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321

Hanna Kinnula

The Influence of Infective Dose, Nutrient Availability and Coinfection on Virulence of *Flavobacterium columnare*

Implications of Intensive Aquaculture on Opportunistic Infections

JYVÄSKYLÄN YLIOPISTO

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ABSTRACT

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Ecological factors are known to affect disease dynamics and even lead to disease emergence. Especially in opportunistic, environmentally transmitted pathogens, the environment may significantly contribute to pathogen virulence. Intensive farming, including aquaculture, has been suggested to create conditions favouring development of highly virulent pathogens. At Finnish fish farms, epidemics caused by opportunistic *Flavobacterium columnare* have been constantly increasing in their prevalence and severity since the 1980's. Yet, factors behind the increased virulence of the pathogen and their mechanisms of action have largely remained unsolved. In this thesis, I explore the effects of infection dose, outside-host nutrients and coinfection with conspecific strains on virulence and disease dynamics of *F. columnare*. Virulence of bacterial strains isolated from fish farms was tested in experimental fish infections, where bacterial dose, water nutrient concentration and number of infecting strains were manipulated. Two fish species, zebra fish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), were used as hosts to confirm the suitability of zebra fish for further infection experiments. Finally, strains isolated during 2003-2010 were studied for temporal and local changes in their virulence, growth and competitive ability. Increase in infection dose was found to increase bacterial virulence in both hosts. High nutrient level increased virulence via enhanced bacterial growth and virulence factor activation. I also found that coinfection can increase *F. columnare* virulence, but the disease outcome depends on the strain characteristics. Finally, it is shown that the aquaculture environment may select for strains with high virulence and enhanced competitiveness. Taken together, the results indicate that all the studied factors can contribute to opportunistic disease outbreaks in aquaculture.

Keywords: Columnaris disease; disease emergence; fish farming; *Flavobacterium columnare*; opportunistic pathogen; virulence; virulence evolution.

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LIST OF ORIGINAL PUBLICATIONS

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

I Kinnula H., Mappes J., Valkonen J.K. & Sundberg L.-R. 2015. The influence of infective dose on the virulence of a generalist pathogen in rainbow trout (*Oncorhynchus mykiss*) and zebra fish (*Danio rerio*). PLoS ONE 10(9): e0139378.

II Kinnula H., Mappes J., Valkonen J.K., Pulkkinen K. & Sundberg L.-R. 2016. Higher resource level promotes virulence in an environmentally transmitted bacterial fish pathogen. Submitted manuscript.

III Kinnula H., Mappes J. & Sundberg L.-R. 2016. Coinfection outcome in an opportunistic pathogen depends on the inter-strain interactions. Submitted manuscript.

IV Sundberg L.-R. Ketola T., Laanto E., Kinnula H., Bamford J.K.H., Penttinen R. & Mappes J. 2016. Intensive aquaculture selects for increased virulence and interference competition in bacteria. Proceedings of the Royal Society B: 283(1826):20153069.

The table shows the contributions of the authors in the original papers. Initials stand for the following authors: Hanna Kinnula, Lotta-Riina Sundberg, Elina Laanto, Reetta Penttinen, Johanna Mappes, Janne Kristian Valkonen, Tarmo Ketola, Katja Pulkkinen, Jaana Bamford.

	I	II	III	IV
Original idea	L-RS, JM, HK	HK, L-RS, JM	L-RS, HK, JM	L-RS, JM, TK, JB
Experimental design	L-RS, JM, HK, JV	HK, L-RS, JM, JV	HK, L-RS, JM	L-RS, JM, TK, JB
Data collection	HK, L-RS	HK, KP, L-RS	HK	EL, HK, L-RS, RP
Data analysis	JV, HK	HK, JV	HK, L-RS	TK, L-RS
Writing	HK, L-RS, JM, JV	HK, L-RS, JM, KP, JV	HK, L-RS, JM	L-RS, JM, TK, EL, HK, RP, JB

1 INTRODUCTION

1.1 Virulence

Parasitism is a common phenomenon in nature. The majority of all living organisms are parasites and thus all free-living organisms, and also their parasites, are parasitized (Windsor 1998). There is evidence showing that free-living organisms may serve as hosts for several parasites at the same time (Windsor 1998, Schmid-Hempel 2011). In a study of four lakes in Central Finland, perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) were found to be parasitized by 42 and 37 species of parasites, respectively (Valtonen *et al.* 1997). Humans are hosts to at least 149 non-microbial species (Windsor 1998). When considering microparasites such as viruses, bacteria, fungi and protozoa, the number is even greater (Schmid-Hempel 2011). These small parasites are usually less than few hundreds of micrometres in size and have short generation times, being thus able to reach high numbers within their host.

As the host and the parasite compete over the same resources (Ebert and Herre 1996), the parasites usually have negative net effect, called virulence, on their hosts (Hamilton 1980, Poulin 2008). Virulence can be defined in many ways. From the view of population biology, virulence is defined as increased host mortality resulting from parasite infection (Anderson and May 1982, May and Anderson 1983), and thus the parasite point of view is disregarded. In medical microbiology, virulence is defined as the harm or morbidity caused to the host by the parasite, but the influence of host on the disease outcome is not always taken into consideration. Instead, the focus is often in determining virulence factors, i.e. elements needed for the virulence but not for the survival of the parasite (Brown *et al.* 2012). These include pathogen organelles (in bacteria) or molecules that help the pathogens to invade the host, to evade the host defences and to cause disease, such as adherence and invasion factors, endo- and exotoxins, capsules and siderophores (Peterson 1996). Virulence factors are often complex and thus considered to have evolved as a result of natural selection (Brown *et al.* 2012).

From a broader perspective, virulence is a product of the host-parasite interaction that depends on both the host and the parasite (Read 1994, Read *et al.* 1999, Casadevall and Pirofski 2003). This view is supported by the observation that the same parasite strain does not cause equal harm to each host genotype, and different parasite strains express different levels of virulence in the same host genotype (Ebert and Hamilton 1996). Also other biotic (living) and abiotic (non-living) factors of the environment may influence virulence, such as host population structure, abundance of predators, temperature, salinity and nutrient concentration. In this thesis, virulence is defined as the harm caused to the host due to pathogen infection, and measured as mortality of hosts in the experimental work. To broaden the approach, interactions of pathogen strains and influence of the outside-host environment on pathogen virulence are studied.

Pathogens can be considered as parasites with an ability to cause a disease (a condition that prevents the organism or its part from working normally), usually viruses, bacteria, fungi or other micro-organisms. However, not all pathogens are solely parasites since opportunistic infections can also be caused by organisms that are normally commensal or mutualistic (Cogen *et al.* 2008), or able to live in the environment outside the host (Casadevall 2008). Virulence varies highly between pathogens: some of them cause only mild symptoms while others, sometimes closely related to the less harmful species, may lead to death of the host (Read *et al.* 1999). Some pathogens even benefit from the death of their host, like anthrax bacteria (*Bacillus anthracis*) and most of the bacterial viruses that are released from the host upon its death (Dimijian 2000). Thus, virulence is not always optimal for the pathogen but can sometimes be too high or too low, for example during transmission to new host species that the pathogen has not adapted to exploit (Ebert 1998, Bull and Ebert 2008). Highly virulent pathogen strains are known to arise when the pathogen infects a new host species: for example Ebola viruses originating from animal hosts are too virulent to humans to effectively spread in the human population (Leggett *et al.* 2013). On the other hand, when the pathogen is not sufficiently virulent, its reproduction rate and consequently transmission are reduced (Ebert and Herre 1996).

1.2 Virulence evolution

Virulence is not a fixed feature of the pathogen but a result of complex host-pathogen interactions which also drive the evolution of virulence (Read 1994, Alizon *et al.* 2009). Nevertheless, as the generation times of parasites are shorter and evolutionary rates consequently higher than that of their host's, evolution of the host is often ignored in the short term (Read 1994, Bull 1994, Ebert and Hamilton 1994). The virulence differs between parasite strains and this variation provides raw material for virulence evolution (Bull 1994). Selection acts on genes that encode virulence determinants, i.e. the traits of a host or a

parasite whose loss attenuates virulence (Read 1994). In bacteria, these traits are encoded by bacteriophages, plasmids and transposons along with the bacterial chromosome (Levin and Svanborg Eden 1990).

Virulence has reached "an optimum" when a decrease or increase in virulence leads to decreased parasite fitness (the organism's reproductive success as compared to competing organisms) (Ebert and Hamilton 1996). To maximize its fitness, the parasite should keep its virulence at this optimal level. The host instead continuously evolves to reduce the virulence of the parasite in order to avoid fitness reduction caused by the parasite. The optimal virulence is thought to increase with horizontal transmission (transmission from one individual to another), and to decrease with vertical transmission (transmission from parents to offspring) (Stewart *et al.* 2005). This is because in the former mode of transmission increased transmission may be favoured even at a cost of increased host morbidity, while in the latter mode of transmission the parasite benefits from keeping its virulence as low as possible to avoid harming the host's reproductive ability.

1.3 Theories of virulence evolution

For most of the 20th century, it was commonly believed that the pathogens should evolve towards benignity and virulence was seen as a maladaptive transition stage in the host-pathogen interaction (Ewald 2004). As parasites have to extract host resources in order to replicate and produce transmission stages, the host suffers damage that can shorten its life-span along with the infectious period over which transmission to another host can occur (Anderson and May 1982, Bull 1994, Frank 1996, Levin 1996). The trade-off theory of virulence suggests that virulence will evolve to an optimum where the virulence and transmission of the pathogen are balanced so that the overall transmission of the pathogen is maximized (Read 1994, Frank 1996). As a result, parasite should evolve an intermediate level of virulence. The trade-off hypothesis is supported by several studies on host–parasite systems (Ebert and Mangin 1997, Mackinnon and Read 1999, Messenger *et al.* 1999, Davies *et al.* 2001, de Roode *et al.* 2005), but it must be noted that according to some studies no link exists between virulence and transmission. For example, in many infectious diseases the virulence is caused by the host immune responses, not the harm caused by the parasite (Graham *et al.* 2005). Therefore, there is no certainty that a virulence-transmission trade-off is common or important to pathogen virulence evolution (Ebert and Bull 2003, Galvani 2003), thus calling for experimental tests for the theory (Brunner and Collins 2009).

Natural selection is dependent on time and place (Levin 1996). This means that the traits that are advantageous for individual organism's survival or replication are expressed in a certain time and environment. They are favoured in that environment, and they do not need to affect the fitness of the organism at other times or in other habitats. For example, virulence sometimes

results from infection of tissues that are a dead-end from which the parasite is not able to transmit forward (Levin 1996). This so called short-sighted virulence evolution theory is necessary for understanding the within-host adaptation of opportunistic bacterial pathogens (Martínez 2014).

The factors responsible for the pathogen virulence in a host may have evolved for other purpose than causing infection, i.e. they are not a result of host-parasite co-evolution (Levin 1996). Thus, pathogen virulence may be an accidental consequence of selection that operates on other traits of the pathogen, leading to coincidental evolution of virulence. For example, lethal toxins produced by soil bacteria *Clostridium botulinum* and *Clostridium tetani* are unlikely targeted to kill humans, but have evolved as a response to selection in the normal life cycle in the soil.

1.4 Ecological factors affect the dynamics of disease

The prevalence and evolution of infectious diseases are dependent on complex interactions among the host and the pathogen and their environment. Thus the environment affects the organisms and, respectively, the organisms influence the environment they live in. Therefore, to understand disease dynamics and to develop functional disease management strategies one needs to understand the biotic and abiotic factors affecting the disease. The environmental conditions are likely to vary in time and space (Meyers and Bull 2002) causing shifts in the characteristics of pathogen-host-environment interactions that may lead to novel transmission patterns or even selection of novel pathogens (Engering *et al.* 2013). Indeed, the ecological factors as such have importance in most emerging diseases, but they can also lead to disease emergence by causing evolutionary changes in host or pathogen (Schrag and Wiener 1995, Engering *et al.* 2013). The pathogens that are most likely to evolve into emerging disease agents, either have a high mutation rate, or are able to acquire genetic material from their environment or infect multiple host species (Engering *et al.* 2013).

Both biotic and abiotic components of the environment may influence the qualities of the host and/or the pathogen and subsequently their evolution. In this thesis the focus is on the pathogen, and the direct or indirect influence of environmental variables on hosts is not discussed. Abiotic components of the environment include temperature, salinity, turbidity, pollution or availability of non-living resources. In bacterial pathogens, temperature and resource availability may affect replication (Shiah and Ducklow 1994, Wedekind *et al.* 2010), diversity (Wilkins *et al.* 2015, Johnson and Carpenter 2010) and virulence (Kimes *et al.* 2012, Ketola *et al.* 2016). For example, the environmental nutrients can increase virulence of the pathogen via enhanced replication (Kisand *et al.* 2001; Farjalla *et al.* 2002; Elser *et al.* 1995) or by virulence factor activation (Somerville and Proctor 2009, Oogai *et al.* 2011, Penttinen *et al.* 2016). Biotic environmental factors include other organisms such as hosts, predatory protozoans, parasitizing bacteriophages, and competing bacteria (Meyers and

Bull 2002). Protozoan predation, for example, can have a positive influence on the evolution of bacterial virulence by promoting expression of bacterial virulence traits (Cirillo *et al.* 1994, Cirillo *et al.* 1997, Greub *et al.* 2004). In more recent studies also opposite results have been found, where a trade-off was observed between bacterial virulence and predation resistance (Friman *et al.* 2009, Friman and Buckling 2014, Zhang *et al.* 2014a, Zhang *et al.* 2014b). Also bacteriophages can lead to increase of bacterial virulence by introducing new genetic material into bacterial genome (Brüssow *et al.* 2004). Phages can also lead to attenuated bacterial virulence when costs of phage-resistance lead to virulence decline (Laanto *et al.* 2012). Competition with other bacteria may lead to different virulence outcomes depending on the relatedness of the interacting genotypes and the type of competition (Frank 1996, Griffin *et al.* 2004, Buckling and Brockhurst 2008).

