Exposure of Atlantic salmon (*Salmo salar* L.) to non-virulent strains of *Y. ruckeri* to protect against subsequent infection with virulent *Y. ruckeri*

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Thesis for the degree Master of Science in Aquamedicine

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01.06. 2022

2022

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Forord

Først og fremst vil jeg rette en stor takk mot veilederen min, Dr. Anita Rønneseth, som med god veiledning, oppmuntring og tålmodighet har hjulpet meg gjennom denne oppgaven. Tusen takk for all tiden du har brukt på planlegging og oppsett av forsøkene mine, og all hjelpen jeg har fått med skrivingen.

Jeg vil også rette en takk mot resten av fiskeimmunologi-gruppen ved UiB, som alltid har stilt opp med godt humør og gitt meg tips og råd da jeg hadde behov for det. En særlig takk går til tekniker Harald Sæbø Lunde som har gitt meg innføring i bruken av en mengde lab-utstyr.

Tusen takk til min familie som har vært en god støtte i denne perioden, jeg er evig takknemlig for all oppmuntring dere har gitt meg og at dere alltid har hatt troen på meg.
Lena, min fantastiske kjæreste, tusen takk for din tålmodighet og urokkelige støtte det siste året. Takk for at du har hjulpet meg gjennom tunge perioder, muntret meg opp og at du alltid er der for meg.

Magnus Ruland
Bergen, 01.06.22

Abstract

Fish that are subjected to non-lethal bacterial infection can develop a greater resistance when exposed to a virulent strain of the same bacteria. This is known as cross-reactivity and occurs when an antibody produced against one specific antigen is capable of binding epitopes of a different antigen or if the same epitope is present on different bacteria. Yersiniose, caused by *Yersinia ruckeri*, in Atlantic salmon has traditionally been regarded as a freshwater disease, but in recent years an increase in disease outbreaks has been observed during the sea water phase. Effective vaccines have already been implemented to reduce disease outbreaks, effective infection models have been established, and factors triggering disease outbreaks of latent infections has been investigated. However, the effect of prior colonization of avirulent *Y. ruckeri* strains in Atlantic salmon on exposure to virulent strains is not yet established.

Atlantic salmon were challenged in freshwater tanks employing a cohabitation model where salmon injected with *Y. ruckeri* acted as shedders. Four isolates were used in the experimental challenge (NVI 10705, 11076, 11587, 11065) and injected shedders were added to separate tanks. Six weeks later new shedders were added to each tank, all injected with the assumed virulent isolate NVI 10705. Additionally, formalin inactivated bacterins from all four *Y. ruckeri* strains were administrated to Atlantic salmon by intraperitoneal injection (i.p.), followed by sera sampling. Analysis of the antisera and their ability to cross-react was done using ELISA.

Analysis of the cumulative mortality percentage in each tank revealed that NVI 10705 was indeed the only virulent strain reaching 70% mortality of cohabitants, and that prior exposure to environmental strains seems to offer some protection of cohabitants when exposed to NVI 10705. The ELISA results shows that vaccines raised against each *Y. ruckeri* strain had the desired effect, a clear antibody response was measured when compared to the non-immunized control group. The ELISA analysis shows a clear cross-reactive ability of antibodies raised against the different isolates, all with significantly higher OD-values compared to the sera collected from non-immunized control group.

The implication of this study is the possibility of immunizing fish by e.g., immersion with avirulent *Y. ruckeri* strains, as opposed to invasive i.p. injected vaccines. However, further research is needed to further confirm the cross-protection ability of the environmental strains and to identify the shared antigenic outer membrane proteins (OMPs).

Sammendrag

Fisk utsatt for en ikke-dødelig bakteriell infeksjon kan bli mer motstandsdyktig når de blir eksponert mot et virulent isolat av samme bakterie. Dette er kjent som kryss-reaktivitet og inntreffer når et antistoff produsert mot et spesifikt antigen er i stand til å binde seg til epitoper til et annet antigen, eller dersom den samme epitopen er til stede hos forskjellige bakterier. Yersiniose, forårsaket av *Y. ruckeri*, i atlantisk laks har tradisjonelt blitt regnet som en ferskvannssykdom, men de siste årene har det blitt observert en økning av sykdomsutbrudd i sjøfasen. Effektive vaksiner har allerede blitt iverksatt for å redusere sykdomsutbrudd, effektive smittemodeller er etablert og utløsende faktorer for sykdomsutbrudd av latente infeksjoner er undersøkt. Men, effekten av tidligere kolonisering av avirulente *Y. ruckeri* isolater av atlantisk laks før eksponering mot virulente isolater er ennå ikke etablert.

Det ble utført smitteforsøk hvor atlantisk laks i ferskvannstanker, ved bruk av en kohabitant-modell ble injisert med *Y. ruckeri* fungerte som sheddere. Fire isolater ble brukt i smitteforsøket (NVI 10705, 11076, 11587, 11065) og injiserte sheddere ble tilsatt separerte tanker. Seks uker senere ble nye sheddere tilsatt hver tank, alle injiserte med det antatt virulente isolatet NVI 10705. I tillegg ble formalin inaktiverte bakteriner fra alle fire *Y. ruckeri* isolater administrert til atlantisk laks ved intraperitoneal (i.p.) injisering, etterfulgt av sera prøvetaking. Analysen av antisera og dens mulighet til å kryss-reagere ble gjort med ELISA.

Analysen av den kumulative dødelighetsprosenten i hver tank viste at NVI 10705 var som antatt det eneste virulente isolatet, og nådde 70% dødelighet hos kohabitanter. Analysen viste videre at tidligere eksponering mot miljøstammer virker å ha beskyttende effekt for kohabitanter ved eksponering mot NVI 10705. ELISA resultatene viser at vaksiner produsert mot hvert *Y. ruckeri* isolat hadde den ønskede effekten, en klar antistoffrespons ble målt sammenlignet med den ikke-immuniserte kontroll-gruppen. ELISA analysen viser en klar kryss-reaksjon av antistoff mot de forskjellige isolatene, alle med signifikant høyere OD-verdi sammenlignet med sera samlet fra den ikke-immuniserte kontrollgruppen.

Implikasjonene av denne studien er muligheten til å immunisere fisk ved f.eks. bad med avirulente *Y. ruckeri* isolater, i motsetning til den invasive i.p. injiserte vaksinen. Men, mer forsking er nødvendig for å videre bekrefte kryss-reaksjonsevnen til miljøstammene og for å identifisere de antigeniske membranproteinene som isolatene har til felles.

Abbreviations

Abbreviation	Clarification
AMP	Antimicrobial peptide
APC	antigen presenting cell
APP	Acute phase protein
BA	Blood agar
BCR	B-cell receptor
BHIB	Brain Heart Infusion Broth
CC	Clonal complex
CDR	Complementarity-determining region
cfu/ml	colony forming units/ml
C.m	Cumulative percent mortality
co	cohabitants
ctr	non-infected controls
DAMP	Danger associated molecular pattern
dpc	days post challenge
ELISA	Enzyme Linked Immunosorbent Assay
ERM	Enteric redmouth disease
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
HK	Head kidney
HRP	Horseradish peroxidase
IAT	Inverse autotransporter
IFAT	Immunofluorescence antibody technique
Ig	Immunoglobulin
ILAB	Industrial and aquatic laboratory
i.p	Intraperitoneal
MHC	Major histocompatibility complex
MLVA	Multilocus Variable-Number Tandem-Repeat
NCC	Non-specific cytotoxic cells
OD	Optical density
OMP	Outer-membrane protein
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
PBS-T	PBS-Tween
PCR	Polymerase chain reaction
PRP	Pattern recognition protein
PRR	Pattern recognition receptor
RPM	Rotation per minute
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide Gel Electrophoresis
T1SS	Type 1 secretion system
T3SS	Type III secretion system
T4P	Type IV Pili
T4SS	Type IV secretion system
T5SS	Type V secretion system
TCR	T-cell receptor

VIE	Visible implant elastomer
YhlBA	*Y. Ruckeri* Pore-forming toxin
Yrp1	*Y. Ruckeri* Protease 1
YrpAB	*Y. Ruckeri* Peptidases AB

Contents

1. Introduction

1.1 Yersinosis

Yersinia ruckeri is the causative agent of enteric redmouth disease (ERM) and yersiniose of salmonids. It was first isolated from farmed rainbow trout (Onchorhynchus mykiss) from the Hagerman Valley in Idaho in the USA during the 1950s (Ross et al., 1966). It has since been found in several fish species: salmonids, eels, goldfish, sole, sturgeon and turbot in South America, Europe, Africa, and Australia. The first isolation of *Y. ruckeri* in Norway was in 1985 from kidney samples collected after an increase in mortality in seawater farmed Atlantic salmon (*Salmo salar* L.) (Sparboe et al., 1986), and it has been one of the most important bacterial pathogens since. While different fish species can be infected by the bacteria, salmonids are the most susceptible. Globally yersiniosis is most economical important in rainbow trout farming, but it is also a significant problem in farmed Atlantic salmon in Norway, Scotland, Australia (Tasmania) and Chile (Gulla et al., 2018).

1.2 Yersinosis in Norwegian aquaculture

Outbreaks of yersiniosis in farmed Atlantic salmon in Norwegian aquaculture has seen a great increase since the early 2000s, with less than 5 cases until 2007. An upward facing trend since 2008 with 16 recorded outbreaks in localities bottomed out at 8 outbreaks in 2011, followed by a considerable increase in 2012 with 16 outbreaks. During the following years yersiniosis-outbreaks continued to rise until 2016 with 34 localities with recorded outbreaks (Gulla, 2016). Almost all the outbreaks were caused by serotype O1 and took place in mid- and northern Norway. Interestingly an increasing amount of disease outbreaks occurred in big salmon in the sea, especially in Mid-Norway. This was a new trend as historically yersinosis in Norwegian farmed salmon has been recognized as a freshwater disease, although sporadically also occurring in on-growth facilities and after seawater transfer. Approximately half of the juvenile fish production sites that has experienced outbreaks of yersinosis since 2007 had recurrent outbreaks the following year (Gulla, 2016). In the following years there were a decline in reported cases, likely caused by an increase in vaccine coverage in mid-Norway. However, this trend seems to have reversed recently with an increase from 14 localities with recorded outbreaks in 2020 to 19 in 2021. The reason for this is not clear as there isn´t available data regarding the number of salmon

vaccinated against *Y. ruckeri* during the sea phase, but if the vaccination coverage indeed has decreased this could be a possible explanation. Yersinosis is not a notifiable disease in farmed fish in Norway, highlighting the issue of possible underreported numbers of cases. As before, yersiniose outbreaks continues to be problematic mainly in mid-Norway, with some sporadic outbreaks in northern Norway (Gulla & Olsen, 2021). The use of mechanical delousing has been raised as possible partly explanation of the increase in cases of yersinosis (Gulla & Olsen, 2021).

