study Name	Infection with *Flavobacterium spp.*	Type of Exposure to Phages	Phages	Type of Preparation
Exp. I Section B	No	Constant	FpV4 and FPSV-D22 (singularly)	Crude lysates and PEG-purified in sterile TYES-B
Exp. II Section B	No	4 h bath	FpV4 and FPSV-D22 (singularly)	Crude lysates
Exp. III Section B	No	30 min bath; constant	FCL-2 and FCOV-F27 (singularly)	Crude lysates
Exp. I Section C	Yes	48 h bath	FpV4 and FPSV-D22 (mixed 1:1)	PEG-purified in SM buffer
Exp. II Section C	Yes	2 h bath; constant	FCL-2	Diafiltration

2.5.2. Bath Exposure of Eyed Eggs to *F. psychrophilum* Phages (Exp. II, Section B)

In this experiment (performed in April 2019), eighty-one rainbow trout eyed eggs were bathed for 4 h at 10 °C at 80–90 RPM either in phage solutions (1.9×10^7 PFU mL^{-1} FpV4 or 8.2×10^7 PFU mL^{-1} FPSV-D22 crude lysates) or in sterile TYES-B, for the control group. The bath procedures were performed in 250 mL sterile glass beakers containing 80 mL of phage or control solution (27 eggs for each treatment) (Figure 1C). After phage exposure, eggs were subdivided into 24-well plates (24 eggs per group) with 2 mL sterile Milli-Q water (one egg per well) using sterile 10 µL inoculation loops. Plates were covered and incubated at 10 °C at 80–90 RPM for 144 h (Figure 1E). At 0 h and 24, 46, and 68 h after the end of phage bath exposure, three eggs and their correspondent well content per group were collected for further phage analysis. At 144 h, the status of three eggs (alive/dead; hatched/not hatched) was characterized, and only the well content was sampled for phage analysis.

2.5.3. Bath and Constant Exposure of Eyed Eggs to *F. columnare* Phages (Exp. III, Section B)

Eyed eggs were exposed to phages FCL-2 or FCO-F27 (1.0×10^9 PFU mL^{-1}; crude lysates) diluted in either sterile distilled water or in Shieh medium at 10 °C. Phage exposure was performed by either a 30 min bath in a Petri dish (40–50 mm Ø, 15 mL medium volume, at 60 RPM) or constant exposure in 24-well plates (no shaking). Eggs without phage treatment and a phage lysate without eggs served as controls. For constant exposure and after the phage bath, eggs (8 per group) were individually placed in 24-well plates containing 2 mL of either sterile distilled water or Shieh medium (similarly as for *F. psychrophilum* in Figure 1E). Bathed eggs were moved into wells with only medium (distilled water or Shieh medium). Eyed eggs for constant exposure experiments were moved directly to wells containing the phages. Eyed eggs were moved using sterile disposable forceps. Survival of the eggs was determined at 0, 24, 48, and 96 h. Phage density was determined both from eggs and the corresponding well content at 0, 24, and 48 h.

2.6. Evaluation of Phages as Pathogen Control Agents (Section C)

In this section, the experiments were aimed at assessing the potential of phages as pathogen control agents (an overview is presented in Table 1). The effects of two selected *F. psychrophilum* phages (FpV4 and FPSV-D22; mixed 1:1) in controlling *F. psychrophilum* 950106-1/1 and the strain 160401-1/5N were tested by a 48 h bath exposure (Exp. I, section C). Similarly, the effects of the *F. columnare* phage FCL-2 in controlling *F. columnare* B185 were tested by either constant or bath phage exposures (Exp. II, section C).

2.6.1. Phage Bath of *F. psychrophilum* Challenged Eggs (Exp. I, Section C)

F. psychrophilum bath-challenged eyed eggs were exposed to a two-component phage solution (phages FpV4 and FPSV-D22 mixed 1:1) for 48 h and then transferred to individual wells for the examination of phage and pathogen abundance (experimental set up presented in Figure A1). The experiment was performed in June 2020.

At first, eyed eggs were bath-challenged (2 h, 10 °C, 80–90 RPM) either with *F. psychrophilum* strain 950106-1/1 or the strain 160401-1/5N at a concentration of 2.0×10^6 CFU mL^{-1}. Control eggs were placed in sterile TYES-B. To perform the challenge, 135 disinfected eyed eggs were placed in 600 mL sterile glass beakers containing 200 mL of bacterial solution or sterile TYES-B (Figure 1B). Subsequently, eyed eggs were moved to 250 mL sterile glass beakers (30 eggs per beaker) containing either the selected phage solution (20 mL) or the phage bath controls (20 mL of sterile SM buffer or Milli-Q water) using sterile 10 µL inoculation loops (SARSTEDT AG & Co. KG, Nümbrecht, Germany) (one per group) (Figure 1C). For phage bath procedures, PEG-purified solutions of phage FpV4 and FPSV-D22 at a concentration of 3.9×10^8 PFU mL^{-1} and 1.3×10^9 PFU mL^{-1}, respectively, were mixed 1:1 to a final concentration of $2.2 \times 10^9 \pm 1.6 \times 10^9$ PFU mL^{-1} (phage bath no. 1) and diluted 10 times in SM buffer for phage bath no. 2 (final concentration of $1.3 \times 10^8 \pm 4.8 \times 10^7$ PFU mL^{-1}). The selected volume (20 mL) was considered enough to cover the eggs during the incubation (Figure 1D). After a 48 h incubation at 10 °C (at 80–90 RPM), eggs were divided in 24-well plates containing 2 mL of sterile Milli-Q water (one egg per well) with the help of sterile 10 µL inoculation loops (one per egg) (Figure 1E). Plates were covered with lids and incubated at 10 °C at 80–90 RPM.

Eyed eggs and the corresponding bath or well content were sampled for bacteria and/or phage quantification at the end of the bacterial challenge (0 h post infection, hpi), during phage exposure (24 and 48 hpi) and during the subsequent incubation in 24-well plates (72 and 144 hpi). For the sampling points 0 and 24 hpi, six eggs were sampled and, during the sampling procedure of three of them, an additional drying step was included. For the following sampling points, three eggs were collected and sampled without any drying step.

2.6.2. Phage Exposure of *F. columnare* Challenged Eggs (Exp. II, Section C)

F. columnare strain B185 and its phage FCL-2 (purified by diafiltration and diluted in NaCl 0.9%) were used in the experiment where eyed eggs were exposed to phages after (upper panel in Figure A2) or before the bacterial challenge (lower panel in Figure A2). Ion-exchanged water was used as a medium for the eggs, and the temperature was 10 °C.

At first, eyed eggs were bathed for 2 h (at 60 RPM): (a) with *F. columnare* B185 (5.0×10^6 CFU mL^{-1}) or with sterile Shieh medium (diluted in ion-exchange water in the same extent as done for the bacterium) in 140 mm diameter Petri dishes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) (97–99 eggs per dish–100 mL volume), or else (b) with the phage FCL-2 (2.5×10^7 PFU mL^{-1}) or with NaCl (0.09%) in 90 mm diameter Petri dishes (24 eggs per dish–35 mL volume). After the baths, the eggs were moved with sterile forceps in 140 mm diameter Petri dishes (22–25 eggs per dish–100 mL volume) containing water and incubated overnight without agitation. In addition, 24 additional eggs (12 per each group) were placed directly in 24-well plates containing water or NaCl (0.09%) without any preliminary bath procedure and observed constantly during the experiment. After the overnight incubation, the viability of all the eggs was checked and, in the bath bacterial challenge groups, 3 eggs and their corresponding well content (per treatment: + or − *F. columnare*) were sampled to quantify the bacterial densities and the phage titers.

Following the overnight incubation, eyed eggs previously exposed to *F. columnare* were either bathed for 2 h or moved directly into 24-well plates in either FCL-2 phage solution (2.5×10^7 PFU mL^{-1}) or NaCl (0.09%) (24 eggs per group). Eyed eggs previously exposed to phages were bath-exposed to *F. columnare* strain B185 (5.0×10^6 CFU mL^{-1}) or sterile Shieh medium for 2 h (12 eggs per group). Bath exposures to either phages or bacteria were performed in 90 mm diameter Petri dishes (35 mL volume) at 60 RPM.

Following the 2 h bath, eyed eggs were transferred to 24-well plates containing 2 mL of water (one per well). In the part of the experiment where eyed eggs were at first exposed to the bacterium and then to the phages (constantly or by bath), the viability of the eggs was observed immediately after transferring the eggs to 24-well plates and then at 24 h intervals until 144 h. In addition, three eggs and their corresponding well content were sampled at 0, 24, and 48 h to quantify the bacterial densities and the phage titers.

2.7. Eyed Eggs Sampling Procedure

The graphical overview in Figure 2 refers to the eyed eggs sampling procedure followed in the experiments concerning *F. psychrophilum* and its phages. Additional information in relation to *F. columnare* are presented at the end of this paragraph.

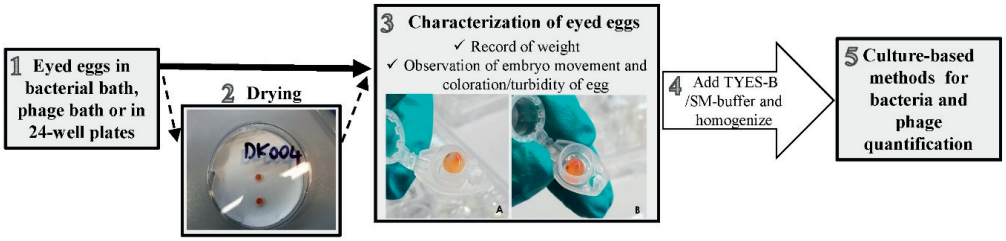

Figure 2. Graphical flow of the eyed eggs sampling procedure in relation to the experiments focused on *F. psychrophilum* and its phages. (**1**) Eyed eggs placed in the bacterial bath, the phage bath, or in 24-well plates were sampled at the selected time points. (**2**) A drying step was included for a selected number of eggs in Exp. I in section C. (**3**) Eyed eggs were characterized (A: example of turbid egg; B: example of normal coloration). (**4**) Sampled eggs were processed and homogenized. (**5**) According to the scope of the experiment, bacteria were enumerated and samples for phage analysis stored.

The sampling procedure was developed based on the previous work of [39–41]. Eyed eggs placed in the bacterial bath, the phage bath, or in 24-well plates (Figure 2 step 1) were collected at the selected time points using a sterile 10 µL inoculation loop (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and placed in pre-weighted sterile 1.5 mL micro tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) (Figure 2 step 3). For the Exp. I in section C, a drying step was included for a selected number of eggs (Figure 2 step 2), which were placed on sterile filter paper for a few seconds (Whatman® cat. no. 1003 090, Cytiva, Marlborough, USA) and then transferred to sterile 1.5 mL micro tubes. The weight was recorded and sampled eggs were characterized by observing the embryo movement and by recording the coloration/presence of turbidity of the egg (Figure 2 step 3). Dead eggs were identified by a whitish/opaque coloration, as previously described [40]. Sampled eggs were then cut and fragmented with the use of sterile scissors, and a fixed volume of TYES-B (experiments section A and Exp. II section C) or SM buffer (Exp. I and II section B) was added according to the scope of the experiment. Samples were thereafter homogenized by vortexing (15–20 s) (Figure 2 step 4). Finally, bacteria were enumerated by CFU counts, and the homogenized content was stored for subsequent phage quantification (Figure 2 step 5).

During Exp. I and II in section B, where our aim was to quantify *F. psychrophilum* phages in connection with the eyed eggs over time, 300 µL of sterile SM buffer was added to the sampled eggs (Figure 2 step 4), and after the homogenization procedure, 5 µL of chloroform was added and samples were stored for further phage analysis. For each sampled egg, the corresponding well content was also collected for phage analysis (300 µL of well content was placed into sterile 1.5 mL micro tubes and 5 µL of chloroform was added). The well content was streaked on TYES-A and Blood-A plates to assess the growth of bacteria/fungi. TYES-A plates were incubated at 15 °C and Blood-A plates at 20 °C for 4–5 days.

During the experiments of section A and C (concerning *F. psychrophilum)*, eyed eggs were sampled to quantify solely the bacterium (section A) or both the bacterium and the phages (Exp. I section C) in connection with the eggs. In this case, after egg status characterization (Figure 2 step 3), a fixed volume of sterile TYES-B (300–1000 µL in experiments of section A; 700 µL in Exp. I section C) was added to each egg sample and homogenized (Figure 2 step 4). Ten-fold serial dilutions were immediately performed and spread on TYES-A plates in order to estimate the bacterial concentration by CFU counts. Sampled eggs from the bacterial control groups (not exposed to *F. psychrophilum*) were also plated on TYES-A (no dilutions). For Exp. I section C, 300 µL of the homogenized egg samples was transferred into new sterile 1.5 mL micro tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and 5 µL of chloroform was added for subsequent phage analysis (Figure 2 step 5). In addition, phages and bacteria were also quantified in the corresponding well or bath content of each sampled egg. The concentration of bacteria was determined performing ten-fold serial dilutions of the well/bath content directly from the 24-well plate or the beaker used for bath procedures and plated on TYES-A plates. Bath/well content of sampled eggs from the bacterial control groups (not exposed to *F. psychrophilum*) were also plated on TYES-A (no dilutions). TYES-A plates were incubated at 15 °C for 4–5 days and CFU per mL of solution was estimated. For the Exp. I section C, 300 µL of the well/bath content was also placed in new sterile 1.5 mL micro tubes, 5 µL of chloroform was added, and the samples were stored at 5 °C in the dark for subsequent phage quantification. Homogenized eggs and the corresponding bath/well content were streaked on Blood-A to assess the growth of other bacteria/fungi and plates were incubated as mentioned earlier (Exp. I section C; only the well content for experiments of section A). The growth of bacteria other than *F. psychrophilum* on TYES-A plates was recorded (section A and C).

In the experiments concerning *F. columnare*, the survival of the eyed eggs was followed by observing the embryo movement and, in the experiments in section A, by observing the blood flow by a light microscope. In addition, and as performed for *F. psychrophilum*, samples for bacterium and phage detection/quantification were collected according to the scope of the experiment. In section A, the well content was streaked on Shieh agar plates, incubated at room temperature for 2 days, and the growth of *F. columnare* colonies recorded. In the Exp. II section B, the egg samples were processed similarly as for *F. psychrophilum*. Briefly, eyed eggs were placed in pre-weighted 1.5 mL Eppendorf tubes and crushed using a Bio Plas homogenization pestle (Thomas Scientific, Swedesboro, NJ, USA). A specific volume of Shieh medium was added (1:10 weight per volume) and the sample was mixed and centrifuged briefly to separate the supernatant, which was stored with chloroform for further phage quantification. For each sampled egg, the corresponding well content was also collected for phage analysis (300 µL media samples were stored at 4–6 °C with 1% chloroform). Finally, in the Exp. II section C, the eggs were not crushed, but were individually vortexed for 10 s in 400 µL of Milli-Q water, of which 100 µL was used to detect *F. columnare* (ten-fold dilutions plated on Shieh agar plates containing 1 µg mL^{-1} of tobramycin), and 150 µL was stored with chloroform for phage titration.

MALDI-TOF MS (Bruker Daltonic GmbH, Bremen, Germany) was used to confirm that the re-isolated bacteria were *F. psychrophilum* in doubtful cases, and to identify some of the background bacteria (if present) [42].

2.8. Detection and Quantification of Bacteriophages

Bacteriophage detection for phages infecting *F. psychrophilum* was performed as described by [24,25]. Egg and well content samples were centrifuged for 10 s at 10,000 RPM at 5 °C to separate chloroform at the bottom of the tube, and a phage spot method was performed [43]. Four milliliters of TYES soft agar (0.4% agar) mixed with 300 µL of a 48 h old *F. psychrophilum* broth culture (in exponential phase) was poured into a TYES-A plate [25,36]. Undiluted samples were then spotted in duplicate (section B) or triplicate (section C) (5 µL) on a bacterial lawn and incubated at 15 °C for 3–4 days. Phages were quantified by counting the plaques in individual spots. In the case of confluent or semi-

confluent clearing areas, samples were diluted 10-fold (180 µL of SM buffer and 20 µL of sample) in triplicates and re-spotted on a bacterial lawn as described above.

Bacteriophage quantification for phages infecting *F. columnare* was performed as previously described by [28]. Three hundred microliters of an overnight-grown *F. columnare* was mixed with 3 mL of melted Shieh soft agar (0.7%) tempered to 47 °C, and poured on Shieh agar plates. Two microliters of the ten-fold dilutions of the phage samples (in sterile Milli-Q water) was spotted on top of the soft agar. Plaques were recorded after incubation for 2 days at room temperature.

2.9. Statistics

Statistical significant differences in the bacterial and phage concentrations were tested with GraphPad Prism version 8.4.0 for Windows, (GraphPad Software, San Diego, CA, USA, www.graphpad.com). For meaningful comparisons of two groups, values were compared with a two-tailed unpaired t-test. For comparison of three or more groups, values were compared with ANOVA. *p*-values for multiple comparisons were adjusted for Dunnet correction (adjusted *p*). *p*-values (*p*) below 0.05 were considered significant.

3. Results

3.1. Establishment of a Bath Bacterial Challenge Method (Section A)

In the first part of our study, we developed an infection bath challenge method for rainbow trout eyed eggs, focusing on *F. psychrophilum*, with the aim of evaluating fish eggs' survival in the established set up, and the bacterial growth and stability associated with fish eggs and different media (Figure 3, Tables A1 and A2). In addition, since the optimum temperature for *F. columnare* is between 22–29 °C (depending on the strain) [44], while rainbow trout eyed eggs are normally incubated between 6 and 12 °C [45], the effects of different temperatures on the eggs' survival were at first evaluated, also in combination with different media (Figure S1).

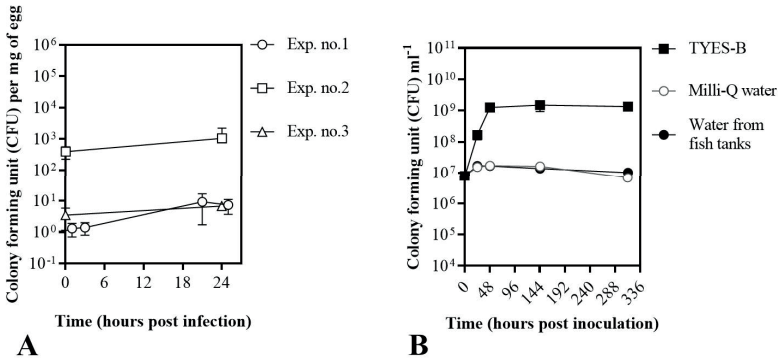

Figure 3. *F. psychrophilum* in connection with the eyed eggs in three independent experiments (section A) (**A**) and *F. psychrophilum* growth in Milli-Q and filter-sterilized water from fish tanks in comparison to TYES-B (**B**). In (**A**), values represent the mean and standard deviation of three biological replicates except in exp. no. 3 at 24 h post infection (n = 2). Control eyed eggs (bathed with sterile TYES-B) were negative to the bacteria for each experiment. In (**B**), values represent the mean and standard deviation of two replicates.

