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Bacteriophage-based control of *Flavobacterium psychrophilum* in rainbow trout

Studies on phage-treatment of rainbow trout at fry and eyed egg stages and effects on gut microbial communities

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PhD thesis

Unit for Fish and Shellfish diseases

National Institute of Aquatic Resources

Technical University of Denmark (DTU)

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Bacteriophage-based control of *Flavobacterium psychrophilum* in rainbow trout. Studies on phage-treatment of rainbow trout at fry and eyed egg stages and effects on gut microbial communities.

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Preface

This PhD research project has been conducted at the Unit for Fish and Shellfish diseases, at the National Institute of Aquatic Resources, Technical University of Denmark (DTU) (Kgs. Lyngby, DK). In addition, the virulence analysis of *F. psychrophilum* genomes was conducted at the Marine Biological Section, University of Copenhagen (Helsingør, DK).

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Manuscripts included in the thesis

I. **Donati V.L.**, Dalsgaard I., Sundell K., Castillo D., Er-Rafik M., Clark J., Wiklund T., Middelboe M., Madsen L. (2021). Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture: *in vivo* experiments to compare delivery methods. Submitted to the journal Frontiers in Microbiology (in review).

II. **Donati V.L.**, Madsen L., Middelboe M., Strube M.L., Dalsgaard I. (2021). The gut microbiota of healthy and *Flavobacterium psychrophilum*-infected rainbow trout fry is shaped by antibiotics and phage therapies. Manuscript.

III. **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. Ready for submission to the journal Microorganisms.

Manuscripts not included in the thesis

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V. Sundell K., Landor L., Nicolas P., Jørgensen J., Castillo D., Middelboe M., Dalsgaard I., **Donati V.L.**, Madsen L., Wiklund T. (2019). Phenotypic and genetic predictors of pathogenicity and virulence in *Flavobacterium psychrophilum*. **Frontiers in Microbiology**, 10, 1711.

VI. Jørgensen J., Sundell K., Castillo D., Dramshøj L.S., Jørgensen N.B., Madsen S.M., Landor L., Wiklund T., **Donati V.L.**, Madsen L., Dalsgaard I., Middelboe M. (2021). Reversible mutations in gliding motility and virulence genes: a flexible and efficient phage defense mechanism in *Flavobacterium psychrophilum*. Manuscript.

Acknowledgments

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To conclude, I would like to express my sincere gratitude to all the academic and commercial partners of the BONUS FLAVOPHAGE project.

Last but not least, I would like to thank my Italian-Danish family and friends for the support, motivation and understanding during the pursuit of my PhD degree.

Summary

The focus of this PhD project is *Flavobacterium psychrophilum*, a Gram-negative bacterium, worldwide-known in salmonid aquaculture as the etiological agent of rainbow trout fry syndrome (RTFS, fry stage) and of Bacterial Coldwater Disease (BCWD, juvenile and adult fish). Due to the rise of antibiotics resistance and the well-known disruptive effects of antibiotics on the host bacterial microbiota and on the environment, novel approaches are of urgent need. Bacteriophages (also called phages) are host-specific viruses of bacteria unable to replicate in eukaryotes and, thanks to their bactericidal activity (they infect, lyse and kill the host bacterium releasing the new phage progeny to the environment), are gaining attention as an environment-friendly alternative to antibiotics or as a prophylactic preventive measure.

Overall, this PhD project brings new knowledge for the development of phage-based control strategies targeting the pathogenic bacterium *F. psychrophilum* that could be applied in aquaculture facilities. The study was part of a larger research project titled "Bacteriophage based technology to control *Flavobacterium* pathogens in aquaculture" (acronym: FLAVOPHAGE), a collaboration of Baltic partners studying bacteriophages specific for *F. psychrophilum* and *F. columnare.*

In **Manuscript I**, we investigated the potential of a two-component mix of bacteriophages in controlling *F. psychrophilum* in rainbow trout fry administered by three methods (oral, bath and injection). For the oral administration experiments, phages were applied on feed pellets by spraying ($1.6*10^8$ PFU g^{-1}), or by irreversible immobilization (produced by Fixed Phage Ltd using their patented corona discharge technology; $8.3*10^7$ PFU g^{-1}). Fish experimental trials were performed, bacteria and phages re-isolated from fish organs by culture-based methods and a survival analysis conducted. Nevertheless phages were constantly detected in the intestine and with a more sporadic occurrence in kidney, spleen and brain, no significant increase was observed on fish survival during *F. psychrophilum* infection. A similar result was obtained when phages were delivered by bath. However, when phages were administered by intraperitoneal injection ($1.7*10^8$ PFU $fish^{-1}$), a significant increase in fish survival was observed in the group exposed to phages (80.0 %) compared to the control group (56.7 %). Additional fish challenge experiments to the ones included in **Manuscript I** were performed and the results are presented in the **Appendix** section of this thesis. In these experiments, phages were administered either via phage-immobilized feed or via a combined approach of phage-immobilized feed and phage bath procedures. No beneficial effect of phages on fish survival was observed.

In **Manuscript II**, we focused on evaluating the effects of *F. psychrophilum* infection, of the oral administration of phages and of the antibiotic florfenicol (antibiotic in use in Denmark for the treatment of RTFS) on the gut microbiota of rainbow trout fry. For this study, phages targeting *F. psychrophilum* (phage-sprayed and phage-immobilized feed) was continuously delivered by feed with a prophylactic period of 12 days.

Florfenicol-coated feed was administered for 10 days starting two days after the infection procedure. Samples of the distal intestine were collected over time and analyzed by community analysis targeting the 16S rRNA gene. Results showed a dysbiosis effect caused both by the infection and by florfenicol administration. Interestingly, phage addition altered the microbiota of the fish independently of the presence of their target bacterium.

In **Manuscript III**, we focused on *F. psychrophilum* infections in rainbow trout eyed eggs. At first we established an infection bath method and evaluated the effects of singular phages on fish eggs (survival of eyed eggs; interaction of phages with eyed eggs). Subsequently, bacterial-challenged eyed eggs were exposed to phages to study their ability to control *F. psychrophilum*. Culture-based methods were used to enumerate the number of bacteria and/or phages. Bacteriophages did not appear to negatively affect the survival of eyed eggs neither to strongly adhere to surface of eyed eggs. Further, the results showed a strong potential of phages in controlling *F. psychrophilium* in short term (24h) experiments.

Sammendrag

Fokus for dette ph.d.-projekt er *Flavobacterium psychrophilum*, en Gram-negativ bakterie, kendt over hele verden som årsag til yngeldødelighedssyndromet (RTFS (Rainbow Trout Fry Syndrome)) hos yngel og Bakteriel Koldtvandssyge (BCWD (Bacterial Coldwater Disease)) hos større fisk i akvakulturopdræt af salmonider (primært regnbueørreder). På grund af stigningen i antibiotikaresistens og de kendte følgevirkninger af antibiotika på værtsbakteriel mikrobiota og på miljøet, er nye kontrolmetoder over for denne bakterie nødvendige. Bakteriofager (også kaldet fager) er bakteriers værtsspecifikke virus, der ikke kan replikere i eukaryoter, og takket være deres bakteriedræbende aktivitet (de inficerer, lyserer og dræber værtsbakterien, der herefter frigiver nye fager til miljøet), er de et potentielt miljøvenligt alternativ til antibiotika eller som profylaktisk foranstaltning.

Samlet set bringer dette ph.d.-projekt ny viden til udvikling af fagbaserede kontrolstrategier rettet mod den patogene bakterie *F. psychrophilum*; strategier der kan anvendes i akvakulturanlæg. Ph.d.-projektet var en del af et større forskningsprojekt med titlen "Bakteriofagbaseret teknologi til kontrol af *Flavobacterium*-patogener i akvakultur" (akronym: FLAVOPHAGE), et samarbejde mellem baltiske partnere omhandlende bakteriofager specifikke over for *F. psychrophilum* og *F. columnare*.

I **Manuskript I** undersøgte vi potentialet for en to-komponent blanding af bakteriofager til kontrol af *F. psychrophilum* i yngel fra regnbueørred administreret ved tre metoder (oral, bad og injektion). Til de orale indgivelseseksperimenter blev fager påført foderpiller ved enten sprøjtning ($1.6*10^8$ PFU g^{-1}) eller irreversibel immobilisering (produceret af Fixed Phage Ltd ved hjælp af deres patenterede corona-discharge teknologi; $8.3*10^7$ PFU g^{-1}). Eksperimentelle forsøg med fisk blev udført, hvor bakterier og fager blev reisoleret fra fiskeorganer ved hjælp af dyrkningsbaserede metoder, og overlevelsesanalyser blev udført. På trods af at der ved alle prøveudtagninger blev påvist fager i tarmen men med en mere sporadisk forekomst i nyre, milt og hjerne, blev der ikke observeret nogen signifikant stigning i fiskens overlevelse under *F. psychrophilum* infektionen i forbindelse med fagbehandlingen. Et lignende resultat blev opnået, når fager blev indgivet ved bad. Når fager blev administreret ved intraperitoneal injektion ($1.7*10^8$ PFU $fisk^{-1}$), blev der imidlertid observeret en signifikant stigning i fiskens overlevelse i gruppen eksponeret for fager (80.0%) sammenlignet med kontrolgruppen (56.7%). Yderligere eksperimenter end dem, der er inkluderet i Manuskript I, blev udført, og resultaterne er præsenteret i tillægssektionen (**Appendix**) til denne afhandling. I disse eksperimenter blev fager indgivet enten via fag-immobiliseret foder eller via en kombination af fag-immobiliset foder og fag-badprocedurer. Der blev ikke observeret nogen gavnlig virkning af fager på fiskens overlevelse ved de afprøvede fagkoncentrationer.

I **Manuskript II** fokuserede vi på at evaluere virkningen af oral administration af fager eller florfenicol (antibiotikum, der anvendes i Danmark til behandling af RTFS) på tarm-mikrobiotaen hos regnbueørredyngel i forbindelse med en *F. psychrophilum* infektion. Til denne undersøgelse blev fager målrettet mod *F. psychrophilum* kontinuerligt leveret via enten fag-sprøjtet eller fag-immobiliseret foder med en profylaktisk periode på 12 dage. Florfenicol-overtrukket foder blev administreret i 10 dage startende to dage efter infektionen. Prøver af den distale tarm blev opsamlet over tid og analyseret ved analyse målrettet mod 16S rRNA-genet. Resultaterne viste en dysbiose-effekt forårsaget både af infektionen og af administration af florfenicol. Interessant nok ændrede fag-tilsætningen fiskens mikrobiota uafhængigt af tilstedeværelsen af deres målbakterie.

I **Manuskript III** fokuserede vi på *F. psychrophilum* infektioner i regnbueørred øjenæg. Først etablerede vi en bakterieinfektions-badmetode og evaluerede virkningen af enkeltfager på fiskeæg (overlevelse af øjenæg; interaktion mellem fager og øjenæg). Efterfølgende blev bakterieinficerede øjenæg udsat for fager for at undersøge deres evne til at kontrollere *F. psychrophilum*. Dyrkningsbaserede metoder blev brugt til at tælle antallet af bakterier og/eller fager. Bakteriofager syntes ikke at have en negativ indflydelse på overlevelsen af øjenæg, og de satte sig ikke fast på overfladen af øjenæg. Resultaterne viste, at fager har potentiale til at kontrollere *F. psychrophilium* i kortvarige (24 timer) eksperimenter.

Abbreviations

16S rRNA	16S ribosomal RNA
AMR	Antimicrobial Resistance
BCWD	Bacterial Coldwater Disease
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
dsDNA	Double-strand DNA
EOP	Efficiency of Plating
GIT	Gastrointestinal Tract
HGT	Horizontal Gene Transfer
IM	Intramuscular
IP	Intraperitoneal
LPS	Lipopolysaccharide
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
QS	Quorum Sensing
RNA	Ribonucleic Acid
RTFS	Rainbow Trout Fry Syndrome
TYES-A	Tryptone Yeast Extract Salts (TYES) agar
TYES-B	Tryptone Yeast Extract Salts (TYES) broth

Terminology

16S rRNA gene: The 16S rRNA gene (~1500 bp) is located in the 30S subunit of the prokaryotic ribosome and consists of nine hypervariable regions (named V1-V9) flanked by highly conserved DNA. The regions V1-V9 represent perfect sites for amplification and sequencing allowing the classification of bacteria and the analysis of diversity (Claesson et al., 2017)

Alpha diversity: Diversity within a group (Christensen et al., 2018).

Antibiotics: Molecules with antimicrobial activity a) originating from the secondary metabolism of bacteria or b) produced partially or entirely by chemical synthesis (semisynthetic or synthetic antibiotics) (Dehò and Galli, 2012).

Bactericidal activity: Causing the death of bacteria (Dehò and Galli, 2012).

Bacteriostatic activity: Inhibition of the growth of bacteria (Dehò and Galli, 2012).

Beta diversity: Diversity between groups (Christensen et al., 2018).

Biofilm: Microbial communities embedded in an extracellular matrix, formed by bacteria from various genera/species but also e.g. yeast, algae and fungi, in a tight contact with the colonized biotic (e.g. teeth) or abiotic (e.g. plastic or other materials) surface (Dehò and Galli, 2012).

Dysbiosis: Status where the bacterial community of the microbiota deviates from its normal composition ("dys"=disrupted; "biosis"=life) (Maresso, 2019).

Gram negative bacteria: Their cell wall is characterized by a thin layer of peptidoglycan (< 10 nm) situated between the inner and the outer membrane of the cell wall (this area is called periplasm). They do not acquire a coloration when incubated with the dye crystal violet (method developed by the Danish microbiologist Hans Christian Gram in 1884 - the dye is washed out by ethanol) but they can be further incubated with a counterstain (e.g. safranin) and acquire a pink-red coloration (Dehò and Galli, 2012; Maresso, 2019).

Gram positive bacteria: Their cell wall is characterized by a thick layer of peptidoglycan (peptides plus sugars/glycan; ~40 nm) external to the cellular membrane. They acquire a blue-purple coloration when incubated with the dye crystal violet (method developed by the Danish microbiologist Hans Christian Gram in 1884) (Dehò and Galli, 2012; Maresso, 2019).

Innate and adaptive immune system: The innate immune system is the first line of defense against external treats providing a quick non-specific response (mediated by phagocytes as macrophages and dendritic cells) while the adaptive or acquired immune system is developed in the late phase of the infections and is responsible for the generation of the immunological memory (mediated by T and B cells with the generation of specific antibodies) (Maresso, 2019).

Microbiome: Collection of the genomes of the microbiota (Lynch and Pedersen, 2016).

Microbiota: Microbial communities inhabiting specific environments (bacteria, archaea, viruses, bacteriophages and fungi) (Lynch and Pedersen, 2016).

Operational taxonomic unit (OTU): In the analysis of microbiome data, sequences that differ by more than 3% are considered as unique OTUs and are likely to represent different species (Maresso, 2019).

Pathogenicity: Ability of bacteria or other microorganisms to interfere with the normal functions of the host causing disease (Dehò and Galli, 2012).

Phage absorption rate: Velocity constant at which a selected phage enters a given bacterium in a certain volume (ml min^{-1}) (Clokie and Kropinski, 2009; Castillo and Middelboe, 2016).

Phage burst size: Number of released new virions (Kosznik-Kwaśnicka et al., 2019).

Phage efficiency of plating (EOP): The titer of the phage on the target bacterium compared to the maximum titer observed (Kutter, 2009).

Phage host range: Which bacterial genera, species and strains a phage can lyse (Kutter, 2009).

Phage latent period: Time between phage absorption and release of phage progeny (Kosznik-Kwaśnicka et al., 2019).

Quorum sensing (QS): Mechanism of communication of bacteria via the secretion and detection of small chemical molecules named inducers or autoinducers (Maresso, 2019).

Sepsis: A syndrome of progressive stages that initiates with symptoms generated by the immune response (system inflammatory response syndrome or SIRS characterized by e.g. increased rate of breathing and heartbeat) and may progress to severe sepsis or septic shock (failure of one or multiple organs), and to multi-organ failure and death (Maresso, 2019).

Taxonomy: Biological classification of all the living organisms on the basis of their similarities [from the largest level of classification to the smallest: domain, phylum, class, order, family, genus and species] (Dehò and Galli, 2012).

Viable but non-culturable (VBNC) bacteria: Living bacterial cells that have lost the ability to grow on routine media and are characterized by e.g. a lower metabolic rate than cells in the exponential phase, changes in morphology. It is considered a long-term survival strategy that is adopted by bacteria in case of unfavorable environmental conditions (e.g. changes in temperature, starvation) (Li et al., 2014).

Virulence: Measure/quantification of pathogenicity by the analysis of specific virulence factors defined as bacterial products enhancing the pathogen ability to cause disease (e.g. secreted toxins, surface proteins). *In vivo* studies can be performed to evaluate virulence e.g.: survival analysis (the proportion of survived host after the infection is plotted over time), measuring the lethal dose 50 or LD50 (dose of infection where 50% of the host population dies) (Maresso, 2019).

Background and outline of the thesis

The aquaculture sector faces a period of constant growth (World Bank, 2013; FAO, 2018). However, the shift to intensive farming, the introduction of recirculating aquaculture systems (RAS) (Bregnballe, 2015) and the administration of antibiotics to combat infectious threats has led to a rise in the occurrence of bacterial diseases and antimicrobial resistance (AMR) in aquatic environments (reviewed by (Preena et al., 2020)). These concerns, together with stricter regulations (e.g. in organic farming (Jokumsen and Svendsen, 2010)) have led to an increased focus on developing more sustainable solutions for disease control in the aquaculture sector (reviewed in (Culot et al., 2019)).

In this study, we focused on rainbow trout (*Oncorhynchus mykiss*, Walbaum), the most dominant species in Danish aquaculture (Jokumsen and Svendsen, 2010), and the prevention and treatment of the freshwater bacterial pathogen *Flavobacterium psychrophilum*. The work focuses on phage therapy (based on the bactericidal activity of lytic phages, natural occurring viruses of bacteria) as an environment-friendly alternative method for the treatment of bacterial infections with the overall aim of bringing new knowledge concerning the application of this new approach in aquaculture facilities. The study was part of a larger research project titled "Bacteriophage based technology to control *Flavobacterium* pathogens in aquaculture" (acronym: FLAVOPHAGE), a collaboration of Baltic partners studying bacteriophages specific for *F. psychrophilum* and *F. columnare* (Middelboe, 2018, 2019, 2020).

The PhD thesis opens with an introduction where the first chapter is dedicated to the bacterium *F. psychrophilum*. Its historical background, clinical signs, transmission and prevention/treatment measures are at first introduced together with other characteristics of this bacterium (cultivation and identification). The chapter concludes with an overview of the experimental infection methods for this bacterium and the ones selected for our experiments.

The second chapter focuses on bacteriophages and phage therapy in the area of aquaculture. This chapter opens with a description of the history of phages, their characteristics and life cycles, and an overview of the steps necessary for the preparation of phages for phage therapy (isolation, characterizations, formulations and routes of administration). Subsequently, a short sub-chapter is dedicated to the distribution of phages in the body from the gastrointestinal tract (oral administration). The challenges of phage therapy are highlighted and the chapter concludes with an overview of phage products concerning aquaculture available or in development, and of the previous experience/experiments concerning phages and *F. psychrophilum.*

As the effects of phages, antibiotics and infection on the fish gut bacterial communities have been investigated in this thesis, the third chapter brings an overview of the fish

gut microbiota. This chapter opens defining the gut microbiota, its role and importance followed by a description of its development in fish and the factors influencing it with a special focus on antimicrobial compounds and dietary supplements (e.g. antibiotics, probiotics and phages). Subsequently, a sub-chapter is dedicated to the gut microbiota of rainbow trout and the following to a short description of other investigated microbiota of fish (gills and skin). Finally, this chapter concludes with a description of how microbiome studies are performed.

An overview of the aims and methods used in this study is presented and the main results summarized including conclusions and future perspectives. Finally, the thesis concludes with the three accompanying manuscripts and some additional experiments concerning phage administration to rainbow trout fry (Appendix).

Introduction

1 *Flavobacterium psychrophilum*

Flavobacterium psychrophilum (Borg, 1948; Bernardet et al., 1996) is a Gram-negative rod-shaped bacterium and the etiological agent of Rainbow Trout Fry Syndrome (RTFS, fry stage) (Lorenzen et al., 1991; Lorenzen, 1994) and of Bacterial Coldwater Disease (BCWD, juvenile and adult fish) (Borg, 1960; Holt, 1988). It belongs to the Family of *Flavobacteriaceae,* Order *Flavobacteriales,* Class *Flavobacteriia*, Phylum *Bacteroidetes* (Bernardet and Bowman, 2006). The characteristics of this bacterium have been reviewed by various authors: Dalsgaard (1993), Nematollahi et al. (2003b); Barnes (2011); Starliper (2011); Holt et al. (2012); Loch and Faisal (2015); Wahli and Madsen (2018).

1.1 Historical background

The first report of *F. psychrophilum* infection was provided by Davis (1946) and the disease was described as "Peduncle disease" since a large number of non-motile rod-shaped bacteria were observed scraping the lesions in the caudal peduncle of rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings in West Virginia (Davis, 1946). Later, Borg (1948) observed a similar disease in coho salmon (*O. kisutch)* in the State of Washington, isolated the bacterium from the kidney and other lesions and reproduced the infection experimentally (Borg, 1948, 1960). The bacterium was at that time named *Cytophaga psychrophila* (Borg, 1960). Subsequently, the bacterium was re-named as *Flexibacter psychrophilus* (Bernardet and Grimont, 1989) and later transferred to the genus *Flavobacterium* and named as *F. psychrophilum* (Bernardet et al., 1996).

In the US, the disease was identified as Bacterial Cold Water Disease (BCWD) since the infection was observed when the temperature was below 10ºC (Borg, 1960; Holt, 1988). In Denmark, a morphologically and physiologically very similar bacterium to *C. psychrophila* was identified in 1991 in fry (Lorenzen et al., 1991) and the name Fry Mortality Syndrome (FMS) was utilized to described the infection. The name Rainbow Trout Fry Syndrome (RTFS) was also used (Lorenzen, 1994). The terms RTFS and BCWD are both in use.

F. psychrophilum has been isolated from freshwater systems worldwide (Nematollahi et al., 2003b; Barnes, 2011).

1.2 Clinical signs, transmission and route of infection

The most susceptible salmonid species to *F. psychrophilum* are rainbow trout (*Oncorhynchus mykiss*, Walbaum) and coho salmon (*O. kisutch*). Depending on the size of the fish, the clinical signs and the degree of mortality vary (reviewed in (Dalsgaard, 1993; Nematollahi et al., 2003b; Barnes, 2011)).

In rainbow trout fry populations (0.5-2 g), mortalities up to 80-90% have been detected during disease outbreaks (Lorenzen, 1994; Nilsen et al., 2011). The fry appear lethargic with abnormal swimming behavior (close to the water surface). Exophthalmia and dark coloration of the skin characterize the diseased fish which manifest poor appetite and severe anemia (very pale gills, kidney and liver). The enlargement of the spleen (splenomegaly) with a change in the coloration towards grey/dark red is also present (Lorenzen et al., 1991) (**Figure 1**). Larger fish are affected differently by *F. psychrophilum* and mortalities of around 20% have been recorded (Lorenzen, 1994; Nilsen et al., 2011). In fingerlings and adult fish, skin ulcerations and dark pigmentation on one side of the body has been detected and the erosion of the tissue in the peduncle area observed with exposure of the spinal cord (Davis, 1946; Nematollahi et al., 2003b; Barnes, 2011; Loch and Faisal, 2015). In some cases, the development of abnormal swimming behavior and spinal deformation have been associated with this bacterial infection (Madsen and Dalsgaard, 1999b; Madsen et al., 2001).

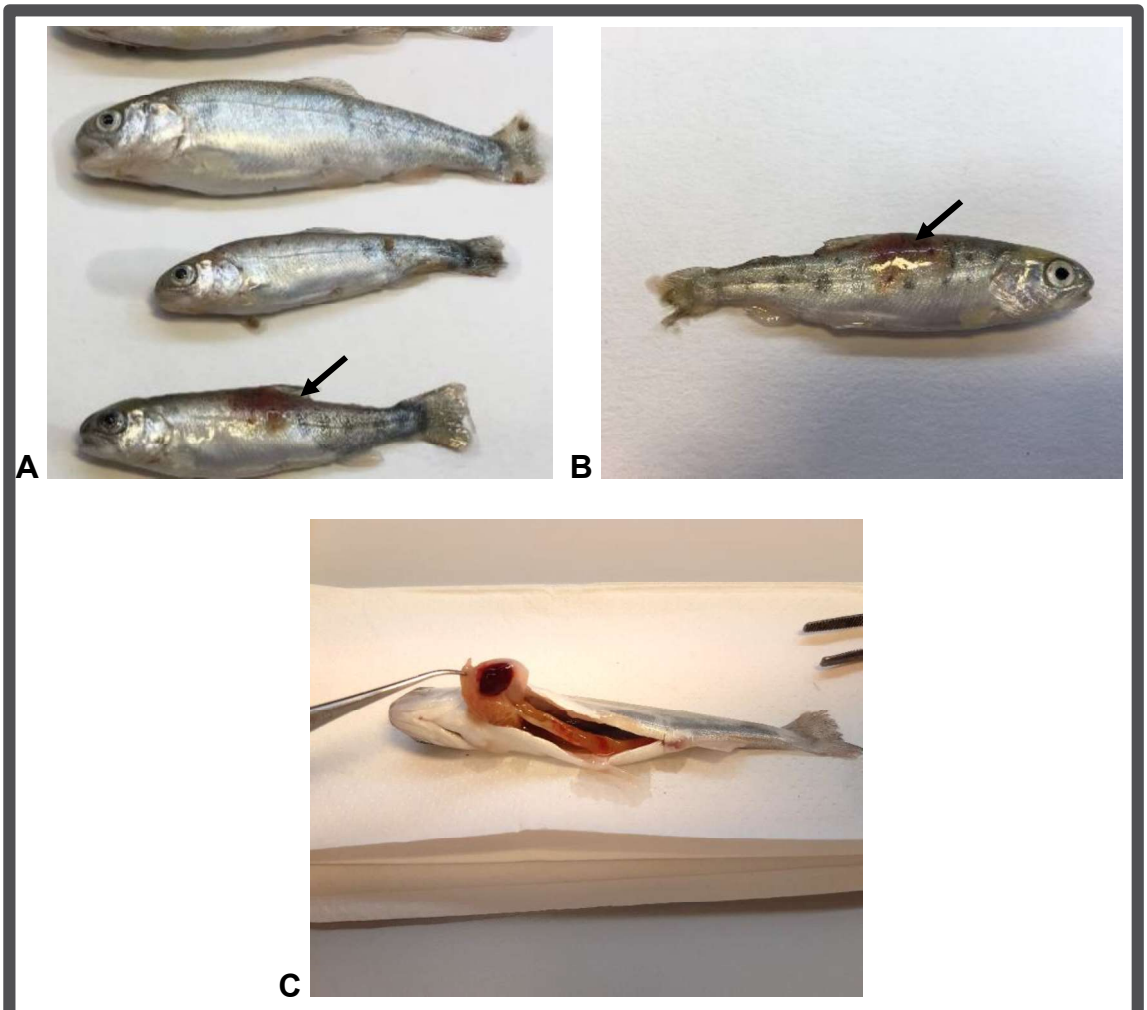

Figure 1. Example of clinical signs caused by *F. psychrophylum.* (**A**), (**B**): external skin lesions (indicated by black arrows); (**C**): splenomegaly and dark coloration of the spleen. Pictures were taken during fish challenge experiments (photos A, B by V.L. Donati; photo C by K.K. Mouritsen).

The transmission of the *Flavobacterium psychrophilum* among fish is not fully understood and both the horizontal and the vertical routes have been suggested to play a role (Brown et al., 1997; Madsen and Dalsgaard, 2000; Nematollahi et al., 2003b; Madsen et al., 2005). Studies report that *F. psychrophilum* has been isolated from the surrounding environment of diseased fish (Madsen et al., 2005; Madsen and Dalsgaard, 2008) but also from milt and ovarian fluids (Rangdale et al., 1996; Brown et al., 1997; Taylor, 2004; Madsen et al., 2005). It has also been demonstrated that *F. psychrophilum* can survive for weeks in starvation conditions in water (Madetoja et al., 2003; Vatsos et al., 2003). Uncertainly exists in defining *F. psychrophilum* as a truly vertically transmitted bacterium. Madsen and Dalsgaard (2008) did not isolate the bacterium from inside the eggs but only in close proximity/on the egg. Some other studies claim the pathogen to be able to penetrate the fish egg before water hardening (Brown et al., 1997; Taylor, 2004; Cipriano, 2005). However, these latter results are in contrast with the fact that, if performed correctly, the disinfection of rainbow trout eyed eggs (with iodine disinfectants) eliminates efficiently the pathogen at this life stage (in Danish aquaculture) (Lone Madsen and Niels Henrik Henriksen personal communication).

Lesions in the skin and in the mouth cavity, and the gills represent the main portals of entry of the bacterium (Lorenzen, 1994; Madsen, 2000; Madetoja et al., 2002; Nematollahi et al., 2003a; Lorenzen et al., 2010). The gastrointestinal tract of the fish is also a possible portal of entry (Lorenzen, 1994; Madsen, 2000).

1.3 Prevention and treatment

Prevention of *F. psychrophilum* infections in aquaculture facilities can be performed by following good management practices and egg disinfection (Madsen and Dalsgaard, 2008; Barnes, 2011). Physical handling procedures and stress should be reduced or avoided, and a quality feed diet should be provided (Barnes, 2011). The quality of the water represents an important parameter in the prevention of RTFS (Barnes, 2011). Finally, the efficient removal of dead and moribund fish from tanks must be performed to reduce the infection pressure during bacterial outbreaks (Madetoja et al., 2000).

Antibiotics have been the most extensively used treatment for RTFS worldwide. In Denmark oxolinic acid, oxytetracycline and amoxicillin, have been used and, since 1996, only florfenicol is allowed for the treatment of this pathogen (Bruun et al., 2000; Dalsgaard et al., 2009). Resistance to oxolinic acid, oxytetracycline and amoxicillin has been detected in Denmark (Bruun et al., 2000, 2003; Dalsgaard and Madsen, 2000; Schmidt et al., 2000; Dalsgaard et al., 2009) and in other countries (Izumi and Aranishi, 2004; Kum et al., 2008; Del Cerro et al., 2010; Sundell and Wiklund, 2011; Ngo et al., 2017). In addition, resistance to the sulphadiazine/trimethoprim, antibiotic used in Danish aquaculture, has been observed in *F. psychrophilum* even if this antimicrobial has not been specifically used for this pathogen (Bruun et al., 2000; Dalsgaard and Madsen, 2000; Dalsgaard et al., 2009). In Bruun et al. (2000), this

feature was hypothesized being an inherent character of this bacterium or a widespread acquired resistance pattern. To conclude, an increased minimum inhibitory concentration (MIC) of 60 *F. psychrophilum* strains to florfenicol was detected in 2009 supporting the need of alternative methods for the treatment of this bacterial infection (Dalsgaard et al., 2009).

Extensive research has been targeting the development of an effective vaccine against *F. psychrophilum* (reviewed in (Nematollahi et al., 2003b; Barnes, 2011; Gómez et al., 2014; Wahli and Madsen, 2018)). A live attenuated immersion vaccine (iron limited B.17 vaccine (B.17-ILM)) was developed and tested by LaFrentz et al. (2008) and Long et al. (2013), further tested and optimized by Sudheesh and Cain (2016) and its efficacy on a diverse group of *F. psychrophilum* isolates evaluated by Ma et al. (2019) [not commercially available]. Additional work has been done by Hoare et al. (2017, 2019) who have investigated the efficacy of a polyvalent, whole cell vaccine containing formalin-inactivated *F. psychrophilum*.

The use of probiotics have also been tested (Korkea-aho et al., 2011, 2012; Madsen et al., 2012).

1.4 Pathogen cultivation and identification

The isolation of *F. psychrophilum* can be performed from skin lesions, spleen, kidney and brain of diseased fish. Samples can be streaked on tryptone yeast extract salts (TYES) agar and incubated at 15-20ºC for 3-6 days (optimal temperature 15ºC) (Holt et al., 1993). *F. psychrophilum* does not grow on rich media as blood-agar but, in addition to TYES medium, other specific media can be used for the growth of this pathogen such as cytophaga agar (Anacker and Ordal, 1959), modified cytophaga agar (Wakabayashi and Egusa, 1974), modified Anacker and Ordal (Lorenzen et al., 1997) and others (reviewed by Nematollahi et al. (2003) and Holt et al. (2012)).

Bright yellow colonies (coloration conferred by the pigment flexirubin) appear on TYES-A after 3-6 days of incubation (**Figure 2A**; 2-3 days in broth cultures, **Figure 2B**). In Lorenzen et al. (1997), *F. psychrophilum* colonies are described as non-adherent, smooth, shiny, convex, circular and opaque colonies that can present regular but also spreading edges with a “cheese-like” odor (**Figure 2A** and **C**). When observed with a stereomicroscope, colonies may be characterized by a blue-green iridescent pattern (Lorenzen et al., 1997) (**Figure 2D**). Smooth and rough colony morphologies have been observed (Högfors-Rönnholm and Wiklund, 2010) (**Figure 2D**). Microscopic observations reveal long rods of 3-10 µm with a diameter of 0.2-0.5 µm. According to the age and the type (broth or agar) of the culture, the rods change their morphology and become shorter and thicker (e.g. old cells or in agar) (Lorenzen et al., 1997). Electron microscopic observations show the typical structure of the cell wall of Gram-negative bacteria (Lorenzen et al., 1997; Rangdale et al., 1999).

To distinguish with other yellow colony forming bacteria, it must be kept in mind that *F. psychrophilum* does not grow on blood-A, and at temperatures higher than 22-25ºC

(Bernardet and Kerouault, 1989; Dalsgaard and Madsen, 2000). In addition, the presence of flexirubin-like pigment can be verified mixing a portion of a colony with a drop of 20% potassium hydroxide (the material will assume a reddish/brown coloration in case of flexirubin presence) (Holt et al., 2012).

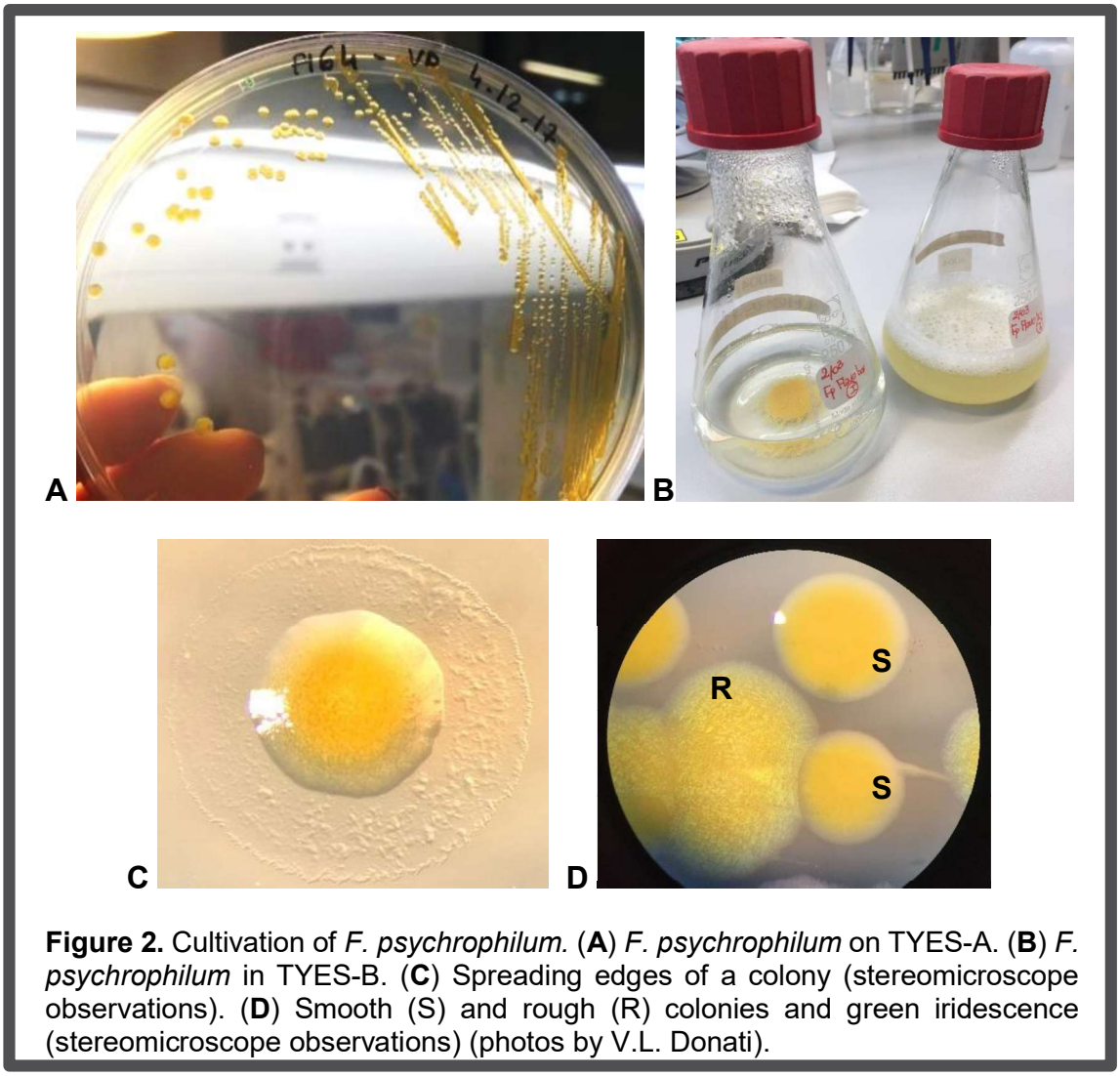

Figure 2. Cultivation of *F. psychrophilum.* (**A**) *F. psychrophilum* on TYES-A. (**B**) *F. psychrophilum* in TYES-B. (**C**) Spreading edges of a colony (stereomicroscope observations). (**D**) Smooth (S) and rough (R) colonies and green iridescence (stereomicroscope observations) (photos by V.L. Donati).

Serological and PCR-based methods can be used for the identification and characterization of *F. psychrophilum* (reviewed by Barnes and Brown (2011) and Holt et al. (2012)). ELISA (enzyme-linked immunosorbent assay) (Lorenzen and Olesen, 1997; Lindstrom et al., 2009; Long et al., 2012), agglutination assay (Lorenzen and Olesen, 1997) and indirect fluorescent antibody test (IFAT) using monoclonal antibodies (MAb FL43) (Lindstrom et al., 2009; Holt et al., 2012) have been developed. A multiplex PCR has also been generated for serotyping *F. psychrophilum* strains (Rochat et al., 2017). PCR reactions for the identification of *F. psychrophilum* target the 16S ribosomal RNA gene (16S rRNA gene) (Toyama et al., 1994; Urdaci et al., 1998) or the β-subunit of the DNA gyrase (*gyr*B gene) (Izumi and Wakabayashi, 2000). Nested PCR (Izumi and Wakabayashi, 1997; Wiklund et al., 2000; Taylor and Winton,

2002) and quantitative PCR methods (Orieux et al., 2011; Strepparava et al., 2014; Jarau et al., 2018) have been developed and their sensitivity assessed.

For the experiments of this thesis and the included manuscripts, MALDI–TOF (matrix assisted laser desorption ionization – time of flight) Mass Spectrometry (MS) (Singhal et al., 2015) was the chosen method for the identification and confirmation of *F. psychrophilum* colonies (Jansson et al., 2015, 2020; Pérez-Sancho et al., 2017). Pérez-Sancho et al. (2017) studied this method in relation to the identification of *F. psychrophilum* and observed that it correctly identified *F. psychrophilum* isolates even if its ability to differentiate among the strains was limited.

1.5 Experimental infection in fish

The intraperitoneal (IP) injection is considered the most reproducible challenge method for studying the infection caused by *F. psychrophilum* in rainbow trout fry (Madsen and Dalsgaard, 1999a). In the standardization studies performed by Madsen and Dalsgaard (1999a), the infection of 1 g fry with a dose of 10^4 CFU $fish^{-1}$ resulted in 60-70% final cumulative mortality and fulfilled the recommendations of Amend (1981) (standard in fish vaccine development: the mortality of the infection group should be between 60-85% in order to evaluate its significance). The fish weight was established being a very important variable. It was necessary to increase the dose up to 10^7 CFU $fish^{-1}$ in larger individuals (28 g) to observe a final cumulative mortality of only 20-30%. This was attributed to the undeveloped immune system of rainbow trout fry and its relation to weight as the body size is a very important parameter in the immunological maturation of the fish (Madsen and Dalsgaard, 1999a). Additional factors to consider are: the density of fish (as this affects the infection pressure), the origin of the fish (as they may have been previously exposed to the pathogen), the virulence of the isolate (Madsen and Dalsgaard, 1999a). Furthermore, a whole 48-hour old culture was injected as additional washing steps did not seem to affect the outcome of the experiment (Madsen and Dalsgaard, 1999a). Additional studies evaluating the IP and Intramuscular (IM) injection as infection methods for *F. psychrophilum* were performed by Garcia et al. (2000). The IP injection was the selected method of infection used for **Manuscript I** and **II, Appendix A** and **B** and the main step of the infection experiment are illustrated in **Figure 3**.

The IP and IM injections bypass the fish defense mechanisms (mucus, skin, gills and gut of the fish) (Madsen and Dalsgaard, 1999a; Nematollahi et al., 2003b). In attempts to establish other reproducible methods closer to the natural way of infection, various studies have been performed. Even if lower mortalities are observed and the standardization is more difficult, a valuable alternative to the IP injection is performing a bath challenge, applying additional stress factors (Madsen and Dalsgaard, 1999a). In Madsen and Dalsgaard (1999a), 1 g fry were bath challenge for 30 minutes in an aerated bacterial solution of 10^8 CFU ml^{-1} after a preliminary treatment with formalin (0.005% formalin for 30 minute) to stress the fish and the obtained final mortality was 66%. Subsequently, hydrogen peroxide (H_2O_2) in different concentrations was studied

as a pre-treatment stress factor before the bath challenge with *F. psychrophilum* (Henriksen et al., 2013). Results showed that it could accelerate the onset of mortality with a 2-fold increase of the final mortality. However, low mortality was obtained (with H_2O_2: 19.2%; without H_2O_2: 9.1%; 1.2 g fry) (Henriksen et al., 2013). The bath or immersion challenge was also tested in other studies (Decostere et al., 2000; Garcia et al., 2000; Madetoja et al., 2000; Aoki et al., 2005; Lorenzen et al., 2010; Long et al., 2014; Chettri et al., 2018). The disruption of the dermal layer was evaluated as additional factor to increase mortality. Madetoja et al. (2000) demonstrated how skin wounded (incision below the dorsal fin) fish bath challenged with *F. psychrophilum* were strongly affected by the infection (95% mortality; 10^6 CFU ml^{-1}; 1 hour). Long et al. (2014) concluded that the disruption of the dermal layer enhances fish mortality in this type of challenge and that the removal of the adipose fin should be preferred over incision type-wounds as, the latter, are more difficult to standardize and repeat among experiments. The bath challenge method was tested in the experiments in **Appendix B** (**Figure 3B**).

Additional studies in developing a reliable method for infection were done performing cohabitation challenges (Madsen and Dalsgaard, 1999a; Madetoja et al., 2000; Chettri et al., 2018), oral challenges by inserting a catheter (Decostere et al., 2000) or by live feed as vector (Madetoja et al., 2000) and anal challenges (Decostere et al., 2000; Chettri et al., 2018). Within these methods, positive results were obtained in the co-habitation infection of skin wounded fish (Madetoja et al., 2000) and in the anal challenge performed by Chettri et al. (2018).

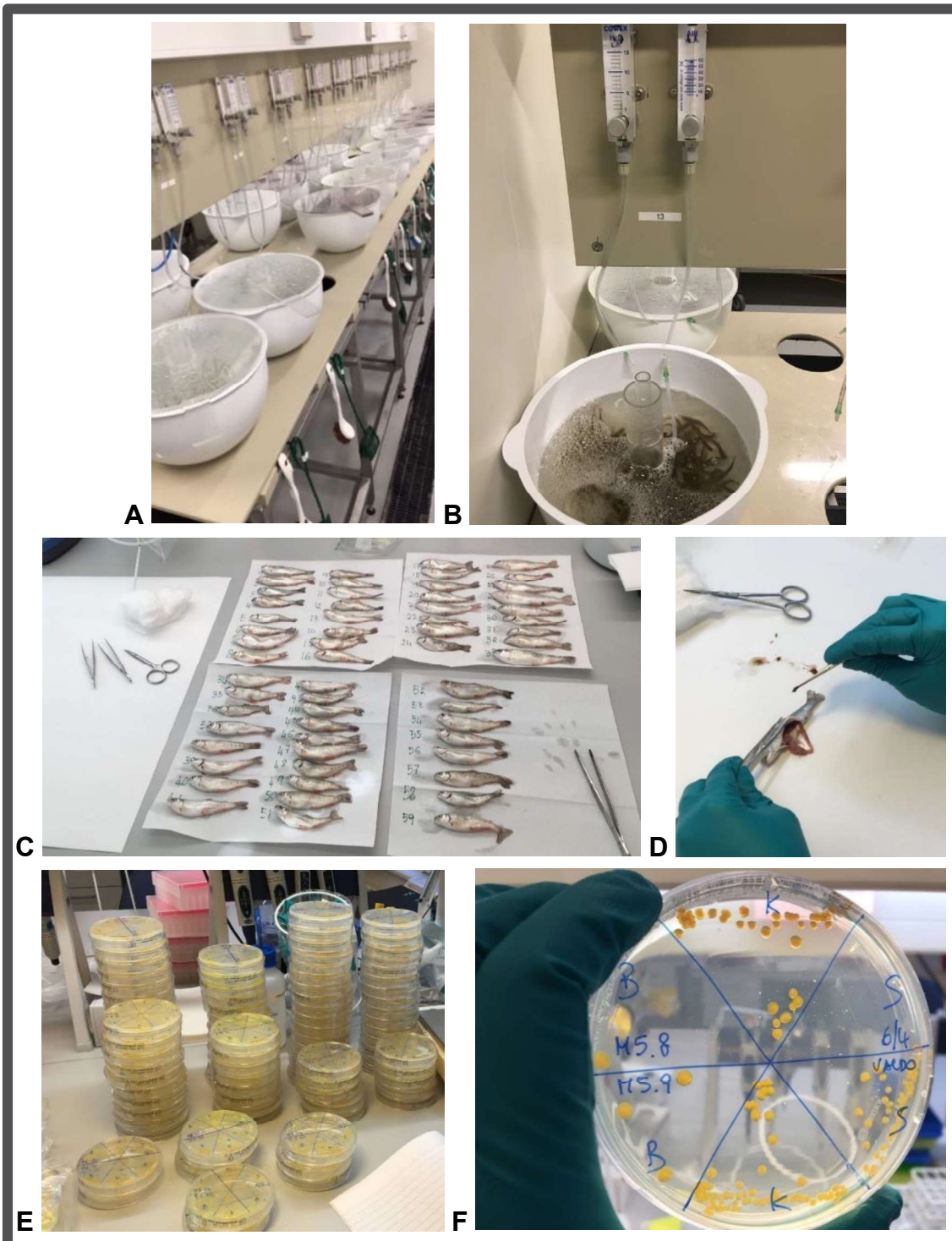

Figure 3. Fish challenge experiments. (**A**) Division of fish in 8 liters aquaria (experimental facilities at DTUAqua). (**B**) Example of bath challenge procedure with *F. psychrophilum* (reduced water volume and increased fish density). (**C**, **D**) Fish sampling. (**E**, **F**) Re-isolation of *F. psychrophilum* colonies from brain, kidney and spleen of dead and moribund fish (photos by V.L. Donati).

2 Phage therapy in aquaculture

Phage therapy relies on the bactericidal activity of virulent bacteriophages (also called phages), natural enemies of bacteria and most abundant entities on our planet (literally "eater of bacteria", from the Greek word φαγειν, *phagein,* to eat). Virulent or lytic phages are viruses that infect and kill specific bacterial hosts by lysing infected cells and releasing newly assembled phages into the surrounding environment (reviewed in (Chanishvili, 2012; Salmond and Fineran, 2015; Dion et al., 2020)). They present various advantages as they are unable to replicate in eukaryotes, easy to isolate and propagate, kill both Gram-negative and Gram-positive bacteria, work synergistically in cocktail preparations and they are Generally Recognized As Safe (GRAS) (Madhusudana Rao and Lalitha, 2015).

In the aquaculture sector, the study of phages and their potential application in the field have been characterized by a steep increase in the period 1988-today (**Figure 4A**). The majority of these studies were focused on *Vibrio spp.* (50%) followed by *Aeromonas* spp. (15%), *Flavobacterium* spp. (13%), *Edwardsiella* spp. (5%), *Pseudomonas* spp. (4%) and *Lactococcus* spp. (3%) (**Figure 4B**). The focus of these research studies varies from e.g. phage isolation and characterization and the formulation of cocktail preparations, where phages work synergistically, to the study of efficient methods of delivery *in vivo*. Fish and crustacean species at different stages of development have been investigated.

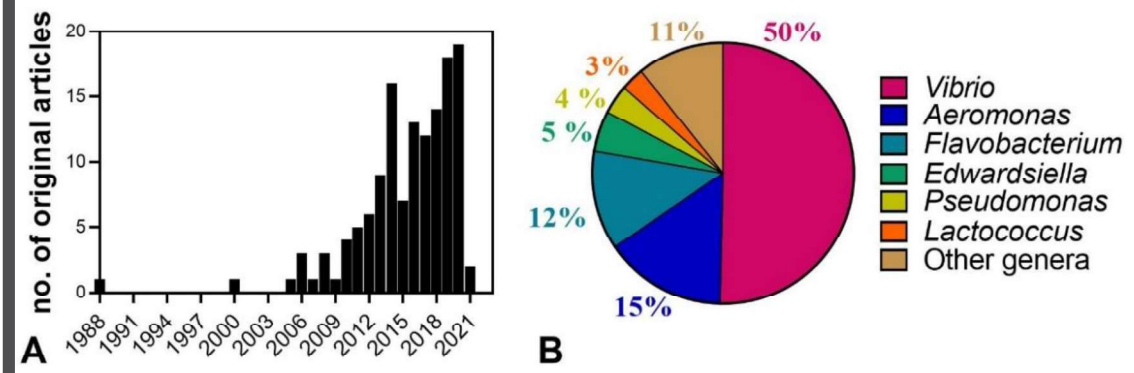

Figure 4. Overview of studies related to aquaculture and phage therapy. (**A**) Number of original studies targeting phage therapy in aquaculture from 1988 until 2021 (in press). (**B**) Percentage of most target bacterial genera are presented. The graphs were built performing a Scopus search TITLE-ABS-KEY (aquaculture AND phage) (Elsevier, 2017), similarly as Culot et al. (2019). Results were screened and only original articles related to phage therapy in aquaculture were considered (135 articles) (The search was performed 16-11-2020).

2.1 Historical background

Bacteriophages were discovered, at the beginning of the 20th century, independently by a British pathologist, Frederik Twort (from the "eaten edges" of *Staphylococcus* colonies) (Twort, 1915), and a French-Canadian microbiologist, Félix d´Hérelle (1917- from patients among French troops with severe hemorrhagic dysentery) (Roux, 2011). In 1919, d´Hérelle applied phages in chickens to treat *Salmonella gallinarum* and subsequently ran the first human trial against bacillary dysentery in Paris (Chanishvili, 2012; Almeida and Sundberg, 2020). In the same period in Brazil, José da Costa Cruz from the Oswaldo Cruz Institute (Rio de Janeiro, Brazil) was the leading researcher in phage therapy and he obtained positive results in the phage treatment of dysentery in humans in Rio de Janeiro in 1923 (Almeida and Sundberg, 2020). One year after in São Paulo during the Paulista revolution, a product named "Bacteriofagina disenterica" was tested on governmental troops (reviewed by (Almeida and Sundberg, 2020)). In the same period (1923), the Eliava Institute (the present George Eliava Institute of Bacteriophages, Microbiology and Virology) was founded in Tbilisi (Georgia) by d'Herelle and his friend and associate George Eliava (director of the institute and subsequently killed in 1937 because of his progressive ideas and collaborations with foreign scientists). The institute is now considered the leader of phage therapy worldwide (Chanishvili, 2012).

D'Hérelle performed various phage therapy clinical trials against cholera and bubonic plague in India (1927) and many others clinical trials in humans were performed by the Soviet Union in Georgia, Russia, Ukraine, Belarus, and Azerbaijan territories (1930–40s) (Chanishvili, 2012). Subsequently, two industrial centers for phage production against cholera (1931) were established in India and various commercial products were prepared by the laboratory of d´Hérelle in Paris and commercialized by a French company (the present L'Oréal). Some of the preparations were named Bacté-rhino-phage, Bacté-pyo-phage, Bacté-coli-phage, Bacté-staphy-phage and Bacté-intesti-phage (Chanishvili, 2012).

Nevertheless these initial positive developments, bacteriophages were subsequently put aside during the antibiotic era, especially in the cold war´s western bloc (Fleming´s discovery of penicillin and the banishment of therapeutic uses of phages in the USA) (Fleming, 1929; Eaton and Bayne-Jones, 1934; Culot et al., 2019). Phage therapy regained interest in the western countries in 1983 and, nowadays, bacteriophage and phage therapy are studied worldwide due to the drawbacks of antibiotic use and overuse (e.g. dispersal in the environment, development of multi-drug resistant bacteria, modulation and disruption of microbiota) (Culot et al., 2019; Almeida and Sundberg, 2020).

2.2 Bacteriophages and their life cycles

The most abundant bacteriophages belong to the order of *Caudovirales*: tailed phages with a dsDNA genome surrounded by an icosahedral or elongated head or capsid (**Figure 5**). This order includes phages belonging to the families of *Siphoviridae* (most numerous; with long, flexible or rigid and not-contractile tails), *Myoviridae* (contractile tails, where the contraction is enabled by an additional sheath/layer surrounding the central tube) and *Podoviridae* (short and non-contractile tails) (Ackermann, 2009; Salmond and Fineran, 2015; Dion et al., 2020). Phages with other morphologies with either single- or double- stranded DNA or RNA exist (Ackermann, 2009; Salmond and Fineran, 2015; Dion et al., 2020).