1.3 Pathogenesis

ERM, in rainbow trout, and yersiniosis in Atlantic salmon is caused by *Y. ruckeri* and can take place both before and after the sea water phase, but the infection likely takes place during the juvenile phase. Although infection by *Y. ruckeri* can develop in several fish species it almost exclusively infects Atlantic salmon in Norway. *Y. ruckeri* can manifest itself as an acute or chronic infection in both seawater and freshwater. The mortality rate is typically low in the initial phase of the disease followed by a rapid increase as it develops, resulting in severe fish losses. Stress from e.g., handling and lice treatment is known to contribute to disease outbreak and further infections (Gulla & Olsen, 2021).

Affected fish generally develops darker skin color, loss of appetite and can be seen swimming isolated from other fish and near the surface. Gross pathology includes exophthalmia, ascites, reddening of the mouth, anus, the fin bases and the area surrounding the lateral line, caused by hemorrhages (Wrobel et al., 2019).

Internal signs of ERM are petechial hemorrhages in liver, pancreas, swim-bladder, stomach, intestine, and muscle tissue. Both the kidney and spleen can be swollen, and the spleen is usually darkened in color (Wrobel et al., 2019). Yersiniose in Atlantic salmon is also characterized by blood spots on the iris of the eye, but without the mandibular hemorrhage that causes the reddening of the mouth.

Y. ruckeri transfers horizontally from fish to fish during contact with diseased fish or asymptomatic carrier fish. Carrier fish has been shown to be important reservoirs for *Y. ruckeri* and their shedding is likely promoted by stress and high temperatures (Hunter et al., 1980). In addition to carriers, *Y. ruckeri* ability to from biofilm may be source for recurrent infection in the same production areas (Tobback et al., 2007).

1.4 Diagnostics

Several diagnostic methods are available for diagnosing yersinosis, such as culturing, serological tests, and molecular biological techniques. Tryptic soy agar is often used to isolate *Y. ruckeri* as it grows rapidly, but blood agar plates is also an option. After 48 h incubation at 25ºC on blood agar opaque colonies, 2-4 mm wide, will appear (Tobback et al., 2007).

Serological tests such as ELISA, agglutination test and immunofluorescence antibody technique (IFAT) are all capable of detecting *Y. ruckeri* (Smith et al., 1987).

Polymerase chain reaction (PCR) is often used as a diagnostic tool, and can detect even low levels of *Y. ruckeri*, in addition to detecting asymptomatic carriers. This makes PCR a useful tool to control and prevent the spread of ERM and yersiniose in salmonid cultivation.

1.5 Characteristics of Yersinia ruckeri

Y. ruckeri cells are gram-negative rods with straight or slightly curved shapes and are a member of the order Enterobacterales. Like the other members of Enterobacterales they are facultative anaerobic, glucose- and mannitol-fermentative, oxidase negative and nitrate reductive. They grow to be 2 to 3 μm in length and 1 μm in width (Ross et al., 1966). They are non-spore-forming and do not have a capsule, but most of them possesses flagella uniformly distributed over the surface, with some strains being the exception (Tobback et al., 2007). To distinguish Y. ruckeri from other bacteria several biochemical tests can be run based on characteristics that will distinguish *Y. ruckeri* from others such as presence of ß-galactosidase, lysine decarboxylase and ornithine decarboxylase, but no H2S and indole. In addition, *Y. ruckeri* doesn't ferment inositol, rhamnose sucrose, melibiose or arabinose. These factors make it possible to identify *Y. ruckeri* with test kits like the API 20E system (Tobback et al., 2007).

1.6 Strain differentiation

Strain differentiation of *Y. ruckeri* is important in disease surveillance as the virulence differs among different strains. Biotype, serotype, and outer-membrane protein (OMP) type can all be used to categorize them. Biotype 1 is positive for lipase secretion and motility (flagellated), whereas biotype 2 is negative for both (Wade, 2019). Strains of *Y. ruckeri* are

categorized into four serotypes with different sub-groups. Serotype O1 is divided into sub-groups O1a ("Hagerman strain"), and O1b; serotype 2 is divided into three subgroups (O2a, O2b, O2c); the remaining two serotypes are O3 and O4 (Wade, 2019).

In rainbow trout serotype O1 biotype 1 and 2 are the most important in ERM development, where serotype O1a is considered the most virulent. Nonmotile *Y. ruckeri* biotype 2 strains have increasingly been dominating disease outbreaks in rainbow trout farming in several countries, indicating an independent evolution caused by flagellar antigens-targeting vaccines (Gulla et al., 2018).

Regarding farmed Atlantic salmon disease outbreaks may be associated with different serotypes, but serotype O1a is considered the most important. In Norway almost all cases of yersiniosis are caused by serotype O1, whereas serotype O2 is sporadically detected (Gulla et al., 2018).

1.7 *Y. ruckeri* virulence factors

Although *Y. ruckeri* has long been known to be responsible for ERM/yersinosis and economic losses in salmonid farming there are still limited numbers of studies investigating the bacteria's virulence mechanisms. The virulence depends on several factors, such as iron availability. temperature, pH and osmolarity (Wrobel et al., 2019).

1.7.1 *Y. ruckeri* toxins

1.7.1.1 *Y. ruckeri* Protease 1 (Yrp1)

Yrp1 is a serralysin metalloprotease which is secreted by an ATP-dependent type 1 secretion system (T1SS). This toxin is produced by some of the most virulent strains at the end of the exponential growth phase. Controlling factors for the production of the toxin is osmolarity and temperature; the highest expression detected at 18°C and during low osmotic pressure. Yrp1 causes typical clinical signs of ERM by digesting a wide range of the hosts matrix and muscle proteins. Inactivated Yrp1 has also been shown to be protective against ERM when its delivered as an immunogen (Wrobel et al., 2019).

1.7.1.2 *Y. ruckeri* Peptidases (YrpAB)

YrpAB is the name of two adjacent genes encoding two peptidases whose operon expression is dependent on available peptone and oxygen (Wrobel et al., 2019). The expression of the YrpAB operon is upregulated under microaerobic conditions, as can be found in the gut of infected fish (Wrobel et al., 2019), which in rainbow trout is the main organ affected during the infection (Navais et al., 2014).

An LD50 experiment has confirmed that at least one of the peptidases, YrpA, contributes to the virulence of *Y. ruckeri* as the deletion mutant of it showed attenuated virulence (Navais et al., 2014).

1.7.1.3 *Y. ruckeri* Pore-forming Toxin (yhlBA)

The yhlBA cluster consists of two adjacent genes, yhlA and yhlB, where yhlA codes for the expression of hemolysin while yhlB is responsible for activating the yhlA gene and is also involved in its secretion. Controlling factors for expression of the operon is temperature and iron availability. Expression of the yhlBA cluster and the following secretion can lead to cytolysis and hemolysis of for example erythrocytes (Wrobel et al., 2019). The hemolysin encoded by yhlA plays an active role in the virulence of *Y. ruckeri* due to its cytopathic activity and possibly its contribution to the acquisition of iron from the host cells (Fernández et al., 2007).

1.7.1.4 *Y. ruckeri* Phospholipase

Phospholipases are enzymes that hydrolyses the fatty acids of phospholipids and have potential to act as exotoxins (secreted toxins) that disrupt the host cell membranes. *Y. ruckeri* biotype 1 phospholipase activity takes place in the bacteria's extracellular fraction and has been implicated in virulence factors, as the products in the fraction are toxic to fish. Secretion is dependent on the flagellar secretion machinery, hence only biotype 1 has this ability (Wrobel et al., 2019).

1.7.2 *Y. ruckeri* secretion systems

1.7.2.1 *Y. ruckeri* Type III Secretion System (T3SS)

The T3SS was recently found in the genome of *Y. ruckeri* SC09 (Wrobel et al., 2019). This secretion system is common among Gram-negative bacteria and is used to transfer toxins into host cells. It consists of a basal body that spans the inner and outer membrane, and a

needle that protrudes out of the cell, making direct contact to the host cell. It's likely that T3SS is required for intracellular survival in fish macrophages, bur more research is needed (Wrobel et al., 2019).

1.7.2.2 *Y. ruckeri* Type IV Secretion System (T4SS)

T4SS can be found in both Gram-negative and -positive bacteria and is used for transporting macromolecules across their cell membrane. This system has been found in several *Y. ruckeri* strains. T4SSs can be divided into three categories, the first group is called conjugative T4SS and transfers DNA from one to cell to another by making direct contact. The second category handles DNA uptake and release into the extracellular environment. The last category transfers virulence proteins and protein complexes into host cells, and thus plays a crucial role in virulency (Wrobel et al., 2019).

1.7.2.3 *Y. ruckeri* Type V Secretion System (T5SS)

T5SSs, also called autotransporters are the most common secretion system among Gram-negative bacteria. As of now five subtypes has been described, where only type Ve or inverse autotransporter (IAT) has been found in *Y. ruckeri* from various strains of serotype O1 and O2 isolated from rainbow trout and Atlantic salmon (Wrobel et al., 2019). IAT contains adhesins associated with virulence, and deletion of IATs has been shown to reduce *Y. ruckeri* ability to form biofilm (Wrobel et al., 2020).

1.7.2.4 Type IV Pili (T4P)

Y. ruckeri possesses T4P, which are filamentous appendages capable of binding to surfaces, perform DNA-uptake and biofilm formation (Wrobel et al., 2019). Unlike other pili T4P are dynamic; their filaments polymerize and depolymerize which enables rapid cycles of extension and retraction that generates mechanical force. Using its T4P, *Y. ruckeri* bacteria can pull itself along mucosal surfaces to host cells, exert forces on them, pull substrates like DNA into its periplasm and export exoproteins across its outer cell membrane (Craig et al., 2019).

1.7.3 Outer Membrane Proteins

OMPs are partly surface-exposed proteins which plays an important role in nutrient uptake, cell survival and host-pathogen interactions. *Y. ruckeri* OMPs are not yet fully characterized, and their contribution to virulence is not determined, however OMPs has potential to be used as a vaccine candidates (Wrobel et al., 2019).