In the case of *F. psychrophilum*, all sampled eggs were characterized as alive based on movement and turbidity indicators (Table A1), and were subsequently recorded to be alive up to 6 days after the start of the experiments (data not shown). *F. psychrophilum* concentrations in connection with the eyed eggs correlated with the initial bacterial concentration of the bath (Figure 3A). After the bath challenge with 8.7×10^4 CFU mL^{-1} (Exp. no. 1, 1 h post infection or hpi) and 1.6×10^5 CFU mL^{-1} (Exp. no. 3, 0 hpi), the

concentration of *F. psychrophilum* detected in connection with the eyed eggs was 1.3 ± 0.6 and 3.5 ± 2.4 CFU mg^{-1} of egg, respectively. When the eyed eggs were bathed in a higher concentration of bacteria (Exp. no. 2: 1.5 × 10^7 CFU mL^{-1}), the bacterial concentration on the eggs had increased to 3.9 × 10^2 ± 1.7 × 10^2 CFU mg^{-1} of egg (0 hpi). The concentration of bacteria detected in connection with eyed eggs was maintained within 24 h in the 24-well plates. The detection of bacteria other than *F. psychrophilum* was recorded, and is presented in Table A2. In additional independent experiments, we observed the growth of *F. psychrophilum* in Milli-Q and filter-sterilized tank water from fish stables (Figure 3B): the bacteria were not able to actively grow under these conditions, but they remained viable for the tested time frame (15 days).

In the experiments concerning *F. columnare* (Figure S1), rainbow trout eyed eggs did not survive at 20 °C, and all movement was lost after 24 h in all the treatments (at 20 °C). Fish eggs were characterized as alive until 96–144 h when placed in water at 5 and 15 °C. The presence of nutrients (Shieh medium) reduced the time of egg survival. When the eggs were spiked with *F. columnare*, their survival was not affected and the bacteria could be isolated in the eggs incubated at 15 and 20 °C up to at least 48 hpi. At 5 °C, *F. columnare* could be isolated only at 24 hpi. Based on these results, the subsequent experiments concerning *F. columnare* and its phages were performed at 10 °C.

3.2. Interactions of Phages with Rainbow Trout Eyed Eggs (Section B)

3.2.1. Constant Exposure of Eyed Eggs to *F. psychrophilum* Phages (Exp. I, Section B)

The tested phages did not seem to negatively affect the eggs' survival (Table 2A). Sampled eggs were characterized as alive up to 49 and 71 h in all groups (only one egg out of 3 exposed to FpV4 in crude lysate was dead at 71 h). However, the embryo movement was not observed for a higher number of eggs exposed to the crude lysate compared to the other groups. In addition, at the termination of the experiment (144 h), most of the eggs in the sampled wells were dead except for two out of three in the PEG-purified FpV4 solution (hatched and alive) and one in the control group (not hatched and alive). Phages were diluted in sterile TYES-B, and this could have stimulated the growth of other bacteria/fungi (Table A3).

Two hours post constant phage exposure (Figure 4A), phages FpV4 and FPSV-D22 were detected in connection with the eyed eggs at a concentration of 4.4 ± 2.7 PFU mg^{-1} (FpV4 in crude lysate), 9.2 ± 3.3 PFU mg^{-1} (PEG-purified FpV4) and 3.2 × 10^3 ± 2.2 × 10^2 PFU mg^{-1} (PEG-purified FPSV-D22). The concentration of phages in connection with the eggs and in the corresponding wells was maintained over time in the groups with one exception (Figure 4A): the concentration of FpV4 associated with the eggs in the PEG-purified solution increased over time (adjusted $p = 0.0184$). No phages were detected in the control group.

To summarize, we observed that the tested phages did not seem to negatively affect the eyed eggs' survival, and that the concentration of phage FpV4 in connection with the eyed eggs increased over time.

3.2.2. Bath Exposure of Eyed Eggs to *F. psychrophilum* Phages (Exp. II, Section B)

Similarly to what was observed during constant phage exposure experiments (Exp. I section B), the survival of eyed eggs was not negatively affected when the eggs were bathed with either FpV4 (1.9 × 10^7 PFU mL^{-1}) or FPSV-D22 (8.2 × 10^7 PFU mL^{-1}) in crude lysates for four hours and then transferred to 24-well plates with sterile Milli-Q water (Table 2B). However, eggs were alive in all groups until the end of the experiment except one in the FPSV-D22 group at 144 h. Bacterial/fungal growth associated with the well content was detected firstly at 68 and 144 h in all three groups (Table A4).

Table 2. Exp. I and II, section B: survival of rainbow trout eyed eggs exposed to phage FpV4 and FPSV-D22. In (**A**), characteristics of the eyed eggs during constant phage exposure (Exp. I). In (**B**), characteristics of the eyed eggs after a 4 h phage bath (**B**, Exp. II). FpV4 and FPSV-D22 were diluted in sterile TYES-B. In yellow: not clear if the egg is alive (no movement and/or light turbidity); in red: the egg is dead (no movement and positive turbidity); not highlighted: the egg is alive. Time = hours of constant phage exposure in **A** and hours post-phage bath in **B**.

(A) Constant Phage Exposure (Exp. I Section B)

Time (h)	Evaluated Parameters	FpV4						FPSV-D22			Control		
		Crude Lysate			PEG-Purified			PEG-Purified					
		no. 1	no. 2	no. 3	no. 1	no. 2	no. 3	no. 1	no. 2	no. 3	no. 1	no. 2	no. 3
2	Movement	+	-	+	(+)	+	+	+	+	(+)	+	+	+
	Turbidity	-	-	-	-	-	-	-	-	-	-	-	-
27	Movement	+	+	-	+	+	+	+	+	+	+	-	+
	Turbidity	-	-	(+)	-	-	-	-	-	-	-	-	-
49	Movement	+	+	-	+	+	+	+	+	+	+	+	+
	Turbidity	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	(+)	(+)
71	Movement	-	-	+	+	+	+	(+)	+	+	+	+	(+)
	Turbidity	+	(+)	-	(+)	(+)	(+)	-	(+)	(+)	-	-	(+)
144	Alive/Dead	Dead	Dead	Dead	Alive	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Alive
	Hatched or not	Yes	No	No	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No

(B) Phage bath exposure (Exp. II section B)

Time (h)	Evaluated Parameters	Crude lysate						Control		
		FpV4			FPSV-D22					
		no. 1	no. 2	no. 3	no. 1	no. 2	no. 3	no. 1	no. 2	no. 3
0	Movement	+	+	+	(+)	(+)	(+)	+	+	+
	Turbidity	-	-	-	-	-	-	-	-	-
24	Movement	(+)	(+)	(+)	+	(+)	(+)	+	+	+
	Turbidity	-	-	-	-	-	-	-	-	-
46	Movement	+	+	+	+	+	+	+	+	+
	Turbidity	-	-	-	-	-	-	-	-	(+)
68	Movement	+	(+)	+	(+)	+	+	+	+	+
	Turbidity	-	-	-	-	-	-	(+)	(+)	(+)
144	Alive/Dead	Alive	Alive	Alive	Alive	Alive	Dead	Alive	Alive	Alive
	Hatched or not	No	Yes	Yes	No	Yes	No	Yes	No	Yes

+ Positive to movement or turbidity; (+) Weak movement/light turbidity; - Negative to movement or turbidity.

The concentration of FpV4 and FPSV-D22 associated with the eyed eggs was 8.2 ± 0.7 PFU mg^{-1} of egg and $3.9 \times 10^2 \pm 1.3 \times 10^1$ PFU mg^{-1} of egg, respectively, at the end of the phage bath (Figure 4B). Subsequently, FpV4 phages were detected only after 24 h (0.9 ± 1.1 PFU mg^{-1} of egg) as no phages were detected in the following samplings. On the contrary, even if the concentration of FPSV-D22 phages in connection with the eggs dropped in the first 24 h (0.1 ± 0.1 PFU mg^{-1} of egg), it subsequently remained stable (46 h: 0.5 ± 0.3 PFU mg^{-1} of egg; 68 h: 0.3 ± 0.1 PFU mg^{-1} of egg). Bacteriophage FpV4 and FPSV-D22 maintained relatively constant concentrations in the well content of the sampled eggs, ranging from $8.0 \times 10^4 \pm 1.3 \times 10^4$ PFU mL^{-1} to $1.2 \times 10^5 \pm 2.5 \times 10^5$ PFU mL^{-1} (FpV4) and from $1.4 \times 10^5 \pm 1.3 \times 10^4$ PFU mL^{-1} to $1.7 \times 10^4 \pm 1.3 \times 10^4$ PFU mL^{-1} (FPSV-D22) during the 144 h incubation (Figure 4B).

To summarize, in this experiment, we observed that the survival of the eyed eggs was not affected by the phage bath (crude lysates) and that the concentration of phages in connection with the eyed eggs decreased over time. While FpV4 phages disappeared after 24 h, it was possible to detect FPSV-D22 phages until the last sampling (68 h).

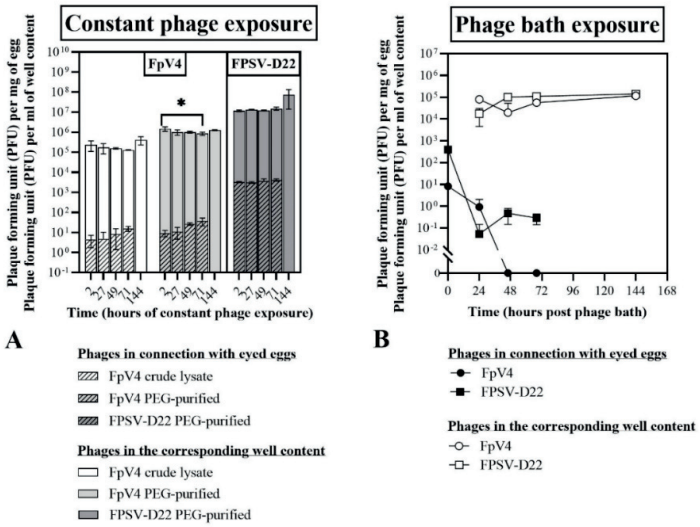

Figure 4. Exp. I and II, section B: phages associated with the eyed eggs and in the corresponding well content (**A**) during constant exposure to phage FpV4 (crude lysate and PEG-purified solutions) and FPSV-D22 (PEG-purified solution) and (**B**) after a 4 h bath exposure to phage FpV4 and FPSV-D22 (1.9×10^7 PFU mL^{-1} FpV4 or 8.2×10^7 PFU mL^{-1} FPSV-D22; crude lysates). Values represent the mean and standard deviation of three biological replicates. At 144 h, phages were quantified only for the well content. In **A**, * = statistically significant differences between the concentration of phages detected at 2 and 71 h in connection with eyed eggs (adjusted $p = 0.0184$) and in the corresponding well content (adjusted $p = 0.0256$). No other statistically significant differences were detected between phage concentrations within each group (**A**).

3.2.3. Bath and Constant Exposure of Eyed Eggs to *F. columnare* Phages (Exp. III, Section B)

The surrounding medium influenced the survival of the eggs (data not shown). While all eggs had died after 96 h incubation in Shieh medium, only 16.67% mortality was observed in water, independent of the presence of phages.

Phages could not be isolated from bath-treated eggs despite the high phage titers in the surrounding liquid (Table 3). Only a few eggs were positive to FCL-2 and FCOV-F27 with a concentration $\leq 10^2$ PFU egg^{-1}. Both phages (FCL-2 and FCOV-F27) could be isolated from the corresponding well content (water/Shieh medium) from the bath, constant phage exposure, and phage control treatments at all the sampling points. The titers varied between 10^5–10^9 PFU mL^{-1} depending on phage, time point, and treatment (Table 3). Shortly, both phages had somewhat higher titers in Shieh medium than in water, FCL-2 had higher titers than FCOV-F27, and constant treatments had higher titers than bath treatments. However, phages did not seem to attach efficiently to the eggs.

Table 3. Exp. III, section B: *F. columnare* infecting phage titers in eggs and the surrounding medium (water or Shieh medium) at 0, 24, and 48 h. Phage counts for two individual samples are provided for each treatment. A "+" indicates a positive detection of phages.

(A) Exposure to Phage FCL-2								
Medium	**Sample**	**Phage exposure**	**Time (h)**					
			0		**24**		**48**	
			No. 1	**No. 2**	**No. 1**	**No. 2**	**No. 1**	**No. 2**
WATER	Well (PFU ml^{-1})	Bath	3.0×10^6	9.0×10^5	3.0×10^6	5.0×10^5	4.0×10^6	3.0×10^6
		Constant	9.0×10^7	1.0×10^9	9.0×10^6	1.0×10^9	3.0×10^7	2.5×10^7
		Control (no egg)	2.3×10^9	1.0×10^9	1.0×10^7	1.0×10^7	1.5×10^7	5.0×10^6
	Egg (PFU egg^{-1})	Bath	0	0	0	0	0	0
		Constant	0	2.0×10^1	2.0×10^2	0	9.3×10^1	5.0×10^1
SHIEH	Well (PFU ml^{-1})	Bath	8.0×10^6	7.0×10^7	2.0×10^8	1.0×10^7	9.0×10^6	7.0×10^7
		Constant	3.5×10^9	2.0×10^9	8.0×10^9	2.0×10^9	4.5×10^9	5.5×10^9
		Control (no egg)	3.5×10^9	2.0×10^9	3.5×10^9	4.0×10^9	2.0×10^9	8.0×10^8
	Egg (PFU egg^{-1})	Bath	0	0	0	0	0	0
		Constant	0	0	0	0	0	0
(B) Exposure to Phage FCOV-F27								
Medium	**Sample**	**Phage exposure**	**Time (h)**					
			0		**24**		**48**	
			No. 1	**No. 2**	**No. 1**	**No. 2**	**No. 1**	**No. 2**
WATER	Well (PFU ml^{-1})	Bath	1.5×10^5	1.0×10^5	+	+	+	4.0×10^5
		Constant	1.3×10^7	5.0×10^6	3.0×10^6	2.0×10^6	+	2.0×10^6
		Control (no egg)	1.5×10^7	2.1×10^7	+	3.0×10^6	1.8×10^7	+
	Egg (PFU egg^{-1})	Bath	0	0	0	0	0	0
		Constant	2.3×10^2	0	5.0×10^0	0	0	0
SHIEH	Well (PFU ml^{-1})	Bath	2.0×10^6	1.0×10^6	6.0×10^6	7.0×10^6	2.0×10^7	1.0×10^7
		Constant	4.5×10^9	1.0×10^9	2.0×10^9	8.0×10^8	1.5×10^9	9.0×10^8
		Control (no egg)	6.0×10^8	5.0×10^8	2.0×10^9	8.0×10^8	3.0×10^9	2.0×10^9
	Egg (PFU egg^{-1})	Bath	0	0	0	0	0	0
		Constant	0	0	0	0	0	0

3.3. Experiments to Evaluate the Use of Phages as Control Agents (Section C)

3.3.1. Phage Bath of *F. psychrophilum* Challenged Eggs (Exp. I, Section C)

After the bacterial challenge with either *F. psychrophilum* 950106-1/1 or the strain 160401/1-5N (sterile TYES-B for the control), eyed eggs were bath-exposed to phages FpV4 and FPSV-D22 (mixed 1:1) for 48 h. Two control baths were included: one containing SM buffer (the buffer where the phages were purified in) and the other with Milli-Q water (to evaluate the effect of the buffer). Subsequently, eyed eggs were moved to 24-well sterile plates containing sterile Milli-Q water (experimental set up in Figure A1). The results of this experiment are presented in Figures 5–7. The time point at which the bacterial bath challenge was finalized is named as 0 h post infection (hpi).

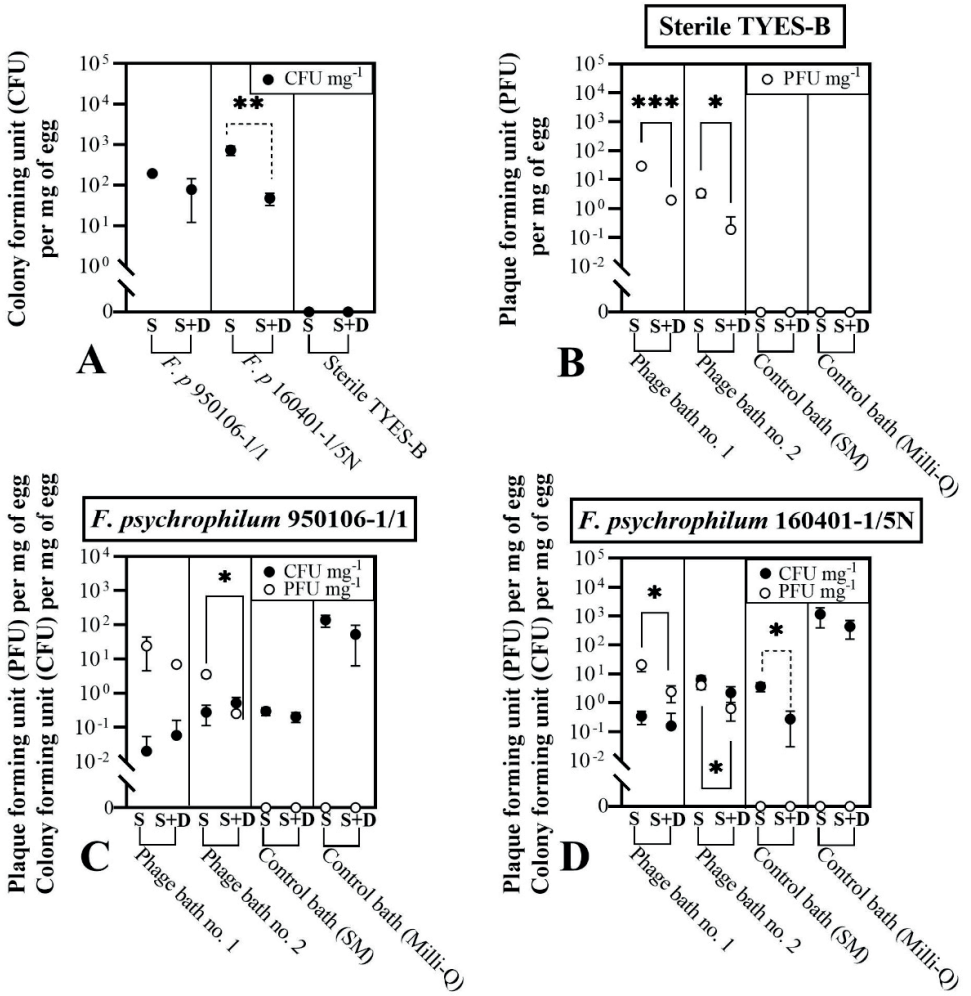

Figure 5. Exp. I, section C: effects of the drying procedure on bacterial and phage concentrations in connection with eyed eggs. Comparison between standard sampling (indicated by "S") and sampling with the additional drying step (indicated by "S + D") for eyed eggs sampled at 0 hpi (**A,** right after the bath challenge) and at 24 hpi (**B**–**D**) that were previously bath-challenged with TYES-B (control, **B**), *F. psychrophilum* 950106-1/1 (**C**) and *F. psychrophilum* 160401-1/5N (**D**). Values represent the mean and standard deviation of three biological replicates. Unpaired t tests of log-transformed values were performed. Statistically significant comparisons (solid lines for phage concentrations and broken lines for bacteria concentrations) are visualized as follows: $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***). Phage bath no. 1: 10^9 PFU mL^{-1}; phage bath no. 2: 10^8 PFU mL^{-1}.