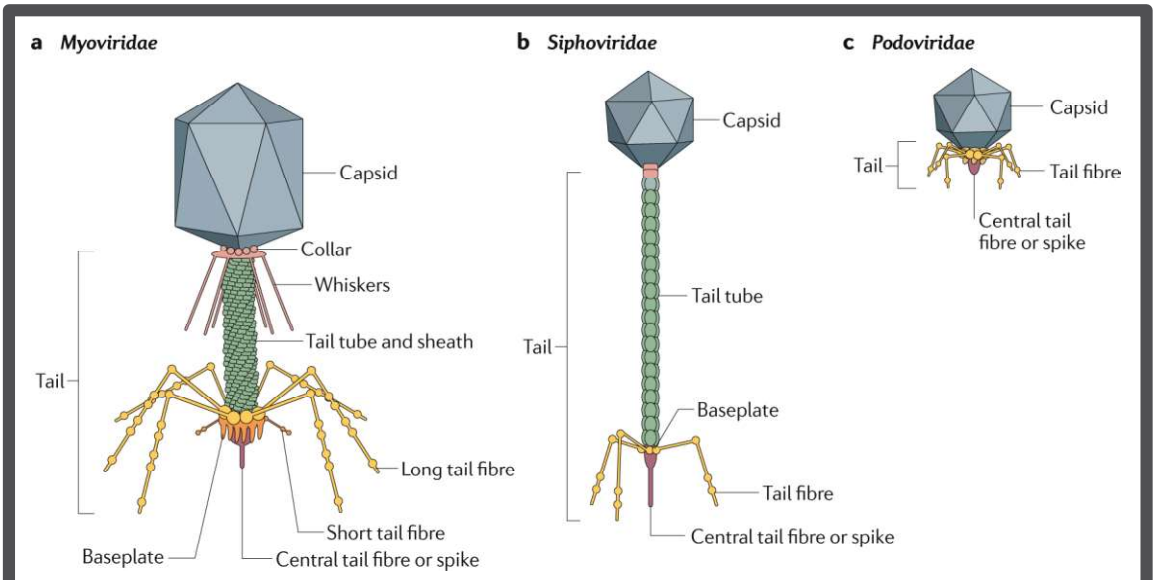

Figure 5. Structure of *Caudovirales* phages. The capsid of tailed phages contains the genome and it connects to the tail. (**a**) *Myoviridae*; (**b**) *Siphoviridae*; (**c**) *Podoviridae*. From Nobrega et al. (2018). *Copyright © 2018, Springer Nature Limited.* A personal license for the reproduction of this figure in this thesis was obtained.

Caudovirales phages infect their target bacteria by at first interacting with the host receptors on the cell surface (e.g. lipopolysaccharide (LPS) and O-antigen in Gram-negative and, teichoic acid and peptidoglycan in Gram-positive bacteria) (**Figure 6**). Specific structures on the phages' tails named receptor binding proteins (RBP) such as tail fibers and tail spikes mediate this interaction. After the establishment of an irreversible binding, phages inject their genome (Salmond and Fineran, 2015; Nobrega et al., 2018). Depending on the nature of the phage (virulent or temperate), two replication strategies can be performed: the lytic (virulent and temperate phages) or the lysogenic (only temperate phages) cycles. In the case of the lytic cycle, the injected phage genome is replicated, transcribed and translated and new virions are assembled. By the actions of phage enzymes (e.g. endolysins), the bacterial cells is lysed and the new progeny released into the environment. In the lysogenic cycle, the injected phage genome is either integrated into the bacterial genome or exist as a

plasmid and it (termed prophage) replicates together with the bacterial cell. Temperate phages follow the latter replication strategy but, under stress conditions, can exit this status and enter the lytic cycle forming new virions (Salmond and Fineran, 2015).

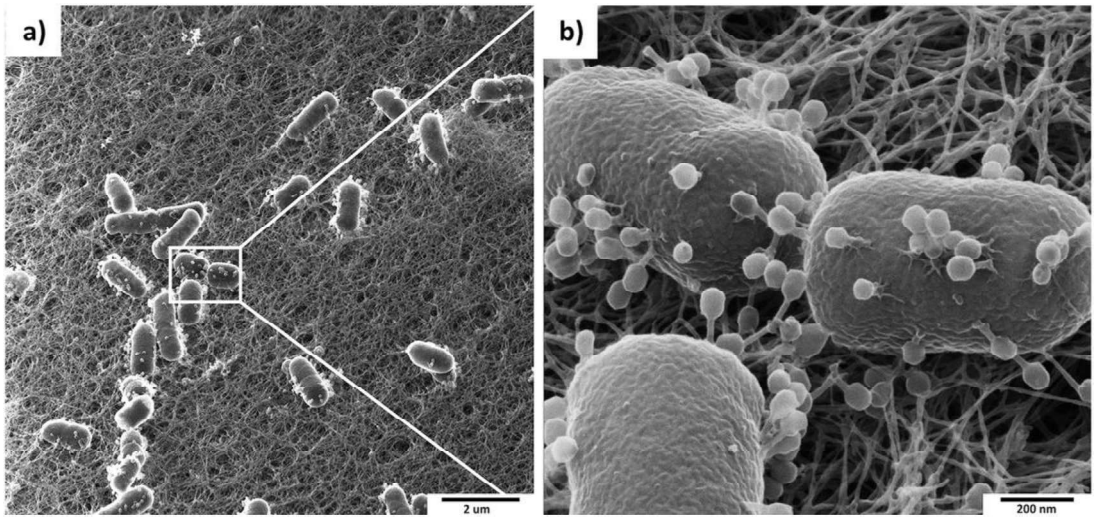

Figure 6. Bacterial cells (*Escherichia coli*) with ongoing phage infections (phage T4) observed with helium ion microscopy or HIM (edge of a plaque present on the *E. coli* bacterial lawn on agar) (**a**); Visualization of T4-phages attached onto the bacterial cell surface (**b**). From Leppänen et al. (2017). *Copyright © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.* A personal license for the reproduction of this figure in this thesis was obtained.

2.3 Phage preparation for phage therapy

Isolation and characterization

Phages can be isolated from the environment that their target bacterium colonizes (e.g. urban sewage, rivers and in the case of aquaculture, diseased fish and fish farm water). The isolation is generally a quick and easy process: 1) centrifugation or filtering of the environmental samples to eliminate other microorganisms, inoculation in a broth culture containing the host strain(s) and plating on a bacterial lawn by the double layer plaque assay to estimate the formation of plaques (indication of phages able to lyse the selected bacterium); 2) transfer single plaques into liquid medium. Additional enrichment steps (culturing with the host bacterium) and concentration procedures (e.g. precipitation by polyethylene glycol (PEG); used in the **Manuscripts** of this thesis and **Appendix B**) can be included. Finally, Mg^{2+} or Ca^{2+} may be added to the selected medium in case the phages are dependent on these ions for attachment or intracellular growth (Kosznik-Kwaśnicka et al., 2019).

Phages can be characterized by assessing their ability to lyse other bacteria than the one used for the isolation (determination of the host range) and by determining other parameters such as the absorption rate (velocity constant at which a certain phage enter a given bacterium; expressed in ml min^{-1}), the burst size (number of released new virions), the latent period (time between phage absorption and release of phage progeny), the morphology of the plaques (temperate phages = turbid plaques; lytic

phages = clear plaques). Genomes should be sequenced and phages presenting antibiotic resistance, virulence or lysogenic genes should be excluded for further utilization in phage therapy studies (Clokie and Kropinski, 2009; Kosznik-Kwaśnicka et al., 2019).

Purification and stabilization (formulations)

Various purification protocols exist and they are generally focused in removing the lipopolysaccharides (LPS) of the membrane of Gram negative bacteria since it is an endotoxin. A: High-speed centrifugation in a cesium chloride gradient, a time consuming, expensive and requiring special equipment procedure; B: extraction with organic solvents (endotoxins accumulate in the solvent); C: anion-exchange chromatography with large pore size; D: alkaline phosphate to inactivate the endotoxins (Kosznik-Kwaśnicka et al., 2019).

Phages are generally stored in aqueous solutions around 4ºC but also at -20 or -80ºC. Glycerol is added to the solution since it renders the structure of the proteins more rigid and avoid their aggregation (Kosznik-Kwaśnicka et al., 2019). Other possible storage methods are lyophilization and spray drying (conventional approaches used in the pharmaceutical industry to produce e.g. vaccines, peptides, and fine powders). Phage lyophilization is based on freeze-drying procedures (dehydratation process based on freezing) which assures long preservation and easy transportation of the phages (briefly, the phage solution is cooled down, ice crystals of pure water form which are then removed by the action of a very low-temperature condenser). This procedure has been used by the former Soviet Union to produce tablets and powders of lyophilized phages (Kosznik-Kwaśnicka et al., 2019; Merabishvili et al., 2019). In spray-drying procedures, the phage solution is sprayed inside a drying chamber containing a hot dry gas. Solvents evaporate and the insoluble phages form a powder. Threalose is generally included in the procedure as protectant. This method, however, has not been used for phage production of commercial scale. Both methods have been characterized to cause a reduction in phage titers because of the freeze-drying procedure and the phage sensibility to thermal and shear stresses (Kosznik-Kwaśnicka et al., 2019; Merabishvili et al., 2019)

Phages can also be encapsulated in protective particles (e.g. liposomes) or droplets (e.g. oil), or coated with polymers or lipids (e.g. alginate, agarose, whey protein, synthetic polymers). Finally, phages may be immobilized on various surfaces by passive adsorption (may cause poor orientation of phages on substrate) or by chemical or electrical methods (more proper orientation of phages, so they can interact with the target bacterium) (Kosznik-Kwaśnicka et al., 2019). In the **Manuscripts I and II** and the **Appendix** of this thesis, phages were either sprayed (passive absorption – applied on feed pellets with the use of a spray bottle) or immobilized on feed pellets. The immobilization was achieved by using the corona discharge method (electrical method) patented by Fixed Phage Ltd (Mattey, 2016, 2018). By this method, the phage capsids are immobilized on feed pellets by the creation of covalent bonds between the

capsid coat protein and the substrate (protein source in feed pellets). This leaves the tails free and able to infect their target bacterium (Mattey, 2018).

Administration

Phages should be administered according to where the infection is localized (Kosznik-Kwaśnicka et al., 2019). In the area of aquaculture research, three ways of phage delivery have been mainly assessed (reviewed in (Kalatzis et al., 2018; Kosznik-Kwaśnicka et al., 2019)): parenteral delivery (intramuscular or intraperitoneal injections: phages reach the systemic circulation very fast but the procedures are laborious and time consuming) (Prasad et al., 2011; Madsen et al., 2013; Sundell et al., 2020; Welch, 2020), oral administration (phage impregnated feed pellets: continuous supply of phages but the phage titers may be reduced by e.g. acid stomach environment, phage permeability to the mucosa in case the phages should reach the systemic circulation) (Park and Nakai, 2003; Prasad et al., 2011; Christiansen et al., 2014; Merabishvili et al., 2019; Ding et al., 2020) and by bath immersion (Prasad et al., 2011; Higuera et al., 2013; Laanto et al., 2015; Kim et al., 2020). The topical application of phages on skin lesions has also been tested (Khairnar et al., 2013).

2.4 Phage distribution in the body: from the gut to the internal organs (oral administration)

The interactions of phages with the secreted mucus layer of the gut epithelial cells have been named as the bacteriophage adherence to mucus or BAM model (**Figure 7A** and **B**) and by this mechanism phages are thought to provide a non-host derived immunity strategy against microbial colonization. If the tail fibers of the phage are involved in the bacterial infection, the proteins displayed in the capsid (Hoc proteins) are the responsible for the weak interactions with mucus glycans and so allow the phages to propagate into the mucus layer increasing the chance of phage-bacteria encounter (Barr et al., 2013; Barr, 2017; Van Belleghem et al., 2019).

When passed the secreted mucus layer, phages can interact with transmembrane mucins present in the apical surface of the enterocytes or be internalized by specific receptors or non-specific uptake mechanisms into the epithelial cells. Phages are then transported and released in the interstitial space or, after internalization, they are degraded and their content released into the cell (**Figure 7A** and **C**). This mechanism that allows phages to cross the intestinal barrier has been termed transcytosis (endocytosis-exocytosis). Furthermore, phages may bypass the intestinal barrier at sites of inflammation or cellular damage (increased gut permeability or “leaky gut”) (Barr, 2017; Nguyen et al., 2017; Van Belleghem et al., 2019).

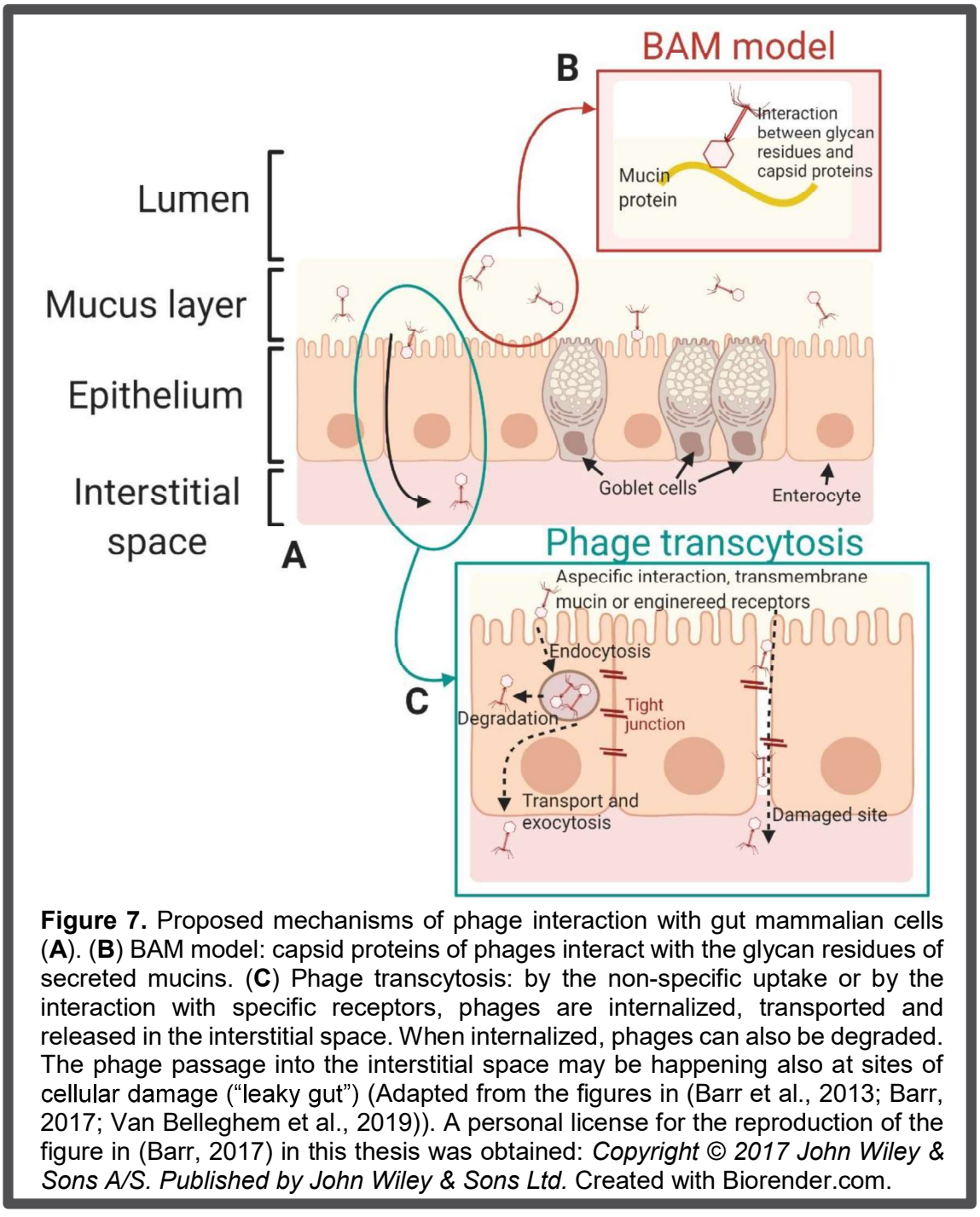

Figure 7. Proposed mechanisms of phage interaction with gut mammalian cells (**A**). (**B**) BAM model: capsid proteins of phages interact with the glycan residues of secreted mucins. (**C**) Phage transcytosis: by the non-specific uptake or by the interaction with specific receptors, phages are internalized, transported and released in the interstitial space. When internalized, phages can also be degraded. The phage passage into the interstitial space may be happening also at sites of cellular damage ("leaky gut") (Adapted from the figures in (Barr et al., 2013; Barr, 2017; Van Belleghem et al., 2019)). A personal license for the reproduction of the figure in (Barr, 2017) in this thesis was obtained: *Copyright © 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.* Created with Biorender.com.

2.5 Challenges of phage therapy

The challenges faced for the utilization of phages as therapeutics are presented in **Table 1**. Among them, the development of phage resistance and the host immune response to phages are further discussed afterwards.

Table 1. Overview of some of the challenges faced by phage therapy (adapted from the review of Ganeshan and Hosseinidoust (2019)).

Challenges of phage therapy

Narrow host-range	This may be a challenge in case of poly-microbial infections (Ross et al., 2016).
Approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA)?	The FDA and the EMA do not approve the administration of phages to humans, because of the increase in regulatory standards, partially linked to the increased knowledge concerning the effect of drugs on the human microbiome and because of the rising concerns in relation to phage resistance and the phages' role in the evolution of the bacterial genomes. However, the FDA have approved phages to be used for environmental prophylaxis, dietary supplements and food decontamination (reviewed by Ganeshan and Hosseinidoust (2019)). Phage can be administered for compassionate use and, in Poland, as an "experimental treatment" when no other options are available. Currently in the EU, phages against pathogens in aquaculture are considered as veterinary medicinal products (De Vos et al., 2019).
Phages as drivers of evolution	Phages can interact with the immune system of the body. They can replicate and evolve within the body as well as their target bacteria can evolve resistance to the administered phages (Koskella and Brockhurst, 2014).

Development of phage resistance

Bacteria can become resistant to phages by a series of mechanisms (**Figure 8**): a) prevent phage attachment by the alteration, loss or blockage by the extracellular matrix of receptors on the bacterial cell surface or the production of competitive inhibitors that bind phage receptors; b) prevent DNA phage entrance into the cell by superinfection exclusion or Sie systems (Sie systems: proteins causing cell surface alterations or inhibition of replication, e.g. the protein Imm encoded by the phage T4 blocks the phage DNA translocation into the cytoplasm); c) inactivation of phage DNA once entered the cytoplasm by restriction-modification (R-M) systems [formed by a methyltransferase which catalyze the methylation of the bacterial DNA and by a restriction endonuclease, which cut the phage, un-methylated DNA] or by the action of the CRISP-Cas system [the clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated (*cas*) genes represent an immunity system targeting foreign nucleic acids as e.g. the phage DNA]; d) abortive infection systems (Abs) (heterologous bacterial proteins that lead to the deliberate death of the infected cell, which prevents phage proliferation and spreading of the infection) (reviewed in (Labrie et al., 2010; Kalatzis et al., 2018)).

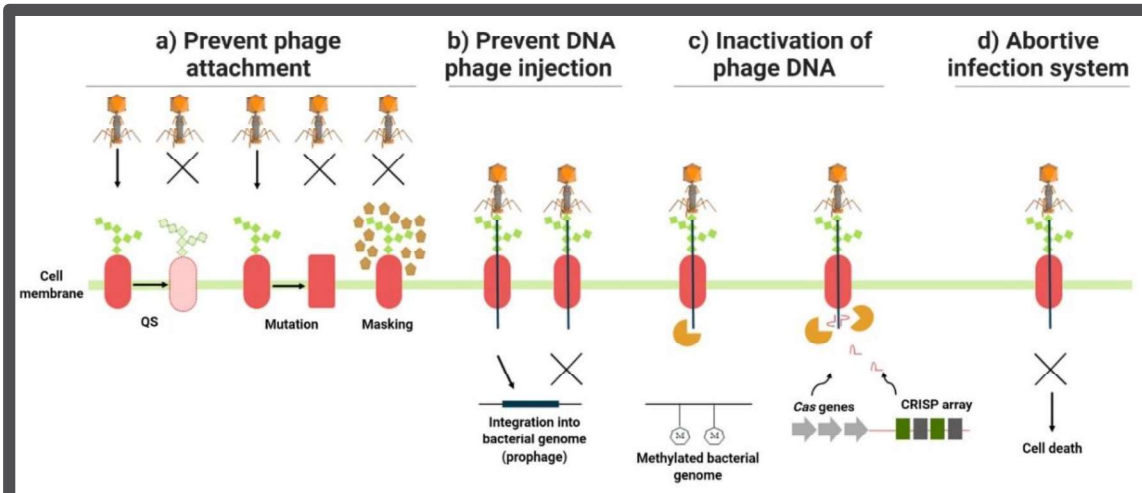

Figure 8. Overview of bacterial defense mechanisms to phage infection. (**a**) Prevent phage attachment by downregulation of receptors by quorum sensing (QS), by mutating of masking the receptors. (**b**) Sie mechanisms: proteins encoded by plasmids or prophages, which may have been previously acquired. (**c**) Digestion of phage DNA by the R-M systems and the CRISPR-Cas system. (**d**) Abortive infection. Adapted from Kalatzis et al. (2018) with Biorender.com. © *2018 by the authors. Licensee MDPI, Basel, Switzerland* [Creative Commons Attribution License (CC BY)].

However, phage resistance appears to be acquired at a cost as the mutation in the surface receptors, which phages bind to, are involved in important functions as motility, attachment to surfaces or nutrients uptake (Middelboe, 2000; Middelboe et al., 2009; Kalatzis et al., 2018). The development of phage-resistance has been linked to a loss in the bacterial virulence (Laanto et al., 2012; Castillo et al., 2015). In *F. psychrophilum* phage resistance have been related to spontaneous mutations (deletions, insertions or point mutations) in genes involved in gliding motility, cell walls properties and lipopolysaccharides biosynthesis and associated with changes in the phenotypic characterization of the isolates (reduced hemolytic, gelatinase and protease activity, and reduced biofilm formation) (Castillo et al., 2015). In addition, *F. psychrophilum* phage-resistant isolates grow at slower growth rates compared to the corresponding phage-sensitive strains (Christiansen et al., 2016). Similar results were obtained in the characterization of phage-resistant *F. columnare* (Laanto et al., 2012; Penttinen et al., 2018; Kunttu et al., 2020) and *Vibrio* sp. (Barbosa et al., 2013).

Host immune response to phages

The mammalian immune response to phages has been reviewed by Van Belleghem et al. (2019). When phages reach the bloodstream and tissues, they meet immune cells, independently of the route they were administered. Phagocytosis of phages alone or phages when bound to their bacterial host operated by the macrophages is well established in mammals. Furthermore, phages can induce cytokine response but the specific responsible immune cells are currently not known. Finally, when antigen presenting cells (APC; e.g., dendritic cells) take up phages, B-cells are activated and

the production of specific antibodies against the phage has been observed (Van Belleghem et al., 2019).

In aquaculture, only few studies have looked into the host immune response following phage administration (reviewed by (Kowalska et al., 2020)). Some experiments in relation to the phage product BAFADOR® (described in the next sub-chapter 2.6) and the immune response are available: Schulz et al., (2019a and 2019b). In these studies, the authors concluded that the administered phage solution was well tolerated by the fish (rainbow trout and European eel, *Anguilla anguilla*) stimulating a cellular and humoral immune response and reducing mortality in relation to the bacterial infection caused by *Aeromonas hydrophila* and *Pseudomonas fluorescens*. No stimulation of the immune system was detected by Nikapitiya et al. (2020a and 2020b) in zebrafish after phage administration by bath (Nikapitiya et al., 2020a) or by oral delivery (Nikapitiya et al., 2020b).

2.6 Phage-based products available or in development

An overview of phage based products already available or in the process to be in the area of aquaculture is presented:

- BAFADOR® (patented in 2017) against *Pseudomonas* spp. and *Aeromonas* spp. and more specifically against *A. hydrophila*, *A. salmonicida* and *P. fluorescens*, via immersion: developed and commercialized by Proteon Pharmaceutical (Poland) (Wojtasik et al., 2017; Proteon Pharmaceuticals, 2019). Additional studies testing this phage-based solution can be found: Schulz et al. (2019b, 2019a).
- CUSTUS®$_{YRS}$ against *Yersinia ruckeri*: a ready to use solution that is added directly to the tank, developed and commercialized by ACD Pharma (Norway) (ACD Pharma, 2020; Norsk Fiskeoppdrett AS, 2020).
- Corona discharge technology to irreversibly bind phages to several type of surfaces, e.g. feed pellets: developed and patented by Fixed Phage Ltd (United Kingdom) (Mattey, 2016, 2018).
- Phage cocktail against *Vibrio tubiashii* and *V. coralliilyticus* infections in oyster: in development by Intralytix (USA) (Intralytix, Inc., 2016).
- Phage-based treatment targeting *V. harveyi* in shrimp: in development by Phage Biotech Ltd (Israel) (Culot et al., 2019; Pereira et al., 2021) [However, their website is now not active and the company seems to be closed].
- LUMI-NIL MBL for prevention and treatment of the luminous bacterium *V. harveyi* in shrimp: Mangalore Biotech Laboratory (India) (Culot et al., 2019; Pereira et al., 2021).

2.7 Previous experience with *F. psychrophilum*

Twenty-two bacteriophages infecting *F. psychrophilum* were at first isolated from Danish rainbow trout farms by Stenholm et al. (2008). The high occurrence of these phages in the selected sample types (water, water with feces or with presence of dead fish) suggested that the Danish trout farms are important reservoirs of *F. psychrophilum* phages, also in periods without RTFS. The isolated phages were divided in 3 groups based on genome size, morphology and host range: group I) large genome (90 kb), with a relatively large host range (among the tested *F. psychrophilum* strains) and belonging to the family of *Podoviridae;* group II) intermediate genome size (48 kb), with the largest host range and belonging to the family of *Siphoviridae*, group III) smallest genome (8.5-12 kb), narrow host range and belonging to the family of *Myoviridae* (Stenholm et al., 2008). Subsequently, the genomes of some selected *F. psychrophilum* phages were sequenced (Castillo and Middelboe, 2016).

Preliminary experiments to evaluate the potential of *F. psychrophilum* phages for treatment and control of this bacterium in aquaculture settings were performed by Castillo et al. (2012). In a small experimental set up, phages were delivered to rainbow trout and Atlantic salmon (10-20 g; 10^9 PFU $fish^{-1}$) by intraperitoneal (IP) injection simultaneously to *F. psychrophilum* (10^8 CFU $fish^{-1}$). The authors were able to detect a reduction in mortality of fish treated with phages (Castillo et al., 2012). Subsequent studies on the dispersal and survival of *F. psychrophilum* phages in rainbow trout showed that infective *F. psychrophilum* phages were recovered from the internal organs of rainbow trout fry after administration by intraperitoneal injection (with and without the bacteria) (Madsen et al., 2013), by bath or via oral administration (oral intubation or by phage-coated feed – without bacterial infection) (Christiansen et al., 2014). The results obtained by Christiansen et al. (2014) suggested that the oral delivery of phages by phage-coated feed was the most promising administration route since providing a constant concentration of phages in the fish (intestine and spleen). Thus, the oral administration of phages was selected and tested in **Manuscript I** and **Appendix A** and **B**, since it was also considered a delivery method that could be used in the field.

3 Fish gut microbiota

The microbiota is defined as the array of microbial communities inhabiting specific environments as, for example, soil, water, skin and gut and including commensal, pathogenic and symbiotic microorganisms (bacteria, archaea, viruses, bacteriophages and fungi). The microbiome is the collection of their genomes (Claesson et al., 2017; Butt and Volkoff, 2019). In the majority of microbiome studies, as in this thesis, the terms microbiota/microbiome are, however, used to refer to only the bacterial communities inhabiting a specific habitat. Other terms, as for example virome, phageome, mycobiome, exist to describe the array of viruses, bacteriophages and fungi, respectively (Claesson et al., 2017).

Although the microbiome research in teleost is behind compared to the one targeting the microbiome in humans and mouse models (Llewellyn et al., 2014; Gallo et al., 2020), a steep increase in this research area have been observed in the period 2011-2018, where the microbiota of the fish belonging to the genus *Oncorhynchus* was the most investigated (Perry et al., 2020). The most studied teleost microbiome is the one characterizing the gut (compared to gills and skin), especially in connection with diet types (Llewellyn et al., 2014; Perry et al., 2020).

3.1 Roles and importance of gut microbiota

The role of the gut microbiota, sometimes described as an “extra organ”, in the development, growth and health of the host is widely acknowledged (reviewed in fish by Llewellyn et al. (2014), Butt and Volkoff (2019), Perry et al. (2020), Legrand et al. (2020)).

Some of the very important roles played by the gut microbiota are the regulation of the appetite/feed intake and the food/feed ingestion, digestion and metabolism. This regulation is achieved by modulating a bi-directional communication channel with the brain, the so-called “microbiota-gut-brain axis”. In fact, the secretions of the gut microbes can influence the digestive process and metabolism as well as the gastro-intestinal tract (GIT) motility and function, the hormone release and feeding behaviors by the interaction with GIT neurotransmitters (e.g. serotonin, catecholamines). On the other hand, enteric neurotransmitters can also regulate the microbial composition of the gut and the microbial secreted compounds (reviewed by (Butt and Volkoff, 2019)). These concepts are not fully understood and clarified in relation to fish since only few studies have been targeting this matter. However, similarities with mammals have been observed (reviewed by (Butt and Volkoff, 2019)). As an example, the work of Borrelli et al. (2016) demonstrated how the administration of the probiotic *Lactobacillus rhamnosus* IMC 501 would cause a shift in the ratio Firmicutes:Proteobacteria in the gut of zebrafish (increase in Firmicutes) together with changes in the shoaling (grouping) behavior, in the brain expression levels of the Brain Derived Neurotrophic Factor (BDNF) gene (*bdnf*) (neurotrophic factor involved in the control of the neuronal function and of cellular activities e.g. differentiation and survival) and of others

important for the regulation of the serotonin signalling/metabolism (Borrelli et al., 2016).

The microbial community of the gut plays also fundamental roles in relation to the development and reproduction of the host and the response to stress conditions (Butt and Volkoff, 2019).

Gut commensal microbes influence the immune system and the immunity of the host in various manners. They represent a defense against pathogens, which inhibits their colonization, passively, by competitive exclusion or, actively, by the production of toxic secondary metabolites. This mechanism has been named as "colonization resistance" (Llewellyn et al., 2014; Butt and Volkoff, 2019). In addition, commensal bacteria of the gut can secrete specific signals stimulating the immune system of the host (Pérez et al., 2010; Perry et al., 2020). As an example, probiotic bacteria of the genus *Bacillus* and *Lactobacillus* have been demonstrated to increase the number of Goblet cells (and so the mucus secretion) in rainbow trout (Topic Popovic et al., 2017), the cytokine secretion in zebrafish (He et al., 2017a) and the phagocytic activity in Nile tilapia (Chen et al., 2019) (reviewed by Perry et al. (2020)).

3.2 Development of fish gut microbiota

The establishment of the microbial flora of teleost initiates as soon as the eggs are laid and, at this stage, the microbes inhabiting the water are the one characterizing the microbiota in connection with the chorion of the eggs (**Figure 9**). During the life stages of the fish, the microbial composition accumulates diversity which is shaped by various biotic and abiotic factors (Llewellyn et al., 2014).

3.3 Factors influencing the microbial composition of the gut

Biotic or intrinsic and abiotic or environmental factors influencing the gut microbiota of fish have been recently reviewed by Butt and Volkoff (2019) and Legrand et al. (2020).

Salinity, temperature and light as well as the water quality and the diet type (abiotic factors) can shape the microbial composition of the gut. As already mentioned, the research targeting the changes of the gut microbiota in relation to diet is the largest (Butt and Volkoff, 2019; Perry et al., 2020). Fishmeal and the substitution or addition of plant-based supplements or diets have been often compared and changes in the microbial community of the gut have been identified e.g. for Atlantic salmon (*Salmo salar*) (Hartviksen et al., 2014; Zarkasi et al., 2016; Gajardo et al., 2017; Egerton et al., 2020) and rainbow trout (*Oncorhynchus mykiss*) (Dalsgaard et al., 2012; Wong et al., 2013; Ingerslev et al., 2014a, 2014b; Gonçalves and Gallardo-Escárate, 2017; Michl et al., 2017; Blaufuss et al., 2020). As an example, a plant-based diet promoted an increase in the number of bacteria with carbohydrate or broad range metabolic activity (e.g. *Aeromonadaceae*, *Thermaceae*) and a decrease in bacteria able to metabolize proteinaceous material (e.g. *Vibrionaceae*, *Pseudomonadaceae*, *Moraxellaceae*) in the gut microbiota of Atlantic salmon at the freshwater stage

(Egerton et al., 2020). This is in relation to the fact that plant-based diets are very rich in carbohydrates compared to fishmeal, which instead is very rich in proteins (Gauthier et al., 2019). In addition, an increase in lactic acid bacteria have been correlated to a shift to plant-based diets (Wong et al., 2013; Ingerslev et al., 2014a, 2014b).

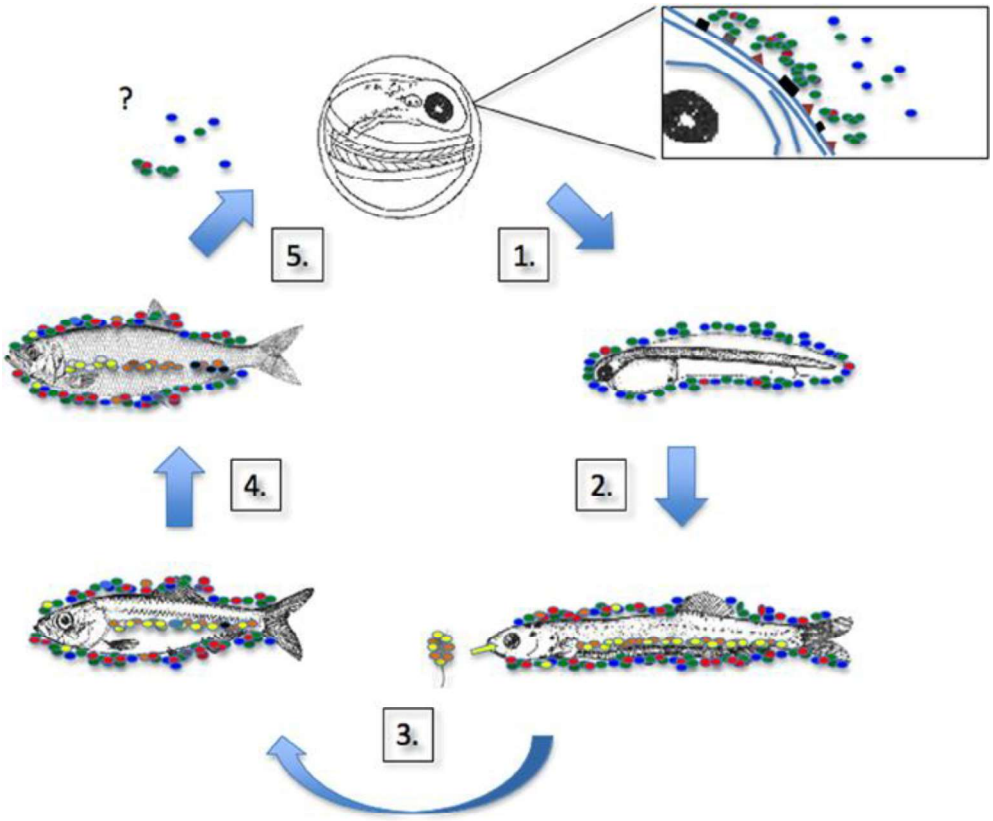

Figure 9. Microbiota establishment and development during the life-stages of teleost. The differently colored ellipses represent different bacterial taxa. (**1**) The egg chorion is colonized by bacteria present in the surrounding environment, which may interact with specific receptors on the egg surface (indicated by squares and triangles). (**2**) After hatching, bacteria previously in connection with the chorion and in the surrounding environment colonize the larvae. (**3**) When external feeding starts the digestive tract is colonized by bacterial taxa associated with feed source. Subsequently, the microbiota develops and accumulates diversity (**4**) reaching a diverse composition in adult individuals (**5**). ?= some microbial components may be vertically transmitted. Adapted from Llewellyn et al. (2014). *Copyright © 2014 Llewellyn, Boutin, Hoseinifar and Derome* [Creative Commons Attribution License (CC BY)].

Rearing conditions, the presence of pollutants or toxins together with the administration of chemicals such as antibiotics can also affect the gut microbiota (Butt and Volkoff, 2019). For example, differences in the gut microbiota between wild and captive fish (Webster et al., 2018), between farmed fish and farmed fish previously transferred to aquaria in research facilities (Lyons et al., 2017) as well as between fish at different rearing densities (Wong et al., 2013; Du et al., 2019) were evaluated. A potential contamination of oil-derived compounds was detected in the analysis of the

gut microbiota of Atlantic cod (*Gadus morhua*) caught in two different areas of Norwegian waters: fish caught in Southern waters were characterized by bacterial genera able to degrade polycyclic aromatic hydrocarbons, PAH (Walter et al., 2019) (PAH = chemicals naturally present in crude oil, coal and gasoline, and also produced by various anthropogenic activities (Wikipedia, 2021)). The effects of antibiotics administration are associated with the disruption of the gut microbiota (Kim et al., 2019) which is discussed in detail in the sub-chapter 3.4.

The intrinsic or biotic factors modulating the gut microbiota concern the genetic background, the gender/reproductive stage, the age and the nutritional status (e.g. feeding habits, starvation). Conditions of stress, disease and the immune system represent biotic factors, too (Butt and Volkoff, 2019). The composition of the gut microbiota at different life stages have been studied by Ingerslev et al. (2014b) in rainbow trout, and by Abdul Razak et al. (2019) in catfish (*Ictalurus punctatus*) larvae. Differences in the microbial composition of the gut between carnivores and herbivores have been studied in various species by Liu et al. (2016), who observed an increased abundance of cellulose-degrading bacteria in herbivores (e.g. *Clostridium*) and an increased abundance of protease-producing bacteria in carnivores (e.g. *Halomonas*). Finally, the relation between gut microbiota and the condition of disease have been reviewed by Legrand et al. (2020), who suggested that the study of overall gut microbial composition may represents also a tool for the identification of emerging diseases and for the assessment of the fish health status.

3.4 Role of antimicrobial compounds and dietary supplements (antibiotics, prebiotics, probiotics, synbiotics and phages)

Antimicrobial compounds and dietary supplements shape the microbial composition of the gut (Butt and Volkoff, 2019).

The administration of broad-spectrum antibiotics is known to cause significant changes in the gut microbiota together with an increased susceptibility to other diseases and the antibiotic bio-accumulation in the tissues (Francino, 2016; Butt and Volkoff, 2019). The condition of disruption of the gut microbiota is commonly named as dysbiosis. This condition is not solely related to antibiotic administration but also to e.g. infections, inflammations, chronic diseases, cancer (in humans (Lynch and Pedersen, 2016)). When the antibiotic-therapy is terminated, the antibiotic-induced dysbiosis is generally recovered as the microbiota is capable of returning to its original state. However, this may not always be the case as these alternations can still be observed after months or years from the termination of the therapy (reviewed by (Francino, 2016)). Further, antibiotics can affect not only the abundances of various bacterial populations but also the status of the immune and metabolic heath (e.g. increased susceptibility to infections, compromised immune homeostasis as, for example, inflammatory diseases) (Francino, 2016).

In fish, various studies have been conducted to evaluate the effects of antibiotics therapies on the gut microbiota (He et al., 2017b; Zhou et al., 2018; Kim et al., 2019). For example, Kim et al. (2019) observed a reduction in the diversity and number of bacteria in the gut microbiome (gut mucus samples) of olive flounder or Japanese halibut (*Paralichtus olivaceus*) after the administration of amoxicillin or oxytetracyclines. The evaluation of the effects of the antibiotic in use in Denmark for the treatment of RTFS (florfenicol) in healthy and infected fish was one of the objectives of **Manuscript II**.

Probiotics (alive or dead bacteria promoting the growth of beneficial species) prebiotics (digestible ingredients promoting the growth of probiotics) and symbiotics (a combination of pro- and prebiotics) are often used to improve the fish health (Zorriehzahra et al., 2016; Butt and Volkoff, 2019). Examples of probiotics used in aquaculture are *Lactobacillus*, *Carnobacterium*, *Lactococcus*, *Leucococcus* and *Clostridium*, and they can act in different manners: a) by the production of inhibitory substances that compromise the growth of pathogenic bacteria; b) by improving fish immunity = higher levels of macrophage activity and antibodies; c) by interfering with quorum sensing = probiotics produce molecules that act as antagonist for this mechanism; d) by competitive exclusion = they colonize the gut and thus block the gut colonization by bacterial pathogens; e) by nutrients competition = probiotics use nutrients and render them unavailable for the pathogenic bacteria (Zorriehzahra et al., 2016). Prebiotics, which have received less attention in aquaculture compared to probiotics, include fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS) and inulin (Butt and Volkoff, 2019). Gonçalves and Gallardo-Escárate (2017) have studied the gut microbiome of rainbow trout juveniles (intestinal mucus and content) in relation to the administration of the probiotic *Saccharomyces cerevisiae* and the prebiotic mannan-oligosaccharides, singularly and combined. They concluded that these diets could modulate the intestinal microbiota altering the bacterial abundances, which might have an impact on the fish physiology.

Bacteriophages (targeting specific pathogenic bacteria not present in the healthy microbiota) have been considered not able to alter the gut microbiota since they target specific bacterial populations. However, the contrary has been recently discussed (Barr, 2019; Ganeshan and Hosseinidoust, 2019; Dahlman et al., 2021). This is one of the objectives of **Manuscript II***,* where we have demonstrated how the phages can impact the overall microbial composition of the distal intestine of rainbow trout fry.

3.5 Gut microbiota of rainbow trout (*Oncorhynchus mykiss*, Walbaum)

The fish gut microbiota is mainly characterized by bacteria belonging to the phylum Proteobacteria, Firmicutes and Fusobacteria, and, in a lower number, by Bacteroidetes, Actinobacteria and Verrucomicrobica (Tarnecki et al., 2017; Egerton et al., 2018). Depending on the section of the intestine investigated and the target community (the allochthonous community, so the transient microbes, or the

autochthonous community, so the one colonizing the mucus), the composition, the function and the density of the microbiota may change (Romero et al., 2014; Tarnecki et al., 2017; Egerton et al., 2018). In rainbow trout, the dominant phyla in the gut microbiome are Proteobacteria and Firmicutes (Ingerslev et al., 2014a, 2014b; Tarnecki et al., 2017) with an increased abundance of Tenericutes and Proteobacteria in the distal content (Desai et al., 2012; Lyons et al., 2017; Villasante et al., 2019). In Wong et al. (2013), the authors have tried to identify a core gut microbiota by sampling the whole mid-intestine of 33 farmed rainbow trout (600-900 g). Results showed it was characterized by bacteria belonging to the class Bacilli (52.2%), Alphaproteobacteria (25.7%), Gammaproteobacteria (16.2%), Betaproteobacteria (2.6%), Flavobacteria (1.4%), Epsilonproteobacteria (0.8%), Clostridia (0.7%) and Sphingobacteria (0.3%).

The development of the microbial community of the gut in rainbow trout was studied by Ingerslev et al. (2014b) in the period from the end of the yolk-sack stage up to seven weeks post first feeding. It was observed that the amount of bacteria and their diversity (measured by Shannon diversity index) in the autochthonous gut microbiome increased significantly after the onset of external feeding no matter the type of diet (marine diet containing fish meal and fish oil or plant-based diet) and it was afterwards maintained. Indeed, before the onset of the external feeding, 43% of the sequence reads belonged to the genus *Sediminibacterium*, bacteria characterizing the aquatic environment, which suggested that the gut bacterial community is at first colonized by bacteria present in the water. It was also observed that the marine diet favored bacteria belonging to the Phylum of Proteobacteria while the plant-based diet to the Phylum of Firmicutes and, in particular, lactic acid bacteria like *Weissella*, *Leucococcus* and *Streptococcus* (Ingerslev et al., 2014b). Similar results were obtained by Ingerslev et al. (2014a).

Another factor modulating the composition of the gut microbiota is the presence of disease/bacterial infection. In Ingerslev et al. (2014a), a radical increase in Proteobacteria and specifically in bacteria belonging to the genus *Aeromonas* was observed 19 days after the bacterial challenge with *Yersinia ruckeri* in the autochthonous gut microbial community of rainbow trout fry. These changes were observed in challenged fish independently the diet type they received (marine or plant-based diets). However, it was suggested the plant-based diet could make the fish less susceptible to *Y. ruckeri* infection (probably because of the higher number of bacteria from the family *Lactobacillaceae*), since the number of fish positive to the bacteria measured by plate count and by number of reads belonging to the genus *Yersina* was higher in case of the marine diet (Ingerslev et al., 2014a). Assessing the influence of the infection caused by *F. psychrophilum* on the microbial composition of the distal intestine of rainbow trout fry was one of the objectives of **Manuscript II**.

3.6 Other studied microbiomes in fish (gills and skin)

The mucosal surfaces of the fish (gut, skin and gills) represent the first lines of defense against the external environment (Ángeles Esteban, 2012). As for the gut, the skin and the gills are inhabited by bacterial communities, whose composition is also shaped by the biotic and abiotic factors described earlier (Gauthier et al., 2019). Nevertheless the gut microbiota have been mostly investigated, several studies have been performed to evaluate the skin and gills microbiome in relation to e.g. antibiotic treatment and disease (Minniti et al., 2017; Legrand et al., 2018; Rosado et al., 2019). As an example, Minniti et al. (2017) studied the skin mucus microbiota of Atlantic salmon (*Salmo salar*). Legrand et al. (2018) assessed the effects of disease (chronic lymphocytic enteritis) on the skin and gills microbiota of Yellowtail Kingfish (*Seriola lalandi*) showing loss of diversity and alterations in the microbial abundances at different stages of the disease. Another study was performed by Rosado et al. (2019), who studied the effects of infection (*Photobacterium damselae*) and antibiotic treatment (oxytetracycline) on the skin and gills microbiota of farmed seabass (*Dicentrarchus labrax*) illustrating how these factors can alter the overall composition and diversity of the bacterial communities inhabiting the skin and the gills.

3.7 How do we study the gut microbiota (16S rRNA community analysis)

Depending on the research questions, various approaches can be used to study the microbiota. Metabarcoding, which answers to the question “who is there?”, is the most common method and the 16S ribosomal RNA (rRNA) gene the most common biomarker used (Claesson et al., 2017; Gauthier et al., 2019). The 16S rRNA gene (~1500 bp) is located in the 30S subunit of the prokaryotic ribosome. The nine hypervariable regions (named V1-V9) flanked by highly conserved DNA, which it consists of, represent perfect sites for amplification and sequencing allowing the classification of bacteria and the analysis of diversity (relative abundances of bacterial taxa, alpha diversity measuring the diversity within a group, beta diversity measuring the diversity between groups) (Claesson et al., 2017; Christensen et al., 2018).The uneven copy number of the 16S rRNA gene among different bacteria can affect this analysis (Claesson et al., 2017). Other biomarkers such as the 18S ribosomal RNA gene (located in the 40S subunit of eukaryotic ribosomes) and the internal transcribed spacer (ITS) regions are also utilized in studies targeting fungi or other single-cell eukaryotes (Claesson et al., 2017).

Metagenomics answer to the question “what can they do?”. This approach consists in the sequencing of the total extracted DNA and requires higher levels of expertise in bioinformatics and costs than the 16S rRNA gene analysis. Metatranscriptomics, even more complicated and expensive procedures, answer to the question “what are they doing?” and allow the prediction of the activity/expression level of a gene by the so-

called RNA-seq (Claesson et al., 2017; Gauthier et al., 2019). No meta transcriptomes have been published in salmonids (Gauthier et al., 2019).

The 16S rRNA community analysis is more accessible considered the lower costs compared to metagenomics approaches and the relatively simple pipeline. Briefly, the DNA from the host samples is extracted, a PCR reaction is performed to amplify single or multiple regions of the 16S rRNA gene and the PCR products including specific DNA "tags" ("per sample" identification) are sequenced simultaneously by the use of a high-throughput machine. The obtained data are then processed with bioinformatics and statistical tools: a) data preprocessing: quality filtering and trimming, error learning, sequence merging; b) data processing: operational taxonomic unit (OTU) clustering and taxonomic assignment; c) statistical analysis: taxonomic comparison, alpha and beta diversity (Claesson et al., 2017; Christensen et al., 2018; Gauthier et al., 2019). However, important considerations must be taken when performing this kind of analysis and when comparing different studies (some examples below).

- Sample type. The first step is to decide whether to analyze the autochthonous (microbes colonizing the mucus) or allochthonous (transient community) by sampling the emptied intestine (so the intestinal mucus) or the gut content, respectively, or both (Tarnecki et al., 2017). In addition, different regions of the intestinal tract can harbor different bacterial communities (Gajardo et al., 2016), so one should consider which part of the intestine to sample also according to fish size.
- DNA extraction. It is important to follow established protocols optimized for bacterial DNA extraction from both Gram negative and positive bacteria. For example, the utilization of bead beading is included to break Gram positive bacteria (Tarnecki et al., 2017). A negative control for the extraction should also be included to check for possible contamination during the process (the DNA extraction kit can be a potential source of contamination).
- PCR conditions. The nine hypervariable regions of the 16S rRNA gene can be target singularly or in combination. In fish research, the region V4 and the combination V3-V4 are the most commonly targeted (Perry et al., 2020).

Aim of the thesis work and overall workflow

The overall aim of the PhD study was to acquire new knowledge regarding the development of a phage-mediated control of the bacterial pathogen *Flavobacterium psychrophilum* in rainbow trout at fry and eyed egg stages. **Figure 10** shows the overall workflow (topic of interests, aims, methods and results/manuscripts).

Building on previous work (Madsen et al., 2013; Christiansen, 2014; Christiansen et al., 2014), the first part of the PhD project focused on evaluating the potential of phages delivered by **phage-treated feed** in the prevention and treatment of infections caused by *F. psychrophilum* in **rainbow trout fry**. The oral delivery was chosen as the main focus since it represents a feasible way that could be applied prophylactically in aquaculture facilities to prevent and control bacterial infections and mortalities caused by this bacterium. For this study, a selected purified two-component mix of phages was applied on feed pellets by irreversible immobilization (corona discharge technology, Fixed Phage Ltd) (Mattey, 2016, 2018) (phage-immobilized feed) or by spraying procedures (phage-sprayed feed). Fish experimental trials were performed with the following aims: a) study the effects of phage delivery in healthy and infected fish comparing the two phage application methods on feed pellets (e.g. fish health status, phage diffusion in internal organs); b) evaluate the effects on fish survival during *F. psychrophilum* infections of phages delivered by feed in comparison to when phages are delivered by repeated bath procedures and by intraperitoneal injection. Culture-based methods were used to enumerate phages and detect bacteria in fish organs and a survival analysis was performed to assess the beneficial effects (if any) of phages on fish survival. The results are included in **Manuscript I**. Additional experiments are presented in the appendix of this thesis where phages were administered by phage-immobilized feed alone (33 days of prophylaxis **– Appendix A**) and in combination with bath procedures (**Appendix B**).

Assessing and understanding the effects of antimicrobial treatments (e.g. antibiotic therapy) on the fish microbiota as a mean to improve the fish health status has had renewed interest (Perry et al., 2020). In the second part of the PhD project, we focused on studying the **gut microbial communities of rainbow trout fry** and on evaluating how these communities are shaped by antibiotic and phage therapies and by *F. psychrophilum* infection. In order to minimize the number of fish utilized in the experiments, we combined this study with the fish experimental trials performed in the first part of the project (oral delivery of phages by **phage-treated feed**). Fish fed with **florfenicol-coated feed** (antibiotic in use in Denmark for the treatment of RTFS) were included. To perform this analysis, DNA was extracted from intestinal samples and a 16S rRNA community analysis (V3-V4 region) was performed with the following aims: a) study the gut microbiome of healthy rainbow trout fry fed with phage-treated feed (after 11 days of prophylaxis) in comparison with control feed; b) evaluate the effects of florfenicol in healthy and infected fish during the treatment and c) characterize the

fish gut microbiome once recovered from the infection under the different feed type regimes. The results are included in **Manuscript II**.

Since the microbial community surrounding fish eggs can harbor pathogenic bacteria, the potential use of phages as bacterial control agents to prevent *F. psychrophilum* infections in connection with **rainbow trout eyed eggs** was the focus of the third part of the PhD project. At first a) we established a bacterial challenge bath method and b) studied the effects of singular phages on eyed eggs (survival of eyed eggs; interaction of phages with eyed eggs). Subsequently, c) we exposed fish eyed eggs to phages to evaluate their efficiency in eliminating the target bacterium. Bacteria and/or phages in connection with eyed eggs and in the surrounding environment were quantified by culture-based methods. The results are included in **Manuscript III**.

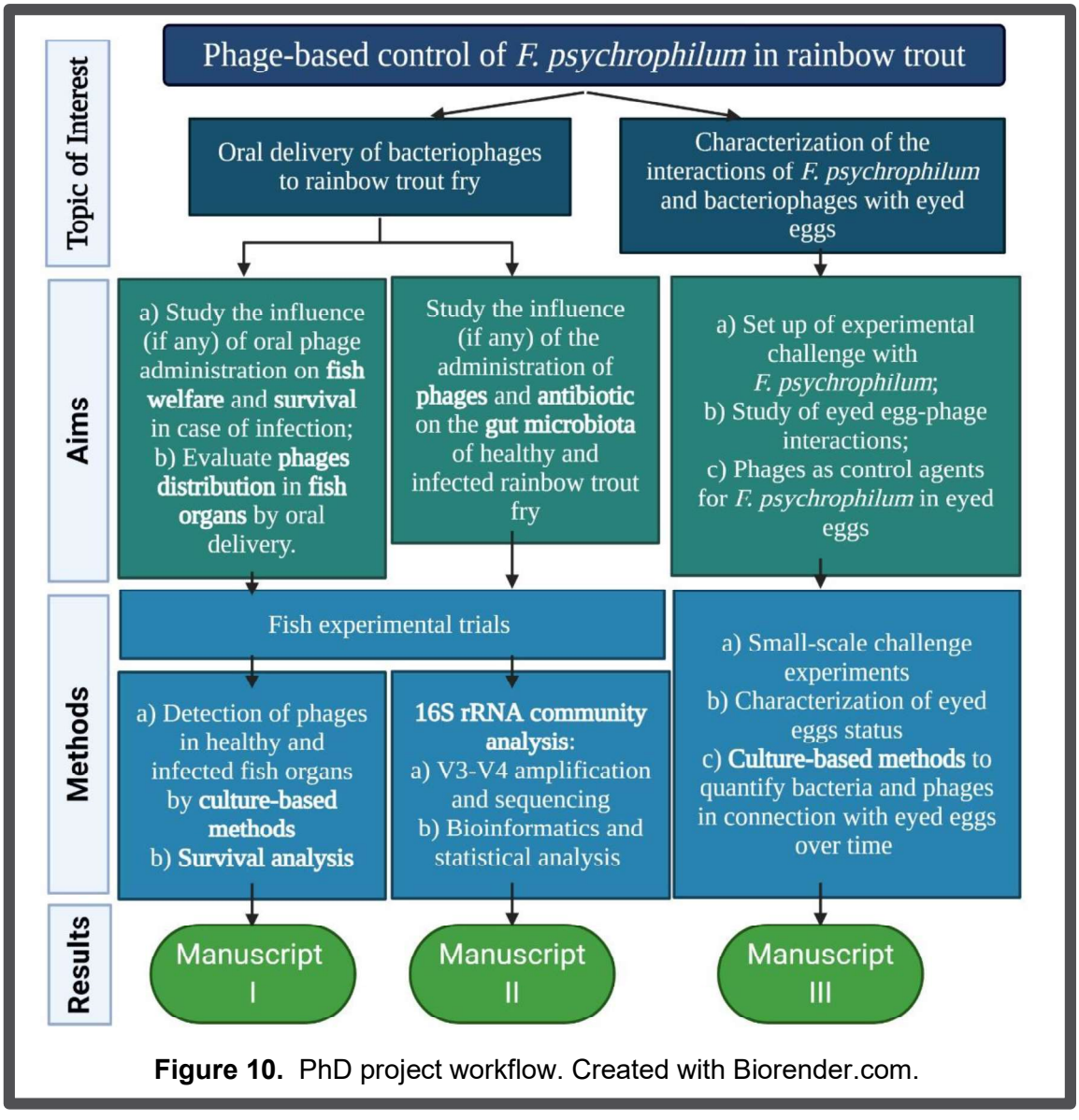

Figure 10. PhD project workflow. Created with Biorender.com.

