Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Immunological and histological responses of Atlantic salmon (Salmo salar**) smolts following a** Yersinia ruckeri **breach in RAS**

Master's thesis in Health Management in Aquaculture
Supervisor: Carlo C. Lazado
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Foreword

This thesis was written for my master's degree in Health Management in Aquaculture (AQUAH) at Norwegian University of Science and Technology (NTNU), Trondheim. The research was conducted as a part of the project PathoRAS – "**Pathogen dynamics and disinfection in salmon recycling facilities"** at Nofima, Ås. This project was funded by FHF (The fisheries and aquaculture industry's research funding) in co-operation with UiT Norway's Arctic University, Sintef Narvik, Skretting and Pharmaq Analytiq. The goal of this thesis is to evaluate the immunological and histological responses of Atlantic salmon smolts when challenged with *Yersinia ruckeri* as a result of biosecurity breach in recirculating aquaculture systems (RAS). The focus of this study is to understand the pathogen dynamics and impact on the health of Atlantic salmon smolts in RAS systems. The increase in use of RAS systems for smolts production and the advancement to maximize the efficiency of RAS, makes this topic relevant in today's aquaculture industry.

I would like to express my heartfelt gratitude to the project manager and my supervisor Carlo Lazado (Senior scientist) at Nofima for giving me this opportunity and your guidance to be a part of this project. Through this project I have learnt a lot with the research work that we have conducted, which has been an exciting journey for me. So thank you so much for the knowledge that you have shared with me throughout the process. I would like to thank Professor Bengt Finstad, my supervisor at NTNU. You have been a great help to me with your guidance throughout the writing process of my thesis and making sure all the communications were smooth. I thank Christian Cordero for helping me with my doubts.

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Abstract

A majority of land-based Atlantic salmon (*Salmo salar*) aquaculture farms in Norway use Recirculating Aquaculture Systems (RAS) which provides a higher degree of biosecurity compared to conventional flow-through systems. However, there are numerous instances of disease outbreaks reported in RAS leading to heavy economic losses. The main objective of this thesis was to simulate a biosecurity breach in RAS using *Yersinia ruckeri* as a model pathogen and study the immune response of Atlantic salmon smolts. *Y. ruckeri* is the causative agent of Enteric Red Mouth (ERM), a disease mostly prevalent during smolt stage but in recent years has become an issue smolts and post-smolts. In addition, two types of diets were tested. There were 3 treatment groups: T1 = received a specialized RAS diet but the system was not infected, T2 = received a plant-based diet and the system was infected, and T3 = received a specialized RAS diet and the system was infected. Infection of the system was carried by spiking the intake water with a fresh culture of *Y. ruckeri* (10^8 colony forming units (cfu)/mL) at 1% (v/v) per total daily volume (ca. 20 L/day). Head kidney and spleen samples for qPCR and histology were collected at time points (e.g. At priming (F1), before infection (F2), 1 day post infection (F3), 3 days post infection (F4), 7 days post infection (F5), 14 days post infection (F6)). Haematopoietic necrosis, fibrous connective tissue surrounding the tubules, presence of melano-macrophages and general cell congestion in the head kidney and mild cell congestion, lymphocytic depletion of the white pulp with the presence of melano-macrophages in the spleen was observed in samples collected over time point F4, F5 and F6. These pathologies are typical to *Yersinia ruckeri* infection, but the tissues were not significantly affected. Gene expression studies revealed a significant downregulation of pro-inflammatory genes *interleukin-1beta* (*il-1b*) and *interleukin-8* (*il-8*) in head kidney. Gene *tumor necrosis factor alpha* (*tnfa*) showed a significant upregulation at 7 dpi compared to before infection and 3 dpi within T3 in spleen while a significant downregulation was observed in head kidney. A significant downregulation in anti-inflammatory gene *interleukin-10* (*il-10*) in T3 at 7 dpi compared to T1 in the head kidney was observed while no changes were seen in the spleen. Membrane bound immunoglobulin (*mIgM*) showed significant downregulation in T2 and T3 compared to T1 in the head kidney, whereas an upregulation of the gene was observed within T3 at 7 dpi compared to before infection time point. *Hepcidin* (*hep*) was significantly downregulated in spleen within T2 and T3. Between tissue comparison revealed a significantly higher gene expression levels of *il-1b, il-8, hep, mIgM* and *tnfa* in head kidney compared to spleen, whereas no significant difference was seen in the genes *lys, cath* and *il-10.* This data reveals a small number of differentially expressed genes to *Yersinia ruckeri* infection as a result of biosecurity breach in RAS. A more comprehensive study is required to fully understand the immune response of Atlantic salmon smolts against *Yersinia* in RAS.

Abstrakt

Et flertall av landbaserte oppdrettsanlegg for atlantisk laks (*Salmo salar*) i Norge bruker Recirculating Aquaculture Systems (RAS) som gir en høyere grad av biosikkerhet sammenlignet med konvensjonelle gjennomstrømningssystemer. Det er imidlertid mange tilfeller av sykdomsutbrudd rapportert i RAS som fører til store økonomiske tap. Hovedmålet med denne avhandlingen var å simulere et biosikkerhetsutbrudd i RAS med *Yersinia ruckeri* som modellpatogen og å studere immunresponsen til atlantisk laksesmolt. *Y. ruckeri* er den forårsakende agenten til rødmunnsyke (EMS), en sykdom som hovedsakelig er utbredt under smoltstadiet, men som i de siste årene har blitt et problem i smolt og postsmoltstadiet. I tillegg ble to typer fôr testet. Det var tre behandlingsgrupper: T1 = mottok et spesialisert RAS-kosthold, men systemet ble ikke infisert, T2 = mottok et plantebasert kosthold og systemet ble infisert, og T3 = mottok et spesialisert RAS-kosthold og systemet ble infisert. Systemet ble infisert ved at inntaksvannet ble tilsatt en fersk kultur av *Y. Ruckeri* (10^8 kolonidannende enheter (cfu)/mL) i en konsentrasjon på 1% (v/v) per totalt daglig volum (ca. 20 L/dag). Prøver av nyre og milt for qPCR og histologi ble samlet inn ved ulike tidspunkter (f.eks. ved forberedelse (F1), før infeksjon (F2), 1 dag etter infeksjon (F3), 3 dager etter infeksjon (F4), 7 dager etter infeksjon (F5), 14 dager etter infeksjon (F6)). Hematopoietisk nekrose, fibrøst bindevev rundt tubuli, tilstedeværelse av melano-makrofager og generell celletetthet i hodenyren pluss mild celletetthet, lymfocyttisk uttømmelse av den hvite pulpa med tilstedeværelse av melano-makrofager i milten ble observert i prøver innsamlet ved tidspunkt F4, F5 og F6. Disse patologiene er typiske for *Yersinia ruckeri*-infeksjon, men vevene var ikke bemerkelsesverdig påvirket. Genekspresjonsanalyse viste en markant nedregulering av de pro-inflammatoriske genene *interleukin 1-beta* (*il-1b*) og *interleukin-8* (*il-8*) i hodenyre. Genet *tumor-nekrosefaktor alfa* (*tnfa*) viste en markant oppregulering ved 7 dpi sammenlignet med før infeksjon og 3 dpi innen T3 i milten, mens det ble observert en markant nedregulering i nyrehode. Det ble observert en markant nedregulering av det betennelsesdempende genet *interleukin-10* (*il-10*) i T3 ved 7 dpi sammenlignet med T1 i hodenyren, mens ingen endring ble observert i milten. *Membranbundet immunoglobulin* (*mIgM*) viste markant nedregulering i T2 og T3 sammenlignet med T1 i hodenyren, mens det ble observert en oppregulering av genet i T3 ved 7 dpi sammenlignet med før infeksjonstidspunktet. *Hepcidin* (*hep*) ble markant nedregulert i milten ved T2 og T3. Sammenligning mellom vevene viste markant høyere nivåer av genuttrykk av *il-1b*, *il-8*, *hep*, *mIgM* og *tnfa* i hodenyre sammenlignet med milten, mens det ikke ble observert noen markant forskjell i genene *lys*, *cath* og *il-10*. Denne dataen viser et lite antall gener som uttrykkes forskjellig i forbindelse med *Yersinia ruckeri*-infeksjon som følge av brudd på biosikkerhet i RAS. En mer omfattende studie er nødvendig for å forstå immunresponsen hos laksesmolt mot *Yersinia* i RAS-systemet.

