Master's Thesis

The influence of competition on the formation of phage resistance in *Flavobacterium columnare*

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Bacteria live in complex communities, where they interact with other bacteria and also bacterial viruses, bacteriophages (phages). During the long coevolution between bacteria and phages, bacteria have developed various immunity mechanisms against phage infections. These mechanisms include CRISPR-Cas and surface modification, which have both been found to be the mechanisms utilized by a fish pathogen, *Flavobacterium columnare*. Although the development of resistance mechanisms has been widely studied, the effect of other bacterial species to their appearance are not yet known. This study aimed to find information about the type of resistance taking place in *F. columnare* in the presence of *Aeromonas* sp. and *Escherichia coli* using a culture condition known to favor CRISPR activity. The study used PCR and gel electrophoresis to detect the acquisition of new spacers in *F. columnare* CRISPR arrays, besides checking colony morphology changes at four timepoints (7, 14, 32 and 56 days). The results showed a clear increase in the number of new spacers in both CRISPR loci. The abundance of wild-type -like rhizoid colonies increased slightly during the experiment, whereas the typically phage resistant rough colonies had a similar decrease. The number of acquired CRISPR spacers in the presence of *Aeromonas* sp. was higher than in the control culture, indicating a connection between the presence of a naturally coexisting bacterium and more competent spacer acquisition in *F. columnare*. These data shows that the occurrence of other bacterial species can influence *F. columnare* strategies against phages, highlighting the importance of studies made in natural communities.

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Luonnossa bakteerit elävät monimutkaisissa eliöyhteisöissä, joissa ne vuorovaikuttavat paitsi keskenään, myös bakteereja infektoivien virusten, (bakterio)faagien kanssa. Bakteerien ja faagien välisen koevoluution aikana bakteereille on kehittynyt useita resistenssimekanismeja faagien torjumiseen. Tällaisia mekanismeja ovat muun muassa CRISPR-Cas-välitteinen ja solun pinnan muutokseen perustuva immuniteetti. Kyseisten mekanismien on aiemmissa tutkimuksissa todettu olevan myös kaloja infektoivan *Flavobacterium columnare* -bakteerin keino torjua faagi-infektioita. Vaikka resistenssimekanismeja onkin tutkittu laajalti, kilpailun vaikutuksia resistenssin kehittymiseen ei vielä juuri tunneta. Tämän tutkimuksen tavoitteena oli saada lisää tietoa resistenssin tyypistä, kun *F. columnaren* annettiin vuorovaikuttaa *Aeromonas* sp.- ja *Eshcerichia coli* -bakteerien kanssa. Uusien CRISPR-toistojaksojen havaitsemiseen käytettiin PCR-menetelmää ja geelielektroforeesia, ja pesäkkeiden morfologiaa tarkasteltiin viljelymaljoilta neljänä ajankohtana 56 vuorokauden aikana. Tulokset näyttivät, että molempien tunnettujen lokusten CRISPR-toistojaksojen määrä kasvoi selvästi kokeen aikana. Faagille alttiiden pesäkkeiden määrä kasvoi, kun taas immuunien pesäkkeiden määrässä havaittiin vastaava lasku. *Aeromonas* sp:n kanssa vuorovaikuttaneella *F. columnarella* havaittiin enemmän CRISPR-toistojaksoja kuin kontrolliviljelmissä, mikä viittaa siihen, että vuorovaikutus muiden bakteerien kanssa parantaa CRISPR-immuniteetin muodostumista *F. columnarella*. Nämä tulokset korostavat luonnollisissa eliöyhteisöissä tehtävien tutkimusten tärkeyttä.

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TERMS AND ABBREVIATIONS

TERMS

Bacteriophage	Viruses capable of infecting bacteria
Morphotype	A group of bacteria within a single species that is distinguishable from other such groups by the morphological characteristics of their colonies
Pathogen	A bacterium, virus, or other microorganism that can cause disease.
Phage therapy	Therapeutic use of bacteriophages to treat pathogenic bacterial infections
Virulence	The ability of a microorganism to infect or damage a host

ABBREVIATIONS

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated (proteins)
LB	Lysogeny broth (medium)

1 INTRODUCTION

Bacteriophages (phages) are viruses that infect bacteria. They are estimated to be the most abundant organisms on Earth, with an estimation of 10^{31} individuals in total, outnumbering prokaryotes by tenfold (Brüssow and Hendrix 2002). The vast majority of known phages (>95%) have linear, double-stranded DNA surrounded by a tailed capsid consisting of proteins (Ackermann 2007).

Phages were discovered twice independently, in 1915 (Twort 1915) and in 1917 (D'Herelle 1917). Their properties as antimicrobial agents were soon discovered and described by d'Herelle, and they were also used to find out some fundamentals of cellular biology, such as the randomness of mutations (Luria and Delbrück 1943), DNA's role as genetic material (Hershey and Chase 1952) and the control of gene expression (Jacob and Monod 1961).

Phages, like other viruses, are parasitic organisms and cannot reproduce without a host. Their infection process starts when a phage binds itself to a specific receptor on the surface of a bacterium and inserts its genome into the host (Guttman et al. 2005). The phage genome is then copied and transcribed inside the bacterium, together with the production of phage proteins. Finally, the genetic material is encapsulated by the capsid proteins, resulting in complete viable phages.

Despite the certain simplicity and lack of consciousness of bacteria, they are not defenseless against phages. There are several resistance mechanisms, both innate and adaptive, that protect bacteria against phage infections. The mechanisms can focus on altering either the surface of the bacteria, blocking phage DNA injection or replication, cleaving the phage genome, or triggering suicide before the phage replication is complete (van Houte et al. 2016). Considering the bacterial resistance mechanisms from the phage's point of view, there are three main components:

receptor binding, genome injection, and intracellular genome replication (van Houte et al. 2016). These three infection stages are the principal options for modifications to take place in order to overcome the resistance developed by the bacterium. The alternating development of features between bacteria and phages to overcome the resistance of the other has led to an arms-race between them (Hampton et al. 2020), and a complex network of competition, trade-offs, and advantages in natural microbial communities.

Bacteria and phages have been shown to coevolve together (Buckling and Rainey 2002a, Laanto et al. 2017). Bacteria develop immunity against phage infections, which obliges phages to evolve ways to overcome those immunities, which leads to an ongoing immunity-driven co-evolution (Buckling and Rainey 2002a). Obtaining and maintaining phage immunity often has a cost for bacteria. Typically, the cost is some type of fitness loss, for example decreased virulence (Heierson et al. 1986), decreased reproductive ability (Meaden et al. 2015), or lower competitiveness for resources (Meaden et al. 2015, Burmeister et al. 2020). The benefits of phage immunity are generally greater than the costs (Burmeister et al. 2020). Additionally, the trade-off between phage resistance and fitness is considered to be beneficial for bacteria when phages are present, but too costly to maintain in the absence of the parasite (Sheldon and Verhulst 1996). Fitness loss in some bacteria can favor other bacteria that have not encountered phages and have therefore not developed phage resistance, and that have better fitness because of that. These competitive dynamics between bacteria can also encourage bacteria to revert back to the ancestral form and phage-susceptibility when phages are absent, which enables phages to infect the bacteria again. In evolutionary sense this fluctuation is described by two theories: a prediction that, over time, phenotypes emerge, disappear, and re-emerge (Red Queen dynamics, Brockhurst et al. 2014), and that the fittest and thereby the most abundant phenotype experiences the most predation by phages, which favors bacteria that are less abundant and have lower fitness (kill-the-winner, Winter et al. 2010). Overall, phages have been found to maintain microbial diversity (Buckling

and Rainey 2002a, Buckling and Rainey 2002b, Rodriguez-Valera et al. 2009), mediate horizontal gene transfer (Canchaya et al. 2003), and alter bacterial competition (Bohannan and Lenski 2000), making them an essential factor of microbial ecology and evolution.