Main findings of the thesis

Manuscript I: Donati V.L., Dalsgaard I., Sundell K., Castillo D., Er-Rafik M., Clark J., Wiklund T., Middelboe M., Madsen L. (2021). Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture: *in vivo* experiments to compare delivery methods. Submitted to the journal Frontiers in Microbiology (in review).

Concerning the first aim of **Manuscript I** (a), we found that the oral delivery of phages using phage-immobilized (produced by Fixed Phage Ltd - final concentration of $8.3*10^7$ PFU g^{-1} of feed pellets) and phage-sprayed in-house feed pellets ($1.6*10^8$ PFU g^{-1}) provided a constant delivery of phages to the fish [stable occurrence in the intestine (~10^3 PFU mg^{-1}) while more sporadic in kidney (~10^1 PFU mg^{-1}), spleen (1-10 PFU mg^{-1}) and brain (~1 PFU mg^{-1})], without a negative effect on fish health or growth. We observed a higher frequency of detection of phages in the internal organs of fish fed with phage-sprayed feed which we hypothesized being partly a direct consequence of the higher phage concentration on the manually sprayed feed and, maybe, of the different phage orientation and detachment properties obtained by the two methods. Nevertheless, we concluded that the application of phages by the corona discharge method reduced the time for feed preparation (compared to spraying procedures) and enabled the stable immobilization of phages on feed pellets.

During *F. psychrophilum* infection, we detected a large variation in the intestinal phage titer of fish sampled in the first 10-20 dpi. We hypothesized this being a direct result of the bacterial infection, since the reduced feed intake is one of the clinical signs of infected/diseased fish. This hypothesis was supported by the lower concentration of phages detected in the intestine of dead/moribund fish. Also, since it was not possible to identify a clear relationship between the presence of the bacteria and the number of phages in the internal organs of the fish, we concluded that the undetected phage proliferation in organs containing *F. psychrophilum* might have been a consequence of the relatively low phage concentration obtained *in situ* by the delivery of phage-treated feed pellets.

Concerning the second aim of **Manuscript I** (b), we found no significant effect on fish survival when phages were delivered by phage-treated feed pellets (final percent survival: 75-80%), nor when delivered by bath (1st and 2nd bath: ~10^6 PFU ml^{-1}; 3rd bath: ~10^5 PFU ml^{-1}) (final percent survival: 42-45%). However, when phages were delivered by intraperitoneal injection ($1.7*10^8$ PFU $fish^{-1}$), the final percent survival observed in the group injected with phages (80.0 %) was significantly higher than in the control group (56.7 %). We concluded that these results support the use of the selected phages to control *F. psychrophilum* and that higher phage dosages may be needed on feed pellets to offer fish an adequate protection against this pathogen.

Manuscript II: Donati V.L., Madsen L., Middelboe M., Strube M.L., Dalsgaard I. (2021). The gut microbiota of healthy and *Flavobacterium psychrophilum*-infected rainbow trout fry is shaped by antibiotics and phage therapies. Manuscript.

Manuscript II explores the gut microbiota of rainbow trout fry in relation to *F. psychrophilum* infection, antibiotics and phage oral administration. In this study, we found that the gut microbiota of rainbow trout fry (distal intestine including the gut content, if present) was characterized by bacteria belonging to the phyla Firmicutes (~50 %) and Proteobacteria (~20 %), which were the most abundant, followed by Actinobacteria (~10 %), Bacteroidetes (~2 %) and Cyanobacteria (~1 %). We concluded that our results were in accordance with the general composition of the gut microbiota of teleost and with previous studies targeting the gut autochthonous microbiome of rainbow trout at early life stages fed with marine diets.

We observed a dysbiosis effect caused both by the infection and by the florfenicol administration. Indeed, shifts in the overall composition were detected by β-diversity analysis, and changes in specific populations were observed during taxonomic mapping (e.g. altered ratio Firmicutes/Proteobacteria in infected fish; variations in the abundances of Lactic Acid Bacteria (LAB) and Actinobacteria during antibiotic therapy). Measures of α-diversity were only affected in infected fish (large variation observed 1 and 8 days post infection (dpi)). These community alterations disappeared again when fish recovered from the infection and the antibiotic treatment was terminated (33 dpi). We concluded that our results illustrate how the gut microbiota of rainbow trout fry is malleable and able to recover from *F. psychrophilum* infections and the florfenicol treatment.

Interestingly, the delivery of phages by feed pellets altered the gut microbiota of the fish independently of the presence of *F. psychrophilum*, their target bacterium. The overall gut bacterial community in fish fed phage-treated feed (phage-immobilized and phage-sprayed feed) was different from the controls at all time points examined (before and during the bacterial infection, and after recovery) as revealed by the β-diversity analysis. However, it was not possible to identify specific bacterial populations responsible of these changes except a rise of the LAB abundance 33 dpi. We concluded that our results indicate that the selected phages might affect the complex network of phage-bacteria interactions in the fish gut. However, we did not observe negative effects on fish health or growth.

Manuscript III: 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. Ready for submission to the journal Microorganisms.

Manuscript III focuses on the potential use of phages to prevent and control *F. psychrophilum* in relation to rainbow trout eyed eggs. In this study, we found 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 (aim a). We concluded that the used experimental set up allowed the study of bacteria/phage interactions with eyed eggs at a small scale under controlled conditions as well as the production of reproducible results. The experimental set up might also be applied for other pathogenic bacteria.

We found that phages did not appear to negatively affect the survival of rainbow trout eyed eggs nor to strongly adhere to the surface of eyed eggs (aim b). However, we concluded that PEG-purified solutions should be chosen over crude lysates for long term exposures since the embryo movement was not observed for a higher number of eyed eggs exposed to the phage in crude lysate. Finally, the results demonstrated a strong potential for short term (24h) phage control of *F. psychrophilum* (aim c). The detection of *F. psychrophilum* after the initial decrease in phage-treated groups may indicate the development of phage-resistant mutants. However, phage-resistant mutants are generally characterized by a loss of virulence.

Conclusions and future perspectives

Phage therapy represents a valuable alternative to antibiotic use and this PhD study aimed to bring the development of this alternative approach in relation to *F. psychrophilum* infections and rainbow trout one step forward for its application in aquaculture facilities. Overall the study has generated important knowledge. However, various challenges were revealed and further studies should be focused on overcoming them.

In our experiments concerning rainbow trout fry (**Manuscript I**), we concluded that the oral administration of phages applied on feed pellets by Fixed Phage Ltd technology represents an effective method of delivering phages, which reduces the time-consuming tasks of spraying and drying feed pellets. In our experiments, the main reason for the lack of a beneficial effect on fish survival was probably the inefficient phage delivery to the fish organs (i.e. the high loss of infective phages across the intestinal barrier and the potential too low phage titer delivered orally and by bath). Indeed, the hypothesis that delivering higher phage dosages at the infection site could positively increase the fish recovery/survival is supported by the significant increase in fish survival after intraperitoneal administration. Thus, further studies should be focused on increasing the concentration of phages applied on feed pellets of maybe a 10-100 times higher concentration. To do so efforts should be placed in creating stable highly concentrated phage solutions. The optimization of phage production for the addition to the feed has been highlighted as a priority for future research in the final summary of the BONUS FLAVOPHAGE Project (Middelboe, 2020).

An alternative option to increasing phage concentration of feed pellets, it may be the combination of phages with other antimicrobial agents or dietary supplements e.g. antibiotics, inorganic solvents (e.g. chlorine), probiotics (Cafora et al., 2019; Knezevic and Aleksic Sabo, 2019). Various studies have evaluated the possibility of combining phages and probiotics (Rasmussen et al., 2019; Grubb et al., 2020; Titze and Krömker, 2020), the latter known to improve the host health (Zorriehzahra et al., 2016; Hoseinifar et al., 2018; Kuebutornye et al., 2020), also in case of *F. psychrophilum* infections (Korkea-aho et al., 2011, 2012). In this case, studies should be focused also in elucidating the effects of this combined approach on the gut microbial communities. In **Manuscript II**, we have found that phages alone can shape the gut microbiota. Thus, additional studies may be focused in trying to elucidate the mechanisms behind these changes (direct action of phages or indirect by the release of cell lysates) by, for example, improving purification procedures, targeting the metagenome (“what can this bacteria do” and “what are they doing” in case of transcriptomics) (Claesson et al., 2017; Gauthier et al., 2019) and the metabolome, defined as the collection of metabolites which gives a direct readout of cellular activity (e.g. performed by mass spectrometry) (Sun and Hu, 2016; Hsu et al., 2019). Finally, the investigation of the fish immune response to phages and/or probiotics should be performed.

An additional area of application of phages in aquaculture facilities may be the immobilization of phages on biological filters by the corona discharge method of Fixed Phage Ltd, thus preventing the circulation of *F. psychrophilum* in the water. Indeed, the results of the BONUS FLAVOPHAGE Project have shown the ability of phages to combat and prevent *F. psychrophilum* biofilm formations (Middelboe, 2020).

To conclude, further studies are needed to better understand the mechanisms of interactions between *F. psychrophilum* and its phages in connection with rainbow trout eyed eggs (e.g. by microscopy-based techniques) and to further explore if phage control can be maintained beyond 24 h (**Manuscript III**). Also in this case, it would be interesting to evaluate if phages targeting *F. psychrophilum* can shape the microbial communities around the eggs and if this may have an impact on the development of the fish.

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Manuscripts

Manuscript I

Phage-mediated control of Flavobacterium psychrophilum in aquaculture: in vivo experiments to compare delivery methods.

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Author contribution statement

VLD: planning and execution of experiments A and B including phage-sprayed feed preparation, fish sampling and phage analysis in fish organs, data preparation and analysis, writing of the manuscript; ID: planning, execution and supervision of experiments A and B, data interpretation and manuscript preparation; KS and TW: planning and execution of experiment C, contribution to manuscript preparation; DC: production of PEG-purified phage solutions applied on feed pellets and contribution to manuscript preparation; MR: TEM imaging of bacteriophages FpV4 and FPSV-D22, contribution to manuscript preparation; JC: production of phage-immobilized feed, contribution to manuscript preparation; MM: data interpretation, manuscript preparation, funding acquisition; LM: planning, execution and supervision of fish experiments A and B, data interpretation and manuscript preparation. The authors have read and approved the final version of the manuscript.

Keywords

Flavobacterium psychrophilum, Rainbow trout fry syndrome (RTFS), Rainbow trout fry, phage-therapy, Bacteriophages

Abstract

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Phage-based approaches have gained increasing interest as sustainable alternative strategies to antibiotic treatment or as prophylactic measures against disease outbreaks in aquaculture. The potential of three methods (oral, bath and injection) for delivering a two-component phage mixture to rainbow trout fry for controlling Flavobacterium psychrophilum infections and reduce fish mortality was investigated using bacteriophages FpV4 and FPSV-D22. For the oral administration experiment, bacteriophages were applied on feed pellets by spraying (1.6*108 PFU g-1) or by irreversible immobilization (8.3*107 PFU g-1), using the corona discharge technology (Fixed Phage Ltd). The fish showed normal growth for every group and no mortality was observed prior to infection as well as in control groups during the infection. Constant detection of phages in the intestine (~103 PFU mg-1) and more sporadic occurrence in kidney, spleen and brain was observed. When fish were exposed to F. psychrophilum, no significant effect on fish survival, nor a direct impact on the number of phages in the sampled organs, were detected. Similarly, no significant increase in fish survival was detected when phages were delivered by bath (1st and 2nd bath: ~106 PFU ml-1; 3rd bath: ~105 PFU ml-1). However, when phages FpV4 and FPSV-D22 (1.0*108 PFU ml-1) were administered by intraperitoneal injection three days after the bacterial challenge, the final percent survival observed in the group injected with bacteriophages FpV4 and FPSV-D22 (80.0 %) was significantly higher than in the control group (56.7 %). The work demonstrates the delivery of phages to fish organs by oral administration, but also suggests that higher phage dosages than the tested ones may be needed on feed pellets to offer fish an adequate protection against F. psychrophilum infections.

Contribution to the field

The utilization of bacteriophages as sustainable antibiotic alternative or prophylactic measure against bacterial infections have gained attention in the growing sector of aquaculture where bacterial diseases can cause important economic losses. This study focuses on the worldwide-known bacterium Flavobacterium psychrophilum, etiological agent of Rainbow Trout Fry Syndrome (RTFS) and Bacterial Coldwater Disease (BCWD). This study builds on previous experiments exploring the potential of bacteriophages targeting F. psychrophilum where phages were delivered to rainbow trout by intraperitoneal injection (in combination with the bacteria) and by bath, oral intubation and by phage-coated feed to evaluate phage diffusion in the internal organs. The current work combines studies focused on phage delivery efficiency and fish mortality in challenge experiments evaluating the potential of three methods (oral, bath and injection) for delivering a two-component phage mixture to rainbow trout fry. Aiming to bring new insights to the development of a bacteriophage-based treatment for F. psychrophilum infections applicable in the field, this work includes oral administration of bacteriophages applied on feed pellets by spraying, or by irreversible immobilization. This study suggests that higher phage dosages may be needed on feed pellets to offer fish an adequate protection against F. psychrophilum infections.

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Ethics statements

Studies involving animal subjects
Generated Statement: The animal study was reviewed and approved by the Animal Experiments Inspectorate of Denmark (Dyreforsøgstilsynet, permission n. 2013-15-2934-00976 until 07-10-2019 and n. 2019-15-0201-00159 from 08-10-2019) (Experiments A and B) and by the National Animal Experimental Board (Eläinkoelautakunta, ELLA) (personal license, under project ESAVI/4225/04.10.07/2017)(Experiment C) .

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Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture: *in vivo* experiments to compare delivery methods.

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Keywords: Phage-therapy, *Flavobacterium psychrophilum*, RTFS, rainbow trout fry, bacteriophages

Abstract

Phage-based approaches have gained increasing interest as sustainable alternative strategies to antibiotic treatment or as prophylactic measures against disease outbreaks in aquaculture. The potential of three methods (oral, bath and injection) for delivering a two-component phage mixture to rainbow trout fry for controlling *Flavobacterium psychrophilum* infections and reduce fish mortality was investigated using bacteriophages FpV4 and FPSV-D22. For the oral administration experiment, bacteriophages were applied on feed pellets by spraying ($1.6*10^8$ PFU g^{-1}) or by irreversible immobilization ($8.3*10^7$ PFU g^{-1}), using the corona discharge technology (Fixed Phage Ltd). The fish showed normal growth for every group and no mortality was observed prior to infection as well as in control groups during the infection. Constant detection of phages in the intestine (~10^3 PFU mg^{-1}) and more sporadic occurrence in kidney, spleen and brain was observed. When fish were exposed to *F. psychrophilum,* no significant effect on fish survival, nor a direct impact on the number of phages in the sampled organs, were detected. Similarly, no significant increase in fish survival was detected when phages were delivered by bath (1st and 2nd bath: ~10^6 PFU ml^{-1}; 3rd bath: ~10^5 PFU ml^{-1}). However, when phages FpV4 and FPSV-D22 ($1.0*10^8$ PFU ml^{-1}) were administered by intraperitoneal injection three days after the bacterial challenge, the final percent survival observed in the group injected with bacteriophages FpV4 and FPSV-D22 (80.0 %) was significantly higher than in the control group (56.7 %). The work demonstrates the delivery of phages to fish organs by oral administration, but also suggests that higher phage dosages than the tested ones may be needed on feed pellets to offer fish an adequate protection against *F. psychrophilum* infections.

Introduction

Phage therapy relies on the bactericidal activity of lytic bacteriophages (also called phages), which infect and kill specific bacterial hosts by lysing infected cells and releasing phage progeny to the environment (Twort, 1915; Roux, 2011) (reviewed in (Salmond and Fineran, 2015; Dion et al., 2020)). In the aquaculture sector, phage therapy efforts have targeted various pathogenic bacteria, such as *Vibrio spp., Aeromonas spp.*, *Flavobacterium spp.*, *Pseudomonas spp.* and *Edwersiella spp.* focusing e.g. on the isolation and characterization of virulent phages (Kalatzis et al., 2016; Kazimierczak et al., 2019), cocktail formulations (Mateus et al., 2014; Duarte et al., 2018), dose and route of phage administration (Laanto et al., 2015; Almeida et al., 2019). However, despite the many benefits of phage therapy, various challenges have been faced such as the development of phage resistant bacteria, the inefficient delivery of phages in high dosages at the infection site, and the phage clearance activity from the organism mediated by the immune cells (reviewed in (Culot et al., 2019; Kowalska et al., 2020)).

Flavobacterium psychrophilum (Borg, 1948; Bernardet et al., 1996) is the etiological agent of Rainbow Trout Fry Syndrome (RTFS, fry stage) (Lorenzen et al., 1991) and of Bacterial Coldwater Disease (BCWD, juvenile and adult fish) (Borg, 1960). Rainbow trout (*Oncorhynchus mykiss,* Walbaum) and coho salmon (*O. kisutch*) are the most susceptible salmonid species to this bacterium (Nematollahi et al., 2003). Despite a strong focus on preventive measures, for example good management practises and egg disinfection (Nematollahi et al., 2003; Madsen and Dalsgaard, 2008), antibiotics have been the most extensively used treatment for RTFS worldwide and resistance to their activity has been detected (Bruun et al., 2000, 2003; Schmidt et al., 2000; Izumi and Aranishi, 2004; Kum et al., 2008; Del Cerro et al., 2010; Sundell and Wiklund, 2011). With the isolation of bacteriophages infecting *F. psychrophilum* (Stenholm et al., 2008), the possibility of developing a sustainable alternative approach to the treatment of RTFS has gained more attention. Castillo et al. (2012) studied the application of phages in rainbow trout and Atlantic salmon delivering bacteriophages (10^9 PFU $fish^{-1}$) by intraperitoneal (IP) injection simultaneously to *F. psychrophilum* (10^8 CFU $fish^{-1}$). The authors were able to detect a reduction in mortality of fish treated with phages (Castillo et al., 2012). Subsequent studies on the dispersal and survival of *F. psychrophilum* phages in rainbow trout showed that infective *F. psychrophilum* phages were recovered from the internal organs of rainbow trout fry after administration by intraperitoneal injection (with and without the bacteria) (Madsen et al., 2013), by bath or via oral administration (oral intubation or by phage-coated feed) (Christiansen et al., 2014). However, in order to assess the potential of phage-based control of *F. psychrophilum* infections in rainbow trout, combined studies on phage delivery efficiency and fish mortality in challenge experiments are required.

Building on previous work (Madsen et al., 2013; Christiansen et al., 2014), this study brings new insights to the development of a bacteriophage-based treatment for *F. psychrophilum* infections in rainbow trout fry applicable in the field. The work includes oral administration of bacteriophages applied on feed pellets by spraying, or by irreversible immobilization, using corona discharge technology (Fixed Phage Ltd) (Mattey, 2016, 2018). The immobilization stabilizes phages at room temperature, simplifying delivery and use of phage products. The use of phage-treated feed could potentially be applied prophylactically in aquaculture facilities to prevent and control bacterial infections and mortalities caused by *F. psychrophilum*. Two additional delivery methods (by bath and by intraperitoneal injection) of the selected purified two-component mix of Danish bacteriophages with a wide host-range among virulent *F. psychrophilum* strains were also included in the study. The aim of the work was to a) evaluate the

effects of the oral administration of phages on healthy and infected fish comparing the two phage application methods on fish pellets (e.g. phage diffusion in internal organs) (Experiment A); b) assess the effects on fish survival of the oral phage administration during *F. psychrophilum* infections (Experiment A) in comparison to when phages are delivered by repeated bath procedures and by intraperitoneal injection (Experiments B and C). The work demonstrates the delivery of phages to fish organs by oral administration, but also suggests that higher phage dosages than the tested ones may be needed on feed pellets to offer fish an adequate protection against *F. psychrophilum* infections for the application in the field.

Materials and methods

Bacterial strain

Flavobacterium psychrophilum 950106-1/1, a well-characterized Danish strain isolated in 1995 from rainbow trout in a freshwater farm, was selected for the experiments (serotype Fd, virulent) (Madsen and Dalsgaard, 1999, 2000; Dalsgaard and Madsen, 2000; Sundell et al., 2019). *Flavobacterium psychrophilum* FPS-S6, a Swedish strain isolated in 2017 from rainbow trout (serotype Th, virulent), was utilized for the propagation of phage FPSV-D22 since it was the most efficient host for producing high phage titers (Sundell et al., 2019). The bacteria were stored at – 80°C in tryptone yeast extract salts medium (TYES: 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2 \times 2H_2O$, 0.05% $MgSO_4 \times 7H_2O$ (pH 7.2)) (Holt, R. A.; Rohovec, J. S.; Fryer, 1993) and glycerol (15-20%). For bacteriophage detection and quantification, *F. psychrophilum* 950106-1/1 was inoculated from a -80°C stock into 5 ml TYES broth (referred as TYES-B), incubated at 15°C at 100 rpm for 48-72 hours and then streaked on TYES agar (TYES-B with 1.1% agar, referred as TYES-A). After 3-4 days at 15°C, single colonies were picked and inoculated in TYES-B for 48 hours. For challenge experiments, *F. psychrophilum* 950106-1/1 was prepared and infection challenge performed as described by Madsen and Dalsgaard (1999). Intraperitoneal (IP) injection was selected as infection method due to its reproducibility when it comes to experimental *F. psychrophilum* infections in rainbow trout (Madsen and Dalsgaard, 1999). According to the established infection dose, appropriate dilutions of the 48-hour culture were performed prior to intraperitoneal (IP) injection and CFU were counted before and after infection.

Bacteriophages

Bacteriophages FpV4 and FPSV-D22 were used in these experiments (**Supplementary Table 1**). FpV4 (lytic phage belonging to *Podoviridae* family, 90kb genome) was isolated in 2005 from water with feces samples (Stenholm et al., 2008; Castillo and Middelboe, 2016) and FPSV-D22 (lytic phage belonging to *Siphoviridae* family), isolated in 2017 from fish tissue samples collected at Danish freshwater farms of rainbow trout (Sundell et al., 2019). Both phages were characterized to have a broad host range among *F. psychrophilum* strains ((Stenholm et al., 2008; Castillo et al., 2014) and unpublished data). High titer solutions of FpV4 and FPSV-D22 were purified 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 (Stenholm et al., 2008; Sundell et al., 2019). The bacteriophages were observed by transmission electron microscopy (TEM) after negative staining with uranyl acetate (**Supplementary Table 1**). To make these observations, 5 µl of the phage solution were deposited onto a freshly glow discharged carbon-covered grid. The bacteriophage solution was left for 2 minutes and the grid was negatively stained with 5 µl uranyl acetate (2% in water) for another minute and finally dried using a filter paper. The grids were observed at 200kV with a Tecnai

G2 (FEI) microscope. Images were acquired with a camera Ultrascan US1000 (Gatan). The concentration of phages FpV4 and FPSV-D22 was 10^9 PFU ml^{-1} in SM buffer with 0.01% gelatin.

Preparation and purification of high-titer phage solutions

Based on previous challenge experiments (data not published and Christiansen, 2014), where we detected an early onset of mortality in fish exposed to phages crude lysates, we decided to PEG purify phage solutions in order to decrease the concentration of compounds that could be toxic for the fish. Gram negative bacteria produce endotoxins which might induce allergic reactions (Kowalska et al., 2020). For the fish experiments, one liter of bacterial cultures (OD 600nm=0.2) were separately infected with the phages FPSV-D22 and FpV4 at MOI=1 (*F. psychrophilum* FPS-S6 and 950106-1/1, respectively) and incubated for ~3 days. The lysed bacteria cultures were centrifuged (9000× g, 10 min, 4 °C) and filtered through a 0.2 µm-pore size sterile filter. Then, the phage stocks FPSV-D22 ($5*10^9$ PFU ml^{-1}) and FpV4 ($3*10^9$ PFU ml^{-1}) were concentrated by adding poly-ethylene glycol 8000 (PEG-8000) and Sodium Chloride (final concentration 10% w/v and 1 M, respectively), followed by incubation at 4 °C for 24 h. Subsequently, phage solutions were centrifuged (10,000× g, 30 min, 4 °C) and the phage pellet was resuspended in 200 mL of SM buffer (Castillo et al., 2019).

Fish (Experiment A and B)

Rainbow trout eyed eggs were purchased at a Danish commercial fish farm (officially registered free of bacterial kidney disease and viral diseases as IPN, VHS and IHN). Eggs were iodophore disinfected, hatched and fish grown at the Unit for Fish and Shellfish Diseases (DTU Aqua, Kgs. Lyngby, Denmark). Fish were initially raised in a recirculation system. When the desired size and weight were reached, fish were transferred to a specific laboratory area used for experimental challenges (flow-through system) and divided randomly in 8-L tanks, each with its own inlet/outlet for water and air-supply. Water temperature was constantly maintained at 13ºC.

Fish (Experiment C)

Rainbow trout fry (1-2 g) were purchased from a commercial fish farm in Finland and kept and reared at the fish facilities of Åbo Akademi University (Turku, Finland) in tanks with flow through of dechlorinated tap water (~12ºC) and continuous aeration.

Experiment A: delivery of phages by phage-sprayed and phage-immobilized feed

I. Preparation of phage feed

Feed pellets (0.8 mm, BIOMAR A/S, Denmark) from the same batch were treated (by spraying or immobilization) with FpV4/FPSV-D22-cocktail (total phage concentration of $3.3*10^9 \pm 6.1*10^8$ PFU ml^{-1} (SD, n=3)), which was prepared mixing 1:1 PEG-purified solutions of FpV4 ($1.2*10^9$ PFU ml^{-1}) and of FPSV-D22 ($4.9*10^9$ PFU ml^{-1}). In the case of phage-sprayed feed, 30 ml of PEG-purified phage preparation containing FpV4 and FPSV-D22 were applied per 100 g of feed pellets with the use of a spray-bottle as previously described (Christiansen et al., 2014). The process was performed in a flow bench where the feed pellets were left to dry. Fixed Phage Ltd produced phage-immobilized feed applying 20 ml of PEG-purified phage preparation per 100 g (Mattey, 2016, 2018). Phage-feed pellets were stored at 5°C before use in the experiments.

II. Detection and quantification of bacteriophages on feed

To verify the presence of bacteriophages on feed, the classical method for phage detection was utilized. Three-hundred microliters of a 48-hour old *F. psychrophilum* broth culture (in exponential phase) were

mixed with 4 ml of TYES soft agar (0.4 % agar) and poured into a TYES-A plate (Stenholm et al., 2008; Madsen et al., 2013). For qualitative detection, feed pellets were spread on the bacterial lawn and plates were incubated at 15ºC for 3-4 days. Phages on feed pellets were quantified according to Christiansen et al. (2014) with some modifications. Three replicates of 0.1 g of feed and 2 ml of SM buffer were prepared in 2 ml sterile micro tubes (SARSTEDT AG & Co. KG, Germany) for each feed type. A sterile 5 mm steel bead (Qiagen, Germany) was added to each micro tube and samples were homogenized with a Qiagen TissueLyser II (1 minute at 20 Hrz; Qiagen, Germany). After storage for 1 hour at 5ºC, samples were transferred to 15 ml sterile Falcon tubes containing 3 ml of sterile SM buffer and vortexed. Phages were quantified by spotting 5µl of serial 10-fold dilutions (180µl of SM buffer and 20µl of sample) of the homogenized solutions in triplicates on a bacterial lawn (TYES soft agar with 48-h old *F. psychrophilum* culture). Plates were incubated at 15ºC for 3-4 days and single plaques were then counted in the preferred dilution to estimate the phage titer per gram of feed pellets (Clokie and Kropinski, 2009; Madsen et al., 2013; Christiansen et al., 2014).

III. Set up and infection method

The first investigated method of phage treatment was through phage application on feed pellets (by spraying or by using the Fixed Phage immobilization technique) (**Table 1** and **Figure 1A**). Rainbow trout fry of 1-2 g were randomly subdivided in 12 x 8 L-aquaria (~50 fish/aquarium). Fish in four aquaria were fed with phage-sprayed feed; fish in other four aquaria with phage-immobilized feed and fish in the remaining aquaria with control (untreated) feed. All groups were fed at 2% of fish weight per day during the experiment. After a 12-day prophylactic treatment period, fish in three of the four aquaria per feed-type group were exposed to the bacterial pathogen, *F. psychrophilum* 950106-1/1, by IP injection (50µl, $1*10^4$ CFU $fish^{-1}$). Fish in the remaining three aquaria (one aquarium per diet group) were injected with sterile TYES-B (as controls for the infection). Prior to IP injection, fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma catalog number A-5040). For each feed-type group, two of the infected aquaria were utilized to follow mortality of fish and the two remaining (one infected with the bacterium and one non-infected) were used for live fish sampling during the experiment. Dead and moribund fish were weighed, their length measured, and bacteriological examination of spleen, kidney and brain performed. If possible, internal organs were also collected and stored for phage detection/quantification. During the experiment, several parameters were considered to evaluate fish health status: feed intake and swimming activity (behavioral observations); fin condition, presence of wounds and coloration (darkening) (external appearance); growth and abnormal mortality (production parameters) (Segner et al., 2019).

III. Fish sampling

Five fish from each sampling aquaria were sampled randomly at 1, 4, 8, 11, 19, 33 and 56 days post infection. Additionally, five fish were collected from the sampling aquaria not infected with *F. psychrophilum* one day before the bacterial challenge. During sampling days, fish were euthanized with an overdose of MS-222. Weight and length of each fish were measured and bacteriological examination of spleen, kidney and brain performed. To assess the spread of phages in fish, internal organs (spleen, kidney, brain and the anterior part of the intestine) were collected in pre-weighted 1.5 ml sterile micro tubes (SARSTEDT AG & Co. KG, Germany) containing 300 µl of SM buffer. After the sampling, micro tubes containing fish organs were re-weighted to determine the weight of the organs and 5 µl of chloroform were added under a fume hood to kill any possible bacteria present in the samples. The used phages are not sensitive to chloroform. Fish were not fed for 24 hours before sampling.

IV. Bacteriological examination

Using 1 µl sterile inoculation loops, samples from spleen, kidney and brain were collected for each sampled and moribund/dead fish and streaked on TYES-A. Agar plates were incubated at 15 °C from 4-5 days up to 4 weeks and *F. psychrophilum* yellow colonies were identified. Randomly chosen yellow colonies were analyzed by MALDI-TOF (Bruker) to confirm that *F. psychrophilum* was the re-isolated bacteria.

V. Bacteriophage detection and quantification

Phage detection in the sampled organs was performed as previously described (Madsen et al., 2013; Christiansen et al., 2014). Briefly, chloroform-fixed fish samples were homogenized by vortexing for 20s and centrifuged for 10s at 10.000RPM at 5ºC (1 minute for intestine samples) to separate chloroform to the bottom of the tube. A spot test method was then performed (Stenholm et al., 2008; Clokie and Kropinski, 2009). For quantification of plaque forming units, five microliters of undiluted sample were spotted on a freshly prepared bacterial lawn (as described above)in triplicate and incubated at 15ºC for 3-4 days. Spots that presented single plaques (from one to 30) were counted and the titer of phages per milligram of tissue quantified. In the case of confluent or semi-confluent clearing areas, samples were 10-fold diluted (180µl of SM buffer and 20µl of sample) in triplicate and spotted on a bacterial lawn. Plates were incubated at 15ºC for 3-4 days, single plaques counted from the preferred dilution and the titer of phages was estimated.

Experiment B: delivery of phages by bath

Rainbow trout fry of 2-3 g were randomly subdivided in 4 x 8 L-aquaria (~30fish/aquaria) (**Table 1** and **Figure 1B**). Fish were fed with commercial feed pellets (0.8 mm, BIOMAR A/S, Denmark) at 2% of fish weight per day during the experiment. Fish in the four aquaria were exposed to the bacterial pathogen, *F. psychrophilum* 950106-1/1, by IP injection (50µl, $1*10^5$ CFU $fish^{-1}$). Based on the results of Experiment A, we decided to increase the infection dose 10 times with the aim of increasing the probability of that bacteria and phages would come into contact with each other. Prior to IP injection, fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma catalog number A-5040). The FpV4/FPSV-D22-mix (total phage concentration of $3.3*10^9 \pm 6.1*10^8$ PFU ml^{-1} (SD, n=3)) was prepared by mixing 1:1 PEG-purified solutions of FpV4 ($1.2*10^9$ PFU ml^{-1}) and of FPSV-D22 ($4.9*10^9$ PFU ml^{-1}). At 48 hours post infection, the water in the aquaria was removed and replaced with 2 liters of cold tap water containing PEG-purified FpV4 and FPSV-D22 (2 ml of phage mix in 2 liters of water – estimated concentration of $3.3*10^6 \pm 6.1*10^5$ PFU ml^{-1}) in two aquaria and 2 liters of cold tap water without bacteriophages in the other two aquaria. Fish were bathed in the phage solution for 1 hour and 30 minutes and subsequently, aquaria were filled up and the flow-through water system was re-established. The same procedure was performed one week after the first phage bath. One week after the second phage bath, phages were directly administered to the selected aquaria (2 ml of phage mix in 8 liters of water – estimated concentration of $8.3*10^5 \pm 1.5*10^5$ PFU ml^{-1}) and the water flow stopped. After 3 hours and 30 minutes, water flow was re-established. The four aquaria were utilized to follow mortality of fish. Dead and moribund fish were weighed, their length measured, and bacteriological examination of spleen, kidney and brain (as for Experiment A) was performed.

Experiment C: delivery of phages through intraperitoneal injection

In experiment C, 120 rainbow trout (~7 g) were randomly divided in six 150 L-tanks (20 fish/aquarium) and fed with commercial feed pellets (1.2 mm, Rehuraisio, Finland) at 2% of fish weight per day (**Table 1** and **Figure 1C**). Fish in the six aquaria were anesthetized with benzocaine (10%) and exposed to *F. psychrophilum* 950106-1/1, by IP injection (100µl, $1.7*10^7$ CFU $fish^{-1}$). A higher bacterial dose,

compared to the previous experiments, was chosen because of the larger fish size. According to Madsen and Dalsgaard (1999), fingerlings have to be challenged with the IP method with an infection dose of 10^7 CFU fish^{-1} or higher to induce mortalities. At 3 days post infection (dpi), fish in three tanks were exposed to PEG-purified bacteriophages FpV4 and FPSV-D22 by IP injection (100 µl, $1.7*10^8$ PFU fish^{-1}). Prior to phage exposure, PEG-purified FpV4 ($1.2*10^9$ PFU ml^{-1}) and FPSV-D22 ($2.2*10^9$ PFU ml^{-1}) solutions were mixed 1:1. Fish in the other three tanks were IP injected with sterile SM buffer as controls. Fish mortality was recorded for 21 dpi and, during this period, dead and moribund fish were removed, weighed and bacteriological examination of spleen and kidney performed. A subset of samples were analyzed and verified as *F. psychrophilum* by PCR (Toyama et al., 1994). To ensure delivery of phages in the fish internal organs by this method, fifteen additional rainbow trout were placed in a 150-L aquarium and injected with bacteriophages alone (100µl, $1.7*10^8$ PFU fish^{-1}). At four and 34 days after exposure, spleen and kidney of five fish were sampled and analyzed for phage detection as described in Experiment A.

Statistical analysis

Phage quantification and survival data were analyzed using GraphPad Prism version 8.4.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. For linear regression analysis of phage concentration detected in sampled organs over time, values were first log transformed. In the Kaplan-Meier survival analysis, data from replicate aquaria were merged together (the difference in survival between replicates was ≤ 20% (Amend, 1981; Midtlyng, 2016)) and comparison of survival curves was performed with the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test.

Results

Phage delivery by feed pellets: phage-immobilized and phage-sprayed feed (Experiment A)

In order to evaluate the potential protection conferred by the phage cocktail targeting *F. psychrophilum*, rainbow trout fry (1.5-2 g) were fed either with phage-immobilized ($8.3*10^7 \pm 2.5*10^7$ PFU g^{-1}) or phage-sprayed ($1.6*10^8 \pm 4.8*10^7$ PFU g^{-1}) feed at 2% of their body weight for 12 days before bacterial challenge (**Table 1**, **Figure 1A** and **Supplementary Figure 1A and B**). Fish growth, abnormal mortalities, feed intake, swimming activity and external appearance (fin condition and coloration) were monitored during the experiment. Positive growth was detected for all groups (**Supplementary Figure 1C** and **Supplementary Table 2**) and no mortalities were observed prior to infection. The addition of phages in either way did not seem to change the taste of the feed for the fish and the fish ate the amount of feed that they were offered (fish not challenged with the bacterium and prior to infection in all groups). Other visual signs of disease conditions, such as destroyed fins, lethargic swimming, color changes and skin ulceration were not seen prior to infection and in non-challenged groups.

Efficiency of phage delivery: percentage of isolation of F. psychrophilum and its phages in fish organs

The qualitative detection of bacteriophages in intestine, kidney, spleen and brain of fish fed with phage immobilized feed, phage sprayed feed and control feed verified the presence of bacteriophages in treated fish and thereby the delivery of phages through the feed pellets before any manipulation (IP injection with either *F. psychrophilum* or sterile TYES-B) (**Figure 2**). The results showed that phages were present in the intestine (100% of sampled fish) and in the internal organs of the fish prior to bacterial challenge (100%, 20% and 40% of kidney, spleen and brain of sampled fish fed with phage-sprayed feed, respectively; 80%, 40% and 20% kidney, spleen and brain of sampled fish fed with phage-immobilized

feed, respectively) (**Figure 2 and 3**). Subsequently, we observed the constant presence of bacteriophages in intestines of fish fed with phage-treated feeds during the experiment with more variable occurrence of phages in the other tested organs (**Figure 2**).

In fish fed with phage-immobilized feed and not exposed to *F. psychrophilum*, bacteriophages were detected in 80% of kidney samples before day 8 and in 40-60 % after 8 days post infection (dpi) (**Figure 3A**). Except for fish sampled 8 dpi, fish challenged with *F. psychrophilum* and fed with phage-immobilized feed (**Figure 3A**) showed a lower percentage of phages in the fish kidney compared to the controls (0-40% at 1, 4 and 11 dpi; frequency of re-isolation of bacterium = 20-60% until 11 dpi). Subsequently, no bacteria were re-isolated from fish kidney and the frequency of isolation of phages changed from 0% at 19 dpi to 80 and 60 % at 33 and 56 dpi, respectively. For spleen samples, we were able to detect phages in 40-60% of fish not challenged with *F. psychrophilum* until 4 dpi and subsequently the percentage of detection dropped to 0-20% (**Figure 3B**). When fish were also exposed to the bacterium, the percentage of phage detection in spleen was between 20 and 60 % until 11 dpi, while *F. psychrophilum* was re-isolated from 40 to 60% of the sampled spleens (**Figure 3B**). Subsequently, no bacteria were re-isolated and phages were detected in 0-20% of the spleen samples. A lower frequency of phage detection was measured in the brain of fish fed with phage-immobilized feed, whether or not fish were challenged with *F. psychrophilum* (between 0 and 20% except for 11 and 33 dpi where 60 and 40 % of sampled brains were positive for phages in non-challenged fish, **Figure 3C**). *F. psychrophilum* was re-isolated only from the brain of one fish sampled 11 dpi.

Fish fed with phage-sprayed feed (negative to *F. psychrophilum)* were characterized by a consistent phage detection in fish kidney (60-100 %; **Figure 3A**), an increase of the frequency of phage isolation in spleen samples from 20 to 60% during the experiment (**Figure 3B**) and more variable measurements in the fish brain (20-60%; **Figure 3C**). When fish were exposed to *F. psychrophilum* (**Figure 3 A and B**), we observed a decrease in phage detection in kidney and spleen samples from 80% to 20% from 1 dpi to 19 and 33 dpi. Subsequently, the frequency of phage detection was increased to 80% for kidney samples (33 dpi) and to 60% for spleen samples (56 dpi). A similar pattern was observed for brain samples (**Figure 3C**) where our measurements showed an initial decrease in phage detection (from 40% to 20%) with a higher detection frequency at 56 dpi. (60%). For *F. psychrophilum*, we detected high frequencies in spleen and kidney (80% for spleen samples and 60% of kidney samples) in the first days after the bacterial challenge, which decreased during the experiment. In brain samples, *F. psychrophilum* was detected at 8 and 11 dpi (40% of sampled fish).

In fish fed with control feed, *F. psychrophilum* was re-isolated from 40 % of kidney samples until 8 dpi and to 20% at 11 and 19 dpi (**Figure 3A**). The frequency of isolation of the bacteria in spleen samples (**Figure 3B**) shifted from 20 % at 1 and 4 dpi to 80, 40 and 20 % at 8, 11 and 19 dpi, respectively. *F. psychrophilum* was only detected in the brain of one fish at 8, 11 and 19 dpi (**Figure 3C**). No phages were detected in fish fed with control feed and no *F. psychrophilum* was re-isolated from fish that served as negative control for the infection fed in any of the three feed groups (IP with sterile TYES-B) (**Figure 3**).

Efficiency of phage delivery in fish organs: quantification

After 12 days of phage prophylactic administration, the concentration of phages detected in the intestine of fish was $2.2*10^3 \pm 1.7*10^3$ PFU mg^{-1} and $1.2*10^3 \pm 1.0*10^3$ PFU mg^{-1} (day -1) in fish fed with phage-

immobilized and phage-sprayed feed, respectively, and these concentrations were maintained over time when fish were not challenged with the bacterium (**Figure 4A and B**). In fish exposed to *F. psychrophilum*, the intestinal phage concentration was also maintained in fish fed with phage-immobilized feed even if we observed a larger variation (SD) among the biological replicates 11 dpi ($1.7*10^3 \pm 1.7*10^3$ PFU mg^{-1}) (**Figure 4A**). A different situation was observed in challenged fish fed with phage-sprayed feed where a decrease in the intestinal phage concentration was detected in the first 8 days after the bacterial challenge. Indeed, the titer of phages per mg of intestine decreased from $9.1*10^2 \pm 5.2*10^2$ PFU mg^{-1} measured at 1 dpi to $2.6*10^2 \pm 3.5*10^2$ PFU mg^{-1} 8 dpi. Subsequently, the number of phages detected in the intestines started to rise even if a large variation among the sampled fish was detected at 11 and 19 dpi. Thirty-three days after the infection the intestinal phage titer raised to $1.0*10^3 \pm 1.2*10^3$ PFU mg^{-1} (**Figure 4B**).

Bacteriophages were detected in the kidney of 57.5% and 75% of non-challenged sampled fish and of 42.9% and 51.4% of challenged sampled fish fed with phage-immobilized and phage-sprayed feed, respectively (**Figure 4C and D**). In fish fed with phage-immobilized feed (**Figure 4C**), the titer of phages in the kidney was ~ 10^1 PFU mg^{-1} one day before the infection and at 1 and 4 dpi. The titer per mg of kidney decreased in the following days reaching its lowest point at 33 dpi (0.5 ± 0.8 PFU mg^{-1}). At the end of the experiment, the concentration of phages in the kidney was restored to $1.4*10^1 \pm 2.1*10^1$ PFU mg^{-1} (56 dpi). When fish fed with phage-immobilized feed were challenged with the bacterium, the concentration of phages in kidney samples over time was on average similar to the non-challenged fish (the lowest concentration, 4.3 ± 9.7 PFU mg^{-1}, was detected 1 dpi and the highest, $3.5*10^1 \pm 4.1*10^1$ PFU mg^{-1}, 8 dpi). For fish fed with phage-sprayed (**Figure 4D**), the concentration of phages in the kidney was constant over time with values as $1.7*10^1 \pm 1.9*10^1$ PFU mg^{-1} (1 day before challenge) and $1.3*10^1 \pm 1.5*10^1$ PFU mg^{-1} (19 dpi). In case of bacterial challenge, the overall concentration of phages per mg of kidney decreased 10 times compared to non-challenged fish (e.g. 2.8 ± 4.7 PFU mg^{-1} detected 11 dpi) except for the initial high phage concentration detected 1 dpi ($6.4*10^1 \pm 7.3*10^1$ PFU mg^{-1}).

Bacteriophages were detected in the spleen of 25% and 35% of non-challenged sampled fish and of 28.6% and 48.6% of challenged sampled fish fed with phage-immobilized and phage-sprayed feed, respectively (**Figure 3**). Quantifying the number of phages per mg of spleen in fish fed with phage-immobilized feed (**Figure 4E**), we observed a decrease from $3.8*10^1 \pm 5.8*10^1$ PFU mg^{-1} detected one day before the bacterial challenge to 0.5 ± 1.1 PFU mg^{-1} and 0.3 ± 0.8 PFU mg^{-1} measured 33 and 56 dpi, respectively. In fish challenged with the bacteria, the number of phages detected over time was stable with values between $2.9*10^1 \pm 4.1*10^1$ PFU mg^{-1} (8 dpi) and 1.1 ± 2.3 PFU mg^{-1} (19 dpi). In fish fed with phage-sprayed feed (**Figure 4F**), the concentration of phages per mg of spleen was $1.2*10^2 \pm 1.3*10^2$ PFU mg^{-1} (+ *F. psychrophilum*) and $1.4*10^1 \pm 1.9*10^1$ PFU mg^{-1} (÷ *F. psychrophilum*) measured one day post infection, and 1.8 ± 3.2 PFU mg^{-1} (+ *F. psychrophilum*) and 2.9 ± 4.6 PFU mg^{-1} (÷ *F. psychrophilum*) 56 dpi.

The number of phages per mg of brain was also quantified (**Figure 4G and H**), although the percentage of detection over time was low (20% and 32.5% of non-challenged sampled fish and 17.1% and 20% of challenged sampled fish fed with phage-immobilized and phage-sprayed feed, respectively - **Figure 3**). In fish fed with phage-immobilized feed (**Figure 4G**), the concentration of phages per mg of brain was 0.9 ± 1.9 PFU mg^{-1} (+ *F. psychrophilum*) and 0.0 ± 0.0 PFU mg^{-1} (÷ *F. psychrophilum*) measured one day post infection, and 0.1 ± 0.3 PFU mg^{-1} (+ *F. psychrophilum*) and 0.7 ± 1.6 PFU mg^{-1} (÷ *F.*

psychrophilum) 56 dpi. In **Figure 4H**, the quantification of phages in the brain of fish fed with phage-sprayed feed is presented. We detected 0.8 ± 1.2 PFU mg^{-1} (+ *F. psychrophilum*) and 1.1 ± 2.4 PFU mg^{-1} (÷ *F. psychrophilum*) measured one day post infection, and 0.7 ± 1.2 PFU mg^{-1} (+ *F. psychrophilum*) and 0.2 ± 0.4 PFU mg^{-1} (÷ *F. psychrophilum*) 56 dpi. The phage propagation rates in fish organs over time, calculated from linear regression analysis, are presented in **Supplementary Table 3**.

Eight and four randomly chosen dead fish were sampled for phage analysis in the groups fed with phage-immobilized and phage-sprayed feed, respectively (**Figure 4 and Supplementary Table 4**). For fish fed with phage-immobilized feed, phage concentration was between 0 and 15.6 PFU mg^{-1} in the intestine, 0 and 45.3 PFU mg^{-1} in the spleen, 0 and 13.3 PFU mg^{-1} in the kidney and, 0 and 65.9 PFU mg^{-1} in the brain. Only two fish (n. 1 and n. 2; **Supplementary Table 4**) showed a higher concentration of phages in kidney and spleen compared to the others ($1.2*10^4$ and 210.5 PFU mg^{-1} in kidney samples; $1.3*10^3$ and 641.9 PFU mg^{-1} in spleen samples). A similar situation was observed for fish fed with phage-sprayed feed where we detected between 0 and 27.5 PFU mg^{-1} of intestine, 0 and 74.2 PFU mg^{-1} of spleen, 0 and 77.8 PFU mg^{-1} of kidney and, 0 and 170.8 PFU mg^{-1} of brain. In the control feed group, only one dead fish was sampled for phage analysis and no phages could be detected.

Effect of phage delivery on fish survival (Experiments A, B and C)

To evaluate the protective effect of bacteriophages and to compare the three phage delivery methods, survival of fish was quantified over time in the three experiments (see experiment set-up in **Figure 1** and **Table 1**). In Experiment A where phages were delivered via the feed, fish mortality started around 10 days post infection (dpi) for the three feed-type groups and it was followed until 56 dpi. The final percent survival for fish fed with phage-sprayed, phage-immobilized and control feed was 75.6%, 80.1% and 76.8%, respectively (**Figure 5A**), with no significant differences among the curves. No mortality was observed in the non-challenged groups of fish fed with the three feed types. When phages FpV4 and FPSV-D22 were administered by bath (Experiment B – **Figure 5B**), the final percent survival was 45.3% in the phage bath group and 42.6% in the control group (no significant difference). When phages FpV4 and FPSV-D22 were administered by intraperitoneal injection three days after the bacterial challenge (Experiment C), the final percent survival observed in the control group (56.7%) was significantly lower than the survival of the group injected with bacteriophages FpV4 and FPSV-D22 (80.0 %) (**Figure 5C**).

To confirm the presence of phages delivered by IP injection (Experiment C), 5 fish were sampled in the control aquaria (only phages) 4 and 34 days after the injection. Four days after IP injection, the concentration of phages detected in the spleen and kidney was 3.3 ± 1.5 and 8.2 ± 3.0 PFU mg^{-1}, respectively. Thirty-four days post inoculation, phages were detected in the kidney of two fish (0.03 and 0.06 PFU mg^{-1}).

Discussion

Phage-therapy has gained increased attention in aquaculture as a sustainable alternative strategy to antibiotic treatment or as a prophylactic measure against disease outbreaks. In our study, we investigated the potential of different methods for delivering a two-component phage mixture to rainbow trout fry to control *F. psychrophilum* infections and to reduce fish mortality.

Phage delivery by edible feed pellets in aquaculture

Oral administration of phages for systemic circulation in fish using phage-immobilized (produced by corona-discharge technology patented by Fixed-Phage Ltd - final concentration of $8.3*10^7$ PFU g^{-1} of feed pellets) and phage-sprayed in-house feed pellets ($1.6*10^8$ PFU g^{-1}) provided a constant delivery of phages to the fish, without a negative effect on fish health or growth. The higher phage density in the internal organs obtained using the phage-sprayed feed compared to the one obtained in fish fed with phage-immobilized feed may partly reflect the higher phage concentration on the manually sprayed feed. However, differences in the orientation of phages on the feed pellets and their detachment and infectivity after the corona-discharge and spray treatment, respectively, might also affect the efficiency of delivery. Leppänen et al. (2019) recently showed that the antimicrobial efficiency was higher for detached phages compared to the attached ones and that covalently bound phages on carboxylate-treated gold had the lowest infectivity even if this surface was characterized by the highest number of attached phages. The authors suggest that the lost infectivity of covalently bound phages might have been caused by chemical interactions or the improper orientation of the phages on the surface (Leppänen et al., 2019). In our study, it does not seem that the covalently bound phages on feed pellets by the corona-discharge method are characterized by a lower infectivity compared to phages applied using a spraying approach, as indicated by a very similar and stable phage number in the fish intestine when fed with the two phage-feed types. It is not clear if the lower phage translocation efficiency in the internal organs in fish fed with phage-immobilized feed compared with the phage-sprayed feed is due to a tighter binding of the phages to the feed pellets, or a lower number of phages attached to the pellets. Nevertheless, the application of phages on feed pellets by the corona-discharge method reduces the time for feed preparation (compared to spraying procedures) and enable the stable immobilization of bacteriophages, with phage activity still present on feed after 1 year storage at 30 °C (Jason Clark personal communication).

The stable concentration of phages detected in the gut after feeding with phage coated pellets indicated a positive gut transit of the selected phages in the gastrointestinal environment, as described previously (Christiansen et al., 2014). However, the detection of phages in the internal organs (spleen, kidney and brain) was not always possible, suggesting inefficient phage penetration from the gastrointestinal tract into the systemic circulation. In a systematic analysis of 144 relevant human and animal experiments, the oral administration route was described as the least effective among the tested methods in delivering active phages that penetrate into the circulatory system (Dąbrowska, 2019) presumably by the transcytosis mechanism (Barr, 2017; Nguyen et al., 2017). Indeed, even if bacteriophages can be isolated in feces/intestine indicating a positive gut transit, it is not always possible to isolate them from blood/internal organs. However, the dose of administration can significantly contribute to phage ability to pass to the systemic circulation (Dąbrowska, 2019). Thus, a key step in delivering phages via feed is the application of very high initial titers. In a recent study, the delivery of *Edwarsiella tarda* phage ETP-1 bio-encapsulated in *Artemia* nauplii (enriched *Artemia* at 10^{11} PFU ml^{-1}) could provide phages with a concentration of 10^4-10^6 PFU mg^{-1} of tissue 10 days after the start of the phage feeding in gut, kidney, spleen and liver of zebrafish (Nikapitiya et al., 2020). Interestingly, it seemed that phage penetration from the gut into the circulatory system was very efficient and only a 10-fold phage decay was observed between the gut and the kidney at the time points examined.

The pH in the gut environment may affect the stability and infectivity of phages as discussed by Madsen et al. (2013) and Christiansen et al. (2014). *In vitro* experiments have shown that the stability of *F. psychrophilum* phages FpV4 and FpV9 were lost completely at pH 3 and in part reduced (a 10 fold reduction over 90 days) at pH 4.5 (Madsen et al., 2013). These pH values resemble the stomach

environment (Bucking and Wood, 2009). FpV9 remained stable at pH 6 and 7.5 (typical of intestine environment) (Bucking and Wood, 2009; Madsen et al., 2013). In our experiment, phages were administered together with the feed, thus the stomach pH would likely have been around 4.9 (Bucking and Wood, 2009; Madsen et al., 2013), potentially causing a minor loss in the amount of infective phages in the stomach. However, other factors like the presence of macromolecules or the bacterial microflora in the gut may potentially protect the phages *in vivo* (Dąbrowska, 2019).

Phage decay in the circulatory system has mainly been attributed to the activity of the innate and the adaptive immune systems, with lymphoid organs considered as the main players in phage clearance and de-activation by phagocytosis (reviewed by (Dąbrowska, 2019; Van Belleghem et al., 2019)). In fish, the main non-mucosal lymphoid organs are the thymus, the kidney (in adult fish mainly the head kidney or *pronephros*) and the spleen (Secombes and Wang, 2012). In our Experiment A, we detected a stable concentration of infective phages (~10^1 PFU mg^{-1}) in the kidney of 57.5 and 75.0% of fish fed with phage-immobilized and phage-sprayed feed, respectively (fish were sampled 24 h after feeding; non-infected fish). These results are in line with previous reported delivery efficiencies using oral intubation (Christiansen et al., 2014) and intraperitoneal injection (Madsen et al., 2013) of *F. psychrophilum* phages in rainbow trout fry, where 75% (24 h after delivery) and 100% (up to 72 h after delivery) of sampled fish showed presence of infective phages in the kidney, respectively (Madsen et al., 2013; Christiansen et al., 2014). The constant detection of infective phages in kidney of fry exposed to the treatment may reflect the role of this well-perfused organ in phage clearance from blood in fish (meaning that phages may be constantly delivered but do not accumulate over time because neutralized), unlike in mammals, where the kidneys have different functions and do not seem to be involved in phage clearance (Dąbrowska, 2019).