1. Introduction

Atlantic salmon (*Salmo salar*) is one of the most important finfish species raised for food worldwide with an estimated 2.7 million tons produced annually. It accounted for 32.5 % of all the marine and coastal water finfish aquaculture production (Fisheries, 2022). Norway alone accounted for about 1.5 million tonnes of Atlantic salmon, thus making it an important species in Norwegian aquaculture (Fisheries, 2022). Atlantic salmon, belonging to the family *Salmonidae* is an anadromous fish and has two different life cycle phases; freshwater phase and marine water phase. Its aquaculture production cycle includes two separate stages: land-based smolts production followed by a grow-out stage in marine cages till market size, where majority of the traditional land based smolts production was in flow through systems (FTS) (Bergheim et al. (2009). About 178.9 million smolts were produced annually in Norway as of 2006. The smolts production accounted for around 415 million individuals as of 2022, which has tripled since 2000 (Fisheries, 2022). The production of salmon has drastically increased over the years due to increased efficiency of the hatcheries. This expansion can only be sustainable through implementation of a more sophisticated system when compared to traditional FTS due to various challenges concerning environmental control, biosecurity, high water requirements and disease. Contrary to FTS, RAS provides a better control and stability of the physiochemical and microbial parameters of the environment, reduced makeup water volumes and better growth performance and survival of the fish before transfer to the open sea cages (Attramadal et al., 2012; Attramadal et al., 2014; Martins et al., 2010; Summerfelt et al., 2001; Summerfelt et al., 2009; Terjesen et al., 2013).

1.1 Recirculatory Aquaculture System (RAS)

RAS is an intensive land based production system that operates on the principle of treating and reusing the water with total water volume replaced per day is less than 10% (Midilli et al., 2012). Generally, it consists of several different components: filtration units (mechanical and biological), sterilization unit, holding tanks, pumps, oxygenation unit and some other treatment components to enhance the water quality parameters and the performance of the fish. In RAS, the water is recirculated throughout the system and the environmental parameters are controlled and maintained up to the optimal levels in the holding tanks where the target species is stocked (Ahmed & Turchini, 2021). The particulate matter such as faeces, uneaten feed and other organic solids are filtered out using a mechanical filtration unit (drum filters, sedimentation, etc) in the form of sludge whereas, the extremely toxic dissolved nitrogenous waste products that fish release, such as ammonia, nitrite, and nitrate (to a certain extent), are broken down by the aerobic bacterial population (*Nitrosomonas* and *Nitrobacter*) in the biofiltration unit by nitrification (Badiola et al., 2018; Ebeling & Timmons, 2012). Biofilters used can be of several types for example, floating bead filter, moving bed bioreactor (MBBR), fluidized-bed biofilter and trickling biofilter depending upon the requirement of the system. Since an intensive aquaculture system has a high demand for dissolved oxygen

for adequate growing condition and optimal biofilter performance, pure oxygen and/or aeration is supplied to the system. Carbon dioxide (CO_2) produced in the biofilter and by the fish respiration can hamper the fish health and can cause a drop in the pH of the water thus, CO_2 stripping is necessary. RAS systems operate at a high stocking densities, where aeration is generally used to eliminate the dissolved carbon dioxide in a small scale RAS system but in a larger scale, degassers are used. Ensuring optimal water quality is essential for the health and growth of fish in the system, and this involves achieving disinfected rearing water. The high stocking densities, fish stressor, and increased nutrient loads inherent in RAS create an environment that is conducive to the proliferation of fish pathogens (Badiola et al., 2018). To ensure high levels of biosecurity, modern RAS systems are equipped with various disinfection units such as ultraviolet (UV) disinfection units and/or ozonators. The treatment and potential disinfection of recirculated water before it is returned to fish culture tanks can be accomplished through the use of ultraviolet (UV) irradiation and/or ozonation. This approach has been explored in various studies, including those by (Brazil, 1997; Christensen et al., 2000; Krumins et al., 2001; Sharrer et al., 2005; Summerfelt et al., 2008; Summerfelt & Hochheimer, 1997; Summerfelt et al., 2009).

In recent times, there has been a notable transition in land-based aquaculture production, moving away from conventional flow-through systems towards the adoption of recirculating aquaculture systems. The rising utilization of recirculating aquaculture systems for smolts and post-smolts production can be attributed to the advantages offered by a carefully controlled production environment. These include the reduction of adverse environmental effects, the flexibility to choose suitable locations, and the implementation of robust biosecurity measures, as emphasized in the works of Lazado and Good (2021); Martins et al. (2010). A study conducted by Roque D'Orbcastel et al. (2009) observed a 17% higher final weight in RAS as compared to FTS, along with a higher carrying capacity of approximately 100 kg/m^3 against 85 kg/m^3 respectively. A higher growth performance of coho (*Oncorhynchus kisutch)* (Walbaum, 1792) and Atlantic salmon (*Salmo salar*) at salinities between 5 to 10 parts per thousand (ppt) in RAS was observed (Fang et al., 2021). This emphasizes on the substantial influence of RAS and elevated stocking densities on fish growth performance, water quality, and feed utilization. This approach also offers a degree of biosecurity by implementing measures to separate the stock from the external environment. This intensification can lead to development of new challenges in RAS. In principle, biosecurity measures are typically more stringent in RAS compared to other production systems like flow-through systems. It should offer a safer environment for fish, free from external stressors like storms, predators, or harmful algal blooms. They are expected to provide superior biosecurity, leading to reduced mortality rates and fewer welfare concerns related to disease. Despite these measures, instances of pathogen breaches still occur, leading to significant mortality events and substantial economic losses (Murray et al., 2014). However, numerous challenges have been encountered in practice.

1.2 Challenges in RAS

While the development of RAS has been positive in European countries, with significant increase in production over the years, many systems have suffered due to poor management or inadequate designs (Delabbio et al., 2004; Timmons et al., 2006). Managing disease outbreaks in Recirculating Aquaculture Systems (RAS) presents unique challenges, as a healthy microbial community is essential for water purification and maintaining water quality (Martins et al., 2009; Martins et al., 2010). In RAS, there is a risk of accumulation of minerals, drug residues, hazardous feed compounds, and metabolites, which can negatively impact the health, quality, and safety of the farmed animals (Martins et al., 2009). Water quality deterioration, low dissolved oxygen levels, high concentrations of fish waste metabolites such as total ammonia nitrogen (TAN), unionized ammonia (NH_3-N), nitrite (NO_2-N), nitrate (NO_3-N) to a lesser extent, dissolved carbon dioxide (CO_2), suspended solids (SS), and non-biodegradable organic matter (Sanni & Forsberg, 1996) are some non-pathogenic factors that can adversely affect the aquatic environment and fish health if not properly managed. Other limitations of recirculating systems include high capital and operational costs, along with the necessity for meticulous management and challenges in disease treatment. To ensure economic viability despite the high investment and operational costs associated when compared to other aquaculture production systems, operators often maintain high stocking densities within the facilities. However, this practice leads to increased loads of metabolic wastes, creating an environment conducive to microbial development (Rurangwa & Verdegem, 2015).