1.7.4 Biofilm as a virulence factor

Y. ruckeri can adhere to several materials common in fish farms such as fiberglass, concrete and wood, and form biofilm. It´s likely that biofilm formation is the main reason for recurrent infections in salmonid aquaculture, as the bacteria are more likely to survive antibiotic treatment and persist in circulating water systems (Wrobel et al., 2019).

1.8 Treatment and Vaccination

1.8.1 Antimicrobial compounds

Antimicrobial compounds, mainly oxolinic acid, is used to some degree as treatment of yersiniosis. However as use of antibiotics carry the risks of resistant bacterial strains its recommended to limit the use to when it's strictly necessary (Gulla & Olsen, 2021).

1.8.2 Vaccination

Vaccination against a variety of bacteria has played a key role for the success of salmon farming in Norway. The fish farming industry suffered enormous economic losses during the 1980s due to disease outbreaks of vibriosis, cold water vibriosis and furunculosis. This led to a major increase in the use of antibacterial drugs. During the 1990s effective vaccines were implemented and greatly reduced the use of antibiotics by more than 90% (Midtlyng et al., 2011). the annual consumption has been less than 1000 kg since the vaccines were implemented, even though the fish production itself has increased vastly (Fig. 2.1).

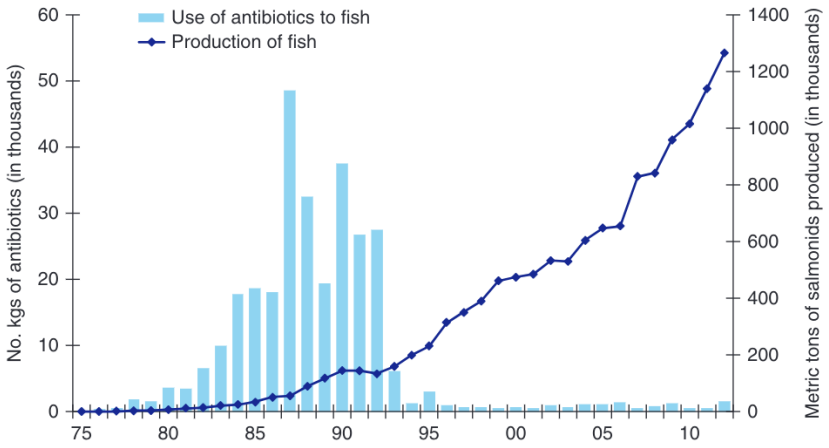

Fig 1.1 The use of antibiotics in Norwegian aquaculture, adapted from Gudding, 2014.

The protection against vibriosis, pasteurellosis and yersiniosis can be achieved by inactivated vaccines administered by immersion or intraperitoneal (i.p) injection. Inactivated vaccines are preferred mainly because of its safety; where attenuated microorganisms might be distributed through water and cause disease in susceptible species, inactivated bacterins are unable to replicate and/or cause disease.

Vaccines against *Y. ruckeri* were the first fish vaccines that were commercially produced in 1976 and were composed of formalin-killed whole bacterial cells (Tobback et al., 2007). The use of water based i.p vaccination has increased recent years due to the problems with yersiniosis in the seawater phase in Mid-Norway, and this seems to have had the desired effect with reduced outbreaks. The vaccine is based on serotype O1, biotype 1, and the flagellar proteins are used as antigens (Gulla & Olsen, 2021).

1.9 The Immune System

Like higher vertebrates fish have innate and adaptive immune systems which protects them from foreign substances. The innate immune system is the first line of defense and has a key role in disease resistance and activation of the adaptive responses. The adaptive immune defense provides a delayed but effective response against pathogenic microbes and are essential for memory and long-lasting immunity.

1.9.1 The innate immune response

The innate system recognizes non-self and danger signals with a limited number of germ-line encoded pattern recognition receptors/proteins (PRRs/PRPs). These receptors and proteins recognize pathogen associated molecular patterns (PAMPs), e.g., bacterial lipopolysaccharides (LPS) and fungal glycoproteins. Such receptors are also able to recognize danger associated molecular patterns (DAMPs) which can be released by host cells after e.g., injury or apoptosis (Magnadóttir, 2006).

The innate immune system can be divided into three sections: physical, humoral, and cellular. The physical section is the barrier that keeps foreign substances from entering the fish, such as epithelium, scales, and the mucous layer (Magnadóttir, 2006)

The cellular section consists of a number of leukocytes: phagocytic cells including macrophages and monocytes, granulocytes (neutrophils, eosinophils and basophils) dendritic cells, and non-specific cytotoxic cells (NCCs) (Magnadóttir, 2006). Unlike in higher vertebrates, B lymphocytes in fish have phagocytic activity in addition to its role in acquired immune system. NCCs are considered have equivalent function as natural killer (NK) cells in higher vertebrates and has cytotoxic activity. These are fast responding cells that can be found circulating the blood stream and in secondary lymphoid organs and are capable of killing a variety of target cells (Secombes & Wang, 2012).

The humoral section consists of molecules present in extracellular fluids, such as complement proteins, cytokines, chemokines, growth inhibitors and lytic enzymes (Magnadóttir, 2006).

1.9.2 The adaptive immune response

The adaptive immune response is more specialized than the innate and activates after contact with an immunogen. Its response can be divided into a humoral response, which produces antibodies, and a cellular response, which activates cytotoxic T-cells.
In antibody responses, the B-cells are activated and differentiate into plasma cells secreting antibodies, which are a soluble analog form of their surface immunoglobulin (Ig) also known as the B-cell receptor (BCR). The antibodies are transported in the bloodstream and will bind specifically to the same antigen which stimulated their production. These antibodies have several functions, such as marking invading pathogens for destruction, or inactivating viruses and microbial toxins. When a foreign substance is marked with Ig molecules it can be recognized by phagocytic cells of the innate immune system through their Fc receptor, which will then try to neutralize it (Secombes & Wang, 2012).

In cell-mediated immune responses, antigen-specific T-cells are activated by being presented a foreign antigen by an antigen presenting cell (APC), via their major histocompatibility complex (MHC) class II proteins. The antigen peptide presented on MHC class II will bind to the T-cell receptor (TCR) of CD4+ T helper cells. This in combination with binding of other co-receptors and secretion of stimulating cytokines will stimulate the differentiation of different subsets of T-helper cells, including Th1 and Th2 cells. Th2 cells are important in the differentiation of plasma cells through the T-cell dependent activation pathway. Th1 cells are important in the activation of cytotoxic T-cells (CD8+ T-cells). CD8+ cytotoxic T-cells are important effector cells in the combat of viral infections as they can destroy infected cells which presents viral antigens on their MHC class I molecules. T-cells can also produce cytokines, signal molecules that activates the innate immune response to kill the invading pathogen (Secombes & Wang, 2012).
An essential part of the adaptive immune system the immunological memory which is maintained by a subset of B- and T-cells called memory cells. These cells can remain dormant for a period and will respond effectively to a subsequent infection. This drastically reduces the response time of the adaptive system (Fig. 1.3) and is the mechanism that makes vaccination possible.

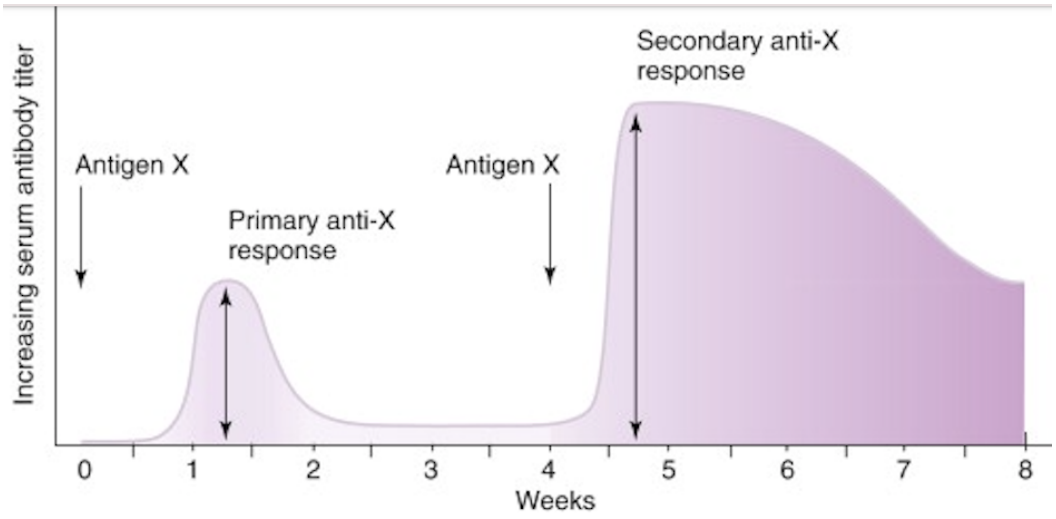

Fig. 1.2 Antibody response during first and second exposure of antigen, adapted from Vendrell, 2005.

1.9.3 Antibodies

The structure of immunoglobulin (Ig) consists of four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains, structured in a way that looks like the letter Y. Both L- and H-chains has one N-terminal variable domains (VH or VL), and at least one C-terminal constant domain that form a constant region (CH or CL). The variable domains are responsible for antigen recognition, while the constant domain mediate effector functions of the antibody molecule. The effector functions include opsonization of pathogens, neutralization of viruses and toxins, and activation of the complement cascade (Mashoof & Criscitiello, 2016).

The variable region, the paratope, which binds to the epitope of antigens is formed by variable domains from VH and VL, one variable region in each chain. Likewise, the constant regions consist of constant domains of both CH and CL (Mashoof & Criscitiello, 2016).

Within each VL and VH there are three hypervariable regions called complementarity-determining regions (CDR). Combined, these 6 CDR segments form the antigen binding site. The different combination of CDR in VL and CDR in VH determines the specificity of the antibody (Mashoof & Criscitiello, 2016).

Inter-chain disulfide bonds between cysteines keeps the IgH and IgL chains together, in addition to the two IgH which are not bound to IgL. Combined with intra-domain disulfide bonds in each domain these bonds give the assembled polypeptide chains the characteristic "Y"-shaped quaternary structure. Here, the amino terminal fragment antigen-binding (Fab) and fragment crystallizable (Fc) stabilize using a flexible hinge region between the first and second CH-domains. It is the flexibility of the hinge region which allows Fab fragments to move independently from each other. allowing the binding of two identical epitopes on multivalent pathogens (Mashoof & Criscitiello, 2016).