The lower detection frequency of active phages in spleen samples compared to intestine and kidney samples, i.e. phage detection in 25 and 35 % of spleen of fish fed with phage-immobilized and phage-sprayed feed, respectively (non-infected fish) was also in agreement with previous findings of 100-1000 fold decrease in phage concentration from intestine to spleen (Christiansen et al., 2014). In addition, the concentration of phages in the spleen appeared to decrease by 10 fold over time (non-infected fish). It is not clear if this is a result of the in-activation of phages in the spleen, or a consequence of the relatively low phage dose delivered. The spleen, a main secondary lymphoid organ (in fish and in mammals) and a reservoir of disease in fish (Secombes and Wang, 2012), has been suggested to play an important role in phage clearance (Dąbrowska, 2019). Previous experiments in rainbow trout (5 g) where bacteriophages were administered by oral intubation (100µl of $1*10^8$ PFU/ml) showed a complete disappearance of viable phages from the spleen 27 hours after phage exposure (Christiansen et al., 2014). Similar results were obtained in mice after the administration of a vibriophage (oral gavage; 100 µl of $1*10^8$ PFU/ml), which showed a very high concentration of infective phages in spleen 6 hours after the administration, which then decreased over time reaching 10^1 PFU mg^{-1} of tissue at 24 hours (Jaiswal et al., 2014). Other studies, however, have demonstrated the presence of active phages in the spleen for a few days after exposure. However, it is unclear how quickly the phages are inactivated (Dąbrowska, 2019).

The detection of phages in 20% and in 32.5% of brain samples of fish fed with phage-immobilized and phage-sprayed feed, respectively documented that phages FpV4 and FPSV-D22 are likely able to cross the blood-brain barrier. However, the simultaneous collection of blood during brain sampling procedures cannot be excluded. Since the dose and the delivery route have been identified as major conditions

572 influencing the diffusion of phages in the brain (Dąbrowska, 2019), we also believe that the low
573 percentage of detection is linked to the relatively low phage dose administered by feed pellets.
574
575 ***Phage delivery methods and fish survival during F. psychrophilum infection***
576 The first indication that the constant delivery of phage FpV9 through feed ($1.5*10^8$ PFU g^{-1}) could
577 provide a decrease in rainbow trout fry (2.5 g) mortality affected by *F. psychrophilum* was provided by
578 (Christiansen, 2014) (final cumulative mortality of 40% against 53% of control fish). In our Experiment
579 A, we did not observe a similar outcome when phages were delivered orally (phage-sprayed feed: $1.6*10^8$
580 PFU g^{-1}; phage-immobilized feed: $8.3*10^7$ PFU g^{-1}). The lack of a significant beneficial effect on fish
581 survival after bacterial challenge, may reflect a low phage density and a reduced phage-pathogen
582 encounter rate in the infected organs. Similar results were obtained when phages were delivered by bath.
583
584 IP injection of bacteriophages has been suggested to be the best route of administration providing a fast
585 and efficient delivery, and is recommended for systemic or localized infections (Dąbrowska, 2019).
586 Phages spread very rapidly in the organs of the fish when delivered with this method but they can also
587 quickly disappear when their target bacteria are not present (IP injection of FpV9 - 10^7 PFU $fish^{-1}$)
588 (Madsen et al., 2013). Previous experiments demonstrated the potential of using *F. psychrophilum* phages
589 to treat RTFS administered by IP injection (phage 1H and 6H delivered together with *F. psychrophilum*)
590 (Castillo et al., 2012). In our Experiment C, phages delivered in high concentrations ($1.0*10^8$ PFU $fish^{-1}$) by intraperitoneal injection three days after bacterial challenge were able to combat the bacterial
592 infection and reduce mortality. These results support the utilization of the selected phages to control *F.*
593 *psychrophilum* when delivered in high dosages. Further, delivering both the pathogen and phages by IP
594 injection likely increases the probability of phage-pathogen encounter in the intraperitoneal cavity.
595
596 Phage-therapy may be more effective on acute than on chronic infections, as the application of an
597 adequate dose of the right phage/phages during early stages of a bacterial infection (during log-phase
598 growth and before the establishment of biofilm), should efficiently eliminate the pathogen (D'Hérrelle
599 and Smith, 1930) (discussed and reviewed by (Abedon, 2014)). Thus, the state and abundance of the
600 bacteria in the body is important to ensure phage-host interaction and phage proliferation in the infected
601 organs. For our experiments, we selected the intraperitoneal injection of *F. psychrophilum* as
602 reproducible infection method, although it is considered relatively harsh for the fish and distant from
603 how it would happen in an aquaculture environment (Madsen and Dalsgaard, 1999). With this method,
604 the injected bacteria spread rapidly in the fish organs (peritoneal cavity, spleen, kidney and brain) in the
605 first 4 days after the infection procedure (Madsen et al., 2013). As expected, we were able to detect *F.*
606 *psychrophilum* in the internal organs of the fish 1 dpi but no difference in the development of the infection
607 in the three feed-groups was observed (Experiment A). Similar results were obtained by Madsen et al.
608 (2013) where the simultaneous IP injection of phage FpV9 did not reduce the occurrence of *F.*
609 *psychrophilum* in the organs. When looking at the concentration of phages, we detected a large variation
610 in intestinal phage concentration among sampled fish in the first 10-20 dpi, which was more prominent
611 in fish fed phage-sprayed feed. This might be the result of the bacterial infection, since the reduced feed
612 intake is one of the clinical signs of infected/diseased fish (Nematollahi et al., 2003). The lower
613 concentration of phages detected in the intestine of dead/moribund fish supports this hypothesis. As
614 observed by Madsen et al. (2013), no increase in phage concentration in the kidney in the presence of *F.*
615 *psychrophilum* was observed. Only few dead fish showed a higher phage concentration indicating phage
616 proliferation in the organ. A similar situation was observed for spleen samples. More markedly for fish

fed with phage-sprayed feed, the overall frequency of phage detection was higher when the bacterium was present (48.6 % against 35% of control fish). We believe that the undetected phage proliferation in organs containing the bacterium may be a consequence of the relatively low concentration of phages obtained *in situ* by the administration of phage-treated feed pellets (in experiment A). To conclude, our understanding of phage replication *in situ* is limited and so, one should focus on optimizing the delivery of high densities of phages with the aim of maximizing phage concentrations at the site of infection, without relying on the self-replicating properties of the phage (Abedon and Thomas-Abedon, 2010; Abedon, 2014). In addition, the role of poorly mixed environments (biofilm) for phage-host encounter in fish organs should be better understood (Abedon and Thomas-Abedon, 2010; Abedon, 2014).

Finally, when comparing our results on fish survival, it is important to mention that the fish used in Experiment A and B were at the fry stage (2-3 g), whereas larger fish (fingerlings, ~7 g) were used in experiment C. This is important as higher mortalities from *F. psychrophilum* infections are generally observed in fry population (80%) than larger fish (fingerlings and bigger; 20%) during disease outbreaks in fish farms (Lorenzen, 1994). Differences in fish size and immune status between experiments (Madsen and Dalsgaard, 1999) thus likely explain why the final mean mortality in the fingerlings in Experiment C was lower than for fish in Experiment B, in control groups, despite that they were challenged with the highest bacterial dose.

Conclusion and future perspectives

Even though phage therapy seems very attractive and straightforward, it presents various drawbacks/challenges. In our experiments, we believe that the delivery of bacteriophages applied by Fixed Phage technology on feed pellets represents an effective method of delivering a product with a feasible application in the field. It also reduces the time-consuming tasks of spraying and drying feed pellets. We believe that the inefficient delivery of phages to the internal organs (i.e. the high loss of infective phages during the delivery process across the intestinal barrier as well as the potential too low phage concentration applied in feed and bath experiments) was the reason for the lack of a beneficial effect on survival of fish challenged with *F. psychrophilum*. The significant increase in fish survival upon IP administration supports the hypothesis that the delivery of higher dosages of phages at the infection site could positively contribute to fish health/recovery, and emphasizes the need for applying higher concentrations of phages on the feed to account of the loss of infective phages during the delivery process. It was recently demonstrated that treatment of *F. psychrophilum*-infected fish (experimental IP infection challenge) by using IP injection of bacteriophages resulted in significantly higher fish survival (lower MOI than in the current study) (Sundell et al., 2020). This work suggested that phages can improve fish survival even when present in relatively low numbers in the peritoneal cavity. We believe, therefore, that the prophylactic approach using phage delivery with feed might be a feasible option, if the phages can be applied to the feed at a 10-100 times higher concentration than the one of the current experiments, as this might sustain sufficient phage concentration (10^4-10^5 PFU mg^{-1}) in the fish intestine to block the early development of the bacterial infection.

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827 # Figures and tables

828 **Table 1. Overview of the studied experimental delivery methods.**
829
830 **Figure 1. Experimental fish trials to test phage delivery by feed (A), by bath (B) and by**
831 **intraperitoneal injection (C). (A)** Eight hundred and ninety-five rainbow trout fry were divided
832 randomly in 12 aquaria (55 fish ±4) which are represented by colored circles. Fish were fed at 2% of
833 their body weight with either control feed (in grey), phage-immobilized (in blue) or phage-sprayed (in
834 red) feed. Bacteriophage drawings indicate FpV4 and FPSV-D22, which were administered to fish by
835 feed. For each group, three of the four aquaria were challenged with *F. psychrophilum* ($1*10^4$ CFU $fish^{-1}$;
836 indicated by a syringe with yellow content because of the coloration of the bacteria). Two of the
837 bacterial challenged aquaria per group were used to follow mortality. The remaining aquaria were
838 dedicated to sampling (5 fish at sampling point). **(B)** Hundred and twenty-five rainbow trout fry were
839 divided in four aquaria (circles; 31 fish ±1 per aquarium) and challenged with *F. psychrophilum* ($1*10^5$
840 CFU $fish^{-1}$; syringe with yellow content). Fish in two aquaria were exposed to three rounds of FpV4 and
841 FPSV-D22 phages bath (blue circles; indicated by bacteriophage drawings). Mortality was followed. **(C)**
842 Hundred and twenty rainbow trout (7g), divided in six aquaria (circles, 20 fish ±0 per aquarium), were
843 IP challenged with *F. psychrophilum* ($1.7*10^7$ CFU $fish^{-1}$; syringe with yellow content). Three days later,
844 fish in three aquaria were exposed to phages FpV4 and FPSV-D22 by IP injection (blue circles; indicated
845 by bacteriophage drawings). This aquaria were used to follow mortality. One additional aquarium was
846 included for few sampling where fish were only exposed to the two component phage mix (light blue
847 circle; 15 fish). Created with BioRender.com.
848
849 **Figure 2. Experiment A. Qualitative detection of bacteriophages in fish organs (brain, spleen,**
850 **kidney and intestine) over time in fish fed with phage-immobilized (PI), phage-sprayed (PS) and**
851 **control (C) feed.** Blue and red colors indicate the presence of phages in organs of fish fed with phage-
852 immobilized and phage-sprayed feed, respectively, challenged with *F. psychrophilum* (full color, no
853 pattern) or not (pattern, striped). Absence of phages in the tested organ is indicated by white/blank.
854 Positive detection = presence of one or more plaques in at least one of the technical triplicates. At each
855 sampling point, five fish were sampled in each group except one day before the infection where samples
856 were collected only from groups not supposed to be challenged with *F. psychrophilum*. dpi= days post
857 infection.
858
859 **Figure 3. Experiment A. Frequency of detection of bacteriophages and of isolation of *F.***
860 ***psychrophilum* in kidney (A), spleen (B) and brain (C) of sampled fish in the different groups over**
861 **time.** No phages were detected in internal organs of fish fed with control feed. Five fish were sampled at
862 each sampling point per group. n.d.= not determined. For each organ of each feed group, the total
863 percentage of phages isolation over time is calculated with and without *F. psychrophilum*.
864
865 **Figure 4. Experiment A. Quantification of bacteriophages FpV4 and FPSV-D22 in intestine (A and**
866 **B), kidney (C and D), spleen (E and F) and brain (G and H) of fish fed with phage-immobilized (A,**
867 **C, E and G) and phage-sprayed (B, D, F and H) feed.** Values represent the average of five biological
868 replicates per time point and error bars the standard deviation. Concentration of phages in the organs of
869 dead fish (dead because of *F. psychrophilum* infection) is included in the graphs for the corresponding

organ and feed type (each symbol in yellow represents a single fish). Simple linear regression lines were calculated from the log-transformed PFU over time and 95% confident bands are also presented.

Figure 5. Percent survival observed in rainbow trout fry exposed to *F. psychrophilum* and bacteriophages in the three experiments. In (**A)**, survival of fish fed with phage-immobilized feed (blue), phage-sprayed feed (red) feed and control feed (grey) are displayed (Experiment A). No mortality was observed in the control aquaria (data not shown). In (**B)**, survival of fish bathed in phage solution (blue) and in control bath (grey) in Experiment B. In (**C)**, bacteriophages were delivered with IP injections three days after the bacterial challenge (blue) while phage control fish were injected with sterile SM buffer (grey) (Experiment C). In all three experiments, moribund and dead fish were positive to *F. psychrophilum*. Final percent survival are presented for each curve in the figures. *= the curves are significantly different. 95% confident interval is presented for each curve.

882 **Table 1. Overview of the studied experimental delivery methods.**

883

Exp.	Phage delivery method	Administered phage titer	Administration time	Fish		Bacterial infection dose (IP) (CFU fish^{-1})
				Weight (g)	Total n. (n. per replicate)	
A	Phage-sprayed feed	$1.6*10^8 \pm 2.5*10^7$ PFU g^{-1} [b]	Continuous feeding started 12 days before IP.*	1.9 (±0.7)[c]	895 (55±4)[d]	$1.0*10^4$
	Phage-immobilized feed	$8.3*10^7 \pm 4.8*10^7$ PFU g^{-1} [b]				
	Control feed[a]	0 ± 0 PFU g^{-1} [b]				
B	Bath	I. ~10^6 PFU ml^{-1} II. ~10^6 PFU ml^{-1} III. ~10^5 PFU ml^{-1}	I. 48 hours after IP.* (1h 30 min); II. One (1h 30 min) and III. 2 weeks (3h 30 min) after 1st bath	2-3	125 (31±1)[d]	$1.0*10^5$
	Control bath	I. 0 PFU ml^{-1} II. 0 PFU ml^{-1} III. 0 PFU ml^{-1}				
C	IP injection	$1.0*10^8$ PFU fish^{-1}	3 days after IP.*	~7	120 (20±0)[d]	$1.7*10^7$
	Control IP injection	0 PFU fish^{-1}				

[a] Non-treated commercial feed from the same batch as the phage-treated feed types.
[b] Average and standard deviation (n=3).
[c] Average weight of fish sampled after 10 days of phage feed prophylaxis (standard deviation in the parenthesis; n=15).
[d] Average number of fish per aquaria and standard deviation in the parenthesis.
* IP= bacterial intraperitoneal injection

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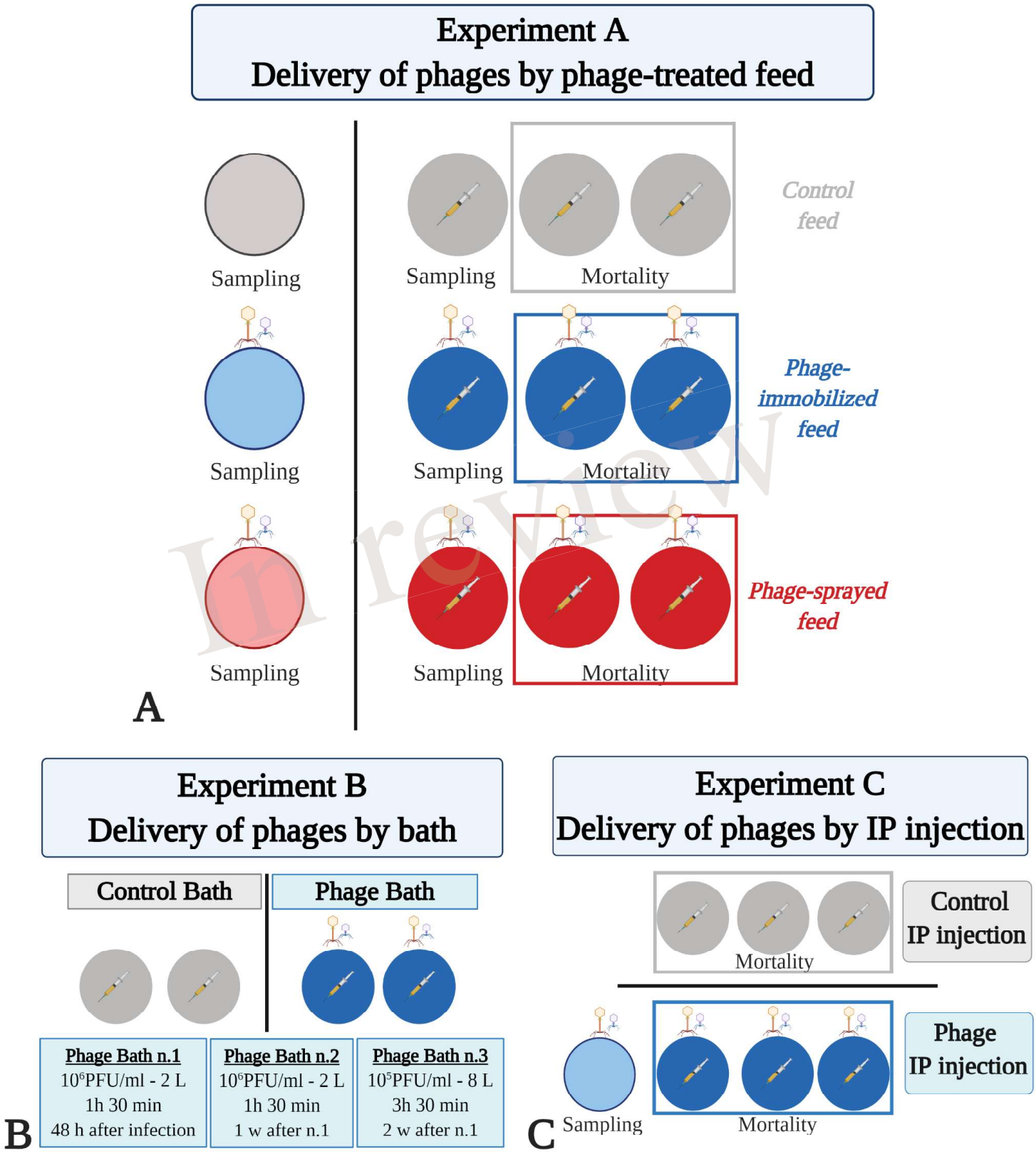
885

Figure 1.JPEG



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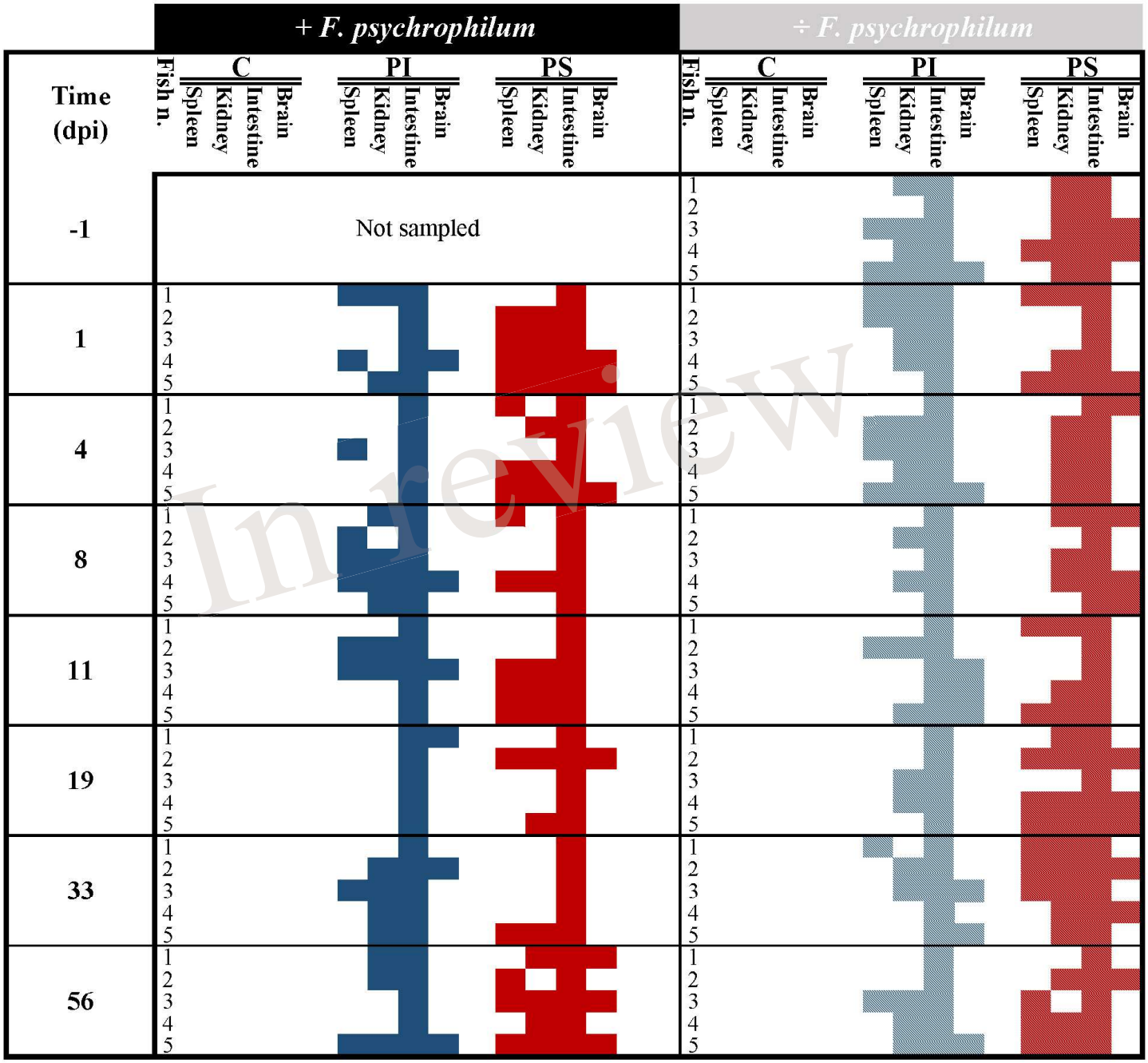

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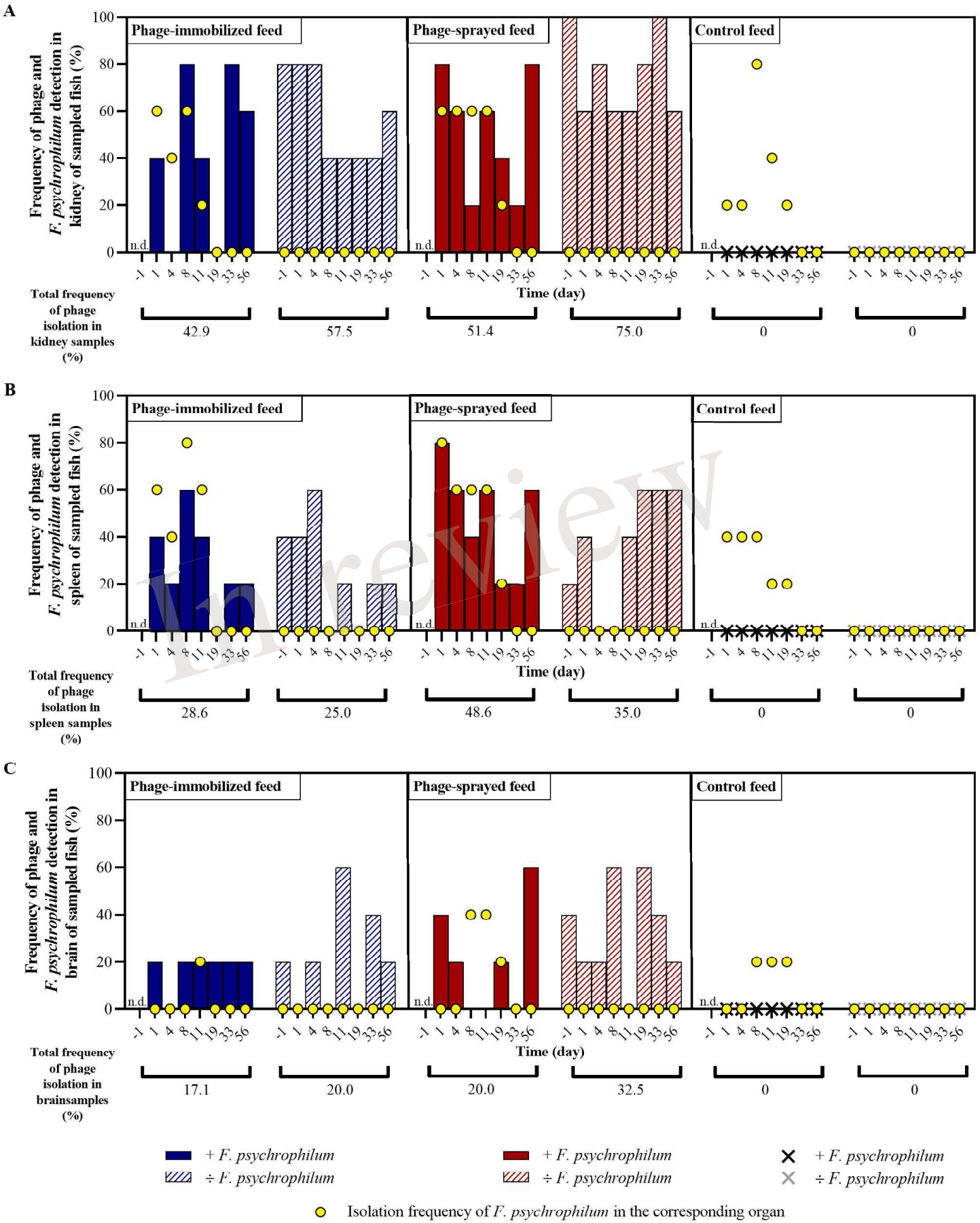
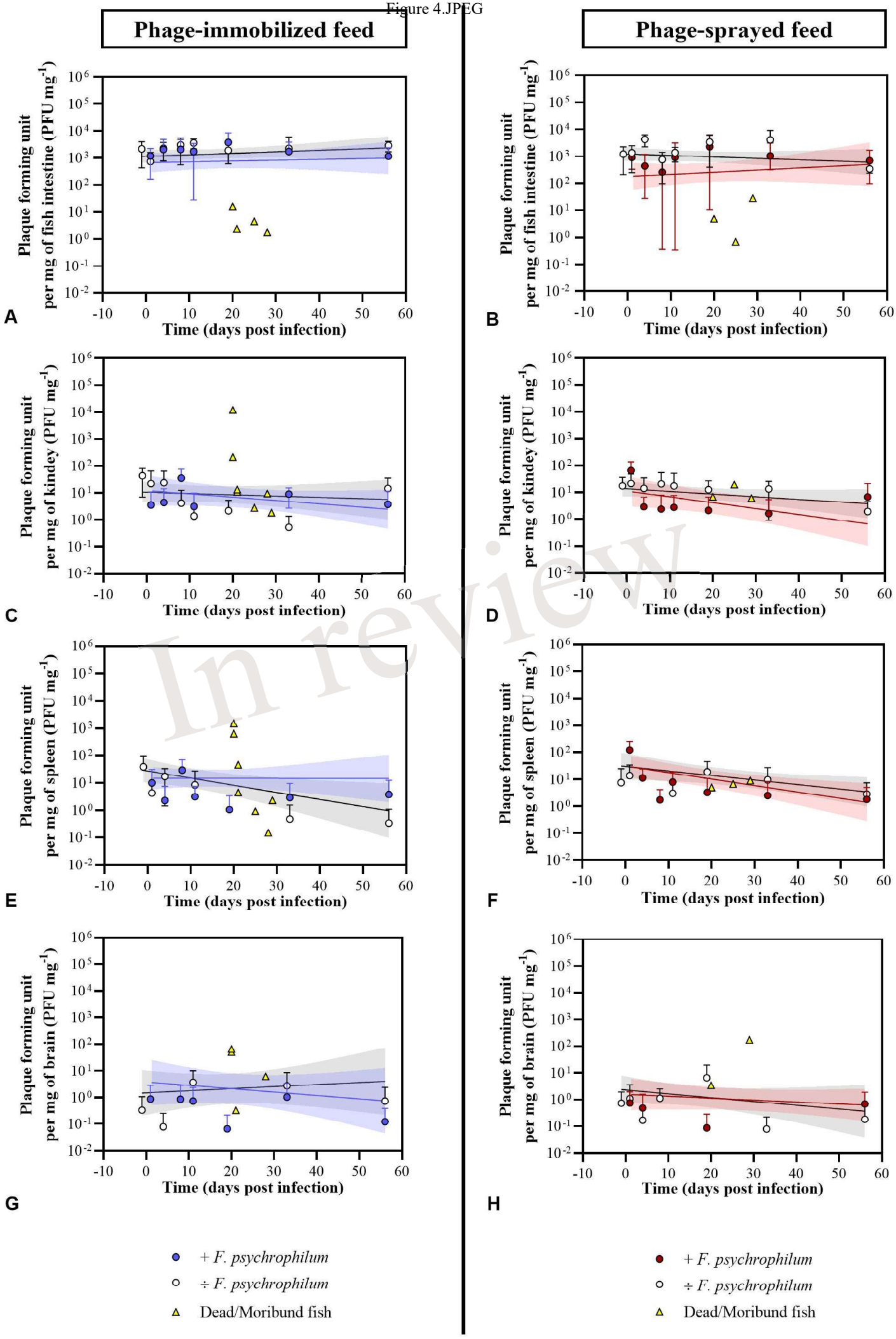


Figure 4.JPEG



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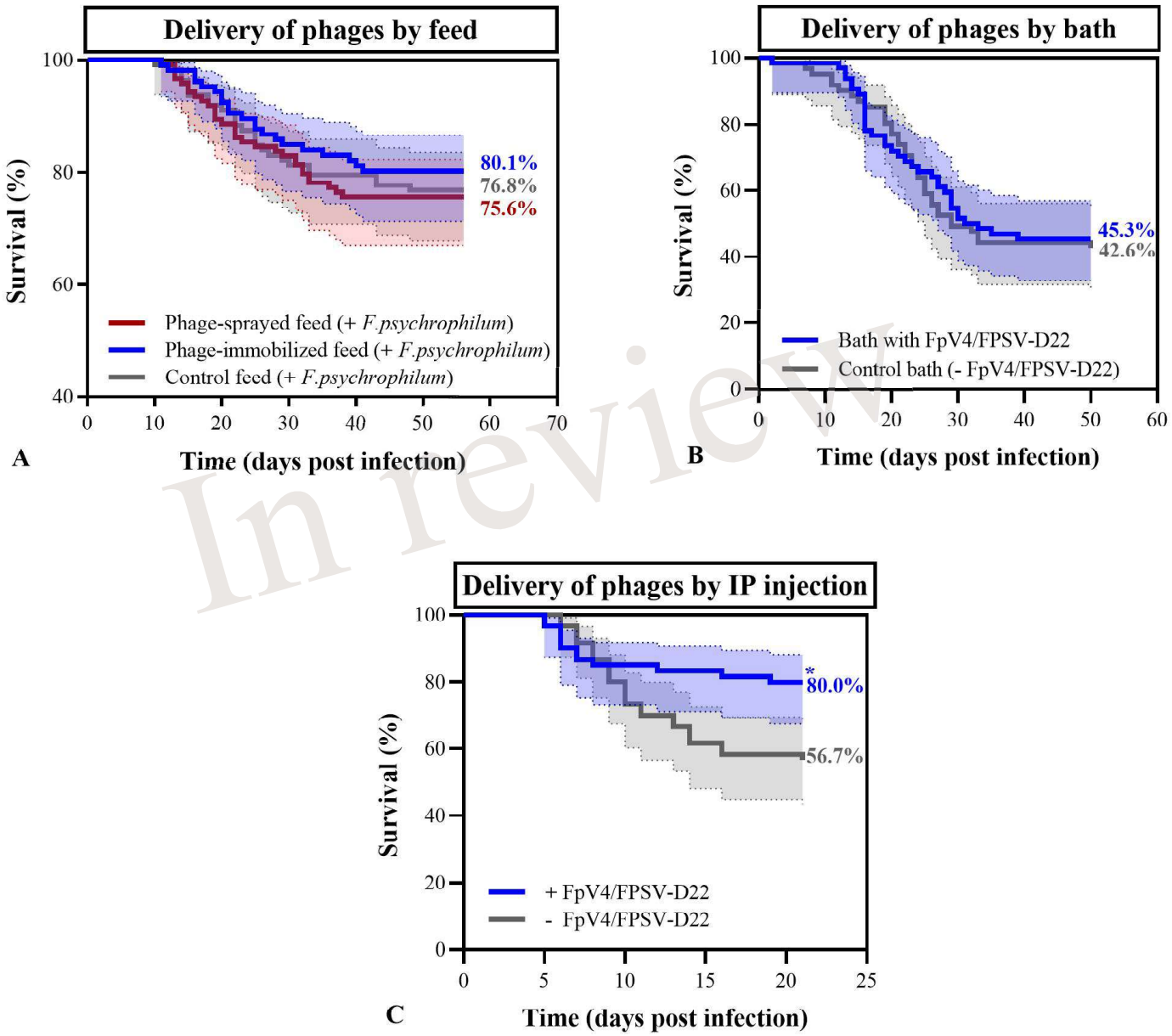
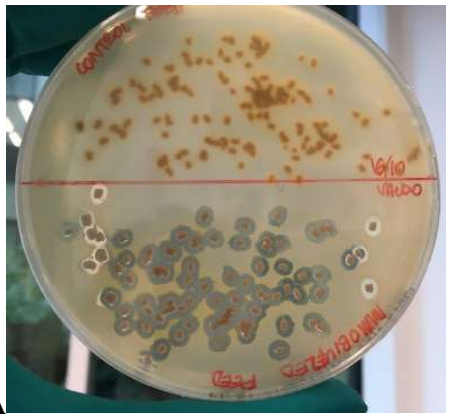


Supplementary Material



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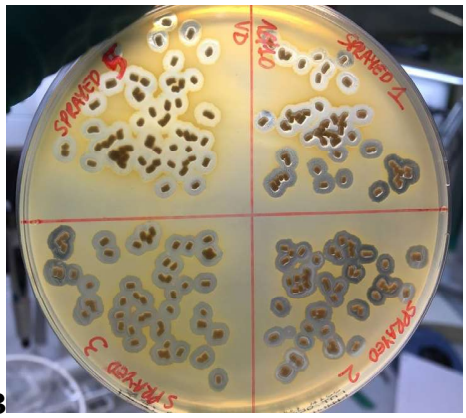

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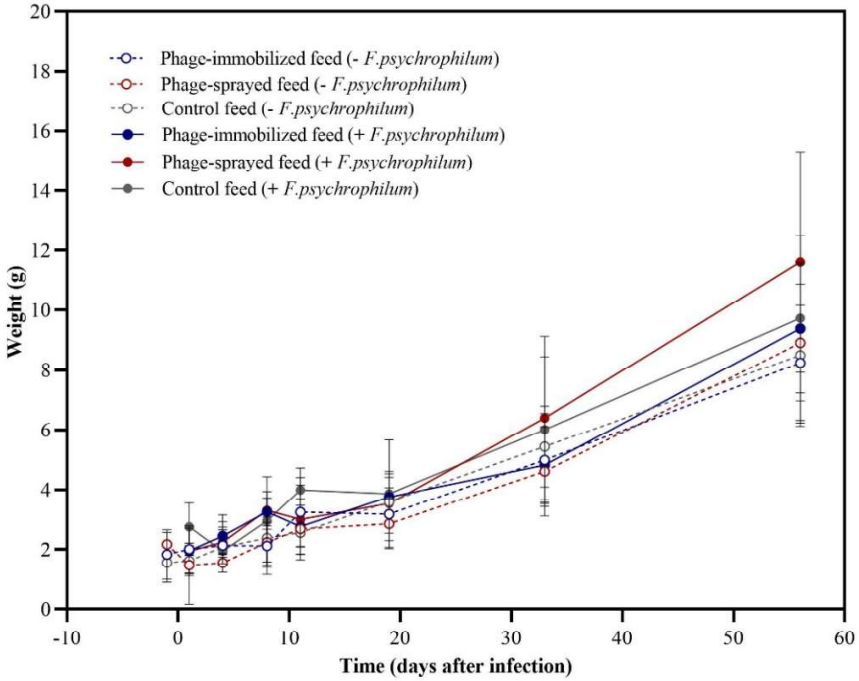
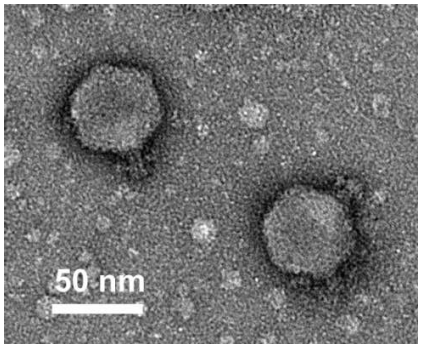
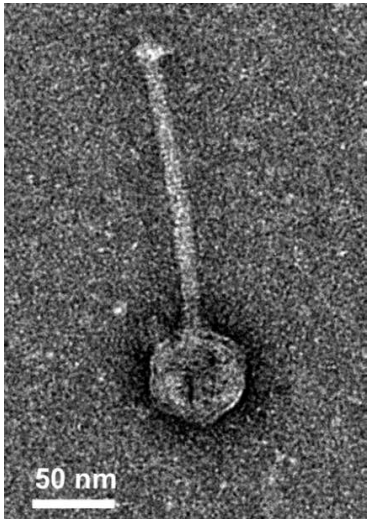


C

Supplementary Figure 1. Experiment A. Double-layer plaque assay of feed pellets treated with phages (A and B) and growth performance based on weight of fish (C) in the three feed groups with (continuous lines) and without the infection (dashed lines). Phage-immobilized feed (lower panel) and control feed (upper panel) are presented in (**A)** and phage–sprayed feed pellets in (**B) (**the four panels on the plate in B represent four batches of phage-sprayed feed**)**. Clearing areas around feed pellets indicate the presence of bacteriophages. Bacterial lawns were prepared with *F. psychrophilum* 950106-1/1. Pictures were taken after five days of incubation at 15ºC.

Supplementary Table 1. Characteristics of selected virulent phages for experiments A, B and C. Isolation: year and source.

Phage	Isolation	Genome size (kb)	Morphology*	Efficiency of plating (EOP)**	Adsorption constant (ml min^{-1})**	Burst size (PFU ml^{-1})**	Latent period (h)**
			Podoviridae [a]				
FpV4[a,b,c]	2005; water with feces [a]	90[a]		$1.4*10^{-3}$ [c]	$3.4 \times 10^{-10} \pm 7.1 \times 10^{-8}$ [b]	101 ± 7[b]	5.5 ± 0.1[b]
			Siphoviridae (this study)				
FPSV-D22[c]	2017; fish tissue [c]	Unpublished data		$2.3*10^{-4}$ [c]	Not determined	Not determined	Not determined

[a] Described in Stenholm et al. (2008); [b] Described in Castillo and Middelboe (2016); [c] Described in Sundell et al. (2019).
*The morphology of phage FpV4 was previously observed in Stenholm et al. (2008). In this study, we repeated the TEM imaging of FpV4 together with the recently isolated phage FPSV-D22.
**Host: *Flavobacterium psychrophilum* 950106-1/1.

Supplementary Table 2. Experiment A. Fish growth parameters. Characteristics of fish sampled at day 1 and day 56 post infection for the three feed groups. Mortalities refer to the ones observed in the sampling aquaria. Initial and final fish weight and length data were tested at first with the Shapiro-Wilk test (to evaluate normality) and then compared with ANOVA or Krustal Wallis in case non-normal data distribution. P-values for multiple comparisons were adjusted for Dunnet (normal distribution) or Dunn's (non-normal distribution) corrections. Significantly different P-values (below 0.05) were not observed. The analysis were performed with GraphPad Prism version 8.4.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Specific growth rate (SGR) was calculated as follows: SGR(%) = [(ln$Weight_i$ - ln$Weight_f$)/feeding days]*100, where $Weight_i$ and $Weight_f$ indicate the initial and final average fish weight (Egerton et al., 2020).

	Phage immobilized feed	**Phage sprayed feed**	**Control feed**	**Phage immobilized feed (+*F. p*)**	**Phage sprayed feed (+*F. p*)**	**Control feed (+*F. p*)**
Initial weight (g) (mean ±SD; n=5)	**2.01**±0.21	**1.48**±1.31	**1.62**±0.52	**1.96**±0.76	**1.94**±0.77	**2.76**±0.80
Initial length (cm) (mean ±SD; n=5)	**5.54**±0.15	**5.02**±0.11	**5.26**±0.58	**5.52**±0.68	**5.46**±0.77	**6.26**±0.54
Final weight (g) (mean ±SD; n=5)	**8.24**±1.92	**8.90**±2.68	**8.50**±2.38	**9.38**±2.16	**11.61**±3.69	**9.73**±2.77
Final length (cm) (mean ±SD; n=5)	**8.7**±0.78	**8.98**±0.74	**8.76**±0.89	**9.38**±0.9	**9.94**±0.98	**9.5**±0.94
Specific growth rate (SGR) (%)	2.57	3.27	3.02	2.85	3.25	2.29
Mortalities	0	0	0	3	4	5

Supplementary Table 3. Experiment A. Phage propagation rate in intestine, kidney, spleen and brain of fish fed with phage-immobilized and phage-sprayed feed (with and without bacterial infection). Values are calculated from linear regression of log transformed PFU over time.

Phage feed type	Bacterial challenge (+ *F. psychrophilum*)	Tissue	Phage propagation	
			Rate day^{-1}	r^2
Phage-immobilized feed	+	Intestine	0.003 (±0.007)	0.005
		Kidney	-0.012 (±0.009)	0.11
		Spleen	-0.0001 (±0.0002)	0.00003
		Brain	-0.012 (±0.011)	0.24
	÷	Intestine	0.006 (±0.005)	0.03
		Kidney	-0.005 (±0.008)	0.01
		Spleen	-0.026 (±0.009)	0.47*
		Brain	0.008 (±0.012)	0.06
Phage-sprayed feed	+	Intestine	0.008 (±0.009)	0.02
		Kidney	-0.022 (±0.009)	0.26*
		Spleen	-0.024 (±0.007)	0.40*
		Brain	-0.008 (±0.006)	0.21
	÷	Intestine	-0.005 (±0.005)	0.03
		Kidney	-0.010 (±0.006)	0.09
		Spleen	-0.017 (±0.007)	0.36*
		Brain	-0.014 (±0.010)	0.15

*=the slope is significantly non-zero (p value <0.05)

Supplementary Table 4. Experiment A. Information of dead/moribund fish sampled for phage analysis. Bacteriological examination: a plus sign (+) indicates that *F. psychrophilum* was re-isolated from the corresponding fish organ.

Feed group	Fish n.	Time of event (dpi)	Fish weight (g)	Organs weight (mg)				Bacteriological examination			Presence of phages (PFU mg^{-1} of tissue)			
				Brain	Kidney	Spleen	Intestine	Brain	Kidney	Spleen	Brain	Kidney	Spleen	Intestine
Phage-immobilized feed	1	20	3.21	12.3	13.2	11.3	26.7	+	+	+	50.7	$1.2*10^4$	$1.5*10^3$	0.0
	2	20	2.66	10.2	11.4	8.1	33.0	+	+	+	65.9	210.5	641.9	15.6
	3	28	5.55	18.7	23.2	40.0	16.8		+	+	6.1	9.3	0.2	1.76
	4	21	3.53	17.0	10.0	14.5	17.7	+	+	+	0.0	10.8	4.6	2.4
	5	21	2.70	18.3	12.2	11.1	29.3		+	+	0.3	13.3	45.3	0.0
	6	25	2.46	16.6	12.2	14.4	21.6	+	+	+	0.0	0.0	0.0	4.4
	7	25	2.97	16.0	8.8	8.6	18.6	+	+	+	0.0	2.7	0.9	0.0
	8	29	5.28	16.2	36.6	28.3	45.5	+	+	+	0.0	1.8	2.3	0.0
Phage-sprayed feed	1	15	5.27	19.2	33.9	26.9	20.1		+	+	0.0	0.0	0.0	0.0
	2	20	4.28	14.1	16.0	22.9	16.2	+	+	+	3.4	6.8	4.9	4.8
	3	25	3.51	18.7	21.8	13.3	60.3	+	+	+	0.0	19.5	6.8	0.7
	4	29	2.74	14.5	15.8	9.4	16.6	+	+	+	170.8	6.1	8.9	27.5
Control feed	1	20	3.46	22.5	12.9	11.8	15.4	+	+	+	0.0	0.0	0.0	0.0

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Manuscript II

The gut microbiota of healthy and *Flavobacterium psychrophilum*-infected rainbow trout fry is shaped by antibiotics and phage therapies

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Keywords

Flavobacterium psychrophilum, microbiome, microbiota, gut, 16S rRNA, phage therapy, antibiotic, florfenicol, RTFS

Abstract

In the aquaculture sector, there is an increased interest in developing environmentally friendly alternatives to antibiotics in the treatment and prevention of bacterial infections. This requires an understanding of the effects of different treatments on the fish microbiota as a measure for improving the fish heath status. In this study, we focused on the freshwater pathogen *Flavobacterium psychrophilum* and investigated the effects of antibiotics (florfenicol) and phage therapies on the gut microbiota of healthy and infected rainbow trout fry (1-2 g). Florfenicol-coated feed was administered for 10 days starting two days after the infection procedure. A two component mix of phage targeting *F. psychrophilum* (FpV4 and FPSV-D22) was continuously delivered by feed with a prophylactic period of 12 days. Samples of the distal intestine were collected over time (day -1 and 1, 8 and 33 days post infection) and analyzed by community analysis targeting the 16S rRNA gene (V3-V4 region). Results showed the dysbiosis effect caused both by the infection and by florfenicol administration. Shifts in the overall composition were detected by β-diversity analysis, and changes in specific populations were observed during taxonomic mapping. Measures of α-diversity were only affected in infected fish (large variation observed 1 and 8 dpi). These community alterations disappeared again when fish recovered from the infection and the antibiotic treatment was terminated (33 dpi). Interestingly, phage addition altered the microbiota of the fish independently of the presence of their target bacterium. The overall gut bacterial community in fish fed phage-treated feed was different from the controls at each time point as revealed by β-diversity analysis. However, it was not possible to identify specific bacterial populations responsible of these changes except an increase of lactic acid bacteria 33 dpi. Overall, the results indicate that the administered phages might affect the complex network of phage-bacteria interactions in the fish gut. Nevertheless we did not observe negative effects on fish health or growth, further studies should be directed in understanding if these changes are beneficial or not for the fish health with an additional focus on the host immune response.

Introduction

The growth in production of the aquaculture sector (World Bank, 2013; FAO, 2018) has led to an increased interest in environmentally friendly alternatives to treat or prevent bacterial infections (reviewed by (Culot et al., 2019; Kowalska et al., 2020)). Moreover, understanding the effects of different treatments on the microbiota of the fish as a measure for improving the fish health status has had renewed interest (Perry et al., 2020). The gut microbiota, defined as the set of commensal, pathogenic and symbiotic microorganisms (bacteria, archaea, viruses, bacteriophages and fungi) inhabiting the gut, plays indeed a very important role in the development, growth and health of the host (metabolic and digestive processes, the energy homoeostasis by feeding regulation and the immune response) (Ingerslev et al., 2014a, 2014b; Butt and Volkoff, 2019). It represents the most investigated microbiota in teleost compared to skin and gills, especially in relation to diet (Perry et al., 2020). Several abiotic and biotic factors can influence the gut microbiota as for example the administration of probiotics (Gonçalves and Gallardo-Escárate, 2017), the presence of disease/infection (Ingerslev et al., 2014a; Nie et al., 2017) and antibiotic therapies (He et al., 2017; Gupta et al., 2019; Kim et al., 2019) (reviewed by (Butt and Volkoff, 2019)).

Flavobacterium psychrophilum (Borg, 1948; Bernardet et al., 1996) is a freshwater pathogen causing important economic losses worldwide and the etiological agent of Rainbow Trout Fry Syndrome (RTFS) and Bacterial Cold Water Disease (BCWD) (Borg, 1960; Lorenzen et al., 1991) (reviewed by (Nematollahi et al., 2003; Wahli and Madsen, 2018)). To overcome the limitations and concerns concerning the antibiotic therapies standardly used, the utilization of bacteriophages (the so called "phage-therapy") is receiving increased attention as alternative method, but also as prophylaxis, for the treatment and prevention of this bacterial infection (Stenholm et al., 2008; Madsen et al., 2013; Christiansen et al., 2014; Donati et al., 2021).

Focusing on *F. psychrophilum* infections, we wanted to evaluate the effects of orally-administered bacteriophages and florfenicol (antibiotic in use in Denmark for the treatment of RTFS) on the gut bacterial community of healthy and infected rainbow trout fry. In order to minimize the number of fish utilized in the experiments, this study was performed in combination with the Experiment A in Donati et al. (2021), where *F. psychrophilum* phages were applied on feed pellets by spraying, or by irreversible immobilization (Mattey, 2016, 2018) and delivered to rainbow trout fry to evaluate the phage diffusion in fish internal organs and to assess the effects on fish survival during *F. psychrophilum* infections. In addition to the phage administration, groups of healthy and infected fish fed with antibiotics were included in the experimental trials and samples of the distal intestine were collected for all groups. A community analysis targeting the 16S rRNA gene (V3-V4 region) of the DNA extracted from intestine samples was performed with the aims of a) characterizing the microbiome, defined as the collection of genomes of the bacterial communities inhabiting the gut, of healthy rainbow trout fry fed with commercial feed and with phage-treated feed (after 11 days of prophylaxis); b) evaluating the effects of florfenicol on the microbiome of healthy and infected fish during the treatment and c) observing the composition of the gut microbiome in fish that have recovered from the infection under the different feed type regimes (commercial, phage-treated and antibiotic-coated feed). The work demonstrates the dysbiosis of the gut microbiome of rainbow trout fry during *F. psychrophilum* infection and florfenicol administration but also suggest that this condition is lost once the fish have recovered from the infection and the antibiotic therapy is terminated. Further, this study demonstrates that orally administered bacteriophages can shape the gut microbial communities independently of the presence of their target pathogen.

Materials and method

Bacterial strain

A well-characterized Danish strain of *Flavobacterium psychrophilum* was selected for the experiment (*F. psychrophilum* 950106-1/1; serotype Fd; virulent) (Madsen and Dalsgaard, 1999, 2000; Dalsgaard and Madsen, 2000; Sundell et al., 2019). The Swedish isolate *F. psychrophilum* FPS-S6 (serotype Th, virulent), was used for the propagation of the phage FPSV-D22, as this isolate is the most efficient host for production of high titers of this specific phage (Sundell et al., 2019). The bacteria were stored in tryptone yeast extract salts medium (TYES) (Holt et al., 1993) and glycerol (15-20%) at – 80°C. For bacteriophage titration, *F. psychrophilum* 950106-1/1 was prepared as described by Donati et al. (2021). A 48-72 h broth culture of *F. psychrophilum* 950106-1/1 was prepared in 5 ml of TYES broth (referred as TYES-B) from a – 80°C stock (incubation at 15°C at 100 rpm) and streaked on TYES agar (referred as TYES-A: TYES-B containing 1.1% agar). Following an incubation period of 3-4 days at 15°C, single colonies were inoculated in TYES-B (5 ml) for 48 hours and then used for the phage quantification assay. For the experimental fish challenge, *F. psychrophilum* 950106-1/1 was prepared and the infection challenge performed as described by Madsen and Dalsgaard (1999). Briefly, a 48-hour culture was diluted in TYES-B and 50 µl of the selected dilution were injected in the peritoneal cavity (intraperitoneal injection, IP) of the fish for a final dose of $1.0*10^4$ CFU $fish^{-1}$. Control fish were injected with 50 µl of sterile TYES-B. CFU count of the injected culture in duplicates was performed before and after infection. This method of infection was selected as considered the most reproducible in the case of experimental *F. psychrophilum* infection challenge in rainbow trout fry (Madsen and Dalsgaard, 1999; Donati et al., 2021).

Bacteriophages

Two Danish lytic bacteriophages infecting *F. psychrophilum* were selected for the experiment: FpV4 (*Podoviridae* family, 90 kb genome, isolated in 2005) (Stenholm et al., 2008; Castillo and Middelboe, 2016) and FPSV-D22 (*Siphoviridae* family, 42 kb genome, isolated in 2017) (Sundell et al., 2019; Donati et al., 2021). Both phages were characterized to have a broad host range among *F. psychrophilum* strains (Stenholm et al., 2008; Castillo et al., 2014; and unpublished data). Purified highly concentrated solutions of FpV4 and FPSV-D22 were stored at -80°C with SM buffer and glycerol (15%) (Stenholm et al., 2008; Sundell et al., 2019).

The quantification of plaque forming units (PFU) in phage solutions was performed by the spot test method (Stenholm et al., 2008; Clokie and Kropinski, 2009). Briefly, a bacterial lawn was prepared by mixing 300µl of a 48-hour old *F. psychrophilum* broth culture with 4 ml of TYES soft agar (0.4 % agar) and the mixture was poured onto a TYES agar plate (TYES-A). Five microliters of undiluted sample were spotted on the bacterial lawn in triplicate and incubated at 15ºC for 3-4 days. Spots with single plaques (from one to 30) were counted. In case of confluent or semi-confluent clearing areas, 10-fold dilutions were performed (180µl of SM buffer and 20µl of sample) in triplicate and spotted on a bacterial lawn. Plates were incubated at 15ºC for 3-4 days, single plaques counted and the phage titer calculated. For quantification of phages on feed pellets (Christiansen et al., 2014; Donati et al., 2021), 0.1 g of feed (n = 3) and 2 ml of SM buffer were mixed in 2 ml sterile micro tubes (SARSTEDT AG & Co. KG, Germany) for each feed type (phage treated pellets and control feed), a sterile 5 mm steel bead (Qiagen, Germany) added and samples were homogenized for 1 minute at 20 Hrz with a Qiagen TissueLyser II (Qiagen, Germany). Homogenized samples were stored for 1 hour at 5ºC and

then transferred to 15 ml sterile Falcon tubes with 3 ml of sterile SM buffer and vortexed. The PFU quantification per gram of feed was performed by the spot test method as described above.