Different micro niches exist within a RAS (Sharrer et al., 2005). In each of these environments, specific groups of microbes thrive, aiding in the breakdown of various organic and inorganic wastes present in the system. In addition to the microbial community responsible for water purification, the microbiota present in RAS can also serve as reservoirs for pathogens (Burr et al., 2012). Even with rigorous environmental control and biosecurity protocols in place, numerous pathogens are naturally present in aquatic environments or can enter the system through various means, including live fish, feed, incoming water, and personnel. Good management and controlling the growth of certain bacteria are crucial for the biosecurity of RAS (Michaud et al., 2009). Ozone and UV treatment, either on their own or combined, are the primary methods preferred for continuous water disinfection in RAS (Rurangwa & Verdegem, 2015). This possesses a challenge to carefully manage the dosage and placement of disinfection to prevent the proliferation of opportunistic organisms within the system as the disinfection process is not selective between pathogens and beneficial microbial populations (Blancheton et al., 2013).

With several challenges associated with the RAS operation, it is a sobering reality that nearly every new recirculating aquaculture system (RAS) has encountered challenges leading to significant losses, if not the entirety of a production cycle. Reports and published disease issues and losses in RAS historically, have been documented (Bostock et al., 2018). Here are a few instances: 600,000 salmon smolts were culled following an outbreak of Infectious Salmon Anaemia (ISA) at two RAS farms in Canada (Woodbury,

2018). Occurrences of *Yersiniosis (Yersinia ruckeri*) and ISA have been reported in RAS facilities in Norway (Hjeltnes et al., 2017). In Denmark, incidents of furunculosis (caused by *Aeromonas salmonicida*) in RAS have led to substantial losses. Common harmful bacteria for fish in RAS include *Aeromonas salmonicida, Vibrio (Listonella) anguillarum, Vibrio (Aliivibrio) salmonicida*, and *Yersinia ruckeri* (Ringø et al., 2010). From maintaining water quality to preventing biofilm formation and implementing effective biosecurity measures, aquaculturists face a multitude of hurdles in safeguarding fish health within these closed-loop systems. Pathogen like *Yersinia ruckeri*, known for its resilience and virulence, underscore the importance of ongoing research, innovation, and collaboration within the aquaculture community. By addressing the specific challenges posed by pathogens such as *Yersinia ruckeri*, we can develop targeted strategies to mitigate risks and ensure the long-term viability of RAS-based aquaculture while safeguarding global food security and environmental sustainability.

1.3 *Yersinia ruckeri*

Yersinia, belongs to the family *Enterobacteriaceae*, is a Gram-negative, rod-shaped facultative anaerobes within constitutes a genus with various pathogenic species capable of inducing diseases in humans and other animal species, including fish. *Y. ruckeri* stands out as the primary reason behind Enteric Red-mouth (ERM) disease, affecting various salmonid species globally. It was originally identified in Rainbow trout within the Hagerman Valley of Idaho, USA during the 1950s (Ross et al., 1966). Presently, *Y. ruckeri* is distributed across diverse regions spanning North and South America, Europe, Australia, South Africa, the Middle East, China, India and Australia (Shaowu et al., 2013; Tobback et al., 2007). First instance of *Y. ruckeri* was reported in 1985 in Norway (Sparboe et al., 1986) which led to losses of Atlantic salmon kept in sea water in northern Norway by extensive petechial haemorrhages and enlargement of the spleen, accompanied by degenerative alterations in muscle and kidney tissues. As of the 2022 report, *Y. ruckeri* was detected in 36 salmon farms along the coast of Norway (Madhun et al., 2022). Various strains of *Y. ruckeri* have been documented and classified according to serotypes, biotypes, and outer-membrane protein types. *Y. ruckeri* distinguishes itself from all other members of the genus *Yersinia* by its ability to produce lysine decarboxylase (Bottone et al., 2005).

Originally, the transmission of *Y. ruckeri* was attributed to the movement of asymptomatic carriers and contaminated eggs among fish populations. However, the presence of the pathogen in mammals raised the possibility of wild animals such as birds, wild fish, and invertebrates, as well as humans, serving as potential vectors (Willumsen, 1989). Asymptotic carrier fish play an important role in *Y. ruckeri* infections (Michel et al., 1986; Wobeser, 1973). Hunter et al. (1980), investigated how stress facilitates the transmission of *Y. ruckeri* from asymptomatic carriers to unaffected fish and found that the pathogen was excreted in the faeces over cycles lasting 36-40 days. The shedding of the pathogen into the environment may be correlated to stressors in the system but further research has to be carried out to confirm this possibility. The microorganism has been detected in water, faeces, and sewage sludge (Willumsen, 1989),

and it readily adheres to surfaces, forming biofilms (Coquet et al., 2002). The pathogen generates a range of toxins, including homolysis, endotoxins, and cytotoxins (Aussel et al., 2000; Fernández et al., 2004; Romalde & Toranzo, 1993; Secades & Guijarro, 1999). Fish become less active, eat less efficiently, and grows slower.

The visible changes resulting from *Y. ruckeri* infection were first described by Rucker (1966). Clinical indications of the disease primarily manifest as generalized haemorrhagic septicaemia. The characteristic red-mouth appearance results from venous and capillary congestion within the vessels of the brain and eyes of infected Rainbow trout. It exhibits melanosis/darkening of the skin, along with ulceration and haemorrhaging (Roberts, 2001). Clinical manifestations can differ, and instances have been documented where the typical "red mouth" symptom is not present (Tobback et al., 2007). Internally, the disease leads to widespread haemorrhaging across the internal organs, accompanied by swelling of the kidney and spleen, stomach may contain watery fluid, while the intestine may contain yellow fluid (ACTION, 2020; Roberts, 2001; Stevenson & Airdrie, 1984; Tobback et al., 2009). Acute condition of the infection can result in heavy infiltration of leukocytes in head kidney, spleen, and liver along with haemorrhaging and necrotic foci but granulomas may be encountered in the kidney during the chronic condition (Roberts, 1983). Numerous studies have been conducted understanding the virulence process of *Yersinia*. The pathogenicity of *Yersinia ruckeri* is influenced by several factors such as stressors, iron availability, temperature, pH and osmolarity (Guijarro et al., 2018). Various different approaches have been used to identify virulent-related genes of *Y. ruckeri.* Molecular approach via in vivo expression technology (IVET), signature-tagged mutagenesis. (STM) (Fernández et al., 2004), Other bioinformatic approaches include proteomic, transcriptomic and genomic analysis (Guijarro et al., 2018; Kumar et al., 2018; Kumar et al., 2019). *Y. ruckeri* is also produces extracellular proteins (ECP) that help the pathogen to colonize and grow in the host body (Madigan et al., 2006). Other factors such as makeup of the pathogen and host pathogen interaction may also contribute to the cause.