Interestingly, it has been shown that some Igs can bind to different antigens, in a process known as cross-reaction (Di Pauli, 1975). The hosts immune response against a pathogen is essentially targeting a small number of epitopes (Yan et al., 2017), which in turn consists of either a linear contiguous sequence of amino acids (linear epitope) or a group of sequentially separated amino acids in a protein sequence brought together by protein folding (conformational epitope) (Negi & Braun, 2017). Cross-reactivity occurs when the epitopes which stimulated the immune response are shared between e.g., bacterial strains, so that B cells stimulated by one strain can protect against another (Yan et al., 2017). Thus, making it possible to immunize fish without using a virulent strain, but rather a closely related one. Unlike linear epitopes the conformational epitopes provides a correct scaffold for an antigen which is important for Ag-Ab interaction, and it is believed that over 90% of clinically important epitopes recognized by antibodies are conformational (Negi & Braun, 2017).

In teleosts, three different Ig classes has been detected: IgM, IgD and IgM. IgM is the only one which is functionally conserved in all jawed vertebrates. IgM, shown in Fig. 1.2, is the most common Ig found in the bloodstream and is expressed as a tetramer when secreted and as a monomer when bound to a membrane. IgM contributes to both innate and adaptive immunity. Its functions include complement activation and facilitating phagocytosis by mediating agglutination (Mashoof & Criscitiello, 2016).

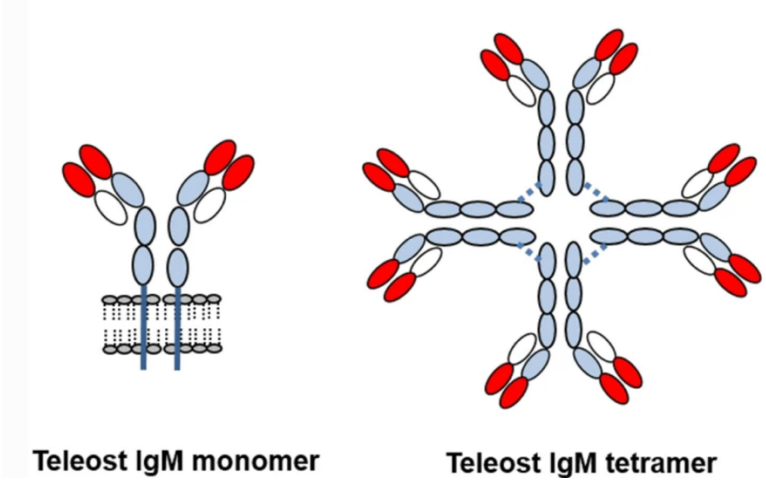

Fig. 1.3 retrieved from Bilal et al., 2021, shows a schematic presentation of membrane bound and secreted IgM in teleosts. Each circle represents a domain; variable domains (red), whereas heavy chain (blue) and light chain (white) are both constant domains (Bilal et al., 2021).

1.10 Aim of the study

The aim of this study was to investigate weather colonization of avirulent strains of *Y. ruckeri* in Atlantic salmon can provide protection after subsequent challenge with a virulent strain. This theory was tested in an experimental challenge experiment and by serological analysis using immune sera harvested from immunized salmon. The challenge trial and the immunization trial were applied for to the Norwegian Food Safety Authorities and designated the approval identification Id: 18792.

Specifically, the aims were:

- To investigate the difference in virulence between virulent and non- virulent strains of *Y. ruckeri*.
- To investigate if previous exposure to non-virulent strains will provide protection upon exposure to virulent strain.
- To formulate vaccines and vaccinate salmon using bacterins from different strains of *Y. ruckeri*.
- To harvest immune sera after vaccination and investigate specific antibody response and cross reactivity of antibodies.

2. Material and methods

2.1 Bacterial cultivation

Four *Y. ruckeri* strains, 10705, 11076, 11587 and 11065, were cultivated for the purpose of this experiment. After being harvested from late exponential growth phase they were grown in 50 ml Bacto™ Brain Heart Infusion (BHIB). They were incubated at 15°C with constant shaking at 140 RPM for 20 hours. The bacterial cultures were then centrifugated at 2500 x g for 10 minutes, followed by the removal of the supernatant and resuspension in RNase/DNase-free distilled water and frozen at -8°C over night.
The frozen bacterial samples were then freeze-dried for 24 hours and stored in weighted tubes.

2.2 Glycerol Stock

Glycerol stocks were established for long time storing. 2 ml ≥ 99% glycerol (Sigma-Aldrich) was added to 8 ml bacterial culture harvested in late exponential growth phase and mixed until the solution was uniform, and no layers could be observed. The glycerol stock was then transferred to cryotubes, each containing 1 ml and stored at -80°C.

2.3 Growth curves

A growth curve was compiled for each isolate to determine their lag-, exponential-, stationary-, and death phase.
1 ml of each *Y. ruckeri* strand were added to 50 mL BHIB in an Erlenmeyer bulb and incubated for eight hours at 15°C and 140 RPM.
1 mL from each pre-culture were then added to an Erlenmeyer bulb containing 100 mL BHIB, becoming the main culture.
Growth curves were then established by measuring OD(A_{600nm}) using a spectrophotometer (Merck Spectroquant® Pharo 300) at one-to-two-hour intervals until the exponential growth phase subsided. The results were then plotted against time using Excel.

2.4 Challenge material

The four *Y. ruckeri* strands were cultivated in BHIB for 24 hours, at 15 °C and continuous shaking at 140 RPM. The bacterial cultures were then centrifugated at 2500 x g for 10 minutes, followed by decantation of the supernatant. The remaining pellets were resuspended in Phosphate buffer saline (PBS, kat.nr. 0000715208). Using CASY cellcounter (CASY

Model TT) the cell concentration was measured for each tube containing the isolates. The bacterial solutions were then diluted in PBS to the wanted concentration of $3x10^7$ cells/ml. To ensure the measurements by CASY was correct, tenfold dilution series was made from each of the isolates, where 100 µl of dilution nr. 7 was smeared onto agar plates, followed by counting of the colonies and calculation of colony forming units/ml (cfu/ml).

2.5 Fish stock and rearing conditions

Atlantic salmon parr (from now on referred to as salmon) were produced and maintained in ILABs facility in Bergen (Vestland, Norway). At the start of the infection trial the average size were 25,36 g ±2,03 g SD (n= 50) and 12,88 cm ±0,37 cm (total length). The fish were kept in six separate tanks of 150 L, each containing 30 salmon, as shown in Figure 2.1. During the trial the fish were automatically fed according to their appetite using an automatic feeder (BioMar). During the challenge experiment the salmon was reared in freshwater tanks with the conditions listed in Table 2.1.

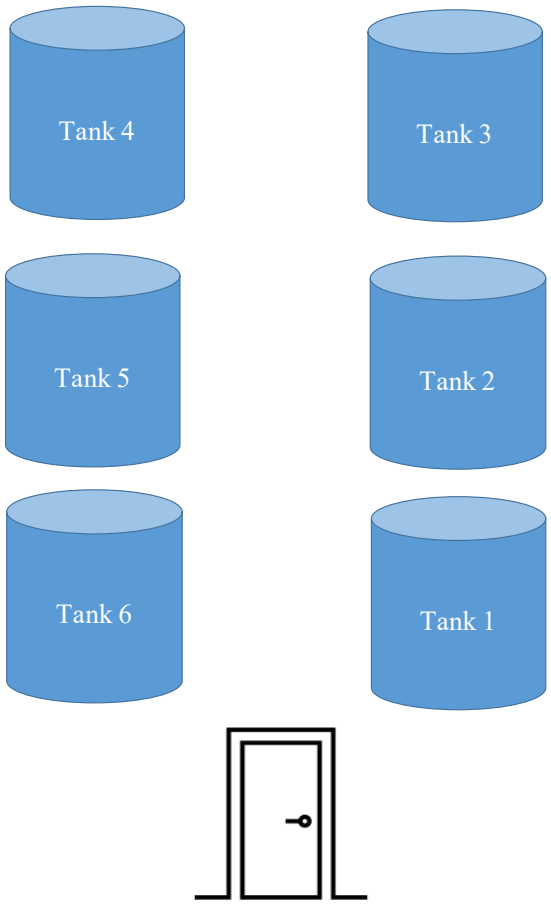

Figure 2.1 Tank setup

Table 2.1 Rearing conditions during the challenge experiment

Parameter	Value
Light/dark	12h/12h
Temperature	14°C
Oxygen saturation	>70%

2.6 Challenge experiment

Using a 6 mm needle, 10 fish in each tank were i.p. injected with 100 µl ($3x10^6$ cfu) bacterial suspension and acted as shedders from that point, making a ratio of 1:2 of shedders and cohabitants. The fish were starved 48 hours prior to infection. Shedders in tank 1-4 were injected with different *Y. ruckeri* strands, while shedders in tank 5 and 6 acted as control and were injected with PBS. The isolate-type in each tank is listed in Table 2.2, along with conditions, average fish length and weight. For identification the shedder fish were marked with Visible Implant Elastomer (VIE) silicone implants (Northwest Marine Technology, Inc.) on the forehead. Each of the eight tanks contained 10 shedder fish and 20 naïve cohabitant fish.

Six weeks later surviving shedders were removed and 10 new shedders were supplied to each tank. The 1:2 ratio was maintained by adding 10 new shedders in each tank, except for tank 1 where three new shedders were added caused by some mortality of the cohabitants. For each tank, except control tank 6, the new shedders were i.p. injected with the virulent isolate NVI 10705. The shedders supplied to control tank 6 were injected with sterile PBS.
The new shedder fish were also marked with VIE for later identification. From dead and moribund fish throughout the challenge experiment, samples for re-isolation of Y. ruckeri were aseptically collected from the head kidney using an inoculation loop for growth on blood agar (BA).

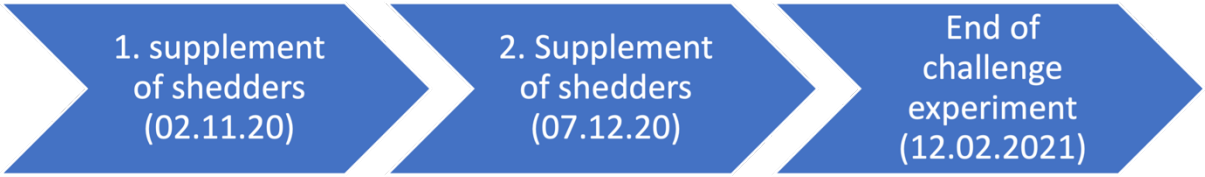

Figure 2.2 Timeline of challenge experiment

Table 2.2 Overview of which *Y. ruckeri* isolate or control shedders in each tank were injected with during first and second infection.