In natural environments, bacteria live in diverse communities and differently detailed conditions, that are virtually impossible to replicate in laboratory settings. This makes it difficult to reliably study the dynamics between a bacterium and its phage as they are in nature. Ecology and evolution of host and parasite is substantially influenced by interactions with surrounding organisms (Parratt and Laine 2016, Betts et al. 2016, Laanto et al. 2017) which further highlights the importance of species diversity microbial research. It has been found by Almeida et al. (2019) that phages and metazoans have a symbiotic relationship. The covering layer of mucus on fish skin does not only protect the fish by forming a physical and chemical barrier against invading pathogens, but also attracts and binds phages into itself. The fish provides phages a medium for interacting with bacteria, and the fish gains an external immunity against pathogenic bacteria.

The genus *Flavobacterium* is widely spread in nature aquatic environments and soils, and some species are pathogenic (Bernardet and Bowman 2006). *Flavobacteria* are heterotrophs, and they are distinct in freshwater (Eiler and Bertilsson 2020) and marine environments (Bartlau et al. 2021). They are present, often in high proportions, during high bacterial production and when resources are plenty, and thereby they have an effect on microbial community dynamics, biogeochemical cycles, and the quality of environments (Bernardet and Bowman 2006, Eiler and Bertilsson 2020, Bartlau et al. 2021). *F. columnare* is known for infecting and killing fish (Suomalainen et al. 2005) and thereby causing financial losses in the fish farming industry. Indefinitely, antibiotics are being used to control outbreaks and fish mortalities in fish farms, which has led to an increasing number of antibiotic-resistant bacteria (Huys et al. 2000). The use of antibiotics becoming more and more ineffective requires alternative methods for preventing and treating bacterial

infections. One alternative for antibiotics is the use of phages as antibacterial treatment. In previous and ongoing research, phages have shown great qualities for fighting bacterial infections caused by *F. columnare*, and the results are promising (Laanto et al. 2015).

As mentioned, bacteria have several phage-resistance mechanisms. This study will focus on two of those mechanisms, both found in the opportunistic aquatic pathogen *Flavobacterium columnare*. The first is surface modification, which can happen by losing or reducing the expression of the surface receptor, mutating the receptor, or blocking/masking the receptor (van Houte et al. 2016), thereby prohibiting the phage from binding to the receptor or injecting its genome into the bacterium. The other mechanism, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), together with CRISPR-associated (Cas) proteins, is based on the recognition of foreign DNA sequences inside the bacterial cell, and cleaving them to prevent their replication (van Houte et al. 2016).

In fish farm studies, it has been found that bacteria tend to rely on different phage resistance mechanisms at the same time (Castillo et al. 2015). It has been shown that a high abundancy of phages may result in the use of other immunity mechanisms than CRISPR-Cas, such as the change of morphotype via surface modifications (Iranzo et al. 2013, Laanto et al. 2012).

1.1 CRISPR-Cas

Bacteria coevolve with bacteriophages, leading to an arms-race between them. CRISPR-Cas is a feature of the bacterial genome and functions as the adaptive immune system of some bacteria (Barrangou et al. 2007). CRISPR-Cas protects bacteria from foreign DNA, giving them a means for stopping phages from infecting them (Makarova et al. 2015).

The CRISPR-Cas system is utilized by approximately 40% of all bacterial species (Burstein et al. 2016), whereas Cas proteins are only found in bacteria with CRISPR

(Jansen et al. 2002). Cas proteins function as helicases and exonucleases. The CRISPR system and Cas act together in a functional relationship to prevent foreign DNA from infecting the host cell (Jansen et al. 2002).

The basic mechanism of CRISPR-Cas immunity includes three steps: adaptation, expression, and interference (Westra et al. 2012, Makarova et al. 2015). Adaptation consists of incorporation of foreign DNA (protospacers) into a CRISPR locus in the bacterial genome. The expression stage involves the production of precursor CRISPR RNA (crRNA) which is matured to crRNA. In the interference stage the target-specific crRNA binds to the Cas-binding trans-activating RNA (tracrRNA) sequence and recognizes the complementary foreign nucleic acid. The Cas nuclease then cleaves the foreign sequence, making it unable to cause an infection (Westra et al. 2012, Makarova et al. 2015).

In the field, all the processes and interactions between species and individuals happen inside complex communities. Phage–bacteria studies are traditionally performed in laboratory conditions, where the natural microbial communities are virtually impossible to reproduce. Therefore, it is important to study the effects of different biotic and abiotic factors together, and not only the individual interactions between host and parasite.

1.2 Model organisms

1.2.1 *Flavobacterium columnare*

Flavobacterium columnare is a Gram-negative rod-shaped bacterium found as bacterioplankton from various habitats worldwide (Bernardet et al. 1996). *F. columnare* is an opportunistic pathogen (Bernardet et al. 2006), which makes it a problem for fish farming in many countries, including Finland (Laanto et al. 2011). There are several isolated strains of *F. columnare*. The strain chosen for this study is strain B245, which was isolated from fish tank water in Finland in 2009, and stored

in -80 °C. The strain is resistant to the antibiotic tobramycin, which was used to separate *F. columnare* from the other bacteria used in the experiment.

The virulence of the strain B245 was investigated by Laanto et al. (2012) and a clear correlation between different morphotypes (Figure 1) and virulence was discovered. It has been found that *F. columnare* representing the rhizoid morphotype was virulent but was not resistant against phage infections, whereas *F. columnare* that had the rough morphotype was non-virulent but had resistance against the phage (Laanto et al. 2012, Kunttu et al. 2021). The morphotype change is often based on mutations in the surface receptor the phage uses to bind to a bacterium. Those mutations are located in genes that encode the type IX secretion system, which is a part of the gliding motility machinery of bacteria (Kunttu et al. 2021). Ergo, phage resistance through surface modification also affects the bacterium's gliding motility and protease activity (Kunttu et al. 2021). Nevertheless, CRISPR mutants could, in theory, remain rhizoid and virulent, but immune to phages.

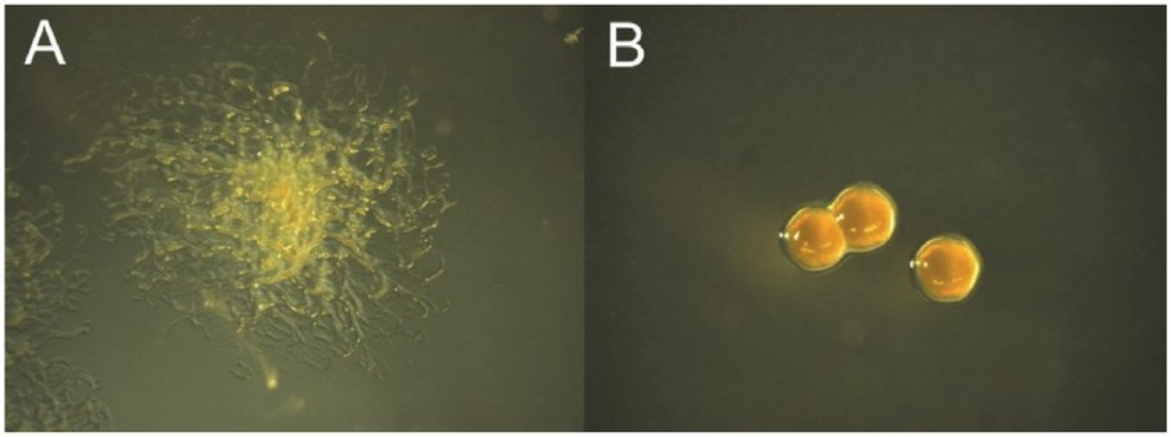

Figure 1. Colony morphotypes of *F. columnare*. Virulent phage-susceptible rhizoid colony (A) and non-virulent phage-resistant rough colony (B) on Shieh agar (Laanto et al. 2012).