1.4 Host-Pathogen Interaction

Host-pathogen interactions involve complex molecular and cellular processes through which pathogens exploit host resources and evade host defences. Upon encountering a host, pathogens employ various strategies to adhere to host tissues, evade immune detection, and establish infection. The host, in turn, mounts an immune response aimed at recognizing and eliminating the invading pathogen through mechanisms such as inflammation, phagocytosis, and antibody production. The outcome of host-pathogen interactions can vary, ranging from successful pathogen clearance by the host immune system to chronic infection or disease progression. Understanding the dynamics of host-pathogen interactions is essential for developing strategies to prevent and treat infectious diseases effectively.

Gram negative bacteria comprises lipopolysaccharides (LPS) in its outer layer which is classified as pathogen associated molecular pattern (PAMP) and is important for the bacterial function (Perez-Perez et al., 1986). The LPS of the pathogen can be smooth or rough depending on the presence or absence of O-antigen side chains respectively (Perez-Perez et al., 1986). These O-antigen side chains in LPS of the pathogen play a crucial role in evading the specific immune response of the fish (Boesen et al., 1999). Based on serotypes and outer membrane protein type *Y. ruckeri* can be divided in two biotypes (1 and 2) and is recognised by the cells using cell surface associated structures, O-antigens and flagellar antigens (Davies & Frerichs, 1989; Ross et al., 1966). This could indicate why there is a lack of a distinct immune response observed in cases of ERM. LPS was indeed regarded as the primary immunogenic molecule in formalin-killed vaccines developed for ERM (Johnson & Amend, 1983). Its variability, attributed to the presence of diverse sugars and sugar linkages, makes it one of the most mutable cellular components, crucial for bacterial evasion of host defense systems (Reeves, 1995). Moreover, *Yersinia* species has three secretion systems (T3SS) (Gunasena et al., 2003), that contains a tube like structure with a series of proteins that facilitates the delivery of effector proteins into the host's cells which aids the pathogen to evade phagocytosis. Toxins produced by the pathogen induce apoptosis, leading to the release of the pathogen from the phagocyte (Monack et al., 1997). The nonspecific immune response has been emphasized as crucial in defending the host against *Y. ruckeri* infections (Raida & Buchmann, 2008). Engulfing and internalization of the bacteria is carried out by neutrophils and macrophages which kills the pathogen through respiratory burst (Neumann et al., 2001). Afonso et al. (1998), found that injection with formalin-killed whole-cell vaccines of *Y. ruckeri* led to a substantial influx of macrophages and neutrophils. Yet there is little work done to determine the role of phagocytosis against the pathogen *Y. ruckeri*. The specific immune system utilizes both humoral components, like antibodies, and cellular components including activated macrophages (Ellis, 1999), however, there is limited evidence suggesting that circulating antibodies contribute to protection against *Y. ruckeri* infections (Cossarini-dunier, 1986). Raida and Buchmann (2008), supported this perspective in their study, where they discovered that passive immunization with antibodies produced against *Y. ruckeri* did not provide any protection. Likewise, Cipriano and Ruppenthal (1987), reported similar findings in brook trout. Vaccine against ERM has shown promising results since its first commercial release in 1970's. By administering a non-virulent antigen preparation, the aim of vaccination is to prompt memory in T and/or B lymphocytes (Lydyard et al., 2006). This ensures that during an infection, the host can rely on a secondary response from the specific immune system, rather than the nonspecific immune system. Overall, vaccination stands as one of the primary methods for preventing infectious diseases (Potter & Babiuk, 2001).

Vaccines certainly are more efficient than the conventional drugs however, there are limited vaccines available commercially. Bath treatment and injection at the fry stage is currently used as the primary strategy to vaccinate against ERM. Tatner and Horne (1985), observed that booster vaccines against *Y. ruckeri* provided increased protection in rainbow trout. The method of vaccine administration can influence

the immune response differently (Palm Jr et al., 1998). Injecting the vaccine intraperitoneally (I.P.) is considered to offer the highest levels of protection (Palm Jr et al., 1998). However, since fish are most vulnerable to ERM at around 4 grams in size, a bath vaccine is recommended (Håstein et al., 2005). In 2008, a bivalent vaccine was released against 2 biotypes of *Y. ruckeri* which contains formalin killed whole cell preparation of the biotypes but the efficacy of this bivalent vaccine is yet to be understood. The nature of the antigen and the dose concentration are important factors affecting the immune response of the fish. In addition to this, Roberts (2001), emphasized that the efficiency of the fish immune response depends on dosage, method of administration and water temperature. Adjuvant vaccines for example Freund's Complete Adjuvant (FCA), are another immunological stimulants widely used against this pathogen but they are not always beneficial as they are known to cause granulomatous side effects to the fish (Secombes & Fletcher, 1992), despite this FCA is still commercially used in Norway.

In present literature, vaccination against ERM has shown relatively promising results, but there remain challenges in achieving widespread efficacy due to factors such as limited vaccine availability, variable immune responses, uncertainties regarding vaccine efficacy, pathogen dynamics and important hotspots for establishment and proliferation of the pathogen in RAS. This makes it imperative to emphasize the importance of good husbandry practices and robust biosecurity measures to complement vaccination strategies and ensure the overall health and well-being of fish populations. This research is aimed at challenging smolts with *Yersinia ruckeri* to mimic the outbreak of the pathogen in RAS as a result of a biosecurity breach to understand the dynamics of pathogen infection.

This study will provide outcomes regarding:

- Influence of the pathogen on the health of the fish and the dynamics of *Yersinia* infection.
- Immune response of Atlantic salmon smolts exposed to *Yersinia ruckeri* simulating a bio-security breach in RAS.

The outcome will be pivotal for developing necessary protocols for better husbandry measures, to understand host-pathogen interaction in RAS and enhance the degree of biosecurity to ensure efficient management of Atlantic salmon in the system.

2. Materials and methods

2.1 Statement of ethics

The infection trials were carried out under Norwegian Food Safety Authority (FOTS) ID 30321. The infection trial did not last longer than 21 days, and severe pathologies were absent. A total of 900 Atlantic salmon smolt were used and the number of fish requested was kept to the minimum number of samples to achieve reliable scientific results and support optimum system performance. There is no alternative technique available at present so the animals in the trails were necessary. The pathogen load to which the fishes were exposed were kept low. No mortality was recorded during the infection trials and all the fish were humanely euthanized by the end of the trials.

2.2 Experimental animal

Atlantic salmon smolts (*Salmo salar*) was the subject species used for this trail. The initial weight before at the start of the trail was *ca* 80 grams. Mixed sex with a 50:50 ratio of male and females belonging to the same age group was used for the trial. The coefficient of variance (CV) was 5% for the weight was set. The fish was procured from a commercial supplier and was transported to Tromsø Aquaculture Research Station. The fishes were quarantined as per the research station's standard protocol and including routine health check-ups. The fishes were briefly reared in a flow through system at approximately 12-13°C under continuous illumination (LD 24:00) and fed till apparent satiation with a commercial diet. The experiment was approved by the Norwegian Food Safety Authority under FOTS ID 30321.