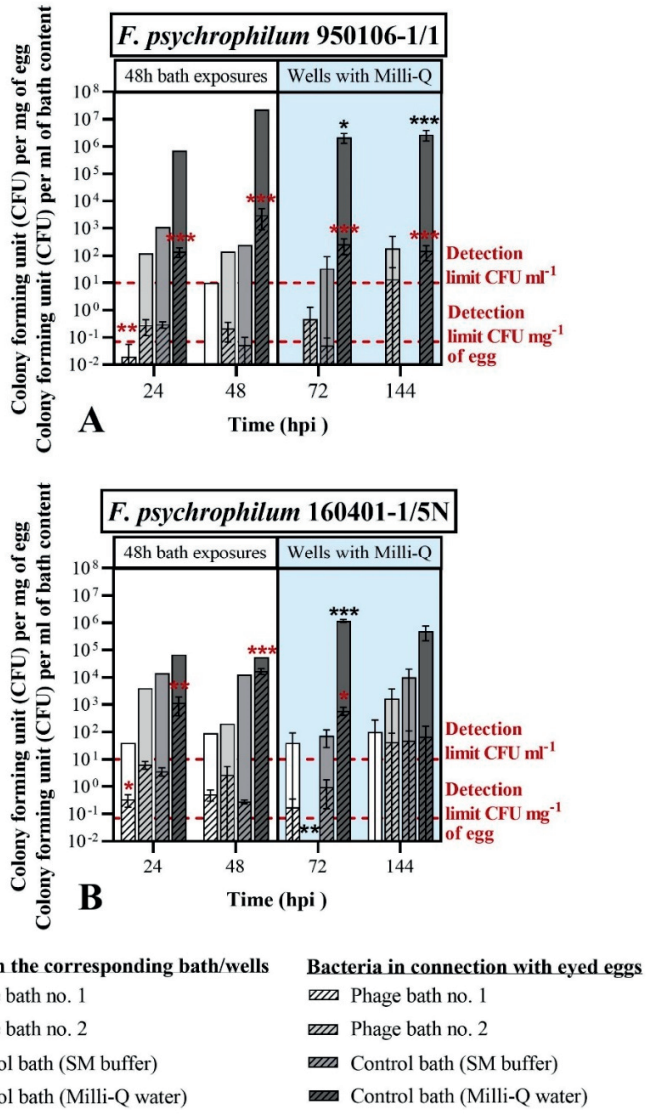

Figure 6. Exp. I, section C: *F. psychrophilum* 950106-1/1 (**A**) and *F. psychrophilum* 160401-1/5N (**B**) in connection with the eyed eggs and in the corresponding bath/well. After the bacterial challenge, eyed eggs were bath-exposed to phages FpV4 and FPSV-D22 mixed 1:1 (phage bath no. 1: 10^9 PFU mL^{-1}; phage bath no. 2: 10^8 PFU mL^{-1}; or control baths containing either SM buffer or Milli-Q water) for 48 h and subsequently moved to 24-well sterile plates containing sterile Milli-Q water (in light blue). Values represent the mean and standard deviation of three biological replicates except for the bath content at 24 and 48 hpi (n = 1). In the control group for bacterial infection (control bath with TYES-B), *F. psychrophilum* was not detected in eyed eggs and in the corresponding bath/wells. For the concentration of bacteria, the detection limit is indicated by red broken lines (calculated as 1 CFU was observed in the undiluted egg (mean weight = 100 mg) or well sample). Unpaired t tests of log-transformed values were performed to compare the tested conditions (phage baths and Milli-Q water-control bath) with the SM buffer-control bath. Statistically significant comparisons are visualized on top of each column (red: eyed egg values; black: well values) as follows: $p < 0.05$ (*), $p < 0.001$ (**), $p = 0.0001$ (***).

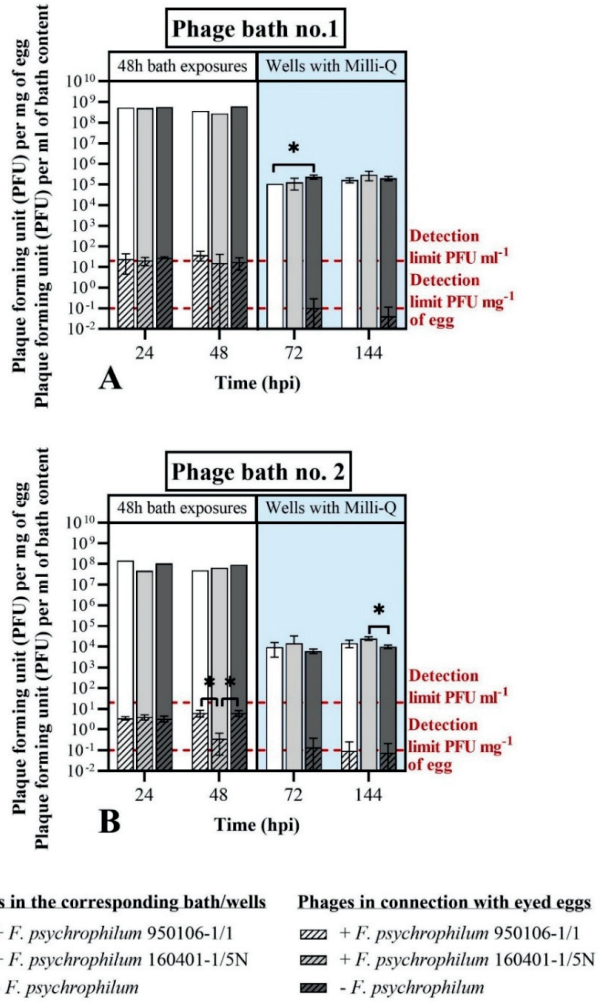

Figure 7. Exp. I, section C: phages FpV4 and FPSV-D22 in connection with the eyed eggs and in the corresponding bath/well following phage bath exposures: (**A**) phage bath no. 1 (10^9 PFU mL^{-1}) and (**B**) phage bath no. 2 (10^8 PFU mL^{-1}). In the phage bath control groups (containing either SM buffer or Milli-Q water), phages FpV4 and FPSV-D22 were not detected. After the bacterial challenge, eyed eggs were bath-exposed to phages FpV4 and FPSV-D22 for 48 h and subsequently moved to 24-well sterile plates containing sterile Milli-Q water (in light blue). Values represent the mean and standard deviation of three biological replicates, except for the bath content at 24 and 48 hpi ($n = 1$). For the concentration of phages, the detection limit is indicated by red broken lines (calculated as 1 PFU was observed in only one of the triplicate spots in the undiluted egg (mean weight = 100 mg) or well sample). Unpaired t tests of log-transformed values were performed to compare the tested conditions. Statistically significant comparisons are indicated as follows: $p < 0.05$ (*), $p < 0.001$ (**), $p = 0.0001$ (***). *F. psychrophilum* 950106-1/1 and the strain 160401-1/5N were used as bacterial hosts for the phage quantification analysis according to which strain was used in the bacterial bath challenge.

The first objective of this experiment was to study the association of phages and bacteria with the surface of the eyed eggs. Thus we compared the number of bacteria and phages per mg of egg at 0 and 24 hpi, sampled with either the standard procedure (S) or including a drying step (S + D), to assess to what extent the bacterial cells and

the phages were firmly attached to the egg surface or associated with the liquid around the eggs (Figure 2 step 2). The results are presented in Figure 5. When the eyed eggs were bath-challenged with *F. psychrophilum* 950106-1/1, $1.9 \times 10^2 \pm 3.2 \times 10^1$ CFU mg^{-1} of egg was found using the standard procedure (S) at 0 hpi, and no significant loss of bacteria by the drying procedure was observed (S + D: $7.7 \times 10^1 \pm 6.5 \times 10^1$ CFU mg^{-1} of egg; $p = 0.0902$) (Figure 5A). This was also observed at 24 hpi (Figure 5C). The bacterial concentrations were 0.3 ± 0.1 and $1.4 \times 10^2 \pm 5.4 \times 10^1$ CFU mg^{-1} of egg in SM buffer and Milli-Q water, respectively, but with no significant difference between the S and S + D treatments. Exposure of the *F. psychrophilum* 950106-1/1-challenged eggs to phages did not influence the effects of the drying step on bacterial abundance.

The concentration of *F. psychrophilum* 160401-1/5N, on the other hand, seemed to be more affected by the inclusion of the drying step, as a 10-fold decrease after drying was detected at 0 hpi (S: $7.3 \times 10^2 \pm 2.0 \times 10^2$ CFU mg^{-1} of egg; S + D: $7.7 \times 10^1 \pm 1.6 \times 10^1$ CFU mg^{-1} of egg; $p = 0.0004$) (Figure 5A). A similar effect was observed at 24 hpi when the eyed eggs were placed in SM buffer (S: 3.6 ± 1.2 CFU mg^{-1} of egg; S + D: 0.3 ± 0.2 CFU mg^{-1} of egg; $p = 0.0056$), but no significant changes were observed in the other groups (Figure 5D). Overall, these findings show that a fraction of the two selected *F. psychrophilum* strains was tightly attached to the eyed eggs' surface and was not detached by the drying step. Additionally, there was a general decrease in egg-associated bacteria over 24 h incubations, even in the SM buffer control groups.

Phages seemed to be less closely attached to the surface of the eyed eggs. The inclusion of the drying step caused a 10- to a 100-fold decrease in phage concentrations in connection with the eyed eggs in each of the tested cases, independent of the presence of the bacteria (Figure 5B–D). For example, the number of phages recorded at 24 hpi in connection with the eyed eggs not exposed to *F. psychrophilum* (sterile TYES-B; Figure 5B) was $2.9 \times 10^1 \pm 2.6$ PFU mg^{-1} of egg for the S procedure compared to 0.7 ± 0.2 PFU mg^{-1} of egg for the S + D procedure ($p < 0.0001$) for eggs bathed in phage bath no. 1 (10^9 PFU mL^{-1}), and 1.4 ± 0.4 PFU mg^{-1} of egg for the S procedure compared to 0.1 ± 0.1 PFU mg^{-1} of egg for the S + D procedure ($p < 0.05$) for eggs bathed in phage bath no. 2 (10^8 PFU mL^{-1}).

To evaluate the ability of phages to control *F. psychrophilum*, the bacterial and phage concentrations in connection with the eyed eggs were measured with the standard sampling procedure (S). Bacteria and phages were quantified on eggs sampled during the phage exposure in the bath treatment (at 24 and 48 hpi) and during the subsequent incubation in wells (at 72 and 144 hpi) (Figures 6 and 7). No negative effect on the eyed eggs' survival was observed in any of the groups, as all the eyed eggs sampled at 24, 48, 72, and 144 hpi were characterized as alive based on movement and turbidity indicators (Figure A3).

The concentration of bacteria per mg of egg was significantly reduced at 24 hpi in the case of phage bath exposure no. 1 (10^9 PFU mL^{-1}) in comparison to the control bath (SM buffer) (Figure 6). In fact, for bath-challenged eyed eggs with *F. psychrophilum* 950106-1/1, the concentration of bacteria associated with the eggs at 24 hpi was 0.02 ± 0.04 CFU mg^{-1} of egg in the phage bath exposure no. 1, compared with 0.3 ± 0.1 CFU mg^{-1} of egg in the case of the SM buffer-bath control ($p < 0.001$), corresponding to a 15-fold reduction in egg-associated bacteria due to the phage treatment (Figure 6A). A similar effect of phage exposure was observed for *F. psychrophilum* 160401-1/5N at 24 hpi, where egg-associated bacteria were reduced from 3.6 ± 1.2 CFU mg^{-1} of egg in the SM buffer-bath control to 0.3 ± 0.2 CFU mg^{-1} of egg in the phage bath exposure no. 1 ($p = 0.0022$, Figure 6B).

Additionally, phage exposure reduced the bacterial abundance at 24 hpi in the bath content for both bacteria (Figure 6A,B). For *F. psychrophilum* 950106-1/1 no bacteria was detected in the phage bath no. 1 at 24 hpi, whereas 1.1×10^3 CFU mL^{-1} ($n = 1$) were present in the SM buffer-bath control (Figure 6A). Similarly, the abundance of strain 160401-1/5N was reduced from 1.4×10^4 CFU mL^{-1} in the SM buffer-bath control to 40.0 CFU mL^{-1} in phage bath no. 1 ($n = 1$, Figure 6B). These findings support the ability of FpV4 and FPSV-D22 to reduce the *F. psychrophilum* abundance at 24 hpi both on the egg surface and in the surrounding water. However, this effect of phage

exposure was only temporary, as no significant difference between the bacterial abundances of the phage baths and the SM buffer-bath controls were observed at the following time points (48, 72, and 144 hpi). In addition, the growth and stability of the bacteria seemed to be increasingly negatively affected by incubation in the SM buffer-control bath compared to the Milli-Q water-control bath over time. As an example, at 72 hpi, the bacterial abundances detected in connection with the eyed eggs was 0.1 ± 0.1 and $2.6 \times 10^2 \pm 1.5 \times 10^2$ CFU mg^{-1} of egg for eyed eggs previously bathed in SM buffer and Milli-Q water, respectively, ($p < 0.0001$) (bath-challenged eyed eggs with *F. psychrophilum* 950106-1/1). The bacterial concentration in the corresponding wells containing the eggs was also significantly decreased (SM buffer bath-control: $3.3 \times 10^1 \pm 5.8 \times 10^1$ CFU mL^{-1}; Milli-Q water-control bath: $2.1 \times 10^6 \pm 8.4 \times 10^5$ CFU mL^{-1}; $p < 0.05$). A similar trend was observed for bath-challenged eyed eggs with *F. psychrophilum* 160401-1/5N at 72 hpi (bacteria associated with eyed eggs: 1.0 ± 0.8 CFU mg^{-1} of egg in the SM buffer bath control and $6.0 \times 10^2 \pm 2.0 \times 10^2$ CFU mg^{-1} of egg in the Milli-Q water control bath—$p = 0.0027$). This was also the case in the corresponding wells where $7.3 \times 10^1 \pm 4.6 \times 10^1$ CFU mL^{-1} were found in the SM buffer bath control compared to $1.2 \times 10^6 \pm 1.4 \times 10^5$ CFU mL^{-1} in the Milli-Q water control bath ($p < 0.0001$). The detection of bacteria/fungi other than *F. psychrophilum* was observed during the CFU enumeration and recorded (Figure A4).

The concentration of the two bacterial strains associated with the bath-challenged eyed eggs at 24 and 48 hpi in the Milli-Q water-control bath varied significantly with *F. psychrophilum* 950106-1/1, occurring in 10-fold lower numbers ($1.4 \times 10^2 \pm 5.4 \times 10^1$ and $3.1 \times 10^3 \pm 2.2 \times 10^3$ CFU mg^{-1} of egg at 24 hpi and 48 hpi, respectively) than strain 160401-1/5N ($1.2 \times 10^3 \pm 7.8 \times 10^2$ and $1.7 \times 10^4 \pm 4.0 \times 10^3$ CFU mg^{-1} of egg at 24 and 48 hpi, respectively) (24 hpi: $p = 0.0030$; 48 hpi: $p = 0.0080$), suggesting different adherence properties of the two strains (Figure 6).

As previously observed and mentioned in this results section (Figure 5), the phages FpV4 and FPSV-D22 did not seem to tightly connect with surface of the eyed eggs in this experiment. Even if the concentration of phages in connection with the eyed eggs was ~10^1 PFU mg^{-1} (Phage bath no. 1) and ~10^0 PFU mg^{-1} (phage bath no. 2) during the 48 h phage bath, very few eggs were positive to phages in the next sampling points (72 and 144 hpi) (Figure 7). However, FpV4 and FPSV-D22 were constantly detected over time, and their concentration was maintained in the baths and the wells, independent of the presence of the bacteria.

To summarize, the findings of this experiment showed that the two selected *F. psychrophilum* strains closely interact with the eyed eggs' surface, but with different efficiencies. Furthermore, exposure of the challenged eggs to phages showed a 12- to 15-fold reduction in egg-associated bacteria for 24 h. However, the growth and stability of the bacteria were negatively affected in the SM buffer bath at all the time points, and the controlling effects of phages on the egg-associated bacteria were not maintained beyond 24 h.

3.3.2. Phage Exposure of *F. columnare* Challenged Eggs (Exp. II, Section C)

The effects of phages on bacteria associated with eggs and their immediate proximity was also assessed with *F. columnare* (experimental set up in Figure A2). As for *F. psychrophilum*, most of the eggs survived until the end of the experiments (Figure S2). Some eggs hatched during the experiment.

In contrast to the experiments in section A (Figure S1), *F. columnare* was not isolated from any of the medium or egg samples taken at any sampling time point. It can thus be inferred that there was no growing or infective *F. columnare* in the treatments during the experiment, probably since the experiment was conducted at 10 °C. However, colonies of other environmental bacteria were observed (data not shown).

Phages were only isolated from egg samples in low titers right after phage bath exposure experiment (Figure 8). One should notice that, in this experiment, eggs were not homogenized as in the previous experiments, but only vortexed in a fixed amount of water, which was then used for the phage and bacterial quantifications. Phages were isolated

from well samples (n = 3) during constant phage exposure at all sampling points with a stable concentration, independent of the presence of *F. columnare* (e.g., 48 h in the case of the bacterial challenge: $9.3 \times 10^4 \pm 7.8 \times 10^4$ PFU mL^{-1}). After the phage bath, it was possible to detect phages at 0 and 48 h in only one of the three wells.

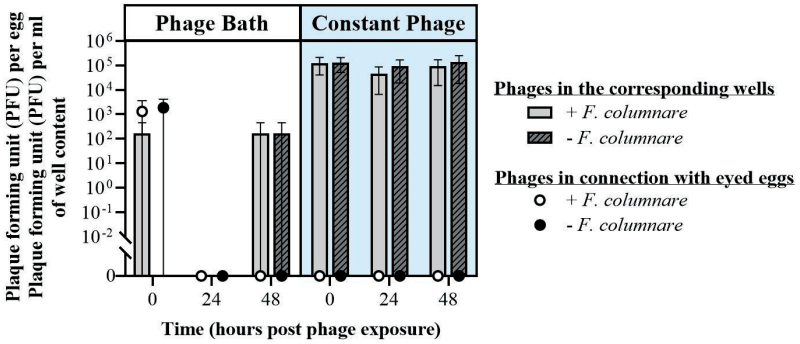

Figure 8. Exp. II, section C: phage FCL-2 in connection with the eyed eggs and in the corresponding wells following phage exposures (bath and constant) with and without bacterial challenge. In the phage control groups (sterile NaCl 0.09%; bath and constant experiments), phage FCL-2 was detected neither in the wells nor in the egg samples. Values represent the mean and standard deviation of three biological replicates. Unpaired t tests of log-transformed values were performed to compare the observed phage titers in the wells with and without bacterial exposure (constant phage exposure). No statistically significant difference was detected ($p > 0.05$).

4. Discussion

Our study aimed to evaluate the interactions between *Flavobacterium* spp. and rainbow trout eyed eggs and the potential of phages as control agents for these pathogens.

4.1. Experimental Infection Method

Although distant from the hatchery environment, the developed experimental set up allows for the study of bacterial and phage interactions with eyed eggs at a small scale under controlled conditions, as well as the production of reproducible results, meaning that the experimental set up might also be applied for other pathogenic bacteria.

No evident negative effects on survival were detected when eyed eggs were exposed to *F. psychrophilum* in our experiments (Section A and C), supporting previous findings of [46], in which no egg mortality was observed prior to hatching in the bacteria-challenged rainbow trout eyed eggs. However, the mortality of post-swim up fry exposed to *F. psychrophilum* was significantly higher than the controls in that study [46]. A different infection method was chosen by Ekman et al. [47], where the nano-injection of *F. psychrophilum* into the yolk of fertilized rainbow trout eggs was performed with the aim of mimicking the vertical transmission of this pathogen. In this study, significantly higher mortality rates were observed for the eggs exposed to the pathogen compared to the controls. However, this method bypasses the immune adaptive response (which is in a stage of development) and the physical barriers of the eyed egg (chorion and membranes). In addition, the vertical transmission and the intra ovum presence of this bacterium in rainbow trout has not been clearly demonstrated [11,48].