Fish are naturally covered with a layer of mucus that protects them from the environment (Bakshani et al. 2018). Although the mucus protects the fish, it also attracts bacteria (Staroscik and Nelson 2008). Staroscik and Nelson (2008) have shown that mucus promotes the growth of *F. columnare*, and also alters its protease production and formation of biofilms.

Mucus affects *F. columnare* in a way that it becomes more virulent and more susceptible to phage infection (Laanto et al. 2012, Almeida et al. 2019). It was also discovered by Almeida et al. (2019) that exposure to mucus causes *F. columnare* to form biofilms and promotes the rhizoid morphotype. As the exposure to mucin favors the rhizoid morphotype regardless of the presence of phages, the expected phage resistance mechanism might be different than surface modification (adopting the rough morphotype). Consequently, CRISPR-mediated resistance could be expected to form.

Previous studies have shown that *F. columnare* is capable of developing CRISPR resistance against its phage (Laanto et al, 2017). There are two known CRISPR loci in *F. columnare* genome: C1 and C2. C1 is a type II-C CRISPR locus and C2 represents a type VI-B locus (Laanto et al. 2017). The phage-targeting CRISPR spacers are located in the variable ends of both loci and usually target the terminal end of the phage genome. Phages, on the other hand, evolve to overcome the CRISPR-mediated immunity. Overcoming the bacterial CRISPR immunity can happen rapidly because the CRISPR spacers target the terminal end of the phage genome, which has been shown to have the most variation over time (Laanto et al. 2017).

1.2.2 Phages infecting *Flavobacterium columnare*

There are many phages that infect *F. columnare*, most of which have been found to belong to the Myoviridae, Podoviridae or Siphoviridae families (Laanto et al. 2011). They all have dsDNA genomes, and all of them are the tailed and they have an icosahedral capsid. It has been found by Laanto et al. (2011) that the host-ranges of *F. columnare* phages are typically narrow, meaning that each can only infect hosts that have a specific genotype.

In Finland, *F. columnare* phages have been isolated only from fish farms during *F. columnare* outbreaks (Laanto et al. 2011), but in India they have been found in natural waters and bottom sediments (Prasad et al. 2011). As well as the phages of *F. columnare*, the bacterium itself is not prevalent in natural fresh waters (Laanto et

al. 2011). However, previous studies by Rickard et al. (2003), Revetta et al. (2005) and Kunttu et al. (2012) show proof of *F. columnare* living in natural waters, which would likely be the initial source of *F. columnare* in fish farms. Furthermore, it has been speculated by Laanto et al. (2011) that *F. columnare* phages might be able to persist inside their hosts during the low-nutrient cold-water period and start proliferation once more resources are available for the host. Thus, the emergence of *F. columnare* in the nutritious fish tank water and its subsequent proliferation could provoke any possible lysogenic phages to initiate reproduction, and thereby considerably increase the number of phages in that environment.

1.2.3 Other bacteria in this study

Microbes in communities interact with each other in many ways and for many functions (Braga et al. 2016). Competition for resources is one of the most fundamental interactions between microbes, and survival is often determined by one's fitness in a community. Microbes can transfer molecular and genetic material to help establish in a community, which can further shape their interactions with their hosts and parasites. The presence of other microbes can also affect the formation of CRISPR immunity in bacteria (Westra et al. 2012). It is important for future phage therapy possibilities to know more about the interactions between bacteria during phage-infections. This study includes two bacteria, *Aeromonas* sp. and *Escherichia coli*, which are found in many of the environments *F. columnare* inhabits.

Aeromonas sp. strain B135 was chosen as the first bacterium to be coupled with *F. columnare* in the experiments. Many *Aeromonas* species are fish pathogens, and they are often found in fish farms (Chen et al. 2019, Borella et al. 2020, Cheok et al. 2020). This shows that *Aeromonas* and *F. columnare* inhabit the same environments and are able to interact with each other in natural communities.

Aeromonas sp. has recently been found to form biofilms in the presence of mucin and behave similarly to *F. columnare* in the formation of biofilm (Almeida et al.

2019). It lacks resistance to tobramycin, which enables the isolation of *F. columnare* from the mixed cultures. On the other hand, *Aeromonas* sp. tolerates salty conditions unlike *F. columnare*, which can be used to isolate *Aeromonas* sp. from the mixed cultures.

The other bacterium that was used in the mixed cultures was *E. coli*. *E. coli* is widely studied, well-known and easily accessible. It can be found in natural waters if they become contaminated by fecal matter (Ishii and Sadowsky 2008). The strain used in this study was DSM613. Like *Aeromonas* sp., it is also susceptible to tobramycin and tolerates salty conditions, so it could be separated from *F. columnare*. *E. coli* and *Aeromonas* sp., however, might not be distinguishable from each other on agar plates with the naked eye.

1.3 Research questions, hypotheses, predictions

The aim of this study was to gather information of the formation of phage resistance in *F. columnare* in the presence of *Aeromonas* sp. and *E. coli*. It has previously been found that the combination of competing bacterial species and host signals can make bacteria favor CRISPR-mediated resistance over surface modification (Alseth et al. 2019). The measurable resistance mechanisms (surface modification or CRISPR-Cas) were aimed to be observed and compared between cultures. The rate of formation of the possible resistance with interaction of other bacteria was also thought to be worth observing.

The hypothesis for this study is that competition with other bacteria does affect the phage resistance mechanisms of *F. columnare*. According to previous research, *F. columnare* is likely to gain some surface resistance (seen as colony morphotype change) morphological resistance against the phage, despite the simulation of host signals (mucin). In cultures with other bacteria the formation of resistance might be different than when *F. columnare* is alone with the phage, due to the bacteria having to compete for nutrition and space.

If morphological resistance is formed, bacterial virulence is lost, as shown in previous studies (Kunttu et al. 2009, Laanto et al. 2014, Zhang et al 2014). Resistance through CRISPR-Cas, on the other hand, could affect the ability to compete against other bacteria, as the sustenance of the system is energetically costly, making replication somewhat slower. However, with CRISPR spacers *F. columnare* would achieve phage resistance which would leave it both immune to phage infection and virulent.

2 MATERIALS AND METHODS

2.1 Bacterial strains used in this study

Shieh media (with no glucose, as described by Song et al. 1988) was used as the growth medium for *F. columnare* and *Aeromonas* sp. *F. columnare* strain B245 has been originally isolated from fish tank water in 2009 in Central Finland. The *Aeromonas* sp. strain used in this experiment (B135) was isolated from a small natural brook in Konnevesi (62°33'08.9"N 26°03'28.7"E), Central Finland. *Escherichia coli* strain DSM613 was obtained from DSMZ GmbH (Braunschweig, Germany). Prior to the experiment, the cultivation of *F. columnare* and *Aeromonas* sp. was done at 25°C and 120 rpm. *E. coli* was cultivated in lysogeny broth (LB) medium at 37°C and 220 rpm. All three bacteria were cultivated from frozen (-80°C) stocks.