2.3 Experimental setup (RAS description)

The experimental trial was carried out at the Fish Health Laboratory of Tromsø Aquaculture Research Station. Figure 1 depicts the RAS facility sketch sourced from the study by Mota et al. (2022). The facility has a biosafety level 2 approval by the Norwegian Food Safety Authority. The experiment was carried in the infection room, housing 9 individual RAS units with cylindroconical experimental tanks with a volume of 0.5 m^3. The tanks have dual outlet drains (a bottom center outlet and a sidewall outlet) leading to a drum filter with a 40 μm microscreen to remove suspended solids and sensors for oxygen and temperature (Oxyguard®, Farum, Denmark. Figure 2 shows the water flow across different RAS units. Backwash discharge for the screen is piped to a septic tank and to an underground pipe circuit (500 m long). The filtrate from the drum filter flows into a moving bed bioreactor (MBBR, V = 0.2 m^3, 50% filled with bio-media). Bio-media (RK BioElements, RK Plast A/S, Skive, Denmark) has a surface area of 750 m^2/m^3. The make-up water is added to the pump sump (V = 0.1 m^3) where, a pH probe (K01SVPHD, Oxyguard® International A/S, Farum, Denmark) and a water level sensor (KQ6001, ifm electronic gmbh, Essen, Germany) are installed. The combined water flow is pumped into 3 loops. In loop 1, the water (3000 L/h) is pumped to the top of the degasser column to remove the carbon-dioxide (CO^2) and exits to a foam

fractionator unit and returns to the MBBR. The backwash foam is collected in the septic tank pipeline. The water (600 L/h) is pumped to a protein skimmer where disinfection by ozone (Ozonizer S 1000, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) is carried out, which then exits back to MBBR by gravity in loop 2. In loop 3, the water (1500 L/h) is pumped to a low-pressure oxygenator cone, where pure oxygen (O_2) is added to the water. The water flows to the chiller and a heater unit (TK- 1000, TECO®, Ravenna, Italy) and then continues to UV-C unit (40 watt or 140-watt lamp, VGE B.V., Schijndel, the Netherlands) and finally to the fish holding unit. The total water volume of RAS is 0.8 m^3. The UV-C units and ozone generator were not used during the initial period of the trails. The system was run for 3-6 weeks for the acclimatization of bio-media using sodium bicarbonate ($NaHCO_3$) and ammonium chloride (NH_4Cl) solutions.

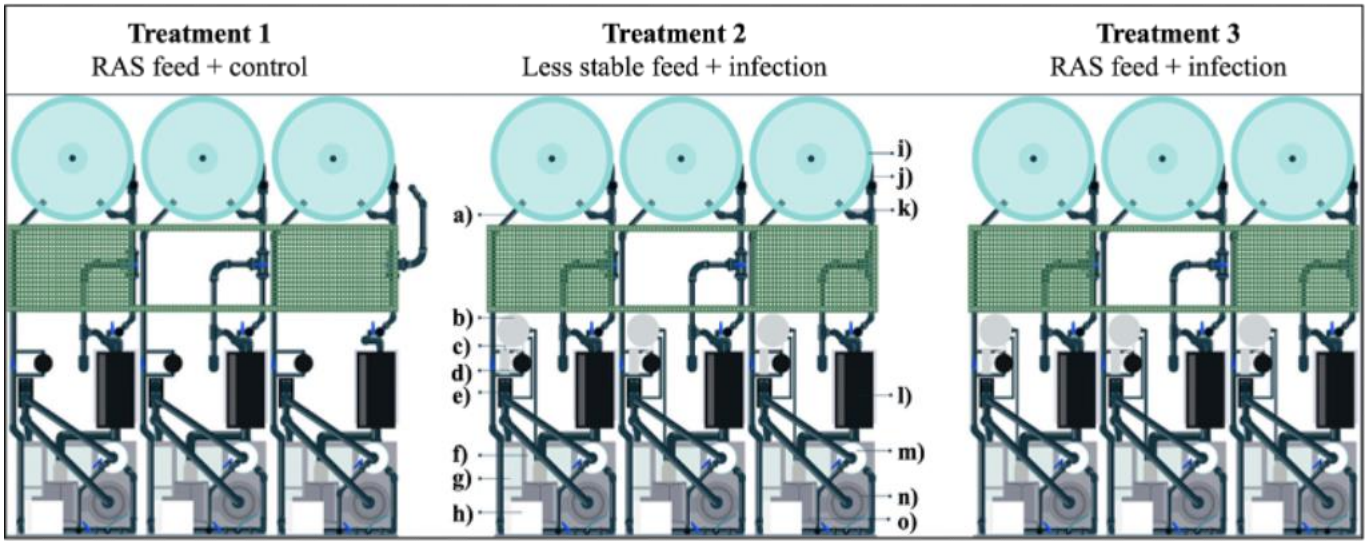

Figure 1. RAS research facility sketch sourced from Mota et al., (2022) with components labelled as a) inlet, b) protein skimmer, c) ozone generator, d) UV-C unit, e) main pump, f) MBBR, g) pump sump, h) chiller/heater i) fish tank, j) centre outlet, k) sidewall outlet, l) drum filter, m) Oxygen generator cone, n) degasser and o) foam fractionator.

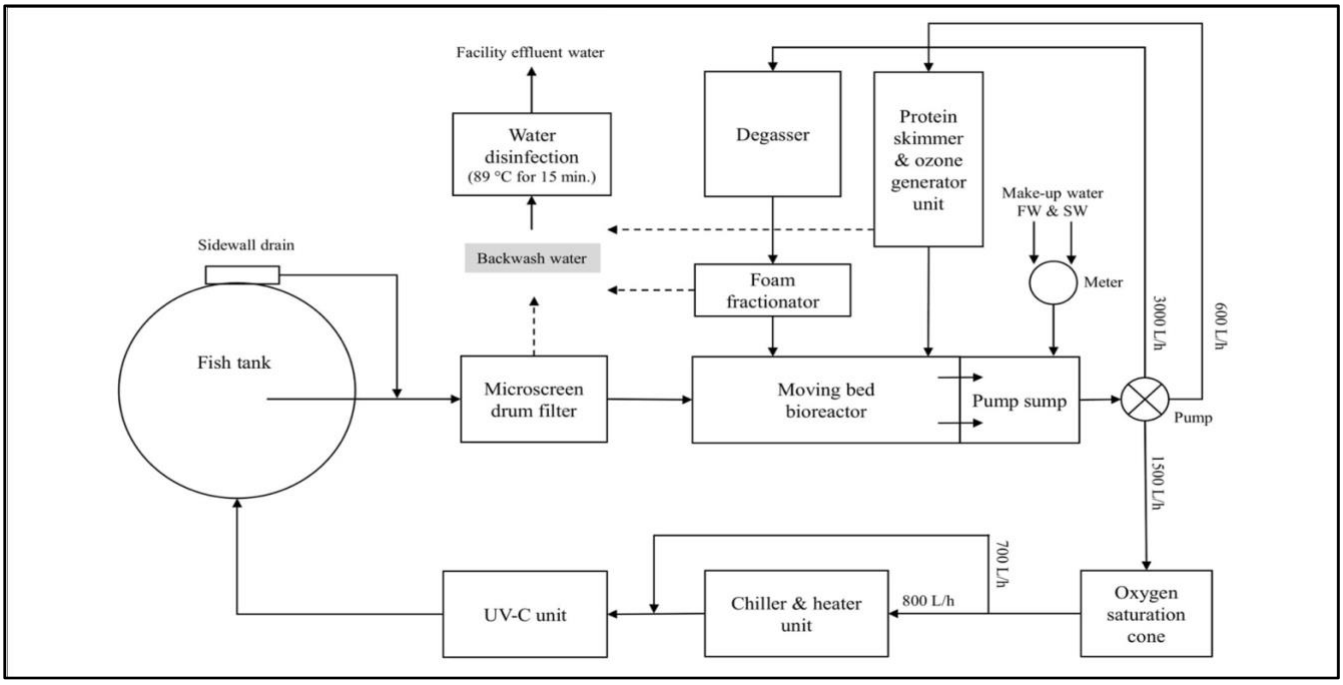

Figure 2. Water flow across different RAS units in three different loops, sourced from Mota et al.,(2022).