Studying the effects of environmental factors on bacterial virulence is difficult in nature, as the interactions are very complex and involve several factors. Therefore, controlled experiments in the laboratory conditions are needed to find out the effects of specific factors on bacterial virulence.

1.5 Intensive farming and disease emergence

Intensive farming creates conditions, where the growth and reproduction of the pathogen are very different than in a wild host due to human actions targeted to increase the production efficiency (Mennerat *et al.* 2010). In natural ecosystems, the host-pathogen coevolution is suggested to favor pathogens with low virulence, but in intensive systems the genetic selection and livestock management provides frequent host-pathogen contact opportunities (Jones *et al.* 2012). Such conditions enable more virulent forms to evolve among the existing pathogens as well as permit invasion and replication of "wild" microorganisms. Consequently, intensive farming may lead to disease emergence (Murray and Peeler 2005, Nowak 2007, Mennerat 2010). Several real-life examples support this suggestion. For example, the risk of zoonotic disease epidemics and disease emergence has been found to increase with intensive farming practices (Jones *et al.* 2012). Also the spread of Nipah virus, that causes respiratory disease in pigs, has been markedly enhanced as a result of high pig density in intensive farming conditions (Daszak *et al.* 2006) and new strains of influenza virus have risen among intensively farmed pigs and poultry (Butler *et al.* 2012).

This thesis focuses on aquaculture that is the fastest growing food production system in the world. Aquaculture conditions are in many ways similar to serial passage experiments that commonly lead to increased pathogen virulence (Nowak 2007). The similarities include increased host density, repeated introduction of new hosts, low genetic variability of hosts and high host growth rate (Ebert 1998, Nowak 2007). Also co-occurrence of pathogen strains differing in their virulence, increase in water temperature related to the global warming, and interactions between co-infecting parasites have been

suggested to lead to selection of more virulent pathogens in aquaculture (Pulkkinen *et al.* 2010, Karvonen *et al.* 2010).

Stressful environmental conditions are generally known to weaken the susceptibility of hosts to pathogens (Llafferty and Holt 2003). High host densities may enhance the invasion of pathogens in many ways. Skin damage resulting from high host densities can markedly enhance pathogen infection in fish farming surroundings (see e.g. Moyer and Hunnicutt 2007, Menanteau-Ledouble *et al.* 2011). Also co-occurring parasite infections may increase the susceptibility of hosts to pathogens (Bandilla *et al.* 2006, Karvonen *et al.* 2010, Louhi *et al.* 2015). In addition to stressing the host, high host density also enhances pathogen transmission by providing easily available resources for the pathogen (Sasal 2003, Ogut *et al.* 2005). As host death does not cause similar cost to the pathogen in a dense host population as when hosts are rare, pathogen virulence increases with transmission (Anderson and May 1982).

The aquatic environment enables pathogens to transmit over long distances even from an immobilized source, such as a dead host (Ewald 1994). This benefits the transmission of the pathogen especially in nature where the host density is often low. Nevertheless, water environment also enhances transmission in fish farming conditions where the host densities are high. In such an environment, fitness of a pathogen may increase, especially if it is able to persist and replicate outside the host. Furthermore, decaying fish, fish feed and accumulating fish feces may serve as reservoirs for pathogens and increase their transmission even more (Wakabayashi 1991, Pulkkinen *et al.* 2010, Kunttu *et al.* 2009a). Additionally, water is a more stable environment than air, and may thus protect the bacteria from sudden temperature changes, promoting their longer survival. The longer survival times increase the probability of a host contact and may even help the pathogen to persist in the water during chemotherapy (Kunttu *et al.* 2009a).

At fish farms, the fish hosts are also commonly infected by multiple pathogen strains or species (Madetoja *et al.* 2001, Karvonen *et al.* 2012). Multiple infections may have either an increasing or decreasing effect on pathogen virulence, depending on the relatedness of the coinfecting pathogens (Lively 2005, Frank 1996). Closely related pathogens are likely to cooperate and exploit their host economically in order to maximize their transmission (Frank 1996), whereas distantly related pathogens are expected to experience intense competition (Frank 1996; Buckling and Brockhurst 2008). Thus, higher relatedness is usually expected to lead to low, and low relatedness to high virulence.

1.6 Opportunistic and generalist pathogens

In contrast to obligatory pathogens, such as viruses, that need a host to survive and replicate, opportunistic pathogens are traditionally described as facultative disease-causing agents that only cause disease in hosts with an impaired

immune defense (Woolhouse *et al.* 2001), or with an altered microbiota (Packey and Sartor 2009, Swe *et al.* 2014). Nevertheless, the ability of a (opportunistic) pathogen to cause a disease is not always dependent on the hosts' immune defence, but also involves the features of the pathogen and is thus more complex (Casadevall and Pirofski 1999). Some pathogens can be considered opportunistic in a sense that they do not need a host to survive but are able to persist in the environment. Thus, a broader definition has been suggested by Brown *et al.* (2012), where opportunists are described as "non-obligate and/or non-specialist parasites of a focal host". Following this definition, opportunistic pathogens can be classified into commensal, environmental and parasitic opportunists.

The class of opportunists with ability to survive and replicate in the outside-host environment is not restricted by the traditional transmission-virulence trade-off (Brown *et al.* 2012). Thus they are not dependent on the longevity of their hosts and may increase their virulence to higher levels than obligate pathogens. The virulence factors in these environmentally proliferating opportunists may have evolved to aid living in the outside-host environment, not necessarily to benefit the pathogen in exploitation of the host. For example, in *E. coli* the virulence towards humans might result from the capacity to resist grazing by protozoa (Adiba *et al.* 2010). Like other microbes, the opportunists are often also phenotypically plastic, i.e. able to change their gene expression as a response to environmental variation (Brown *et al.* 2012). This ability helps the microbes to quickly adapt to changing conditions. For example the opportunistic pathogen *Pseudomonas aeruginosa* is able to adjust its siderophore (iron binding agent) production as a response to environmental iron availability (Kümmerli *et al.* 2009), and some bacteria are able to change their phenotypic features (such as cell organization) as a response to stressful conditions like antibiotic treatment, resulting in altered colony morphology (Sousa *et al.* 2012). This phenotype switching may secure bacterial virulence, resistance to antimicrobial substances or persistence in the environment.

The above-mentioned broad definition of opportunistic pathogens includes a concept of host generalism. Only a minority of all pathogens are strictly specialists that only infect one species of hosts. Most pathogens are able to infect several host species, and many of these are also able to transmit from multiple hosts (Woolhouse *et al.* 2001). Generalism of a pathogen influences both pathogen epidemiology and evolution of virulence, lowering the predictability of both (Woolhouse *et al.* 2001). Indeed, being able to infect several hosts has been thought to have both ecological and evolutionary costs in pathogen virulence (Benmayor *et al.* 2009). The first occurs when pathogen transmission in a novel host is reduced, and the latter when pathogen fitness is reduced in the original host. Thus specialist pathogens are expected to replicate and transmit more efficiently than generalists. Some experimental studies have shown that the aforementioned costs of generalism also apply within one host species when the pathogen expresses different levels of virulence in different host clones or genotypes (Ebert 1994, Bruns *et al.* 2012, Kubinak *et al.* 2014). Overall, the existing evidence suggests that host range is narrower in more-

virulent compared to less-virulent pathogens (Garamszegi 2006, Agudelo-Romero and Elena 2008). Nevertheless, host generalism may also be advantageous. As generalist pathogens are able to infect several host species, they are likely to encounter suitable hosts more often than specialists. Thus they are also expected to cause multiple infections in hosts more frequently than specialists (Leggett *et al.* 2013). Instead, environmental generalists that thrive in various environmental conditions are often expected to have a selective advantage in changing environmental conditions while specialists are more prone to get eliminated (Colles *et al.* 2009).

1.7 *Flavobacterium columnare*

Flavobacterium columnare is an environmentally transmitted bacterial fish pathogen that occurs in natural inland waters worldwide (Pulkkinen *et al.* 2010). It is a few micrometres long thin Gram-negative rod belonging to the family *Flavobacteriaceae*, Bacteroidetes (Bernardet and Grimont 1989, Declercq *et al.* 2013). *F. columnare* strains have been divided into three genomovars (I-III) based on 16S-restriction fragment length polymorphism (Triyanto and Wakabayashi 1999), of which only genomovar I has been observed in Finland. The Finnish strains can be further genotyped into eight genetically different groups (A-H) with automated ribosomal intergenic spacer analysis (Suomalainen *et al.* 2006) and multilocus sequence analysis (Ashrafi *et al.* 2015). In laboratory, *F. columnare* can be cultured in a low-nutrient, peptone-based media (Shieh 1980). When isolated from diseased fish or lake water, it exhibits yellow, rhizoid colony morphology with an ability to glide on agar (Kunttu *et al.* 2009b). On plate culture, also two other avirulent colony types are observed, that may help the bacteria to persist in the environment (Sundberg *et al.* 2014). The rhizoid type is the causative agent of columnaris disease, typical clinical symptoms of which include skin lesions, fin erosion and gill necrosis (Declercq *et al.* 2013). The common cause of death of the infected fish is respiratory distress and suffocation resulting from severe gill damage involving column-like formations around gill filaments. The virulence factors of *F. columnare* are poorly known, but the bacterium is known to produce collagenase and chondroitinase targeted to degrade host connective tissue components (Olivares-Fuster and Arias 2008), and its gliding motility has been suspected to be related to its pathogenicity (Kunttu *et al.* 2011, Laanto *et al.* 2012). Yet, only chondroitinase has been connected to *F. columnare* virulence (Stringer-Roth *et al.* 2002, Suomalainen *et al.* 2006, Kunttu *et al.* 2011, Penttinen *et al.* 2016) and its production was recently shown to increase as a response to increased nutrient availability (Penttinen *et al.* 2016).

F. columnare is able to persist in the aquatic environment and can transmit from biofilms (Cai *et al.* 2013) and via water from infected host to another (Kunttu *et al.* 2012). Thus the bacterium can be classified as an environmental opportunistic pathogen that does not need a host to survive and replicate (see

Brown *et al.* 2012). Nevertheless, *F. columnare* is able to infect several cold-water and temperate fish species, such as carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), trout (*Salmo trutta*), arctic charr (*Salvelinus alpinus*), salmon (*Salmo salar*), pikeperch (*Sander lucioperca*) and grayling (*Thymallus thymallus*) (Decostere *et al.* 2002, Suomalainen *et al.* 2006, Soto *et al.* 2008). The bacterium is also infectious against some tropical aquarium species such as black molly (*Poecilia sphenops*) and platy (*Xiphophorus maculatus*) (Decostere *et al.* 1998), indicating it has a host-generalist nature. Additionally, *F. columnare* has been shown to transmit more intensely from a dead than a living host, indicating that the pathogenicity of this originally harmless water bacterium may have evolved following a saprotrophic transition stage (Kunttu *et al.* 2009a), i.e. with an ability to feed on dead organic matter. The saprotrophic lifestyle of *F. columnare* not only enables an effective transmission even after host's death, but also provides the bacterium an opportunity to increase its virulence without a cost related to death of its host.

Columnaris disease is mainly a problem of cultured freshwater fish, leading to substantial economic loss in fish farming worldwide (Wagner *et al.* 2002, Pulkkinen *et al.* 2010). Outbreaks in natural waters are rare, but have been observed occasionally (e.g. Morley and Lewis 2010). In the US, columnaris outbreaks are a remarkable threat to channel catfish industry (Wagner *et al.* 2002, Olivares-Fuster et al 2007), whereas in Finland the disease is especially problematic in salmonid fingerling and fry production leading up to 100 % mortality during summer when the water temperature exceeds 20 °C (Suomalainen *et al.* 2005). Search of efficient methods to prevent and cure the disease are still in progress. At the moment, only antibiotic treatment with oxytetracyclin has proven to be an effective treatment against columnaris outbreaks (Rach *et al.* 2008). Also recent experimental trials of phage therapy have shown promising results as an alternative treatment against columnaris disease (Laanto *et al.* 2015).

In Finland the severity and incidence of columnaris outbreaks have been increasing since 1990's, but the reasons for the observed emergence have largely remained unclear. The intensive aquaculture practices selecting for *F. columnare* strains with higher virulence have been proposed as an explanation (Pulkkinen et al 2010). Indeed, the strains isolated from Finnish lakes and rivers have been shown to cause less mortality in fish when compared to bacteria isolated from fish farms (Kunttu *et al.* 2012). *F. columnare* strains also express variable levels of virulence within the fish farming units, where the co-occurrence of genetically different strains enables strain competition and coinfection of fish (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2010). This is important, as the coexistence of genetically different strains has been suggested to promote virulence in conditions where the more virulent strains have a competitive advantage (Frank 1996, Gandon 2001). Regardless of antibiotic treatments that began in 1993, the prevalence of columnaris disease outbreaks has been constantly increasing, and thus also chemotherapy has been suspected to select for more virulent *F. columnare* strains, at least when combined with the saprotrophic lifestyle of the pathogen (Pulkkinen *et al.* 2010). Additionally, the high fish

density in the farming units enhances the transmission of the disease. The above-mentioned factors make *F. columnare* a good model organism for studying the evolution of bacterial virulence. In this thesis, *F. columnare* is used as a model to study the influence of ecological factors on virulence of opportunistic, environmentally transmitted pathogens.