Feed types

Four feed types were selected for our experiment: control feed (named C); florfenicol-coated feed (named An, short for "Antibiotic"); phage-immobilized feed (named PI) and phage-sprayed feed (named PS). Feed pellets (0.8 mm, Inicio Plus, BIOMAR A/S, Denmark) were used as control feed and for applying either the antibiotic or the selected phages. The commercial feed type was selected as starter feed for the rainbow trout fry. The composition of the commercial feed pellets Inicio Plus used for the groups C, An, PI and PS is the following (BioMar A/S): 60.3% protein, 33% lipid, 6.7% starch plus Bactocell®, a probiotic lactic acid bacterium (*Pediococcus acidilactici* MA 18/5M), and B-WYSE^{TM} (BioMar Whole Yeast Synergistic Extracts), a yeast-based additive (*Cyberlindnera jadinii* + two *Saccharomyces cerevisiae* strains) developed by BioMar's partner Lallemand Animal Nutrition (Canada).

The preparation of phage-treated feed pellets was done as follows (for more details, see description of experiment A in Donati et al. (2021)). Briefly, high titer solutions of phages FpV4 and FPSV-D22 were prepared from crude lysates following infection of *F. psychrophilum* strains FPS-S6 (for FPSV-D22 propagation) and 950106-1/1 (for FpV4 propagation), filtered through a 0.2 μm-pore size sterile filter, concentrated by adding poly-ethylene glycol 8000 (PEG-8000) and sodium chloride (final concentration 10% w/v and 1 M, respectively), centrifuged (10,000× g, 30 min, 4 °C) after 24 h incubation at 4ºC and re-suspended in sterile SM buffer (Castillo et al., 2019; Donati et al., 2021). The purified high titer solutions of FpV4 ($1.2*10^9$ PFU ml^{-1}) and FPSV-D22 ($4.9*10^9$ PFU ml^{-1}) were mixed 1:1 (total phage concentration of $3.3*10^9 \pm 6.1*10^8$ PFU ml^{-1} (mean ± SD, n=3)). The two-component phage mix was applied on control feed pellets from the same batch by irreversible immobilization using the corona discharge technology by Fixed Phage Ltd (20 ml per 100 g; phage-immobilized feed, PI) (Mattey, 2016, 2018) or by spraying (30 ml per 100 g; phage-sprayed feed, PS) (Christiansen et al., 2014; Donati et al., 2021). Phage-treated feed pellets were stored at 5°C in the dark. The measured final concentration of phages on feed pellets (performed as described in the previous paragraph) was $8.3*10^7 \pm 2.5*10^7$ PFU g^{-1} of PI (n = 3) and $1.6*10^8 \pm 4.8*10^7$ PFU g^{-1} of PS (n = 3).

Florfenicol-coated feed (An) was purchased and stored at 5°C in the dark. For this feed type, florfenicol (Aquaflor®, Intervet Inc., a subsidiary of Merck & Co. Inc., USA) was applied on Inicio Plus (BIOMAR A/S, Denmark) feed pellets for a final concentration of 0.2% (veterinarian Thomas Clausen personal communication). The recommended administration for rainbow trout fry was 2% of fish weight per day for 10 consecutive days.

Experimental set up

Rainbow trout eyed eggs were purchased at a Danish commercial fish farm, iodophore disinfected, hatched and the fish grown at the Unit for Fish and Shellfish Diseases (DTU Aqua, Kgs. Lyngby, Denmark). Initially raised in a recirculation system, fish were then transferred to a dedicated experimental area (flow-through system, no recirculation of water) used for experimental work when the desired size/weight were reached. Fish were divided randomly in 8-L tanks, each with a separate inlet/outlet for water (13ºC) and air-supply.

Rainbow trout fry (1-2 g) were divided in 16 X 8 L-aquaria (~50 fish/aquarium) and the four feed groups (C, An, PI, PS) with four aquaria per group. All groups were fed at 2% of fish weight per day. PI and PS were administered continuously from 12 days before the bacterial

challenge. The florfenicol-coated feed was administered to the fish for 10 days starting two days after bacterial exposure. When not fed with antibiotic-coated feed, fish in this group (An) were fed with control feed (**Figure 1**). Fish in three of the four aquaria per treatment group were challenged with *F. psychrophilum* 950106-1/1 by intraperitoneal (IP) injection as described above (indicated by "feed type/Fp"). As controls for the infection, fish in one aquaria per treatment group were injected with sterile TYES-B. Prior to IP injection, fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma catalog number A-5040). Survival of fish was followed for each group in two of the infected aquaria. Fish sampling was performed in the two remaining (one infected with the bacterium and one non-infected) during the experiment. Dead and moribund fish were collected and euthanized with an overdose of MS-222. Length and weight of each fish were recorded and bacteriological examination performed as described for sampled fish (see below).

The results of the fish survival (C, PI and PS incubations), and the re-isolation/quantification of bacteria and phages in the intestine and in the internal organs of the fish fed PI and PS, are presented in Donati et al. (2021) (Experiment A). No mortality was recorded in fish fed antibiotic feed.

The use of fish in this study complied with Danish and EU legislation (Directive 2010/63/EU) on animal experimentation and it was approved by the Animal Experiments Inspectorate of Denmark (Dyreforsøgstilsynet, permission number 2013-15-2934-00976 until 7/10-19, and 2019-15-0201-00159 from 8/10-19).

Sampling for microbiome analysis

One day before the bacterial exposure, five fish were sampled from each feed-type group from the sampling aquaria not supposed to be infected with *F. psychrophilum* (C, An, PI, PS). Following the bacterial challenge, five fish from each sampling aquaria (C, An, PI, PS, C/Fp, An/Fp, PI/Fp, PS/Fp) were sampled at 1 and 33 days post infection (dpi) (**Figure 1**). Eight dpi, five fish from the control and the antibiotic groups with and without the infection were also sampled (C, An, C/Fp, An/Fp). Fish were euthanized with an overdose of MS-222, weighed and their length measured. Bacteriological examination of spleen, kidney and brain was performed by streaking samples of organs on TYES agar plates. Plates were incubated at 15ºC for 3-5 days until a maximum of 4 weeks and the growth of yellow *F. psychrophilum* colonies was recorded. Randomly chosen colonies were then analyzed by MALDI-TOF (Bruker) to confirm that *F. psychrophilum* was the re-isolated bacteria (Donati et al., 2021). For microbiome analysis, the distal part of the intestine was aseptically removed together with the fecal content, if present, and placed in a sterile 1.5 ml micro tube (SARSTEDT AG & Co. KG, Germany). Intestine samples were stored at -20ºC (Ingerslev et al., 2014b, 2014a). Fish were not fed as from 24 hours before sampling.

DNA extraction

DNA extraction was performed using the Maxwell LEV Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA) (Ingerslev et al., 2014b, 2014a; Strube et al., 2018). After thawing, samples were transferred to sterile 2 ml Eppendorf tubes containing a sterile 5 mm steel bead (Qiagen, Germany) and incubated with 100 µl of lysozyme mixture (25 mg/ml lysozyme, 1.2% Triton X, 2 mM EDTA, 20 mM Tris-HCl (pH 8)) for 1 hour at 37 ºC. Subsequently, 350 µl of lysis buffer were added and each sample was homogenized by using a Qiagen TissueLyser II (2 minutes at 20 Hrz; Qiagen, Germany). Samples were incubated overnight at 56ºC with 20 µl Proteinase K (20 mg/ml). A Maxwell 16 Research Instrument System (Promega Corporation, WI, USA) was used following the manufacturer's instructions.

The concentration and quality of the extracted DNA was measured by a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and with a Qubit™ 1X dsDNA HS (High Sensitivity) assay kit (Thermo Fisher Scientific catalog number Q33231) in the Invitrogen Qubit™ 4 Fluorometer (Invitrogen, USA). A negative control (empty 2 ml vial in which the bead and the various solutions for the DNA extraction and purification were added during the procedure) was included in the DNA extraction and purification protocol.

Library preparation

Extracted DNA was sent to DNAsense (Aalborg, Denmark) for 16s DNA library preparation, sequencing and bioinformatics, which were performed as follows. Bacterial 16S rRNA gene region V3-4 sequencing libraries were prepared by a custom protocol based on an Illumina protocol (Illumina, 2015). 15 ng of extracted DNA was used as template for PCR amplification of the Bacteria 16S rRNA gene region V3-4 amplicons. Each PCR reaction (25 μL) contained (12.5 μL) PCRBIO Ultra mix (PCR Biosystems, USA) and 400 nM of each forward and reverse tailed primer mix. PCR was conducted with the following program: initial denaturation at 95°C for 2 min, 30 cycles of amplification (95 °C for 15 s, 55 °C for 15 s, 72 °C for 50 s) and a final elongation at 72 °C for 5 min. Duplicate PCR reactions were performed for each sample and the duplicates were pooled after PCR. The forward and reverse tailed primers were designed according to (Illumina, 2015) and contain primers targeting the Bacteria 16S rRNA gene region V3-4: [341F] CCTACGGGNGGCWGCAG and [805R] GACTACHVGGGTATCTAATCC (Herlemann et al., 2011). The primer tails enable attachment of Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 μL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries. Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 μL) contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U/reaction) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was conducted with the following program: Initial denaturation at 95 °C for 2 min, 8 cycles of amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 60 s) and a final elongation at 72 °C for 5 min. The resulting sequencing libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 μL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

DNA sequencing and bioinformatics processing (DNAsense)

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. >10% PhiX control library was spiked in.

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al., 2014) with the settings SLIDINGWINDOW: 5:3 and MINLEN: 275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011) with the

settings -m 10 -M 250. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using –confidence 0.8 and the SILVA database, release 132 (Quast et al., 2013). The results were analysed in R v. 4.0.2 (R Core Team, 2017) through the Rstudio IDE using the ampvis package v.2.6.5 (Albertsen et al., 2015).

Statistics

The analyses were performed with GraphPad Prism version 8.4.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. OTUs relative abundances, the Shannon diversity index, the Chao1 richness index and fish size were analyzed to assess if there was any statistically significant difference among the groups. First, normality was evaluated with the Shapiro-Wilk test. Comparisons were then performed with ANOVA or Krustal Wallis in case non-normal data distribution. P-values (P) below 0.05 were considered significant. P-values for multiple comparisons were adjusted for Dunnet (normal distribution) or Dunn's (non-normal distribution) corrections. Shannon diversity index values are based on 10.000 reads per sample. For comparison of the abundance of specific bacteria of interested between one group at two different time points (e.g. the genus *Rhodococcus* in C), the Mann-Whitney test was used ($P < 0.05$ is considered significant).

Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) was used to asses β-diversity and group-wise differences were tested with PERMANOVA using the adonis function from the vegan package (Oksanen et al., 2020).

Results

Library preparation and sequencing (DNAsense)

Library preparation for bacterial sequencing (V3-4) was successful for all samples except one which yielded 30 reads after QC and bioinformatic processing (and so it was excluded). Seven low input samples yielded only ~4.600 - 8.000 reads, but the general sequencing outcome was ~10.000 or more reads. One sample produced >400.000 reads, which was attributable to overloading of that particular sample when pooling sequencing libraries (**Supplementary Table 1**). The negative controls implemented in the project revealed a minor reagent contamination from 2 specific OTUs namely OTU 1 belonging to the genus *Pseudomonas* and OTU 4 belonging to the genus *Sphingobacterium*. Noteworthy, the fraction of total OTU counts attributed to the two contaminating OTUs showed an inverse relationship with PCR amplicon yield, i.e. the two OTUs were only an issue in samples with low bacterial DNA input material. Comparing laboratory journals across projects processed in parallel identified the likely source as a batch PCR primers provided by the supplier. However, the two OTUs had negligible effect on project outcome, and the OTUs were excluded from downstream analyses.

Microbial gut community of rainbow trout fry and effects of phage prophylaxis (day -1)

At first, the gut microbiome of rainbow trout fry was studied one day prior to infection (fish negative to *F. psychrophilum*). At this time point, C and An were fed with non-treated feed while PI and PS had received bacteriophages for 11 days (**Figure 1**).

The taxonomic mapping at phylum and class level (**Figure 2A and B)** did not reveal any statistically significant difference among the top-5 and top-7 most abundant phyla and classes, respectively (**Supplementary Table 2 and 3)**. The dominant phyla were Firmicutes (C: 52.4 ± 11.4%), Proteobacteria (C: 23.0 ± 16.0%) and Actinobacteria (C: 16.0 ± 12.0 %) followed by Bacteroidetes (C: 2.4 ± 2.6 %) and Cyanobacteria (C: 1.3 ± 1.4 %). The top-7 most abundant classes were Bacilli (C: 42.6 ± 9.6%), Gammaproteobacteria (C: 17.6 ± 13.5%), Actinobacteria (C: 13.8 ± 12.1%), Alphaproteobacteria (C: 4.9 ± 2.8%), Clostrida (C: 9.7 ± 5.6%), Bacteroida (C: 2.4 ± 2.6%) and Oxyphotobacteria (C: 1.3 ± 1.4 %). Furthermore, no statistically significant differences in the Shannon diversity index (C: 3.6 ± 0.9), the Chao1 richness index (C: 138.6 ± 31.6) and the fish size (C: 1.7 ± 0.7 g) were observed among the feed groups (**Figure 2C, D and E)**.

The top-30 most abundant genera are presented in **Figure 3** and **Supplementary table 4** and, among them, *Lactobacillus* (C: 13.4 ± 6.4%), *Rhodococcus* (C: 10.2 ± 13.6%), *Pediococcus* (C: 9.9 ± 15.5%), *Acinetobacter* (C: 5.0 ± 5.0%), *Vagococcus* (C: 5.0 ± 1.5%) and *Thermomonas* (C: 2.7 ± 2.6%) were observed as the most copious. Even though fish in C and An received the same feed type at this time point and no significant differences were observed at phylum or class level, differences were observed: a higher abundance of *Staphylococcus* (Phylum: Firmicutes; Class Bacilli; Order Bacillales) (2.7 ± 0.9%; adjusted $P < 0.0001$) and a lower abundance of *Weissella* (Phylum: Firmicutes; Class Bacilli; Order Lactobacillales) (0.5 ± 0.3%; adjusted $P = 0.024$) were observed in An relative to the control (C). Additional differences concerning fish fed with phage-treated feed pellets compared to C were revealed by the taxonomic mapping at genus level as significantly higher abundances of *Enhydrobacter* (Phylum Proteobacteria; Class Alphaproteobacteria; Order Rhodospirillales) and *Stenotrophomonas* (Phylum Proteobacteria; Class Gammaproteobacteria; Order Xanthomonadales) were observed for PS (2.5 ± 2.1%; adjusted $P = 0.049$) and, PS and PI (in PS: 1.7 ± 1.7%, adjusted $P = 0.048$; in PI: 2.9 ± 4.5%, adjusted $P = 0.046$), respectively.

The identification of similarities/dissimilarities among the microbial communities of the fish gut (β-diversity) was performed using Principal Coordinates Analysis (PCoA) (**Figure 4A**). The clusters formed by fish fed phage-treated feed (PS and PI) were separated from the clusters of C and An suggesting an effect of the phage treatments on the microbial community ($P = 0.002$, PERMANOVA). No statistically significant difference was detected between the microbiome of C and An ($P = 0.256$, PERMANOVA), and between PI and PS ($P = 0.326$, PERMANOVA).

Effects of bacterial infection and infection procedure (microbial gut community 1 dpi)

One day post infection, fish were sampled to evaluate the effect of the infection and at this time point, 60, 60 80 and 100 % of sampled fish in the groups C/Fp, An/Fp, PI/Fp and PS/Fp, respectively, were positive to *F. psychrophilum* (**Figure 1**). Sampled fish in control fish without IP infection with *F. psychrophilum* (C, An, PI and PS) were all negative to the bacterium. As for day -1, fish in C, An, C/Fp and An/Fp were fed with non-treated feed.

PCoA analysis revealed dissimilarities among the bacterial community between the feed groups (**Figure 4B)**. Fish in An were characterized by a different community than C ($P = 0.033$, PERMANOVA). The clusters formed by PS and PI were divergent from the cluster of C and An ($P = 0.028$, PERMANOVA), indicating also a larger in-group variation among the microbial communities of PI and PS. No statistically significant difference was detected between the microbiome of PI and PS ($P = 0.133$, PERMANOVA). Furthermore, a diversification of the bacterial communities was observed when fish were exposed to *F. psychrophilum* (C/Fp,

An/Fp, PI/Fp and PS/Fp) compared to non-challenged fish (P = 0.016, PERMANOVA). The groups C and PS/Fp formed two separated independent clusters. While no statistically significant difference was detected between C/Fp and An/Fp (P = 0.091, PERMANOVA), this was not the case between PI/Fp and PS/FP (P = 0.018, PERMANOVA).

Dissimilarities were also revealed by the taxonomic mapping and alpha diversity values (**Figure 5 and 6; Supplementary Table 5, 6 and 7**). Indeed, the Shannon diversity and the Chao1 richness indexes were significantly reduced in PS/Fp compared to C/Fp (adjusted P ≤ 0.01) (**Figure 5C and D**). In contrast to the other time points, a variation in relation to fish size was also observed at 1 dpi (**Figure 5E**) as fish in An (3.3 ± 0.3 g) were larger than fish in C (1.6 ± 0.5 g; adjusted P = 0.0389), likely contributing to the variation observed in the PCoA plot between these two groups.

Compared to the Firmicutes abundance in the control group C (66.0 ± 12.0%), a general drop was observed in the groups PI (26.4 ± 24.5%), C/Fp (36.0 ± 15.3%), An/Fp (32.7 ± 18.0%) and, with the largest and significant decrease, in PS/Fp (10.2 ± 12.0%, adjusted P = 0.0020) (**Figure 5A**; **Supplementary Table 5**). These changes were reflected at class level in the abundances of Bacilli and Clostridia, which were significantly lower in PS/Fp than in C (Bacilli: PS/Fp: 8.7 ± 9.1%; C: 53.1 ± 11.7%; adjusted P = 0.0056) (Clostridia: PS/Fp: 1.3 ± 2.7%; C: 12.8 ± 3.8%; adjusted P = 0.0459) and, than in PS in case of Clostridia (12.7 ± 5.3%; adjusted P = 0.0259) (**Figure 5B**; **Supplementary Table 6**). The general decrease in Bacilli abundance in PI, C/Fp, An/FP and PS/Fp was observed at genus level at different significant levels among the genera *Lactobacillus*, *Pediococcus*, *Vagococcus* and *Weissella* (Order Lactobacillales) (**Figure 6**; **Supplementary Table 7**). Specifically, the genus *Lactobacillus* was reduced in PI, C/Fp, An/FP and PS/Fp compared to C (adjusted P ≤ 0.01). A significant decline in *Vagococcus* was detected in PI (1.8 ± 2.6 %; adjusted P = 0.0264) and PS/Fp (0.6 ± 1.3 %; adjusted P = 0.0025) compared to C (7.2 ± 2.9 %). The genus *Pediococcus* was reduced in PI, C/Fp and PS/Fp and a significant difference was detected between PS/Fp (0.05 ± 0.1%) and PI/Fp (14.8 ± 8.7%) (adjusted P = 0.0116). Further, similarly as observed at day -1, the genus *Weissella* was characterized by lower abundances in An (0.8 ± 0.8 %) and An/Fp (0.3 ± 0.4 %) compared to C (4.1 ± 0.6 %) (An/Fp vs C: adjusted P = 0.0200). The differences observed among the genus *Staphylococcus* at day -1 between C and An were not observed at this time point as its relative abundance was around 1% in all groups except in An/Fp where it was 4.4 ± 6.6% (adjusted P > 0.05).

The decline in Firmicutes seemed to favor a rise in Proteobacteria (**Figure 5A and B**). Compared to the Gammaproteobacteria abundance in the control group C (15.7 ± 5.1%), a general increase was detected in PI (44.7 ± 26.3%), C/Fp (31.7 ± 12.7%), An/Fp (34.9 ± 15.9%) and, with the largest increment, in PS/Fp (50.0 ± 17.4%, adjusted P = 0.0224) (**Supplementary Table 6**). These changes were reflected in the abundance of the genera *Stenotrophomonas*, *Thermomonas*, *Acinetobacter* and *Photobacterium* (**Figure 6**; **Supplementary Table 7**). The abundance of the genus *Thermomonas* (Order Xanthomonadales) was significantly increased in C/Fp (5.8 ± 2.5%; adjusted P = 0.0301) and PS/Fp (6.0 ± 4.2%; adjusted P = 0.0211) compared to C (1.7 ± 0.8%) as well as the genus *Stenotrophomonas* (Order Xanthomonadales) in PI (11.4 ± 10.8%), as at day -1. The genus *Acinetobacter* (Order Moraxellaceae) significantly expanded in PS/Fp (14.0 ± 4.5%) compared to An (3.0 ± 2.0%) and PI/Fp (3.7 ± 3.4%) (adjusted P < 0.05). Finally, fish in PS were characterized by a higher abundance of the genus *Photobacterium* (Order Vibrionales) compared to PS/Fp (PS: 5.7 ± 4.5%; PS/Fp: 0.1 ± 0.2%, adjusted P = 0.0048). Differently from the observations at day -1, no significant differences were observed in the genus *Enhydrobacter* between PS and the other feed groups even if a larger variation was observed for PS (0.9 ± 1.3%). Generally, a higher abundance of this genus

was observed in fish exposed to *F. psychrophilum* (except PI/Fp) and a significant increment was detected in An/Fp (2.3 ± 1.5%) compared to PI (0.2 ± 0.3%; adjusted P = 0.0467).

Additional variations in the taxonomic mapping were revealed among the phyla Actinobacteria, Bacteroidetes and Cyanobacteria (**Figure 5A**; **Supplementary Table 5**). A greater abundance of Actinobacteria was detected in An (An: 21.6 ± 12.1%; C: 7.0 ± 3.9%; adjusted P = 0.037). At genus level (**Figure 6**; **Supplementary Table 7**), variations were observed in the abundances of *Rhodococcus* and *Gordonia* (Class Actinobacteria; Order Actinomycetales). Compared to day -1 where the abundance of the genus *Rhodococcus* was 10.2 ±13.6% in C, lower values were observed 1 dpi (C: 2.1 ± 3.2%) (Mann-Whitney test; P > 0.05). The highest quantity was detected in An (9.5 ± 10.3%) and the lowest in C/Fp (0.5 ± 0.2%) and PS/Fp (1.0 ± 2.2%). A rise in *Gordonia* was recorded in C/Fp (4.2 ± 2.5%) compared to C (0.6 ± 0.6%) (adjusted P < 0.05). Even if no significant differences in the phylum Bacteroidetes and in the class Bacteroida were detected among the groups, the abundance of an unidentified bacteria belonging to the family *Weeksellaceae* (Class Bacteroida, Order Flavobacteriales) was incremented in C/Fp (C/Fp: 3.5 ± 1.9%; C: 1.0 ± 0.8%; adjusted P < 0.05). Finally, a significant decrease in the phylum Cyanobacteria reflected in the class Oxyphotobacteria was observed in PS/Fp compared to PS (PS: 2.0 ± 1.8%; PS/Fp: 0.02 ± 0.1%; adjusted P = 0.0346). However, these variations may be due to the presence of algae in the samples as a significant increment of the "*o_Chloroplast_OTU_7*" were detected in PS (adjusted P = 0.0322).

Changes in the composition after the bacterial infection and in relation with antibiotic administration (microbial gut community 8 dpi)

Two dpi and for 10 consecutive days, the antibiotic florfenicol was administered in An and An/Fp. Eight dpi, fish in C, An, C/Fp and An/Fp were sampled. At this time point, 80 and 0 % of sampled fish in C/Fp and An/Fp were positive to *F. psychrophilum*, respectively (**Figure 1**). Sampled fish in C and An were negative to the bacterium.

PCoA analysis (**Figure 7**) showed a clear shift in the microbial composition of the gut during the bacterial infection (C/Fp and An/Fp) and in relation to the antibiotic treatment (An and An/Fp) compared to fish in C. Indeed, four separated independent clusters can be observed (P = 0.001, PERMANOVA).

Dissimilarities were also revealed by the taxonomic mapping (**Figure 8 and 9**; **Supplementary Table 8, 9 and 10**). However, no statistically significant differences in the Shannon diversity index (C: 3.3 ± 0.3), the Chao1 richness index (C: 155.7 ± 35.1) and the fish size (C: 2.4 ± 1.0 g) were observed between the groups (**Figure 8C, D and E**). A large variation in the Shannon diversity index was detected for C/Fp (2.8 ± 1.5) and in the Chao1 richness index for An/Fp (108.8 ± 65.0).

The antibiotic administration caused variation in the abundances of bacteria within the phylum Firmicutes. Differently from the previous time points, variations in the genera *Streptococcus*, *Vagococcus* (Phylum Firmicutes; Class Bacilli; Order Lactobacillales) and *Clostridum sensu stricto 7* (Phylum Firmicutes; Class Clostridia; Order Clostridiales) were recorded in An and An/Fp compared to C and C/Fp (**Figure 9**; **Supplementary table 10**). The genus *Streptococcus* was more copious in An (7.1 ± 2.9%) and An/Fp (8.8 ± 7.9%) (An/Fp vs C/Fp: adjusted P = 0.0333). The genera *Vagococcus* and *Clostridum sensu stricto 7* were instead strongly reduced as their values were close to zero in An and An/Fp (adjusted P < 0.05). In addition, other differences can be observed within the Firmicutes compared to the previous time points. The reduced abundance of the genus *Lactobacillus* observed 1 dpi in An/Fp compared to C was not observed 8 dpi as well as no difference was recorded in the genus *Weissella* (at day -1 and 1

dpi, it was reduced in An and An/Fp). The genus *Pediococcus* dropped to zero in An, C/Fp and An/Fp. In C/Fp, these values were already observed 1 dpi. Unlike the high abundance of the genus *Staphylococcus* detected in An at day -1 and the 1-2% abundance detected 1 dpi for all groups, its abundance was ~ 0% 8 dpi (data not presented).

The bacterial infection induced changes among the phylum Proteobacteria as a general increase in the class Gammaproteobacteria was detected in infected fish (C: 9.5 ± 2.25%; An: 12.7 ± 12.5%; C/Fp: 27.3 ± 17.5%; An/Fp: 34.4 ± 15.9%) (C vs. An/Fp: adjusted P = 0.0274). However, no significant difference was observed at genus level among the most abundant bacteria belonging to this class. This was different to what observed 1 dpi where the genus *Thermomonas* was significantly increased in C/Fp compared to C (**Figure 8 and 9**; **Supplementary table 8, 9 and 10**).

The effects of the bacterial infection were also observed in other phyla (**Figure 8**; **Supplementary table 8 and 9**). Indeed, the bacterial challenged fish were also characterized by an inferior abundance of the phylum Actinobacteria compared to non-challenged fish (C: 33.1 ± 7.9%; An: 39.4 ± 26.0%; C/Fp: 9.2 ± 7.7%; An/Fp: 8.6 ± 1.5%) (adjusted P < 0.05), and by a significant rise of the phylum Bacteroidetes when not fed with the antibiotic-coated feed (C: 0.5 ± 0.6%; C/Fp: 32.6 ± 39.3%; adjusted P = 0.008). These changes were reflected in the class Actinobacteria and Bacteroida. At genus level (**Figure 9; Supplementary table 10**), the mean abundances of *Rhodococcus* and *Gordonia* (phylum Actinobacteria) were affected by the infection. The genus *Rhodococcus* dropped to zero in C/Fp and An/Fp, similarly to what observed 1 dpi, compared to C (28.5 ± 9.5%) and An (37.4 ± 26.7%) (adjusted P < 0.05). The abundance of this genus in C was higher at this time point than what observed in C 1 dpi (Mann-Whitney test; P = 0.0079). The rise in the genus *Gordonia* in C/Fp observed 1 dpi was lost. However, a significant increase was detected in An/Fp (4.5 ± 0.5%) compared to the other groups (adjusted P ≤ 0.02). The genus *Flavobacterium* (Class Bacteroida, Order Flavobacteriales) became bigger in C/Fp (28.5 ± 41.7%; adjusted P ≤ 0.02) as, probably, a direct consequence of the higher percentage of detection of *F. psychrophilum* in C/Fp sampled fish 8 dpi (80% against 0% in An/Fp). Among the Class Bacteroida, the abundance of an unidentified bacteria belonging to the family *Weeksellaceae* was also affected by the infection as higher values were recorded in C/Fp and An/Fp, similarly to what observed 1 dpi.

Similarly as observed 1 dpi for PS, an increased abundance within the phylum Cyanobacteria and the class Oxyphotobacteria was recoded in fish subjected to the antibiotic therapy (An: 2.3 ± 1.4%; An/Fp: 2.7 ± 2.0%; adjusted P ≤ 0.02 vs C/Fp) (**Figure 8**; **Supplementary table 8 and 9**). In this case, these variations may be due to the presence of algae in the samples as a significant increment of the "*o_Chloroplast_OTU_35*" was detected (adjusted P < 0.05) (**Supplementary Table 10**).

Gut microbial community of rainbow trout fry 33 dpi: changes in the composition after recovery from the bacterial infection and in relation with antibiotic or phage administration.

To assess the composition of the gut microbial community after recovery from the bacterial infection and in relation to antibiotic and phage administration, fish were sampled 33 dpi (all negative to *F. psychrophilum*) (**Figure 1**).

As performed in the other time points, the microbial communities of the fish gut were visualized in a PCoA plot (**Figure 10**). No statistically significant difference was observed in the microbial composition in the groups that have been subjected to the bacterial infection in comparison the non-challenged groups (P = 0.135, PERMANOVA). Among the non-challenged fish, the gut

microbial communities in the four groups were significantly different (P = 0.003, PERMANOVA), even if the microbiome of fish in C seemed to be fairly diverse. No differences were recorded between C and An (P = 0.115, PERMANOVA) while PI and PS were statistically different between each other (P = 0.025, PERMANOVA) and in comparison with C and An (P = 0.045, PERMANOVA). A similar pattern was observed in fish that had recovered the infection in the four groups (P = 0.017, PERMANOVA). No differences were recorded between C/Fp and An/Fp (P = 0.241, PERMANOVA) while PI/Fp and PS/Fp were statistically different between each other (P = 0.032, PERMANOVA) and in comparison with C/Fp and An/Fp (P = 0.014, PERMANOVA). In addition, fish in PS and PS/Fp were characterized by a smaller in-group variation compared to the other groups.

The taxonomic mapping did not reveal dissimilarities at phylum (top-5) and class (top-6) level except that a higher abundance of Cyanobacteria reflected in the class Oxyphotobacteria was detected in the group PS/Fp (6.4 ± 3.0%) compared to C/Fp (0.5 ± 0.5%) (**Figure 11A and B**; **Supplementary Table 11 and 12**). Similarly at the previous time points, these variations may be due to the presence of algae in the samples as e.g. a significant increment of the "*o_Chloroplast_OTU_27*" (adjusted P value PS/Fp vs C/Fp = 0.0254) was detected. The Shannon diversity index, the Chao1 richness index and fish size are presented in **Figure 11C, D and E**, respectively. No statistically significant difference was observed between the groups likely due to the large variation observed between replicates.

The top-30 most abundant genera are shown in **Figure 12** and **Supplementary Table 13**. Among the Firmicutes, some of the previously observed differences were restored. In contrast to what observed 8 dpi, the abundance of the genera *Vagococcus*, *Streptococcus* and *Clostridium sensu stricto 7* were restored in An and An/Fp as no significant difference was detected with C and C/Fp (adjusted P > 0.05). Also, the reduced abundances compared to C of *Vagococcus* in PI and PS/Fp and, of *Lactobacillus* in PI, An/Fp and PS/Fp observed 1 dpi were also re-established. Nevertheless this trend, new differences among the feed types were detected: the genera *Pediococcus* and *Carnobacterium* were increased in PS (*Pediococcus*: 10.0 ± 9.7%; *Carnobacterium*: 3.4 ± 2.0%) compared to fish fed C (*Pediococcus*: 0.1 ± 0.1%; *Carnobacterium*: 0.8 ± 0.8%) (adjusted P: *Pediococcus* = 0.0012*; Carnobacterium* = 0.0264). Finally, similarly to what observed at day -1 and 1 dpi, the genus *Weissella* was characterized by the lowest values in An and An/Fp. However, no statistically significant difference was measured with C and C/Fp. Unlike the high abundance of the genus *Staphylococcus* detected in An at day -1 and the 1-2% abundance detected 1 dpi for all groups, its abundance was ~ 0% as at 8 dpi (data not presented).

Similarly as for bacteria belonging to the phylum Firmicutes, some of the differences observed at the previous time points disappeared. For example the increased abundance of *Thermomonas* detected in C/Fp and PS/Fp measured 1 dpi was lost as no significant difference was detected 33 dpi. A similar pattern was observed for the genera *Stenotrophomonas* and *Acinetobacter.* However, new dissimilarities were measured: the genus *Pseudorhodobacter* (previously only observed in few replicates; no significant differences detected at day -1 and, 1 and 8 dpi) was significantly increased in PI and An/Fp (compared to PS and PS/Fp; adjusted P < 0.05) and characterized by a large variation in the feed groups C/Fp and PI/Fp; the genus *Photobacterium* (significantly increased in PS 1 dpi) was more copious in the group PS/Fp (10.5 ± 6.3%) compare to C (adjusted P = 0.0306); a higher abundance of the genus *Phreatobacter* was detected in the groups C (8.8 ± 11.5%) and An (7.0 ± 6.1%) compared to PS/Fp (adjusted P < 0.05). Differently than what observed at day -1, no significant differences were observed in the genus *Enhydrobacter* between PS and the other feed groups.

Among the phylum Actinobacteria, the genus *Rhodococcus* dropped to 2.3 ± 1.1% in PS compared to An (18.4 ± 13.6 %) and PI (17.2 ± 10.4 %) (adjusted $P < 0.05$). Similarly as 8 dpi, the abundance of this genus in C was higher 33 dpi than what observed in C 1 dpi (Mann-Whitney test; $P = 0.0317$). Dissimilarities in the genus *Gordonia* observed 1 and 8 dpi were lost (0-1 % in all groups). Finally, previously observed dissimilarities of the genus *Flavobacterium* (~0 %) and of an unidentified bacteria belonging to the family *Weeksellaceae* (*f_Weeksellaceae_OTU_25*) (0-1 %) were not observed.

Discussion

With the aim of studying the microbial composition of the gut of rainbow trout fry exposed to bacteriophage and antibiotic therapies, the intestine including the gut content (if present) of healthy and infected fish exposed to the different feed regimes were sampled. The sampling of the intestine including the gut content was performed, necessitating mixing of autochthonous and allochthonous communities, due to the small size of the fish in order to avoid any unintentional manipulation/modification of microbial community. In addition, fish were not fed for 24 hours before sampling to diminish the possibility of presence of gut content in the fish.

Microbial gut community of rainbow trout fry (day -1)

The gut microbiota of fish sampled before performing the bacterial challenge was characterized by bacteria belonging to the phyla Firmicutes (~50 %) and Proteobacteria (~20 %), which were the most abundant, followed by Actinobacteria (~10 %), Bacteroidetes (~2 %) and Cyanobacteria (~1 %). These results are in accordance with previous research focused on the gut autochthonous microbial composition of rainbow trout at early life stages fed with marine diets (Inicio plus not including the yeast-based additive, BioMar A/S, Denmark) (Ingerslev et al., 2014a, 2014b) but also with the general composition of the gut microbiota of teleost (reviewed by (Tarnecki et al., 2017; Egerton et al., 2018)). In addition to these phyla, a higher abundance of Tenericutes and Proteobacteria has been detected in the distal content of rainbow trout juveniles (Lyons et al., 2017; Villasante et al., 2019).

When looking at genus level, the genera *Pediococcus*, *Lactobacillus*, *Vagococcus* (belonging to the phylum Firmicutes), *Acinetobacter*, *Thermomonas* (phylum Proteobacteria) and *Rhodococcus* (phylum Actinobacteria) were the most abundant. The high abundance of the genus *Pediococcus* might be linked to the presence of the probiotic lactic acid bacterium *Pediococcus acidilactici* MA 18/5M (Bactocell®) in the selected commercial feed. Thus, differences in the abundance of this genus during the experiment may be related to the impaired feed intake normally observed in diseased fish.

Changes in the composition after the bacterial infection and in relation with antibiotic administration

Infection and antibiotic therapy are known factors able to alter the composition of the gut microbiota (reviewed in teleost by (Butt and Volkoff, 2019)). In our experiment, the effects of the infection in fish fed control feed (C/Fp) were observed 1 and 8 dpi as dissimilarities were revealed by the β-diversity analysis and by the taxonomical mapping. Alpha diversity measures were not affected by the infection even if a lower (but not statistically significant) Chao1 richness index values were recorded for fish exposed to *F. psychrophilum* 8 dpi.

In our study, the taxonomic mapping revealed that the infection caused by *F. psychrophilum* altered the ratio Firmicutes/Proteobacteria. Indeed, a decrease in Firmicutes (at genus level:

Lactobacillus 1 dpi) and a rise in the Proteobacteria (at class level: Gammaproteobacteria 1 and 8 dpi; at genus level: *Thermomonas* 1 dpi) were observed. Previous experiments investigating the effects of *Yersinia ruckeri* infection on the autochthonous gut microbial community of rainbow trout fry were performed by Ingerslev et al. (2014a) and changes in the bacterial abundances were observed in challenged fish. For example, an increase in Proteobacteria reflected in a higher number of bacteria belonging to the genus *Aeromonas* (causing a decrease in the abundance of Firmicutes) was observed 19 dpi with *Y. ruckeri* (the genus *Aeromonas* contains opportunistic pathogens for fish). In humans, the increased abundance of bacteria belonging to the phylum Proteobacteria has been suggested as an indication of infection, since this group is commonly associated with human diseases (e.g. metabolic diseases, cardiovascular diseases, gut inflammation) (as reviewed by (Rizzatti et al., 2017)). Proteobacteria are Gram negative bacteria (lipopolysaccharide, LPS, in the outer membrane) that represent the broadest phylum in the domain of Bacteria. The LPS has been shown to cause low-grade inflammation (named endotoxemia) and to play a role in the development of metabolic disorders (Rizzatti et al., 2017). However, it should be stated that the overall composition of the human gut (90% Bacteroidetes and Firmicutes; 10 % Actinobacteria and Proteobacteria) (Arumugam et al., 2011) is different from the one of fish.

Actinobacteria are Gram positive non-motile rods, mainly anaerobic (Rizzatti et al., 2017) and, in our experiment, their abundance was impaired by the infection, i.e. the genus *Rhodococcus* was lower in infected fish (1 and 8 dpi) while the genus *Gordonia* increased (highest abundance when combined with antibiotic administration 8 dpi). Bacteria belonging to the genus *Rhodococcus* (one of the most abundant genera observed in this study) have been detected in the gut microbiome of rainbow trout fry also by Ingerslev et al. (2014b, 2014a). This genus includes Gram-positive cocci/rod-shaped anaerobic bacteria (Walsh et al., 1993) and some studies have looked into using *Rhodococcus* spp. as probiotics in aquaculture (e.g. for prevention of bacterial infections) (Boutin et al., 2013; Sharifuzzaman et al., 2018). Thus, the infection caused by *F. psychrophilum* clearly affect a population of bacteria that could be beneficial for the fish. Concerning the increased abundance of *Gordonia* (phylum Actinobacteria), bacteria belonging to this genus (suborder *Corynebacterineae*) are actinomycetes containing mycolic acid (long chain fatty acids) and of biotechnological interest because of the various range of chemical compounds they produced. However, some are considered opportunistic pathogens (Arenskötter et al., 2004). In a recent study, Qiao et al. (2019) associated the increased abundance of the genus *Gordonia* in the gut of Zebrafish with the ingestion of microplastics (so in case of intestinal dysbiosis).

Florfenicol is a broad-spectrum antibiotic (sensitive bacteria include Gram negative bacilli, Gram positive cocci and other bacteria such as mycoplasma; principle of action: inhibition of protein synthesis) (Papich, 2016) that has been used in Danish fish farms since 1996 to control RTFS (Bruun et al., 2000). Antibiotics with a wide broad spectrum are known to cause alternations in the bacterial community (e.g. reduced microbial diversity, increased chance that opportunistic pathogens proliferate) and various studies have been targeting this topic in teleosts (Gupta et al., 2019; Kokou et al., 2020). In our study, the gut microbial community was clearly affected by the antibiotic therapy as revealed by the β-diversity analysis and the taxonomic mapping 8 dpi. In contrast, measures of α-diversity were not. These results are in line with what observed by Kokou et al. (2020) in seabass (study of microbiota of the pyloric caeca, mid- and hind gut; however, it is not explained if gut content was removed and when precisely samples were collected in relation to the antibiotic treatments). Other studies, however, have recorded a higher α-diversity after antibiotics treatment. Gupta et al. (2019) studied the autochthonous communities of the gut of Atlantic salmon after the administration of florfenicol and oxolinic acid and observed a higher α-diversity in the distal part of the

intestine following florfenicol administration. No increase was observed in case of oxolinic acid treatment.

The immediate effect of florfenicol was observed in relation to the genus *Flavobacterium,* which was not detected among the most abundant genera in infected fish fed with antibiotics suggesting that 8 dpi fish had already recovered (0% of fish in An/Fp were positive to the bacterium). Indeed, this genus was highly increased in challenged fish fed control feed (80% of fish in C/Fp were positive to the bacterium 8 dpi). Further, when looking at the taxonomic mapping during the administration of florfenicol, we recorded variations in the genera *Streptococcus*, *Vagococcus* (class Bacilli, phylum Firmicutes), *Clostridium sensu strictu 7* (class Clostridia, phylum Firmicutes) and, as already mentioned in combination with the infection, *Gordonia* (class Actinobacteria, phylum Actinobacteria). Changes in the microbial abundances related to antibiotic administration were also observed by Gupta et al. (2019) and Kokou et al. (2020), which noticed that the tested antibiotics affected more markedly the composition of the distal gut microbiota than the mid gut community. In Kokou et al. (2020), the authors also observed that antibiotics with the same principle of action can affect the gut microbial communities in different manners (e.g. in how broad they are).

Streptococcus and *Vagococcus* are lactic acid bacteria (LAB) (Gram positive cocci) performing homolactic fermentation that have been associated with the commensal gut microbiota of salmonids (Ringø and Gatesoupe, 1998; Ingerslev et al., 2014a, 2014b). An increased abundance of these genera have been associated with plant-based diets and suggested to be beneficial for the fish immune system since these bacteria may help to protect from pathogens than could penetrate the intestinal barrier (Ingerslev et al., 2014a, 2014b). In our experiment, we observed a decrease in the number of bacteria belonging to the genus *Vagococcus* during florfenicol administration suggesting that the treatment may negatively affect part of the beneficial bacterial community. The number of bacteria belonging to the genus *Streptococcus* was instead increased. One should remember that this genus as well as other LAB also contains pathogenic bacteria (Ringø and Gatesoupe, 1998).

The gut microbial community is malleable and able to recover from infections and antibiotics treatment (Francino, 2016). In our study, previously observed dissimilarities between the microbial community of non-challenged and recovered fish were lost at 33 dpi. No statistically significant difference was detected in the β-diversity analysis (+Fp vs –Fp). Also, the taxonomic mapping revealed that the previously observed differences in the genera *Streptococcus*, *Vagococus*, *Clostridium sensu strictu 7* (Firmicutes), *Rhodococcus*, *Gordonia* (Actinobacteria), *Flavobacterium* and the unidentified bacterium belonging to the family *Weeksellaceae* (Bacteroidetes) were recovered.

Changes in the composition in relation to phage administration in healthy and infected rainbow trout fry.

Bacteriophages are species-specific viruses of bacteria and they are studied in the gut microbiome therapy research to target specific gut pathogens and so restore beneficial bacteria (Zheng et al., 2019; Dahlman et al., 2021). Since they target specific bacterial populations, lytic phages are generally considered not able to alter the intestinal bacterial communities. However, these considerations are being revised/discussed as new studies have demonstrated, at different levels of analysis (alpha- or beta-diversity and taxonomic mapping), the ability of lytic phages to alter the gut microbiome independently of the presence of their target bacteria (Silva et al., 2016; Tetz et al., 2017; Barr, 2019; Febvre et al., 2019; Hsu et al., 2019). Other studies have instead not revealed changes in the gut microbiome as a result of phage therapy (Richards et

al., 2019). In the current work, we were not expecting any significant change in the overall gut microbiome of fish exposed to phages by oral administration, since our phages were specific for the freshwater pathogen *F. psychrophilum* (not considered part of the normal microbiome in high abundances). However, this was not the case as after 11 days of phage administration via phage-immobilized or phage-sprayed feed (day -1), differences in the gut microbial composition compared to the control groups were revealed by the β-diversity analysis and the same pattern was observed 1 and 33 dpi, independently if the fish were exposed or not to *F. psychrophilum*. Further, the two phage-treated feed types caused different changes in the microbial community 33 dpi independently of the infection.

The reasons of these effects of phages on gut community composition may relate to indirect effects of phage infections of *F. psychrophilum*. The release of cell lysates from infected cells has been shown to favor specific bacteria (Middelboe et al., 2003), and may thus affect the composition of microbiota. Alternatively, other phage-susceptible Flavobacterial populations may have been present in the gut. Also, it has been hypothesized that phages may evolve to become able to infect other bacteria than their original target (reviewed by (Ganeshan and Hosseinidoust, 2019)). Overall, the gut harbors a large population of bacteria (10^{13}-10^{14} bacterial cells in the human colon (Sender et al., 2016)) and phages (> 10^{10} g^{-1} of human gut (Sutton and Hill, 2019)), which interact in a complex network. Consequently, the delivery of a high quantity of specific phages may disturb these interactions directly or indirectly.

The observed alteration in the overall population identified by the β-diversity analysis was not revealed by the taxonomic mapping, suggesting that the administration of phages was mostly influencing the richness of low abundant bacteria and/or the dynamics between gut bacteria and phage populations (i.e. different bacteria were enhanced or decreased at different time points in non-infected fish fed phage-treated feed). One day prior to the infection, the taxonomic mapping revealed dissimilarities at genus level as significantly higher abundances of the genera *Enhydrobacter* and *Stenotrophomonas* (phylum Proteobacteria) were detected for fish fed phage-sprayed feed and for both phage-treated feed types, respectively (compared to the control). The higher abundance of *Stenotrophomonas* was maintained in fish fed phage-immobilized feed 1 dpi (non-infected fish; but not at 33 dpi) while the differences in *Enhydrobacter* were not further observed. Another example is the enhanced abundances of various LAB 33 dpi by phage-treated feed, more markedly for phage-sprayed feed (non-infected fish) (not previously observed). Among them, we found the genus *Carnobacterium*. Bacteria belonging to the this genus have been tested as probiotics against various fish bacterial infections (Ringø et al., 2010).

These observed changes in the gut microbiota in response to phage-treated feed administration did not affect the fish growth and no negative fish health parameters were recorded (Donati et al., 2021). Consequently, even though the selected phages did affect the gut microbiome, no negative implications were observed. However, further research should be targeting e.g. the metabolome (= collection of metabolites that provides a direct readout of cellular activity (Sun and Hu, 2016)) and the immune response that phages can trigger as a limited number of studies have been conducted in relation to phage therapy and their effects on the immune response in fish (e.g. (Schulz et al., 2019b, 2019a)).

Conclusion

The gut microbiota composition of rainbow trout fry observed in this study is in line with what previously observed. The bacterial infection and the antibiotic administration caused changes in the microbial composition of the gut which were then lost once fish recovered from the

infection and the antibiotic treatment was terminated. Interestingly, the administered phages changed the overall composition of the gut microbiota independently of the infection. Thus, future studies should try to resolve the mechanism of phage-driven changes in the microbiota and understand how they impact the immune response of the fish.

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Figures

Figure 1. Timeline of the experiment. PI: phage-immobilized feed group; C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. The results of the bacteriological examination performed on sampled fish are presented (% of fish positive to re-isolation of *F. psychrophilum*). Created with Biorender.com

Figure 2. Microbial community (day -1). Characterization of mean relative abundance at phylum (A) and class (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean of five samples except for group fed with phage-sprayed feed (n=4). In C, D and E, error bars represent the standard deviation. Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group.

Figure 3. Top-10 most abundant plus six additional genera selected among the top-30 (day -1). The selection of the additional genera was based on abundances presenting statistically significant differences. Values represent the mean and SD of five samples except for group fed with phage-sprayed feed (n=4). Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group.

Figure 4. Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) 1 day before (A, 19 samples and 423 OTUs) and 1 day after (B; 40 samples and 478 OTUs) the infection. Prior to the analysis, OTU's that are not present in more than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*.

Figure 5. Microbial community 1 dpi. Characterization of mean relative abundance at phylum (A) and class (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean of five samples. In C, D and E, error bars represent the standard deviation and statistically significant differences are indicated by * (adjusted p value < 0.05). Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*.

Figure 6. Top-10 most abundant plus five additional genera selected among the top-30 1 dpi in fish fed with control, antibiotic, phage-immobilized and phage-sprayed feed with (C/Fp; An/Fp; PI/Fp; PS/Fp) and without (C; An; PI; PS) bacterial exposure. The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.

Figure 7. **Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) of 20 samples and 310 OTUs (8 dpi)**. Prior to the analysis, OTU's that are not present in more than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles. C: control feed group; An: antibiotic feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*.

Figure 8. Microbial community (8 dpi). Characterization of mean relative abundance at phylum (A) and class (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean of five samples. In C, D and E, error bars represent the standard deviation. Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*.

Figure 9. Top-10 most abundant plus five additional genera selected among the top-30 in the control and antibiotic feed groups with (C/Fp and An/Fp) and without (C and An) bacterial exposure (8 dpi). The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.

Figure 10. Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) of 40 samples and 448 OTUs (33 dpi). Prior to the analysis, OTU's that are not present in more than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum.*

Figure 11. **Microbial community 33 dpi.** Characterization of mean relative abundance at phylum (A) and class (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean of five samples. In C and D, error bars represent the standard deviation. Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*.

Figure 12. Top-10 most abundant plus five additional genera selected among the top-30 33 dpi in fish fed with control, antibiotic, phage-immobilized and phage-sprayed feed with (C/Fp; An/Fp; PI/Fp; PS/Fp) and without (C; An; PI; PS) bacterial exposure. The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.

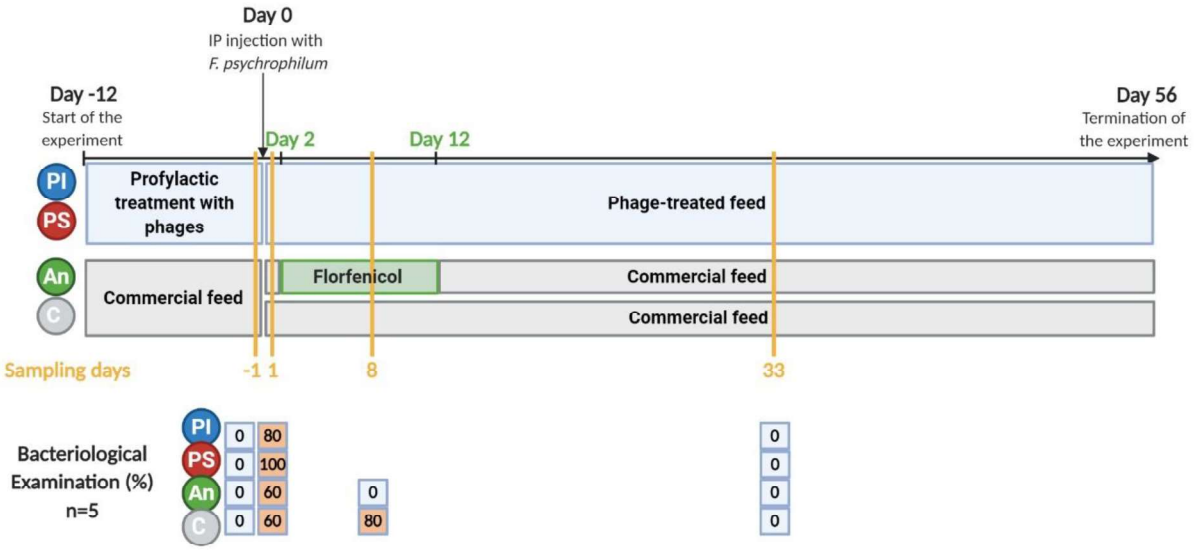
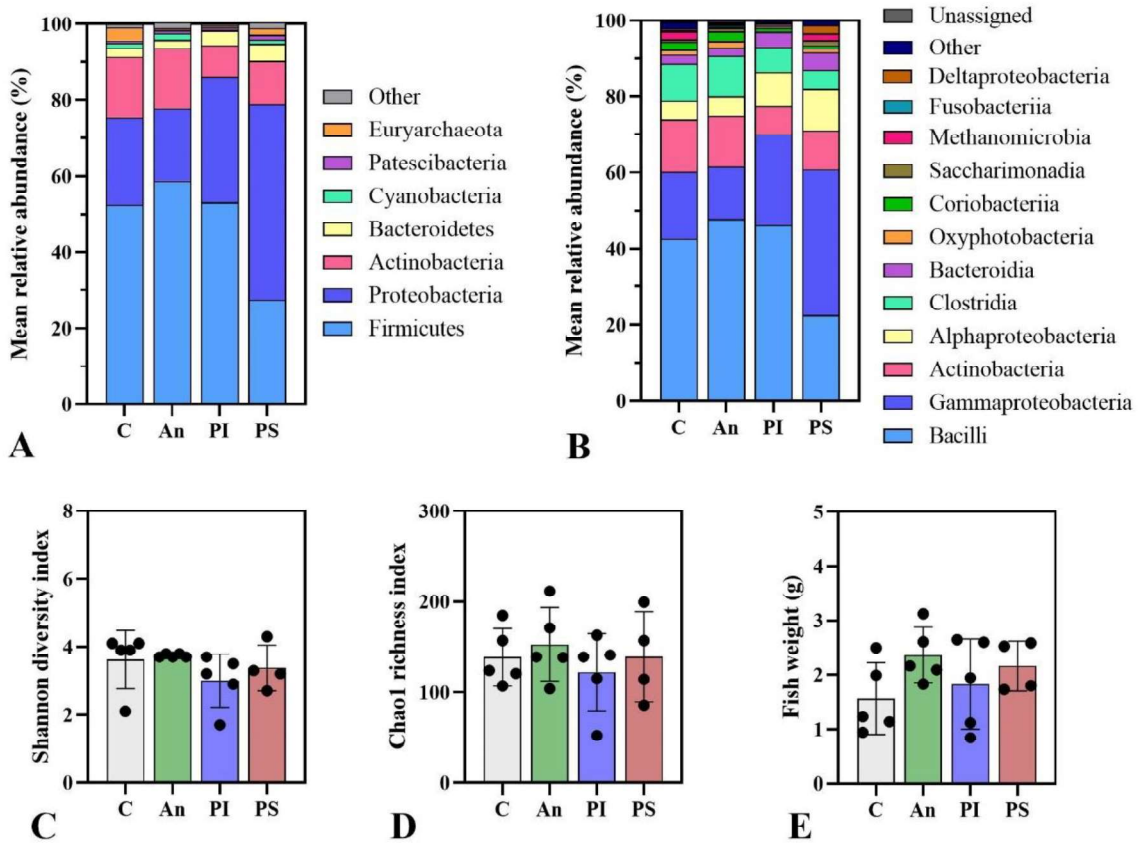


Figure 1. Timeline of the experiment. PI: phage-immobilized feed group; C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. The results of the bacteriological examination performed on sampled fish are presented (% of fish positive to re-isolation of *F. psychrophilum*). Created with Biorender.com.