As mentioned, the phages infecting *F. columnare* are genotype-specific (Laanto et al. 2011). Therefore, it is important to choose a phage that infects the bacterial strain used in the experiments. For that reason, for *F. columnare* strain B245, phage V156 was chosen. The phage was previously isolated from the same fish farm as *F. columnare* strain B245 in 2009.

2.2 Experimental set-up

In order to find answers to the question "Does the presence of other bacteria (*Aeromonas* sp. and *Escherichia coli*) affect the phage resistance mechanisms of *F. columnare*?", the plan was to cultivate bacteria in growth medium in pairs with and without the phage. All conditions were tested in triplicates.

The bacteria were first cultivated in growth media and then mixed with phage V156 according to Table 1. In addition, one set of triplicates was made as a bacteria-free phage control. The experimental cultures were made in autoclaved lake Jyväsjärvi water (Jyväsjärvi, 13.12.2018) supplemented with autoclaved 0.1% porcine mucin (Sigma, 2% w:v stock).

Table 1. The experimental setup with four phage-treated samples and one phage-free control condition for each sample.

Condition	Treatment	Phage-free control
1	*F. columnare* + *Aeromonas* sp. + *E. coli* + phage	*Aeromonas* sp. + *E. coli* + *F. columnare*
2	*F. columnare* + *Aeromonas* sp. + phage	*Aeromonas* sp. + *F. columnare*
3	*F. columnare* + *E. coli* + phage	*E. coli* + *F. columnare*
4	*F. columnare* + phage	*F. columnare*

The cultures were made in the volume of 5 ml, and with an inoculum of $5*10^4$ colony-forming units (cfu) of each bacterium and $5*10^3$ plaque-forming units (pfu) of the phage. The bacterial density was determined by an optical density measurement with the wavelength of 595 nm (Thermo Scientific, Multiskan FC, Shanghai, China). The cultures were incubated in a shaking incubator at 25°C at 120 rpm. Sampling was done at four timepoints, days 7, 14, 32 and 56, by removing 20% of each culture and replacing it with an equal volume of fresh autoclaved lake water supplemented with 0.1% mucin. Bacterial titrations were made with the removed

supernatants (1 ml) forthwith by making a dilution series of 10^{-2}, 10^{-4} and 10^{-6} from each of them in Shieh and plating 100 µl on Shieh and LB agar plates. The Shieh plates were supplemented with 1 µg/ml tobramycin to select *F. columnare* and the LB plates were chosen to select *Aeromonas* sp. and *E. coli*. The sample plates were incubated in room temperature for three days, and then additionally at 25°C overnight to get visible colonies.

2.3 Analysis of bacterial counts and phage resistance

The Shieh plates were used for counting the colonies of *F. columnare* and differentiation of rhizoid and rough colonies. The colonies of *E. coli* and *Aeromonas* sp. were counted on the LB plates. Four individual *F. columnare* colonies (two rhizoid and two rough when possible) were picked from each of the phage-infected culture plates for CRISPR analysis. The analysis was done after each sampling by resuspending the collected colonies in 50 µl Shieh and using the resuspensions as templates for PCR designed to identify the possible addition of new CRISPR spacers in both loci (C1 and C2). To replicate the variable ends of the loci, the following primer sequences were used (Hoikkala et al. 2021):

C1 forward: CTGTTTTGTTTCATTTGGTAAATCA

C1 reverse: CCCTAAAGCACCACAACCCA

C2 forward: GGTCTAAATACAATTGCTCTTTGACATT

C2 reverse: GATGTAGAAATACTTAGCGACAATGTAG

The PCR mixtures were made before each run according to Table 2, pipetted onto a PRC plate together with the template, and run in a PCR machine (Bio-Rad T100, Foster city, CA, U.S.A.). The used PCR program is presented in Table 3. The PCR-products were kept at -20 °C until running a 2% agarose gel electrophoresis to check if the PCR-products had any increase in size compared to control. The gel run was carried out in 1x TAE buffer with a sample volume of 15 µl, and a 1 kb ladder, at

100 V for 1,5-2 hours. Ethidium bromide was used as the stain. The imaging was done right after the gel run with a Bio-Rad ChemiDoc MP imaging system (Carlsbad, CA, U.S.A.). In practice, half a 96-well plate was needed for the colonies, one PCR plate for two sets of PCR and one agarose gel with 100 wells per sampling. The collected colonies were stored for later use.

Table 2. The PCR mixtures were made separately for the two known CRISPR loci, using different primers for C1 and C2. The table represents the ingredients for one reaction.

Reagent	Locus C1	Locus C2
H_2O	12.75 µl	12.75 µl
Green buffer	2 µl	2 µl
dNTP 2mM	1 µl	1 µl
Forward primer	1 µl	1 µl
Reverse primer	1 µl	1 µl
Dream Taq (Thermo Fisher, 5 U/µl)	1.25 U	1.25 U
Template	2 µl to each reaction	2 µl to each reaction

Table 3. The PCR program used to amplify the potential CRISPR spacers in the collected colonies.

Step	Temperature	Duration
1.	95°C	3 min
2.	95°C	30 s
3.	60°C	30 s
4.	72°C	30 s
5.	repeat 2-4	30x
6.	72°C	15 min
7.	12°C	∞

With the planned setup, there was a total of 24 sample cultures with sets of three replicates, and three controls with the phage and no bacteria. The plan was to test four colonies from each treatment sample culture, which meant 12 colonies per condition and 48 colonies in total for each sampling.

2.4 Phage counts

Phage titrations were made after each sampling by adding 10% of the bacterial sample volume of chloroform to the samples and the dilutions (10^{-2}, 10^{-4}, 10^{-6}). Chloroform was added to kill the bacteria and preserve the phages for phage titration. The chloroform-treated supernatant dilutions were added to double-layer Shieh agar plates containing *F. columnare*. The plates were made with a 3 ml layer of 47 °C Shieh soft agar supplemented with 0.1% mucin and 100 µl of an overnight culture of *F. columnare*, poured on top of a Shieh agar layer. The chloroform-treated samples were pipetted onto the Shieh soft agar forthwith as 10 µl drops. The plates were incubated at room temperature for 2-3 days before counting the phage plaques.

2.5 Data analysis

The quantities of *F. columnare*, *Aeromonas* sp. and the phage were observed. The different morphologies of the *F. columnare* colonies were verified and presented in graphs and compared with each other. Agarose gel images were taken for the CRISPR analysis. The spacer acquisition data from the CRISPR analysis was presented in figures showing the number of new spacers and mutants in each of the tested conditions. The data was analyzed using Microsoft Excel, and GraphPad Prism 9.1.2 software was used for drawing the figures. The significance of the differences in spacer acquisition between culture conditions was tested with a non-parametric Kruskal-Wallis ANOVA analysis with pairwise comparisons using IBM SPSS Statistics 28.0 software. The test included all analyzed colonies from every timepoint.