2.4 Experimental trial description

The trail consisted of 3 treatment groups. Each treatment group was divided into three experimental RAS units. Each treatment unit was stocked with 100 fishes, with a total of 300 fishes in each treatment group, hence, 900 Atlantic salmon smolts were used in total. Treatment 1 consisted of a control group that was fed with a RAS feed designed by Skretting and was not infected throughout the trial period. In treatment 2, the fishes were fed with a plant based commercial diet with low water stability and were infected. Treatment group 3 was also infected with the pathogen *Yersinia ruckeri* and fed a RAS diet similar to treatment group 1. Groups 1 and 2 simulated a system with a low organic load. The trail was carried out in 2 phases distributed over a period of 6 weeks. Acclimation and priming was carried out in phase I for the first 3 weeks followed by disease development in phase II where, the system was spiked with the pathogen *Yersinia ruckeri* at 10^8 cfu/mL through the makeup water at 1% (v/v) per total daily volume (ca 20 L/day) at the beginning of the 4th week and the disease will allowed to develop for 3 weeks (Figure 3) . Note that the treatment group 1 (control) was not infected as it served as a control group. During the two phases, the fishes were fed with experimental diets. At the end of phase II, all the remaining fishes were euthanized using a Benzocaine (Benzoak vet, 200 mg/ml, EuroPharma, Leknes, Norway) overdose bath. The target organs analyzed for this trial were head kidney and spleen. The sample organs were collected carefully at predetermined time points throughout the experimental duration (Table 1). All the physicochemical water quality parameters required to support the fish biological requirement were maintained up to optimum levels throughout the trial duration. Fish behaviour and appetite was monitored daily throughout the experimental duration[1].

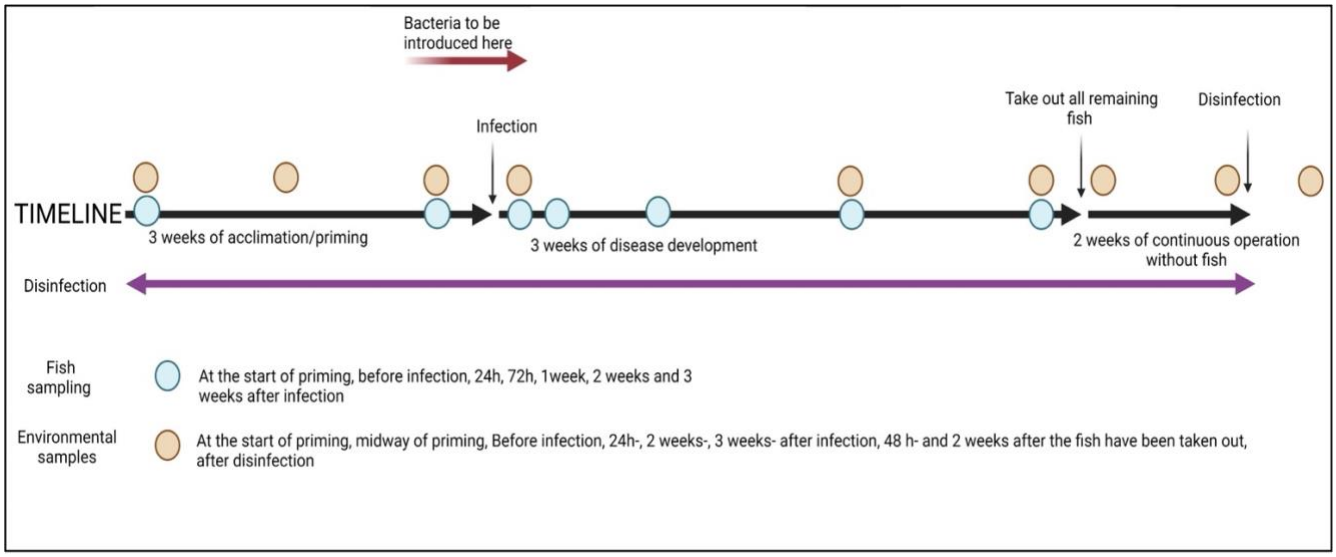

Figure 3. This figure indicated the timeline of the trial progression.

[1] *The growth performance parameters including but not limited to weight, length was recorded as a part of another research study.*

Table 1. Sample collection time points.

Sample groups	Time points
F1	At the start of priming
F2	Before infection
F3	24 h after infection
F4	72 hr after infection
F5	1 week after infection
F6	2 weeks after infection
F7	3 weeks after infection

2.5 Histological analysis

2.5.1 Tissue processing

Three samples from each tank (total 9 tanks) were selected across 5 time points (F1, F2, F4, F5 and F6) accounting to 135 samples for each tissue (i.e., head kidney and spleen). The tissue samples were carefully added to the embedding cassettes and were labelled. The head kidney and spleen samples of the same individual fish were added in the same cassette. These cassettes containing samples were kept in 70% ethanol solution before further processing . Before paraffin embedding, the sample tissues fixation, dehydration, clearing and impregnation was carried out using Leica T1020 Tissue Processor (Leica Biosystems, Germany). Freshly prepared solutions were used to process the samples. Programme followed for tissue processing is given in Table 2.

2.5.2 Paraffin embedding

Embedding of the tissue cassettes in paraffin was carried out using Leica EG1150H Modular Tissue Embedding Centre machine (Leica Biosystems, Germany). The molten wax was first poured into the mould just adequate to cover the base of the mould. Using pre-heated forceps, the tissues were carefully lifted from the cassettes and added to the mould and positioned to the centre so as to avoid any improper embedding of the block. Once the tissue was positioned in the mould, the mould was hovered over the cooling plate so the wax within would solidify resulting in stabilizing the tissue in the position intended. Then the labelled cassette top was placed on the mould and molten wax was poured so that it just covers the top of the cassette. The mould was then placed onto the cooling plate to completely solidify. After solidification of the wax block with the tissues, the mould was separated from the block carefully. Care was taken to avoid any damage to the block.

2.5.3 Sectioning

Leica RM2165 Rotary Microtome (Leica Biosystems, Germany) was used to prepare the sections of the samples. The sample embedded blocks were carefully fixed onto the block holder of the microtome.

A fresh blade was used for the sectioning and changed frequently. The distance between the block and the knife was adjusted just so it almost seemed to be in contact with the knife. The trimming thickness was set to 10 μm to trim away the surplus wax so the suitable area of the tissue was exposed. Once the surplus wax was trimmed off, the section thickness was set to 4 μm using the control panel. Using the rotating handle the block was moved to prepare the tissue section ribbons of 4 μm. The ribbon was picked carefully with the help of a forceps and was floated on the warm water in a thermostatically controlled water bath. Leica HI1210 Water bath (Leica Biosystems, Germany) was used for the paraffin section and the water temperature was maintained at 40°C. The unwanted sections from the ribbon were teased apart using a seeker and the desired sections were split into groups of sections. The section was picked up using a labelled glass slide by slightly immersing 3/4th length of the slide just below the floating section. Once the section was in contact with the slide, it was vertically lifted from the water. The slide was tapped gently to remove the excess water and increase the contact between the section and slide.