2 AIMS OF THE STUDY

Aquaculture is the fastest expanding food production sector in the world, but increasing prevalence and severity of infectious diseases cause a significant constraint to its development. The intensive farming practices can lead to evolution of increased pathogen virulence, most likely in opportunistic pathogens that can proliferate in both within- and outside-host environments, when their transmission is not restricted by their host's death. The purpose of this study was to find out whether specific biotic and abiotic factors, such as i) infection dose, ii) environmental nutrient availability, and iii) coinfections with conspecific strains can lead to increase of virulence in environmental opportunists, and to delve deeper into the mechanisms when virulence increase was observed. The final study was conducted to find out whether the intensive fish farming environment as a whole can alter virulence of opportunistic pathogens. The specific aims for each chapter are presented below.

I) Outside-host pathogen density and genetic diversity are unstable environmental variables that may have unexpectedly strong effects on disease dynamics. Increasing pathogen dose is usually expected to lead to increased disease virulence (Regoes *et al.* 2002), as the higher pathogen numbers cause more harm to the host. While this hypothesis seems self-evident, I examined whether increasing dose increases virulence also in the opportunistic fish pathogen *F. columnare*, that is able to infect several fish species (Declercq *et al.* 2013) and may thus be considered a host-generalist. Also coinfection of hosts by genetically different pathogen strains often leads to virulence increase (Read and Taylor 2001), but their significance for host-generalist opportunistic pathogens has remained uncertain although host-generalists may come across with potential hosts more often than specialist pathogens (Brown *et al.* 2012, Leggett *et al.* 2013). Here I ask how the infective dose and co-infection (with two bacterial strains differing in their virulence) affect bacterial virulence in two phylogenetically different hosts, and whether the effects of dose and coinfection interact. Moreover, as rainbow trout is a cold-water species and temperatures above 20 ˚C are needed for *F. columnare* to successfully cause infection, the

suitability of zebra fish as a model for experimental columnaris infections is confirmed.

II) Environmental nutrients have been linked with the abundance and severity of parasitic diseases, but the existing information on the effects of nutrients on microparasitic diseases is scarce (Johnson and Carpenter 2010). However, the opportunistic microbial pathogens are often able to persist in and transmit via environment (Brown *et al.* 2012), and nutrient availability in the outside-host environment is thus likely to influence their pathogenic features. As all organisms, also bacteria need nutrients for growth and survival, increased resources could be expected to increase bacterial growth and consequently virulence. Here, I study how environmental nutrient levels affect virulence in opportunistic pathogens, by infecting fish with *F. columnare* in different nutrient concentrations (low and high). I use different doses and study the growth of *F. columnare* strains *in vitro* in the same nutrient concentrations in order to separate the effects of dose and nutrients on virulence.

III) Coinfections by two or more pathogen strains are rather a rule than an exception in nature (Woolhouse *et al.* 2002, Balmer and Tanner 2011, Karvonen *et al.* 2012). However, the role of multiple-strain interactions in many diseases is still unclear. Coinfections may have particular importance in disease dynamics of environmentally growing opportunistic pathogens, as unlike obligatory pathogens, they do not suffer costs of transmission-virulence trade-off (Brown *et al.* 2012) and may thus evolve high levels of virulence. *F. columnare* strains differing in their growth and competitiveness are known to co-occur at fish farms (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2010), but it is not yet known how coinfection of multiple strains affects the virulence of the pathogen, or if and how growth and competition traits of the strains affect coinfection. Here, I study how coinfection with two and three conspecific strains affects virulence of *F. columnare*, and whether the coinfection outcome can be explained with bacterial growth in liquid co-culture and interference competition on agar.

IV) Intensive aquaculture has been suggested to select for increased pathogen virulence (Nowak 2007, Mennerat *et al.* 2010, Pulkkinen *et al.* 2010). Yet, only little is known about the influence of intensive farming practices on disease evolution, and the experimental studies are often unrelated to the food production systems. Also strains of *F. columnare* have been hypothesized to evolve higher virulence in the intensive aquaculture environment (Pulkkinen *et al.* 2010), and the strains isolated from nature have indeed been shown to be less virulent than the strains isolated from fish farms (Kunttu *et al.* 2012). The aim of this study is to confirm the previous observations by exploring the effect of intensive aquaculture on virulence, growth and competitive ability of *F. columnare* strains on both long (2003-2010) and short (summer 2010) timescales.

3 SUMMARY OF THE MATERIALS AND METHODS

3.1 Study species

3.1.1 Bacterial strains

Thirty-four *F. columnare* strains isolated from different fish farms in Central and Northern Finland during years 2003-2010 were used in all experiments (chapters I-IV) (more detailed information of the strains is given in Table 1). Pure cultures of the strains were stored frozen at -80 °C in 500 µl stocks containing 10% glycerol and 10% fetal calf serum (FCS, Gibco, BRL Co., United Kingdom). Before the experiments, all strains were grown in 5 ml of modified Shieh medium (Song *et al.* 1988) at 26 °C under constant agitation of 150 rpm (110 rpm in IV) for 48 h (24 h in IV). Then the cultures were enriched (1:10) overnight and grown in the same conditions to an early log phase (16-21 hours). To calculate the bacterial doses for the experimental infections, the optical densities (OD) of the over-night cultures were measured at wavelength of 570 nm with a spectrophotometer (VICTOR X Multilabel Plate Reader, Perkin-Elmer, USA). The bacterial numbers were expressed as colony forming units per millilitre (CFU ml^{-1}) based on a previously determined OD vs. CFU relationship.

TABLE 1 *Flavobacterium columnare* strains used in the studies I–IV. Year of isolation, fish farm ID and source of the isolate (fish species or water sample) are given for each strain. The genetic groups of the strains based on ARISA genotyping are also included in the table. The strains used in study IV have been underlined (strains isolated from disease outbreaks at different fish farms during 2003-2010) or marked with an asterisk (strains isolated from inlet and outlet water of fish farm B during one outbreak season in 2010).

Strain	Year of isolation	Fish farm	Source	Genetic group
B395*	2010	Farm B, Central Finland	River, biofilm	G
B396*	2010	Farm B, Central Finland	River, biofilm	A
B397*	2010	Farm B, Central Finland	River, free water	C
B398*	2010	Farm B, Central Finland	River, free water	A
B400*	2010	Farm B, Central Finland	River, free water	A
B404*	2010	Farm B, Central Finland	River, biofilm	C
B355*	2010	Farm B, Central Finland	Inlet water	A
B406*	2010	Farm B, Central Finland	River, free water	C
B407*	2010	Farm B, Central Finland	River, free water	G
B339*	2010	Farm B, Central Finland	Outlet water	C
B340*	2010	Farm B, Central Finland	Outlet water	C
B350*	2010	Farm B, Central Finland	River, biofilm	E
B351*	2010	Farm B, Central Finland	River, biofilm	E
B366*	2010	Farm B, Central Finland	Outlet water	C
B370*	2010	Farm B, Central Finland	River, free water	E
B374*	2010	Farm B, Central Finland	River, free water	E
B375*	2010	Farm B, Central Finland	River, free water	E
B379*	2010	Farm B, Central Finland	Outlet water	E
B067	2007	Farm A, Central Finland	Trout/*Salmo trutta*	A
B185	2008	Farm A Central Finland	Tank water	G
B424	2007	Farm C, Northern Finland	Atlantic salmon/*Salmo salar*	C
B259	2009	Farm B, Central Finland	Tank water	C
B431	2003	Farm A, Central Finland	Grayling/*Thymallus thymallus*	A
H2	2003	Farm B, Central Finland	Rainbow trout/*Oncorhynchus mykiss*	H
B429	2003	Farm B, Central Finland	Pikeperch *Zander lucioperca*	H
B430	2003	Farm B, Central Finland	Pikeperch *Z.lucioperca*	E
B425	2007	Farm B, Central Finland	Rainbow trout *O. mykiss*	
B245	2009	Farm B, Central Finland	Tank water	C
B402	2010	Farm B, Central Finland	Whitefish *Coregonus lavaretus*	C
B405	2010	Lake water, nature	Lake Jyväsjärvi	C
B428	2006	Farm C, Northern Finland	Atlantic salmon *S. salar*	
B426	2006	Farm C, Northern Finland	Atlantic salmon *S. salar*	C
B420	2009	Farm C, Northern Finland	Atlantic salmon *S. salar*	G
B421	2009	Farm C, Northern Finland	Atlantic salmon *S. salar*	C

3.1.2 Fish hosts

The cold-adapted rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) occur naturally in the Pacific Ocean and cold streams in Asia and North America. The species was introduced into Finland around 1900, after which it has become the most important commercially farmed fish species in our country (Finnish Game and Fisheries Research Institute 2008). Since the 1990's, the fish farms culturing rainbow trout have suffered yearly economical losses due to outbreaks of columnaris disease during warm water periods (Pulkkinen *et al.* 2010). For the studies I-II, fingerling rainbow trout with no known history of with *F. columnare* and without clinical signs of disease were obtained from a fish farm in Central Finland. The fish were obtained from the farm during a cold water season in early spring, kept in treated (pathogen-free) ground water in 250-l flow-through tanks, and fed with commercial trout feed (Nutra Parr, Skretting, Norway). The fish were maintained this way for at least two months at 15.0-16.0 °C before conducting the experiments. The oxygen concentration of the water was continuously monitored. Before the experimental infections, the fish were transferred to 60-l aquaria supplemented with constant aeration, and acclimated to laboratory conditions for 2-4 weeks before the experiments. After acclimatization, the water temperature was gradually lifted to 25 °C during a period of three to five days, i.e. maximum two and half degrees per day. This temperature was necessary for a successful infection with *F. columnare* that has the best ability to cause infections at temperatures higher than 20 °C (Declercq *et al*. 2013).

The zebra fish (*Danio rerio*, Hamilton 1822) is a well-established laboratory species that has a similar temperature optimum as the pathogen (Lawrence 2007). As a tropical species it does not have a history with the Finnish bacterial strains used in this study and can thus be considered as a novel host. For the studies III-IV, adult, unsexed, disease-free wild-type zebra fish were obtained from Core Facilities (COFA) and Research Services of Tampere (University of Tampere, Finland). The fish were maintained in 60-l aquaria in stagnant ground water at a constant temperature of 25.0 °C with constant aeration, and fed daily with commercial pellets (Scientific Fish Food, Special Diet Services, United Kingdom). To keep the water clean, half of it was changed twice a week, and the fish excrements and excess feed were removed by siphoning.

3.2 Experimental design

3.2.1 Effect of infective dose on virulence (I)

To study how the infection dose, coinfection and host species affect *F. columnare* virulence, three treatment groups were formed with bacterial dose, infection type (a high-virulence strain B185, a low-virulence strain B398 and their 1:1 coinfection) and host species (rainbow trout or zebra fish) as variables. The dose

had nine levels (5.0×10^5, 1.0×10^6, 3.0×10^6, 6.0×10^6, 9.0×10^6, 1.2×10^7, 1.6×10^7, 2.0×10^7 and 3.0×10^7 CFU per ml) and it was treated as a continuous variable. Thus, in total, 18 replicate fish were used per species per each treatment group. Additionally, five replicates of negative controls were used per species. Thus, there were altogether 118 fish in the experiment.

3.2.2 Effect of outside-host nutrients on virulence (II)

To investigate the role of outside-host nutrient level on the virulence of *F. columnare*, and to separate the effects of nutrients and bacterial dose from each other, the virulence of three bacterial strains (the low-virulence strain B398 and high-virulence strains B067 and B185) was studied using three different doses (1.0×10^4, 1.0×10^5 and 1.0×10^6 CFU per ml) in two nutrient levels (high and low). The high and low nutrient levels were obtained by adding sterilized growth medium (to reach 1.28 % and 0.64 % growth medium concentrations) to the fish aquaria. Each treatment (strain + dose + nutrient level) was replicated five times with zebra fish and three times with rainbow trout. All three strains were used to infect zebra fish but only two of them to infect rainbow trout (the low-virulence strain B398 and a high-virulence strain B067). Thus, the zebra fish had 18 treatment groups (90 fish individuals) and the rainbow trout 12 treatment groups (36 fish individuals), totaling 126 fish. Additionally, the effect of nutrient level on bacterial replication was studied in three concentrations of modified Shieh media. Two of these were equivalent to the concentrations used in the virulence experiment. In addition to these low and high nutrient levels, a baseline level (with no added nutrients) was included in the experiment. The highest bacterial dose (1.0×10^6 CFU per ml) of strains B067 and B398 were cultured in dechlorinated tap water with three replicates per strain, and the bacterial count was determined by plate counting once a day for four days after inoculation.