3 RESULTS

The bacterial cultures were successful. *F. columnare* and *Aeromonas* sp. survived well through the experiment, whereas *E. coli* seemed to die out soon after the beginning of the experiment. Bacterial titrations (Figure 2) indicate the evolution of the total number of *F. columnare*, and the number of *Aeromonas* sp. throughout the experiment. The phage stayed viable in all cultures with bacteria, though decreased in number towards the end of the experiment (Figure 3). The phage in the bacteria-free phage control disappeared before the first sampling on day seven. The culture condition that initially included all three bacteria had *E. coli* seemingly die out before the first sampling. The same occurrence took place in the condition where *F. columnare* and *E. coli* were together. Therefore, the two conditions including *Aeromonas* sp., should, in theory, yield similar results, as well as the *F. columnare* + *E. coli* + phage, and *F. columnare* + phage conditions.

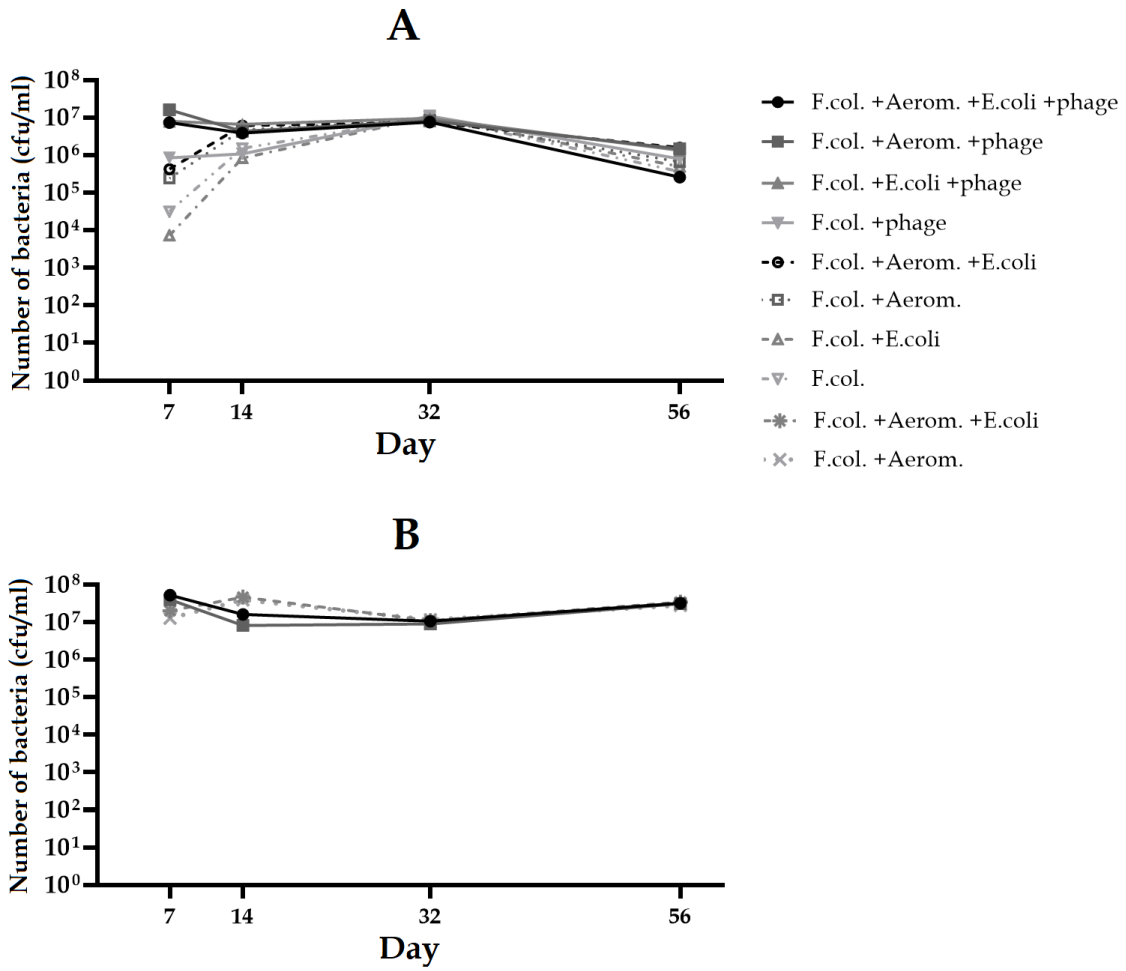

Figure 2. Bacterial numbers in the experiment. Replicate averages of *F. columnare* (A), and Aeromonas sp. (B) by condition. Solid lines represent cultures with phage, and dashed lines without phage.

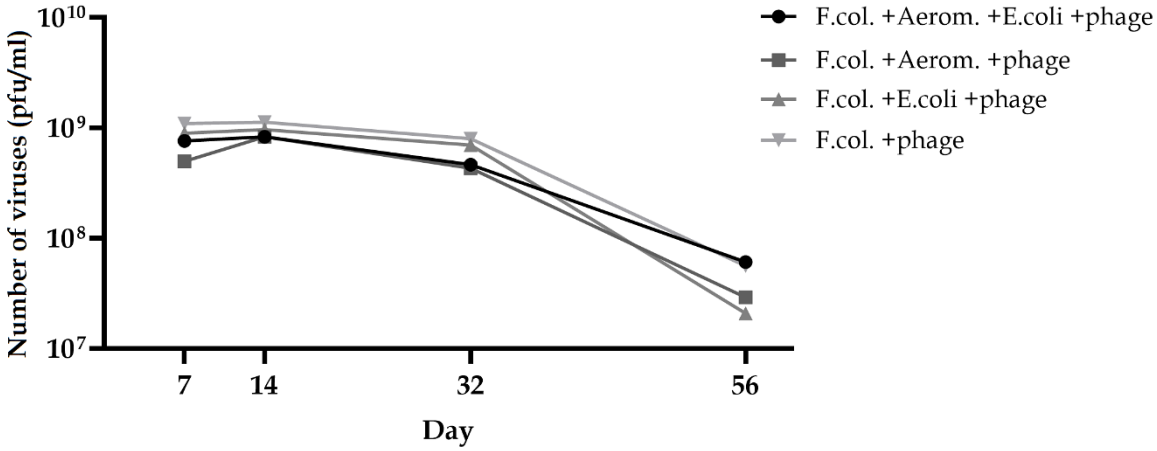

Figure 3. Phage numbers in each culture condition. Drawn with triplicate averages.

The morphology of *F. columnare* colonies was followed throughout the experiment to see if the bacteria used surface modification to defend themselves against phage predation (Figure 4). The initial inoculation in the beginning of the experiment had *F. columnare* in the virulent but phage-exposed rhizoid morphotype. The first sampling on day 7, however, showed that more than 90% of the bacteria in the phage-containing cultures expressed the rough morphotype. In the phage-free cultures the corresponding percentage was less than four. At the end of the experiment on day 56, the percentage of rough colonies in all of the cultures had decreased by 3.6-23.4% from the beginning. Consistently, the number of rhizoid colonies had an increase of 4.4-20.5%. An exception was found in the *F. columnare* + *Aeromonas* sp. + *E. coli* + phage -condition, which had a high abundance of rhizoid bacteria in one of the replicates in the first sampling, resulting in a smaller average in the last sampling. Additionally, rhizoid colonies were not found in seven of the twelve phage-containing cultures in the first sampling, while in the last sampling they were present in all but one culture.