1073

1074 **Figure 2. Microbial community (day -1).** Characterization of mean relative abundance at phylum (A) and class
1075 (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean
1076 of five samples except for group fed with phage-sprayed feed (n=4). In C, D and E, error bars represent the standard
1077 deviation. Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An:
1078 antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group.

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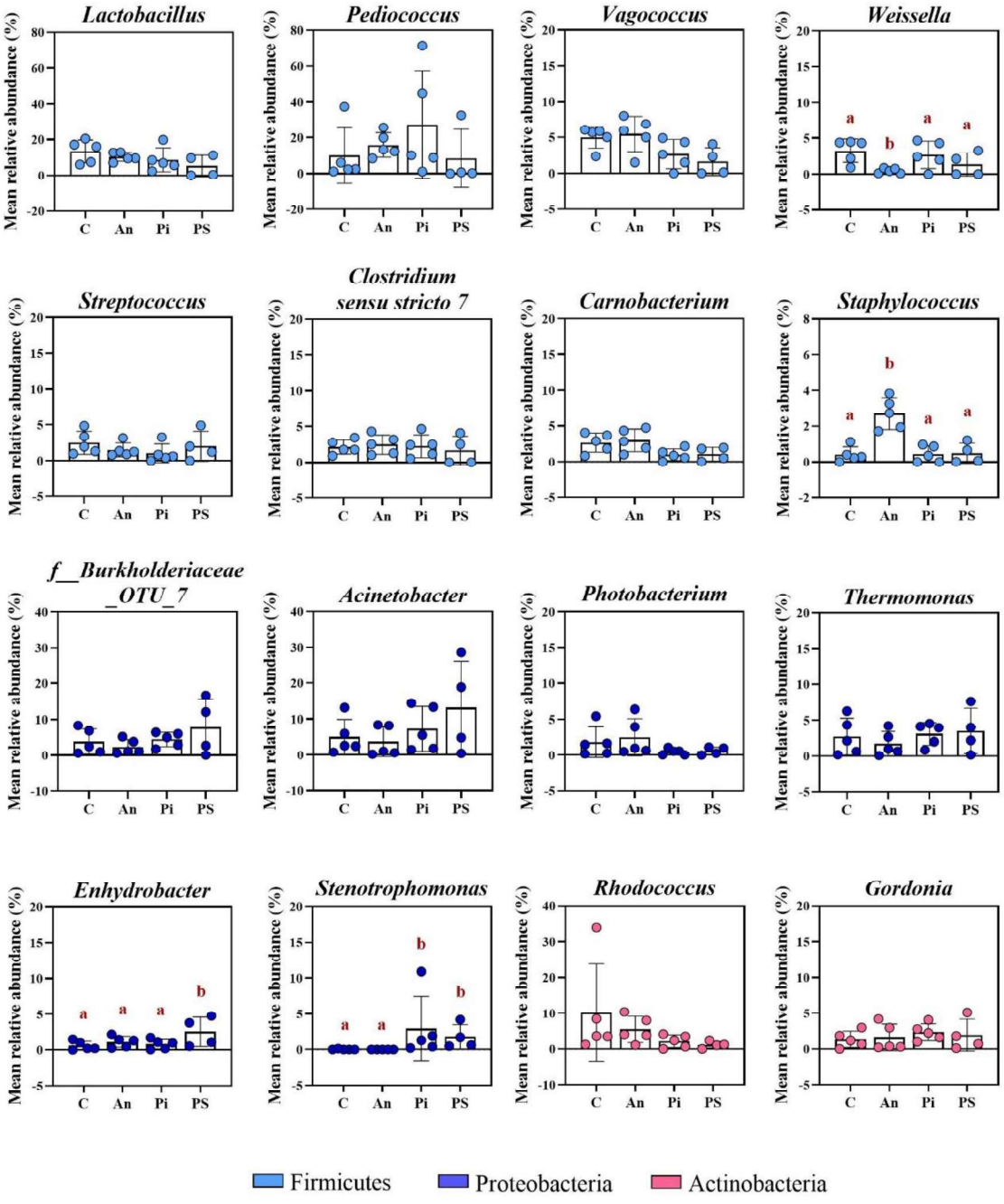
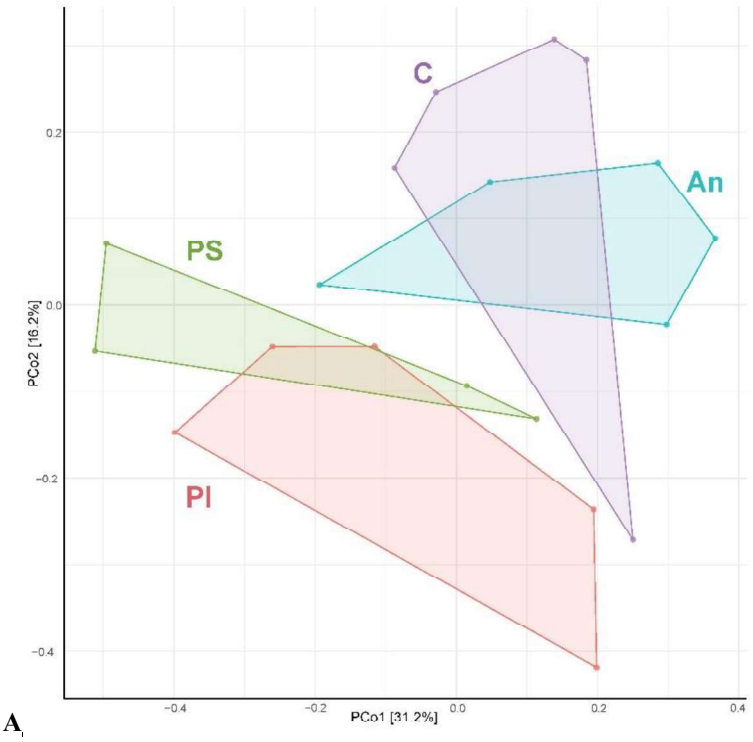
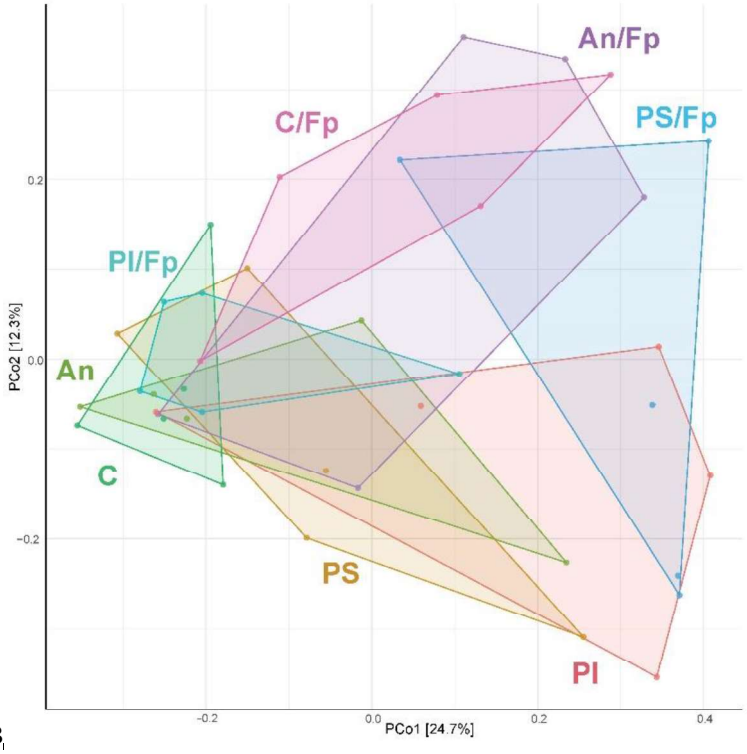


Figure 3. Top-10 most abundant plus six additional genera selected among the top-30 (day -1). The selection of the additional genera was based on abundances presenting statistically significant differences. Values represent the mean and SD of five samples except for group fed with phage-sprayed feed (n=4). Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group.



A



B

Figure 4. Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) 1 day before (A, 19 samples and 423 OTUs) and 1 day after (B; 40 samples and 478 OTUs) the infection. Prior to the analysis, OTU's that are not present in more than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*.

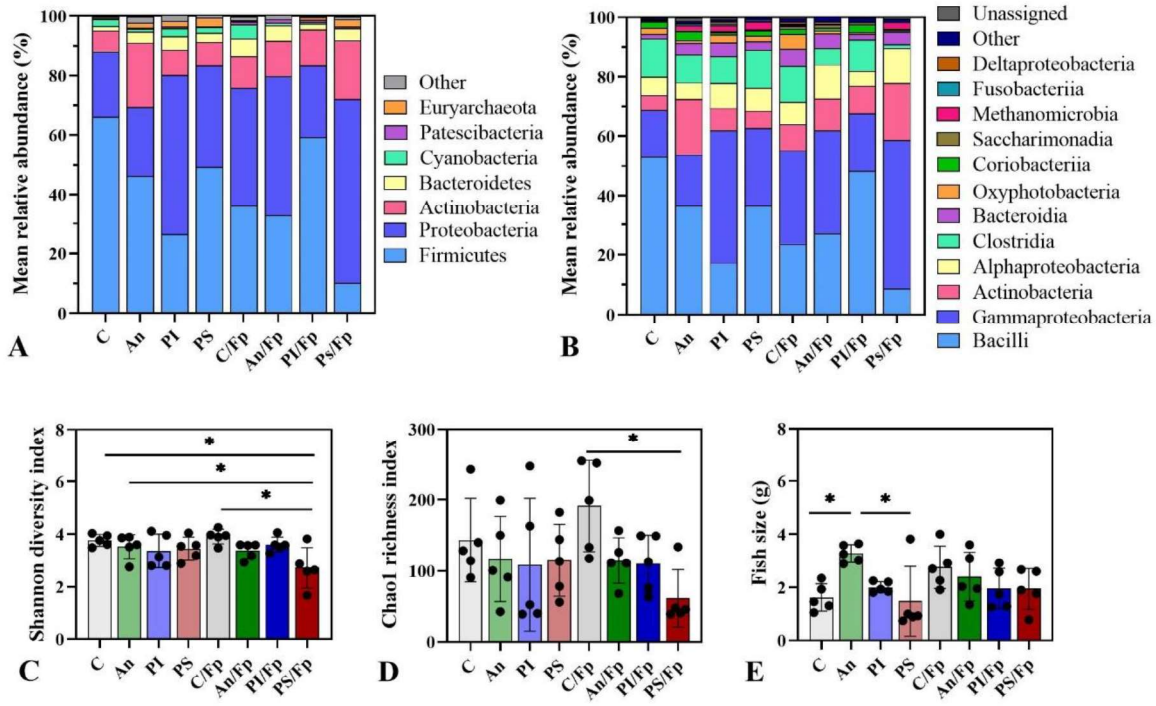


Figure 5. Microbial community 1 dpi. Characterization of mean relative abundance at phylum (A) and class (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean of five samples. In C, D and E, error bars represent the standard deviation and statistically significant differences are indicated by * (adjusted p value < 0.05). Shannon diversity index values are based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*.

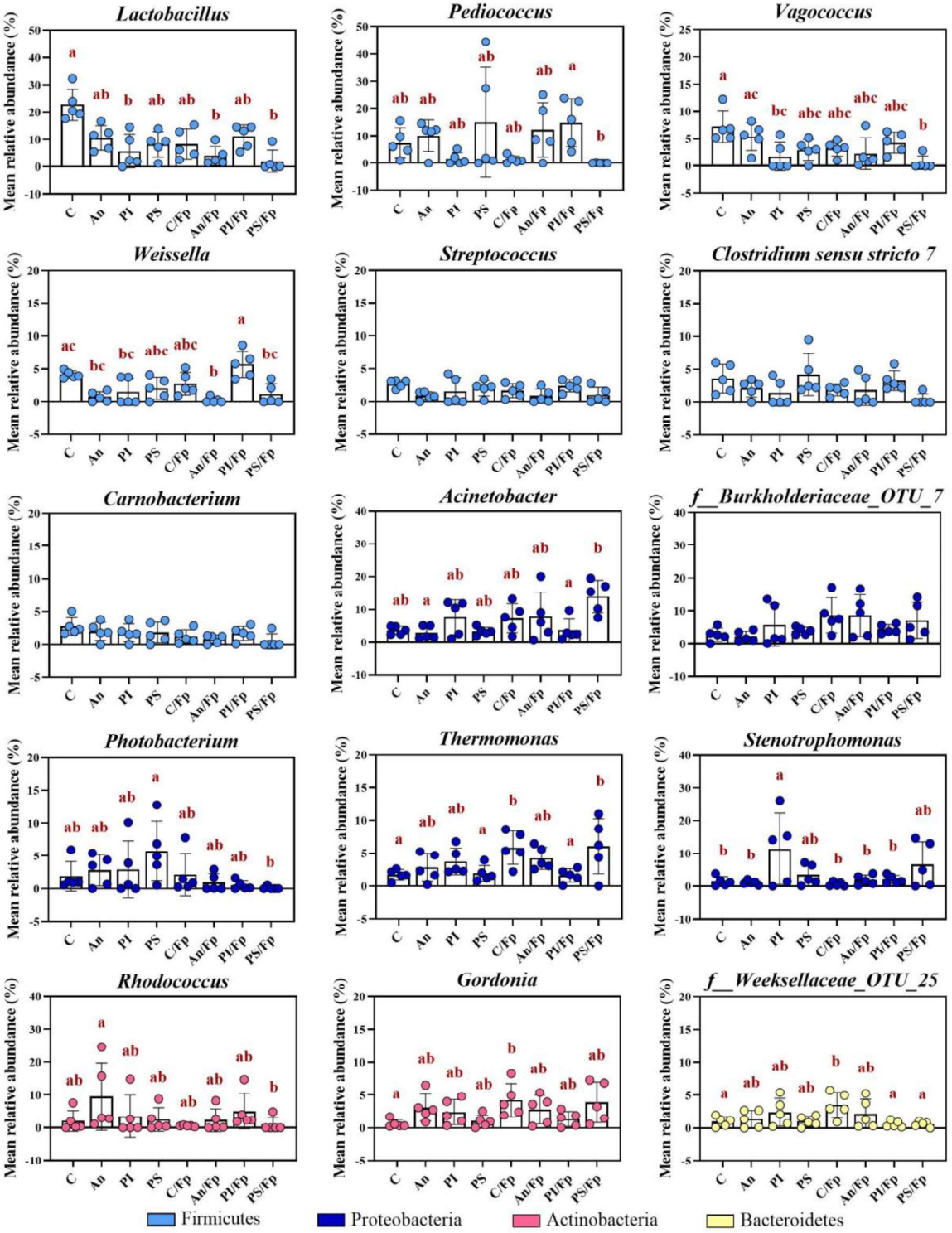


Figure 6. Top-10 most abundant plus five additional genera selected among the top-30 1 dpi in fish fed with control, antibiotic, phage-immobilized and phage-sprayed feed with (C/Fp; An/Fp; PI/Fp; PS/Fp) and without (C; An; PI; PS) bacterial exposure. The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.

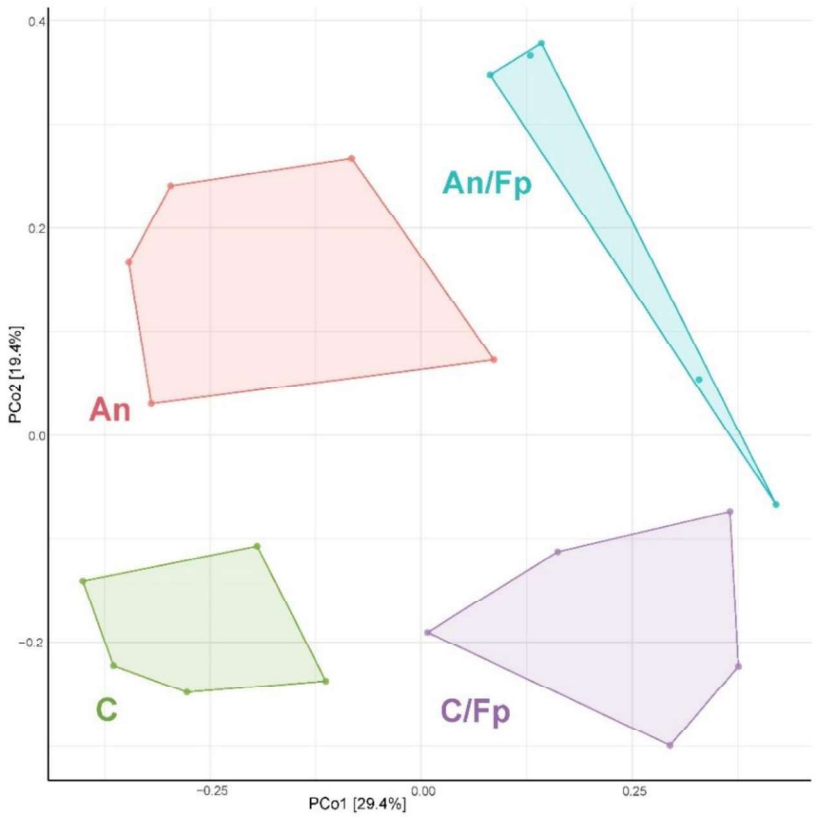
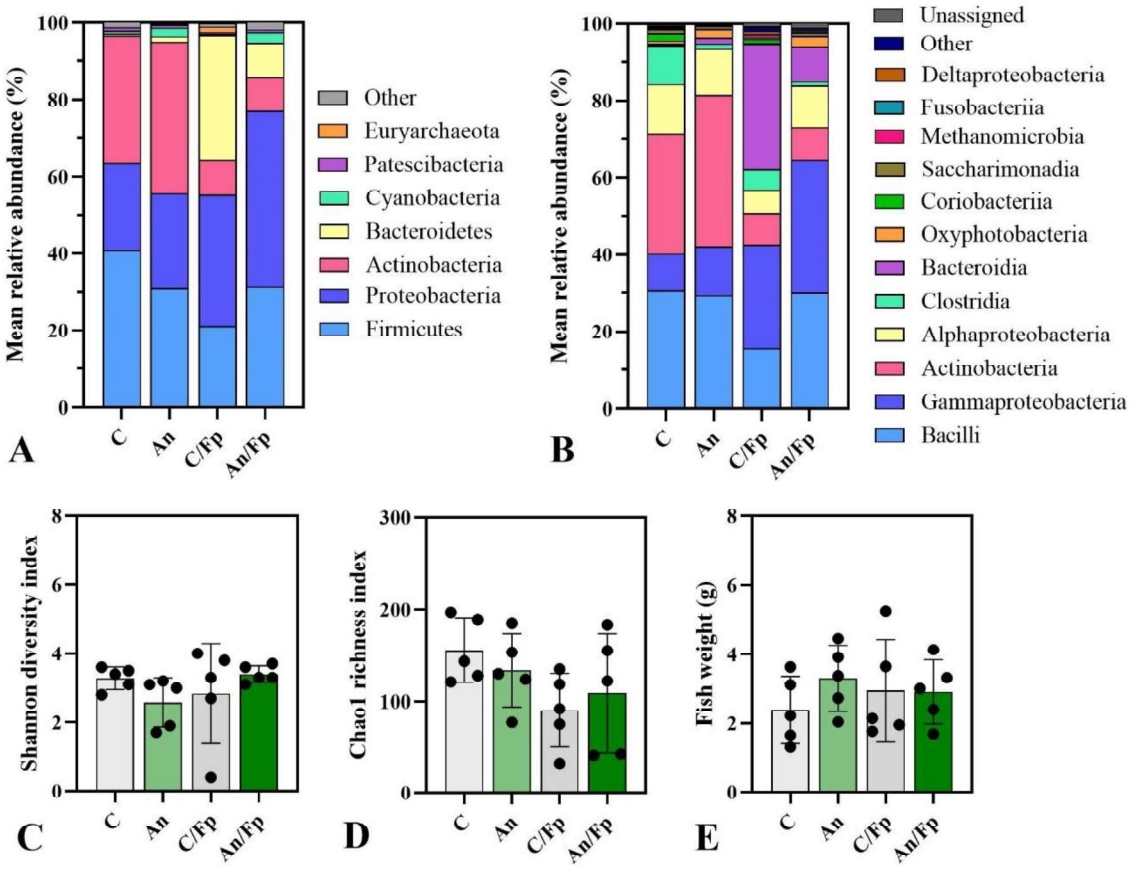


Figure 7. **Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and Curtis, 1957) of 20 samples and 310 OTUs (8 dpi)**. Prior to the analysis, OTU's that are not present in more than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles. C: control feed group; An: antibiotic feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*.



1121

1122 **Figure 8. Microbial community (8 dpi).** Characterization of mean relative abundance at phylum (A) and class
1123 (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean
1124 of five samples. In C, D and E, error bars represent the standard deviation. Shannon diversity index values are
1125 based on 10.000 reads per sample. C: control feed group; An: antibiotic feed group; C/Fp: control feed group +
1126 *F. psychrophilum*; An/Fp: antibiotic feed group + *F. psychrophilum*.

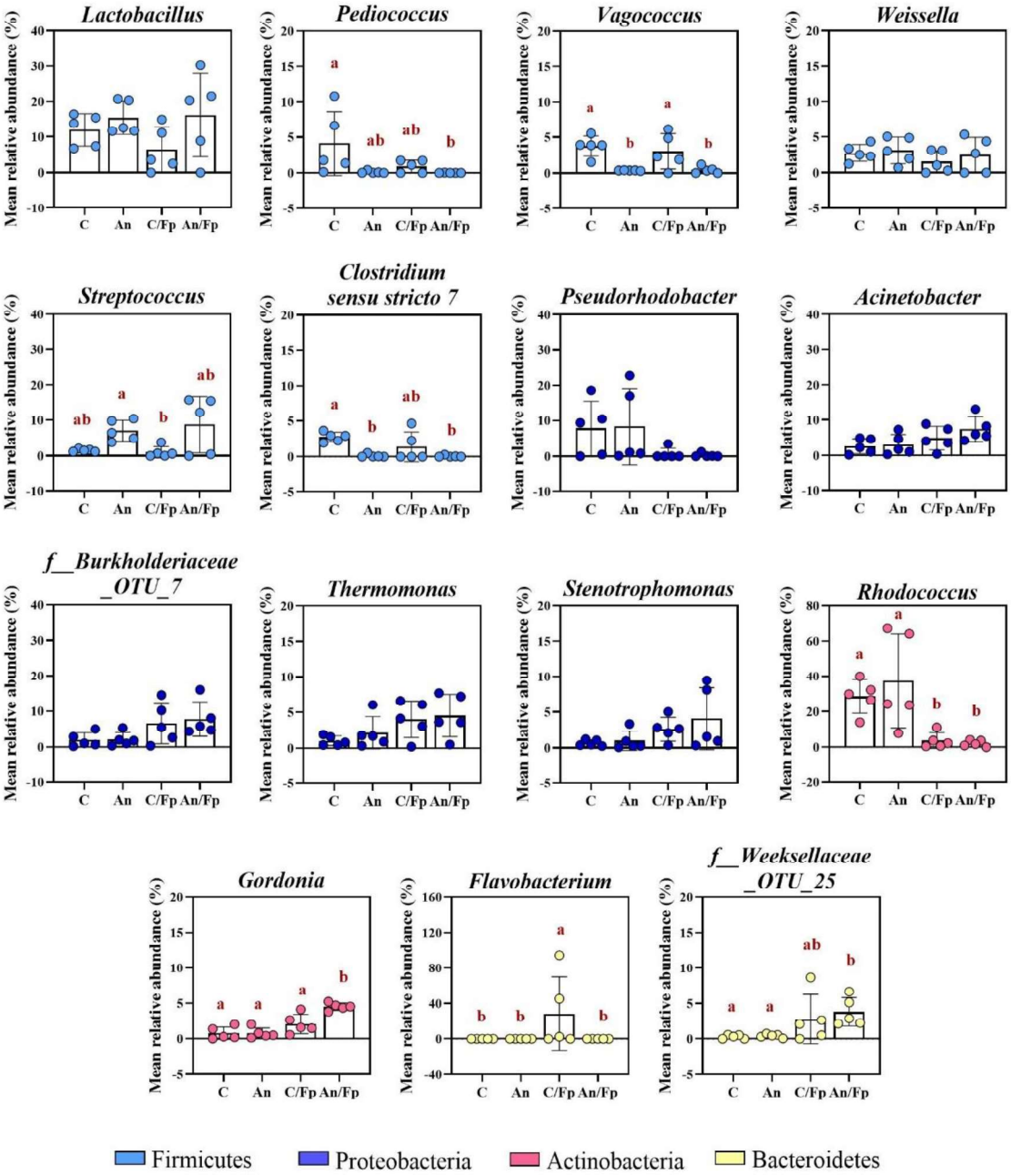
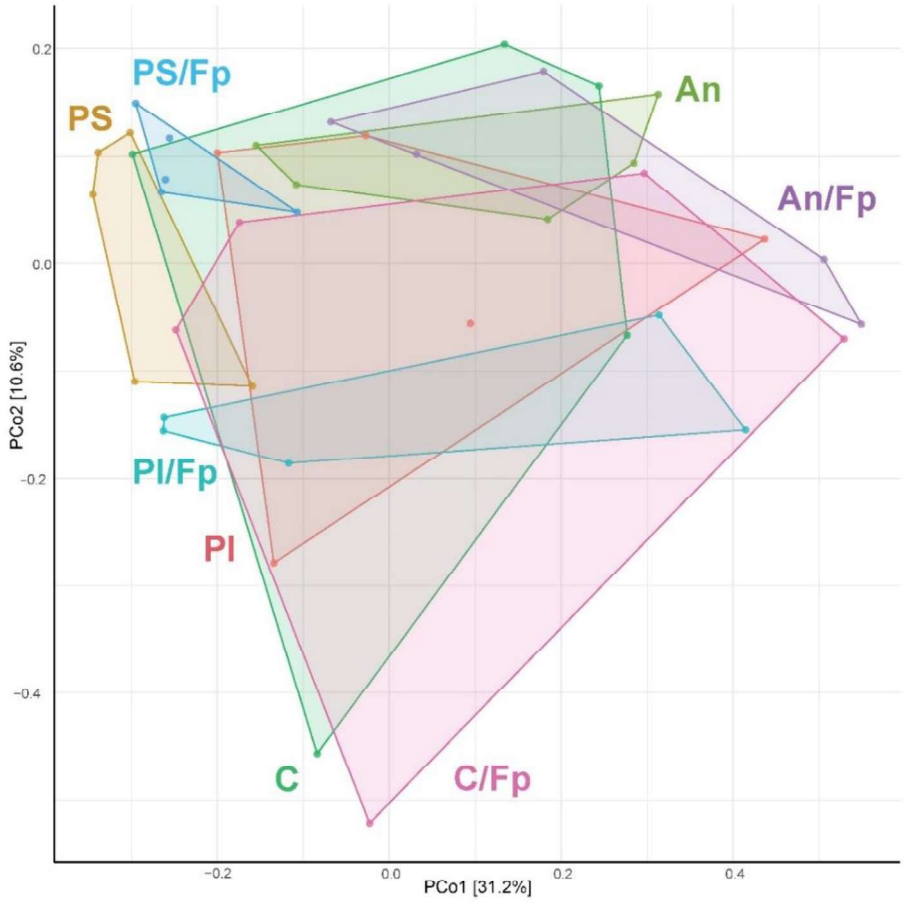


Figure 9. Top-10 most abundant plus five additional genera selected among the top-30 in the control and antibiotic feed groups with (C/Fp and An/Fp) and without (C and An) bacterial exposure (8 dpi). The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.



1134

1135 **Figure 10. Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance measure (Bray and**
1136 **Curtis, 1957) of 40 samples and 448 OTUs (33 dpi).** Prior to the analysis, OTU's that are not present in more
1137 than 0.1% relative abundance in any sample have been removed. No initial data transformation has been applied.
1138 The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis
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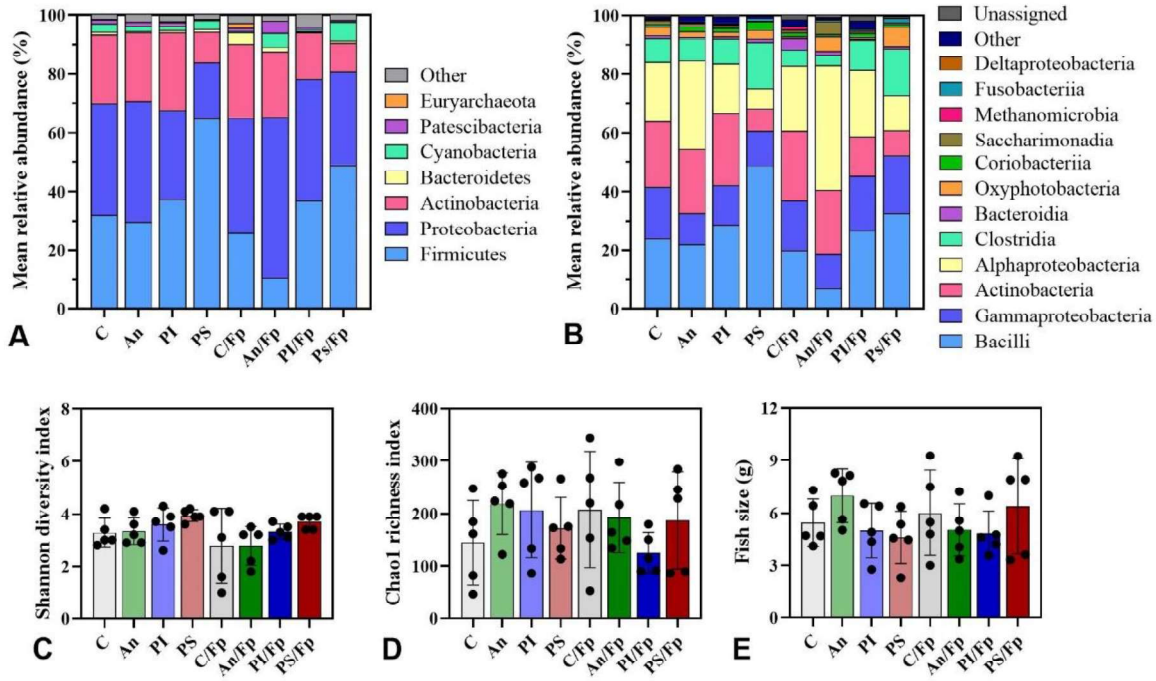

1142

1143 **Figure 11**. **Microbial community 33 dpi.** Characterization of mean relative abundance at phylum (A) and class
1144 (B) level, Shannon diversity index (C), Chao1 richness index (D) and fish weight (E). Values represent the mean
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1147 PS: phage-sprayed feed group; C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group + *F.*
1148 *psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F.*
1149 *psychrophilum*.

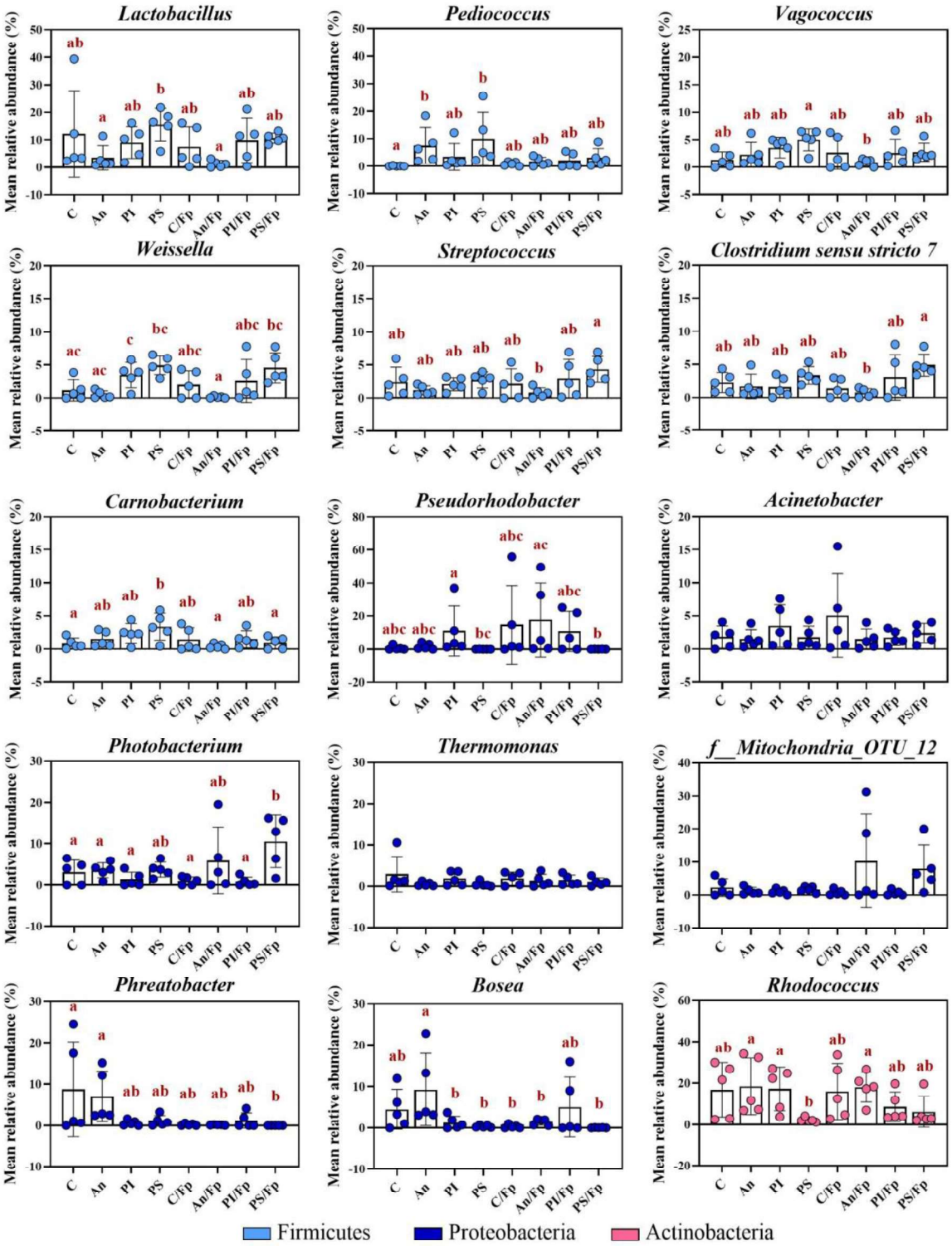

Figure 12. Top-10 most abundant plus five additional genera selected among the top-30 33 dpi in fish fed with control, antibiotic, phage-immobilized and phage-sprayed feed with (C/Fp; An/Fp; PI/Fp; PS/Fp) and without (C; An; PI; PS) bacterial exposure. The selection of the additional genera was based on abundances presenting statistically significant differences or on previously observed abundances. Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis (P-values are adjusted for multiple comparison). When significant, differences are presented with different red letters.

Supplementary material

Supplementary Table 1. Characteristics of sequenced samples. C: control group; An: antibiotic (florfenicol) feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. Red: failed sequencing; yellow: low output samples; green: highest number of reads obtained for one sample. Dpi: days post infection.

Feed group	Bacterial infection	Dpi	Sample no.	Number of reads	Observed OTUs
C	NO	-1	1	51143	142
C	NO	-1	2	57811	183
C	NO	-1	3	52746	104
C	NO	-1	4	43455	122
C	NO	-1	5	55967	120
C	NO	1	1	26690	110
C	NO	1	2	19515	89
C	NO	1	3	39887	205
C	NO	1	4	20454	112
C	NO	1	5	25004	131
C	NO	8	1	34420	176
C	NO	8	2	38885	178
C	NO	8	3	29163	118
C	NO	8	4	30443	121
C	NO	8	5	36302	125
C	NO	33	1	4624	43
C	NO	33	2	35193	236
C	NO	33	3	45460	154
C	NO	33	4	29804	75
C	NO	33	5	41789	181
C	YES	1	1	27126	247
C	YES	1	2	27076	190
C	YES	1	3	24700	126
C	YES	1	4	16905	116
C	YES	1	5	32112	246
C	YES	8	1	20236	116
C	YES	8	2	19638	87
C	YES	8	3	28374	31
C	YES	8	4	14904	61
C	YES	8	5	24858	130
C	YES	33	1	40387	210
C	YES	33	2	6520	43
C	YES	33	3	39938	150
C	YES	33	4	41062	259
C	YES	33	5	36001	217
An	NO	-1	1	48950	136
An	NO	-1	2	56185	200
An	NO	-1	3	21620	100
An	NO	-1	4	49981	136
An	NO	-1	5	58179	164
An	NO	1	1	24880	143
An	NO	1	2	17206	84
An	NO	1	3	22151	100
An	NO	1	4	37486	188
An	NO	1	5	6492	40
An	NO	8	1	48384	177
An	NO	8	2	26360	122
An	NO	8	3	27416	113

Feed group	Bacterial infection	Dpi	Sample no.	Number of reads	Observed OTUs
An	NO	8	4	41703	146
An	NO	8	5	17010	63
An	NO	33	1	37556	189
An	NO	33	2	22129	207
An	NO	33	3	34473	250
An	NO	33	4	23269	119
An	NO	33	5	32966	239
An	YES	1	1	27299	107
An	YES	1	2	24421	58
An	YES	1	3	18268	107
An	YES	1	4	30170	146
An	YES	1	5	26023	111
An	YES	8	1	13908	41
An	YES	8	2	39186	148
An	YES	8	3	38832	161
An	YES	8	4	32874	118
An	YES	8	5	7944	41
An	YES	33	1	46642	276
An	YES	33	2	35065	209
An	YES	33	3	31231	146
An	YES	33	4	44012	149
An	YES	33	5	48113	129
PI	NO	-1	1	25820	120
PI	NO	-1	2	31213	154
PI	NO	-1	3	9843	49
PI	NO	-1	4	35043	133
PI	NO	-1	5	19509	101
PI	NO	1	1	5013	33
PI	NO	1	2	10610	37
PI	NO	1	3	437110	244
PI	NO	1	4	13322	51
PI	NO	1	5	31065	158
PI	NO	33	1	41832	275
PI	NO	33	2	31321	115
PI	NO	33	3	30043	248
PI	NO	33	4	43626	248
PI	NO	33	5	15936	79
PI	YES	1	1	15344	63
PI	YES	1	2	24950	112
PI	YES	1	3	21601	76
PI	YES	1	4	32256	131
PI	YES	1	5	36352	140
PI	YES	33	1	34263	136
PI	YES	33	2	37301	171
PI	YES	33	3	21890	76
PI	YES	33	4	19135	77
PI	YES	33	5	35283	87
PS	NO	-1	1	34055	146
PS	NO	-1	2	11940	57
PS	NO	-1	3	22176	196
PS	NO	-1	4	22972	107
PS	NO	-1	5	FAILED	FAILED
PS	NO	1	1	9573	53
PS	NO	1	2	17074	77
PS	NO	1	3	18206	108

Feed group	Bacterial infection	Dpi	Sample no.	Number of reads	Observed OTUs
PS	NO	1	4	32840	135
PS	NO	1	5	26061	176
PS	NO	33	1	32386	165
PS	NO	33	2	22952	110
PS	NO	33	3	22403	130
PS	NO	33	4	31773	158
PS	NO	33	5	29542	254
PS	YES	1	1	13236	38
PS	YES	1	2	23924	132
PS	YES	1	3	5407	33
PS	YES	1	4	8158	36
PS	YES	1	5	19737	39
PS	YES	33	1	22432	84
PS	YES	33	2	31855	215
PS	YES	33	3	19640	248
PS	YES	33	4	23998	75
PS	YES	33	5	29967	234

6

Supplementary Table 2. Top five most abundant phyla at day -1 in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples except for group fed with phage-sprayed feed (n=4). Differences are tested by ANOVA or Kruskal-Wallis. (**B**) P-values adjusted for multiple comparison are also presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. Dpi: days post infection.

A

% mean abundance at phylum level

Feed group	Dpi	Firmicutes		Proteobacteria		Actinobacteria		Bacteroidetes		Cyanobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	-1	52.4	11.4	23.0	16.0	16.0	12.0	2.4	2.6	1.3	1.4
An	-1	58.7	17.3	19.0	13.0	16.0	2.8	2.2	2.8	1.8	1.5
PI	-1	53.1	31.3	33.0	24.1	8.4	3.4	3.8	4.9	0.2	0.1
PS	-1	27.4	31.5	51.6	25.7	11.3	4.2	4.6	3.2	1.1	1.1

B

P-values adjusted for multiple comparison

	Firmicutes	Proteobacteria	Actinobacteria	Bacteroidetes	Cyanobacteria
C vs An	>0.9999	0.9798	0.5886	>0.9999	0.8593
C vs PI	>0.9999	0.7755	0.5323	>0.9999	0.3400
C vs PS	0.7807	0.1240	>0.9999	0.9810	0.9913

Supplementary Table 3. Top seven most abundant classes at day -1 in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples except for group fed with phage-sprayed feed (n=4). Differences are tested by ANOVA or Kruskal-Wallis. (**B**) P-values adjusted for multiple comparison are also presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. Dpi: days post infection.

% mean abundance at class level

Feed group	Dpi	Bacilli		γ-proteobacteria		Actinobacteria		α-proteobacteria		Clostridia		Bacteroidia		Oxyphotobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	-1	42.6	9.6	17.6	13.5	13.8	12.1	4.9	2.8	9.7	5.6	2.4	2.6	1.3	1.4
An	-1	47.9	14.1	13.6	8.5	13.4	3.9	5.2	5.8	10.6	3.9	2.2	2.8	1.8	1.5
PI	-1	46.2	31.9	23.8	16.4	7.5	3.5	8.9	11.1	6.7	2.9	3.8	4.9	0.2	0.1
PS	-1	22.5	26.5	38.3	25.6	10.2	4.4	10.9	6.8	4.9	5.5	4.6	3.2	1.1	1.1

A

P-values adjusted for multiple comparison

	Bacilli	γ-proteobacteria	Actinobacteria	α-proteobacteria	Clostridia	Bacteroidia	Oxyphotobacteria
C vs An	0.9650	0.9643	0.857	>0.9999	0.9771	>0.9999	>0.9999
C vs PI	0.9881	0.8830	>0.9999	>0.9999	0.6174	>0.9999	0.7473
C vs PS	0.4192	0.1890	>0.9999	0.4929	0.3090	0.9810	>0.9999

B

Supplementary Table 4. Top-30 most abundant genera at day -1 in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). Values represent the mean and SD of five samples except for group fed with phage-sprayed feed (n=4). C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. Dpi: days post infection. Light blue = Firmicutes; Blue = Proteobacteria; Pink = Actinobacteria; Yellow = Bacteroidetes, Orange = Euryarchaeota.

No.	*Genus*	C		An		PI		PS	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
1	*Pediococcus*	9.9	15.5	15.9	6.8	27.3	30.1	8.4	16.1
2	*Lactobacillus*	13.4	6.4	10.3	2.5	8.7	6.6	5.3	5.9
3	*Acinetobacter*	5.0	4.9	3.7	4.2	7.3	6.2	13.2	13.0
4	*Rhodococcus*	10.1	13.6	5.5	3.7	2.1	1.8	1.2	1.0
5	*f__Burkholderiaceae_OTU_7*	3.8	3.5	2.3	2.0	4.4	2.0	7.9	7.8
6	*Vagococcus*	5.0	1.5	5.5	2.5	2.7	2.0	1.6	1.9
7	*Thermomonas*	2.7	2.6	1.7	1.7	3.1	1.6	3.5	3.2
8	*Clostridium sensu stricto 7*	2.1	1.0	2.5	1.4	2.2	1.6	1.6	2.0
9	*Weissella*	3.3	1.6	0.5	0.3	2.7	1.9	1.4	1.6
10	*Carnobacterium*	2.7	1.3	3.0	1.6	1.0	0.8	1.1	0.9
11	*Gordonia*	1.3	1.1	1.6	1.8	2.3	1.2	1.9	2.2
12	*Streptococcus*	2.5	1.6	1.5	1.0	1.0	1.3	2.0	2.1
13	*f__Weeksellaceae_OTU_25*	1.3	1.2	1.7	2.7	1.8	1.7	1.7	2.6
14	*Paracoccus*	1.0	1.5	1.2	1.5	1.1	1.3	3.1	2.9
15	*Photobacterium*	1.8	2.1	2.5	2.6	0.5	0.4	0.6	0.5
16	*Enhydrobacter*	0.6	0.6	1.1	0.8	0.8	0.7	2.5	2.1
17	*Pseudorhodobacter*	0.0	0.1	0.3	0.5	3.9	8.6	0.2	0.6
18	*f__Mitochondria_OTU_12*	1.6	2.6	1.9	2.4	0.4	0.5	0.2	0.1
19	*Corynebacterium 1*	0.5	0.4	1.2	0.3	1.3	2.0	1.8	1.8
20	*Stenotrophomonas*	0.0	0.1	0.0	0.0	2.9	4.5	1.8	1.7
21	*Clostridium sensu stricto 18*	1.8	1.4	1.0	0.6	0.5	0.3	1.1	1.4
22	*f__Coriobacteriales Incertae Sedis_OTU_23*	1.3	0.7	1.6	1.2	0.7	0.7	0.5	0.7
23	*Staphylococcus*	0.4	0.4	2.7	0.9	0.4	0.5	0.5	0.6
24	*Peptoniphilus*	0.8	0.7	1.4	0.4	0.8	0.4	0.3	0.4
25	*Methanosaeta*	1.5	3.4	0.3	0.4	0.0	0.0	1.8	3.5
26	*Sphingomonas*	0.0	0.1	0.0	0.1	0.2	0.3	3.7	6.5
27	*Tepidimicrobium*	1.1	1.4	1.0	0.8	0.4	0.4	0.5	0.7
28	*Enterococcus*	0.9	0.6	1.2	0.6	0.4	0.3	0.4	0.4
29	*Pseudomonas*	0.4	0.7	0.1	0.1	1.2	2.4	1.4	1.9
30	*Leuconostoc*	0.9	0.5	0.7	0.5	0.4	0.4	0.8	1.1

Supplementary Table 5. Top five most abundant phyla 1 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples. Differences are tested by Kruskal-Wallis. When significant, differences are presented with different letters in red. (**B**) P-values adjusted for multiple comparison are presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection. Significant P values are in bold.

% mean abundance at phylum level

Feed group	Dpi	Firmicutes		Proteobacteria		Actinobacteria		Bacteroidetes		Cyanobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	1	66.0 [a]	12.0	22.1	8.2	7.0 [a]	3.9	1.5	0.1	2.2 [ab]	4.5
An	1	46.2 [ab]	24.0	23.2	11.1	21.6 [b]	12.1	3.7	4.0	1.0 [ab]	1.2
PI	1	26.4 [ab]	24.5	53.9	27.8	8.3 [ab]	6.0	4.7	3.4	2.6 [ab]	3.5
PS	1	49.2 [ab]	23.9	34.1	18.8	8.0 [ab]	5.8	2.9	2.6	2.0 [a]	1.8
C/Fp	1	36.0 [ab]	15.3	39.9	15.7	10.7 [ab]	3.1	5.8	2.7	4.9 [ab]	9.8
An/Fp	1	32.7 [ab]	18.0	47.1	15.1	12.0 [ab]	5.7	5.0	5.3	1.0 [ab]	1.7
PI/Fp	1	59.1 [ab]	18.4	24.3	15.6	12.0 [ab]	6.0	1.9	1.0	0.6 [ab]	0.4
PS/Fp	1	10.2 [b]	12.0	61.8	23.4	19.8 [ab]	28.0	4.1	4.2	0.02 [b]	0.1

A

P-values adjusted for multiple comparison

	Firmicutes	Proteobacteria	Actinobacteria	Bacteroidetes	Cyanobacteria
C vs An	>0.9999	>0.9999	**0.0373**	>0.9999	>0.9999
C vs PI	0.0712	0.3835	>0.9999	0.6925	>0.9999
C vs PS	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs. C/Fp	0.3172	0.9577	>0.9999	0.1615	>0.9999
C vs. An/Fp	0.2441	0.3835	0.6547	>0.9999	>0.9999
C vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs. Ps/Fp	**0.0020**	0.0519	>0.9999	>0.9999	0.6309
C/Fp vs. An/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C/Fp vs. PI/Fp	0.7000	0.7387	>0.9999	0.3088	>0.9999
C/Fp vs. Ps/Fp	0.6275	>0.9999	>0.9999	>0.9999	0.2422
An vs. An/Fp	>0.9999	0.3951	>0.9999	>0.9999	>0.9999
PI vs. PI/Fp	0.1956	0.2898	>0.9999	>0.9999	>0.9999
PS vs. Ps/Fp	0.0660	0.5005	>0.9999	>0.9999	**0.0346**

B

Supplementary Table 6. Top seven most abundant classes 1 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples. Differences are tested by Kruskal-Wallis. (**B**) P-values adjusted for multiple comparison are also presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection.

A

% mean abundance at class level

Feed group	Dpi	Bacilli		γ-proteobacteria		Actinobacteria		α-proteobacteria		Clostridia		Bacteroidia		Oxyphotobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	1	53.1 [a]	11.7	15.7 [a]	5.1	4.9	4.1	6.3	5.3	12.8 [a]	3.8	1.5	0.1	2.2 [ab]	4.5
An	1	36.4 [ab]	18.0	17.4 [ab]	9.6	18.6	13.4	5.6	3.8	9.5 [ab]	5.8	3.7	3.9	1.0 [ab]	1.2
PI	1	17.2 [ab]	18.4	44.7 [ab]	26.3	7.6	5.2	8.5	5.2	8.9 [ab]	8.0	4.7	3.4	2.6 [ab]	3.5
PS	1	36.3 [ab]	27.7	26.3 [ab]	15.3	5.9	4.9	7.8	8.1	12.7 [a]	5.3	2.9	2.6	2.0 [a]	1.8
C/Fp	1	23.5 [ab]	12.0	31.7 [ab]	12.7	8.9	3.3	7.4	3.1	12.2 [ab]	6.9	5.8	2.7	4.9 [ab]	9.8
An/Fp	1	27.1 [ab]	13.5	34.9 [ab]	15.9	10.7	5.6	11.5	6.1	5.4 [ab]	5.6	5.0	5.3	1.0 [ab]	1.7
PI/Fp	1	48.4 [ab]	14.6	12.3 [ab]	14.1	9.3	6.8	4.9	4.3	10.5 [ab]	4.6	1.9	1.0	0.6 [ab]	0.4
PS/Fp	1	8.7 [b]	9.1	50.0 [b]	17.4	19.2	28.1	11.8	13.7	1.3 [b]	2.7	4.1	4.2	0.02 [b]	0.1

B

P-values adjusted for multiple comparison

	Bacilli	γ-proteobacteria	Actinobacteria	α-proteobacteria	Clostridia	Bacteroidia	Oxyphotobacteria
C vs An	>0.9999	>0.9999	0.0968	>0.9999	>0.9999	>0.9999	>0.9999
C vs PI	0.0519	0.1211	>0.9999	>0.9999	>0.9999	0.6925	>0.9999
C vs PS	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs. C/Fp	0.1991	0.4338	>0.9999	>0.9999	>0.9999	0.1615	>0.9999
C vs. An/Fp	0.3381	0.3381	0.5839	>0.9999	0.6924	>0.9999	>0.9999
C vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs. Ps/Fp	**0.0056**	**0.0224**	>0.9999	>0.9999	**0.0459**	>0.9999	0.6309
C/Fp vs. An/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C/Fp vs. PI/Fp	0.3287	0.5936	>0.9999	>0.9999	>0.9999	0.3088	>0.9999
C/Fp vs. Ps/Fp	>0.9999	>0.9999	>0.9999	>0.9999	0.0862	>0.9999	0.2422
An vs. An/Fp	>0.9999	0.3719	>0.9999	0.5611	>0.9999	>0.9999	>0.9999
PI vs. PI/Fp	0.0964	0.1828	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
PS vs. Ps/Fp	0.2388	0.5005	>0.9999	>0.9999	**0.0259**	>0.9999	**0.0346**

Supplementary Table 7. Top-30 most abundant genera 1 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). Values represent the mean and SD of five samples. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection. Light blue = Firmicutes; Blue = Proteobacteria; Pink = Actinobacteria; Yellow = Bacteroidetes, Green = Cyanobacteria.

No.	Genus	C		An		PI		PS		C/Fp		An/Fp		PI/Fp		PS/Fp	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
1	*Lactobacillus*	22.7	5.8	10.6	4.5	5.7	6.2	8.3	4.6	8.4	5.5	4.0	3.5	11.1	4.2	2.1	4.1
2	*Pediococcus*	7.4	5.5	10.1	5.7	1.6	2.3	14.9	20.1	1.3	1.4	12.2	9.9	14.8	8.7	0.0	0.1
3	*Acinetobacter*	3.7	1.2	3.0	2.0	7.6	5.3	3.4	1.2	7.4	4.4	7.8	7.5	3.7	3.4	14.0	5.0
4	*f__Burkholderiaceae_ OTU_7*	2.8	2.0	2.2	1.6	5.7	6.4	3.9	1.1	8.7	5.4	8.5	6.3	4.5	1.4	7.0	5.5
5	*Stenotrophomonas*	1.5	1.5	1.1	0.7	11.4	10.8	3.4	3.2	0.6	0.7	1.9	1.5	2.1	1.2	6.6	6.9
6	*Thermomonas*	1.7	0.8	2.8	2.1	3.7	2.0	1.9	1.2	5.8	2.5	4.2	1.7	1.6	1.0	6.0	4.2
7	*Vagococcus*	7.2	2.9	5.4	2.5	1.8	2.6	3.0	1.9	3.3	1.4	2.3	2.9	4.3	1.9	0.6	1.3
8	*Rhodococcus*	2.0	3.2	9.5	10.3	3.5	6.4	2.5	3.6	0.5	0.2	2.3	3.5	5.0	5.5	1.0	2.2
9	*Gordonia*	0.6	0.6	3.1	2.1	2.4	1.9	1.0	1.0	4.2	2.5	2.8	2.2	1.4	1.1	3.9	3.1
10	*Clostridium sensu stricto 7*	3.6	2.2	2.1	1.3	1.4	1.9	4.2	3.2	1.9	0.9	1.8	2.3	3.3	1.5	0.4	0.9
11	*Weissella*	1.4	0.6	0.8	0.8	1.5	2.1	2.1	1.6	2.7	1.7	0.6	0.4	5.7	2.1	1.2	1.6
12	*Paracoccus*	0.5	0.3	2.0	3.7	1.9	2.6	0.6	0.4	2.6	1.8	2.4	2.1	2.3	3.3	5.1	10.9
13	*Photobacterium*	1.9	2.3	2.8	2.4	2.9	4.3	5.7	4.5	2.1	3.2	0.9	1.3	0.5	0.7	0.1	0.2
14	*Rothia*	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.5	1.2	12.6	28.2
15	*Delftia*	0.6	0.4	1.5	2.4	2.3	3.2	2.8	3.2	0.4	0.6	1.9	1.8	1.4	1.8	2.6	3.5
16	*Streptococcus*	2.7	0.6	0.9	0.6	1.6	2.0	2.0	1.2	1.7	1.1	0.9	1.1	2.4	0.9	1.0	1.2
17	*f__Weeksellaceae_ OTU_25*	1.0	0.8	1.4	1.3	2.4	2.1	1.0	0.8	3.5	1.9	2.2	2.2	0.6	0.5	0.6	0.4
18	*Carnobacterium*	2.8	1.3	2.0	1.4	1.8	1.4	1.8	1.6	1.2	1.0	0.8	0.6	1.8	1.1	0.5	1.1
19	*Staphylococcus*	0.5	0.2	1.6	1.4	0.5	0.5	0.5	0.9	1.2	0.9	4.4	6.6	1.8	1.0	1.1	1.6
20	*Clostridium sensu stricto 18*	2.5	1.1	1.3	1.2	0.8	1.2	2.1	1.3	1.2	0.9	0.3	0.6	1.9	1.7	1.2	0.4
21	*Pseudomonas*	1.4	1.1	0.4	0.1	3.0	2.8	0.9	0.9	0.6	0.5	0.7	0.5	0.4	0.3	2.1	2.0
22	*f__Coriobacteriales Incertae Sedis_ OTU_23*	1.1	0.6	2.4	1.7	0.5	0.7	1.2	1.5	1.3	0.5	0.6	1.1	2.2	1.7	0.2	0.5
23	*Enhydrobacter*	0.2	0.4	0.4	0.4	0.2	0.3	0.9	1.3	1.4	0.9	2.3	1.5	0.4	0.4	2.2	2.3
24	*Diaphorobacter*	0.6	0.3	1.1	1.4	0.5	0.4	0.7	1.1	1.1	0.6	0.6	0.7	0.3	0.4	3.1	2.4
25	*o__Chloroplast_ OTU_35*	0.5	0.8	0.5	0.5	0.9	1.4	0.9	1.0	4.3	9.0	0.2	0.1	0.4	0.5	0.0	0.1
26	*Tepidimicrobium*	1.5	0.6	0.9	1.1	1.0	1.5	1.0	0.8	1.2	0.8	0.4	0.9	1.5	1.0	0.2	0.5
27	*f__Mitochondria_ OTU_12*	0.6	1.0	2.0	3.4	2.2	3.3	1.0	1.2	0.6	1.6	0.4	0.8	0.4	0.4	0.0	0.0
28	*Pseudorhodobacter*	3.1	4.2	0.2	0.3	0.4	0.7	2.9	6.5	0.2	0.4	0.0	0.0	0.2	0.5	0.0	0.0
29	*o__Chloroplast_ OTU_27*	1.7	3.8	0.5	0.9	1.7	2.5	1.1	1.2	0.5	0.7	0.8	1.7	0.2	0.1	0.0	0.0
30	*Peptoniphilus*	1.2	0.7	1.1	1.0	0.3	0.5	0.9	1.4	1.5	1.1	0.4	0.4	0.5	0.4	0.1	0.3

Supplementary Table 8. Top five most abundant phyla 8 dpi in the control and antibiotic feed groups. (**A**) Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis. When significant, differences are presented with different letters in red. (**B**) P-values adjusted for multiple comparison are presented. C: control feed group; An: antibiotic feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; Dpi: days post infection. Significant P values are in bold.