3.2.3 Effect of coinfection on virulence (III)

To explore the effects of coinfection on *F. columnare* virulence, three strains (B259, B350 and B424) were used to infect zebra fish individually and in combinations of two and three. A fixed dose of 4×10^5 CFU per ml administered in 600 µl of growth media was used in all treatments, and thus the proportion of one strain in a coinfection treatment was either 2×10^5 or 1.3×10^5 CFU per ml depending on the number of the strains. To evaluate the virulence of *F. columnare* at the lower doses, dose control groups were included in the study, where either 2×10^5 or 1.3×10^5 CFU per ml was administered to fish hosts in 300 µl or 200 µl of growth media. The study consisted of seven treatment groups and six dose control groups, each having seventeen fish replicates. In addition, a group of ten negative control fish exposed to 600 µl of growth medium was included in the experiment. In total, 231 fish were used in the third experiment.

3.2.4 Effect of intensive farming on virulence (IV)

To study evolution of virulence and competitive ability at both temporal and spatial scale, two sets of *F. columnare* strains were used. The first set consisted of 17 strains that were isolated from fish farms in Central and Northern Finland during 2003–2010 (the strains underlined in Table 1). The second set consisted of 18 strains isolated from the inlet and outlet water from a fish farm in Central Finland (farm) during one outbreak season in summer 2010 (the strains marked with an asterisk in Table 1). The virulence of both sets of strains was studied in zebra fish, in the first set in 10 replicate fish per strain and in the second set in 14 replicate fish per strain. Strain growth rate and ability to inhibit the growth of the other strains were compared to study whether they change in time and place. Additionally, bacterial competition in finite resources was studied using the strains isolated during 2003–2010, as well as growth in two different resource concentrations using the inlet water and outlet water strains.

3.3 Infection experiments (I-IV)

Two different infection methods were used in this thesis. In study I, a previously optimized bath immersion method was used, where the fish were individually challenged in 50 millilitre volume of aerated ground water for two hours and then transferred into individual 1 l aquaria with 0.5 l of clean ground water for the rest of the experiment (Laanto *et al.* 2012). In studies II-IV, the fish were challenged using a continuous infection method where the bacteria were added directly into the fish aquaria (see Laanto *et al.* 2015). In more detail, the fish were challenged in individual aquaria containing 0.5 l of aerated ground water, except in study II, where the rainbow trout were challenged in a 1.0 l volume. The bacteria were added into aquaria in a fixed volume of growth media depending on the experimental set-up. After challenging, the fish were monitored for clinical signs of disease and mortality for 2-11 days depending on the progression of the disease. For the first 48 hours, the fish were monitored at intervals of one or two hours, and after the progression of the epidemic ceased, the monitoring points were reduced but the fish were checked at least twice a day. When the fish did not react to external stimuli they were considered dead, removed from the experiments and euthanized under terminal anesthesia with MS-222 (Sigma). Also the controls and the fish surviving the infection were put to sleep in the end of the experiment. The water temperature was maintained between 25.0–26.3°C during all experiments, and to reduce the differences between aquaria, they were randomly placed on shelves in the experimental room. To verify that the reason of fish mortality was indeed columnaris infection, skin or gill cultivations from fish were spread on Shieh agar supplemented with tobramycin (Decostere *et al.* 1997), and checked for yellow colonies with the rhizoid morphology typical to virulent *F. columnare* (see Kunttu *et al.* 2009b). The experiment was conducted under permission ESAVI-

2010-05569/Ym-23, granted by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

3.4 Growth measurements (III-IV)

To monitor the growth of the bacterial strains, a small amount of an overnight-grown bacterial culture was inoculated on fresh Shieh medium on a BioScreen Honeycomb plate (100-well plate, Oy Growth Curves Ab Ltd). The growth was measured as a change in the optical density of the growth medium. Before the measurements, the optical densities of the bacterial cultures were adjusted between 0.15-0.20 at wavelength of 570 nm (VICTOR X Multilabel Plate Reader, Perkin-Elmer, USA) to minimize the inter-strain differences in turbidity. The growth data was recorded at 25 °C for 96 h at 5 min intervals (absorbance at 420–580 nm) with Bioscreen™ spectrophotometer (Growth Curves Ltd., Helsinki, Finland).

To study the effect of co-culture on the strains used in coinfections (study III), three strains (B259, B350 and B424) were cultured individually and in combinations of two and three in seven replicates per treatment. To achieve this, a 30 µl inoculum was supplemented with three hundred microlitres (300 µl) of Shieh media. Accordingly, the proportion of one strain in a co-culture of two strains was 15 µl, and 10 µl in a co-culture of three strains. Similarly to the coinfection experiment, the half and third "doses" were included as controls. To study the effects of resource level and competition in liquid culture on the growth of *F. columnare* strains (study IV), a 40 µl inoculum of individual bacterial strain or a 1:1 mixture were inoculated onto 400 ml of Shieh media in five replicates per strain.

The growth parameters (maximum growth rate, maximum biomass yield, time to maximum yield and area under curve) were calculated from the raw data using a MATLAB script (Mathworks, MA, United States) created by Tarmo Ketola.

3.5 Measuring interference competition (III-IV)

The ability of interference competition of *F. columnare* strains was studied reciprocally using a standard double layer method. To minimize the turbidity differences of bacterial cultures, the optical densities of the fresh overnight cultures were adjusted to 0.29 at wavelength of 570 nm (VICTOR X Multilabel Plate Reader, Perkin-Elmer, United States). To obtain the "recipient" bacterial lawn, three hundred microlitres (300 µl) of bacterial culture was mixed with three millilitres (3 ml) of soft Shieh agar (0.7 %) that was tempered to 52 °C, and poured on 1 % Shieh agar plates. To prepare the "donor" bacteria, one millilitre (1 ml) of the remaining culture was centrifuged for 3 min at full speed (13 000

G) to separate the bacterial cells from the liquid. After this, five hundred microlitres (500 µl) of the supernatant was carefully collected into a new Eppendorf tube to avoid dissolving the pellet. Ten microlitres (10 µl) of the supernatant were spotted on the recipient bacterial lawn and let dry under a hood. The plates were incubated at room temperature (ca. 21 ˚C) for two days, after which clear growth inhibition zones caused by the "donor" strains were monitored. All assays were replicated three times.

3.6 Genetic analyses (IV)

The strains collected in 2003–2010 (the strains underlined in Table 1) and the inlet water strains (Table 1) were genetically characterized in an earlier study using multilocus sequence analysis (MLSA) method (Ashrafi *et al.* 2015) and automated ribosomal intergenic spacer analysis (ARISA) (Kunttu *et al.* 2012). The outlet water strains and the inlet water strain B355 were analyzed in this study using ARISA, as described in (Suomalainen *et al.* 2006, Kunttu *et al.* 2012). The genetic clustering produced by MLSA method has been shown to be comparable with the ARISA method (Ashrafi *et al.* 2015) and thus the results are congruent.

4 RESULTS AND DISCUSSION

4.1 Virulence of an opportunistic pathogen increases with dose in a novel and a native fish host

Pathogen density fluctuates in the outside-host environment and thus affects the onset and severity of epidemics, especially those caused by opportunistic, environmentally growing pathogens (e.g. Merikanto *et al.* 2012). The pathogen dose to which the host is exposed, influences many epidemiological processes, such as within-host reproduction of the pathogen, progress of infection and survival and reproduction of the host (Regoes *et al.* 2002). Observations from experimental studies indicate that pathogen virulence usually increases with the dose, as the higher pathogen density is a higher burden to the host immune system and thus causes more harm to the host. Nevertheless, as the infective dose (the number of cells that are needed to infect a host) has a great variability between pathogen species (Leggett *et al.* 2012), it is not always clear which doses are high enough to infect a host, or if different doses are needed to infect different host species by a generalist pathogen. Furthermore, the infective doses of opportunistic pathogens have not been extensively studied using different host species. It is also likely that the doses needed to establish an infection differ between pathogen strains, as the strains are known to vary also in their growth rates.

Here, to explore how the dose affects virulence in opportunistic pathogens, rainbow trout and zebra fish were exposed to *F. columnare* strains expressing different levels of virulence (studies I-III). The consistency of the results of the three separate studies confirms that the virulence of *F. columnare* is strongly dose-dependent (Figure 1 in I, Figure 1 in II, and Figure 1 in III). This observation suggests that like in obligatory, host-dependent pathogens such as bacterium *Pasteuria ramosa* (Ebert *et al.* 2000) and microsporidium *Vavraia culicis* (Fellous and Koella 2009), higher dose also leads to virulence increase in environmentally transmitted opportunistic pathogens. This is a significant finding, as the ability to survive and proliferate outside-host (Brown *et al.* 2012)

can bring a great advantage to the environmental infective bacteria that are able to produce a high amount of infective units in suitable conditions. Understanding the importance of bacterial dose for disease virulence is therefore of high relevance, and controlling the factors contributing to bacterial replication in the outside-host environment plays a key role in preventing disease outbreaks caused by environmentally proliferating opportunists. However, the higher infection dose typically led to higher virulence only in virulent *F. columnare* strains with rhizoid morphology (the only exception is discussed in the next chapter), supporting the observations from earlier studies on *F. columnare* where only the rhizoid colony morphology was connected to virulence (Kunttu *et al.* 2009a, Kunttu *et al.* 2011, Laanto *et al.* 2012).

In epidemiological models of microparasitic infections it is often assumed that pathogen virulence is a linear function of the infection dose, i.e. the virulence increases linearly along with the dose increase (Regoes *et al.* 2002). Here, the virulence was found to be an exponential function of the dose, increase in host mortality risk exponentially growing at the highest doses (Figure 1 in studies I-III). In accordance, a nonlinear relationship between pathogen dose and host infection rate has been commonly observed in experimental studies (Agnew and Koella 1999, Little and Ebert 2000, Ebert *et al.* 2000, McLean and Bostock 2000, Brunner *et al.* 2005). A possible explanation to the observed exponential increase in virulence could be related to the host immune system, which is able to prevent the infection until the pathogen numbers infecting the host grow high enough (Regoes *et al.* 2002). This kind of dose-dependent virulence is suggested to produce an epidemiological Allee effect, where the low bacterial density limits the bacterial growth and the higher density promotes it, leading to even higher bacterial numbers. Indeed, the lower infection doses were not always successful in causing host mortality, indicating that a certain threshold bacterial density needs to be reached before the infection is established. Additionally, when three *F. columnare* strains were individually cultured *in vitro* starting from three different inoculum sizes (study III), the highest inoculum size substantially increased the maximum yield and growth rate in two out of three strains (the most virulent one reached high densities independent on the inoculation dose) (Figure 6 in III). This observation further supports the density-dependent growth of *F. columnare*. Unfortunately, pathogen load in the infected hosts was not measured in this study, and thus a threshold density needed for a successful columnaris infection cannot be determined. However, the results indicate that pathogen infective dose (see Leggett *et al.* 2012) is both strain-specific and dependent on the infection conditions such as water quality or nutrient availability.

The mechanism behind the density-dependent growth could be related to bacterial cooperation. Microbes are known to produce enzymes and signals that can promote the growth of the neighbouring cells (Waters and Bassler 2005, Little *et al.* 2008, D'Onofrio *et al.* 2010, Strassmann *et al.* 2011). However, no studies on *F. columnare* growth stimulation related to interstrain interactions have yet been published. Nevertheless, growth stimulation of *Flavobacteria* was observed when *Bacillus cereus* strain was introduced to soybean rhizosphere to

control root-rot and damping-off diseases (Gilbert *et al.* 1993). In a later study conducted by Peterson *et al.* (2006), this growth-promoting effect was found to be caused by peptidoglycan-mediated commensalism between the studied bacterial species. Also virulence factor expression in bacterial pathogens can be controlled via density-dependent communication between bacterial cells (Rutherford and Bassler 2012). In this so called quorum sensing, the bacteria detect and produce signalling molecules that accumulate in the environment as the bacterial density increases. Quorum sensing –mediated control of virulence has been observed from several bacterial pathogens, such as *B. cereus* (Bouillaut *et al.* 2008), *Staphylococcus aureus* (Thoendel *et al.* 2011), *P. aeruginosa* (Passador *et al.* 1993) and *Vibrio cholerae* (Higgins *et al.* 2007). Although no quorum sensing has yet been reported from *F. columnare*, the possibility that quorum sensing might contribute to the bacterium's virulence factor activation cannot be excluded.

Infective doses are likely to differ between host species, as pathogen strains are differently virulent towards different host genotypes (Ben-Ami *et al.* 2008, Duneau *et al.* 2011, Bruns *et al.* 2012, Hall and Ebert 2012, Leggett *et al.* 2013, Hotson and Scneider 2015). Here, the rainbow trout that is naturally infected by *F. columnare* at fish farming facilities, was found to be more sensitive to experimental columnaris infections than the zebra fish that had no recent history with the pathogen (Figure 1 in studies I and II). Although the sensitivity of the rainbow trout is partly explicable by the experimental conditions (e.g. high water temperature) that are more stressful for this cold-adapted species (Chen *et al.* 2015) than to the tropical zebra fish (Lawrence 2007), it might also be possible that *F. columnare* could be better adapted to exploit its natural host than zebra fish (see Ebert 1994). As support for this finding, similar associations have been found between *F. columnare* strains and salmonid species as well as between *F. columnare* and channel catfish (Shoemaker *et al.* 2008, Olivares-Fuster *et al.* 2011, LaFrentz *et al.* 2012).

Infection dose has also been found to affect the outcome of within-host competitive interactions between parasites in coinfections (Fellous and Koella 2009). When the hosts were coinfected with a high-virulence and a low-virulence strain (study I), the virulence of the coinfection treatment was found to be intermediate of the individual strains (Figure 1 in I). As the infection dose was the same in both single-strain and coinfection, the result indicates that the low-virulence strain may have had a dilutive effect on the virulence in the coinfection treatment. Thus, the virulence of the coinfection treatment was dependent on the doses of the coinfecting strains.