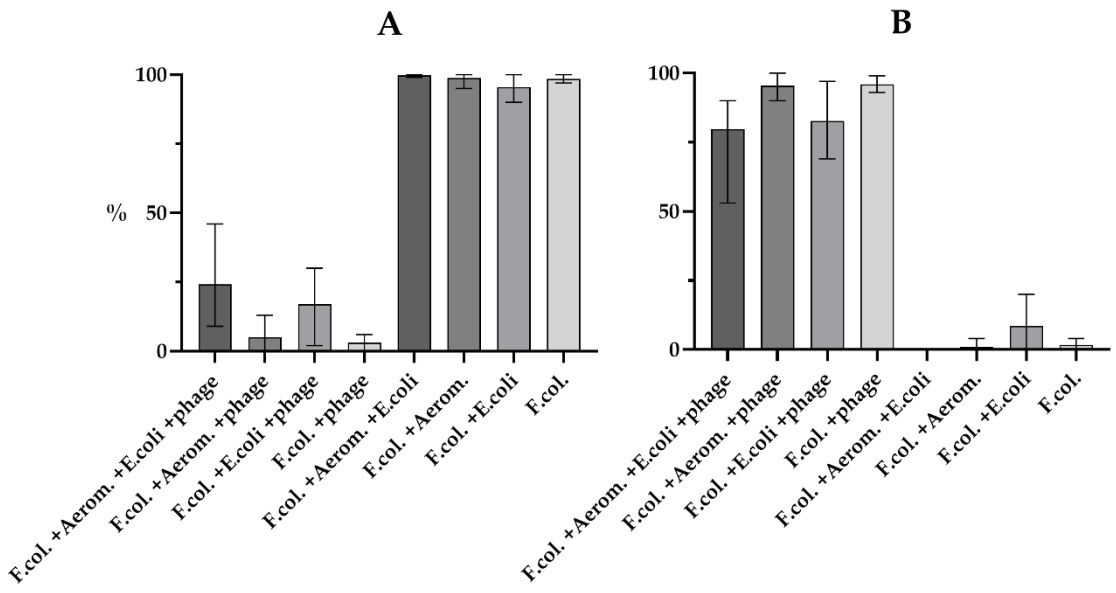

Figure 4. *F. columnare* colony morphologies from all experimental conditions. Each bar represents the average proportion of A) rhizoid or B) rough colonies found in each condition, all sampling time points and replicates combined. The error bars show the standard deviations of sample distribution.

The proportion of CRISPR mutants increased through the experiment. The first sampling at day seven revealed one mutant in locus C1 and ten mutants in locus C2. The three following samplings had 5, 19 and 22 mutants in C1, and 14, 22 and 24 mutants in C2, respectively, out of 48 analyzed colonies. Out of all found mutations, 40% were found in locus C1 and 60% in locus C2. An individual colony had a mutation only in locus C1 in 11% of the tested colonies, whereas 41% of the mutants had acquired spacers only in locus C2. 48% of the mutants had acquired spacers in both loci. The majority of all mutants were found from rhizoid colonies. Approximately 74% of all mutants were rhizoid and the remaining 26% had the rough morphotype.

The number of new CRISPR spacers in isolated bacterial colonies (4 per culture) (Figure 5) increased considerably in both loci during the experiment. There were notable differences in spacer acquisition between culture conditions. A non-parametric Kruskal-Wallis ANOVA analysis with pairwise comparisons gave a test statistic value of 17.742, three degrees of freedom, and asymptotic significance (2-sided test) $p<0.001$. The pairwise comparisons are shown in Table 4.

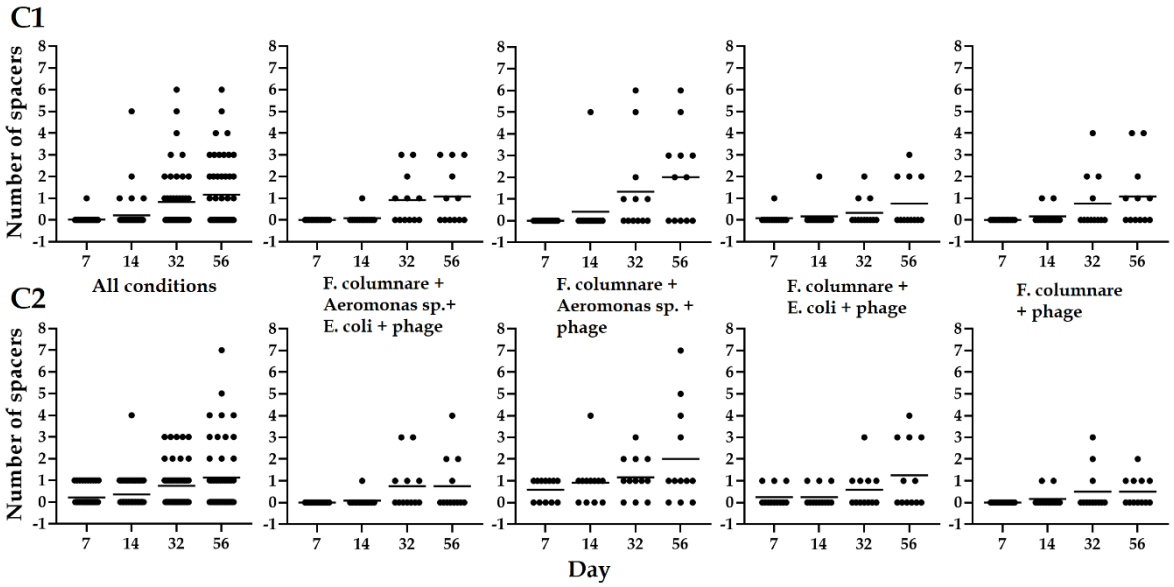

Figure 5. CRISPR spacer counts by condition at each time point for locus C1 (above) and C2 (below). Each dot represents one analyzed colony and shows the number of acquired spacers. Averages are indicated with horizontal lines.

Table 4. Pairwise comparisons of the number of acquired spacers in the experimental conditions from the Kruskal-Wallis ANOVA analysis. The table shows the test statistic value and the significance value (p-value) for each pair.

Comparison	Test statistic	Significance
[F.col + Aerom. + E. coli] – [F.col + Aerom.]	-36.292	<0.001
[F.col + Aerom. + E. coli] – [F.col + E. coli]	-6.229	0.536
[F.col + Aerom. + E. coli] – [F.col]	-0.146	0.988
[F.col + Aerom.] – [F.col + E. coli]	30.063	0.003
[F.col + Aerom.] – [F.col]	36.146	<0.001
[F.col + E. coli] – [F.col]	6.083	0.546

In the condition with all bacterial species (*F. columnare*, *Aeromonas* sp., *E. coli,* and the phage) present, *F. columnare* had a slight increase in the number of new CRISPR spacers in locus C1 between the first and the second sampling, and a greater increase between the second and the third sampling, however remaining the same in the fourth sampling (Figure 6). Overall, the ratio of C1 mutants in this condition went from 0% to 50% during the experiment. The number of C2 CRISPR mutants had a very similar evolution until the third sampling, when 42% of the analyzed bacteria were mutants. However, the number of C2 mutants in condition number one decreased to 33% in the fourth sampling. This decrease was the only negative progression in the mutation acquisition data, including all four conditions.

The condition with *F. columnare* and *Aeromonas* sp. and the phage, presented in Figure 6, had identical spacer acquisition rate in locus C1 as the condition with all three bacteria, starting from no mutations and ascending to 50%. However, C2 CRISPR mutants were highly more abundant than in when all bacterial species were present. In the first sampling, 58% of the analyzed bacteria were mutants, 67% in the second sampling, and 75% in the third and fourth sampling.