F. psychrophilum did not grow actively in water, but its concentration remained stable up to 13 days after inoculation (Figure 3B). This was in agreement with previous studies [49,50], where the concentration of *F. psychrophilum* in stream water and sterilized natural freshwater (measured by CFU count) remained stable for 116 days [49] and for 300 days [50], respectively. However, an initial drop in the bacterial concentration was detected in [50]. In [49], the authors observed that the number of viable bacterial cells was

higher (viable but non-culturable; measured by a viability assay) than the one enumerated with CFU count, suggesting that *F. psychrophilum* may undergo a starvation phase. Even after 9 months, cells were resuscitated in *Cytophaga* broth, regaining their initial morphology [49]. Similar observations have been recorded for *F. columnare* [51].

Experiments with eggs and *F. columnare* showed that the presence of nutrients (Shieh medium) had an adverse effect on egg survival, and maybe more importantly, that their optimum temperature does not match. While *F. columnare* grows well in high (above +18 °C) temperatures, this is not a suitable temperature for egg viability (the incubation of eyed eggs at temperatures higher than 12 °C can cause the development of skeletal deformities [52]). It is thus unlikely that *F. columnare* would cause problems in rainbow trout eyed eggs. However, different bacterial strains may have the ability to also grow at these lower temperatures [44], therefore the interactions between *F. columnare* and rainbow trout eggs might occur [53]. Furthermore, since this pathogen is also present in warm countries and tropical fish [54,55], and *F. columnare* has been previously found in association with Chinook salmon eggs [56], it remains relevant to study the association of bacteria and their phages in fish eggs. Indeed, *F. columnare* was isolated from all treatments (Figure S1), suggesting potential interactions in the hatchery conditions where conditions favor the presence of this bacterium.

Growth of bacteria/fungi other than the one of interest was detected (section A,B and C) and it was more prominent in experiments performed during late spring (including not published data), suggesting seasonal changes in the microbial community surrounding the chorion of the eyed eggs [57]. In addition, the lysis of bacterial cells caused by the phages releases nutrients and may stimulate the growth of other bacteria, as suggested by [58]. However, bacterial growth other than *F. psychrophilum* was detected independently of phage presence in the case of bacteria-challenged eggs (Exp. I section C, Figure 4B). Less detection of bacteria other than *F. psychrophilum* was observed in the control group for the bacterial infection. It is known that the iodine disinfection, a standard disinfection method for salmonid eyed eggs in hatcheries, does not create a sterile environment [56,59]. However, the use of higher iodine concentrations than the ones used are not recommended since this may compromise the survival of the eggs after the treatment. The growth of a background bacterial community was previously observed in phage studies in challenge experiments with *Vibrio* spp. and fish larvae [58,60]. Here, a positive effect of phages on the survival of *Vibrio*-challenged turbot and cod larvae were detected, despite a relatively high mortality caused by the background larval-associated bacterial community [36]. In our experiments, a correlation between higher mortality and the detection of other bacteria was not observed.

It is important to be aware that our experimental approach is only valid for short-term disinfection efficiency experiments, and does not consider the effects on overall survival or the hatching rate of the eyed eggs, and other factors, e.g., oxygen requirements (in salmonid eggs the hatching of the eggs happens faster in conditions of asphyxia) [61], may be influencing these parameters.

4.2. Rainbow Trout Eyed Eggs–Bacteriophage Interactions

The virulent phages FpV4 and FPSV-D22 targeting *F. psychrophilum* do not seem to affect the survival of rainbow trout eyed eggs (Table 2), as rainbow trout eyed eggs could tolerate the presence of bacteriophages under the tested conditions. These effects were observed for up to 71 h when phages were diluted in TYES medium (Exp. I section B), and up to 144 h when in Milli-Q water (Exp. II section B), and thus indicated that phage applications for *F. psychrophilum* control do not have a negative impact on egg survival for the tested time period. Similar results were obtained by Silva et al. [60], where the exposure of zebrafish larvae to *Vibrio* phages did not negatively affect the survival of the larvae. However, the embryo movement was not observed for a higher number of eyed eggs exposed to the phage FpV4 in crude lysate compared to the other groups (Exp. I

section B), suggesting that PEG-purified solutions should be chosen over crude lysates for long-term exposures.

The qualitative and quantitative analysis of phages showed that it was possible to detect FpV4 and FPSV-D22 associated with eyed eggs after both constant (Exp. I section B) and short-term bath exposure (Exp. II section B). While the concentration of phages associated with the eggs was maintained over time during constant exposure (Figure 4A), it decreased after 24 h post-phage bath (Figure 4B) suggesting that phages do not tightly interact with the surface of the eggs. In particular, while a significant increase in the phage FpV4 concentration was detected during constant PEG-purified phage exposure, FpV4 in connection with eyed eggs was detected at very low titers 24 h after the phage bath, and could not be detected in the following samplings. The concentration of phages in the surrounding medium was constant. In contrast, FPSV-D22 in connection with the eyed eggs was detected for a longer period after the bath procedure and in a higher concentration compared to FpV4 during constant exposure experiments. These differences likely reflect differences in adherence and stability of the two phages during the interactions with the egg membranes. FpV4 belongs to the Podoviridae family with very short tails, whereas FPSV-D22 is a Siphoviridae [27,36,62] with long flexible tails, and these differences in phage morphology may affect their adherence to biotic surfaces. In addition, the time of exposure seems to represent an important variable.

Similar results were obtained with phages infecting *F. columnare* (FCL-2 and FCOV-F27): phages were detected from eggs at very low titer, while maintaining high concentrations in the surrounding medium. Further investigations are needed to shed light on this matter. Indeed, the binding of *F. columnare* phages on mucins found in mucosal surfaces have provided promising results for phage-based bacterial control and prophylaxis [63]. However, it is unclear if similar mucin glycoproteins are present on egg surfaces, which also is distinct to the mucosal secretion of fish skin. Therefore, the phages may not strictly bind to the chorion, but rather survive in the surrounding environment. Nevertheless, the presence of pathogen-targeting phages in the proximity of the eggs may prevent the bacterial infection after hatching.

4.3. Phages as Control Agents for F. psychrophilum in Eyed Eggs

The combined action of FpV4 and FPSV-D22 demonstrated the ability to reduce the number of bacteria associated with the eyed eggs and contained in the corresponding bath/well during the first 24 hpi (Figure 6, Phage bath no. 1: 10^9 PFU mL^{-1}). However, this controlling effect of the phages was only temporary, and the observed negative effect of the SM buffer on *F. psychrophilum* growth (more markedly for *F. psychrophilum* 950106-1/1), likely overshadowed the effects of the phage treatment after 24 h. The inhibiting effect of the buffer was thought to be related to the NaCl concentration in this buffer (0.6%). Previous studies have shown that *F. psychrophilum* can tolerate NaCl concentrations in the range 0.5–1.0%, but these properties vary among strains [29,64]. However, more studies are required to assess the potential of phage control on time scales beyond 24 h, using different incubation media that do not inhibit bacterial growth in the control cultures. The detection of *F. psychrophilum* colonies after the initial decrease in phage-treated groups may indicate the development of phage-resistant mutants (Figure 6A). In [60], zebrafish larvae (chosen as biological model system) exposed to phage VP-2 were characterized by a significantly lower mortality than the ones challenged with *Vibrio* only. The authors also observed the growth of some phage-resistant mutants of the pathogenic bacteria with a different morphology, which are generally characterized by a loss of virulence.

If *F. psychrophilum* growth was negatively affected by the SM buffer, a 10-fold increase in *F. psychrophilum* cells associated with the eyed eggs was instead detected during the 48 h of bathing in the Milli-Q water-control bath (Figure 6). Knowing that this bacterium does not grow actively in water, the reduced water flow was thought to stimulate the overgrowth of the pathogen on the egg surface. In addition, starved cells of *F. psychrophilum* have been shown to adhere to unfertilized eggs in higher numbers [65]. Moreover, no significant

difference in the *F. psychrophilum* concentration was detected when including a drying step in the sampling procedure (Figure 5). All these findings suggest that these bacteria were indeed directly associated with the egg surface. Moreover, cells of *F. psychrophilum* 160401-1/5N adhered to the eyed eggs in a higher number than *F. psychrophilum* 950106-1/1 (Figure 5), suggesting that strain-specific differences in cell-adherence properties may be due to specific properties of the isolates. A previous study have shown large differences in adherence properties between different *F. psychrophilum* strains [31], but that analysis based on using polystyrene surfaces did not find different adhesion properties of the strains 160401-1/5N and 950106-1/1 used in the current study. Despite that, the ability to adhere to polystyrene surfaces is likely not directly comparable to their ability to colonize fish eggs.

4.4. Phages as Control Agents for F. columnare in Eyed Eggs

F. columnare has been found in the eggs and ovarian fluids of Chinook salmon (*Oncorhynhus tshawytscha*) [66,67]. Here, we tested if phage baths can control *F. columnare* in relation to eyed eggs, either given as prophylactic treatments or following exposure to bacteria. Although detected in the first experiments described above (Figure S1), *F. columnare* was not isolated from any of the medium or egg samples taken at any sampling time point in later experiments (Table 3 and Figure 8). This was probably caused by the experimental temperature (10 °C) in those experiments, which was too low for the bacterium. Similarly, in a study by Barnes et al. (2009) [56], *F. columnare* was found to interact with eggs, but the bacteria had no effect on salmonid egg survival at temperatures between 10–12 °C. Indeed, the adhesion, replication, and virulence characteristics of this bacterial species are strongly dependent on temperature [16,68,69], and the lack of bacterial growth in our experiment hampered the assessment of the effect of phages on the prevention of this bacterium. Yet, as an encouraging fact, neither bacterial nor phage addition had any adverse effects on egg survival. Constant phage treatments yielded 10^5 PFU per mL titers up to 48 h. However, as mentioned above, while the optimum temperatures for *F. columnare* and salmonid eggs do not match, the results obtained in this study may be beneficial for warm water fish species, suggesting a need for similar experiments in such species.

5. Conclusions

To the authors' knowledge, the present work represents the first study exploring the potential of using bacteriophages to control *Flavobacterial* pathogens in relation to salmonid eyed eggs. The results demonstrated a strong potential for short term (24 h) phage control of *F. psychrophilum* colonization of rainbow trout fry eggs. However, further studies are needed to explore if phage control can be maintained beyond 24 h and to better understand the mechanisms of interaction between flavobacteria and their phages in connection with rainbow trout eyed eggs. For example, microscopy based methods to visualize the interactions could be used.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9050971/s1. Figure S1: Survival of rainbow trout eggs at different temperatures with and without exposure to *F. columnare*, in either water or Shieh medium (Section A); Figure S2: Exp. II, section C: survival and hatching percentage recorded at the end of the experiment (144 h).

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Data Availability Statement: The data presented in this study are available in this article and in the supplementary material.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Experiments in section A concerning *F. psychrophilum*. Characteristics of sampled eggs during Exp. no. 1, no. 2, and no. 3. hpi = hours post infection. In exp. no. 1, movement and turbidity indicators of sampled eggs were not recorded at 1 and 3 hpi.

	Time (hpi)	Evaluated Parameters	*+ F. psychrophilum*			*− F. psychrophilum*		
			No. 1	**No. 2**	**No. 3**	**No. 1**	**No. 2**	**No. 3**
Exp. no. 1	21	Movement	(+)	+	+	+	+	+
		Turbidity	(+)	-	(+)	-	-	-
	25	Movement	+	+	+	+	+	+
		Turbidity	(+)	(+)	(+)	-	-	-
Exp. no. 2	0	Movement	+	+	+	+	+	+
		Turbidity	-	-	-	-	-	-
	24	Movement	+	-	+	+	+	+
		Turbidity	-	(+)	-	-	-	-
Exp. no. 3	0	Movement	+	+	+	+	+	+
		Turbidity	-	-	-	-	-	-
	24	Movement	+	-	+	+	+	+
		Turbidity	-	-	-	-	-	-

+ Positive to movement or turbidity; (+) Weak movement/light turbidity; - Negative to movement or turbidity.

Table A2. Experiments in section A concerning *F. psychrophilum*. Bacterial growth other than *F. psychrophilum* in sampled eyed eggs and the corresponding bath/well. A plus symbol with an orange background indicates a positive detection of bacterial colonies in TYES-B and/or on Blood-A ("-" = no growth).

	Time (hpi)	Sample (Medium Type)	*+ F. psychrophilum*			*− F. psychrophilum*		
			No. 1	**No. 2**	**No. 3**	**No. 1**	**No. 2**	**No. 3**
Exp. no. 1	**25**	Egg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		Well (Blood-A)	-	-	-	-	-	-
Exp. no. 2	**0**	Egg (TYES-A)	+	-	+	+	+	-
		Bath (Blood-A)		-			-	
	24	Egg (TYES-A)	-	-	-	+	+	-
		Well (TYES-A; Blood-A)	-; -	-; -	-; -	+; -	-; -	-; -
Exp. no. 3	**0**	Egg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		Bath (Blood-A)		-			-	
	24	Egg (TYES-A)	+	-	-	+	+	+
		Well (TYES-A)	-	-	-	+	+	+

n.d = not determined.

Table A3. Exp. I section B. Bacterial growth in the well of sampled eyed eggs was assessed during the experiment. A plus symbol with an orange background indicates a positive detection of bacterial colonies in TYES-B and/or on Blood-A ("-" = no growth). *F. psychrophilum* was not detected.

Time (h)	Medium Type	+ FpV4						+ FPSV-D22			Control		
		Crude Lysate			PEG-Purified			PEG-Purified					
		No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
2	TYES-A	-	-	-	-	-	-	-	-	-	-	-	-
	Blood-A	-	-	-	-	-	-	-	-	-	-	-	-
27	TYES-A	+	-	-	+	-	-	-	-	-	-	-	-
	Blood-A	+	-	-	+	-	-	-	-	-	-	-	-
49	TYES-A	-	-	-	-	-	-	-	-	-	-	-	-
	Blood-A	+	-	+	-	-	-	+	-	+	-	+	-
71	TYES-A		n.d.			n.d.			n.d.			n.d.	
	Blood-A	-	-	-	-	-	-	-	-	-	-	-	-
144	TYES-A		n.d.			n.d.			n.d.			n.d.	
	Blood-A	-	-	-	-	-	+	+	-	-	+	-	-

n.d. = not determined.

Table A4. Exp. II section B. Bacterial growth in the well of sampled eyed eggs was assessed during the experiment. A plus symbol with an orange background indicates a positive detection of bacterial colonies in TYES-B and/or on Blood-A ("-" = no growth). *F. psychrophilum* was not detected.

Time (h)	Medium Type	+ Crude Lysate						Control		
		+ FpV4			+ FPSV-D22					
		No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
0 *	TYES-A		-			-			-	
	Blood-A		-			-			-	
24	TYES-A	-	-	-	-	-	-	-	-	-
	Blood-A	-	-	-	-	-	-	-	-	-
46	TYES-A	-	-	-	-	-	-	-	-	-
	Blood-A	-	-	-	-	-	-	-	-	-
68	TYES-A		n.d.			n.d.			n.d.	
	Blood-A	-	-	-	-	-	-	-	+	-
144	TYES-A		n.d.			n.d.			n.d.	
	Blood-A	-	-	+	+	-	-	+	+	-

* Bath content; n.d. = not determined, not sampled.

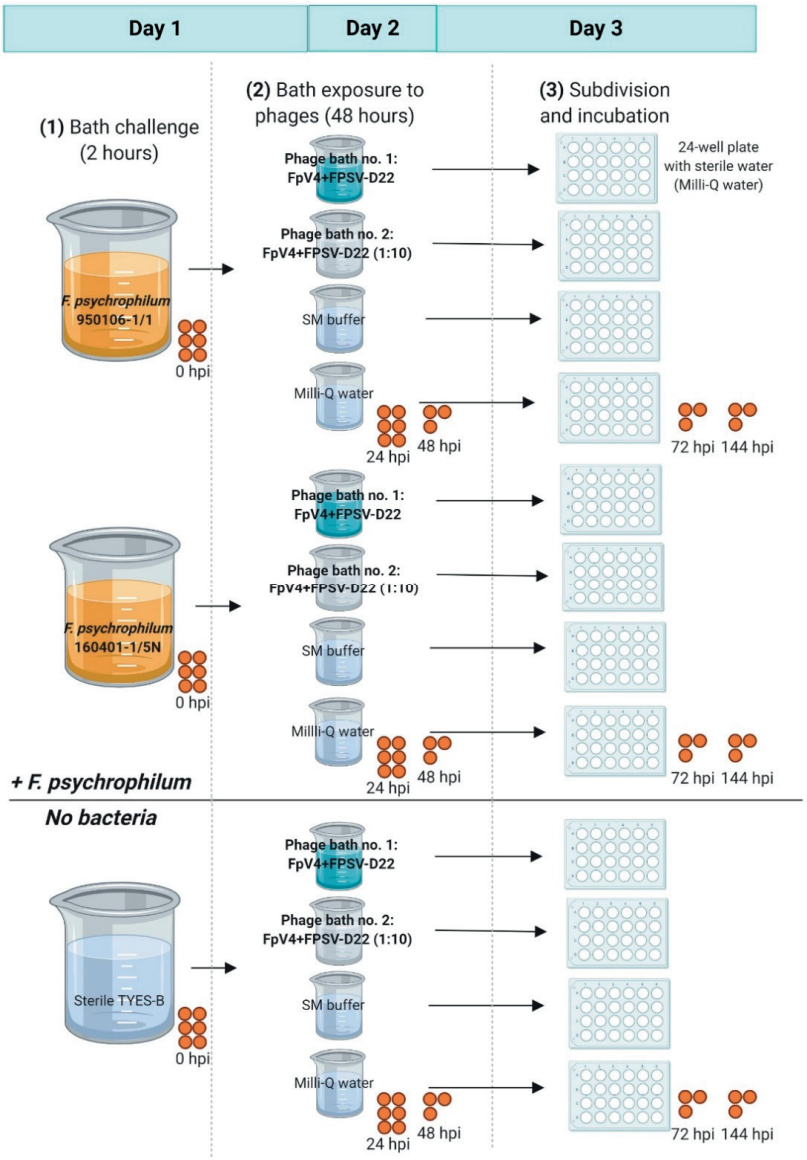

Figure A1. Exp. I section C: experimental set up. (**1**) Rainbow trout eyed eggs were bath-challenged for 2 h with either *F. psychrophilum* strain 950106-1/1 or the strain 160401-1/5N (2.0×10^6 CFU mL^{-1}), while control eggs were placed in sterile TYES-B. The procedure was performed in 600 mL sterile glass beakers containing 200 mL of the selected solution (135 eggs per beaker). (**2**) Eyed eggs were exposed to phages FpV4 and FPSV-D22 (mixed 1:1) for 48 h by bath: phage bath no. 1 (10^9 PFU mL^{-1}) and phage bath no. 2 (10^8 PFU mL^{-1}). Phage bath controls with SM buffer and Milli-Q water were included. The procedure was performed in 250 mL sterile glass beakers (30 eggs per beaker) containing the selected solution (20 mL). (**3**) Eyed eggs were divided in 24-well plates containing 2 mL of sterile Milli-Q water (one egg per well). For each step, eyed eggs were incubated at 10 °C at 80–90 RPM. Eyed eggs were sampled during the experiment as indicated by the round orange circles resembling trout eyed eggs. At 0 and 24 hpi, six eggs were sampled to compare the standard sampling procedure to the one including a drying step. hpi = hours post infection. Created with Biorender.com (the figure was exported under a paid license subscription).