4.2 Virulence of opportunistic pathogen depends on the nutrient availability in the outside-host environment

Availability of nutrients is a significant driver of growth, survival and reproduction in all living organisms. Thus, it is not surprising that the availability of nutrients has also been linked with the abundance and severity of diseases caused by both micro- and macroparasites (Johnson and Carpenter 2010). In aquatic systems, the increased nutrient availability causes diverse effects on disease that depend on the pathogen, species and condition of the host, qualities of the ecosystem, and the level of nutrient enrichment (Johnson *et al.* 2010, Johnson and Carpenter 2010). Nutrient inputs can lead to alterations in disease dynamics in many different ways ranging from changes in abundance and distribution of hosts to infection resistance and pathogen virulence.

The studies on the effects of environmental nutrient levels on microbial pathogens have remained rather limited and disease outbreaks in most of the existing studies have been related to nutrient-induced stress such as hypoxia (Johnson and Carpenter 2010), leaving the interactions between disease and nutrients unclear. Here, zebra fish and fingerling rainbow trout hosts were exposed to a low-virulence and two high-virulence strains of *F. columnare* in low and high nutrient concentrations (study II). It was found that the high level of nutrients in the outside-host environment significantly increased the virulence (measured as host mortality risk) of *F. columnare* as compared to the low nutrient level (Figure 1 and Table 3 in II). Although the nutrient-related virulence increase has been commonly linked with nutrient limitation (see e.g. McKenney and Allison 1995, Jensen *et al.* 2006, Cornforth and Foster 2013), the result of this study is in accordance with observations from some other microbial pathogens. Increase in environmental nutrients has been shown to increase the severity of aspergillosis caused by a fungus *Aspergillus sydowii* and yellow band disease caused by *Vibrio* bacteria in corals (Bruno *et al.* 2003). Based on the experiments by Wedekind *et al.* (2010), increase in environmental nutrients may also increase the virulence of opportunistic bacterial pathogens. Indeed, especially the opportunistic pathogens able to infect multiple host species have been suggested to benefit from increased availability of nutrients (Johnson *et al.* 2010, Johnson and Carpenter 2010), as they have the capacity to exploit outside-host nutrients for growth, while the survival of obligatory pathogens is dependent solely on their host (Brown *et al.* 2012, Leggett *et al.* 2013). Thus, the nutrient availability may have a high relevance for disease outbreaks and emergence caused by opportunists. The finding of this thesis highlights the importance of outside-host nutrient levels for the disease-causing ability of the environmental opportunistic pathogens.

The mechanisms by which the enhanced availability of nutrients affects virulence increase are not yet well known. Here, the mechanism behind the observed virulence increase was studied by culturing a high-virulence and a low-virulence strain of *F. columnare* in the same nutdrient concentrations as

used in the virulence experiment (low and high), and in an environment without any added nutrients (baseline). It was found out that the low and high nutrient additions increased bacterial replication compared to the baseline (Figure 2 in II), indicating that the enhanced bacterial growth leading to a higher infective dose could lead to an increased host mortality risk. As support to this observation, nutrient increase has been found to increase bacterial replication in other studies on aquatic bacteria (Sipura *et al.* 2005, Forehead *et al.* 2012). However, the increased bacterial dose could not have been the only reason for the increased virulence in this study, as no difference in bacterial growth was found between the low and high-nutrient additions, although the difference in pathogen virulence was significant between these nutrient conditions (Figure 1 in II). Thus, the virulence-increasing effect is likely to involve also other explanatory factors. In a recent study, high-nutrient culture conditions were found to promote gene expression of tissue degrading enzymes collagenase and chondroitinase in *F. columnare* (Penttinen *et al.* 2016). The observation of dose-independent virulence increase between the low and high nutrient levels in this study supports the suggested role of outside-host nutrients in virulence gene activation of this bacterium.

The effect of the nutrient enrichment on virulence was found to be strain-specific (Figure 1 in II). The low-virulence strain that was not virulent towards fish at the low nutrient level, became highly virulent in the high-nutrient treatment. At the same time, the responses of the high-virulence strains were not as remarkable, although significant. The more notable increase in virulence of the low-virulence strain could also be partly caused by the continuous infection method used in this study compared to bath immersion method used in study I. The continuous infection is likely to increase the frequency of host-pathogen contacts, thus increasing the infection probability (see Aiello *et al.* 2016). It is also possible that the prolonged infection time switches on virulence factors in the low-virulence strain that is adapted to live in natural waters where the hosts are rare and thus might not normally express virulence. Indeed, it is not necessarily beneficial for the pathogen to express virulence factors when there are no hosts available. Also the growth phases of the strains were different in the studied nutrient concentrations (Figure 2 in II), indicating that pathogen strains differ in their growth rate in a strain-specific manner. The differences in growth of the strains co-occurring in the fish rearing units could ensure that infective bacterial populations are constantly present at fish farms during the outbreak season, and the high resource availability in fish tanks could boost this effect even more.

Compared to natural water systems such as lakes and rivers, the aquaculture environment is nutrient-rich due to accumulation of uneaten feed and fish excrements. The results of this study indicate that the increased availability of nutrients in tank water in the fish farming units may contribute to the severity of opportunistic disease outbreaks in two ways: by stimulating pathogen replication and via virulence factor activation. Therefore, controlling the level of organic nutrients in aquaculture is of high importance in order to prevent the disease epidemics caused by opportunistic pathogens.

4.3 Strain interactions contribute to opportunistic pathogen virulence

Coinfections by two or more pathogen strains or species are very common in nature (Read and Taylor 2001, Woolhouse *et al.* 2002, Malpica *et al.* 2006, Karvonen *et al.* 2012, Susi *et al.* 2014). The coinfecting strains inhabit the same ecological niche, i.e. utilize the same resources (the host), and thus are affected by the presence of others (Read and Taylor 2001, Balmer and Tanner 2011). This naturally leads to within-host interactions between the co-occurring strains, which have been suggested to contribute to the severity and epidemiology of disease (Read and Taylor 2001, Galvani 2003), as well as to evolution of virulence (Read and Taylor 2001, Alizon *et al.* 2013, Ben-Ami and Routtu 2013). However, the role of multiple-strain interactions in many diseases has remained unsolved, including those caused by opportunistic pathogens.

It has previously been suspected that *F. columnare* strains with variable levels of virulence co-occurring in the same population could contribute to the increased severity of columnaris disease outbreaks (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2010). Nevertheless, no studies on *F. columnare* strain interactions and virulence have previously been done. Here, the influence of coinfection on virulence of this bacterium was studied by infecting zebra fish with three strains in single and in coinfection using two- and three-strain combinations (study III). The number of strains was found to affect the overall bacterial virulence (Table 7 in III). More precisely, the coinfection of three strains was significantly more virulent towards fish than the two-strain or single-strain infections, while no difference was found between the two latter infection types. The individual strains differed significantly in their virulence and had diverse effects on the outcome of the coinfection treatments (Figure 1 in III): the infection treatments containing the most virulent strain (B259) were the most virulent of all treatments, while presence of the avirulent strain (B424) in coinfection treatment led to decreased virulence of coinfection (but only in absence of the most virulent strain). These findings indicate that coinfections can indeed lead to increased virulence in opportunistic pathogens, but the infection outcome depends on the characteristics of the coinfecting strains. Generally, both growth-stimulating mutualistic interactions (West and Buckling 2003) and competitive interactions (Frank 1996, Buckling and Brockhurst 2008) can lead to increased virulence, but in this study, bacterial growth seemed to have no overall effect on virulence of coinfection (Figures 3-5, Supplementary Table 1 in III). Although the exact mechanism behind the increased virulence of the three-strain coinfection was not found in this study, it is possible that the high virulence resulted from difficulty of the host immune system to handle the heterogeneous infection (Davies *et al.* 2002). Multiple-genotype coinfection has previously been reported to increase virulence in a *Daphnia*-infecting bacterium *Pasteuria ramosa* (Ben-Ami and Routtu 2013). Besides pathogen genotypes, the virulence of coinfection in this host-pathogen system was dependent on the

bacterial dose (Ben-Ami and Routtu 2013). Similarly here, in study I, the virulence in coinfection of a high-virulence strain and a low-virulence strain was almost exactly an average of that of individual strains, indicating that the low-virulence strain rather diluted the infective dose than had any additive effect on the coinfection outcome. Thus, the results indicate that the individual strains differ a lot, and the coinfection outcome is always unique.

Bacteriocins are toxic compounds produced by a wide variety of bacteria (Riley and Wertz 2002) commonly targeted to hamper the growth of conspecific strains (Jack *et al.* 1995, Riley and Gordon 1999, Riley *et al.* 2003). The most extensively studied bacteriocins may be the colicins produced by *E. coli* (see e.g. James *et al.* 1996, Gouaux 1997, Cascales *et al.* 2007, Mader *et al.* 2015). In this study (III-IV), *F. columnare* strains were found to have an ability to inhibit the growth of conspecific strains by production of bacteriocidal substances (Supplementary Figure S1 in IV). Colicin-like bacteriocin production has been reported from *F. columnare* in an earlier study by Anacker and Ordal (1959), but their role in virulence of the bacterium has remained unknown. Here, the ability to produce bacteriocins was found to be strain-specific, some of the strains being able to inhibit the growth of a co-cultured strain and some not (Table 8 in III). Interestingly, in study IV, the more virulent strains isolated from outlet water from a fish farm were observed to have a greater ability for interference competition via bacteriocins than the less virulent inlet water strains originating from river upstream the fish farm. The results of study III are not in discordance with these observations, as the other of the toxin-producing strains was still virulent towards fish, and the other had lost its virulence due to colony morphology change. Additionally, no clear associations were found between growth-inhibiting ability and bacterial growth. Thus, the observations in this thesis indicate that the ability for interference competition might not have a trade-off with growth or virulence in *F. columnare*, but is instead more prevalent in the more virulent strains. This is in contradiction with studies from *E. coli* where cell lysis is needed to release toxins (Pugsley 1983, Mader *et al.* 2015), and *P. aeruginosa*, where bacteriocin production is traded off with growth of the producing strain (Inglis *et al.* 2009). Furthermore, it was observed that a direct cell-cell contact is likely needed for production of bacteriocins in *F. columnare* (studies III and IV), i.e. they are not released without a stimulus by competing bacteria. In contrast, sterile-filtered supernatant from an overnight-grown bacterial culture was rather found to have a possible growth-promoting effect on the recipient bacterial lawn, that should be confirmed in future experiments.

When the strains were grown in liquid individually and in co-cultures, the population growth seemed to be dependent on the strain characteristics (Figures 3-5 in III). In some strain combinations, co-culture in liquid medium changed the bacterial growth dynamics, which may support the suggestion that interstrain interactions can contribute to changes in pathogen virulence when virulence is related to replication of the pathogen (see e.g. Read *et al.* 1999, Regoes *et al.* 2002). On the other hand, bacterial virulence may sometimes be independent on growth, if a fast-growing strain lacks a virulence factor that is needed for a successful infection. The observed changes in growth in co-culture

are likely related to the interactions between the studied strains, such as interference competition discussed above, resource competition where the faster-growing strain outgrows the other (Buckling and Brockhurst 2008) or cooperative interactions that can either promote or decrease the growth of a co-cultured strain depending on the mode of cooperation (Frank 1996, Brown *et al.* 2002, Buckling and Brockhurst 2008). When strains were studied for their resource competition abilities in co-culture (study IV), the competition was found to be more intense between the more virulent than less virulent strains. When the resources are limited, competition is generally expected to lead to lower growth when the interacting strains have the ability for interference competition (Brown *et al.* 2009, Hibbing *et al.* 2010). However, no indications of cooperation between the studied *F. columnare* strains were found. In order to find out the mechanisms affecting the bacterial growth dynamics in co-culture, more detailed studies are needed on the interactions between *F. columnare* strains differing in their relatedness, growth and virulence.

The interactions of the genetically different *F. columnare* strains co-occurring in the fish farming units are complex and depend on the interacting strains. The strains were shown to be able to compete with each other by producing growth-inhibiting toxins, some strains probably having an ability to outgrow other strains, as they are more dominant in coinfections. The strain interactions can lead to increase in bacterial density (i.e. infection dose) and thus to increased disease severity also in a short timescale. Furthermore, it is possible that the coexistence of genetically different strains with different growth rates and competitive abilities lead to virulence decrease, although the opposite might be expected in the intensive aquaculture conditions that have been suggested to favour selection of increased virulence. It cannot be ruled out that in the long term, the strain interactions might have a role in evolution of virulence.