The third experimental condition consisted of *F. columnare*, *E. coli* and the phage (Figure 6). In the first and second sampling, new C1 spacers were found in 8% of

the analyzed bacteria, whereas the third and fourth sampling showed 25% and 33% of C1 CRISPR spacers, respectively. Locus C2 had a similarly moderate increase in the number of CRISPR mutants. The first two samplings had 25% mutants, and the third and fourth sampling had 42% and 50% mutants of the analyzed colonies, respectively.

The evolution of spacer acquisition in the fourth condition (*F. columnare* and the phage), presented in Figure 6, exhibited a relatively linear increase in the number of spacer mutants in both loci. C1 mutants started at 0% in the first sampling, ascending to 17%, 33% and 50% in the three next samplings. Mutations in the C2 locus started at 0% as well, and the ratio increased to 17%, 25% and 42% in the subsequent samplings, respectively.

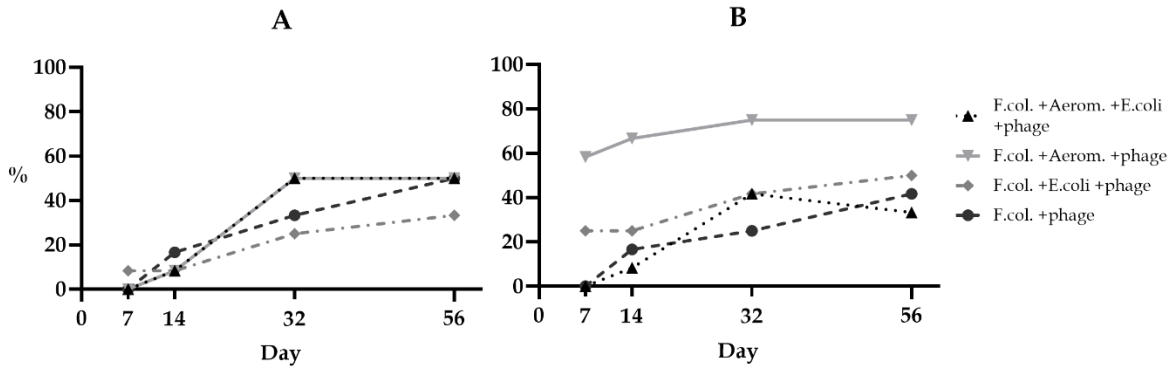

Figure 6. The proportions of colonies with new CRISPR spacers at each sampling time point. A) Mutants with at least one CRISPR spacer in locus C1 and B) mutants with at least one CRISPR spacer in locus C2.

The distribution of mutants between the two morphotypes had a clear trend. All C1 mutants had the rhizoid morphotype, also those that had concurrent spacer addition in locus C2. C2 mutants, on the other hand, had the rhizoid morphotype in 57% of cases when new spacers were obtained, and only in 6% when there was a mutation only in C2. In other words, when CRISPR spacers had been acquired in locus C2 but not in C1, the colony morphotype was rough in 94% of the cases. Importantly, 70% of the C2 mutants that had the rough morphotype were found in the condition where *F. columnare* was interacting with *Aeromonas* sp. and the phage.

The remaining 27% and 3% were found in conditions 3 (*F. columnare* with *E. coli* and phage) and 4 (*F. columnare* with phage), respectively.

The overall combined numbers of bacterial colonies obtaining new CRISPR spacers in Figure 7 show the differences in the quantity and distribution of mutants between the culture conditions. Noteworthy are the high numbers of mutants that had acquired CRISPR spacers either only in locus C2 or in both loci, C1 and C2, especially in the *F. columnare* + *Aeromonas* sp. + phage cultures, compared to the considerably lower numbers of corresponding CRISPR mutants in the *F. columnare* + phage cultures. Interestingly, the quantity of C1-only mutants in the *F. columnare* + phage cultures was four times higher than that of the *F. columnare* + *Aeromonas* sp. + phage cultures.

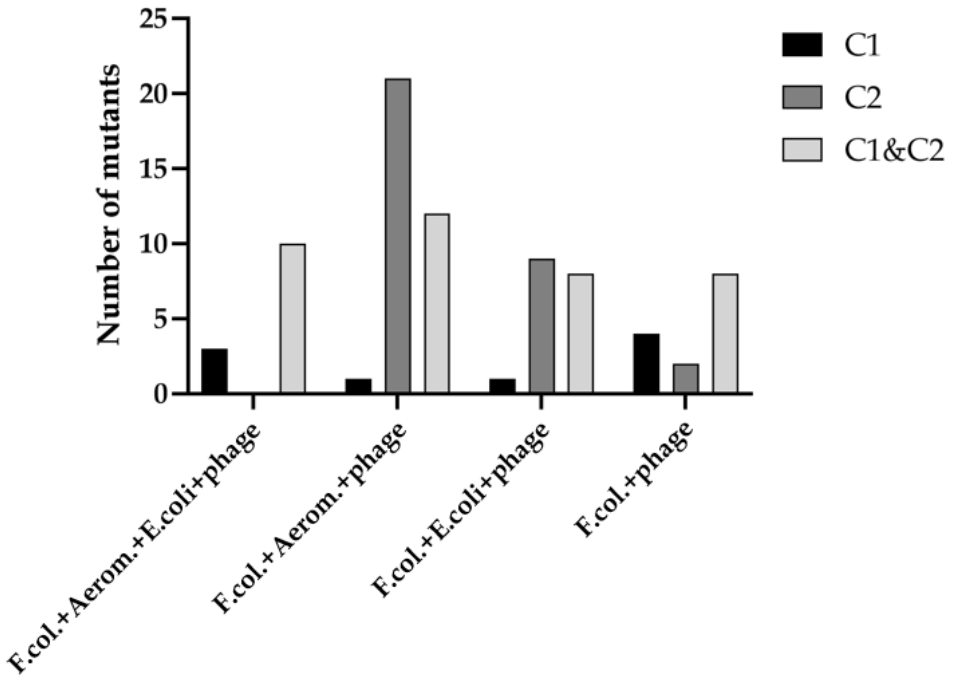

Figure 7. Numbers of bacterial colonies with new CRISPR spacers in *F. columnare* when cultured in competition with other bacterial species. Each bar indicates the CRISPR mutants found, combining all four sampling time points. The black bars represent the colonies that had acquired CRISPR spacers only in locus C1, while the dark grey bars indicate the corresponding numbers of C2-only mutants. The light grey bars represent the colonies with acquired CRISPR spacers in both loci.

A phage-free experimental control treatment done with the same mixtures of bacteria in similar culture conditions showed no CRISPR mutants in any of the cultures on a period of three weeks and three samplings (data not shown). Accordingly, the phage-free cultures of this experiment were also assumed to develop no CRISPR mutants.

4 DISCUSSION

The relationships between hosts and parasites are affected by their own characteristics but presumably also by their interactions with the surrounding communities. The aim of this experiment was to study the effects of competition on two phage resistance mechanisms, surface modification and CRISPR-Cas. This was studied in a long-term experiment, where *F. columnare* was exposed to its virulent phage in combination cultures with *Aeromonas* sp. and *E. coli*. The evolution of both surface modification and CRISPR spacer acquisition was observed throughout the experiment. This was done by plating the bacterial cultures and defining colony morphotypes and analyzing chosen colonies with PCR and gel electrophoresis to detect the addition of new potential CRISPR spacers in the bacterial genome.