Tank	Date	NVI	Date	NVI
1	02.11.2020	10705	07.12.2020	10705
2	02.11.2020	11076	07.12.2020	10705
3	02.11.2020	11587	07.12.2020	10705
4	02.11.2020	11065	07.12.2020	10705
5	02.11.2020	PBS	07.12.2020	10705
6	02.11.2020	PBS	07.12.2020	PBS

2.7 Vaccine production

Vaccines were made against formalin inactivated whole bacteria (WB) from strains 10705, 11076, 11587 and 11065. A new batch of bacterial medium of each isolate were cultivated, and 150 ml of each were placed in four weighed tubes. The precise weight of the medium solutions was then used to calculate the amount of formalin needed to inactivate the bacteria. After adding formalin, the mediums were incubated at 12°C for 15 minutes. The suspensions were then transferred to new tubes and continued incubating for a total amount of 90 minutes. To verify formalin inactivation of bacterial cells 100 µl from each suspension were spread on blood agar plates and left for incubation at 15°C for three days.

On the day of immunization 50 ml of the inactivated bacteria were transferred to a 50 ml tube and centrifuged for 10 minutes, at 4°C and 4000 x g. Following decantation of the supernatant the pellets were resuspended in 50 ml of PBS. In the first immunization trial the bacterial suspension was mixed in a 1:1 relation with Freund´s incomplete adjuvant (Sigma), and then shaken for 10 seconds at 4 m/s until the inactivated bacterial culture was homogenized with the adjuvant.

Table 2.3 Overview of vaccination trial

Date	Injection volume (µl)	Vaccine type
16.03.2021	100	Freund´s incomplete adjuvant
13.03.2021	100	Without adjuvant
27.04.2021	100	1:1 dilution with PBS

2.8 Vaccination

Vaccination was performed by i.p. injection 100 µl vaccine suspension, using a 0,5 mm diameter needle. Each fish group were vaccinated three times (Table 2.3). The first vaccination trial contained inactivated bacteria emulsified in a mineral adjuvant (Freuds incomplete), whereas the second trial four weeks after the initial vaccination and the third trial two weeks after the second vaccination were carried out without adjuvants.
The fish group (n=46) were starved for 24 hours prior immunization to prevent injection in distended stomachs or intestines. Fish were then transferred to an oxygenated tank, while small portions were moved to a bucket (10L) with sedation (Finquel). For identification purposes the fish were marked with VIE on the forehead, each colour representing a bacterial strain. The control group was injected with PBS. Using a sterile needle each fish was immunized with 100 µl by i.p. injection.

2.9 Sera

Sera was collected 500 d°C after the third vaccination. After euthanizing the fish by an overdose of Finquel, blood was sampled from *Vena caudalis* and quickly placed in blood collection tubes without additives (BD Vacutainer). Blood samples were stored over night at 4°C to allow coagulation, followed by centrifugation at 1300 x g for 10 minutes at 4°C. The serum fractions were then extracted and stored at -20°C in aliquots.

2.10 Antigen stock preparation

The antigen stock solutions were prepared by making a 10 mg/ml solution with freeze dried bacteria and PBS supplied with 5 mM EDTA. The weight of the freeze-dried bacteria was found by subtracting the weight of the tubes, which then determined the volume of PBS w/ EDTA needed for a 10 mg/ml concentration for each isolate as listed in Table 2.4 After sonicating the bacterial solutions for 2 minutes at 20 kHz, they were diluted to 1 mg/ml in PBS w/EDTA and placed in aliquots and frozen at -20°C.

Table 2.4 List of freeze-dried *Y. ruckeri* isolates, their weight and volume of PBS w/EDTA needed for a 10 mg/ml concentration.

NVI	Weight (mg)	PBS w/EDTA (mL)	Final concentration
11076	110	11	10 mg/ml
10705	130	13	10 mg/ml
11587	100	10	10 mg/ml
11605	120	12	10 mg/ml

2.11 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was used to test the effect of the immunization by measuring levels of specific antibodies in fish sera sampled after the vaccination. In addition, ELISA was used to investigate potential cross-reactivity between specific antibodies targeting the bacterial isolates.

The bacterial stock solutions (1 mg/ml) were diluted to 10 µg/ml in PBS and coated the 98 well immunoplate (Nunc MaxiSorp^{TM}) by adding 150 µl of the bacterial suspension in each well. The coated immunoplates were then covered by an empty plate, wrapped in plastic, and incubated over night at 4°C.

After washing the plates three times with 200 µl PBS-Tween (PBS-T) per well using a microplate washer (BioTek 405 LS), empty sites on plates were blocked by adding 200 µl of blocking solution (3% skimmed milk powder in PBS-T) followed by an hour incubation at room temperature. The washing, covering, and wrapping procedure were repeated for each step.

The salmon antisera to *Y. ruckeri* and the control sera were diluted 1:100 in PBS-T. Further a twofold dilution series of 10 dilutions was made for each serum, which was then added to the wells in duplicates before incubation over night at 15°C.

After washing the plates 50 µl rabbit-salmon IgM were diluted 1:2000 and added to each well. The plates were incubated for 2 hours at room temperature and washed. 50 µl of goat-rabbit Ig conjugated with horseradish peroxidase (HRP) (Dako) diluted 1:2000 was then added per well and incubated for 1 hour at room temperature. After a final washing, a peroxidase substrate solution (o-Phenyleneidamine, OPD) was prepared by adding 15 mg of o-Phenyleneidamine (P-4664, Sigma) to 100 ml phosphate-citrate buffer (pH 5,0), followed by adding 15 µl of hydrogen peroxide (H_2O_2) (Sigma Aldrich), just before 50 µl of the peroxidase solution was added to each well. The substrate reaction was stopped after six minutes by adding 50 µl of 2.5 N H_2SO_4 to every well. Using a spectrophotometric microplate reader (Tecan SpectraFluor) the absorbance was measured at 492 nm, and the OD readings was used to compare the results between different sera.

3. Results

3.1 Growth curves

As presented in Figure 3.1 the growth curves compiled clearly demonstrate the bacteria's lag, log- and early stationary phase. All four isolates follow a similar growth pattern where the exponential growth phase is reached after approximately 7 hours of incubation. Throughout isolate NVI 10587 (red line) has the highest measured OD and reaches its stationary phase after 20 hours, followed by NVI 11065 (yellow line) which also reaches its stationary phase after 20 hours. NVI 11076 (green line) and 10705 (orange line) has similar OD-values throughout the experiment and reaches their stationary phase after 20 hours.

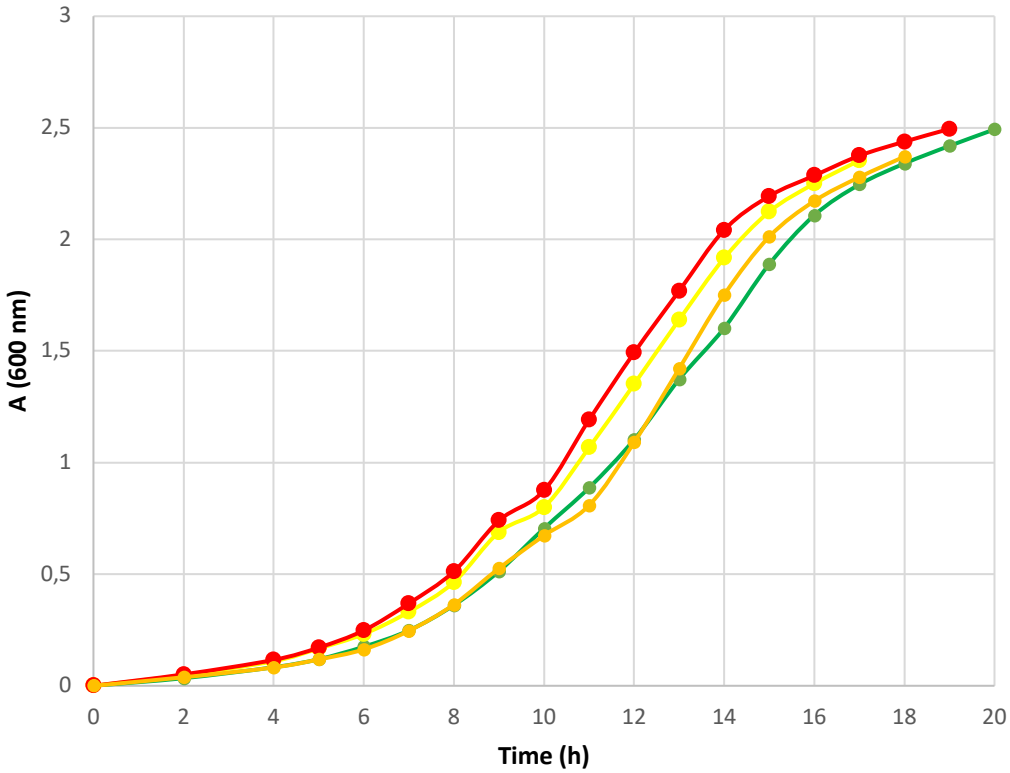

Figure 3.1 Growth curves of *Y. ruckeri* NVI 11076 (green), 11065 (yellow), 10587 (red) 10705 (orange) grown in BHIB. Time (h) represents hours of incubation; OD was measured at a wavelength of 600 nm.

3.2 Mortality after experimental challenge

Mortality during the challenge experiment was recorded daily and plotted as cumulative percent mortality against time post challenge (Figure 3.2). Mortalities were observed in shedders in all four tanks for the first shedder group (VIE#1) receiving *Y. ruckeri*, and in the shedders supplied as the second shedder group (VIE#2) (five tanks). Mortalities were also observed in respective cohabitants in all challenge tanks. Throughout the experiment there were no mortality in tank 6 where both shedder groups where injected with sterile PBS (figure not shown).