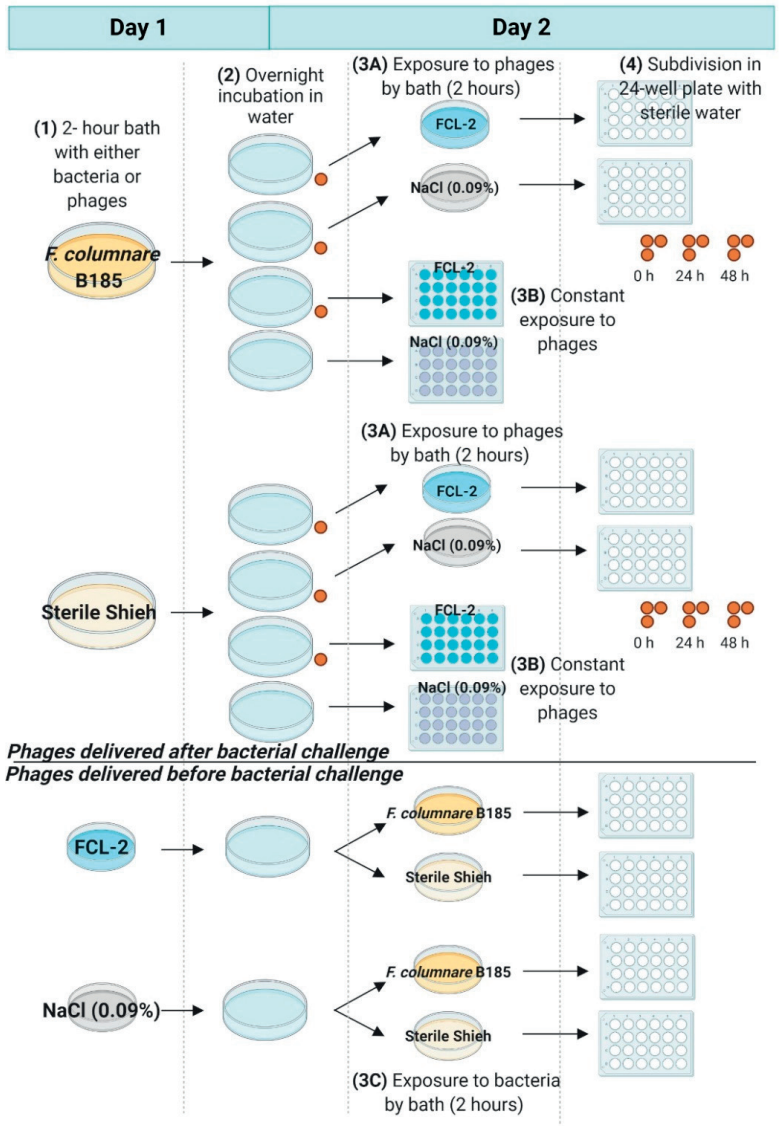

Figure A2. Exp. II section C: experimental set up. Rainbow trout eyed eggs were exposed to phages after (upper panel) or before bacterial challenge (lower panel). (**1**) Eyed eggs were bathed for 2 h with *F. columnare* B185 (5.0×10^6 CFU mL^{-1}) or sterile Shieh medium (upper panel) in 140 mm Ø Petri dishes (97–99 eggs per dish–100 mL volume), and with the phage FCL-2 (2.5×10^7 PFU mL^{-1}) or NaCl (0.09%) (lower panel) in 90 mm Ø Petri dishes (24 eggs per dish–35 mL volume). (**2**) Eggs were moved into 140 mm Ø Petri dishes (22–25 eggs per dish–100 mL volume) with water and incubated overnight. (**3**) Eyed eggs previously exposed to *F. columnare* were either (**3A**) bathed for 2 h or (**3B**) moved directly into 24-well plates in either FCL-2 phage solution (2.5×10^7 PFU mL^{-1}) or NaCl (0.09%) (24 eggs per group) (upper panel). (**3C**) Eyed eggs previously exposed to phages were bath-exposed to *F. columnare* strain B185 (5.0×10^6 CFU mL^{-1}) or sterile Shieh medium for 2 h (12 eggs per group) (lower panel). Bath exposures to either phages or bacteria were performed in 90 mm Ø Petri dishes (35 mL volume). (**4**) Following the 2 h bath, eyed eggs were transferred into 24-well plates containing 2 mL of water (one per well). Eyed eggs were sampled during the experiment as indicated by the round orange circles resembling trout eyed eggs. h = hours post phage exposure. Created with Biorender.com (the figure was exported under a paid license subscription).

Time (hpi)	Evaluated parameters	*F. psychrophilum* 950106-1/1 Bath no. 1 Egg no. 1 2 3	Bath no. 2 Egg no. 1 2 3	Bath no. 3 Egg no. 1 2 3	Bath no. 4 Egg no. 1 2 3	*F. psychrophilum* 160401-1/5N Bath no. 1 Egg no. 1 2 3	Bath no. 2 Egg no. 1 2 3	Bath no. 3 Egg no. 1 2 3	Bath no. 4 Egg no. 1 2 3	Negative control (without *F. psychrophilum*) Bath no. 1 Egg no. 1 2 3	Bath no. 2 Egg no. 1 2 3	Bath no. 3 Egg no. 1 2 3	Bath no. 4 Egg no. 1 2 3
24	Movement	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	Turbidity	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
48	Movement	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	Turbidity	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
72 (24 h in wells)	Movement	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	Turbidity	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
144 (96 h in wells)	Movement	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	Turbidity	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -

\+ Positive to movement or turbidity
(+) Weak movement/light turbidity
\- Negative to movement or turbidity

Figure A3. Exp. I section C: characteristics of sampled eggs. hpi = hours post infection. Bath no. 1: phage bath with of 10^9 PFU mL^{-1}; Bath no. 2: phage bath with 10^8 PFU mL^{-1}; Bath no. 3: control bath with SM buffer; Bath no. 4: control bath with Milli-Q water.

Time (hpi)	Sample	Type of medium	*F. psychrophilum* 950106-1/1 Bath no. 1 no. 1 2 3	Bath no. 2 no. 1 2 3	Bath no. 3 no. 1 2 3	Bath no. 4 no. 1 2 3	*F. psychrophilum* 160401-1/5N Bath no. 1 no. 1 2 3	Bath no. 2 no. 1 2 3	Bath no. 3 no. 1 2 3	Bath no. 4 no. 1 2 3	Negative control (without *F. psychrophilum*) Bath no. 1 no. 1 2 3	Bath no. 2 no. 1 2 3	Bath no. 3 no. 1 2 3	Bath no. 4 no. 1 2 3
24	Egg	TYES-A	+ - -	- - -	- - -	- + -	- - -	+ + +	- - +	- - -	- - -	- - -	- - -	- - -
		Blood-A	- - -	+	- - -	- - -	+	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	Bath	TYES-A	-	-	-	-	-	+	-	-	-	-	-	-
		Blood-A	-	-	-	-	-	+	-	-	-	-	-	-
48	Egg	TYES-A	- - -	- - -	- + -	- - -	+ + +	+ + +	- - -	+ - +	- - -	- + -	- - -	- - -
		Blood-A	- - -	- - -	+ - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	Bath	TYES-A	-	-	+	-	-	+	-	+	-	-	-	-
		Blood-A	-	-	-	-	-	+	-	-	-	-	-	-
72 (24 h in wells)	Egg	TYES-A	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	- + -	+ +	- - -	+ + +
		Blood-A	+ + +	+ + +	+ + +	+ + +	+ + -	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	- + -
	Well	TYES-A	- + +	- - -	- - +	- - -	+ - -	+ + +	- - -	+ + +	- - -	- - -	- - -	- - -
		Blood-A	- - -	- - -	- - -	- - -	- - -	+ + +	- - -	- - -	- - -	- - -	- - -	- - -
144 (96 h in wells)	Egg	TYES-A	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		Blood-A	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ - +	+ + +	+ + -	+ + +
	Well	TYES-A	+ - -	- + -	+ + +	+ + +	+ + +	+ + +	- - -	+ + +	- - -	- - -	+ - -	+ + +
		Blood-A	+ + +	- + -	+ + +	+ + +	+ + +	+ + +	- + -	+ + +	- - -	- - -	- - -	- - -

Figure A4. Exp. I section C: bacterial growth other than *F. psychrophilum* in sampled eyed eggs and the corresponding bath/wells was recorded. A plus symbol with an orange background indicates a positive detection of bacterial colonies in TYES-B and/or on Blood-A ("-" = no growth). Bath no. 1: phage bath with of 10^9 PFU mL^{-1}; Bath no. 2: phage bath with 10^8 PFU mL^{-1}; Bath no. 3: control bath with SM buffer; Bath no. 4: control bath with Milli-Q water.

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Supplementary Material: Interactions between Rainbow Trout Eyed Eggs and *Flavobacterium* spp. Using a Bath Challenge Model: Preliminary Evaluation of Bacteriophages as Pathogen Control Agents

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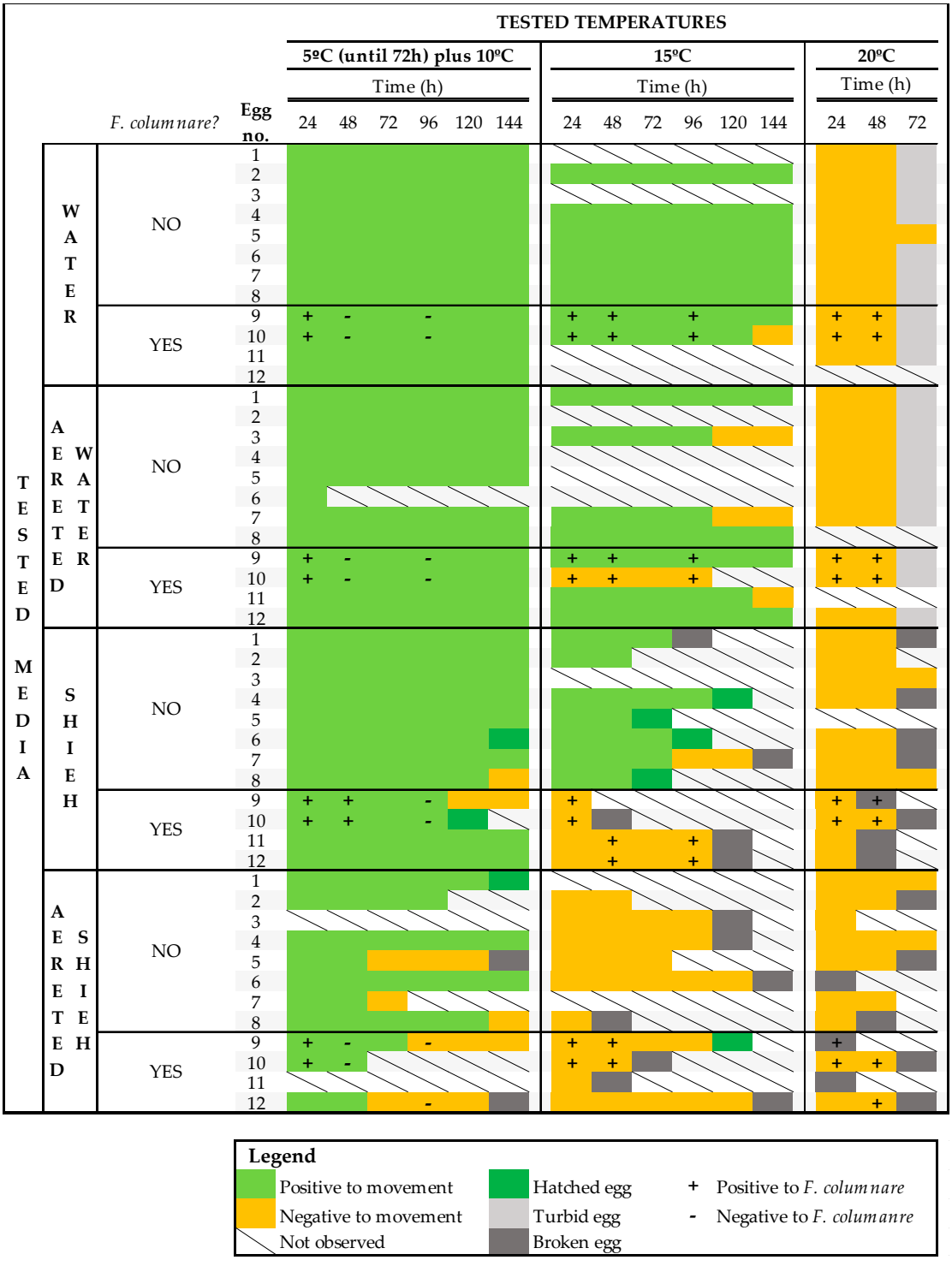

Figure S1. Survival of rainbow trout eggs at different temperatures with and without exposure to *F. columnare*, in either water or Shieh medium (Section A).

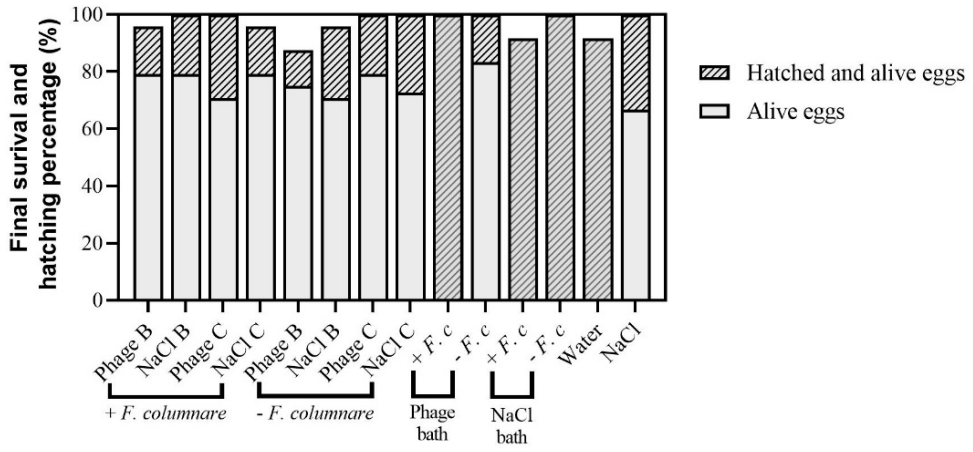

Figure S2. Exp. II, section C: survival and hatching percentage recorded at the end of the experiment (144 h). B: bath exposure, C: constant exposure, *F. c* = *F. columnare* B185.

V

COMPARISON OF DELIVERY METHODS IN PHAGE THERAPY AGAINST *FLAVOBACTERIUM COLUMNARE* INFECTIONS IN RAINBOW TROUT

by

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Comparison of Delivery Methods in Phage Therapy against *Flavobacterium columnare* Infections in Rainbow Trout

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Abstract: Viruses of bacteria, bacteriophages, specifically infect their bacterial hosts with minimal effects on the surrounding microbiota. They have the potential to be used in the prevention and treatment of bacterial infections, including in the field of food production. In aquaculture settings, disease-causing bacteria are often transmitted through the water body, providing several applications for phage-based targeting of pathogens, in the rearing environment, and in the fish. We tested delivery of phages by different methods (via baths, in phage-coated material, and via oral delivery in feed) to prevent and treat *Flavobacterium columnare* infections in rainbow trout fry using three phages (FCOV-S1, FCOV-F2, and FCL-2) and their hosts (FCO-S1, FCO-F2, and B185, respectively). Bath treatments given before bacterial infection and at the onset of the disease symptoms were the most efficient way to prevent *F. columnare* infections in rainbow trout, possibly due to the external nature of the disease. In a flow-through system, the presence of phage-coated plastic sheets delayed the onset of the disease. The oral administration of phages first increased disease progression, although total mortality was lower at the end of the experiment. When analysed for shelf-life, phage titers remained highest when maintained in bacterial culture media and in sterile lake water. Our results show that successful phage therapy treatment in the aquaculture setting requires optimisation of phage delivery methods in vivo.

Keywords: aquaculture; bacteriophage; bacterial infection; columnaris disease; *Flavobacterium columnare*; phage therapy; phage delivery; rainbow trout; treatment; virulence

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1. Introduction

The aquaculture industry is under increasing pressure to produce food for the constantly growing world population. Intensive fish farming practices are required to provide increasing quantities of high-quality dietary protein. However, this creates favourable conditions for outbreaks of infectious diseases. To combat bacterial infections, vast amounts of antibiotics are used in aquaculture, leading to antibiotic leakage to natural waters [1,2]. Due to the increased risk of development of antibiotic resistance among bacteria at farms and in the environment, alternative ways of treating and preventing bacterial infections are urgently needed.

In the recent years, the need for alternatives to antibiotics has given a new push to phage therapy research in both human and veterinary medicine [3]. Viruses that infect bacteria, bacteriophages, were discovered at the beginning of the 20th century, and then quickly used to treat bacterial infections, i.e., phage therapy. Since the discovery of antibiotics, interest in the development of methods using phages as therapeutic agents decreased [4–6].

In the field of aquaculture, the lack of efficient vaccines against some diseases threatening fish fry presents a challenge to preventing infectious diseases [7]. Phage therapy has been studied as an alternative for antibiotics to prevent and cure bacterial diseases. Promising results have been obtained, e.g., for *Aeromonas salmonicida* infections in brook trout (*Salvelinus fontinalis*) [8] and Senegalese sole (*Solea senegalensis*) [9], *Flavobacterium columnare* infections in zebra fish (*Danio rerio*) and rainbow trout [10,11], *Flavobacterium psychrophilum* infections in rainbow trout [11], *Pseudomonas plecoglossicida* infections in ayu (*Plecoglossus altivelis*) [12,13], and *Vibrio anguillarum* infection in Atlantic salmon (*Salmo salar*) [14].

There are several key considerations when practically applying phage therapy in aquaculture to attain the desired outcome, reduction or elimination of mortality caused by bacterial infection. First, the timing and frequency of phage delivery should be planned carefully, keeping in mind the virulence characteristics of the bacterial pathogen and nature of the outbreaks [7]. Second, the optimal route of phage administration must be identified for each bacterial infection [7,15]. Since pathogenic bacteria have different infection routes (internal or external), it is clear that some bacterial infections are treatable by phages given orally, via intraperitoneal (i.p.) or intramuscular (i.m.) route, whereas others require bath or topical application. Third, an appropriate dose of phages must be determined, which will depend on the expected number of target bacteria, i.e., multiplicity of infection (MOI) is of great importance [7,15,16]. The principle would be to keep the MOI as low as possible while still allowing sufficient bacterial clearance to prevent or stop an outbreak, since using unnecessarily high phage doses is not cost effective for fish farmers. Furthermore, long-lasting stability and resultant infectivity are essential features for phages to be used as therapeutics [16]. Optimal storage conditions are phage-dependent and should be investigated for each potential therapeutic agent.

In aquaculture systems, the real situation often is much more complicated than "one pathogen—one disease". Fish may suffer from multiple simultaneous infections caused by many bacterial species, or co-infecting parasites, which can influence the outcome of the disease [17–19]. Several bacterial strains can be present during disease outbreaks [20], causing further challenges for phage therapy. Since most of the phages usually are very host-specific, infecting only one bacterial species or subspecies, mixture of multiple phages with overlapping host ranges are normally used (reviewed by, e.g., [21,22]).

Flavobacterium columnare causes epidermal infections in farmed fish worldwide and is associated with repeated antibiotic treatments and high mortality [20,23–25]. *F. columnare* isolates from Finland can be divided into different genetic groups A–H [26–28], and phages infecting *F. columnare* are very host-specific, usually infecting only one genetic group [29]. *F. columnare* belonging to several genetic groups can be isolated from the same outbreak and even from the same rearing tank [20,26]. This means that in order to tackle columnaris infection at the farms, phages infecting several genetic groups should be applied simultaneously.

While it has been shown that phages can significantly reduce mortality of zebra fish and rainbow trout in laboratory-induced columnaris disease [10,30], optimal phage mixture, dose, and delivery methods of *F. columnare* phages have remained unclear. In this study, we addressed these issues in three phage therapy experiments with rainbow trout fry, which were treated with phage either prophylactically or after bacterial exposure. In addition, the shelf-life of phages used for treating the fish was studied in different storage conditions. Our results show, that *F. columnare* phages maintain high infectivity in either lake water or *F. columnare*-specific growth medium, and that the most effective phage therapy method against columnaris infections in rainbow trout is phage bath-exposure immediately after the first symptoms of the disease appear in the fish population.