4.4 Intensive aquaculture selects for increased virulence in bacteria

Intensive farming practices have been proposed to select for more virulent parasite and pathogen strains in aquaculture (Nowak 2007, Mennerat *et al.* 2010), also in the case of *F. columnare* (Pulkkinen *et al.* 2010). The main reason for this lies in the environmental conditions at farming units that are extremely different from what pathogens experience in nature, and may change the selection on pathogen virulence and eventually lead to evolution of more virulent pathogens (Mennerat *et al.* 2010). In fish farming, host densities are high and genetic diversity of hosts is decreased compared to nature, which may facilitate pathogen transmission (Ebert 1998, Nowak 2007, Mennerat *et al.* 2010, Pulkkinen *et al.* 2010, Kennedy *et al.* 2016). Additionally, hosts are treated against diseases, which may also select for increased virulence (Mennerat *et al.*

2010, Pulkkinen *et al.* 2010, Kennedy *et al.* 2016). Also other factors have been suggested to contribute to increased pathogen virulence in fish farming, such as interactions between co-occurring strains (Pulkkinen *et al.* 2010), and the level of environmental nutrients (Penttinen *et al.* 2016). Yet, only little is known how intensive farming affects the evolution of virulence, although this information is needed to understand disease evolution and to develop efficient methods to prevent and to treat diseases. Here (in study IV), the influence of intensive aquaculture on bacterial virulence, growth and interference competition ability was studied at short and long timescales using *F. columnare* as a model organism.

To explore the influence of location of isolation (short timescale), virulence of 18 strains isolated from inlet and outlet water of a fish farm during summer 2010 were studied using zebra fish as an infection model. It was found out that the outlet water strains were significantly more virulent than the inlet water strains (Figure 1b in IV), which indicates that intensive aquaculture conditions may select for more virulent strains already at short timescale. When the inlet and outlet water strains were cultured in two resource concentrations, the outlet water strains were found to be able to grow to higher population sizes than the inlet water strains. This observation indicates that the aquaculture environment may select for strains with increased growth as suggested in (Nowak 2007, Pulkkinen *et al.* 2010, Kennedy *et al.* 2016). The finding is also in accordance with the literature, as pathogen virulence is often linked with growth (see e.g. Schmid-Hempel and Frank 2007, Frank and Schmid-Hempel 2008, Leggett *et al.* 2012,), although the relationship differs between pathogen species (Casadevall and Pirofski 2001). In previous studies, growth and virulence of *F. columnare* have been positively connected (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2010). The bacteria with an ability to produce higher population sizes are better able to exploit host populations, and thus gain the most in intensive aquaculture conditions where hosts are reared in high densities and treated against diseases (Pulkkinen *et al.* 2010). Furthermore, the outlet water strains seemed to be better able to inhibit the growth of inlet water strains than each other. Interestingly, the outlet water strains were more sensitive to changes in resource availability, which may indicate that they have already adapted to the more resource rich conditions at the fish farm (high host density, uneaten feed, fish excrements) compared to river water from where the bacterial strains enter the farm. When the strains were genotyped, the outlet water strains were found to be genetically more homogenous than the inlet water strains. As some of the genotypes found among the inlet water strains were not observed in the outlet water strains, the results may mean that the aquaculture environment selects for certain genotypes that are better adapted to the intensive farming conditions.

To explore how the year of isolation influences bacterial virulence and competitive abilities, *F. columnare* strains isolated from three fish farms during eight consecutive years (2003-2010) were compared. The strains isolated earlier caused less mortality in zebra fish than the strains isolated later (Figure 1a in IV), indicating that virulence of *F. columnare* has been increasing during the

years of observation. Here, bacterial growth was not associated with the year of isolation nor bacterial virulence, although the individual strains varied in their growth. However, the strains isolated later were more efficient in interference competition compared to the strains isolated earlier (Figure 2 in IV), supporting the results above and indicating that the strains that are better adapted to the farming conditions benefit the most from the ability for interference competition, as more bacteria seem to be present at the farming environment than in nature. Indeed, columnaris outbreaks are common in freshwater aquaculture but rare in nature. Together with the results discussed above, these findings support the suggestion that aquaculture conditions select for increased virulence in *F. columnare* (Pulkkinen *et al.* 2010). As selection for increased virulence was observed already at a short timescale, the results indicate that selection pressures at fish farms may lead to rapid changes in populations of opportunistic bacterial pathogens that may have long-lasting effects on virulence of pathogens. Yet, the genetic mechanisms behind the observed virulence increase remain to be solved.

Combined with the results of studies I-III, the findings from study IV indicate that aquaculture conditions combined with the intensive farming practices can lead to increased severity of disease outbreaks, and create circumstances where evolution of higher virulence of opportunistic bacterial pathogens is enabled. One reason for this might be that the ability of outside-host survival in opportunistic pathogens (Brown *et al.* 2012) relaxes the trade-off between virulence and transmission that is commonly experienced by obligate pathogens that are dependent on their hosts (Bull 1994, Frank 1996, Levin 1996, de Roode *et al.* 2008). The saprotrophic nature of *F. columnare* decreases the virulence-transmission trade-off even more (Kunttu *et al.* 2009a). The costs of high virulence are further relaxed by repeated introductions of new, susceptible hosts, increased host density, decreased host heterogeneity and decreased parasite genetic diversity (Ebert 1998, Nowak 2007). Also chemotherapy of infected fish hosts is likely to favour selection of more virulent strains, as treated fish do not remain infectious but dead fish can provide enhanced transmission opportunities for saprotrophic *F. columnare* (Pulkkinen *et al.* 2010). Thus, limiting the virulence-promoting environmental factors (such as organic nutrient levels in the tank water) is of great importance in controlling disease outbreaks and virulence evolution of this opportunistic pathogen.

5 CONCLUSIONS

The results of this PhD study show that the environmental factors in the fish farming conditions can contribute to pathogen virulence, and are consistent with the previous studies suggesting that intensive aquaculture is likely to select for increased pathogen virulence (Mennerat *et al.* 2010, Pulkkinen *et al.* 2010, Kennedy *et al.* 2016). The bacterial density in the water has a significant effect on virulence of environmental opportunistic pathogens (see also Merikanto *et al.* 2012), as they can increase their virulence to high levels without suffering a cost due to their host's death (Brown *et al.* 2012). Here, it is shown that the pathogen density in the aquatic environment can be elevated as a response to increased availability of nutrients in the outside-host environment. Interestingly, nutrient input increases pathogen virulence by stimulating pathogen growth which leads to increased bacterial densities and higher infective doses in the water environment, but also via virulence factor expression (see also Penttinen *et al.* 2016). Additionally, it was found out that the virulence of *F. columnare* can increase when the host is coinfected with several pathogen strains, possibly due to difficulty of the host to cope with multiple-strain infections, or due to strain interactions during coinfection. To find out the exact mechanisms influencing the overall virulence in coinfection, more detailed studies are needed on the strain interactions, both in laboratory and in a living host. Finally, intensive fish farming was shown to select for *F. columnare* strains with high virulence and increased competitiveness in both long and short time scales, indicating that the more virulent strains perform the best in the intensive farming environment. Although this PhD work was conducted using *F. columnare* as a model, the results may have value in understanding the disease dynamics of other environmentally growing bacterial pathogens. The main findings of each chapter are listed below.

I) It was shown that higher infection dose leads to virulence increase also in opportunistic bacteria, similarly to obligatory pathogens. The effect of dose increase on virulence of *F. columnare* was qualitatively similar in both fish species studied, but lower doses were needed to infect rainbow trout than zebra

fish. Based on this study zebra fish is a suitable model host in experimental *F. columnare* infections. Coinfection with a low-virulence and a high-virulence resulted in decreased overall virulence due to dilutive effect of the low-virulence strain.

II) Enhanced availability of outside-host nutrients can lead to virulence increase in opportunistic bacteria in two ways: via increased replication leading to higher infective dose, and virulence factor activation. Thus, enhanced nutrient availability increases virulence also independent of bacterial dose. The high nutrient conditions can also promote virulence increase in the low-virulence bacterial strains. The findings of this chapter highlight the importance of environmental nutrient availability for opportunistic infections in aquaculture.

III) Coinfection may lead to virulence increase in opportunistic pathogens, but the infection outcome depends on the characteristics of the coinfecting strains. *F. columnare* strains were found to differ in their virulence, growth and ability for interference competition in a strain-specific manner. The virulence was dependent on the number of infecting strains, three-strain coinfection being more virulent towards fish than two-strain and single-strain infections. The increased virulence of the three-strain coinfection was independent on the bacterial growth, indicating that the higher virulence results from other factors, such as within-host competition of the coinfecting strains or factors related to the host immune system.

IV) When the effect of isolation year on bacterial virulence was studied, strains isolated more recently were more virulent than strains isolated earlier. Similarly, the strains isolated from the outlet water of a fish farm during one outbreak season were more virulent than strains isolated from the inlet water. The more recently isolated strains were most capable for interference competition, and the outlet water strains were able to inhibit the growth of the inlet water strains. The strains isolated from the outlet water were genetically more homogeneous than the strains isolated from the inlet water, and some genotypes found from inlet water were not detected in the outlet water. This finding indicates that the aquaculture environment may select for specific genotypes. As a conclusion, intensive aquaculture seems to select for strains with higher virulence and enhanced ability for interference competition at both short and long timescales.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Infektioannoksen, ravinteiden ja yhteisinfektion vaikutus *Flavobacterium columnare*n taudinaiheuttamiskykyyn kalanviljelyolosuhteissa

Tartuntataudit ovat riippuvaisia isännän, taudinaiheuttajan ja ympäristön välisistä monimutkaisista vuorovaikutuksista. Täten yksittäisetkin ympäristötekijät voivat vaikuttaa tautien dynamiikkaan. Joissakin tapauksissa ekologiset tekijät voivat johtaa jopa uusien tautien ilmaantumiseen tai olemassa olevien tautien esiintyvyyden kasvuun. Ympäristötekijöiden merkitys on erityisen suuri opportunististen, ympäristön välityksellä leviävien taudinaiheuttajien (patogeenien) taudinaiheuttamiskyvylle eli virulenssille. Tämä johtuu siitä, ettei näiden taudinaiheuttajien elinkierto ole riippuvainen isännästä, vaan ne pystyvät elämään ja monistumaan myös isännän ulkopuolella ja siirtymään uuteen isäntään (transmissio) ympäristön välityksellä. Tällaisten patogeenien tutkiminen on tärkeää siksi, että niiden evoluutiosta on toistaiseksi hyvin vähän tietoa. On myös otettava huomioon, että opportunistien kyky kasvaa isännän ulkopuolella vaikeuttaa niiden aiheuttamien tautien torjuntaa ja hävittämistä, mikä voi johtaa vakavien tautitapausten ilmaantumiseen.

Kasvavaan tuotantotehokkuuteen tähtäävä tehoviljely luo olot, joissa patogeenien elinkierto on erilainen kuin luonnonvaraisissa isännissä. Tästä syystä tehoviljelyn, kalanviljely mukaan lukien, on ehdotettu edesauttavan korkean virulenssin kehittymistä taudinaiheuttamiskykyisillä bakteereilla. *Flavobacterium columnare* on yleinen tautiepidemioita aiheuttava bakteeri kalanviljelyssä. Se on vakava uhka etenkin suomalaiselle lohikalojen poikastuotannolle, mutta aiheuttaa kalantuottajille taloudellisia tappioita myös maailmanlaajuisesti. Sekä *F. columnare*n esiintyminen että taudinaiheuttamiskyky ovat lisääntyneet maassamme merkittävästi 1980-luvun jälkeen. Tarkkaa syytä tälle ei tiedetä, mutta sekä kalojen tehoviljelyssä että *F. columnare*n elinkierrossa on esitetty olevan piirteitä, jotka voivat lisätä bakteerin taudinaiheuttamiskykyä. Kalanviljelyssä esimerkiksi suurten isäntätiheyksien ja kalojen toistuvan lääkitsemisen on oletettu kasvattavan bakteerien virulenssia. Lisäksi *F. columnare* kykenee opportunistina elämään myös isännän ulkopuolella ja käyttämään ravinnokseen kuolleita kaloja (saprotrofismi) sekä leviämään niistä. Korkeasta virulenssista ei siis koidu kustannuksia bakteerin leviämiselle, minkä seurauksena sen taudinaiheuttamiskyky voi kasvaa korkeammaksi kuin obligatorisilla (ehdottomilla) patogeeneilla, joille isännän hengissä pysyminen on transmission kannalta välttämätöntä.

Tämän väitöskirjatutkimuksen tavoitteena oli tutkia biologisten ja ympäristötekijöiden, erityisesti bakteeritiheyden (infektioannoksen), isännän ulkopuolisten ravinteiden (veteen lisätty elatusaine) ja bakteerikantojen yhteisinfektion, vaikutuksia *F. columnare*n virulenssiin ja infektiodynamiikkaan. Paneuduin tutkimuksessa myös virulenssin taustalla oleviin mekanismeihin. Tutkin suomalaisilta kalanviljelylaitoksilta eristettyjen bakteerikantojen virulenssia infektiokokeissa, joissa altistin kirjolohen (*Oncorhynchus mykiss*) poikasia ja

seeprakaloja (*Danio rerio*) eri bakteeriannoksille, eri ravinnepitoisuuksissa tai eri bakteerikantojen yhdistelmille. Lopuksi tarkastelen yleisemmin kalanviljelyolojen vaikutusta opportunistipatogeenien virulenssiin.

Obligatoristen taudinaiheuttajien virulenssi yleensä kasvaa infektioannoksen lisääntyessä, mutta opportunistien kohdalla asiaa ei ole kattavasti tutkittu. Väitöskirjan ensimmäisessä osatyössä tutkin kuinka kasvava bakteeriannos vaikuttaa *F. columnare*n virulenssiin. Käytin tutkimuksessa isäntinä kahta eri kalalajia selvittääkseni kuinka seeprakala soveltuu käytettäväksi infektiomallina kirjolohen sijasta. Tutkimuksessa havaittiin, että *F. columnare*n virulenssi lisääntyi merkitsevästi infektioannoksen kasvaessa molemmissa isännissä. Lisäksi todistettiin, että seeprakala on toimiva tautimalli columnaris-taudin tutkimuksessa, ja infektion kulku ja vaste kasvavaan bakteeriannokseen ovat seeprakalalla kvalitatiivisesti samanlaisia kuin kirjolohella. Tämä tulos on menetelmällisesti tärkeä, koska kirjolohi kylmän veden lajina sietää infektiokokeiden oloja huonommin kuin seeprakala, jonka optimilämpötila on samanlainen kuin patogeenilla itsellään.