As illustrated in Figure 3.2 the mortalities in the first group of shedders reached 100% shortly after i.p. injection (Figure 3.2 A-D). The cohabitants exposed to shedder fish i.p. injected with the virulent strain NVI 10705 (Figure 3.2 A) reached a mortality rate of 70% at 17 days post challenge (dpc), whereas cohabitant groups exposed to shedders i.p. injected with non-virulent strains had zero mortality (Figure 3.2 B-D). The second group of shedders (VIE#2), supplied to the tanks 37 days after the first group (VIE#1), were i.p. injected with isolate NVI 10705 for all tanks except for one of the control tanks. Mortalities after introduction of the second group of shedders were high and acute in all the i.p. injected shedder fish (VIE#2). Cohabitants in tank 1-5 were now exposed to virulent strain NVI 10705 though the introduced shedders (VIE#2). The cohabitant fish group in tank 1 (Fig 3.2 A), which already had been exposed to this isolate showed no increase in mortality. Cohabitant fish in tank 2 had a notable mortality rate of 35%, whereas fish groups in tanks 3-5 had a somewhat lower mortality rate at 5% (tank 3), 15% (tank 4) and 10% (tank 5). Infection was confirmed in all dead and moribund fish by re-isolation of *Y. ruckeri* from head kidney samples on BA.

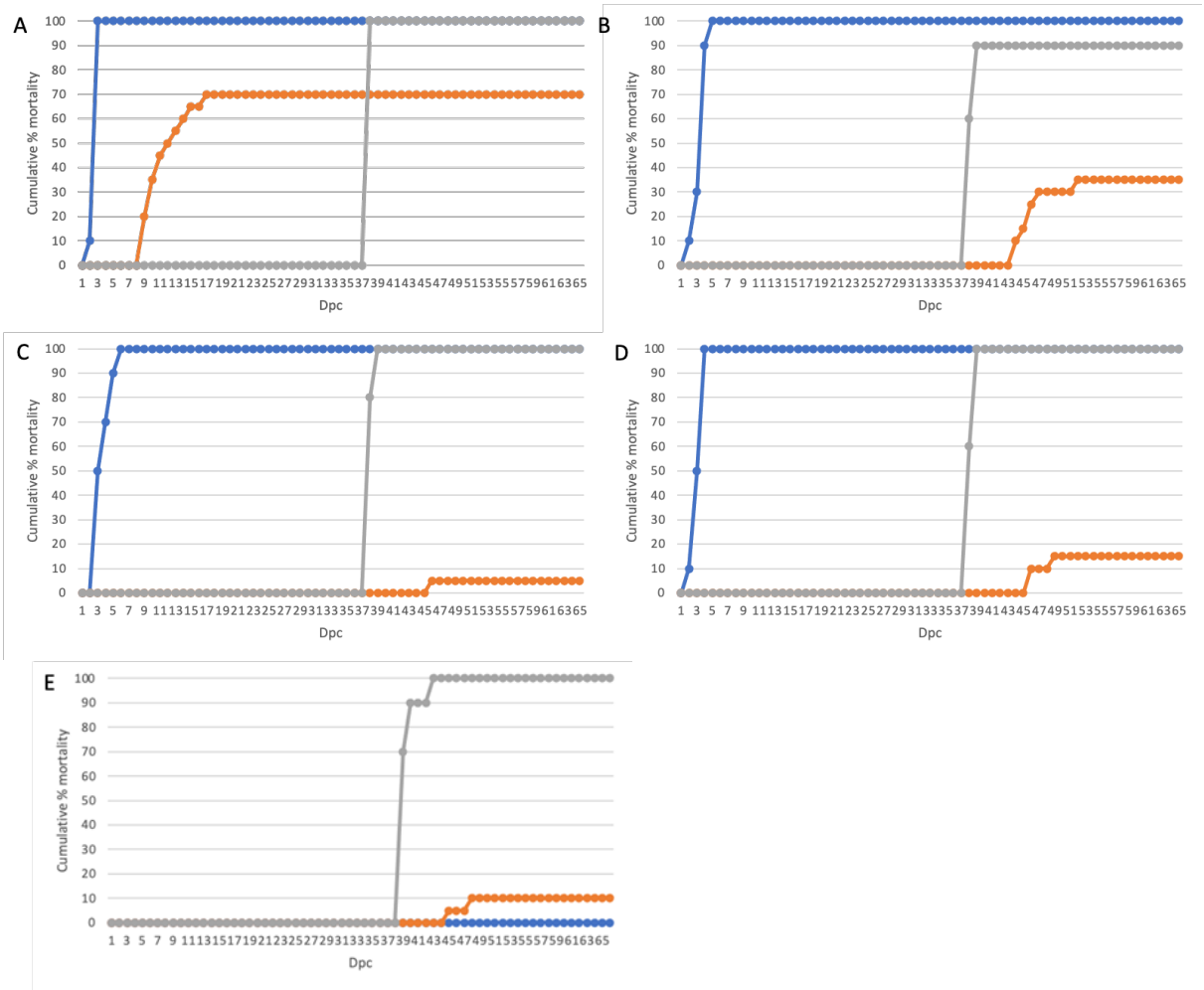

Figure 3.2 Mortality rates (cumulative percent mortality) over time in tanks 1-5 during the challenge trial. A) Tank 1; B), Tank 2; C) Tank 3; D) Tank 4; E) Tank 5. Blue line (first supplement of shedders), grey line (second supplement of shedders), orange line (cohabitants).

Using statistic software (R core team 2020) the statistical differences in cumulative mortality in percentage (%) between the different treatment groups were calculated and compared at day 20 dpc (#1) and day 20 dpc (#2). At 20 dpc (#1), as shown in Table 3.1, there were significant differences in mortality between i.p. injected salmon and cohabitants, and i.p. injected salmon and non-infected controls in tank 2-4, whereas no significant difference was found between cohabitants and non-infected controls. In tank 1 there were significant differences between i.p. injected salmon and non-infected controls, and cohabitants and non-infected control, but no significant difference between i.p. injected and cohabitants.

At 20 dpc (#2) there were significant differences between i.p. injected salmon and cohabitants, and i.p. injected salmon and non-infected controls in all tanks except tank 2, where all three treatment groups showed significant differences in cumulative mortality.

Table 3.1: Statistical analyses from the challenge experiments. Statistical differences in cumulative mortality between the different treatment groups (ip= i.p. injected, co= cohabitants, ctr= non-infected controls) on day 20 post supplement of first groups of shedders (#1) and on day 20 post second supplement shedders (#2). C.m. is cumulative percent mortality in the treatment groups assigned * or **. X^2 is Yates´ continuity corrections in Pearson´s Chi-Squared test. In R differences are significant at significance level $\alpha<0,05$. All significant values are in bold. NA= Not available. NaN= Not a number.

		20 dpc #1				20 dpc #2			
Tank	Group	C.m.*	C.m.**	X^2	p-value	C.m.*	C.m.**	X^2	p-value
1	*Ip vs. **co	100	70	2.2094	0.1464	100	0	10.815	**0.001007**
	*Ip vs **ctr	100	0	34.844	**3.57E-09**	100	0	22.009	**2.71E-06**
	*Co vs **ctr	70	0	25.798	**3.79E-07**	0	0	NaN	NA
2	*Ip vs. **co	100	0	25.669	**4.05E-07**	90	35	6.0435	**1.40E-02**
	*Ip vs **ctr	100	0	34.844	**3.57E-09**	90	0	29.869	**4.62E-08**
	*Co vs **ctr	0	0	NaN	NA	35	0	9.4754	**0.002083**
3	*Ip vs. **co	100	0	25.669	**4.05E-07**	100	5	18.906	**1.37E-05**
	*Ip vs **ctr	100	0	34.844	**3.57E-09**	100	0	34.844	**3.57E-09**
	*Co vs **ctr	0	0	NaN	NA	5	0	0.042517	0.8366
4	*Ip vs. **co	100	0	25.669	**4.05E-07**	100	15	18.906	**1.37E-05**
	*Ip vs **ctr	100	0	34.844	**3.57E-09**	100	0	34.844	**3.57E-09**
	*Co vs **ctr	0	0	NaN	NA	15	0	2.497	0.1141
5	*Ip vs. **co	0	0	NaN	NA	100	10	18.906	**1.37E-05**
	*Ip vs **ctr	0	0	NaN	NA	100	0	34.844	**3.57E-09**
	*Co vs **ctr	0	0	NaN	NA	10	0	1.0634	0.3024

3.3 Bacteriology

Samples collected for PCR and histopathology (not included in this thesis) and smears from head kidney (HK) on blood agar (BA) (1.5% NaCl) confirmed infections by *Y. ruckeri* in all fish that died throughout the experiment. Samples were also collected from all surviving fish at termination of the experiment in challenge tanks and from 15 non-infected control fish, all negative for bacterial growth on BA.

3.4 Vaccination

There were no mortalities after vaccination. Smears of vaccine antigens on BA were all positive for bacterial growth prior to inactivation, and negative after, which confirmed successful inactivation of the vaccine antigens.

3.5 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was used to determine the presence of antigen specific antibodies in sera harvested post immunization. The results are shown as titration curves of sera harvested from immunized fish (n=10/group) compared to sera from non-immunised fish (n=10) (Figure 3.3). When compared to control sera from non-immunized fish, each individual fish and immunization group shows a clear antibody response. The response varies somewhat between the different groups, and within fish, the highest response was measured in antisera from fish immunized against isolate NVI 10705. The response within each group also varied, where some individuals where high responders and some were low responders.

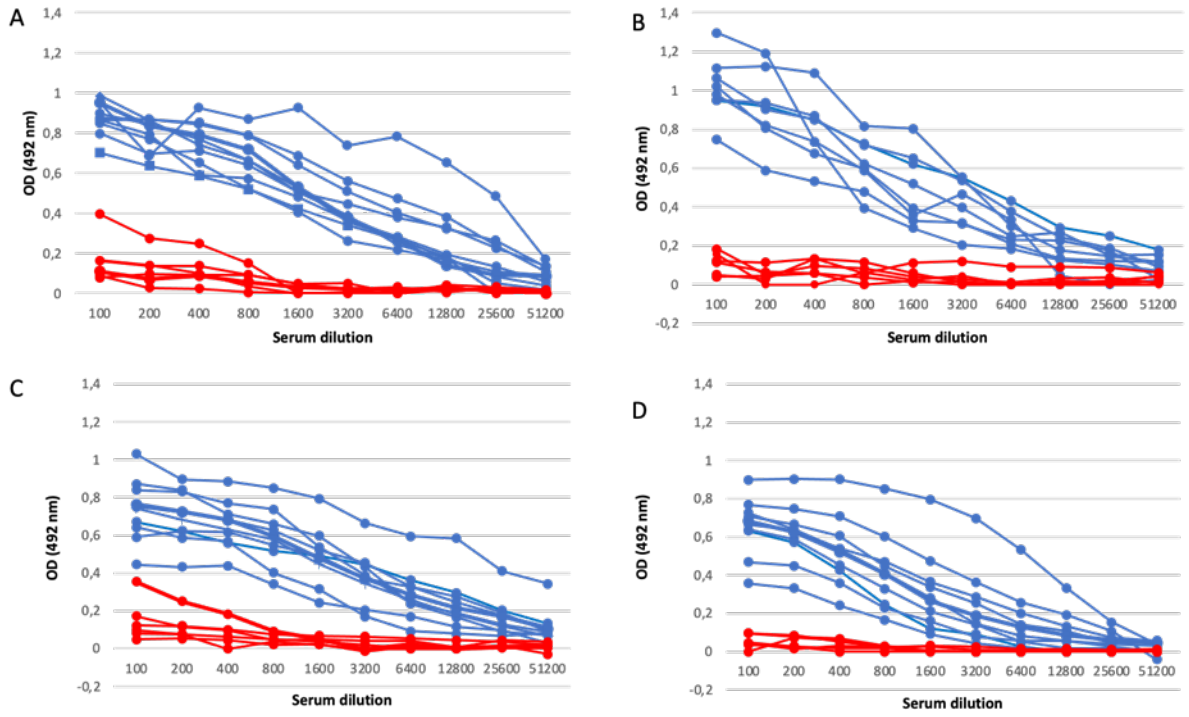

Figure 3.3: Sera antibody-dilution curves for the immunized fish groups measured using ELISA. A) Antisera: NVI 11076; B) Antisera: NVI 10705; C) Antisera: 10587; D) Antisera: NVI 11065. Blue lines: vaccinated fish, red lines: non-vaccinated control fish.