2. Results

2.1. Phage Therapy Experiment I: Individual Phages and Phage Mixes

In the first experiment, we tested how the presence of individual phages and phage mixes influence *F. columnare* infection in rainbow trout. Three bacterial strains (FCO-S1, FCO-F2, and B185) and their phages (FCOV-S1, FCOV-F27, and FCL-2, respectively) were used at MOI 1 [10,31]. Fish in negative control groups, without bacterial challenge, survived significantly longer than all other treatments where bacteria were present (Figure 1a). Compared to the fish having received only bacterial infection, the phage treatments did not have any effect on mortality in fish infected with *F. columnare* strain FCO-S1 (Figure 1a).

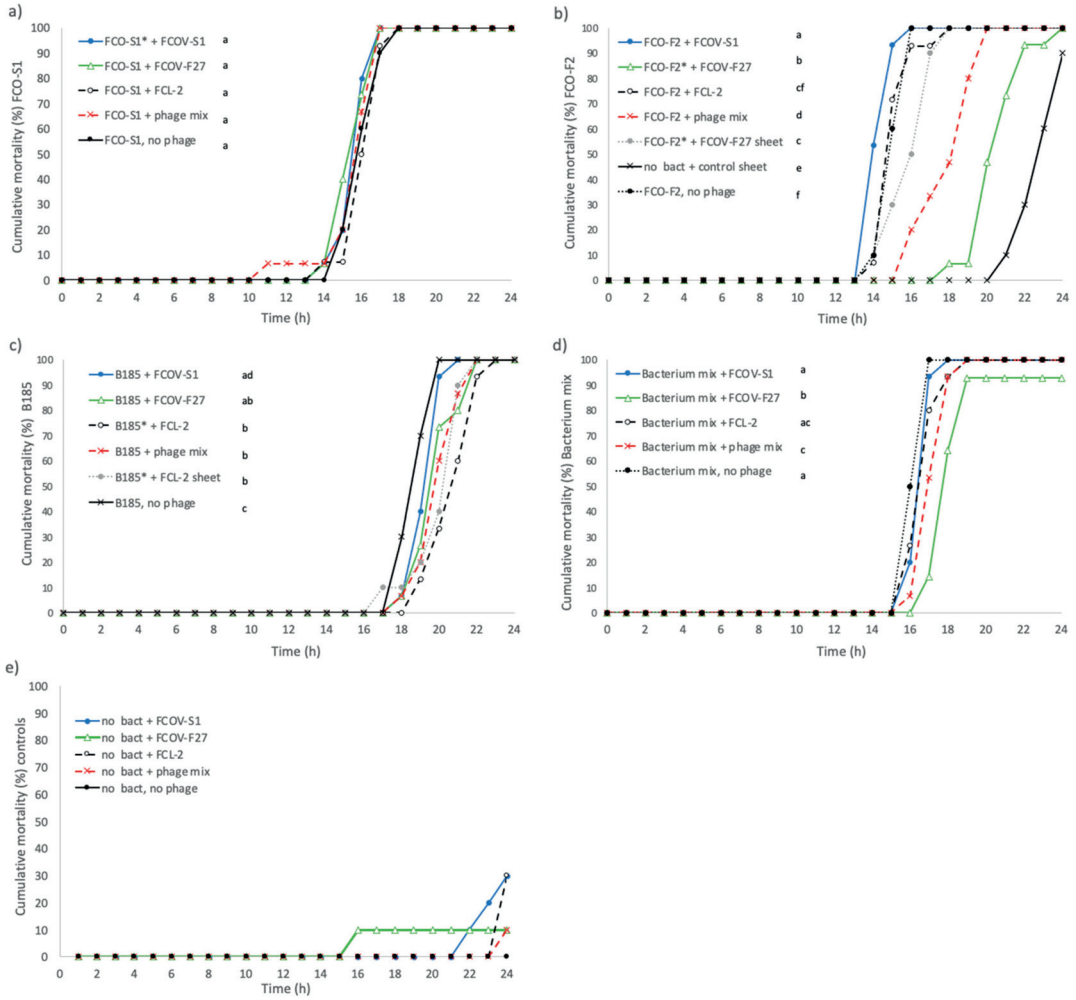

Figure 1. Experiment I: Cumulative mortalities (%) of rainbow trout fry infected with *Flavobacterium columnare* isolates (**a**) FCO-S1, (**b**) FCO-F2, (**c**) B185, and (**d**) their mix, and treated with *F. columnare* phages FCOV-S1, FCOV-F27, and FCL-2, their mix and plastic sheets coated with FCOV-F27 and FCL-2. Mortality related to control treatments without bacterial infections are presented in panel (**e**), except for the plastic control sheet (no phage coating) presented in panel (**b**). The bacterial host of the phage is indicated by an asterisk in each panel. Different lower-case letters a-f indicate statistical difference ($p < 0.05$, Kaplan–Meier survival analysis, log rank pairwise comparisons, Mantel–Cox) between the treatment groups ($n = 15$ rainbow trout in treatments receiving both phage and bacteria, and 10 fish in sheet and control treatments, total n of fish = 360).

Among the fish infected with strain FCO-F2, phage treatments caused a significant decrease in progression of the mortality rates ($p < 0.05$), although the endpoint mortality reached 100% in all cases. Mortality was slowed down the most by phage FCOV-F27 (infecting FCO-F2), which caused approximately a five-hour delay in reaching 50% mortality compared to the phage-free control. With phage mix (a combination of all 3 phages) 50% mortality was reached around 3 h later than in the control, and in FCOV-F27-coated sheet treatment one hour later (Figure 1b). Treatment with phage FCL-2 did not have significant effect; phage FCOV-S1 increased the mortality rate compared to the fish with no phage treatment.

In fish infected with bacterial strain B185, the mortality rate decreased similarly in all other phage treatments except FCOV-S1, which did not have any effect (Figure 1c). In infections induced by mixtures of bacteria, cumulative mortality rate was decreased the most by FCOV-F27 and then by phage mix treatment (Figure 1d). Treatments with FCOV-S1 and FCL-2 did not affect the mortality rates when compared to the fish with only bacterial infection. On the other hand, mortality rates of the fish with FCOV-F27 and FCL-2 treatments did not differ from each other. Among fish having received only phage treatments, cumulative mortality rates did not differ from that of negative control fish with no bacterial infection and phage treatment. Plastic control sheet treatment, however, caused a higher mortality rate than the only phage and negative control treatments. *F. columnare* was isolated from all fish with bacterial infection but not from fish in the infection control (no bacteria).

The number of phages in aquarium water was monitored in all treatments where they were added (Figure 2). From samples taken at 0 h and 12 h, phages were present in all the treatments with a bacterial infection (except phage FCOV-S1, which was not detected in FCO-F2 infection). At the end of the experiment (24 h), phages were only detected in samples where their own host was present, either alone or in a mixture with other bacteria.

Phage titers generally increased in treatments where the host bacterium was present, indicating successful phage replication during the bacterial infection. Titers of phages FCOV-F27 and FCL-2 increased by 3–4 logs (from the initial 5×10^3 PFU mL^{-1}) when fish were infected with their host bacteria alone or if it was present in the bacterial mixture. With phage FCOV-S1, the titer increase was more subtle (2 logs) and was not observed in the bacterium mix. If phages were delivered as a mix (total MOI 1), individual phage titers increased most efficiently in single-host bacterium infections, but if the infection was done with the bacterial mixture, only FCOV-F27 titers increased (4 logs).

2.2. Phage Therapy Experiment II: Effect of Phage Dose

In this experiment, the effect of phage mix dose (MOI 10, 1, and 0.1) when given at the time of bacterial infection was tested. The same bacterial strains (FCO-S1, FCO-F2, and B185), and their phages (FCOV-S1, FCOV-F27, and FCL-2, respectively) were used as described previously. With MOI 1, the timing of phage application was also examined; phage treatment was given before or 2 h after bacterial exposure. The only treatment that decreased the cumulative mortality rate of FCO-S1-infected fish was a pre-infection phage bath (Figure 3a). Pre-infection bath treatments were also the most effective in reducing mortality rate in fish infected with B185 (Figure 3c) and the bacterial mix (Figure 3d). In FCO-F2 infected fish treated with phage mix at MOI 10, the overall mortality was reduced to 86.7% (compared with 100% in all other treatments) (Figure 3b), and this treatment also had the strongest effect on cumulative mortality rates. Other phage treatments, excluding MOI 0.1, significantly decreased the mortality rate of FCO-F2-infected fish.

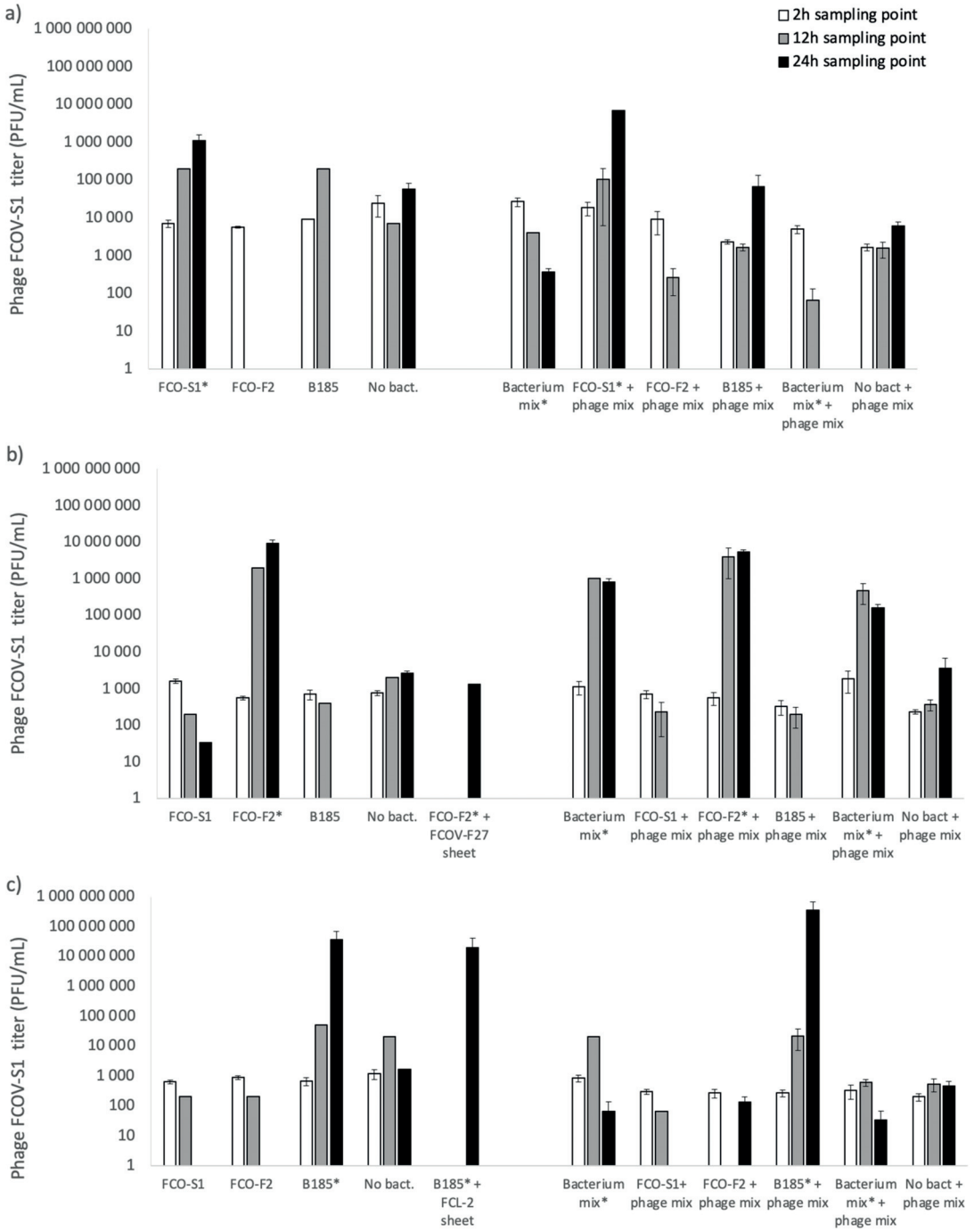

Figure 2. Mean titers (PFU mL^{-1}) of phages (**a**) FCOV-S1, (**b**) FCOV-F27, and (**c**) FCL-2 in water samples during bacterial infection of rainbow trout with *F. columnare* strains FCO-S1, FCO-F2, B185, and their mixes. Phage titers were monitored at 0 h (white bars), 12 h (dark grey bars), and 22 h (black bars). Error bars indicate standard error of mean (not available for all 12 h samples). The presence of the phage host is indicated by an asterisk (*).

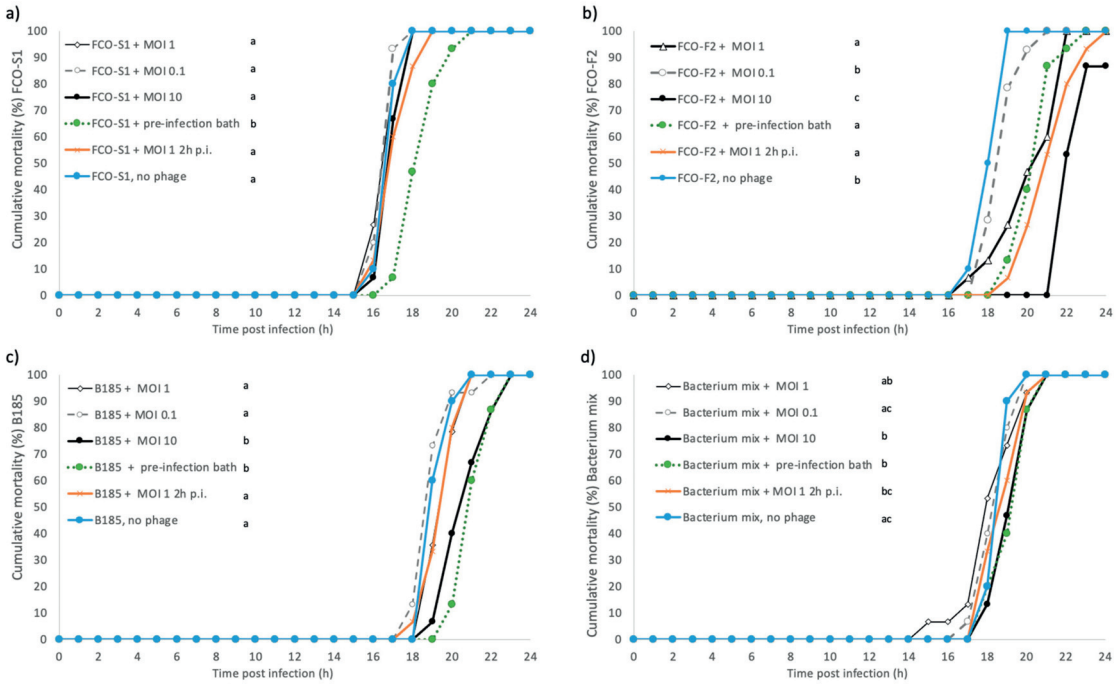

Figure 3. Experiment II: Cumulative mortalities (%) of rainbow trout fry infected with *Flavobacterium columnare* isolates (**a**) FCO-S1, (**b**) FCO-F2 (**c**) B185, and (**d**) their mix, and treated with a mix of phages FCOV-S1, FCO-F27, and FCL-2. Phage mixes were added at the same time as bacteria (0 h) at MOI 1, 0.1, and 10, 2 h post-bacterial infection at MOI 1 (MOI 1 2 h post-infection, p.i.) or given as a 2-h bath exposure at MOI 1 before adding the bacteria (pre-infection bath). Different lower-case letters a-c indicate statistical difference ($p < 0.05$, Kaplan–Meier survival analysis, log rank pairwise comparisons, Mantel–Cox) between the treatment groups ($n = 15$ in phage treatments, $n = 10$ in controls, total $n = 400$).

There was no mortality among negative control fish (no bacterial infection and phage treatment) or among fish in pre-infection phage bath treatment without bacterial infection (results not shown). However, only-phage mix treatments (no bacteria added) with MOI 1, MOI 0.1, MOI 10, and post-infection bath MOI 1 2 h caused reduced mortality, with mortality rates of 90.0, 60.0, 60.0, and 20.0%, respectively. In other treatments without bacterial infection (results not shown), the cumulative mortality rates did not differ between phage-mix-only treatments, with MOI 1, 0.1, and 10, between pre-infection bath, post-infection bath, and negative control, and on the other hand between MOI 0.1 0 h, MOI 10 0 h, and post-infection (MOI 1 2 h) phage treatments. *F. columnare* could be cultured from the groups with dead fish with bacterial infection but were not detected in control fish without bacterial exposure.

2.3. Phage Therapy Experiment III: Phage Delivery in Flow-Through System

The third experiment was done in a more realistic flow-through system with rainbow trout populations. Here, we tested phage delivery via feed, plastic sheets with immobilised phages, or baths (before and after bacterial infection, or as a treatment at the start of the outbreak). Mortality was lowest (56.5%) among fish that received a 2-h phage treatment when the first symptoms of columnaris disease appeared (post-infection phage bath) (Figure 4). A 2-h phage mix bath treatment before bacterial infection (pre-infection phage bath) slowed down the cumulative mortality (endpoint mortality 92.7%), which did not differ from that of fish exposed to phage delivery by coating on plastic sheets. Surprisingly, among the fish receiving the phage orally via feed, the cumulative mortality increased the fastest during the first two days but stabilised after that reaching the endpoint mortality

of 88.3%. However, cumulative mortality of phage-fed fish did not differ statistically from that of infection control (bacteria only) or control sheet (with no phage attached). Phage-coated sheet treatment somewhat slowed down the mortality rate compared to the infection control and control sheet treatments ($p < 0.05$).

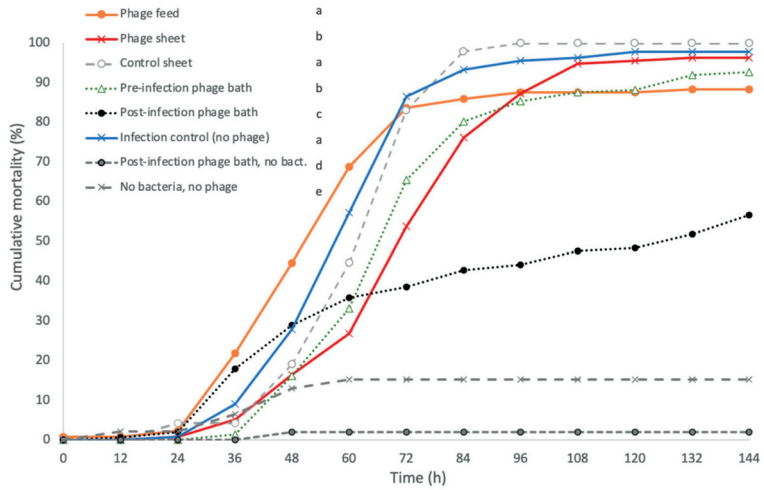

Figure 4. Experiment III: Cumulative mortalities (%) of rainbow trout fry infected with a mix of *Flavobacterium columnare* isolates FCO-F2 and B185. Rainbow trout were kept in three replicate aquaria per treatment (48 fish each) except for controls, which included only a single aquarium (total *n* of fish in experiment = 960). The fish were fed 7 days before the infection with phage mix-coated (FCOV-F27 and FCL-2) feed (phage feed), kept with the phage mix-coated sheet (phage sheet), or a control sheet without phage mix (control sheet) during the whole experiment starting from 7 days before bacterial infection, or treated with a 2-h phage mix bath one day before bacterial infection (pre-infection phage bath) and right after the first symptoms of columnaris disease appeared after the bacterial infection (post-infection phage bath). Negative controls for pre-infection phage bath and phage feed did not cause any mortality and are not shown in the graph. Different lower-case letters indicate statistical difference ($p < 0.05$, Kaplan–Meier survival analysis, log rank pairwise comparisons, Mantel–Cox) between the treatment groups.