The cultures sustained *F. columnare* and *Aeromonas* sp., but *E. coli* perished soon after starting the experiment. The reason behind *E. coli* not surviving could be that the conditions regarding temperature, nutrients, and shaking frequency were not optimal for it. *E. coli* might have lower fitness in the tested conditions compared to *F. columnare* and *Aeromonas* sp., and therefore it might have been the weakest competitor of the three bacteria. The phage multiplied constantly, signifying successful predation on *F. columnare,* and, additionally, competent proliferation of the host bacterium, denoting that neither extinguished the other. This is further substantiated by the extinction of the phage in the bacteria-free phage control.

The morphology changes of the *F. columnare* colonies evolved as expected in all conditions. The predicted trend was that the phage-infected bacteria would be in the shielded rough form and that the bacteria in the phage-free conditions would be in the infective rhizoid form (Laanto et al. 2012, Kunttu et al. 2021). It was also anticipated that the number of rhizoid colonies would grow with the increasing number of CRISPR spacers, because resistance through surface modification is no longer essential for surviving encounters with the phage. All these trends were observed in this experiment.

Defense via surface modification has been shown to have a fixed fitness cost on *F. columnare*, as it not only reduces the virulence on the bacterium, but also decreases its gliding motility and protease activity (Kunttu et al. 2021). CRISPR-mediated resistance, on the other hand, has fitness costs from maintaining and using the system, but instead of a fixed cost, costs vary as the system is inducible and can be activated if needed (Vale et al. 2015, Westra et al. 2015). The choice between surface modification and CRISPR would then depend on the probability of infection, that is to say, the frequency of phage encounters (Westra et al. 2015), with the fixed surface modification serving better for high concentration of phages and the inducible CRISPR being better suited for less frequent phage encounters. In this experiment, *F. columnare* was seen to utilize both resistance mechanisms, of which surface modification was observed more often. This implies that the phage load on the host bacteria was relatively high. However, the fact that CRISPR spacers were detected increasingly during the experiment, and in higher numbers in the *F. columnare* + *Aeromonas* sp. + phage condition than in the *F. columnare* + phage condition, could signify that CRISPR-mediated resistance offers bacteria a selective advantage for competition with other bacterial species in the presence of phages and host signals (mucin).

Acquisition of CRISPR spacers was observed in both loci, suggesting that *F. columnare* probably developed CRISPR-mediated resistance against the phage. The number of spacers in both loci increased consistently through the experiment, locus

C2 containing a slightly higher number in every sampling. At the end of the experiment, CRISPR spacers were found from locus C1 in 22 colonies, and locus C2 in 24 colonies, out of 48 tested colonies. Overall, this gives a 46% coverage for acquisition of spacers in locus C1 and a 50% coverage in locus C2.

A previous study on *Pseudomonas aeruginosa* by Alseth et al. (2019) has shown that phage predation and the presence of competing species has ecological effects on the bacterium so that it favors CRISPR-Cas over surface modification during host colonization. In the phage-infected cultures of this experiment, the number of CRISPR spacer mutants increased concurrently with the number of (presumably virulent) rhizoid colonies, resulting in phage-resistant and virulent bacteria. Furthermore, the acquisition of new CRISPR spacers in *F. columnare* was significantly most pronounced when *F. columnare* had competition over resources with *Aeromonas* sp., suggesting that *F. columnare* can favor CRISPR-Cas over surface modification in certain conditions despite frequent phage encounters.

The evolved CRISPR mutants (i.e. colonies with new spacers) had differences in which locus the spacer or spacers had been added to. The number of C2 mutants was considerably higher in the culture where *F. columnare* was competing with *Aeromonas* sp., compared to the no-competition culture, implying that competition induced CRISPR mutations in locus C2. Overall, there were nearly four times more C2-only mutants compared to C1-only mutants. The reason for the more pronounced acquisition of C2 mutants is not yet known but it could be related to acquisition conditions and locus activity. In previous research on *F. columnare*, locus C2 has been shown to be dependent on the CRISPR adaptation genes (Cas1 and Cas2) of locus C1 to acquire spacers (Hoikkala et al. 2021). The same study also showed that nearly half of new spacers in locus C2 were self-targeting, drastically differing from locus C1, where only a fraction of new spacers targeted self. This is well explained by the nature of the two loci. C1 targets DNA, and self-DNA-targeting spacers are usually lethal to the bacterium, whereas spacers in locus C2 target mRNA, which probably only disturbs normal gene expression (Hoikkala et

al. 2021). The acquisition of self-targeting CRISPR spacers could be the result of an infection by a phage that contains genes of bacterial origin from previous rounds of infection, or simply an error in spacer incorporation (Stern et al. 2010). Self-targeting CRISPR spacers have a negative effect on the host, as they might lead to gene deletion and even death (Stern et al. 2010), in addition to a larger genome being a negative trade-off for reproductive ability. This in mind, the copious C2-only mutants in this experiment are likely to include self-targeting genes (negative effect) as well as phage-targeting ones (positive effect). Overall, depending on the nature of the spacer, acquisition of new CRISPR spacers could be either a positive or a negative trade-off for the bacteria.

In addition to CRISPR-mediated immunity, resistance through surface modification was also prominent in the results of the study. The morphotype of the colonies that had acquired CRISPR spacers was rhizoid (infective, but susceptible to phage-infection without CRISPR- mediated immunity) in 74% of the cases, indicating that spacer acquisition offered the bacteria an effective protection against phage infection, allowing them to retain their potential for infecting a fish host themselves.

The reason for the more profuse spacer acquisition with *Aeromonas* sp. could lie in the competitional setting between the two bacteria. Both bacteria are found in aquatic environments, and they inhabit similar ecological niches. They are likely to compete for resources, such as space and nutrients, which would make it beneficial for *F. columnare* to remain in the rhizoid morphotype. Since the mucin in the culture medium can act as a virulence-boosting host signal for bacteria (Staroscik and Nelson 2007, Almeida et al. 2019), it may implicate a benefit for remaining in the infective rhizoid form. To survive phage infections caused by the infective phage present in the cultures, *F. columnare* would need to achieve phage resistance through the inducible CRISPR system, in opposition to the constitutive rough morphotype, that would be phage resistant but uninfective, immobile and likely a poor competitor. Interaction with *Aeromonas* sp. in the experimental conditions was therefore likely to amplify the spacer acquisition in *F. columnare*, because obtaining

rapid phage resistance through CRISPR-Cas while maintaining infectivity was a less costly trade-off than immunity through surface modification and loss of virulence.

The use of phages to prevent and treat bacterial outbreaks has previously shown promising results (Nakai and Park 2002, Higuera et al. 2013, Khairnar et al. 2013). Studies on *F. columnare* have also shown that phages have good potential for being an alternative treatment method against columnaris disease instead of antibiotics (Laanto et al. 2012, Prasad and Kumar 2012). Not only do phages kill bacteria, but they also decrease their virulence by inducing the change of colony morphotype from the virulent rhizoid form to the non-infectious rough type. However, as the results of this experiment suggest, competition with other bacteria can increase the formation of CRISPR immunity, which would decrease the effectivity of phage therapy with the same phage. To overcome this problem of CRISPR resistance, different phage mixtures could be used, or phage therapy could be used together with antibiotic treatments (Laanto et al. 2015).

4 CONCLUSIONS

According to this study, competition with other bacteria can have an effect on the formation of phage resistance. This is supported by the significantly higher abundance of CRISPR spacers in bacteria that were cultivated together with *Aeromonas* sp., compared with the control cultures that had *F. columnare* alone with the phage. Phage resistance was achieved by both methods, CRISPR-Cas and surface modification, but the latter did not seem to be affected by competition. These results support and are supported by previous research, and they are promising in respect of future research on microbial interactions in natural communities.

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