3.6 Cross-reactivity tests

Cross-reactivity of Yersinia-specific antibodies in sera from vaccinated and non-vaccinated fish was measured using ELISA. Antisera collected from all vaccine groups cross-reacted to the isolates not included in the vaccine. All antisera irrespective of coating antigen provided significantly higher OD values when compared to sera from non-vaccinated control fish (Figure 3.4).

When measured against NVI 10705 as coating antigen (Figure 3.4 A), the anti-NVI 10705 sera had a significantly higher absorbance compared to the sera from fish vaccinated with environmental non-virulent isolates ($p \leq 0.001$). There was no significant difference in measured absorbance between sera collected from fish vaccinated using the environmental non-virulent isolates towards NVI 10705 as coating antigen (Fig. 3.4 A).

When measured against NVI 11076 as coating antigen (Figure 3.4 B) the anti-NVI 11076 sera had a significantly higher measured absorbance compared to NVI 10705 ($p \leq 0.001$) and 11065 ($p \leq 0.001$), and also NVI 11587 ($p \leq 0.01$).

When measured against NVI 11587 as coating antigen (Figure 3.4 C) the anti-NVI 11587 sera had a significantly higher absorbance measured compared to NVI 10705 ($p \leq 0.01$) and also compared to 11065 ($p \leq 0.01$), while no significant difference was detected when compared to anti-NVI 11076 sera.

When measured against NVI 11065 as coating antigen (Figure 3.4 D) the anti NVI 11065 sera had a significantly higher absorbance measured compared to anti-NVI 11587 ($p \leq 0.05$), while no significant difference was detected when compared to anti-NVI 10705 and anti-11587 sera. Results from statistical analysis is summarized in Table 1 in appendix.

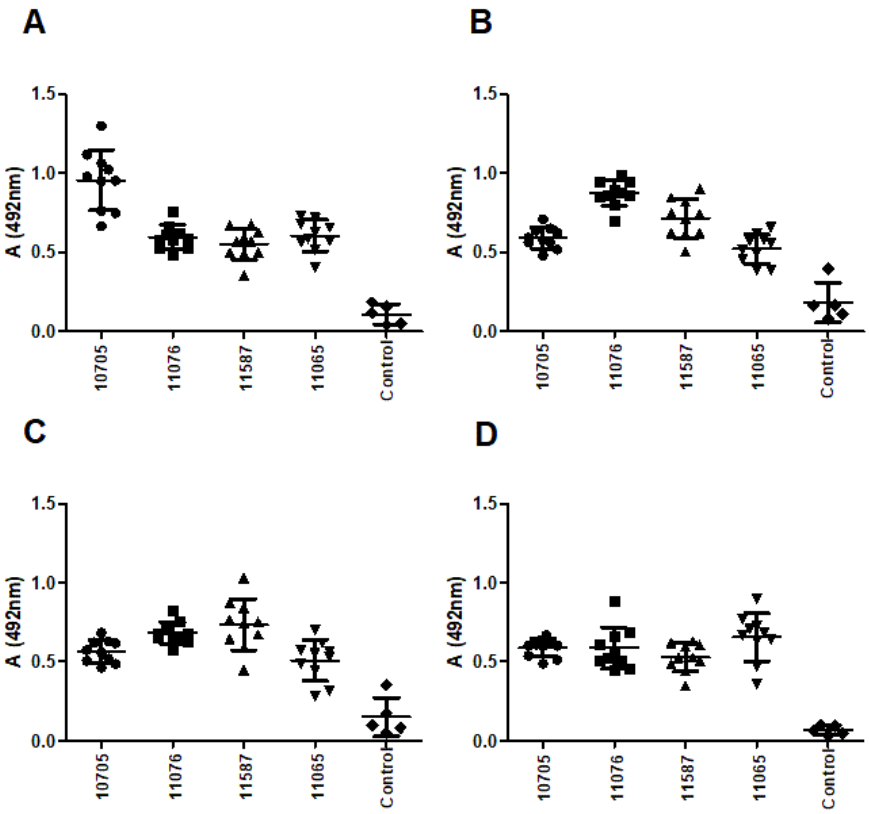

Figure 3.4: Cross-reactive antibodies in antisera measured with ELISA. A) Antisera raised against NVI 10705; B) Antisera raised against NVI 11076; C) Antisera raised against NVI 10587; D) Antisera raised against NVI 11065.

3.7 Statistical analysis of cross-reactivity test

Two-tailed unpaired t-tests were performed for analysis of the ELISA results, using GraphPad Prism 5. The results were considered significant when $p<0.05$. In Prism 5, $p>0.05$ = n.s., *$p<0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4. Discussion

This study was carried out to validate the difference in virulence between virulent and putatively non-virulent strains of *Y. ruckeri*, and importantly to investigate weather previous exposure to non-virulent strains will provide protection upon exposure to a virulent strain. To pursue this a challenge experiment was conducted. To further investigate cross reactivity and possible cross protection, vaccines were formulated and used for vaccination of Atlantic salmon to produce immune sera. The immune sera were investigated for specific antibody response and cross reactivity of antibodies. The experimental challenge indicates that NVI 10705 is the only virulent isolate tested, and that exposure to non-virulent strain provides some protection against exposure to a virulent strain. The serological tests demonstrate a clear specific antibody response for all isolates, including cross-reactivity between them.

4.1 Mortality after experimental challenge

The mortality rates during the experimental challenge turned out as expected for the most part. The shedders quickly reached a high cumulative percent mortality due to the invasive i.p. injection. Mortalities in i.p. injected shedders were first registered at 2 and 3 dpc and reached 100% at 3-6 dpc. This is similar to results from previous studies on experimental challenge using *Y. ruckeri* where i.p. injected or bath immersed rainbow trout showed mortality 3 dpc (Monte et al., 2016). This implies that within that timeframe *Y. ruckeri* establishes within its hosts and cause lethal disease. In the current study mortalities in i.p. infected fish were obtained in all groups including the groups injected with the putative non-virulent strains, most likely caused by the invasive i.p. injection which bypasses the hosts first line of defense e.g., the skin and mucosal layer. The lack of mortality of the cohabitants in these groups indicates that the bacteria is not spread through the water from the shedders, whereas the high mortality of cohabitants in tank 1 indicates bacterial shedding from fish injected with the virulent strain NVI 10705.

Cohabitation is well suited as a delivery route for pathogens as it is similar to the transmission route in fish farms, compared to the more in invasive i.p. injection (Monte et al., 2016). Because of this previous studies of vaccine efficacy (Monte et al., 2016) and virulence comparison (Haig et al., 2011) on *Y. ruckeri* have also used cohabitation as an infection model.

The findings of the present challenge experiment aligns with previous studies that have demonstrated that *Y. ruckeri* spread between infected or carrier fish (Tobback et al., 2007), in the case of the present study through i.p. injected shedder fish, likely by shedding through feces (Monte et al., 2016).

A recent study by Gulla et al (2018) places NVI 10705 in serotype O1 clonal complex 1 (CC 1) using Multilocus Variable-Number Tandem-Repeat Analysis (MLVA) and shows that this complex dominates the disease outbreaks in Norwegian salmon aquaculture (Gulla et al., 2018). The three putatively non-virulent isolates included in this challenge experiment were collected from freshwater farms without disease outbreaks and therefore presumed avirulent or less-virulent. Using MLVA, Gulla et al (2018) categorizes these strains in serotype O1, but in different clonal complexes not associated with virulence; NVI 11076 in CC 7, NVI 10587 in CC 8, while NVI 11065 is categorized as a minor clonal complex (Gulla et al., 2018). In the current experiment, during the first exposure to shedders, mortality of cohabitants could only be observed in tank 1, which supports the notion that the only virulent strain used in the challenge was NVI 10705.

Following the second addition of i.p. injected shedders no mortality was observed in cohabitants in tank 1, indicating immunity. This was the expected outcome as the survival cohabitants were exposed to the same isolate once more (NVI 10705). Fish in tank 2-4 (previously exposed to non-virulent *Y. ruckeri*), now exposed to the virulent strain, all showed an increase in mortality. Interestingly, the mortality rates of fish in tanks 2-4 differs from that of fish in tank 1 during the first introduction to the shedders; the highest mortality was observed in tank 2 with 35% mortality, significantly lower than the 70% mortality rate observed in tank 1. These results might suggest that exposure to non-virulent isolates provides some cross-protection against a virulent isolate. According to this experiment the most cross-protective isolate seems to be NVI 10587 (tank 3) followed by NVI 11065 (tank 4), and NVI 11076 (tank 2).
During the challenge experiment *Y. ruckeri* was re-isolated on agar plates from all dead fish, confirming the bacteria as the cause of mortality.