Vesiympäristön ravinteiden tiedetään vaikuttavan loistautien vakavuuteen, mutta niiden merkityksestä mikrobien infektiodynamiikalle on vain niukasti tietoa. Toisessa osatyössä tutkin kuinka isännän ulkoisen ympäristön ravinnepitoisuus vaikuttaa opportunistipatogeenien taudinaiheuttamiskykyyn altistamalla kirjolohia ja seeprakaloja *F. columnare*lle kahdessa eri ravinnepitoisuudessa. Virulenssi kasvoi merkittävästi vesiympäristön ravinnepitoisuuden noustessa. Kun kohonneen virulenssin taustalla olevia tekijöitä tutkittiin tarkemmin nesteviljelmässä, ravinnelisäyksen havaittiin lisäävän bakteerien kasvua ravinneköyhään ympäristöön verrattuna. Kohonneen ravinnepitoisuuden stimuloima lisääntynyt kasvu ei kuitenkaan selitä nimenomaan tässä tutkimuksessa havaittua virulenssin muutosta, sillä nestekasvatuksessa bakteerien solumäärässä ei havaittu eroa niiden kahden ravinnetason välillä, jotka infektiokokeessa johtivat merkitsevään eroon virulenssissa. Ympäristön ravinnepitoisuus voi siis vaikuttaa opportunististen patogeenien virulenssiin myös aktivoimalla virulenssitekijöitä, eli taudinaiheuttajien virulenssiin vaikuttavia ominaispiirteitä. Virulenssitekijät voivat edesauttaa esimerkiksi isäntään kiinnittymistä tai isännässä leviämistä, tai parantaa patogeenin kykyä suojautua isäntänsä immuunipuolustukselta.

Luonnossa infektio on vain harvoin yhden patogeenikannan tai -lajin aiheuttama ja kolmannessa osatyössä tutkinkin yhteisinfektion vaikutusta *F. columnare*n taudinaiheuttamiskykyyn altistamalla seeprakaloja yhdelle, kahdelle tai kolmelle bakteerikannalle samanaikaisesti. Lisäksi tarkastelin tutkittavien kantojen interferenssikilpailua (muiden kantojen kasvua estävien toksiinien tuottoa) ja vertailin näiden kantojen kasvua yksittäin ja yhteiskasvatuksissa nesteviljelmässä. Tutkimuksen perusteella *F. columnare*n taudinaiheuttamiskyky voi kasvaa, jos isäntä altistuu usealle bakteerikannalle samanaikaisesti. Yhteisinfektion virulenssi kuitenkin riippuu infektioon osallistuvista kannoista ja niiden välisistä keskinäisistä vuorovaikutuksista. Vaikka tutkitut kannat tuottivat toistensa kasvua estäviä yhdisteitä maljalla, korkeimpaan virulenssiin joh-

taneen yhteisinfektion tulos ei ollut selitettävissä pelkästään bakteerikantojen kasvulla. Kohonneen virulenssin taustalla olevia syitä on siksi etsittävä muista tekijöistä, kuten esimerkiksi kantojen keskinäisistä kilpailevista vuorovaikutuksista tai isännän immuunipuolustukseen liittyvistä tekijöistä.

Aikaisemmissa tutkimuksissa on esitetty, että kalanviljely-ympäristö voisi johtaa virulentimpien *F. columnare* -kantojen valikoitumiseen, mutta asiaa ei ole pystytty kokeellisesti todistamaan. Väitöskirjan neljännessä osatyössä tarkastelin kotimaisilta kalanviljelylaitoksilta vuosina 2003–2010 eristettyjä *F. columnare* -kantoja ja vertailin niiden virulenssia, kasvua ja kilpailukykyä ajan (eristysvuosi) ja paikan (kalanviljelylaitoksen tulovesi tai poistovesi) suhteen. Myöhemmin eristettyjen kantojen virulenssin havaittiin olevan korkeampi kuin aikaisemmin eristettyjen kantojen. Samoin poistovedestä eristettyjen kantojen virulenssi oli tulovedestä eristettyjen kantojen virulenssia korkeampi. Lisäksi niin myöhemmin eristetyt kuin poistovedestä eristetyt kannat kykenivät parhaiten estämään kilpailevien kantojen kasvua. Kiinnostavaa on myös se, että poistovedestä eristetyt kannat olivat geneettisesti homogeenisempia kuin tulovedestä eristetyt, eikä joitakin tulovedessä esiintyviä genotyyppejä havaittu poistoveden kantojen joukossa lainkaan. Tutkimuksen tuloksista voidaan vetää se johtopäätös, että kalanviljely-ympäristö voi niin lyhyellä kuin pitkälläkin aikavälillä suosia bakteerikantoja, joiden taudinaiheuttamiskyky on korkea. Lisäksi voidaan todeta, että tehotuotanto-olot toimivat hyvänä empiirisenä mallina tukemassa teoreettista tutkimusta.

Väitöskirjatutkimukseni tulokset osoittavat, että tutkitut ekologiset tekijät (bakteeritiheys, ympäristön ravinnetaso ja eri bakteerikantojen yhteisinfektio) todella voivat edistää korkean virulenssin kehittymistä opportunistisilla taudinaiheuttajilla. Työssä kerätty aineisto tukee aikaisempia tutkimuksia, joissa tehoviljelyn on ehdotettu johtavan patogeenien taudinaiheuttamiskyvyn kasvuun. Työssä saatiin lisäksi selville *F. columnare*n virulenssin taustalla olevia mekanismeja, kuten ravinteiden kahtalainen virulenssia kasvattava vaikutus, ja bakteerikantojen ominaisuuksien (kuten vuorovaikutusten laadun ja bakteerikannan genotyypin) merkittävä vaikutus yhteisinfektion lopputulemaan. Tulosten perusteella voidaan päätellä, että sekä ympäristön ravinteet että samanaikaisesti esiintyvien bakteerikantojen väliset vuorovaikutukset voivat johtaa bakteeriannoksen kasvamiseen kalanviljelyaltaissa ja sitä kautta voimakkaisiin tautiepidemioihin. Näiden tekijöiden vaikutuksia voitaneen vähentää sekä pienempiä isäntätiheyksiä suosimalla että välttämällä kalojen liiallista ruokintaa. Viljelyolojen vaikutukset patogeenien virulenssiin ovat kuitenkin seurausta useiden elottomien ja elollisten tekijöiden vuorovaikutuksista, jotka kaipaavat vielä lisätutkimuksia: esimerkiksi lääkitsemisen ja bakteerin kasvunvaiheen vaikutuksista *F. columnare*n virulenssiin ei vielä ole kokeellista tietoa. Vaikka tutkimuksessa käytettiin mallilajina *F. columnare*a, voidaan sen tulosten avulla ymmärtää opportunistipatogeenien aiheuttamien tautiepidemioiden puhkeamisen syitä ja taudinaiheuttajien evoluutiota ruoantuotannossa ja muissa tiheissä isäntäpopulaatioissa.

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ORIGINAL PAPERS

I

THE INFLUENCE OF INFECTIVE DOSE ON THE VIRULENCE OF A GENERALIST PATHOGEN IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND ZEBRA FISH (*DANIO RERIO*)

by

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RESEARCH ARTICLE

The Influence of Infective Dose on the Virulence of a Generalist Pathogen in Rainbow Trout (*Oncorhynchus mykiss*) and Zebra Fish (*Danio rerio*)

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Abstract

Pathogen density and genetic diversity fluctuate in the outside-host environment during and between epidemics, affecting disease emergence and the severity and probability of infections. Although the importance of these factors for pathogen virulence and infection probability has been acknowledged, their interactive effects are not well understood. We studied how an infective dose in an environmentally transmitted opportunistic fish pathogen, *Flavobacterium columnare*, affects its virulence both in rainbow trout, which are frequently infected at fish farms, and in zebra fish, a host that is not naturally infected by *F. columnare*. We used previously isolated strains of confirmed high and low virulence in a single infection and in a co-infection. Infection success (measured as host morbidity) correlated positively with dose when the hosts were exposed to the high-virulence strain, but no response for the dose increase was found when the hosts were exposed to the low-virulence strain. Interestingly, the co-infection resulted in poorer infection success than the single infection with the high-virulence strain. The rainbow trout were more susceptible to the infection than the zebra fish but, in both species, the effects of the doses and the strains were qualitatively similar. We suggest that as an increase in dose can lead to increased host morbidity, both the interstrain interactions and differences in infectivity in different hosts may influence the severity and consequently the evolution of disease. Our results also confirm that the zebra fish is a good laboratory model to study *F. columnare* infection.

Introduction

Virulence (the harm caused to the host by a pathogen) is influenced by several ecological and evolutionary processes that often involve a trade-off between host exploitation and pathogen reproduction [1, 2]. The key factors driving the virulence of a pathogen (e.g. host susceptibility, pathogen growth rate and host-specificity) coevolve in an arms race between the pathogen and its host [1]. Opportunistic pathogens are often host-generalists and can have the ability to

survive and replicate outside the host, thus not being restricted by the transmission-virulence trade-off [3]. Despite the opportunists having a great impact on general health, their infection dynamics and infectivity in different host species are poorly characterized, and not covered by the traditional theories of virulence.

In nature, populations of hosts and their pathogens are diverse, and the hosts are often infected by several pathogen genotypes or species, which often leads to increased virulence [4, 5, 6, 7]. Interactions of co-infecting pathogens can have a significant role in virulence evolution, either due to strain competition or by facilitating infection with cooperative interactions [4, 8, 9, 10]. Co-infections may be especially important for generalist pathogens that have a wide host range and, thus, a higher likelihood of coming across potential hosts than host-specialists [3, 11]. However, pathogen virulence may face trade-offs as a result of the ecological and evolutionary costs of generalism [12], which could lead to higher pathogen doses needed for the initiation of an infection. As a consequence, the virulence of a generalist pathogen in different hosts may not always be easy to predict [11].

Infective dose (the number of cells needed to infect a host) varies greatly among pathogen species [13]. The infective dose is generally recognized to influence disease dynamics and severity [14, 15], as virulence typically increases with the dose [8, 14]. Although dose effects in multiply infected hosts can have important evolutionary consequences [16], the strain interactions in the context of dose effect are still poorly understood.

Previous studies on co-infections with eukaryotic parasites have demonstrated the virulence increase in the fish pathogen *Flavobacterium columnare* [17, 18]. However, it has remained unknown how the interstrain interactions of different *F. columnare* strains affect the virulence during co-infection. Using two host species we investigate how an increasing pathogen dose and co-infection (with two bacterial strains differing in their virulence), affect the virulence of this host-generalist pathogen in two phylogenetically distant host species. Our aim is to shed light on how the infective doses and co-infections in opportunistic pathogens shape the disease outcome in different host species, and thus increase the present understanding of disease evolution and how disease epidemics emerge in differing conditions. As an infection model we use the opportunistic fish pathogen *F. columnare*, and as hosts the rainbow trout (*Oncorhynchus mykiss*), a Salmonid host frequently infected in fish farms, and the zebra fish (*Danio rerio*), a Cyprinid host.

Materials and Methods

Pathogen

Flavobacterium columnare is a globally important fish pathogen in freshwater aquaculture [19, 20] and known to affect several fish species in fish farming and in the wild as a causative agent of columnaris disease [21, 22, 23]. The common clinical signs of the disease include gill necrosis, fin erosion and skin lesions such as the typical saddleback symptom around the dorsal fin [20, 24]. The disease is transmitted from infected fish via water and biofilms [25, 26]. In Europe, *F. columnare* is an especially difficult pathogen in salmonid fish farming, where it can cause severe fish mortality within the rearing units [20, 25]. The disease outbreaks occur in the summer when the water temperature naturally rises above 20°C [27]. Two previously isolated *F. columnare* strains were used in this study: a high-virulence strain B185 isolated during a columnaris disease outbreak at a salmonid fish farm in Central Finland (farm L, see details on the strain isolation and virulence in our previous studies [28, 29]) and a low-virulence strain B398 isolated from the inlet water of another salmonid fish farm in the same area (farm V, see [25]). Pure cultures were stored frozen at -80°C in a stock containing 10% glycerol and 10%

fetal calf serum. For the experiments, the bacterial strains were grown in modified Shieh medium [30] at 26°C with constant agitation (150 rpm).

Host species

The rainbow trout is a cold-adapted fish species, occurring naturally in the Pacific Ocean and cold streams in the North American continent from Alaska to Mexico [31]. After introduction into Finland around 1900, the rainbow trout has become the most important commercially farmed fish species in the country [32], and since the 1990's has been severely affected by columnaris disease during warm water periods [20]. As *F. columnare* is prevalent at salmonid farms and their inlet waters in Finland, we used rainbow trout as a model species representing a natural host of *F. columnare*. For the study, apparently healthy fingerling rainbow trout with no known history with *F. columnare* were obtained from a stock of a fish farm (farm V) in Central Finland. The fish were obtained from the farm during a cold water season (when no outbreaks occur), brought to our fish rearing facilities where *F. columnare*-free well water is used, and maintained for two months at 15.0–16.0°C before conducting the experiments. The average weight of the fish was 1.25 g.