Bacterial infection caused 96–100% mortality among fish in all treatments: phage sheet (96.3%), infection control (97.7%), and control sheet (100%) (Figure 4).

No mortality was observed in the bacterium-free pre-infection phage bath and phage feed treatments, but some background mortality was observed in the post-infection phage bath control and in phage-free control. *F. columnare* was isolated from all the dead fish exposed to bacteria. Phages, however, were only detected in three out of nine sampled fish fed for one week with phage-coated feed (Table S1): FCOV-F27 was detected in skin mucus and intestine of two fish and from kidney of one fish, and FCL-2 from skin mucus of two and intestine of three fish. Both phages were detected in the water only in two phage bath-treated aquaria in samples taken at the end of the experiment.

2.4. Shelf-Life of Phages in Different Storage Conditions

Two experiments focusing on the shelf-life of phages in different storage conditions were conducted. When testing suitability of different buffers for phage preservation, all phages maintained the highest infectivity in Shieh medium (Figure S1). In other buffers, the infectivity of phages infecting genetic groups C and G decreased toward the end of the experiment, although higher titers at some testing points were observed in certain buffers. The variability in results from different sampling points are most likely caused by variability in bacterial growth during titration.

In a second experiment, phage shelf-life was tested at different temperatures (−20 °C, 7 °C, and 20 °C) using Shieh medium, lake water (from Lake Jyväsjärvi), and 0.9% NaCl as preservation media. In general, phage survival was best in Shieh medium and in lake water (Figure 5). FCOV-F27 and FCL-2 titers did not drop more than approximately 1 log during the 125 days in all conditions if stored in Shieh medium or lake water. However, in 0.9% NaCl, the infectivity of phages remained stable at room temperature and at 7 °C up to 32 days (second sampling timepoint), after which the phage titers started to decrease, leading to a titer of approximately 10^2 PFU mL^{-1} on day 125 (Figure 5). FCOV-S1 titers could not be determined at room temperature or at 7 °C during the first sampling point days due to poor growth of the host, but high titers (around 10^6 PFU mL^{-1}) in all media were detected on day 125. All three phages could be revived from 149 and 468 days of dry conditions (data not shown).

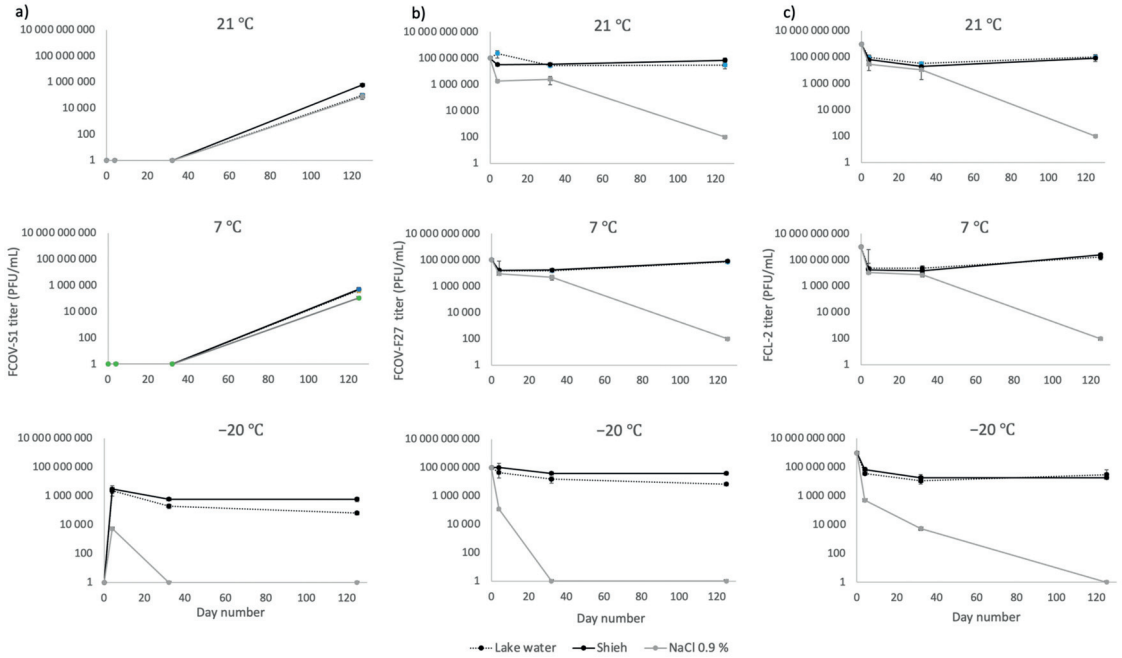

Figure 5. Shelf-life, determined as mean plaque forming units (PFU) mL^{-1}, of Flavobacterium columnare phages (**a**) FCOV-S1, (**b**) FCOV-F27, and (**c**) FCL-2 at different storage temperatures and in different media. Error bars indicate standard error of mean. Number of replicate measurements was 3 for lake water and 2 for Shieh medium and NaCl 0.9%.

3. Discussion

As pathogen-specific, self-templating, self-enriching, and naturally occurring biological entities, lytic phages are appealing tools for biocontrol in aquaculture systems. Indeed, phages can be used in treatment of existing disease outbreaks, but also in disinfection of water and biofilms to prevent disease development and transmission. Phages have been observed to effectively reduce bacterial growth in in vitro studies with fish pathogens *A. salmonicida* [32,33] and *Vibrio parahaemolyticus* [34]. Environmentally transmitting pathogens often have heterogeneous populations where several genetically different strains co-exist, so the development of phage mixtures with coverage of the desired target strains is essential. Furthermore, optimal phage dosing and delivery methods are critical for success of both preventive and treatment approaches. Choosing the best delivery approach is especially important for aquaculture settings, where the disease agents can spread via water or fish faeces and form biofilms on rearing equipment. In this study, we tested different approaches to target *F. columnare* strains. Using rainbow trout fry as an

infection model, we tested different delivery methods, such as bathing before and after infections, and oral administration of phages via feed. Our results showed mortality related to columnaris disease was reduced most efficiently by phage bath treatment, applied to the fish when the first disease symptoms had appeared, although other delivery methods also slowed down the progression of the disease.

From the perspective of choosing phages to include in a mixture, our results show that with *F. columnare* the protective effect of phages depends on their infection efficiency in their specific host, but that no clear pattern could be seen with the applied phage-to-bacterium doses (multiplicities of infection) tested. Timing of phage treatment (at the same time or 2 h after the bacterial infection) or delivery (bath or constant) also did not substantially affect outcomes in this study. Mortality related to mixed bacterial infection was slowed by both phage mix (MOI 1 given 2 h after bacterial exposure, or MOI 10 at the same time with bacteria) and FCOV-F27 alone. Since the bacterial strain FCO-F2 was the most virulent of the three strains, the efficiency of phage in prevention of fish mortality indicates FCOV-F27 has a strong lytic activity during bacterial infection. A phage mix containing all three phages (FCOV-S1, -F27, and FCL-2) slowed the mortality rate when fish were infected with bacterial strains B185 and FCO-F2, and similar results were obtained when fish infected with these strains were treated with their specific phage. Surprisingly, FCO-S1 infection was not affected by its own phage FCOV-S1, and this phage-bacterium pair was not used in further experiments.

When different phage doses were studied, it was found that an MOI of 0.1 did not provide protection against the disease in any of the cases. MOI 10 (given at 0 h) had the strongest effect on cumulative mortality rate in fish infected with FCO-F2 and B185 (Figure 3c). In previous experiments with FCL-2 and B185, no clear difference was found in the survival of zebra fish between MOI 1 and MOI 100 (continuous phage exposure) or in rainbow trout between MOI 1 and MOI 10 (phage exposure by bathing) [10]. However, it should be noted that here our first experiments were done in stagnant water, where a high bacterial dose was maintained. This type of condition does not completely reflect to disease dynamics happening at fish farms. Subsequently, phage treatments were also tested in flow-through conditions. Furthermore, compared to antibiotics, where the initial dose is central for successful treatment, phages are self-replicating, which changes the phage-to-bacterium ratio when the bacterial hosts are present.

In the continuous bacterial exposure in stagnant water, the only truly effective treatment was a two-hour phage-mixture bath (MOI 1) given before the bacterial infection. In this treatment, fish were pre-colonised with phage, which has shown to be efficient to slow down the onset of disease [30]. Some tailed phages, including *F. columnare* phages, have the capacity to bind to mucin glycoproteins via Ig-binding domains [35,36] and be maintained in the mucosa for up to 7 days [30]. Since *F. columnare* bacteria has a strong chemotaxis towards fish mucosa, preventive phage baths allow replication of phage immediately upon bacterial colonisation, which delays the onset of the disease. In real-life farming systems, the effect of introduced mucosal phages might even be stronger as the bacterial numbers initiating the infection are likely to be lower than in the experimental infection used here. Pre-colonisation of vertebrate hosts with phages has been shown to be efficient also in preventing *Vibrio cholerae* infections in mice [37], and the approach has been used also in humans in the former Soviet Union [38].

Conducting experiments in conditions resembling the real-life rearing environment is important to allow a better understanding of the efficiency of phage treatments. In the flow-through experimental system (experiment III, which most closely resembled the real-world situation) the phage bath treatment significantly reduced fish mortality. The greatest effect was observed when the bath treatment was given when the first symptoms of columnaris disease were observed. Phage bath treatment has previously been shown to decrease and slow down the mortality of *F. columnare* infected rainbow trout [10,30], but in these cases the phage was delivered prior to the symptoms of bacterial infection appearing. Here, phage treatment at the onset of epidemic probably targeted both bacteria

replicating in the fish and also those transmitting in the water. Furthermore, it is possible, that the increased replication rate of bacteria on the fish mucosa exposed them to phage infections, leading to efficient reduction of bacterial burden and transmission (see also [30]). Nevertheless, using phage baths as "medication" seems an efficient way to stop progression of the outbreak. It remains to be studied how such treatment influences the development of phage resistance in target bacteria, and if a subsequent phage treatment should include a mixture ("cocktail") of different phages.

Administration of phages via water is conceptually simple, but in commercial-scale aquaculture the volumes of water requiring phage treatment can be impractically large. Attaching phages on surfaces to provide materials that bioactivate upon contact with the host bacteria is therefore an alternative option to efficiently deliver phages in rearing systems. Here, we tested two approaches of attached phages: plastic sheets and fish feed. Phage-coated plastic sheets delayed fish mortality significantly both in stagnant water (experiment I, Figure 1) and in the flow-through system, although other approaches were more efficient. Nevertheless, the results suggest that immobilisation of phages on aquaculture-relevant surfaces, e.g., biofilters, could be an efficient way to reduce the effect of environmentally transmitting pathogens in inlet and tank water at flow-through fish farms. This would allow maintaining a "biobank" of pathogen-targeting phages that are able to interact with target bacteria in the water and initiate infection cycles to enrich and spread phage progeny in the bacterial population at farms during the rearing cycle. Phage immobilisation techniques have several generalisable downstream applications in biomedicine and food production. For example, phage coating of catheters efficiently prevents *Staphylococcus epidermitis* biofilm formation [39] and prevents *Pseudomonas aeruginosa* in wound dressing [40], and phage coating of food packing materials has been applied to prevent food from spoiling [41].

The effect of prophylactic oral administration of a phage-mix via coated feed was tested against columnaris disease. Interestingly, phage feed did not have a major protective effect on fish mortality, though in experiment III the endpoint mortality was lower than in the control group. However, in the beginning of the experiment mortality increased rapidly in phage-fed groups, resulting in endpoint mortality being higher than in groups having received phage bath treatment after the bacterial infection. In a recent study where a similar phage immobilisation technique on fish feed was used, oral phage delivery did not protect fish from *F. psychrophilum* infection [42]. In other studies, phage-impregnated feed has been shown to have a protective effect against bacterial infections, e.g., in *P. plecoglossicida* infections of ayu [12,43] and *Lactococcus garvieae* infections in yellowtail (*Seriola quinqueradiata*) [44]. The most probable reason for the inefficiency of phage-coated feed against columnaris disease is the external infection route and symptoms of *F. columnare*. The bacterium is probably not cleared by orally applied phages as efficiently as by phages applied from outside the body. While phages were detected in the organs of fish fed phage-immobilised feed, this was at relatively low numbers and transmission of phages through the gut appears to be relatively inefficient.

However, other explanations are worth considering. In our flow-through experiment (experiment III), the feed was coated with a crude phage lysate that would include bacterial debris, possibly containing intra- and extracellular bacterial toxins, which could affect the overall welfare of fish and make them more susceptible to experimental bacterial infection. In this light, it might be possible that the primary immune system of small fish reacts heavily to parenterally delivered phages and this kind of energy allocation weakens their ability to resist bacterial infections, as has been shown with immunostimulant delivery to rainbow trout fry [45]. Phage therapy is known to have variable immunomodulatory effects, e.g., changes in cytokine and C-reactive protein responses, in humans (reviewed by [1]). Immune responses in the fish were not examined in this study, but based on two independent experiments, it is clear, however, that phage exposure via oral route does not improve the recovery of rainbow trout fry from *F. columnare* infection.

All the three phages could be isolated from the aquarium water in all the treatments of experiments I and II (stagnant water experimental system) for 24 h (excluding treatments with other than phage's own host). However, phages could only be found in intestine, skin mucus, and kidney in a minority of the sampled fish after one-week prophylactic feeding with phage-coated feed in the flow-through experimental system (experiment III). No phages were detected in spleens or in the aquarium water before the bacterial infection in the phage-fed fish. Additionally, no phages were detected from treatments with sheet or bath pre-infection, which contrasts with recent results using *F. columnare* phages that were found to maintain their activity in fish skin mucus for up to one week [30] and in aquarium water for two days [10,30] in flow-through systems. However, in these previous studies, the bacterial infection had been applied before phage sampling, allowing phage replication. Our present results also differ from those observed in rainbow trout using *F. psychrophilum* phages in which phages were not only detected in the intestine and kidney but also from the spleen after one-week feeding [46]. However, when using oral intubation and bathing [46] or intraperitoneal injection [47] as application routes, *F. psychrophilum* phages were cleared from intestine, spleen, and i.p. cavity of rainbow trout after three or four days if no host bacteria were present.

One major criticism against phage therapy is the rapid development of phage resistance. It has been suggested that one way to reduce this issue is the simultaneous use of multiple phages that target different bacterial receptors [21,48,49]. It has been shown in in vitro studies using other aquatic pathogens that, compared to single phages, phage mixtures more efficiently inhibit both the growth and development of phage resistance in *Vibrio* [34] and *A. salmonicida* [32,33]. In this study, only phage mixtures against infections of bacteria belonging to different genetic groups were tested. Thus, in the future, the effect of mixtures with phages infecting one genetic group of bacteria but targeting different receptors should be examined.

Compared to a previous study on the shelf-life of *F. columnare* phage, where FCL-2 maintained high (10^9–10^{10} PFU mL^{-1}) titers for six weeks in modified Shieh medium, Tris-HCl and KH_2PO_4 at 6 °C [10], the infectivity of phages (incl. FCL-2) decreased clearly in most of the buffers after one week in the present study. On the other hand, regardless of the temperature (−20 °C, 7 °C, and 21 °C), *F. columnare* phages maintained their infectivity for over four months in Shieh medium and autoclaved lake water. Phages could also be recovered from over 15-month desiccation in Shieh medium (data not shown). Similar results have been obtained in *F. psychrophilum* phages, which could maintain infectivity both after two- and eight-month desiccations on feed pellets [46,47], and when preserved in autoclaved fish pond water [47,50] or *F. psychrophilum* -specific growth medium [50]. Also, in *Vibrio* phages, it was shown that the conditions resembling the natural environment of the phage, i.e., marine aquaculture water, favour phage stability [34].

Our study gives encouraging results for the practical development of phage therapy against *F. columnare* infections in fish farming. We show that bath treatment, especially after the first symptoms of columnaris disease appear in the fish population, is efficient in treating the disease in rainbow trout. This delivery method is most likely successful due to the epidermal nature and environmental transmission of the disease. Furthermore, *F. columnare* phages maintain their infectivity at simple storage conditions.

4. Materials and Methods

4.1. Bacteria and Phage Isolates

Bacteria and phages used in this study were isolated from water samples collected at Finnish and Swedish fish farms during columnaris outbreaks and have been characterised previously [29] (Table 1 and Table S2). The bacterial isolates FCO-S1, FCO-F2, and B185 were specifically infected by the phages FCOV-S1, FCOV-F27, and FCL-2, respectively.

Table 1. *Flavobacterium columnare* and their phages used in this study. Bacteria and phages were isolated from Finnish and Swedish fish farms. *F. columnare* isolates have been previously categorised into genetic groups by restriction fragment length polymorphism analysis of the internal transcribed spacer region between 16S and 23S rRNA genes.

Bacterial Strain and Genetic Group	Phage Isolate and Genetic Group of the Host	Isolation Farm	Isolation Year	Experiment
FCO-S1 (A)		1	2017	I, II
FCO-F2 (C)		2	2017	I–III
B185 (G)		3	2008	I–III
	FCOV-S1 (A)	1	2017	I, II
	FCOV-F27 (C)	2	2017	I–III
	FCL-2 (G)	3	2008	I–III

4.2. Bacterial Cultures and Phage Purification

All bacterial and phage cultures mentioned in this article were grown in modified Shieh medium [51], called "Shieh" for simplicity.

F. columnare isolates were inoculated from cryopreserved (−80 °C) stocks into Shieh medium and grown for 48 h at 25 °C with 120 rpm agitation. Afterward, subcultures were made in Shieh medium and grown for 24 h at 25 °C with 120 rpm agitation. The optical density (OD) of the bacterial broth suspensions was measured spectrophotometrically at 595 nm and adjusted to give a *F. columnare* concentration of 5×10^6 colony-forming units (CFU) mL^{-1} (based on previously determined OD/CFU relationship).

Unless otherwise mentioned, phage lysates were prepared according to [52], were purified by the polyethylene glycol (PEG)-NaCl-method according to [53], and adjusted to 5×10^7, 5×10^6, and 5×10^5 plaque forming units (PFU) mL^{-1} in Shieh medium.

4.3. Fish

Rainbow trout fry were received from a fish farm in central Finland where they had hatched about two months before transferring to the laboratory. Fish were held in bore hole water at 16 °C with constant flow-through and aeration and fed with commercial feed pellets. For the experiments, the water temperature in holding aquaria was increased 0.5–1.0 °C per day up to 25 °C.

4.4. Phage Therapy Experiments I and II: Effect of Phage Dose and Delivery Method in Constant Exposure

Rainbow trout fry, averaging 0.73 g in Exp I and 0.71 g in Exp II, were placed individually into 0.75-L aquaria containing 500 mL of aerated, 25 °C bore hole water. In Exp I (Table 2) the fish were divided into 28 aquaria. Bacterial infection with three different strains (see Table 1), either individually or as a mixture, was made by pipetting 0.5 mL of bacterial solution of 5×10^6 CFU mL^{-1}, giving a constant infection dose of 5×10^3 CFU mL^{-1}. Phage treatment was given at the same time with bacterial infection at a MOI of 1 (final dose 5×10^3 PFU mL^{-1} for each individual phage). In the phage mixture, the dose of each phage was 1/3, resulting to total MOI of 1.