A

% mean abundance at phylum level

Feed group	Dpi	Firmicutes		Proteobacteria		Actinobacteria		Bacteroidetes		Cyanobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	8	40.8	15.8	22.7	9.6	33.1 [a]	7.9	0.5 [a]	0.6	0.7 [ab]	0.6
An	8	31.0	9.9	24.6	17.9	39.4 [a]	26.0	1.5 [ac]	2.1	2.3 [a]	1.4
C/Fp	8	21.1	18.3	34.0	21.7	9.2 [b]	7.7	32.6 [b]	39.3	0.1 [b]	0.2
An/Fp	8	31.4	25.1	45.8	17.7	8.6 [b]	1.5	9.1 [bc]	6.8	2.7 [a]	2.0

B

P-values adjusted for multiple comparison

	Firmicutes	Proteobacteria	Actinobacteria	Bacteroidetes	Cyanobacteria
C vs. An	0.7271	0.9961	0.8223	>0.9999	0.6463
C vs. C/Fp	0.2391	0.6102	**0.0425**	**0.0083**	0.5345
C vs. An/Fp	0.7478	0.1239	**0.0375**	**0.036**	0.5884
An vs. C/Fp	0.7257	0.7257	**0.0102**	**0.0485**	**0.0292**
An vs. An/Fp	>0.9999	0.1683	**0.0089**	0.1630	>0.9999
C/Fp vs. An/Fp	0.7048	0.5874	0.9999	>0.9999	**0.0249**

Supplementary Table 9. Top seven most abundant classes 8 dpi in the control and antibiotic feed groups. (**A**) Values represent the mean and SD of five samples. Differences are tested by ANOVA or Kruskal-Wallis. When significant, differences are presented with different letters in red. (**B**) P-values adjusted for multiple comparison are presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection. Significant P values are in bold.

A

% mean abundance at class level

Feed group	Dpi	Bacilli		γ-proteobacteria		Actinobacteria		α-proteobacteria		Clostridia		Bacteroidia		Oxyphotobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	8	30.7	12.7	9.5 [a]	5.2	31.1 [ab]	9.3	12.9	10.6	10.5 [a]	3.2	0.5 [a]	0.6	0.7 [ab]	0.6
An	8	29.5	9.5	12.7 [ab]	12.5	39.3 [a]	26.0	11.8	12.1	1.5 [b]	0.4	1.5 [ac]	2.1	2.3 [a]	1.4
C/Fp	8	15.6	14.0	27.3 [ab]	17.5	8.1 [b]	6.5	6.1	4.0	5.4 [ab]	4.7	32.6 [b]	39.3	0.1 [b]	0.2
An/Fp	8	30.2	24.0	34.4 [b]	15.9	8.4 [b]	1.4	10.8	4.7	1.1 [b]	1.0	9.1 [bc]	6.8	2.7 [a]	2.0

B

P-values adjusted for multiple comparison

	Bacilli	γ-proteobacteria	Actinobacteria	α-proteobacteria	Clostridia	Bacteroidia	Oxyphotobacteria
C vs. An	0.9987	0.9658	0.6889	0.9948	**0.0007**	>0.9999	0.6463
C vs. C/Fp	0.3413	0.1407	0.0536	0.4718	0.0529	**0.0083**	0.5345
C vs. An/Fp	>0.9999	**0.0274**	0.0562	0.9650	**0.0005**	**0.0360**	0.5884
An vs. C/Fp	0.4052	0.2618	**0.0084**	0.5942	0.1213	**0.0485**	**0.0292**
An vs. An/Fp	0.9997	0.0569	**0.0089**	0.9956	0.9950	0.1630	>0.9999
C/Fp vs. An/Fp	0.3673	0.7264	>0.9999	0.7149	0.0858	>0.9999	**0.0249**

Supplementary Table 10. Top-30 most genera phyla 8 dpi in the control and antibiotic feed groups. Values represent the mean and SD of five samples. C: control feed group; An: antibiotic feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; Dpi: days post infection. Light blue = Firmicutes; Blue = Proteobacteria; Pink = Actinobacteria; Yellow = Bacteroidetes, Green = Cyanobacteria.

No.	Genus	C		An		C/Fp		An/Fp	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
1	*Rhodococcus*	28.5	9.5	37.4	26.7	3.6	4.4	2.1	1.7
2	*Lactobacillus*	12.0	4.6	15.4	4.7	6.4	6.3	16.2	11.8
3	*Flavobacterium*	0.0	0.0	0.0	0.0	28.5	41.6	0.0	0.0
4	*f__Burkholderiaceae_OTU_7*	2.0	2.0	2.1	1.9	6.6	5.8	7.8	4.8
5	*Streptococcus*	1.5	0.3	7.1	2.9	1.0	1.6	8.8	7.9
6	*Acinetobacter*	2.6	2.1	3.0	2.9	4.9	3.3	7.4	3.5
7	*Pseudorhodobacter*	7.8	7.7	8.4	10.7	0.7	1.6	0.3	0.5
8	*Thermomonas*	1.0	0.7	2.1	2.3	4.0	2.6	4.5	3.0
9	*Weissella*	2.7	1.2	3.1	1.9	1.5	1.5	2.5	2.5
10	*Stenotrophomonas*	0.7	0.4	1.0	1.4	2.6	1.7	4.1	4.3
11	*Gordonia*	0.8	0.9	0.8	0.7	2.1	1.4	4.5	0.5
12	*Vagococcus*	3.8	1.4	0.4	0.0	3.0	2.5	0.4	0.5
13	*f__Weeksellaceae_OTU_25*	0.3	0.3	0.4	0.3	2.8	3.5	3.8	2.0
14	*o__Chloroplast_OTU_35*	0.5	0.4	2.0	1.3	0.1	0.3	2.6	2.0
15	*Pediococcus*	4.1	4.5	0.1	0.2	0.9	0.9	0.0	0.0
16	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	1.2	1.8	0.9	0.7	0.4	0.6	2.5	2.3
17	*Paracoccus*	0.3	0.4	0.8	1.0	1.7	0.8	1.7	0.8
18	*Clostridium sensu stricto 7*	2.8	0.7	0.1	0.2	1.4	2.1	0.1	0.1
19	*Delftia*	0.3	0.4	0.3	0.4	1.0	0.7	2.5	3.0
20	*Pseudomonas*	0.5	0.4	1.0	1.9	1.5	1.9	1.1	2.2
21	*Clostridium sensu stricto 18*	2.1	1.4	0.0	0.1	1.4	1.5	0.0	0.1
22	*Carnobacterium*	1.9	0.8	0.3	0.3	1.1	1.0	0.1	0.2
23	*Bosea*	1.6	1.4	0.3	0.6	1.6	0.8	0.7	0.4
24	*Diaphorobacter*	0.3	0.4	0.4	0.8	1.1	0.7	0.9	1.1
25	*f__Rhizobiaceae_OTU_81*	0.2	0.3	0.0	0.1	0.8	1.2	1.5	1.3
26	*Enhydrobacter*	0.2	0.2	0.1	0.2	1.4	1.3	0.8	0.7
27	*Bacillus*	0.6	0.3	0.8	0.7	0.4	0.6	0.7	0.7
28	*f__Xanthomonadaceae_OTU_65*	0.1	0.2	0.9	1.8	1.3	0.4	1.1	1.7
29	*Peptoniphilus*	1.1	0.8	0.0	0.1	1.0	0.9	0.2	0.3
30	*Leadbetterella*	0.1	0.1	0.7	1.6	0.0	0.1	1.4	3.2

Supplementary Table 11. Top five most abundant phyla 33 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples. Differences are tested by Kruskal-Wallis. When significant, differences are presented with different letters in red. (**B**) P-values adjusted for multiple comparison are presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection. Significant P values are in bold.

A

% mean abundance at phylum level

Feed group	Dpi	Firmicutes		Proteobacteria		Actinobacteria		Bacteroidetes		Cyanobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	33	32.1	23.3	37.9	13.3	23.4	9.5	0.9	0.5	2.9 [ab]	2.4
An	33	29.9	21.5	40.8	16.8	23.3	13.6	0.5	0.3	2.0 [ab]	1.6
PI	33	37.3	19.7	30.3	15.3	26.4	11.9	0.8	0.4	1.6 [ab]	1.9
PS	33	65.0	10.9	18.8	9.4	10.5	2.7	1.0	1.0	3.1 [ab]	1.4
C/Fp	33	25.9	25.7	39.1	19.1	25.1	12.8	3.8	5.6	0.5 [a]	0.5
An/Fp	33	10.7	8.1	54.5	10.8	22.6	7.2	1.3	1.2	4.8 [ab]	5.6
PI/Fp	33	36.9	29.8	41.4	20.2	15.8	6.4	0.5	0.4	0.6 [ab]	1.4
PS/Fp	33	49.0	2.7	31.8	7.5	9.8	9.0	0.8	0.7	6.4 [b]	3.0

B

P-values adjusted for multiple comparison

	Firmicutes	Proteobacteria	Actinobacteria	Bacteroidetes	Cyanobacteria
C vs An	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs PI	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs PS	0.3602	0.2974	0.2282	>0.9999	>0.9999
C vs. C/Fp	>0.9999	>0.9999	>0.9999	>0.9999	0.8613
C vs. An/Fp	0.8167	>0.9999	>0.9999	>0.9999	>0.9999
C vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	0.7317
C vs. Ps/Fp	>0.9999	>0.9999	0.1211	>0.9999	0.7317
C/Fp vs. An/Fp	>0.9999	0.9100	>0.9999	>0.9999	0.3716
C/Fp vs. PI/Fp	>0.9999	>0.9999	>0.9999	0.6271	>0.9999
C/Fp vs. Ps/Fp	>0.9999	>0.9999	0.0611	>0.9999	**0.0093**
An vs. An/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
PI vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
PS vs. Ps/Fp	0.9100	>0.9999	>0.9999	>0.9999	>0.9999

Supplementary Table 12. Top seven most abundant classes 33 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). (**A**) Values represent the mean and SD of five samples. Differences are tested by Kruskal-Wallis. (**B**) P-values adjusted for multiple comparison are also presented. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection.

% mean abundance at class level

Feed group	Dpi	Bacilli		γ-proteobacteria		Actinobacteria		α-proteobacteria		Clostridia		Bacteroidia		Oxyphotobacteria	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
C	33	24.0	19.6	17.6	7.2	22.4	10.2	20.2	17.7	8.1	7.2	0.9	0.5	2.9 [ab]	2.4
An	33	22.2	16.3	10.5	4.0	21.7	14.2	30.2	17.7	7.5	5.5	0.5	0.3	2.0 [ab]	1.6
PI	33	28.8	14.4	13.4	9.1	24.5	12.2	16.9	17.1	8.4	6.0	0.8	0.4	1.6 [ab]	1.9
PS	33	48.9	7.2	11.9	5.8	7.4	2.9	6.9	4.8	15.9	3.8	1.0	1.0	3.1 [ab]	1.4
C/Fp	33	20.0	19.7	17.0	17.3	23.7	13.5	22.0	24.5	5.7	5.9	3.8	5.6	0.5 [a]	0.5
An/Fp	33	6.8	5.4	12.1	10.2	21.7	7.1	42.6	19.3	3.8	2.9	1.3	1.2	4.8 [ab]	5.6
PI/Fp	33	26.6	21.4	19.6	11.3	23.6	6.1	22.7	23.8	10.7	9.0	0.5	0.4	0.6 [ab]	1.4
PS/Fp	33	32.8	3.9	19.5	5.5	8.6	8.3	11.9	6.3	16.1	2.5	0.8	0.7	6.4 [b]	3.0

A

P-values adjusted for multiple comparison

	Bacilli	γ-proteobacteria	Actinobacteria	α-proteobacteria	Clostridia	Bacteroidia	Oxyphotobacteria
C vs An	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs PI	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
C vs PS	0.2282	>0.9999	0.1615	>0.9999	0.5839	>0.9999	>0.9999
C vs. C/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.8613
C vs. An/Fp	0.8167	>0.9999	>0.9999	0.7321	>0.9999	>0.9999	>0.9999
C vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.7317
C vs. Ps/Fp	>0.9999	>0.9999	0.1302	>0.9999	0.408	>0.9999	0.7317
C/Fp vs. An/Fp	>0.9999	>0.9999	>0.9999	0.4453	>0.9999	>0.9999	0.3716
C/Fp vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.6271	>0.9999
C/Fp vs. Ps/Fp	>0.9999	>0.9999	0.0769	>0.9999	0.0611	>0.9999	**0.0093**
An vs. An/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
PI vs. PI/Fp	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
PS vs. Ps/Fp	0.5301	0.663	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

B

Supplementary Table 13. Top-30 most abundant genera 33 dpi in the feed groups (note that fish in the control and antibiotic groups are fed with non-treated feed at this time point). Values represent the mean and SD of five samples. C: control feed group; An: antibiotic feed group; PI: phage-immobilized feed group; PS: phage-sprayed feed group. C/Fp: control feed group + *F. psychrophilum*; An/Fp: antibiotic feed group+ *F. psychrophilum*; PI/Fp: phage-immobilized feed group + *F. psychrophilum*; PS/Fp: phage-sprayed feed group + *F. psychrophilum*. Dpi: days post infection. Light blue = Firmicutes; Blue = Proteobacteria; Pink = Actinobacteria; Green = Cyanobacteria; Purple = Patescibacteria.

No.	Genus	C		AN		PI		PS		C/Fp		AN/Fp		PI/Fp		PS/Fp	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
1	*Rhodococcus*	16.8	13.1	18.4	13.6	17.2	10.4	2.3	1.1	16.0	13.4	18.0	7.1	8.6	7.1	6.3	7.5
2	*Lactobacillus*	12.2	15.7	3.5	4.5	9.1	5.8	15.6	6.0	7.6	7.2	1.3	1.2	9.8	8.1	10.7	1.8
3	*Pseudorhodobacter*	0.7	1.3	1.7	1.8	11.0	15.1	0.0	0.0	14.7	23.7	17.7	22.4	10.7	12.1	0.0	0.1
4	*Photobacterium*	3.1	3.0	3.6	1.9	1.4	1.8	3.7	1.8	1.0	1.0	6.0	8.1	0.8	1.1	10.5	6.3
5	*Pediococcus*	0.1	0.1	7.6	6.6	3.5	4.9	10.0	9.7	0.9	0.7	1.7	1.6	2.1	2.7	3.1	3.4
6	*f__Mitochondria_ OTU_12*	2.2	2.6	1.2	1.1	1.0	0.8	1.7	0.9	0.7	0.9	10.3	14.1	0.6	0.8	7.9	7.2
7	*Bosea*	4.5	4.8	9.3	8.7	1.3	1.6	0.4	0.3	0.4	0.5	1.5	0.7	5.1	7.2	0.1	0.1
8	*Vagococcus*	1.4	1.5	2.3	2.3	3.6	1.9	5.0	2.0	2.7	3.0	0.9	0.6	2.6	2.5	2.8	1.7
9	*Weissella*	1.2	1.6	0.5	0.6	3.5	1.9	5.0	1.4	2.0	2.1	0.1	0.1	2.6	3.2	4.6	2.3
10	*Phreatobacter*	8.8	11.5	7.0	6.1	0.6	0.7	1.1	1.3	0.3	0.3	0.2	0.1	1.2	1.9	0.0	0.0
11	*Acinetobacter*	1.8	1.7	1.5	1.4	3.5	3.1	1.7	1.7	5.1	6.3	1.5	1.5	1.7	1.1	2.4	1.5
12	*Clostridium sensu stricto 7*	2.3	1.5	1.7	1.8	1.6	1.5	3.4	1.3	1.4	1.4	0.7	0.6	3.1	3.5	4.9	1.7
13	*Streptococcus*	2.4	2.3	1.2	0.7	2.1	1.0	2.7	1.2	2.2	2.3	0.8	0.8	2.9	2.9	4.3	2.0
14	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	0.9	1.0	8.6	6.9	0.3	0.6	0.4	0.6	0.5	0.7	6.2	3.6	0.2	0.3	0.9	0.5
15	*f__Burkholderiaceae_ OTU_7*	1.7	1.8	1.1	1.1	2.0	1.7	1.1	1.4	3.3	3.9	1.5	1.7	1.8	1.6	2.2	1.8
16	*Carnobacterium*	0.8	0.8	1.5	1.2	2.3	1.5	3.4	2.0	1.4	1.8	0.4	0.3	1.5	1.3	0.9	0.9
17	*Tepidimicrobium*	1.3	2.0	1.0	0.8	1.5	1.3	2.7	0.3	0.8	1.0	0.4	0.4	1.6	1.5	2.7	2.5
18	*f__Microbacteriaceae_ OTU_47*	1.1	1.0	1.4	1.7	3.9	2.6	0.6	0.9	2.6	4.1	1.7	1.1	0.5	0.7	0.4	0.4
19	*Thermomonas*	2.9	4.3	0.5	0.5	1.9	1.7	0.5	0.6	1.8	1.6	1.4	1.5	1.4	1.3	1.0	1.0
20	*o__Chloroplast_ OTU_27*	1.3	0.8	0.6	0.3	0.5	0.7	1.1	0.6	0.3	0.3	3.5	5.2	0.0	0.0	4.1	2.1
21	*o__Chloroplast_ OTU_35*	1.5	1.5	1.4	1.6	1.1	1.6	2.0	0.9	0.2	0.2	1.1	1.0	0.1	0.1	2.1	1.8
22	*Clostridium sensu stricto 18*	0.8	1.0	0.6	0.4	1.2	1.0	2.7	0.9	0.9	1.1	0.4	0.4	0.9	1.3	1.7	0.8
23	*f__Coriobacteriales Incertae Sedis_OTU_23*	0.6	0.7	1.1	1.4	0.9	0.6	2.0	1.2	0.9	1.1	0.6	0.7	1.1	1.0	0.7	0.7
24	*Stenotrophomonas*	1.5	2.6	0.3	0.2	0.9	1.0	0.9	1.0	1.2	1.2	0.1	0.1	2.4	2.7	0.3	0.1
25	*Enhydrobacter*	0.3	0.4	1.1	0.1	0.2	0.2	0.2	0.3	0.4	0.3	0.1	0.1	6.0	12.7	0.5	0.6
26	*Paracoccus*	0.4	0.6	0.1	0.1	0.9	1.0	0.3	0.6	2.2	3.7	0.3	0.3	1.6	1.9	0.2	0.2
27	*Bacillus*	0.5	0.8	0.4	0.5	0.6	0.7	1.8	0.6	0.5	0.6	0.1	0.1	0.3	0.3	1.9	1.3
28	*f__Saccharimonadaceae_ OTU_50*	0.8	0.7	1.2	0.9	1.0	0.4	0.1	0.2	0.8	0.3	1.2	1.0	0.7	0.8	0.2	0.2
29	*Lactococcus*	2.1	3.4	0.4	0.4	0.7	0.5	0.9	0.4	0.4	0.4	0.2	0.2	0.7	0.9	0.4	0.2
30	*Pseudomonas*	0.4	0.5	1.3	3.5	0.1	0.1	0.4	0.6	1.3	2.3	0.1	0.1	0.8	1.1	0.3	0.4

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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 at first established and the effects of singular phages on fish eggs was assessed (survival of eyed eggs; interaction of phages with eyed eggs). Subsequently, bacterial-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 (24h) 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 pathogens transmission [7].

The transmission of the freshwater pathogen *Flavobacterium psychrophilum* [8], [9] 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 as *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 [16]. The implementation of good husbandry management and eggs disinfection has been highlighted as methods to reduce the development of *F. psychrophilum* infections among fish in hatcheries [12].

The utilization of virulent bacteriophages [17] to reduce mortalities and prevent the spread of bacterial populations among fish and crustacean at different stages has gained increased attention (reviewed by [18], [19]). 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 [20]).

Previous studies of phage control of *Flavobacterial* pathogens in rainbow trout have focused on fry and juvenile stages [21]–[25]. Here we report for the first time on 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, a) we established a bacterial challenge bath method (Section A) and b) evaluated the effects of phage addition on eyed eggs (Section B). Subsequently, c) 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], [26]–[28] while *F. psychrophilum* 160401-1/5N was isolated in 2016 and recently characterized (serotype Th, virulent) [28]. 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 [24], [28]. The strains were stored at – 80°C in tryptone yeast extract salts medium (TYES: 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2 \times 2H_2O$, 0.05% $MgSO_4 \times 7H_2O$ (pH 7.2))[29] 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 hours (15°C; 100 rpm) and then streaked on 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 hours [24]. For bath challenge experiments, the selected bacteria were prepared according to [27]. Specifically, 0.5 ml of a 72-hour bacterial culture (5 mL) were transferred into 100 ml TYES-B and incubated at 15°C. After 48 hours of incubation, appropriate dilutions depending on the selected dose of infection were performed prior to 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 [30]. B185 was isolated from rearing tank water in 2008 [31]. Bacterial cultures have been stored frozen at -80 ℃ with 10 % glycerol and 10 % fetal calf serum. For the experiments, bacteria were revived from -80 ℃ by inoculation into 5 ml of Shieh medium [32] and cultured overnight at 25 ℃ 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) and colony forming units per ml (CFU ml^{-1}) estimated based on our previously determined OD-CFU relationship.

2.2 Bacteriophages

F. psychrophilum-targeting lytic bacteriophages FpV4 and FPSV-D22 were selected for the studies. FpV4, isolated in 2005 from water with feces samples, belongs to the *Podoviridae* family [33], [34]. FPSV-D22, isolated in 2017 from fish tissue samples from a freshwater Danish rainbow trout farm, belongs to the *Siphoviridae* family [24], [28]. Solutions of FpV4 and FPSV-D22 were purified (0.2 μm-pore size sterile filter) and stored in SM buffer (8 mM MgSO4, 50 mM Tris-Cl [pH 7.5], 99 mM NaCl, 0.01% gelatin) and glycerol (15%) at -80°C [28], [33]. For the experiments in section B, phage high titer solutions were prepared from crude lysates following 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 an incubation of 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). For the experiment 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 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 [24], [35].

Two previously isolated *Myoviridae* phages infecting *F. columnare* were used in the experiments: FCL-2 and FCOV-F27. Both originate from fish farms in Finland, and have been isolated from tank water during columnaris disease outbreaks. FCL-2 has been isolated in 2008, and infects *F. columnare* strains belonging to genetic group G [31]. The phage has previously been shown effective against columnaris infections in rainbow trout [25]. FCOV-F27 (isolated in 2017) infects hosts in genetic group C [30]. To test the interaction with rainbow trout eggs (section B, experiment III), crude lysates of each phage were produced, as described earlier [25]. 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, three-hundred ml of the crude lysate were 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 diafiltated by using ultrafiltration membranes (PES, 100 kDa pass) to completely remove or lower the concentration of salt, solvent and metabolites by exchanging three times the volume of the lysate to 0,9% NaCl.

2.3 Rainbow trout eyed eggs

Rainbow trout (*Oncorhynchus mykiss*) eyed eggs (>200 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, where after the eggs were disinfected according to standard procedures done at Danish rainbow trout hatcheries (10-15 minutes 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.

For the experiments concerning *F. columnare* and its phages, rainbow trout eyed eggs (>200 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 minutes treatment), cold-transported to the lab, and used immediately in the experiments. Before the start of an experiment, six eggs were sampled for presence of *F. columnare* and its phages and found negative.

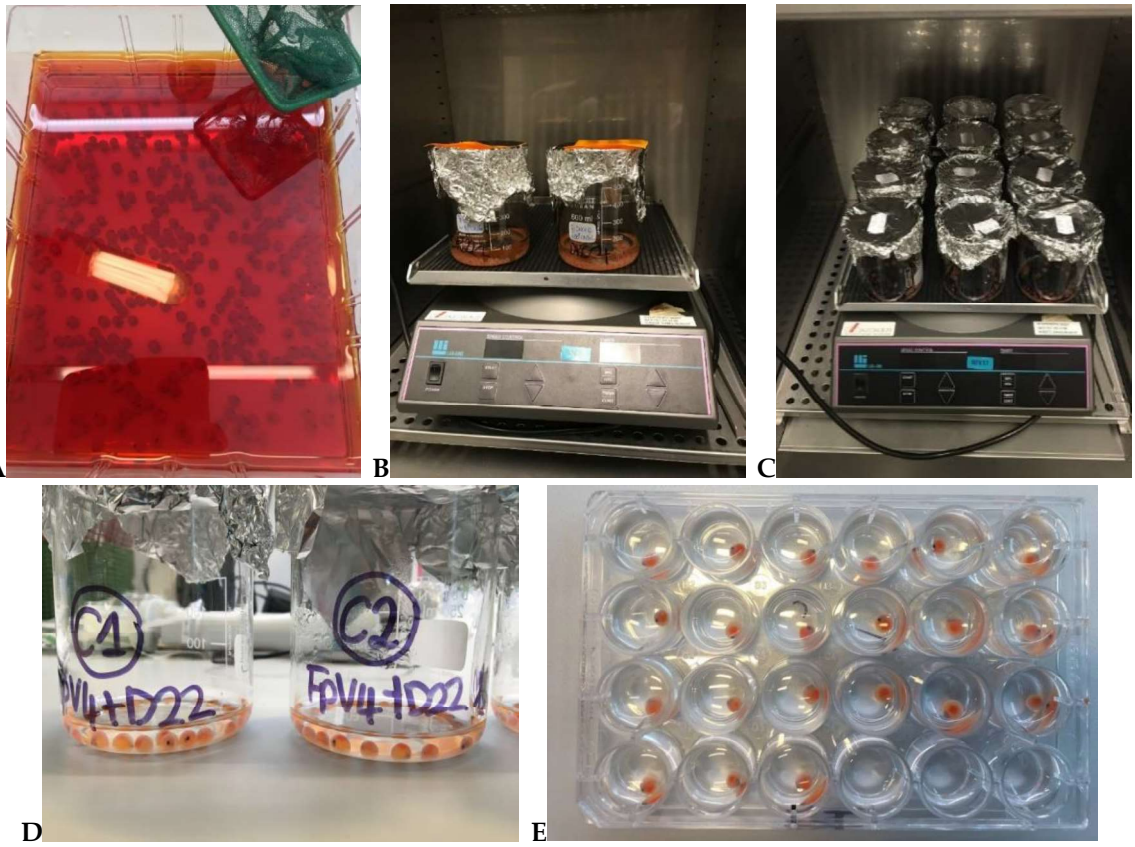


Figure 1. Illustrated overview of the experimental procedure followed in the various experiments concerning *F. psychrophilum* and its phages. (**A**) Disinfection of eyed eggs following standard procedures used in hatcheries facilities (iodine-based solution) performed at the start of each experiment; (**B**) Eyed eggs during the two-hour bacterial bath challenge with *F. psychrophilum* and incubation at 10ºC (experiments section A and C); (**C**) Eyed eggs during phage bath and incubation at 10ºC (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).

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 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 eggs' survival.

F. psychrophilum 950106-1/1 was chosen for these three preliminary experiments. Disinfected eyed eggs were placed in 500 ml sterile glass beakers containing either 200 ml of 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 hours at 10°C and 80-90 RPM (**Figure 1B**). After the bath challenge procedure, eyed eggs were moved to sterile 24-well plates (one egg per well) containing 2 ml of sterile TYES-B (Exp. no. 1), sterile Milli-Q water (Exp. no. 2) or sterile SM buffer 10 times diluted in Milli-Q water (Exp. no. 3). Eyed eggs were transferred using sterile 10µl inoculation loops (**Figure 1E**). The plates were covered with lids and incubated at 10°C (at 80-90 RPM) for 24 hours. In Exp. no. 1, three eggs were sampled at 1, 3, 21 and 25 hours 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 hours of incubation. Exp. no. 1 was performed in December

2018, Exp. no. 2 in Maj 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-hour bacterial culture (5 mL) were transferred into either 100 ml of Milli-Q water, 100 ml of water from fish experimental facilities or sterile TYES-B and incubated at 15°C. The experiment was performed in duplicates and CFU count was performed at various time points.

In 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, or non-aerated sterile distilled water; 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™) 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 *F. columnare* strain B480 by adding 10 µl of overnight culture ($1.0*10^8$ CFU ml^{-1}) directly in the wells, giving a final density of $5.0*10^5$ CFU ml^{-1}. Survival of eggs (embryo movement and blood flow observed under a light microscope) was followed in 24-hour intervals for 144 hours except for the experiments performed at 20°C which were carried out until 72 hours. In 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 hours.

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 interacted 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**.

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-hour 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-hour bath	FpV4 and FPSV-D22 (mixed 1:1)	PEG-purified in SM buffer
Exp. II Section C	Yes	2-hour bath; constant	FCL-2	Diafiltration

2.5.1 Constant exposure of eyed eggs to *F. psychrophilum* bacteriophages (Exp. I, section B)

In this experiment (performed in April 2019), 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. 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 10µl 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, plates were incubated at 10°C at 80-90 RPM. After 2, 27, 49 and 71 hours of incubation, three eggs and their correspondent well content per group were collected. At 144 hours 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.

2.5.2 Bath exposure of eyed eggs to *F. psychrophilum* bacteriophages (Exp. II, section B)

In this experiment (performed in April 2019), eighty-one rainbow trout eyed eggs were bathed for 4 hours 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-wells plates (24 eggs per group) with 2 ml sterile Milli-Q water (one egg per well) using sterile 10µl inoculation loops. Plates were covered and incubated at 10°C at 80-90 RPM for 144 h (**Figure 1E**). At 0 hour and 24, 46 and 68 hours 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* bacteriophages (Exp. III, section B)

In this experiment, 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-minute 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, 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. The effects of two selected *F. psychrophilum* bacteriophages (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* bacteriophage FCL-2 in controlling *F. columnare* B185 were tested by either constant or bath phage exposures (Exp. II, section C). An overview of the experiments performed in this section is presented in **Table 1**.

2.6.1 Phage bath of *F. psychrophilum* challenged eggs (Exp. I, section C)

For this experiment (performed in June 2020), eyed eggs were at first bath challenged for 2 hours with one of two selected *F. psychrophilum* strains, then exposed to bacteriophages FpV4 and FPSV-D22 at two concentrations (phage bath no. 1 and phage bath no. 2) for 48 hours and finally transferred to individual wells for examination of phage and pathogen abundance (experimental set up presented in **Figure B1**).

Specifically, 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, 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 $2.2*10^9 \pm 1.6*10^9$ PFU ml^{-1} (phage bath no. 1) and diluted 10 times in SM buffer for the phage bath no. 2 (final concentration of $1.3*10^8 \pm 4.8*10^7$ PFU ml^{-1}). The selected volume (20 ml) was considered enough to cover the eggs during the incubation (**Figure 1D**). After a 48-hour 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 hours 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 B2**) or before the bacterial challenge (lower panel in **Figure B2**). 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 hours (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, 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 hours 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 hours (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-hour bath, eyed eggs were transferred in 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 in 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.

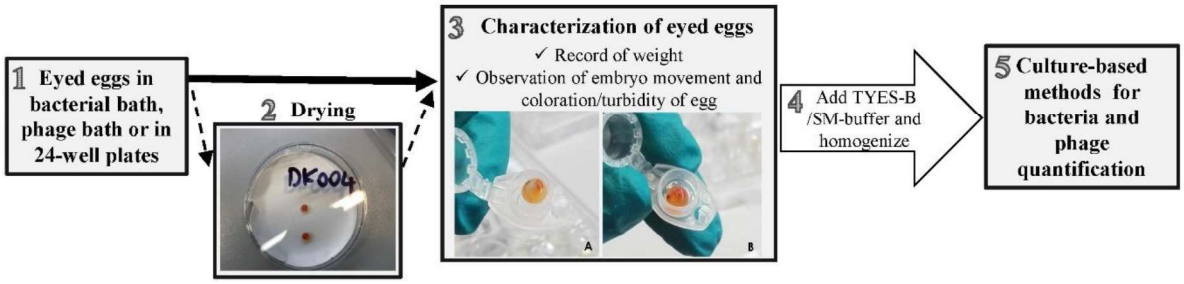


Figure 2. Graphical flow of the eyed eggs sampling procedure in relation to the experiments focused on *F. psychrophilum* and its phages. (**1**) Eyed eggs placed in the bacterial bath, the phage bath or in 24-well plates were sampled at the selected time points. (**2**) A drying step was included for 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 [36]–[38]. 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, Germany) and placed in pre-weighted sterile 1.5 ml micro tubes (SARSTEDT AG & Co. KG, 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) 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 [37]. Sampled eggs were then cut and fragmented with the use of sterile scissors, 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 stored for subsequent phage quantification (**Figure 2 step 5**).

During the 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 were added to the sampled eggs (**Figure 2 step 4**) and after the homogenization procedure, 5 µl of chloroform were added and samples stored for further phage analysis. For each sampled egg, the corresponding well content was also collected for phage analysis (300 µl of well content were placed in sterile 1.5 ml micro tube and 5 µl of chloroform were 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 15ºC and Blood-A plates at 20ºC 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 eggs status characterization (**Figure 2 step 3**), a fixed volume of sterile TYES-B (300-1000 µl in experiments of section A; 700µl 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 the Exp. I section C, 300 µl of the homogenized egg samples were transferred into new sterile 1.5 ml micro tubes (SARSTEDT AG & Co. KG, Germany) and 5 µl of chloroform were 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 15ºC

for 4-5 days and CFU per ml of solution were estimated. For the Exp. I section C, 300 µl of the well/bath content were also placed in new sterile 1.5 ml micro tubes, 5 µl of chloroform were 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 earlier mentioned (Exp. I section C; only the well content for experiments of section A). The growth of other bacteria 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 phages 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). A specific volume of Shieh medium was added (1:10 weight per volume) and the sample was mixed and centrifuged briefly to separate 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 4-6ºC with 1% chloroform). Finally, in the Exp. II section C, the eggs were not crushed, but were individually vortexed for 10 seconds in 400 µl of Milli-Q water, of which 100 µl were used to detect *F. columnare* (ten-fold dilutions plated on Shieh agar plates containing 1 µg ml^{-1} of tobramycin), and 150 µl were stored with chloroform for phages titration.

MALDI-TOF MS (Bruker) 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) [39].

2.8 Detection and quantification of bacteriophages

Bacteriophage detection for phages infecting *F. psychrophilum* was performed as described by [21], [22]. Egg and well content samples were centrifuged for 10s at 10.000 RPM at 5ºC to separate chloroform at the bottom of the tube and a phage spot method performed [40]. Four milliliters of TYES soft agar (0.4 % agar) mixed with 300 µl of a 48-hour old *F. psychrophilum* broth culture (in exponential phase) were poured into a TYES-A plate [22], [33]. Undiluted samples were then spotted in duplicate (section B) or triplicate (section C) (5 µl) on a bacterial lawn and incubated at 15ºC 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 10-fold diluted (180µl of SM buffer and 20µl of sample) in triplicates and re-spotted on a bacterial lawn as described above.

Bacteriophage quantification for phages infecting *F. columnare* was done as previously described by [25]. Three hundred microliters of an overnight-grown *F. columnare* were mixed with 3 ml of melted Shieh soft agar (0.7%) tempered to 47ºC, and poured on Shieh agar plates. Two microliters of ten-fold dilutions of the phage samples (in sterile Milli-Q water) were 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, California 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 built 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 in different media (**Figure 3, Table A1 and A2**). In addition, since the temperature optimum of *F. columnare* is between 22-29°C (depending on the strain) [41]while rainbow trout eyed eggs are normally incubated between 6 and 12°C [42], the effects of different temperatures on the eggs' survival were at first evaluated, also in combination with different media (**Table 2**).

In 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 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 hour 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 other bacteria than *F. psychrophilum* was recorded and it 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 at these conditions, but they remained viable for the tested time frame (15 days)**.**

In the experiments concerning *F. columnare* (**Table 2**), rainbow trout eyed eggs did not survive at 20 °C, and all movement was lost already 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.

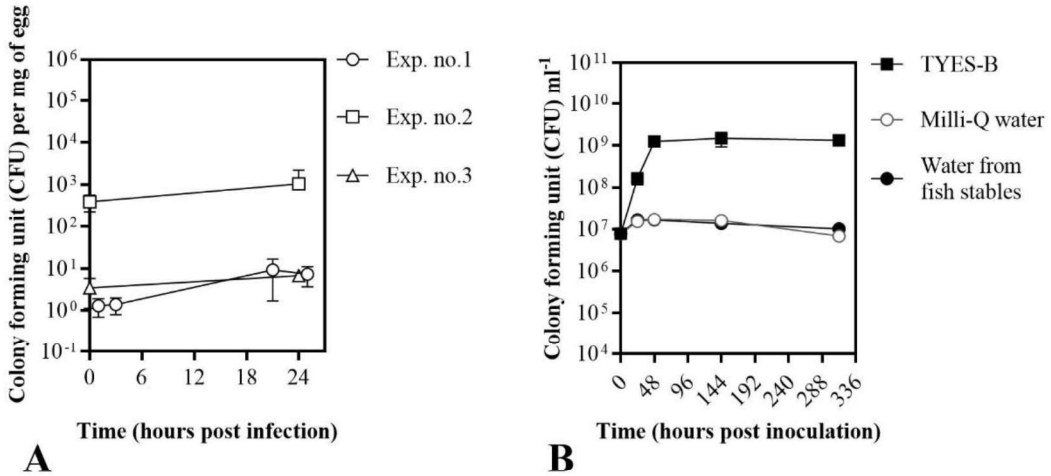
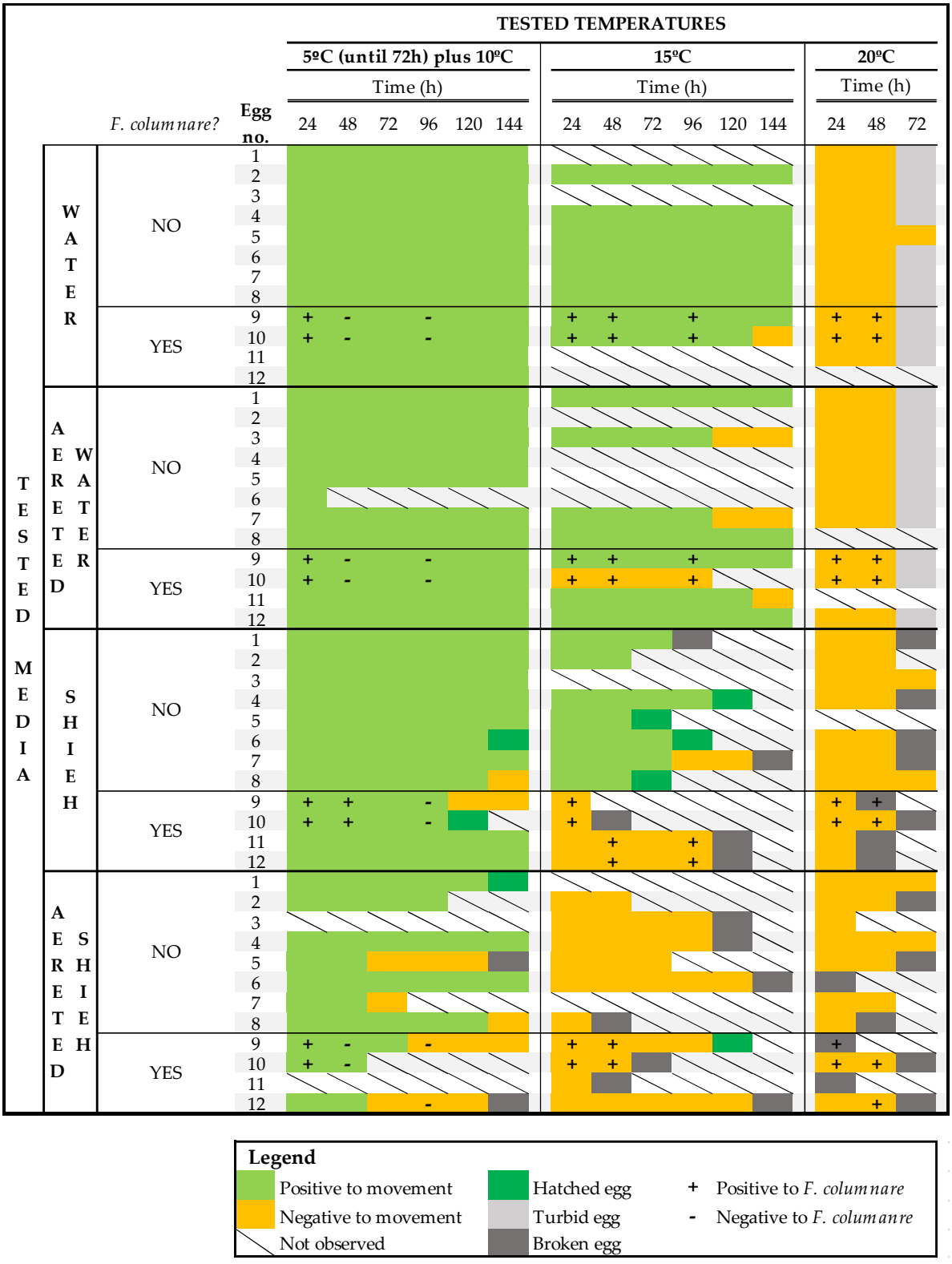


Figure 3. *F. psychrophilum* in connection with eyed eggs in three independent experiments (section A) (**A**) and *F. psychrophilum* growth in Milli-Q and filter-sterilized water from fish stables in comparison to TYES-B (**B**). In (**A**), values represent the mean and standard deviation of three 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.

Table 2. Survival of rainbow trout eggs at different temperatures with and without exposure to *F. columnare*, in either water or Shieh medium (Section A).



3.2 Interactions of phages with rainbow trout eyed eggs (section B)

3.2.1 Constant exposure of eyed eggs to *F. psychrophilum* bacteriophages (Exp. I, Section B)

The tested phages did not seem to negatively affect the eyed eggs survival (**Table 3A**). Sampled eggs were characterized as alive up to 49 and 71 hours 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 eyed 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 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 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 $3.2*10^3 \pm 2.2*10^2$ 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 different groups with one exception (**Figure 4A**): the concentration of FpV4 associated with the eyed eggs in the PEG-purified solution increased over time (adjusted $P = 0.0184$). No phages were detected in the control group.

To summarize, in this experiment 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.

Table 3. Exp. I and II, section B: survival of rainbow trout eyed eggs exposed to phage FpV4 and FPSV-D22. In **A**, characteristics of eyed eggs during constant phage exposure (Exp. I). In **B**, characteristics of eyed eggs after a 4-hour phage bath (**B**, Exp. II). FpV4 and FPSV-D22 were diluted in sterile TYES-B. In yellow: not clear if the egg is alive; in red: the egg is dead; 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

Legend

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

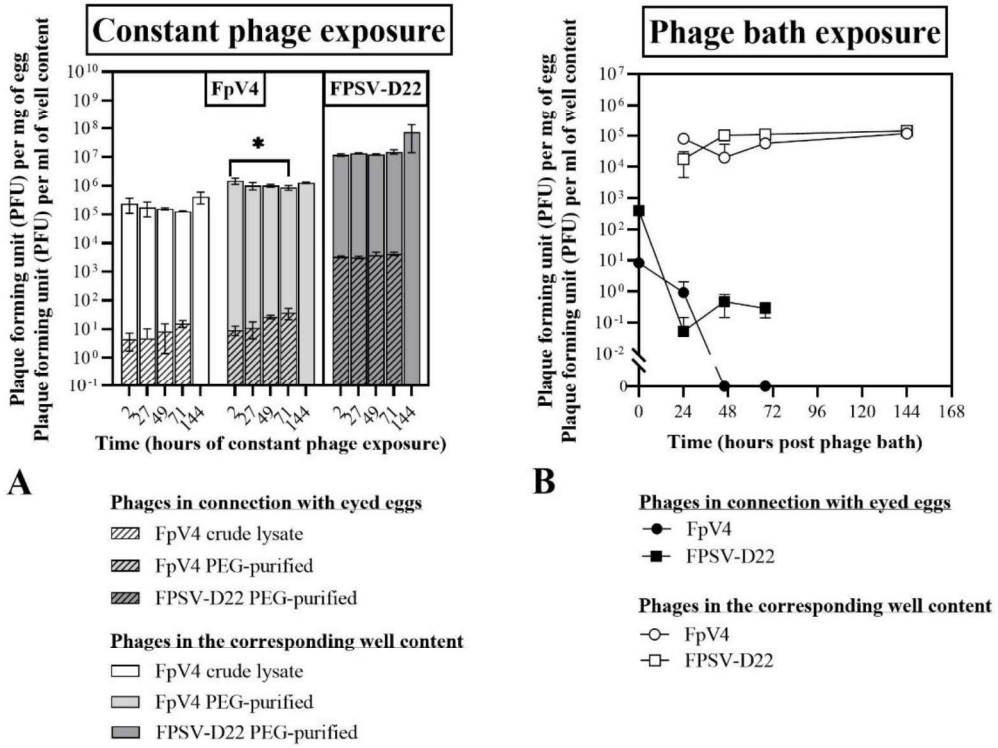


Figure 4. Exp. I and II, section B: phages associated with eyed eggs and in the corresponding well content (**A**) during constant exposure to phage FpV4 (crude lysate and PEG-purified solutions) and FPSV-D22 (PEG-purified solution) and (**B**) after a 4-hour bath exposure to phage FpV4 and FPSV-D22 ($1.9*10^7$ 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, phage 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**).

3.2.2 Bath exposure of eyed eggs to *F. psychrophilum* bacteriophages (Exp. II, Section B)

Similarly to what 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 3B)**. 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**).

The concentration of FpV4 and FPSV-D22 associated with the eyed eggs was 8.2 ± 0.7 PFU mg^{-1} of egg and $3.9*10^2 \pm 1.3*10^1$ PFU mg^{-1} of egg, respectively, at the end of the phage bath (**Figure 4B**). Subsequently, FpV4 phages were detected only after 24 hours (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 eggs dropped in the first 24 h (0.1 ± 0.1 PFU mg^{-1} of egg), subsequently it 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 sampled eggs, ranging from $8.0*10^4 \pm 1.3*10^4$ PFU ml^{-1} to $1.2*10^5 \pm 2.5*10^5$ PFU ml^{-1} (FpV4) and from $1.4*10^5 \pm 1.3*10^4$ PFU ml^{-1} to $1.7*10^4 \pm 1.3*10^4$ 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).

3.2.3 Bath and constant exposure of eyed eggs to *F. columnare* bacteriophages (Exp. III, Section B)

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

Phages could not be isolated from bath-treated eggs despite the high phage titers in the surrounding liquid (**Table 4**). Only few eggs were positive to FCL-2 and FCOV-F27 with a concentration ≤ 10^2 PFU egg^{-1}. Both phages (FCL-2 and FCOV-F27) could be isolated from the corresponding well content (water/Shieh medium) from 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 4**). 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 4. 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 "+" indicate 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 hours. 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 B1**). The results of this experiment are presented in **Figure 5, 6 and 7**. The time point at which the bacterial bath challenge is finalized is named as 0 hours post infection (hpi).

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 extend the bacterial cells and the phages were firmly attached to egg surface or rather 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, $1.9*10^2 \pm 3.2*10^1$ CFU mg^{-1} of egg were found using the standard procedure (S) at 0 hpi, and no significant loss of bacteria by the drying procedure was observed (S+D: $7.7*10^1 \pm 6.5*10^1$ CFU mg^{-1} of egg; $P = 0.0902$) (**Figure 5A**). This was also observed 24 hpi (**Figure 5C**). The bacterial concentrations were 0.3 ± 0.1 and $1.4*10^2 \pm 5.4*10^1$ CFU mg^{-1} of egg in SM buffer and Milli-Q water, respectively, but with no significant difference between S and S+D treatment. 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: $7.3*10^2 \pm 2.0*10^2$ CFU mg^{-1} of egg; S+D: $7.7*10^1 \pm 1.6*10^1$ 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 did not detached by the drying step. Also, 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 independently of the presence of the bacteria (**Figure 5B, C and 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 $2.9*10^1 \pm 2.6$ 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 S procedure compared to 0.1 ± 0.1 PFU mg^{-1} of egg for 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 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) (**Figure 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 (**Table B1**).