It is worth noting that the mortality rates in tank 5 were unexpectedly low. The cohabitants in tank 5 were not exposed to any *Y. ruckeri* strains during the first introduction of shedders, when only fish injected with sterile PBS were supplied to the tanks, and one could therefore expect a similar mortality rate as observed in tank 1 during the first challenge. The reason for the

mortality rate of only 10% is not clear. Prior to supplement of group two of i.p. injected shedders, all previously i.p. injected fish were removed from the tanks and euthanized. The number of supplied new shedders to the tanks were adjusted for each tank to make sure that the ratio between i.p. injected fish and cohabitants was the same for all tanks. This resulted in the same number of fish in all tanks, except for in tank 1 which held fewer fish due to high mortality in cohabitants after exposure to the first group of shedder fish. This implies that challenge pressure and ratio between i.p. injected fish and cohabitants have not influenced on the low mortality rate observed in tank 5. Throughout the experiment, no mortality was observed in tank 6, holding non-challenged fish.

Y. ruckeri serotype 1 are antigenically homogeneous and cross-protection between isolates has been shown in existing literature (Cipriano & Ruppenthal, 1987). The results of the current challenge experiment is supported by the findings of McCarthy and Johnson (1982) which indicated that rainbow trout could be protected from infection with various strains of Serotype 1, using a cross-protection tests (McCarthy & Johnson, 1982).

4.2 Antibody response

The Atlantic salmon were immunized three times, following an established protocol (Rønneseth et al 2015). No mortality, abnormal behavior or other negative influences on the fish were observed as a result of the vaccination procedure. The sera collected 14 days post the third immunization were analyzed using ELISA to measure the level of specific antibodies produced. The salmon were immunized three times to maximize the level of specific antibodies produced. The first immunization initiates the primary immune response where memory B-cells will be produced (Stosik et al., 2021). The second and third immunization served as booster doses and initiated the secondary immune responses, which is faster in response and produces a higher concentration of antibodies with an increased affinity to the antigens (Stosik et al., 2021). Specific antigen producing B cells (plasma cells) plays a similar part as the mammalian long-lived plasma cells, they are responsible for maintain a protective level of antibodies in the blood (Stosik et al., 2021). In 2015 an experiment indicated that a booster vaccination may increase the survival and the protection period against ERM in rainbow trout, and that fish receiving the booster dose showed superior protection compared to fish receiving only one dose (Chettri et al., 2015). In case of the current study, the immune sera produced will serve as a laboratory

reagent and the high level of antibodies produced is important to ensure measurable results in following analysis.

The results of the ELISA show a clear antibody response in each vaccine group, compared to the non-immunized control group. This show that the vaccines have stimulated the immune response of the fish, and that B-cells of the adaptive branch of the immune system has developed into antibody producing plasma cells. This is consistent with existing literature describing inactivated *Y. ruckeri* vaccines as giving good or adequate protection without any major side effects (Magnadottir, 2010). A recent study showed that in addition to initiating B-cell development i.p. vaccination of rainbow trout also induces expression of pro- and anti-inflammatory cytokines and adaptive cytokines in the spleen. A heightened expression of acute phase proteins (APPs) and antimicrobial peptides (AMPs) was also detected in both spleen and gills, presumably caused by the induced cytokines (Wangkahart et al., 2019).

The highest specific antibody levels post vaccination of salmon in the current study is seen in sera raised against NVI 10705, followed by NVI 11076, NVI 10587, and NVI 11065. Individual differences in levels of antibodies produced between fish is detected, this is as expected for Atlantic salmon. Studies has shown that for fish in general, the activity of different immune parameters, like antibody production, can vary greatly between individuals of the same species (Magnadottir, 2010).

4.3 Cross-reactivity

The ELISA performed to investigate cross-reactivity of antibodies across the *Y. ruckeri* isolates shows that all four antisera collected from the different vaccine groups cross-reacts with the other *Y. ruckeri* isolates, with significantly higher OD-values compared to sera from non-vaccinated control fish. When measured against their “native” coating antigen (isolate) all antisera had significantly higher reactivity compared to the other antisera, except for NVI 11065, where there was no significant difference in OD-value between anti-NVI 11066, anti-10705 and anti-11587. The lack of difference in reactivity between the antisera might indicate that NVI 11065 shares a larger amount of OMPs with the other isolates, thus stimulating production of more efficient cross-reactive antibodies in its hosts. However, the fact that NVI 11065 was also the isolate which measured the least antibody response when used for coating in ELISA could be a more probable explanation.

Perhaps most interesting is the antisera raised against the non-virulent isolates all reacted significantly higher to the virulent isolate than the control sera from non-vaccinated fish. This fits well with the results from the experimental challenge, and further indicates that non-virulent environmental isolates could play a prophylactic role against disease-causing *Y. ruckeri* in the future.

The presence of cross-reactivity between strains indicates a shared range of OMPs or at least OMPs with similar structure, which acts as antigens in the vaccines. Previous studies has suggested that the cross-reactivity of OMPs are widely present in Gram-negative bacteria in general (Xu et al., 2005). In salmonid aquaculture cross-protection by vaccines has been detected in e.g., *A. salmonicida* ssp., the causative agent of furunculosis (Gudmundsdóttir & Gudmundsdóttir, 1997) and *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (Ma et al., 2019).

5. Conclusions and future perspectives

This project aimed to find out if a previous exposure to non-virulent *Y. ruckeri* strains will provide protection upon exposure to a virulent strain. Based on the results from the challenge experiments and ELISA analysis, it can be concluded that antibodies made against non-virulent strain can cross-react to a virulent strain to some degree.

The main conclusions of this work are as follows:

- *Y. ruckeri* strain NVI 10705 seems to be the only virulent isolate out of the isolates tested. It was the strain causing mortality during the experimental challenge, reaching 70 cumulative % at 17 dpc. This concurs with existing literature placing it in CC 1, the clonal complex that is dominating disease outbreaks in Norwegian aquaculture.
- Results from the experimental challenge indicates that prior exposure to non-virulent strains provides some protection upon exposure to a virulent strain. Cohabitants exposed to environmental strains prior to virulent strain were significantly less susceptible for infection and showed far less mortality compared to the fish group without this prior exposure.
- The vaccines formulated with bacterins from the different *Y. ruckeri* strains had the desired effect in all fish groups, as demonstrated with ELISA.
- Using ELISA, the current work has established that *Y. ruckeri* strains from different clonal complexes in serotype O1 cross reacts with each other *in vitro*. Sera from all immunized fish groups produced significantly higher OD-values when compared to non-vaccinated control group.

This work indicates that vaccines against *Y. ruckeri* can potentially be replaced by exposure to non-virulent strains, which in turn could reduce the handling and stress which is involved in an i.p. vaccination. Further research is needed to determine the effectiveness of exposure to environmental isolates and the isolate which is the most suited for the task.

For future work more in-depth research on the OMPs of *Y. ruckeri* may be suggested. Identification of immunogenic proteins using SDS-PAGE and Western blotting may provide more information for the selection of vaccine isolates, especially regarding cross-protective immunization. If or when this is established it could be used to formulate a recombinant protein vaccine, which when administrated would produce a more targeted immune response as it

consists solely of the most immunogenic protein or proteins. Further research on cross protection *in vivo* is necessary to confirm this work. The low mortality in the control group during the experimental challenge is a weakness, and although the ELISA results from the cross-reactivity tests are promising, the presence of antibodies established in the lab does not necessarily correlate with protection against an infection in nature/aquaculture.

6. References

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7. Appendix

7.1 ELISA preparation

7.1.1 PBS, pH 7,3

0,72 g $Na_2HPO_4x2H_2O$
0,27 g KH_2PO_4
8,5 g NaCl
MilliQ water until 1000 ml

7.1.2 PBS-Tween (PBS-T)

50 µl Tween 20 per 100 ml PBS (0,05%)

7.1.3 Blocking solution

3 g dried skimmed milk
100 ml PBS-T

7.1.4 0,1M citric acid

21,0 g citric acid
MilliQ water until 1000 ml

7.1.5 Phosphate citrate buffer, pH 5,0

24,3 ml 0,1 M citric acid
25,7 ml 0,2 M M Na_2HPO_4 x 2 H_2O
50,0 ml MilliQ water

7.1.6 Substrate solution

15 mg o-Phenyleneidamine (P-4664, Sigma)
37,5 ml phosphate-citrate buffer
15 µl 30% H_2O_2

7.1.7 Stop solution

6,94 ml H_2SO_4
93,06 ml MilliQ water

7.1.8 Bacterial dilution 10 mg/ml

10 ml MilliQ water
10 mg freeze-dried, sonicated bacteria
Diluted in 10 ml MilliQ water
(Resulting in a 10 mg/ml concentration, using $C_1V_1=C_2V_2$)

7.2 Statistical analysis

Table 7.1 Statistically analysis of cross-reactivity, showing significance and p-value

Antigen	Serum vs. Serum	sign	p-value
10705	10705 vs. 11076	***	P<0.0001
10705	10705 vs. 11587	***	P<0.0001
10705	10705 vs. 11065	***	P<0.0001
10705	10705 vs. Control	***	0,0007
10705	11076 vs. 11587	ns	0,4813
10705	11076 vs. 11065	ns	0,7959
10705	11076 vs. Control	***	0,0007
10705	11587 vs. 11065	ns	0,2176
10705	11587 vs. Control	***	0,0007
10705	11065 vs. Control	***	0,0007
11076	11076 vs. 10705	***	0,0002
11076	11076 vs. 11587	**	0,0052
11076	11076 vs. 11065	***	P<0.0001
11076	11076 vs. Control	***	P<0.0001
11076	10705 vs. 11587	*	0,0312
11076	10705 vs. 11065	ns	0,1211
11076	10705 vs. Control	**	0,0027
11076	11587 vs. 11065	**	0,0021
11076	11587 vs. Control	***	0,0007
11076	11065 vs. Control	**	0,0027
11587	11587 vs. 10705	**	0,0089
11587	11587 vs. 11076	ns	0,3527
11587	11587 vs. 11065	**	0,0021
11587	11587 vs. Control	***	0,0007
11587	10705 vs. 11076	**	0,0015
11587	10705 vs. 11065	ns	0,3150
11587	10705 vs. Control	***	0,0007
11587	11076 vs. 11065	***	0,0007
11587	11076 vs. Control	***	0,0007
11587	11065 vs. Control	**	0,0027
11065	11065 vs. 10705	ns	0,0524
11065	11065 vs. 11076	ns	0,1655
11065	11065 vs. 11587	*	0,0115
11065	11065 vs. Control	***	0,0007
11065	10705 vs. 11076	ns	0,6305
11065	10705 vs. 11587	ns	0,1230
11065	10705 vs. Control	***	0,0007
11065	11076 vs. 11587	ns	0,4813
11065	11076 vs. Control	***	0,0007
11065	11587 vs. Control	***	0,0007