In experiment II (Table 3), fish were divided into 30 treatment groups of ten or 15 fish per group depending on the treatment. Fish were again exposed to three bacterial strains (Table 1) in single infections or as a mix. Phage treatment was given at the same time as bacteria or 2 h after adding the bacteria. For each phage, three doses were used. 0.5 mL phages and their mixtures with titers of 5×10^7, 5×10^6, and 5×10^5 PFU mL^{-1} were added into aquaria, giving a final dose of 5×10^4, 5×10^3, and 5×10^2 PFU mL^{-1}, respectively, and multiplicities of infection 10, 1, or 0.1. One group of fish was bath-exposed to a phage mixture of 5×10^3 PFU mL^{-1} for 2 h before moving to experimental aquaria and performing the bacterial infection (MOI 1). In phage-coated sheet treatments, the plastic sheets (approx. 21 cm^2, 3.3×10^5—5.0×10^7 PFU cm^{-2}, manufactured and supplied by Fixed Phage Ltd, Glasgow, Scotland, UK) were placed into aquaria just before the addition of bacteria. In control treatments, Shieh medium replaced phage and/or bacteria

and plastic sheets without phage-coating were applied. Survival of fish was monitored hourly for 24 h after addition of bacteria. Morbid fish that did not respond to stimuli were considered dead, removed from the experiment, and euthanised by decapitation. Bacterial cultivations from gills of all the dead fish were made on Shieh agar supplemented with tobramycin [54] to confirm the presence/absence of the pathogen. In experiment I, water samples (500 µL) for phage titer determination were taken from three replicate tanks per treatment at 0 h (right after the phage addition), 12 h, and 22 h (at the end of the experiment = 24 h after the bacterial infection) after the phage addition. At the end of the experiment, surviving fish were euthanised using 0.008% benzocaine.

Table 2. Setup of experiment I. Rainbow trout fry were infected individually with three *Flavobacterium columnare* isolates (FCO-S1, FCO-F2, and B185) (5×10^3 CFU mL^{-1}) and their mix. For phage treatment, purified phages (FCOV-S1, FCOV-F27, and FCL-2) (MOI 1) or their mix were added 2 h after and phage-coated plastic sheets (FCOV-F27, FCL-2, and control without phage coating) just before adding the bacteria. – = Shieh medium added instead of bacterium and/or phage.

Bacterial Isolate/mix	Phage Isolate/Mix	Fish n:o.	
FCO-S1	FCOV-S1	15	
FCO-S1	FCOV-F27	15	
FCO-S1	FCL-2	15	
FCO-F2	FCOV-S1	15	
FCO-F2	FCOV-F27	15	
FCO-F2	FCL-2	15	
B185	FCOV-S1	15	
B185	FCOV-F27	15	
B185	FCL-2	15	
Mix	FCOV-S1	15	
Mix	FCOV-F27	15	
Mix	FCL-2	15	
FCO-S1	Mix	15	
FCO-F2	Mix	15	
B185	Mix	15	
Mix	Mix	15	
FCO-F2	FCOV-F27-coated sheet	10	
B185	B185-coated sheet	10	
FCO-S1	–	10	No phage control
FCO-F2	–	10	No phage control
B185	–	10	No phage control
Mix	–	10	No phage control
–	FCOV-S1	10	No bacteria control
–	FCOV-F27	10	No bacteria control
–	FCL-2	10	No bacteria control
–	Control sheet	10	No bacteria control
–	Mix	10	No bacteria control
–	–	10	No treatment control
Total number of fish		360	

4.5. Phage Therapy Experiment III: Phage Delivery in Flow-Through System

Water temperature in holding aquaria of fish was increased 1 °C day^{-1} from 15 to 20 °C, after which the fish were transferred to experimental aquaria. The experiment was started one day after transferring the fish, and the temperature increase continued one day after that by 1 °C day^{-1}, from 20 to 25 °C.

Rainbow trout fry, averaging 3.03 g, were divided into 20 11-L flow-through aquaria supplied with aerated bore hole water. The 20 tanks were divided into five treatment groups, four aquaria with 48 fish in each (Table 4). The first group received phage lysate mix-coated (FCOV-F27 and FCL-2) feed (0.8 mm pellet size, 6×10^6 PFU g^{-1}), manufactured and supplied by Fixed Phage Ltd, Glasgow, Scotland, UK, daily for 7 days at 2% of body weight. Into three aquaria of the second fish group, phage mix-coated plastic sheets

(A4 = 29.7 × 21.0 cm, 5×10^5 PFU cm^{-2}) were placed 7 days before bacterial infection (see below) for 2 weeks. One aquarium of the second group received a control sheet with no phage coating. One day before bacterial infection, a third group of fish (see below) was treated with a 2-h bath treatment in 9-L aquaria in aerated 25 °C bore hole water containing 1.5×10^6 PFU mL^{-1} of phage mix, giving an MOI of 1. A fourth group received 1.5×10^6 PFU mL^{-1} phage mix treatment right after the first symptoms of columnaris disease after bacterial infection was observed; the water flow from experimental aquaria was closed, phage mix solution added, and water flow opened again after 2 h. The fifth group of fish did not receive any phage treatment.

Table 3. Setup of experiment II. Rainbow trout fry were infected individually with three *Flavobacterium columnare* isolates (FCO-S1, FCO-F2, and B185) (5×10^3 CFU mL^{-1}) and their mix. For phage-treatment, a phage mix of three phages (FCOV-S1, FCOV-F27, and FCL-2) was added at MOI 1, 0.1, or 10 at the same time (0 h) or at MOI 1, 2 h after adding the bacteria. One group of fish was bath-exposed for 2 h to the phage mix (MOI 1) before transferring to experimental aquaria and adding the bacteria. − = Shieh medium added instead of bacterium and/or phage.

Bacterial Infection	Phage Treatment	Fish n:o
FCO-S1	MOI 1 0 h	15
FCO-S1	MOI 0.1 0 h	15
FCO-S1	MOI 10 0 h	15
FCO-S1	MOI 1 bath	15
FCO-S1	MOI 1 2 h	15
FCO-F2	MOI 1 0 h	15
FCO-F2	MOI 0.1 0 h	15
FCO-F2	MOI 10 0 h	15
FCO-F2	MOI 1 bath	15
FCO-F2	MOI 1 2 h	15
B185	MOI 1 0 h	15
B185	MOI 0.1 0 h	15
B185	MOI 10 0 h	15
B185	MOI 1 bath	15
B185	MOI 1 2 h	15
Mix	MOI 1 0 h	15
Mix	MOI 0.1 0 h	15
Mix	MOI 10 0 h	15
Mix	MOI 1 bath	15
Mix	MOI 1 2 h	15
FCO-S1	−	10
FCO-F2	−	10
B185	−	10
Mix	−	10
−	MOI 1 0 h	10
−	MOI 0.1 0 h	10
−	MOI 10 0 h	10
−	MOI 1-bath	10
−	MOI 1 2 h	10
−	−	10
Total number of fish		400

On day 8, three fish from three tanks of each treatment group were anesthetised with 0.008% benzocaine, and kidney, spleen, intestine, and skin mucus (carefully scraped with a scalpel from both sides of the fish) were collected for phage detection. At the same time, and also at the end of the experiment, three replicate water samples of 400 µL were taken from the same tanks. Water samples from one tank with only phage bath treatment were also taken after the experiment (Table 4). After the sampling on day 8, the fish from all the treatment groups were transferred to receive a 2-h bacterial infection in 9-L aquaria of aerated 25 °C bore hole water containing 1.5×10^6 CFU mL^{-1} of a bacterium mix (FCO-

F2 and B185). One tank per treatment received Shieh medium instead of bacteria. The exception was the treatment with the control plastic sheet that was also given a bacterial infection. The survival of fish was monitored for 6 days in 12 h intervals. Morbid fish that did not respond to stimuli were considered dead, removed from the experiment and euthanised by decapitation. Bacterial cultivations from gills of all the dead fish were made and live fish euthanised at the end of the experiment, as described above.

Table 4. Setup of experiment III. First group (feed) received feed coated with a phage mix (FCOV-F27 and FCL-2), daily for 7 days at 2% of body weight. Phage mix-coated plastic sheets were placed in tanks 7 days before bacterial infection for two weeks. One aquarium of the second group received a control sheet with no phage coating (control sheet). A third group of fish was placed one day before bacterial infection in a 2-h phage mix bath (pre-infection bath). The fish were infected with a bacterium mixture (FCO-F2 and B185) on day 8 in a bath for 2 h. A fourth group received a 2-h phage mix bath right after the first symptoms of columnaris disease after the bacterial infection were observed (bath post-infection). The fifth group of fish did not receive any phage mix treatment (control). Just before the bacterial infection, three fish from three tanks of each treatment group were anesthetised, and kidney, spleen, intestine, and skin mucus were collected for phage enumeration. At the same time and after the experiment, water samples were taken from the same tanks. + = bacterial infection/sampling for phage detection, − = no bacterial infection/no sampling for phage detection, [a] = only water sample after the experiment.

Delivery of Phage Mix	Bacterial Infection	Replicate	Fish n:o	Phage Sample
Feed	+	1	48	+
Feed	+	2	48	+
Feed	+	3	48	+
Feed	−	4	48	−
Sheet	+	1	48	+
Sheet	+	2	48	+
Sheet	+	3	48	+
Control sheet	+	4	48	−
Pre-infection bath	+	1	48	+
Pre-infection bath	+	2	48	+
Pre-infection bath	+	3	48	+
Pre-infection bath	−	4	48	−
Post-infection bath	+	1	48	+
Post-infection bath	+	2	48	+
Post-infection bath	+	3	48	+
Post-infection bath	−	4	48	+ [a]
Control	+	1	48	+
Control	+	2	48	+
Control	+	3	48	+
Control	−	4	48	−

4.6. Phage Presence in Tissues Samples

Organs were aseptically removed, placed into 1.5 mL Eppendorf tubes, and smashed with plastic rods, after which 400 µL of Shieh medium was added. Skin mucus was transferred into Eppendorf tubes containing 200 µL of Shieh medium. Chloroform was added to organ, mucus, and water sample tubes at end concentration of 10%, tubes were vortexed and preserved at 7 °C for six days. Afterward, the samples were shortly centrifuged to separate chloroform and debris from the phage-containing Shieh phase that was collected and diluted tenfold in Shieh medium. Drop titrations (2 µL drops) were made on double-layer Shieh agar and plaques counted after two days to determine the presence of phages in the samples.

4.7. Statistical Analyses

Kaplan–Meier survival analysis (IBM SPSS Statistics version 24), log rank pairwise comparisons (Mantel–Cox), was used for analysis of cumulative mortality data.

4.8. Shelf-Life of Phages in Different Storage Conditions

Forty-six previously isolated phages [31] were used in the first shelf-life experiment (Table S1). High titer lysates (10^{10}–10^{11} PFU mL^{-1}) of phages were diluted into tenfold series with different buffers (2 mM Tris-HCl, $MgCl_2$, and $CaCl_2$) of three different pH (6.5, 7.5, and 8.0). Shieh medium (pH 7.5) was used as a control. Dilutions were applied on 96-well (1000 µL) storing plates, sealed with a parafilm, protected from light, and stored in a cold room (7 °C). Phage infectivity was tested after 1, 3, 5, 7, 14, 21, and 89 days: drop titrations (2 µL) were made on double-layer Shieh agar using the original host bacteria. The highest dilutions with clear plaques were recorded, and results expressed as an average of all the phages with the same original host.

Afterward, it was tested how phages used in therapy experiments (FCL-2, FCOV-S1, and FCOV-F27) tolerate different temperatures in different storage media. Phage titers were adjusted to 1×10^9 PFU mL^{-1} by diluting high titer lysates in 10 mL of Shieh medium (duplicate samples), autoclaved lake water (triplicate samples), or 0.9% NaCl (duplicate samples). Phage suspensions were stored at 21 °C, 7 °C, and −20 °C. Sampling for phage infectivity was conducted after 1, 4, 32, and 125 days by making tenfold dilutions from phage suspensions in original media. Samples in 0.9% NaCl, however, were diluted in autoclaved Milli-Q® water. Suspensions preserved in −20 °C were thawed in a water bath (20 °C) before sampling. Drop titrations (2 µL) were made on double-layer Shieh agar and titers read after 2 days incubation in RT. We also tested if phages FCL-2, FCOV-S1, and FCOV-F27 could tolerate dehydration. Aliquots (500 µL) of phage lysates (in Shieh medium) were air-dried in a cold room (7 °C) in open Eppendorf tubes for two months, after which the tubes were closed. Phage infectivity was tested after 149 and 468 days by detaching a small amount of dry material from the bottom of the Eppendorf tube with 1 µL inoculation loop and suspending it to 200 µL of Shieh medium for PFU analysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10080914/s1, Figure S1: Shelf-life of *Flavobacterium columnare* phages in different buffers, Table S1: *Flavobacterium columnare* phages detected from fish organ and water samples in experiment III, Table S2: *Flavobacterium columnare* strains and phages used in phage shelf-life experiment with buffers.

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Conflicts of Interest: J.C. was employed by the company Fixed Phage Ltd. (Glasgow, United Kingdom). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

Table S1. *Flavobacterium columnare* phages detected from fish organ and water samples in Experiment III. Samples from the organs were taken before the bacterial infection, water samples after the experiment. + phage detected, – = no bacterial infection, () = sample taken after the experiment, and i = sample inhibits bacterial growth. The number of + and i in phage columns indicate the number of replicate samples giving the particular result. Three fish were sampled from each replicate aquarium, + indicates positive results from an individual fish.

			FCOV-F27				FCL-2			
Delivery of phage mix	Bacterial infection	Replicate aquarium number	Water	Skin mucus	Intestine	Kidney	Water	Skin mucus	Intestine	Kidney
Feed	yes	1	i				i		+	ii
Feed	yes	2		+	+			+	+	ii
Feed	yes	3		+	+	+		+	+	
Sheet	yes	1								
Sheet	yes	2								
Sheet	yes	3								
Bath pre-infection	yes	1				i				
Bath pre-infection	yes	2	(+++)							
Bath pre-infection	yes	3	(+)			i				
Bath post-infection	yes	1	(+++)							
Bath post-infection	yes	2								
Bath post-infection	yes	3								
Bath post-infection	no	4				i	(+)			i
Control	yes	1								
Control	yes	2								
Control	yes	3				ii				i

Table S2. *Flavobacterium columnare* strains and phages used in phage shelf life experiment with buffers. Phages are grouped according to their host bacterium (genetic group of the bacterium in parentheses).

Host bacterium (genetic group)	B534 (A)	B537 (C)	FCO-F2 (C)	B185 (G)
Phage	FCOV-S1	FCOV-F1	FCOV-F10	FCOV-F13
		FCOV-F2	FCOV-F11	FCOV-F14
		FCOV-F3	FCOV-F18	FCOV-F15
		FCOV-F4	FCOV-F22	FCOV-F16
		FCOV-F5	FCOV-F24	FCOV-F45
		FCOV-F6	FCOV-F26	
		FCOV-F7	FCOV-F27	
		FCOV-F8	FCOV-F31	
		FCOV-F9	FCOV-F32	

FCOV-F12	FCOV-F39
FCOV-F17	FCOV-F40
FCOV-F19	FCOV-F41
FCOV-F20	FCOV-F42
FCOV-F21	FCOV-F43
FCOV-F23	FCOV-F44
FCOV-F25	
FCOV-F28	
FCOV-F29	
FCOV-F30	
FCOV-F33	
FCOV-F34	
FCOV-F35	
FCOV-F36	
FCOV-F37	
FCOV-F38	

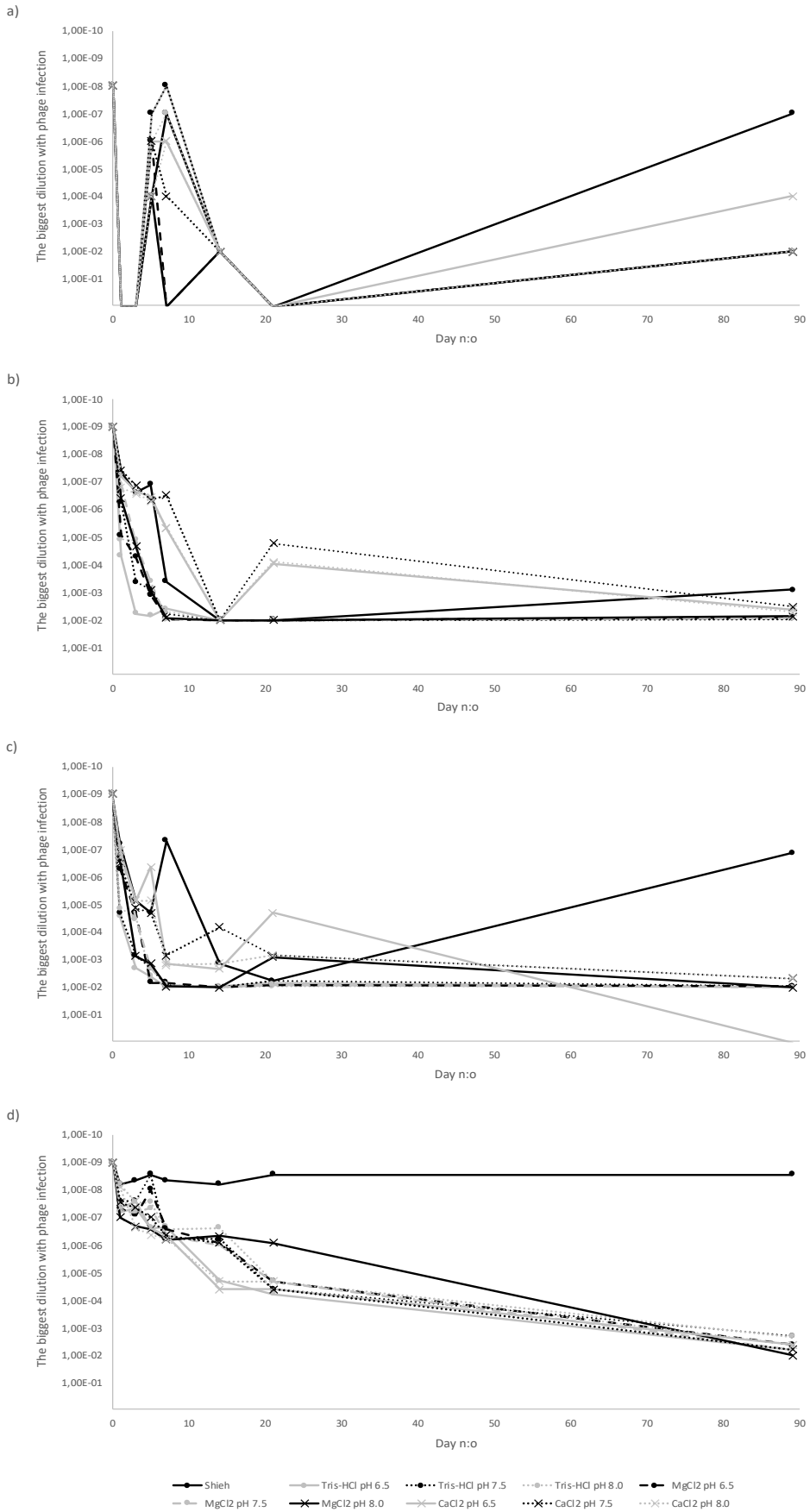

Figure S1. Shelf life (represented as the biggest dilution of phage sample able to infect host bacterium) of *Flavobacterium columnare* phages preserved in different buffers and infecting different host bacteria belonging to genetic groups a) A (host bacterium B534), b) C (host bacterium B537), c) C (host bacterium FCO-F2) and d) G (host bacterium B185).