2.5.4 Drying

The slide was immediately placed on a slide warmer plate, Adamas SW 85 slide warmer (Adamas Instrumenten B.V, Netherlands) and the temperature was maintained at 40°C. The slides were then incubated at 35°C overnight in an incubator. Binder ED 23 incubator (Binder GmbH, Germany) was used for the incubation.

2.5.5 Deparaffinization and Staining

The slides were retrieved from the incubator and were arranged vertically in the slide holder. The deparaffinization and HE staining was carried out using Leica Autostainer XL (Leica Biosystems, Germany) slide stainer. Fresh solutions were prepared. The slides were loaded into the machine and ran through a series of solutions for predetermined time intervals, approximately for about 40 mins. The programme followed is detailed in Table 3. Once the staining process was completed the stained slides were collected and the stains were checked. The slides were then loaded in Leica CV 5030 Automated Glass Coverslipper to secure the section with a coverslip. DPX mountant was used as the mounting liquid for the coverslip.

Table 2. Tissue processing programme followed.

Solutions	Time (h)
Phosphate Buffer Saline(PBS)	1
Ethanol 50%	1
Ethanol 70%	1
Ethanol 96%	1
Ethanol 100%	0.5
Ethanol 100%	0.5
Ethanol 100%	0.5
Xylene	0.5
Xylene	0.5
Xylene	0.5
Paraffin	1
Paraffin	2

Table 3. Tissue staining process followed.

Solutions	Time (min)
Xylene	5
Xylene	5
Absolute alcohol	3
Absolute alcohol	3
Ethanol 96%	2
Ethanol 96%	2
Ethanol 70%	2
Distilled water	3
Haematoxylin	4
Distilled water	2
Ammonia solution[2]	1
Distilled water	0.3
Hydrochloric acid[3]	0.05
Distilled water	0.3
Eosin Y	0.3
Distilled water	0.35
70% Ethanol	0.15
96% Ethanol	0.15
Absolute Alcohol	0.3
Absolute Alcohol	1
Xylene	1
Xylene	Exit

[2] Ammonia solution: 150 µL of ammonia was added to 400 mL of distilled water.

[3] Hydrochloric acid (HCL) solution: 600 µL of HCL was added to 400 mL of 70% alcohol.

2.5.6 Scanning, imaging and scoring.

The slide scanning and imaging was performed using an Aperio CS2 (Leica Biosystem, Germany) high quality digital slides scanner. The slide analysis was carried out using Qupath Software (version: 0.5.1) (Bankhead et al., 2017). For histopathological evaluation, a semi-quantitative scoring system was followed. The scoring was based on percentage damage in a predetermined region of the tissue and a tissue as whole and were categorised from a score of 0 (no damage to the tissue or non-specific damage) to 3 (more than 20% damage to the tissue/ severe necrosis). The tissues were scored twice based on a predetermined region and once as a whole tissue to reduce the bias in the scoring process. A detailed scoring category is described in Table 4 and Table 5

Table 4 . Histopathological scoring for spleen.

Score	Spleen	Changes
0	Normal, no structural changes,	No damage/changes, well defined structure, non-specific pathological changes <5%
1	Mild changes, early signs of structural damage	Haemorrhages ranging between 5-10% of the tissue, including mild presence of leukocytes, general congestion of cells.
2	Moderate to high structural changes	10-20% Haemorrhages, necrosis, including changes in RE cells (destruction), moderate presence of leukocytes.
3	Severe damage, including necrosis	>20% structural changes/damage, multifocal necrosis,

Table 5 . Histopathological scoring for head kidney.

Score	Head Kidney	Changes
0	Normal, no structural changes,	No damage/changes, well defined structure, non-specific pathological changes <5%
1	Mild changes, early signs of structural damage	Haemorrhages ranging between 5-10% of the tissue, including mild presence of melano-macrophages, general congestion of cells.
2	Moderate to high structural changes	10-20% Haemorrhages, necrosis, including structural damages to the tubules (dilation), moderate presence melano-macrophages.
3	Severe damage, including necrosis	>20% necrotic damage including high presence of melano-macrophages

2.6 Gene expression.

2.6.1 Sample preparation and lysis.

A total of 81 samples of each tissue, head kidney and spleen covering time points F2, F4 and F5 were used for the RNA extraction. Two 3 mm stainless steel beads were added to the 1.2 mL tubes in Qiagen collection microtubes (Qiagen N.V, Germany) using a bead dispenser and 400 µL of lysis LBE buffer (Thermo Fisher Scientific, USA) to each tube. A small piece of the sample tissue (approximately 5-10 mg) was cut using a sterile scalpel blade and was added to the lysis buffer. A new sterile blade was used for every sample and the forceps used to pick the samples was cleaned between every sample with 70% ethanol and ddH_2O. Care was taken to make sure that the tissue is submerged in the buffer. Once the plate was ready with all the samples, 20 µL of Proteinase K (REF C42150) was added to each sample. Push-cap lids were put on each tube to secure the mixture in the tubules. The plate was centrifuged for 30 seconds to ensure proper mixing of the sample with the lysis PBE buffer and the enzyme. The lysis of the samples was done with FastPrep 96 High-throughput homogenizer (M.P Biomedicals, USA) at 1800 rpm for 120 seconds. The plate was then centrifuged for a minute and incubated at 37°C for approximately 30 minutes.

2.6.2 RNA extraction

Around 300 to 350 µL of supernatant form the lysate was pipetted to a new deep well plate. Care was taken to avoid pipetting out debris from the lysate. Once all the lysate was transferred, the plate was covered with a PCR film and centrifuged for 1 minute to ensure proper mixing. The RNA extraction was carried out in the Biomek 4000 Automated RNA extraction robot (Beckman Coulter Inc., USA) .

Figure 4. Biomek 4000 robot used for the extraction of RNA.

For each sample, 2.5 mL of 70% Ethanol, 0.42 mL of Bind solution and 0.17 mL DNAse enzyme solution was added. All the solutions were prepared according to the Agencourt RNAdvance Tissue kit. The sample plate was loaded and the reagents were added to their specific plastic ware in the deck of the robot. The volume of the elution (RNAse free water) was calculated by the computer to be added to the robot. The RNA extraction was completed in approximately 3 hours. Upon completion of the extraction process, the RNA integrity was determined using a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, USA).

2.6.3 cDNA synthesis.

High-capacity cDNA Reverse Transcription (RT) Kit (Thermo Fisher Scientific, USA) was used to reverse transcribe the RNA into cDNA using the guidelines provided by the kit. Normalization of the samples based on the RNA integrity was calculated using Microsoft Excel software (Version 16.83) for all the samples. A total of 81 samples were selected from both the tissues (head kidney and spleen tissue), 3 samples from each tank across 3 time points F2, F4 and F5. A normalization plate was prepared by diluting the selected samples to 500 ng per 20 µL of Nuclease free water (VWR Life Scientific, USA). To prepare the sample plate for PCR reaction, 10 µL of sample was mixed with 2 µL of 10X RT buffer, 0.8 µL of 25X dNTP mix , 2 µL of 10X Random primers, 1 µL of MultiScribe ^{TM} Reverse Transcriptase enzyme and 4.2 µL of Nuclease free water to a total volume of 20 µL. For positive control, 10 µL of pooled samples was added to the same volume of master mix and a negative control reaction with 10 µL of pooled samples, 2 µL of 10X RT buffer, 0.8 µL of 25X dNTP mix , 2 µL of 10X Random primers and 5.2 µL of Nuclease free water was included. The plate was centrifuged for 1 minute to avoid any air bubbles and ensure proper mixing. Veriti 96 well Thermocycler (Thermo Fisher Scientific, USA) was used for cDNA synthesis by denaturing at 25°C for 10 minutes, annealing at 37°C for 120 minutes, elongation at 85°C for 5 minutes and hold at 4°C. The cDNA was kept at -21°C until further use.