The zebra fish is a well-established laboratory animal that shares the temperature optimum of the pathogen (for zebra fish, see [33]; for *F. columnare*, see [24]). It is a tropical species indigenous to South Asia, and thus it does not have a recent co-evolutionary history with the bacterial strains used in this study. The adult, unsexed, disease-free zebra fish (average weight 0.21 g) were obtained from Core Facilities (COFA) and Research Services of Tampere (University of Tampere, Finland).

Both rainbow trout and zebra fish have been previously used as experimental hosts for *F. columnare* [21, 25, 34, 35], but how the bacterial strain and the dose affect the onset of columnaris disease has not yet been thoroughly studied. If the zebra fish are found to respond to the experimental columnaris infection in a similar way to the rainbow trout, they could be used as a reliable model in further columnaris disease experiments.

Infection treatments

To examine the interactions between virulence, infection dose, and host species, the fish were infected with the two *F. columnare* strains, and with a 1:1 mixture of these strains, by bath challenge [29]. The fish were individually challenged in 50 ml of aerated ground water with $5.\times10^5$, 1.0×10^6, 3.0×10^6, 6.0×10^6, 9.0×10^6, 1.2×10^7, 1.6×10^7, 2.0×10^7 and 3.0×10^7 CFU (colony forming units) ml^{-1} of overnight-grown bacteria for 2 hours at 25°C in two fish per dose. The dose was treated as a continuous variable, totaling 18 replicate fish per species per each treatment group (high-virulence strain, low-virulence strain and co-infection). Per species, 5 replicates of negative control fish (sham-exposed to sterile Shieh medium) were used. After being challenged, the fish were transferred individually into 1 liter aquaria with 0.5 liter of ground water, and monitored for clinical signs of disease and morbidity for 5 days, the first 48 hours at 2-hour intervals. The water temperature was maintained at 25.0–26.3°C throughout the experiment. The fatally moribund fish were euthanized by decapitation. Also the surviving and the control fish were euthanized in the end of the experiment. To verify the columnaris infection, cultivations from gills were spread on Shieh agar supplemented with tobramycin [36]. The yellow colonies with the rhizoid morphology typical to *F. columnare* were considered as an indicator of columnaris infection. The experiment was conducted under permission ESAVI-2010-05569/Ym-23, granted by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Statistical analysis

The data were analyzed using a generalized linear model (GLM) for binomial distribution. Two factors ('host species' and 'treatment' (high-virulence strain, low-virulence strain or co-infection)), a continuous covariate ('dose'), and all their possible interactions were included as variables to explain the fate of the fish (dead or surviving) within the time from the beginning of the experiment (see [37]). All the infected rainbow trout 'died' during the experiment. The last moribund rainbow trout were euthanized 42 hours before the end of the experiment. As the gill cultures taken from the infected and moribund fish were positive for *F. columnare* and cultures from the surviving fish were negative, it can rather safely be assumed that zebra fish surviving up to 141 hours (i.e. until the end-point of the experiment) were able to resist, or tolerate and survive, the infection. Thus, we did not consider the surviving individuals as censored cases. The model selection was based on Akaike information criteria (AIC; Table 1) and the analysis was conducted with the software R 2.15.2 and the package Lme4. When interpreting the effects of the terms included in the model, a significance level of 0.05 or less was used.

Results

The risk of fatal infection of the host was significantly influenced by the dose, the treatment (high-virulence strain, low-virulence strain or co-infection) and the host species (Fig 1, Tables 2 and 3). We found that 1) the increase in the dose correlates positively with the host morbidity risk when the hosts are exposed to the high-virulence strain or the mixture; 2) the infection success in the co-infected hosts is approximately an average of that of hosts infected with the high-virulence and the low-virulence strains (Fig 1), indicating that only the high-virulence strain is responsible for the host morbidity; and 3) the rainbow trout is more susceptible to the columnaris infection than the zebra fish (Fig 1), but both hosts respond to the bacterial doses and strains qualitatively similarly. All the moribund hosts were found positive for *F. columnare* in bacterial culture taken from fish gills, whereas the unexposed hosts and hosts surviving the infection were found negative.

Discussion

Opportunistic pathogens are often host-generalists and may survive and replicate outside the host [3], thus having different environmental dynamics than obligate pathogens. Pathogens that are durable in the outside-host environment may not have high fitness costs related to virulence [38], which has also been observed for *F. columnare* [39, 40]. The ability to survive and

Table 1. Model selection based on Akaike information criteria (AIC).

Model	AIC	df	P
host*treatment*dose	234.23		
host+treatment+dose+host:treatment+host:dose+dose:treatment	231.22	2	0.611
host+treatment+dose+host:treatment+dose:treatment	230.72	2	0.173
host+treatment+dose+host:treatment	232.93	2	0.104
host+treatment+dose+dose:treatment	236.78	1	0.009

The model with smallest AIC value estimating the morbidity risk of the host (rainbow trout or zebra fish) within time is underlined.

The degrees of freedom (df) and significance levels (P) are given for the goodness of fit compared to the next higher level model.

Single- and co-infections are included in the term 'treatment'.

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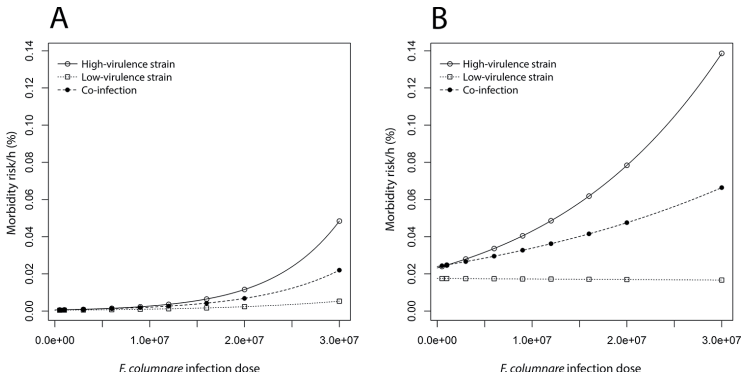

Fig 1. Estimated mortality risk per hour of A) zebra fish (*Danio rerio*), and B) rainbow trout (*Oncorhynchus mykiss*) infected with a high-virulence (continuous line) and a low-virulence (dotted line) strain of *F. columnare*, and their mixture, i.e. co-infection (dashed line).

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replicate outside the host can contribute significantly to the infective bacterial populations in the environment, and therefore information on the relationship between the number of free-living bacteria (the infection dose) and disease virulence is needed. Although the influence of dose on disease dynamics has been widely acknowledged, its effect on parasite virulence and reproduction is sometimes unclear or even contradictory [14]. Additionally, the experimental evidence from the infective doses of opportunistic pathogens is scarce, especially in different host species. We addressed these issues by infecting two host species with increasing doses of a high-virulence and a low-virulence bacterial strain in a single and in a co-infection.

We found a strong positive relationship between the dose and the host morbidity risk in the treatments in which the high-virulence strain was involved (Fig 1). As the host morbidity risk in this study is a measure of pathogen virulence (as demonstrated in e.g. [2, 41, 42, 43]), our result suggests that the virulence of *F. columnare* is strongly dose-dependent. This finding is in agreement with the experimental evidence from obligatory pathogens [14, 44]. Interestingly, in contradiction with numerous previous studies [5, 7, 45, 46, 47], we did not observe any additive effects of co-infection on the host morbidity risk. This indicates that the outcome of the infection in this study is affected by the interplay between the bacterial strain and the dose. Previous studies (e.g. [48, 49]) have shown that the more virulent strains have a competitive advantage in mixed infections, whereas in some systems, like *Schistosoma mansoni*, co-infections may favor the less virulent strains [50]. Our result suggests that the presence of a low-virulence

Table 2. The significance and test values of the bacterial dose, the treatment and the host species on the morbidity risk of the hosts.

Source	Df	Deviance	Residual deviance	P
Host	1,106	84.350	116.663	**<0.001**
Dose	1,105	9.606	107.057	**0.002**
Treatment	2,103	14.654	92.403	**<0.001**
Host:Dose	1,102	10.205	82.198	**0.002**
Dose:Treatment	2,100	4.522	77.677	0.104

Significant P values are denoted in bold.

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Table 3. The effect of the bacterial dose, the treatment and the host species on the host morbidity risk.

Source	Estimate	SE
(Intercept) [a]	-3.738	0.390
Host(Zebra fish)	-3.633	0.571
Dose	6.372^{-8}	2.805^{-8}
Treatment(Co-infection)	3.337^{-2}	0.505
Treatment(Single infection, low)	-2.861^{-1}	0.496
Host(Zebra fish):Dose	8.272^{-8}	3.197^{-8}
Dose:Treatment(Co-infection)	-2.832^{-8}	3.353^{-8}
Dose:Treatment(Single infection, low)	-6.542^{-8}	3.186^{-8}

[a] Intercept includes the effects of the host (rainbow trout) and the treatment (single infection, high).

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strain may significantly alter the co-infection outcome, most likely by diluting the infection dose. Indeed, if the low-virulence strain lacks the ability to produce essential virulence factors needed for a successful infection, its presence may reduce the total severity of the disease outbreak. This is an important finding as the interactions between high-virulence and low-virulence strains are generally poorly understood. Yet, pathogen strains with variable levels of virulence often co-occur in the environment [20, 22], thus influencing the onset of disease outbreaks or host immune response.

Also interference competition via antimicrobial compounds like colicins (i.e. inhibitory compounds targeted to hamper the growth of other conspecific strains) may have trade-offs with virulence [51]. *F. columnare* has been reported to produce bacteriocins that are equivalent to colicins, as demonstrated in [52]. However, in order to find out if the mechanism leading to reduced virulence in our system builds upon the competitive interactions between the bacterial strains, more studies are needed in the context of virulence evolution.

Although maintaining the ability to infect multiple host species can be an efficient survival strategy, it may result in a trade-off, leading to lower pathogen virulence [11, 12, 53]. We found the two host species to respond qualitatively similarly to the increase in the infection dose, but the rainbow trout was more sensitive to the increase than the zebra fish. Similar associations between *F. columnare* strains and the host species have also been found in salmonids in general and in channel catfish [35, 54, 55]. However, more extensive studies on a variety of host species would be needed to find out if our results are due to adaptation of the strains to the rainbow trout, or if host generalism has trade-offs with virulence of *F. columnare*. Nevertheless, our result has important implications because *F. columnare* populations encounter a wide range of host species both in the wild and at fish farms [21, 22, 23, 56].

The sensitivity of the rainbow trout in this study can also be partly caused by the experimental conditions. The water temperature during the experiment was not optimal for the cold-adapted rainbow trout, although it still was within the temperature range naturally occurring in fish farming conditions. Indeed, columnaris disease outbreaks at fish farms are typically prevalent during the warm water season [20, 57]. Yet, our findings confirm that zebra fish is a suitable model species for experimental studies of *F. columnare* infections. Zebra fish has been successfully used as an infection model for columnaris disease already in prior studies [34, 35], but the infection dynamics of *F. columnare* in zebra fish compared to rainbow trout has remained unclear. Information about the comparability of the dose responses in these two species is therefore intensely needed to be able to replace the stress-sensitive rainbow trout in the demanding laboratory experiments. Unlike the rainbow trout, the zebra fish is well suited to

laboratory conditions; it is a small-sized species that thrives in warm temperatures (as does the pathogen) and does not require constant water flow [33]. Additionally, zebra fish are available year-round.

Our results suggest that an increase in dose can lead to more severe disease and poorer host survival in host-generalist opportunistic pathogens, but the host survival may be dependent on the original ability of each bacterial strain to cause disease in a strain-specific manner. For the same reason, different pathogen strains may not necessarily have additive effects on disease virulence. Based on our results, it seems that the interactions between the dose and the pathogen strains are important drivers of infection in different host species, and warrant for more studies for evolution of virulence and pathogen host range. Furthermore, from an applied perspective, using zebra fish as an infection model can provide valuable information on the virulence of *F. columnare*, as the zebra fish shares the temperature optimum of the pathogen and tolerates the experimental conditions well.

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Author Contributions

Conceived and designed the experiments: LRS HK JM JKV. Performed the experiments: HK LRS. Analyzed the data: JKV HK. Contributed reagents/materials/analysis tools: LRS. Wrote the paper: HK LRS JM JKV.

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II

HIGHER RESOURCE LEVEL PROMOTES VIRULENCE IN AN ENVIRONMENTALLY TRANSMITTED BACTERIAL FISH PATHOGEN

by

Hanna Kinnula, Johanna Mappes, Janne K. Valkonen, Katja Pulkkinen & Lotta-Riina Sundberg 2016

Submitted manuscript

III

COINFECTION OUTCOME IN AN OPPORTUNISTIC PATHOGEN DEPENDS ON THE INTER-STRAIN INTERACTIONS

by

Hanna Kinnula, Johanna Mappes & Lotta-Riina Sundberg 2016

Submitted manuscript

IV

INTENSIVE AQUACULTURE SELECTS FOR INCREASED VIRULENCE AND INTERFERENCE COMPETITION IN BACTERIA

by

Lotta-Riina Sundberg, Tarmo Ketola, Elina Laanto, Hanna Kinnula, Jaana Bamford, Reetta Penttinen & Johanna Mappes 2016

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