The concentration of bacteria per mg of egg was significantly reduced at 24 hpi in 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 case of the SM buffer-bath control ($P < 0.001$),

557 corresponding to a 15-fold reduction in egg-associated bacteria due to the phage treatment (**Figure**
558 **6A**). A similar effect of phage exposure was observed for F. *psychrophilum* 160401-1/5N at 24 hpi where
559 egg-associated bacteria were reduced from 3.6± 1.2 CFU mg^{-1} of egg in the SM buffer-bath control to
560 0.3 ± 0.2 CFU mg^{-1} of egg in the phage-bath exposure no. 1 (P = 0.0022, **Figure 6B**).
561 Also, phage exposure reduced the bacterial abundance 24 hpi in the bath content for both
562 bacteria (**Figure 6A and B**). For *F. psychrophilum* 950106-1/1 no bacteria was detected in the phage
563 bath no. 1 at 24 hpi whereas $1.1*10^3$ CFU ml^{-1} (n=1) were present in SM buffer-bath control (**Figure**
564 **6A**). Similarly, the abundance of strain 160401-1/5N was reduced from $1.4*10^4$ CFU ml^{-1} in the SM
565 buffer-bath control to 40.0 CFU ml^{-1} in phage bath no. 1 (n=1, **Figure 6B**). These findings support the
566 ability of FpV4 and FPSV-D22 to reduce the *F. psychrophilum* abundance at 24 hpi both on the egg
567 surface and in the surrounding water. However, this effect of phage exposure was only temporary as
568 no significant difference between the bacterial abundances of phage baths and SM buffer-bath
569 controls were observed at the next time points (48, 72 and 144 hpi). In addition, the growth and
570 stability of the bacteria seemed to be increasingly negatively affected by incubation in the SM buffer-
571 control bath compared to the Milli-Q water-control bath over time. As an example, at 72 hpi the
572 bacterial abundances detected in connection with eyed eggs was 0.1 ± 0.1 and $2.6*10^2 \pm 1.5*10^2$ CFU
573 mg^{-1} of egg for eyed eggs previously bathed in SM buffer and Milli-Q water, respectively, ($P < 0.0001$)
574 (bath-challenged eyed eggs with *F. psychrophilum* 950106-1/1). The bacterial concentration in the
575 corresponding wells containing the eggs was also significantly decreased (SM buffer bath-control:
576 $3.3*10^1 \pm 5.8*10^1$ CFU ml^{-1}; Milli-Q water-control bath: $2.1*10^6 \pm 8.4*10^5$ CFU ml^{-1}; $P < 0.05$). A similar
577 trend was observed for bath-challenged eyed eggs with *F. psychrophilum*160401-1/5N at 72 hpi
578 (bacteria associated with eyed eggs: 1.0 ± 0.8 CFU mg^{-1} of egg in the SM buffer bath-control and $6.0*10^2$
579 $\pm 2.0*10^2$ CFU mg^{-1} of egg in the Milli-Q water-control bath – P = 0.0027). This was also the case in the
580 corresponding wells where $7.3*10^1 \pm 4.6*10^1$ CFU ml^{-1} were found in the SM buffer bath-control
581 compared to $1.2*10^6 \pm 1.4*10^5$ CFU ml^{-1} in the Milli-Q water-control bath ($P < 0.0001$). The detection of
582 other bacteria/fungi than *F. psychrophilum* was observed during the CFU enumeration and recorded
583 (**Table B2**).
584 The concentration of the two bacterial strains associated with the bath-challenged eyed eggs at
585 24 and 48 hpi in the Milli-Q water-control bath varied significantly with *F. psychrophilum* 950106-1/1
586 occurring in 10-fold lower numbers ($1.4*10^2 \pm 5.4*10^1$ and $3.1*10^3 \pm 2.2*10^3$ CFU mg^{-1} of egg at 24 hpi
587 and 48 hpi, respectively) than strain 160401-1/5N ($1.2*10^3 \pm 7.8*10^2$ and $1.7*10^4 \pm 4.0*10^3$ CFU mg^{-1} of
588 egg at 24 and 48 hpi, respectively) (24 hpi: P = 0.0030; 48 hpi: P = 0.0080), suggesting different
589 adherence properties of the two strains (**Figure 6**).
590 As previously observed and mentioned in this results section (**Figure 5**), the phages FpV4 and
591 FPSV-D22 did not seem to tightly connect with surface of the eyed eggs in this experiment. Even if
592 the concentration of phages in connection with the eyed eggs was ~10^1 PFU mg^{-1} (Phage bath no. 1)
593 and ~10^0 PFU mg^{-1} (Phage bath no. 2) during the 48 h phage bath, very few eggs were positive to
594 phages in the next sampling points (72 and 144 hpi) (**Figure 7**). However, FpV4 and FPSV-D22 were
595 constantly detected over time and their concentration was maintained in the baths and the wells
596 independently of the presence of the bacteria.
597 To summarize, the findings of this experiment showed that the two selected *F. psychrophilum*
598 strains closely interact with the eyed eggs' surface but with different efficiencies. Further, exposure
599 of the challenged eggs to phages showed a 12- to 15-fold reduction in egg-associated bacteria for 24
600 h. However, the growth and stability of the bacteria were negatively affected in the SM buffer-bath
601 at all the time points, and the controlling effects of phages on the egg-associated bacteria was not
602 maintained beyond 24 h.
603

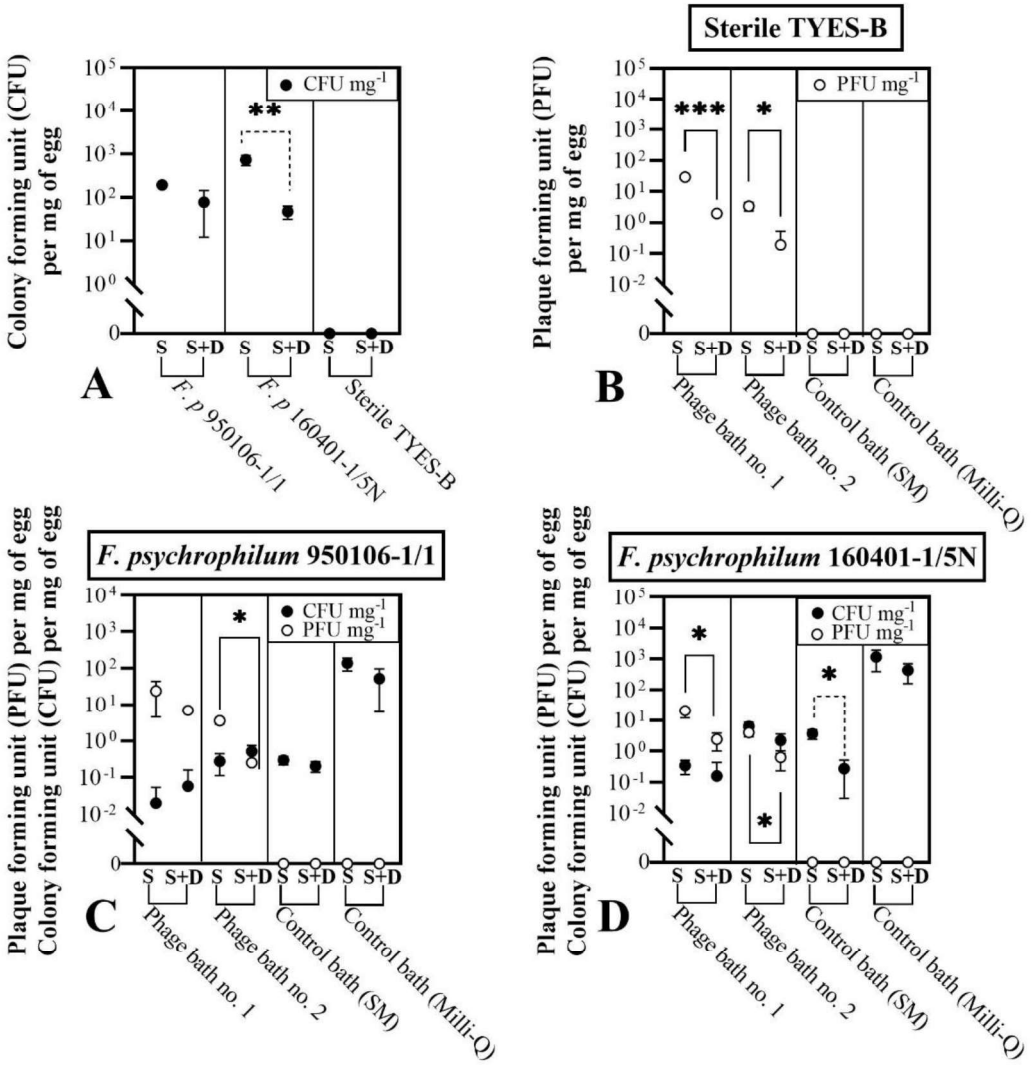


Figure 5. Exp. I, section C: effects of drying procedure on bacterial and phage concentrations in connection with eyed eggs. Comparison between standard sampling (indicated by "S") and sampling with the additional drying step (indicated by "S+D") for eyed eggs sampled at 0 hpi (**A,** right after the bath challenge) and at 24 hpi (**B**, **C**, **D**), 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}.

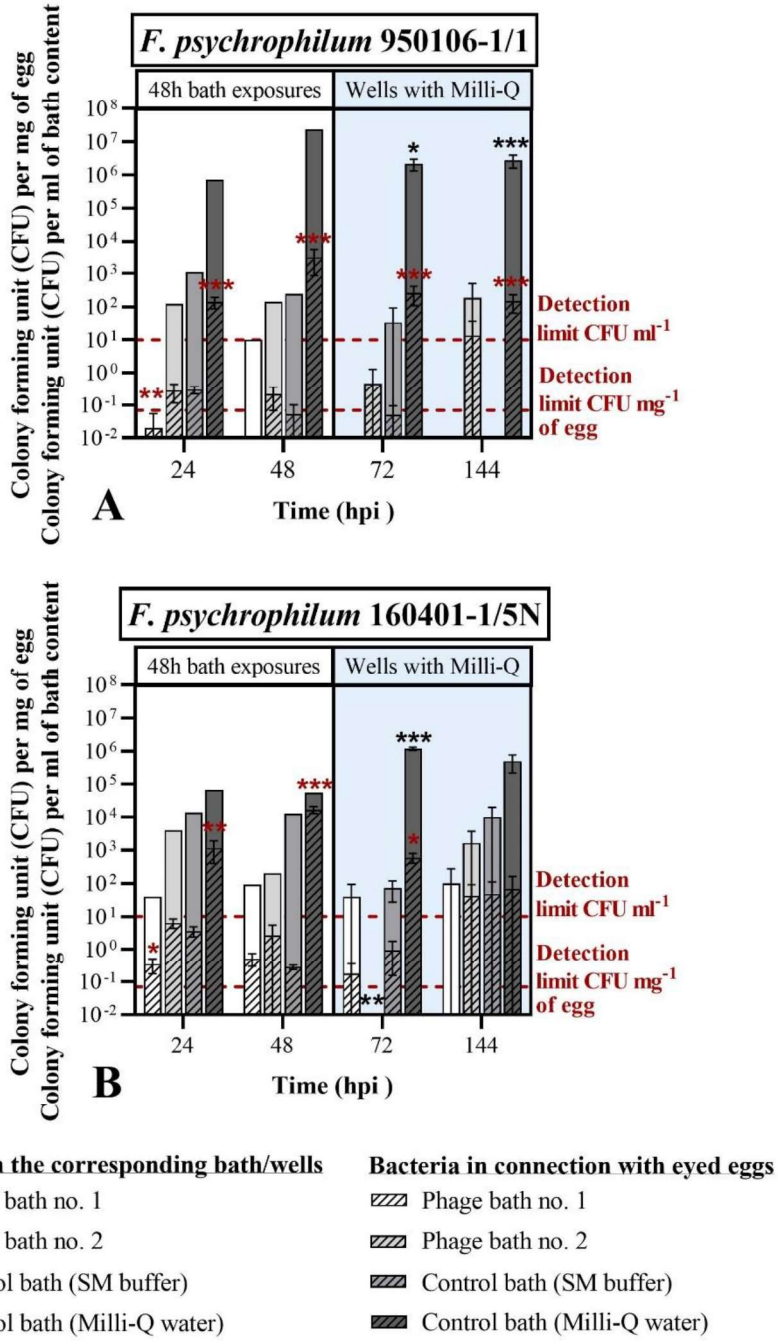


Figure 6. Exp. I, section C: *F. psychrophilum* 950106-1/1 (**A**) and *F. psychrophilum* 160401-1/5N (**B**) in connection with the eyed eggs and in the corresponding bath/wells. After the bacterial challenge, eyed eggs were bath-exposed to phages FpV4 and FPSV-D22 mixed 1:1 (Phage bath no. 1: 10^9 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 hours 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 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 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 eggs values; black: wells values) as follows: $P < 0.05$ (*), $P < 0.001$ (**), $P = 0.0001$ (***).

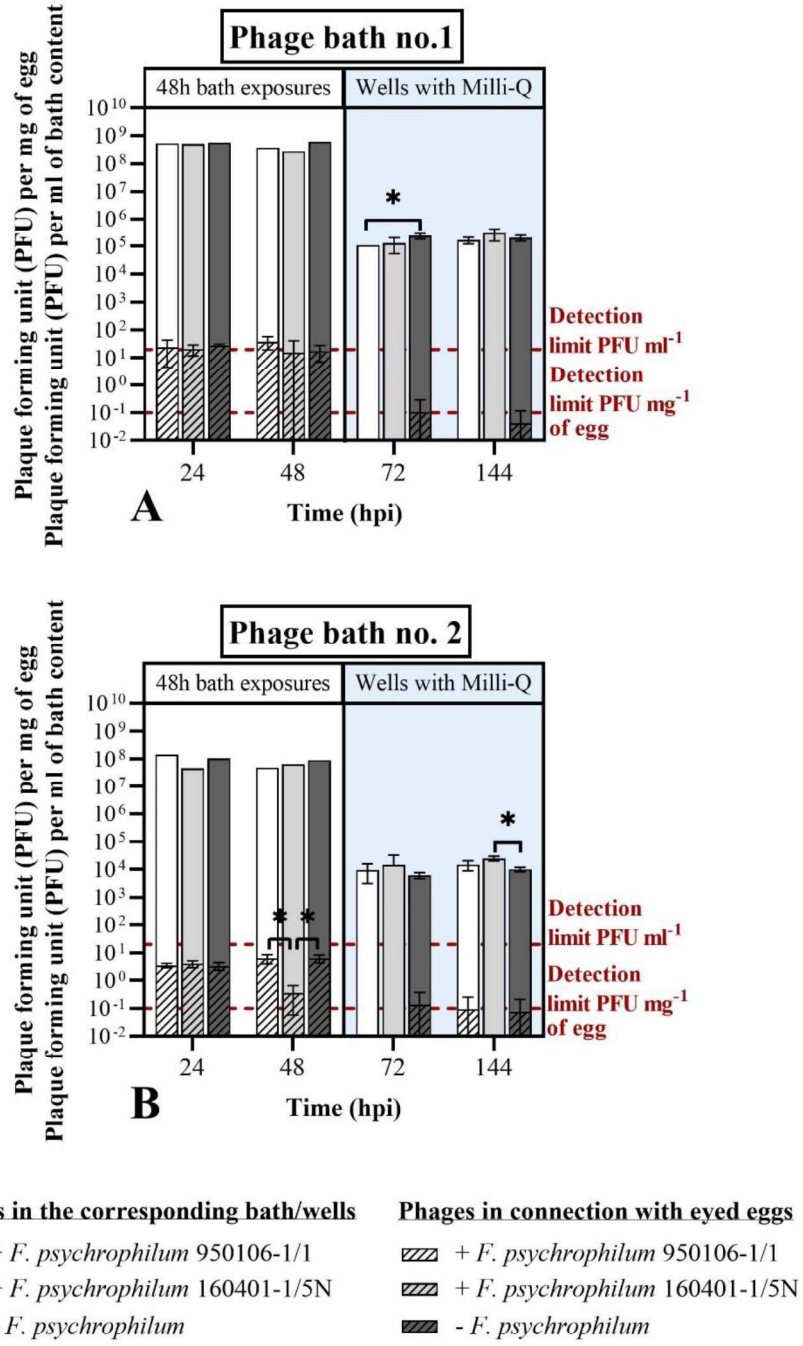


Figure 7. Exp. I, section C: phages FpV4 and FPSV-D22 in connection with the eyed eggs and in the corresponding bath/wells following phage bath exposures: (**A**) Phage bath no. 1 (10^9 PFU ml^{-1}) and (**B**) Phage bath no. 2 (10^8 PFU ml^{-1}). In the phage bath control groups (containing either SM 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 hours 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 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 with strain was used in the bacterial bath challenge.

3.3.2 Phage exposure of *F. columnare* challenged eggs (Exp. II, section C)

The effects of phage on bacteria associated with eggs and their immediate proximity was assessed also with *F. columnare* (experimental set up in **Figure B2**). As for *F. psychrophilum*, most of the eggs survived until the end of the experiments (**Figure 8**). Some eggs hatched during the experiment.

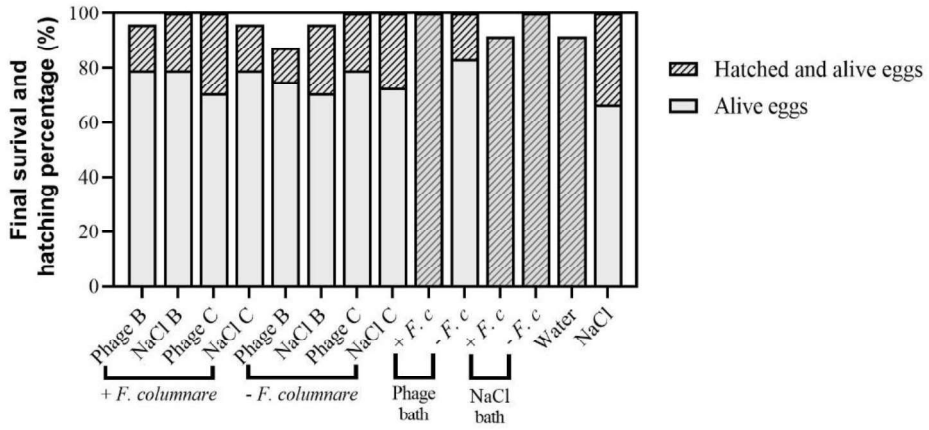


Figure 8. Exp. II, section C: survival and hatching percentage recorded at the end of the experiment (144 h). B: bath exposure, C: constant exposure, *F. c* = *F. columnare* B185.

In contrast to the experiments in section A (**Table 2**), *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 isolated from egg samples in low titers only right after phage bath exposure experiment (**Figure 9**). One should notice that, in this experiment, eggs were not homogenized as 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, independently of the presence of *F. columnare* (e.g., 48 h in case of bacterial challenge: $9.3*10^4 \pm 7.8*10^4$ PFU ml^{-1}). After phage bath, only in one of three wells was possible to detected phages at 0 and 48 h.

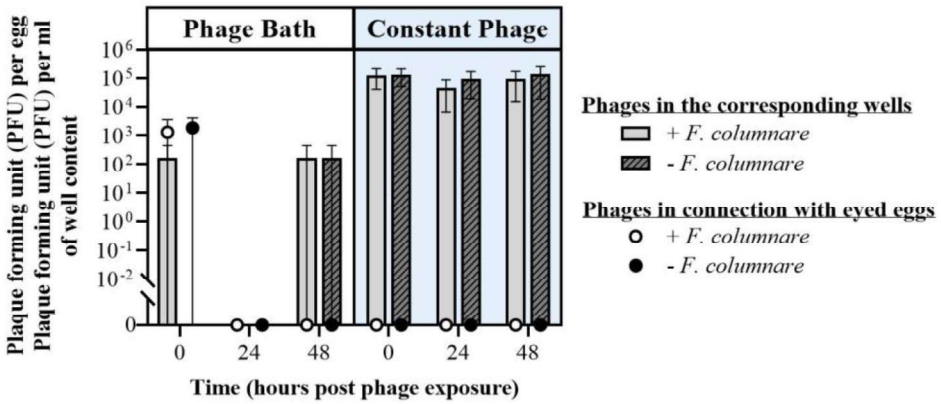


Figure 9. Exp. II, section C: phage FCL-2 in connection with the eyed eggs and in the corresponding wells following phage exposures (bath and constant) with and without bacterial challenge. In the phage control groups (sterile NaCl 0.09%; bath and constant experiments), phage FCL-2 was not detected neither in wells or eggs 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.

Experimental infection method

Although distant from the hatchery environment, the developed experimental set up allows 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 [43], in which no egg mortality was observed prior to hatching in bacterial 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 [43]. A different infection method was chosen by Ekman et al. [44], where the nano-injection of *F. psychrophilum* in the yolk of fertilized rainbow trout eggs was performed with the aim of mimicking vertical transmission of this pathogen. In this study, significantly higher mortality rates were observed for 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], [45].

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 [46], [47], where the concentration of *F. psychrophilum* in stream water and sterilized natural freshwater (measured by CFU count) remained stable for 116 days [46] and for 300 days [47], respectively. However, an initial drop in the bacterial concentration was detected in [47]. In [46], 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 [46]. Similar observations have been recorded for *F. columnare* [48]. Finally, it was previously observed that *F. psychrophilum* cannot survive in distilled [46] and in salt water (6%) [47].

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 temperature optimum does not match. While *F. columnare* grows well in high (above +18°C) temperatures, this is not a suitable temperature for egg viability. It is thus unlikely, that *F. columnare* would cause problems in rainbow trout hatchery conditions. However, since this pathogen is present in also warm countries and tropical fish [49], [50] and *F. columnare* has been previously found in association of Chinook salmon eggs [51], it remains relevant to study the association of bacteria and their phages in fish eggs. Indeed, *F. columnare* was isolated from all treatments (**Table 2**), suggesting potential interactions in the hatchery conditions where conditions favor the presence of this bacterium.

Growth of other bacteria/fungi 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 [52]. In addition, the lysis of bacterial cells caused by the phages releases nutrients and may stimulate the growth of other bacteria as suggested by [53]. However, bacterial growth other than *F. psychrophilum* was detected independently of phage presence in case of bacterial-challenged eggs (Exp. I section C, **Table B2**). Less detection of other bacteria than *F. psychrophilum* was observed in the control group for the bacterial infection. It is known that the iodine-disinfection, standard disinfection method for salmonid eyed eggs in hatcheries, does not create a sterile environment [51], [54]. However, the use of higher iodine concentrations than the one 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 [53], [55]. 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 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 effects on overall survival or the hatching rate of the eyed eggs and other factors, e.g. oxygen requirement (in salmonid eggs the hatching of the eggs happens faster in conditions of asphyxia) [56], may be influencing these parameters.

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 3 and 4**), as rainbow trout eyed eggs could tolerate the presence of bacteriophages under the tested conditions. These effects were observed for up to 71 hours when phages were diluted in TYES medium (Exp. I section B) and up to 144 hours 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. [55], 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 both after 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 already after 24 hours post phage-bath (**Figure 4B**) suggesting that phages do not tightly interact with the surface of the eggs. In particular, while a significantly 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* [24], [33], [57] 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 [58]. However, it is unclear if similar mucin glycoproteins are present on egg surfaces, which also is distinct to mucosal secretion of fish skin. Therefore, the phages may not strictly bind to the chorion, but rather survive in the surrounding environment. Nevertheless, presence of pathogen-targeting phages in the proximity of the eggs may prevent the bacterial infection after hatching.

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/wells 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 varies among strains [59], [60]. However, more studies are required to assess the potential of phage control on time scales beyond 24 h, using different incubation media which 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 [55], 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 hours of bath 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 a higher number [61]. 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 [28] 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.

Phages as control agents for F. columnare in eyed eggs

F. columnare has been found from eggs and ovarian fluids of chinook salmon (*Oncorhynhus tshawytscha*) [62], [63]. 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 (**Table 2**), *F. columnare* was not isolated from any of the medium or egg samples taken at any sampling time point in later experiments (**Table 4** and **Figure 9**). 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) [51], *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, adhesion, replication and virulence characteristics of this bacterial species are strongly dependent on temperature [16], [64], [65], and the lack of bacterial growth in our experiment hampers assessing the effect of phages on 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 48h. However, as mentioned above, while the temperature optimum of *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.

Conclusion

At 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 (24h) phage control of *F. psychrophilium* 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 interactions

between flavobacteria and their phages in connection with rainbow trout eyed eggs. For example, microscopy based methods to visualize the interactions could be used.

Author Contributions: Conceptualization, L.M., I.D., L.S. and M.M.; methodology, V.L.D., A.R., H.K., L.M., I.D., L.S. and M.M.; formal analysis, V.L.D.; investigation, V.L.D., H.K., A.R., J.J. and D.C.; data curation, V.L.D, A.R. and H.K.; writing—original draft preparation, V.L.D and L.S.; writing—review and editing, all authors; visualization, V.L.D.; supervision, L.M., I.D., L.S. and M.M.; project administration, L.M., I.D., L.S. and M.M.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

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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 recorder 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/wells. A plus symbol with 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 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 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.

Appendix B

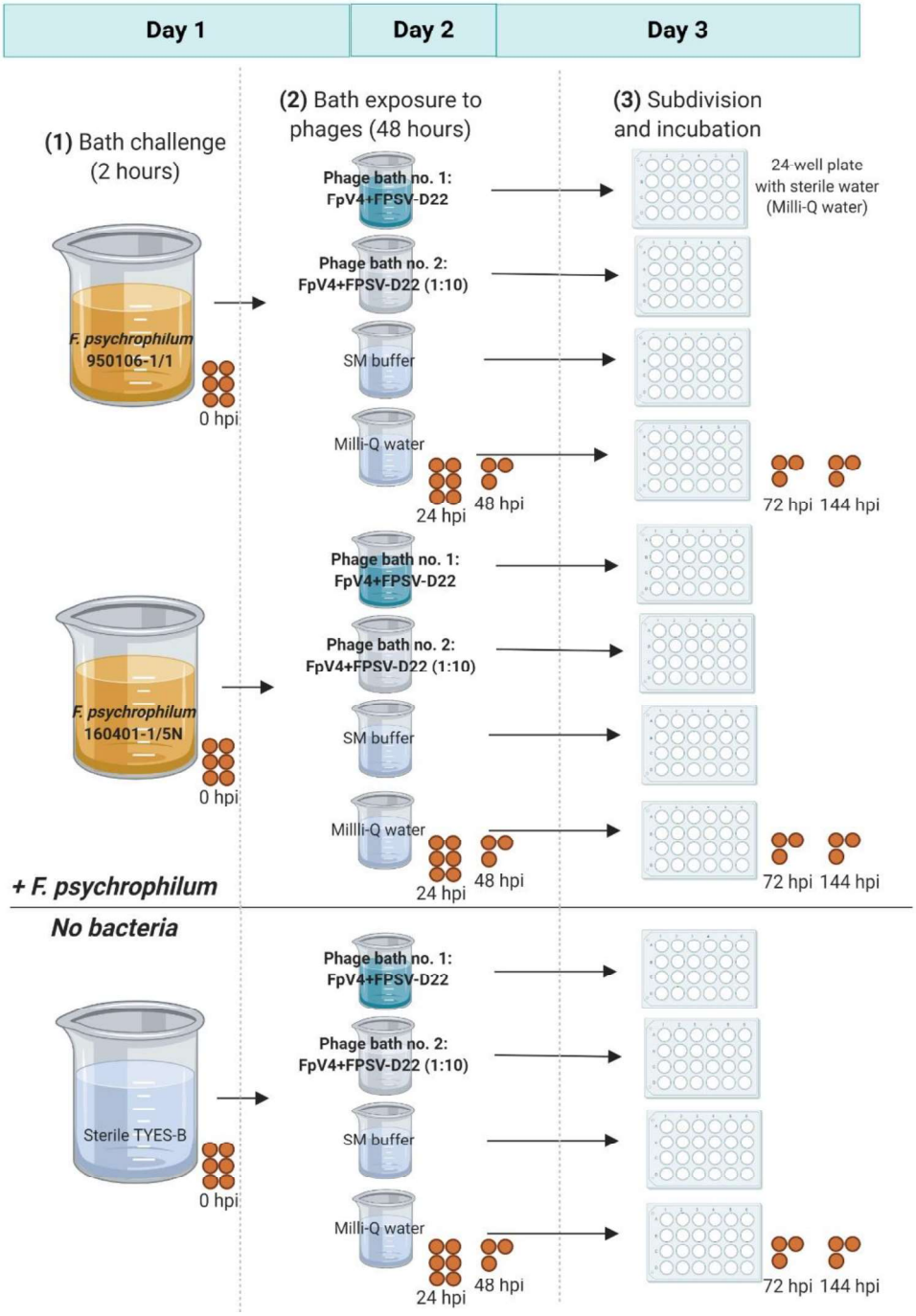

Figure B1. Exp. I section C: Experimental set up. (**1**) Rainbow trout eyed eggs were bath challenged for 2 hours with either *F. psychrophilum* strain 950106-1/1 or the strain 160401-1/5N ($2.0*10^6$ CFU ml^{-1}) while control eggs were placed in sterile TYES-B. The procedure was performed in 600 ml 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 hours 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.

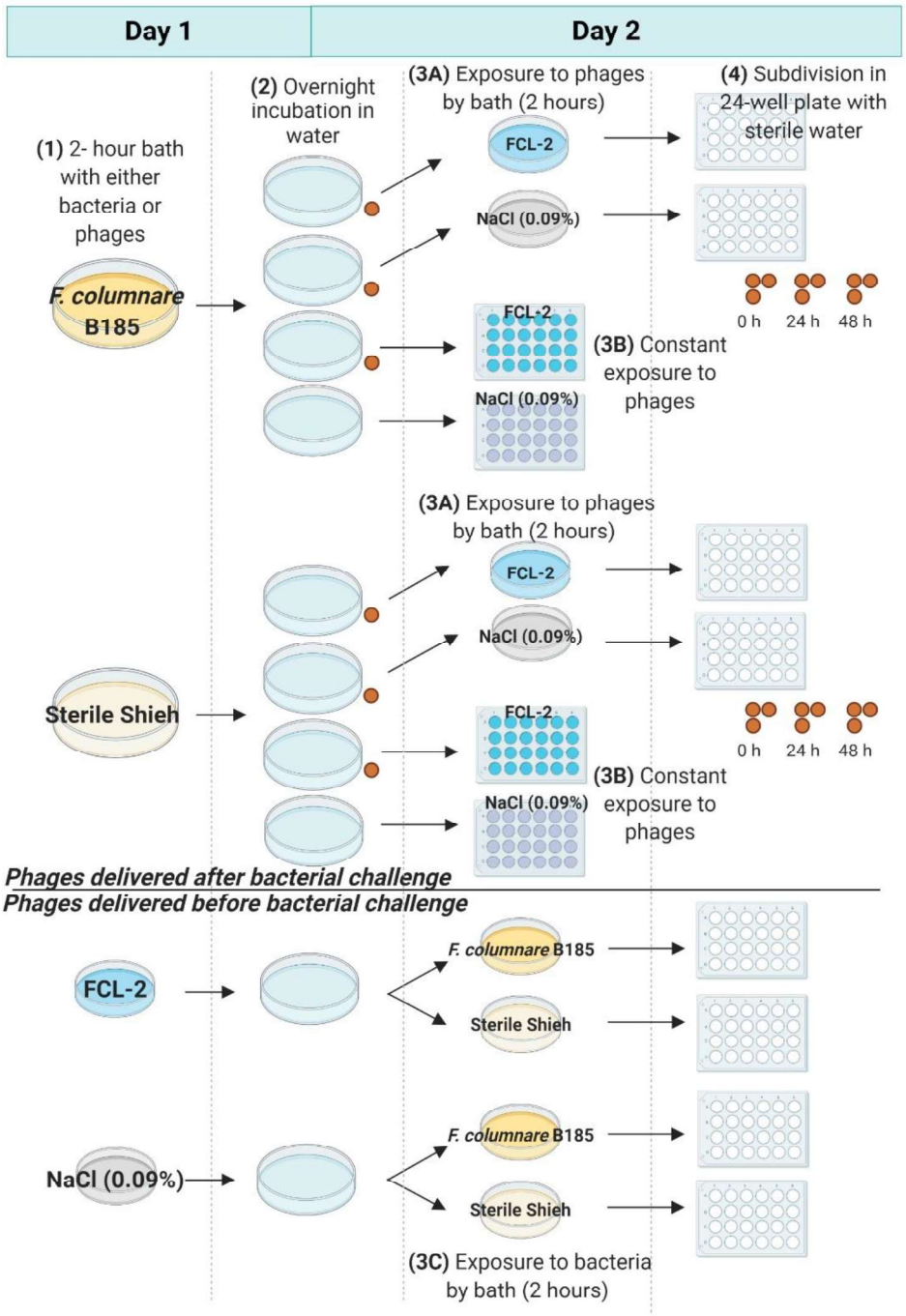

Figure B2. Exp. II section C: Experimental set up. Rainbow trout eyed eggs were exposed to phages after (upper panel) or before bacterial challenge (lower panel). (**1**) Eyed eggs were bathed for 2 hours with *F. columnare* B185 ($5.0*10^6$ CFU ml^{-1}) or sterile Shieh medium (upper panel) in 140 mm 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 in 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 hours 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 hours (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-hour bath, eyed eggs were transferred in 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.

Table B1. 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)	Evaluated parameters	*F. psychrophilum* 950106-1/1				*F. psychrophilum* 160401-1/5N				Negative control (without *F. psychrophilum*)			
		Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4	Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4	Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4
		Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	Egg no. 1 2 3	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

Table B2. 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 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.

Time (hpi)	Sample	Type of medium	*F. psychrophilum* 950106-1/1				*F. psychrophilum* 160401-1/5N				Negative control (without *F. psychrophilum*)			
			Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4	Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4	Bath no. 1	Bath no. 2	Bath no. 3	Bath no. 4
			no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	no. 1 2 3	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	+ + +	- + -	+ + +	+ + +	+ + +	+ + +	- + -	+ + +	- - -	- - -	- - -	- - -

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Appendix A: Effects of phages delivered by phage-immobilized feed (33-day prophylaxis) on rainbow trout survival after bacterial challenge with *F. psychrophilum*.

Overview

The following experiment was performed preliminary to the ones in **Manuscript I**. In this experiment, phage-immobilized feed (prepared by Fixed Phage with the corona discharge technology) was administered prophylactically to rainbow trout (~2 g) for 33 days before the bacterial challenge. The crude lysate of the phage FPSV-D22 was immobilized on feed pellets. The long period of prophylaxis was chosen due to the relatively low concentration of phage per gram of feed ($2.1*10^5$ PFU g^{-1}). For the same reason, a low dose of infection was selected. The results of the experiment confirmed that the concentration of phages on feed pellets was not efficient in controlling the bacterial infection.

Materials and methods

Bacteria and phages

The Danish strain *F. psychrophilum* 950106-1/1 was selected for the infection and the Danish phage FPSV-D22 to be immobilized on feed pellets (0.8 mm, BIOMAR A/S, Denmark). Their characteristics are illustrated in **Manuscript I**. Bacterial growth and phage proliferation were performed as for **Manuscript I**.

Phage administration by phage-immobilized feed (produced by Fixed Phage)

Phage-immobilized feed were produced with the corona discharge method by applying the phage FPSV-D22 in crude lysate ($2*10^{10}$ PFU ml^{-1}) on feed pellets. The final titer of phages in the feed ($2.1*10^5 \pm 4.4*10^4$ PFU g^{-1}) was measured according to Christiansen et al. (2014).

Experimental set-up

Rainbow trout were raised as described for Experiment A in **Manuscript I**. An overview of the experiment is presented in **Figure A1** and **Table A1**. Rainbow trout fry (~2 g) were divided in 8 x 8L-aquaria (~60 fish per aquaria) and fed with either phage-immobilized feed (4 aquaria) or control feed (4 aquaria). All groups were fed at 2% of fish weight per day. Thirty-three days after the start of the phage feeding, fish in three of the four aquaria per feed-group (aquaria no. 2, 3 and 4, **Figure A1**) were exposed to the bacterium by intraperitoneal (IP) injection (100 µl, 10^4 CFU $fish^{-1}$) (Madsen and Dalsgaard, 1999a). Fish in the remaining aquarium were IP injected with sterile TYES-B as controls for the infection (aquaria no. 1, **Figure A1**). Prior to IP injection, fish were anaesthetized with 3-aminobenzoic acid ethyl ester. For each group, two of the infected aquaria were used to follow mortality (aquaria no. 3 and 4, **Figure A1**) and

one for sampling (aquaria no. 2, **Figure A1)**. Five fish from each sampling aquaria (no. 2) were sampled randomly before (day 3, 10, 24, and 31) and after the infection (day 35, 38, 45, 52 and 61). In addition, three fish were sampled from aquaria no. 1 after the bacterial challenge (controls for the infection). Sampled, dead and moribund fish were weighed, their length measured, and bacteriological examination of spleen, kidney and brain was performed. Internal organs were also collected and stored for phage detection (Christiansen et al., 2014) from sampled fish (day 3, 31, 35, 38 and 45) and, if possible, for dead/moribund fish. Bacteriological examination, sampling and analysis of phages in sampled organs were performed as in **Manuscript I**.

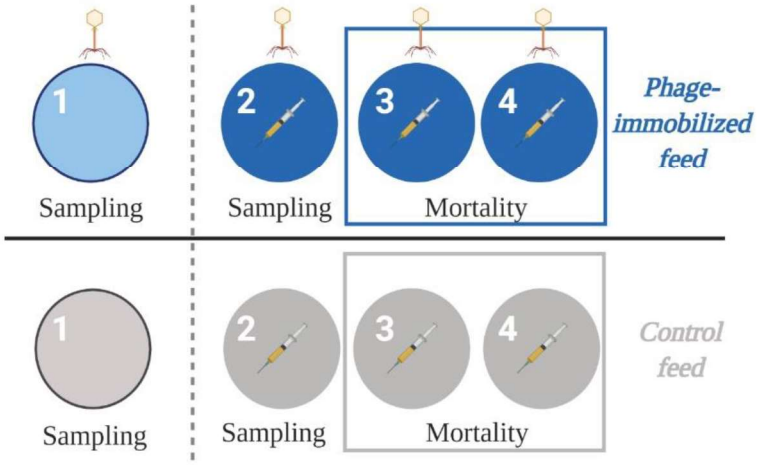

Figure A1. Schematic overview of the experiment set up. Rainbow trout fry were divided randomly in 8 aquaria (60 fish ±4) which are represented by colored circles and numbered. Fish were fed at 2% of their body weight with either phage-immobilized (in blue) or control feed (in grey). Phage drawings indicate FPSV-D22. For each group, three of the four aquaria were challenged with *F. psychrophilum* ($1.9*10^4$ CFU $fish^{-1}$; indicated by a syringe with yellow content because of the bacteria color). Two of the bacterial challenged aquaria per group were used to follow mortality (aquaria no. 3 and 4). The remaining aquaria were dedicated to sampling (5 fish at each sampling point in aquaria no. 2, and 3 fish in aquaria no. 1 after the bacterial challenge). Created with BioRender.com.

Table A1. Overview of the experiment.

Phage delivery method	Administered phage titer	Administration time	Fish		Infection dose (CFU $fish^{-1}$)
			Weight (g)	Total n. (n. per replicate)	
Phage-immobilized feed	$2.1*10^5 \pm 4.4*10^4$ PFU g^{-1} [b]	33 days before IP.; continuous feeding	1.7 (±0.6)[c]	477(60±4)[d]	$1.9*10^4$
Control feed[a]	0 ± 0 PFU g^{-1} [b]				

[a] Non-treated commercial feed from the same batch as the phage-treated feed.
[b] Mean and standard deviation (n=3).
[c] Mean weight of fish sampled after 3 days of phage feed prophylaxis (standard deviation in the parenthesis; n=10); two days before bacterial challenged, fish weight was 2.6 ± 1.0 g (SD, n=10).
[d] Mean number of fish per aquaria and standard deviation in the parenthesis.

Results

Positive growth was detected for all groups during the experiment (**Figure A2 A**). When fish fed with phage-immobilized feed were challenged with *F. psychrophilum*¸ no beneficial effect was observed on fish survival (**Figure A2 B**). It was possible to re-isolate phages from the internal organs of the fish before and after the infection, mostly from intestinal samples (**Figure A3 A**). *F. psychrophilum* was re-isolated from the internal organs of the fish after the bacterial challenge (**Figure A3 B**). The quantification of phages in the fish organs showed their presence in the intestine (10^1-10^2 PFU mg^{-1} of tissue) with a minor occurrence in the inner organs (spleen and kidney: 1-10 PFU mg^{-1}) (data not shown).

Conclusion

The results of the experiment showed that the immobilization of phages on feed pellets performed by Fixed Phage Ltd did not affect the fish growth. However, the concentration of phages on the feed was too low to control the bacterial infection.

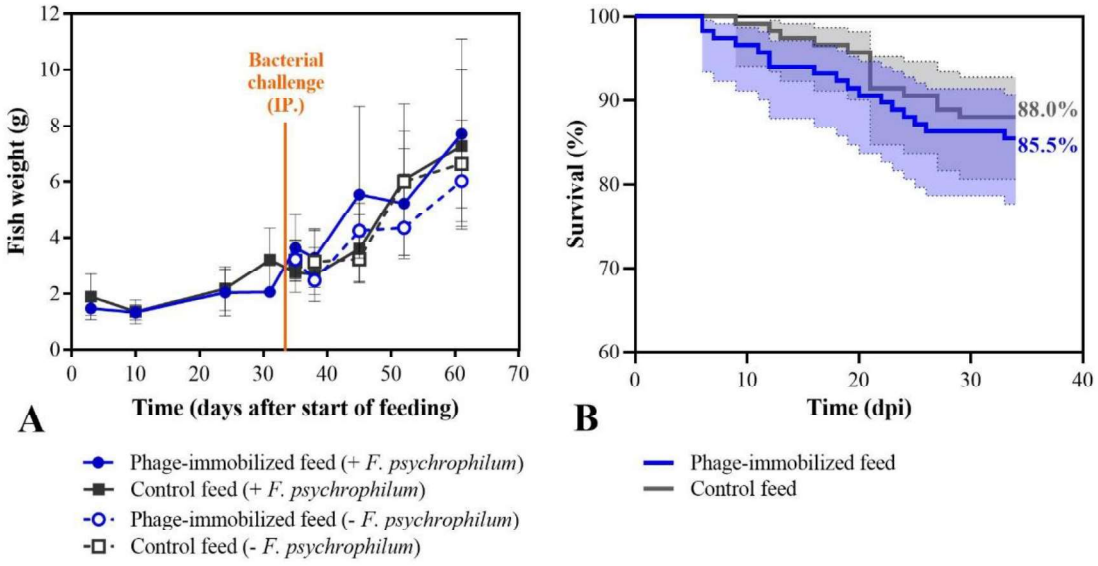

Figure A2. Growth performance based on fish weight in the two feed groups with (continuous lines) and without the infection (dashed lines) (**A**) and percent survival observed in rainbow trout fry exposed to *F. psychrophilum* and phages (**B**). In **A**, values represent the mean and standard deviation of five fish (+ *F. psychrophilum*) and of three fish (- *F. psychrophilum*). In **B**, moribund and dead fish were positive to *F. psychrophilum*. Final percent survival are presented for each curve. 95% confidence interval is presented for each curve. The Kaplan-Meier survival analysis was performed (GraphPad Prism version 8.4.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com): data from replicate aquaria were merged together (the difference in survival between replicates was ≤ 20% (Amend, 1981; Midtlyng, 2016)) and comparison of survival curves was performed with the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. The curves were not significantly different. dpi = days post infection.

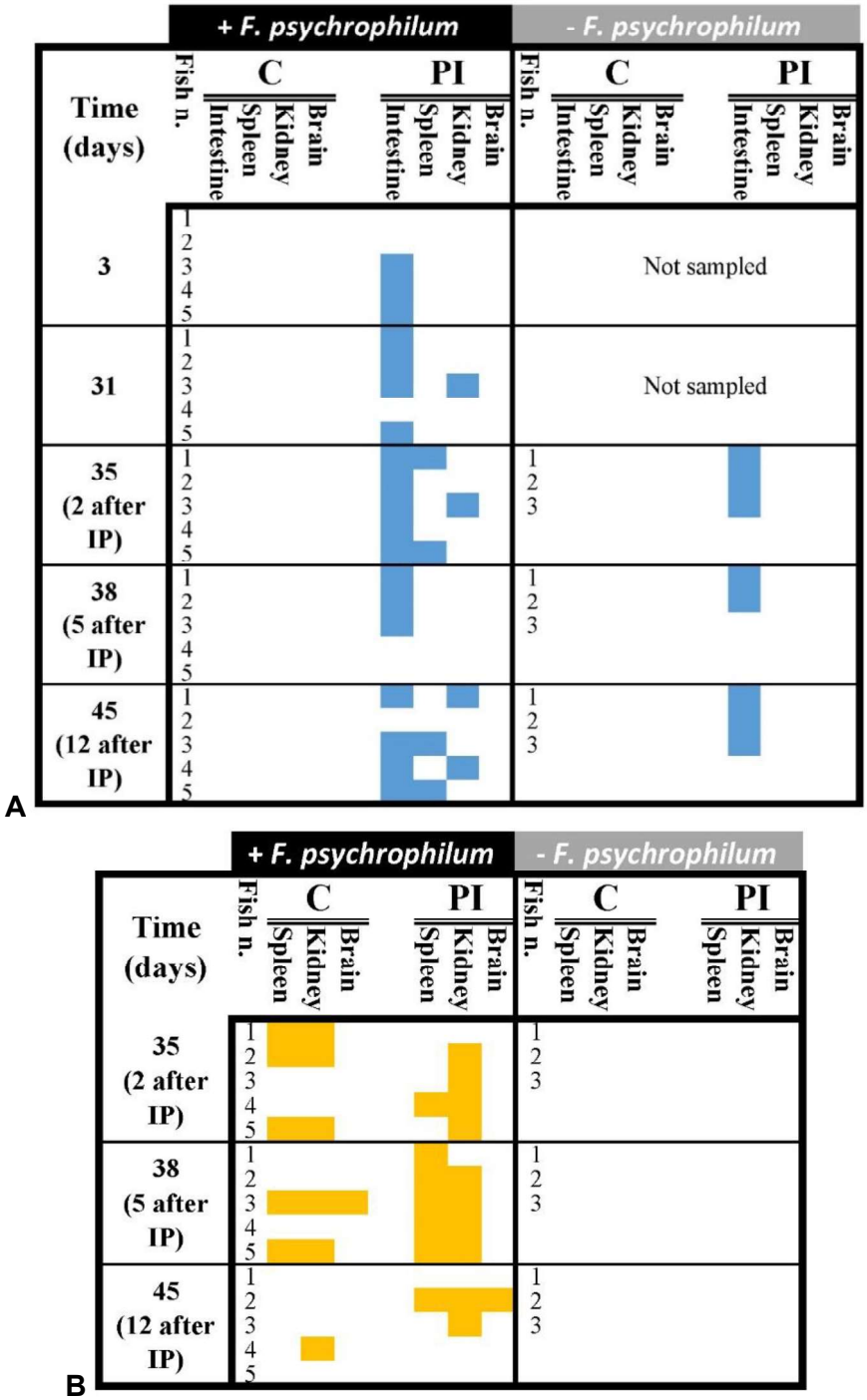

Figure A3. Qualitative detection of phages (**A**) in fish organs (brain, spleen, kidney and intestine) and re-isolation of *F. psychrophilum* in spleen, kidney and brain (**B**, after bacterial challenge) over time in fish fed with control (C) and phage-immobilized (PI) feed. The blue color (**A**) indicates the presence of phages in organs of fish and the yellow color (**B**) the presence of the bacterium. Absence of phages/bacterium in the tested organ is indicated by white/blank. In **A**, positive detection = presence of one or more plaques in at least one of the technical triplicates. In **B**, fish sampled prior to the infection as well as after 45 days were all negative to *F. psychrophilum* (data not included). At each sampling point, five fish were sampled in each group except in groups not challenged with *F. psychrophilum* (n =3). Time = days after the start of feeding; IP = intraperitoneal injection (bacterial challenge).

Appendix B: Effects of phages delivered by bath and by phage-immobilized feed combined on rainbow trout survival after bacterial challenge with *F. psychrophilum*.

Overview

Rainbow trout fry (1-2 g) were challenged with *F. psychrophilum* by bath or by intraperitoneal injection. Five days after, a two component phage mix (FpV4 and FPSV-D22) was administered by phage-immobilized feed and by repeated bath procedures (three times a week). A PEG-purified solution of bacteriophages FpV4 and FPSV-D22 was utilized. Fixed Phage Ltd prepared the phage-immobilized feed. The results of the experiment showed that phages (in the used concentrations) were not able to control the bacterial infection.

Materials and methods

Bacteria and phages

The Danish strain *F. psychrophilum* 160401-1/1N was selected for the infection and the phages FpV4 and FPSV-D22 to be immobilized on feed pellets (0.8 mm, BIOMAR A/S, Denmark) and for the bath procedures. The characteristics of the selected strain are illustrated in **Manuscript III** while FpV4 and FPSV-D22 are described in **Manuscript I**. Bacterial growth and phage proliferation were performed as for **Manuscript I**.

Phage administration by phage-immobilized feed (produced by Fixed Phage Ltd)

Phage-immobilized feed (0.8 mm feed pellets) was produced by applying 20 ml per 100 g of feed of a phage solution ($3.1*10^9$ PFU ml^{-1}) prepared by mixing 1:1 PEG-purified solutions of FpV4 ($1.2*10^9$ PFU ml^{-1}) and of FPSV-D22 ($4.9*10^9$ PFU ml^{-1}). The obtained final FpV4/FPSV-D22 concentration on feed pellets was $8.3*10^7 \pm 4.8*10^7$ PFU g^{-1}. For bath procedures, the PEG-purified phage solutions of FpV4 ($1.2*10^7$ PFU ml^{-1}) and FPSV-D22 ($1.2*10^9$ PFU ml^{-1}) were mixed 1:1 for a final phage concentration of $6.1*10^8$ PFU ml^{-1} ($6*10^6$ PFU ml^{-1} of FpV4 and $6*10^8$ PFU ml^{-1} of FPSV-D22) and used for phage bath administration. Phage quantification in PEG-purified solutions and on feed pellets was performed as described in **Manuscript I**.

Experimental set-up

Rainbow trout were raised as described for Experiment A in **Manuscript I**. Rainbow trout fry of 1-2 g were randomly divided in 16 x 8L-aquaria (~50fish/aquarium) (**Figure B1**). All groups were fed at 2% of fish weight per day. Fish were exposed to the bacterial pathogen, *F. psychrophilum* 160401-1/1N, either by intraperitoneal (IP) injection (50µl, $2.8*10^4$ CFU $fish^{-1}$) (four aquaria) or by bath (5 hours in $8.1*10^7$ CFU ml^{-1} in a total of 4 L volume) (four aquaria) (Madsen and Dalsgaard, 1999a). Controls

for the bacterial infection were included: fish in the remaining eight aquaria were injected (four aquaria) or bathed (four aquaria) with sterile TYES-B. Prior to IP injection, fish were anaesthetized with 3-aminobenzoic acid ethyl ester. For the bath challenge, fish were moved to one single aquarium containing either the bacteria or the sterile TYES-B (3.6 L of water plus 0.4 L of bacterium/sterile broth), and water flow was stopped during the procedure. Five days after, phages were administered by phage-immobilized feed and by repeated bath procedures (three times a week) to fish in two of the four bacterial challenged aquaria (IP and bath challenge) and in two of the four controls for the infections (IP and bath challenge controls) (**Table B1 and B2**). The remaining fish were fed with commercial feed not treated with phages and exposed to control bath procedures (only SM buffer). All aquaria were utilized to follow mortality of fish. Dead and moribund fish were weighted, their length measured, and bacteriological examination of spleen, kidney and brain was performed. If possible, internal organs were also collected and stored for phage detection (Christiansen et al., 2014). Bacteriological examination, sampling and analysis of phages in sampled organs were performed as in **Manuscript I**.

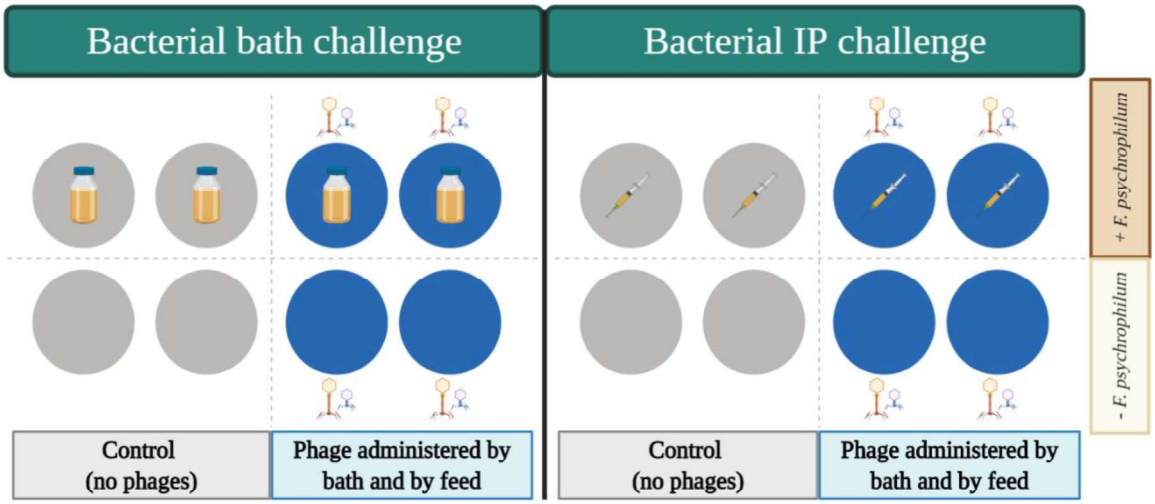

Figure B1. Experimental set-up. Eight hundred rainbow trout fry were divided in sixteen aquaria (circles; ~50 fish per aquarium). Fish in eight aquaria were challenged with *F. psychrophilum* either by IP injection (syringe with yellow content) or by bath (bottle with yellow content). Five days after the bacterial challenge, fish in eight aquaria were exposed to phages FpV4 and FPSV-D22 by feed and by repeated bath treatments (blue circles; indicated by bacteriophage drawings). Aquaria containing fish not exposed to phages are represented by grey circles. Mortality was followed. Created with Biorender.com.

Table B1. Timeline for phage administration (bath treatment plus oral by phage-immobilized pellets) for fish challenged with *F. psychrophilum* by intraperitoneal injection. Dpi = days post infection.

IP challenged groups			
Dpi	**Bath n. and procedure**	**Estimated final phage concentration (PFU ml^{-1})**	**Duration**
5	1st bath – 20 ml in 2 l	2.1*10^{6}	2 hours
7	2nd bath – 2 ml in 2 l	2.1*10^{5}	2 hours
9	3rd bath – 2 ml in 8 l	1.6*10^{5}	Phages added to aquaria
Phage-immobilized feed was administered from day 5 until day 15 included.			

Table B2. Timeline for phage administration (bath treatment plus oral by phage-immobilized pellets) for fish challenged with *F. psychrophilum* by bath. Dpi = days post infection.

Bath challenged groups			
Dpi	**Bath n. and procedure**	**Estimated final phage concentration (PFU ml^{-1})**	**Duration**
5	1st bath – 20 ml in 2 l	2.1*10^{6}	2 hours
7	2nd bath – 2 ml in 2 l	2.1*10^{5}	2 hours
9	3rd bath – 2 ml in 8 l	1.6*10^{5}	Phages added to aquaria
12	4th bath – 10 ml in 8 l	7.9*10^{5}	Water flow stopped for 2 hours
14	5th bath – 10 ml in 8 l	7.9*10^{5}	Water flow stopped for 2 hours
16	6th bath – 10 ml in 8 l	7.9*10^{5}	Water flow stopped for 2 hours
19	7th bath – 10 ml in 8 l	7.9*10^{5}	Water flow stopped for 2 hours
Phage-immobilized feed was administered from day 5 until day 23 included.			

Results

No significant beneficial effect on fish survival was observed in case of phage treatment (**Figure B2**). The analysis for phage detection of the internal organs of few dead fish in the IP challenge group showed the presence of very low concentrations of phages (**Table B3**).

Conclusion

The phages FpV4 and FPSV-D22 administered orally and by repetitive bath procedures were not able to control the bacterial infection neither when fish were challenged by intraperitoneal injection nor when challenged by bath (which was included to mimic a more natural way of infection).

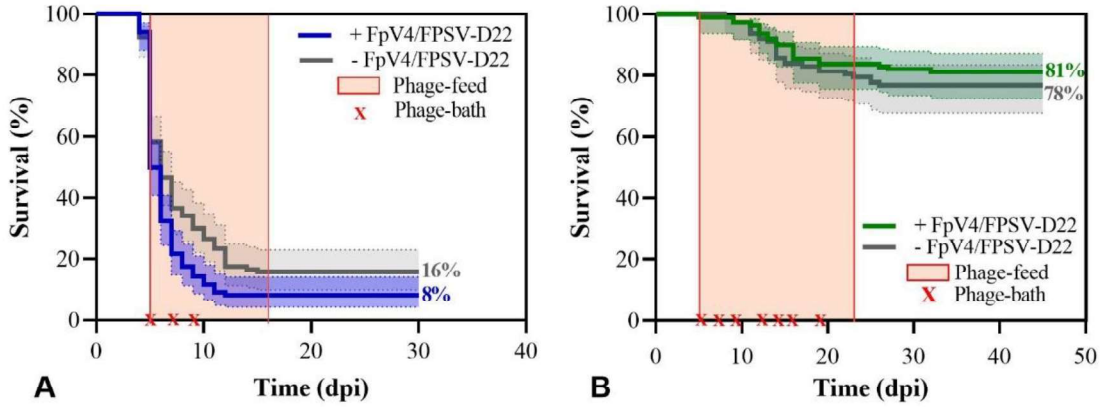

Figure B2. Percent survival observed in rainbow trout fry exposed to *F. psychrophilum* by IP injection (**A**) and by bath challenge (**B**) with (in blue and in green) and without (in grey) phage treatment. Moribund and dead fish were positive to *F. psychrophilum*. dpi = days post infection. Final percent survival are presented for each curve in the figures. 95% confidence interval is presented for each curve. The Kaplan-Meier survival analysis was performed (GraphPad Prism version 8.4.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com): data from replicate aquaria were merged together (the difference in survival between replicates was ≤ 20% (Amend, 1981; Midtlyng, 2016)) and comparison of survival curves was performed with the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. The curves were not significantly different.

Table B3. IP challenged groups: information about dead/moribund fish sampled for phage analysis (dpi= days post infection).

IP groups	Fish n.	Time of event (dpi)	Fish weight (g)	Bacteriological examination			Presence of phages (PFU mg^{-1} of tissue)			
				Brain	Kidney	Spleen	Brain	Kidney	Spleen	Intestine
Phage-immobilized feed + phage bath	1	6	4.69	+	+	+	0	1.17	0	0.05
	2	6	2.75	+	+	+	2.17	8.57	0	6.04
	3	6	3.70	+	+	+	1.02	1.94	0	0.09
	4	6	4.81	+	+	+	0.24	0	0	2.83
	5	6	3.63	+	+	+	6.09	0	0	0.77
	6	6	2.38	+	+	+	0	0	2.4	0
Control feed + control bath	1	6	2.79	+	+	+	0	0	0	0
	2	6	3.64	+	+	+	0	0	0	0
	3	6	5.08	+	+	+	0	0	0	0
	4	6	3.32	+	+	+	0	0	0	0