2.6.4 *Yersinia ruckeri* detection by qPCR.

A 1:10 dilution of cDNA was prepared using nuclease free water for each solution. A 384 well plate was used for the qPCR standard amplification curve. The primers (conc.100mM) were first diluted to 1:10 with nuclease free water. A 6 µL master mix containing 0.5 µL of each, forward and reverse primer, 5 µL of SYBR Green master mix (Applied Biosystem, USA) was made. The reaction was run in duplicates. The master mix was added to the wells of the qPCR plate and 4 µL of cDNA sample was added to each well accounting to a total of 10 µL volume. A positive control with pooled samples and a negative control without the template was included. Master mix with *Y. ruckeri* DNA sample was also included in duplicates for the reference. The plates were sealed and spun down for 1 minute. QuantStudio 5 Real Time qPCR System (Applied Biosystems, USA) was used for the detection of *Y. ruckeri.* The programme used is shown in Table 6.

Table 6. Thermo-cycle programme for RT-qPCR.

Steps	Temperature (°C)	Duration
Hold	50	2 mins
	95	2 mins
PCR	95	1 second
	60	30 seconds
Melt Curve	95	15 seconds
	60	1 minute
	95	15 seconds
Cooling	40	1 minute

2.6.5 Primer selection and efficiency.

Two potential housekeeping genes were selected as the reference genes and 8 target genes were selected from previous publications based on *Yersinia ruckeri* infection in salmonids. The genes markers selected representing inflammatory process, immunoregulatory response, antimicrobial peptides and antigen recognition are mentioned in Table 7. To prepare a working cDNA sample solution to check the primer efficiency, 1 µL of 20 random samples were pooled together and diluted to 1:10 ratio with nuclease free water. Using this 10X dilute sample, a further dilution was prepared with nuclease free water, being 1:20, 1:40, 1:80, 1:160 and 1:320 ratios. The primers were diluted to 1:10 dilution before preparing the master mix. The PCR plate (The Standard 384-well PCR plate, Thermo Fisher Scientific, USA) was prepared by adding 0.5 µL of each, forward and reverse primers, 5 µL of SYBR Green Master mix (Applied Biosystems, USA) and 4 µL of diluted sample to a total volume of 10 µL for each sample. Each gene was run in duplicates with each dilution point. The plate was sealed with the PCR cover and centrifuged for a minute to ensure proper mixing and to avoid any air bubble. The standard curve for primer efficiency was generated by running the plates in QuantStudio 5 Real-Time PCR Systems (Thermo Fisher Scientific, USA). The programme followed for amplification is mentioned in Table 6. The cycle threshold (Ct) values generated from the qPCR run was used to calculate the primer efficiency (E) using the Equation *1*. The percentage efficiency was converted by using Equation 2. A converted primer efficiency value of 2 indicates a 100% efficiency.

Equation 1

$$\textbf{Primer efficiency \%} = \left(-1 + \left(10^{\left(-\frac{1}{slope}\right)}\right)\right) \times 100$$

Equation 2

$$\textbf{Converted primer efficiency (E)} = \left(\frac{Primer\ efficiency\ (\%)}{100}\right) + 1$$

2.6.6 Gene expression study by RT-qPCR

The gene expression reactions were carried out in duplicates for all the selected samples mentioned in 2.5.3 subsection cDNA synthesis. The samples were diluted to 1:10 ratio and 6 µL of each sample was added to the new 384 well PCR plate in duplicates. The primers were also diluted to a 10x dilution before preparing the master mix. To each well 0.5 µL of each primer (forward and reverse), and 5 µL of SYBR Green master mix was added with the combined volume of 10 µL. The plate was sealed with a PCR cover and centrifuged for a minute and was loaded into the qPCR system. The reactions were carried out using a similar programme as mentioned in Table 6. The process was repeated for all the 10 genes (Table 7) using the same programme and the amplification curve was generated.

Table 7. Genes evaluated by RT-qPCR.

Reference genes	Primer sequence	Reference
Beta actin (*actb*)	F: CCAAAGCCAACAGGGAGAA	Sanden and Olsvik (2009)
	R: AGGGACAACACTGCCTGGAT	
18s ribosomal RNA (*18s rRNA)*	F: TGTGCCGCTAGAGGTGAAATT	Jorgensen et al. (2006)
	R: GCAAATGCTTTCGCTTTCG	
Target genes	**Primer sequence**	**Reference**
Interleukin-1-beta (*il-1b*)	F: AGGACAAGGACCTGCTCAACT	Ingerslev et al. (2009)
	R: CCGACTCCAACTCCAACACTA	
Interleukin-10 (*il-10*)	F: GGGTGTCACGCTATGGACAG	Ingerslev et al. (2009)
	R: TGTTTCCGATGGAGTCGATG	
Interleukin-8 (*il-8*)	F: GAAAGCAGACGAATTGGTAGAC	Zanuzzo et al. (2020)
	R: GCTGTTGCTCAGAGTTGCAAT	
Cathelicidin (*cath*)	F: ACACCCTC AACACTGACC	Holm et al. (2017)
	R: CCTCTTCTGTCCGAATCTTCT	
Hepcidin (*hep*)	F: TTCAGGTT CAAGCGTCA GAG	Holm et al. (2017)
	R: AGGTCCTC AGAATTTGC AGC	
Lysozyme (*lys*)	F: CACCGACTATGGCATCTTCC	Mutoloki et al. (2010)
	R: CTGACCGCCACTGTGATGTC	
Membrane bound immunoglobulin (*mIgM*)	F: TCTGGGTTGCATTGCCACTG	Mutoloki et al. (2010)
	R: GTAGCTTCCACTGGTTTGGAC	
Tumour Necrosis Factor-alpha (*tnfa*)	F: AGCATGGAAGACCGTCAA	Crespo et al. (2010)
	R: TTCGTTTACAGCCAGGCT	

***The primers (conc.100mM) were diluted to 1:10 ratio before preparing the master mix.**

2.6.7 Data calculations.

After running all the plates in the RT-qPCR, the Ct value was generated for all the samples and the genes of interest. The method followed for the gene expression studies was Pfaffl method (Pfaffl, 2004) as it requires to calculate the primer efficiency and allows normalization with multiple reference genes and gene specific amplification efficiencies. The standard mean Ct values of sample replicates was calculated using the average function in Microsoft excel software. The standard curve mean Ct values for different dilution was used to calculate the primer efficiencies (Equation 1 and Equation 2). Maximum value from the mean Ct values of the treatments was used as a calibrator value to calculate the delta Ct

value for each sample (Equation 3). Using the delta Ct values and the primer efficiency, the relative quantity (RQ) for each sample was calculated using the Equation 4. Using the geomean function in Microsoft excel, the geometric means for the two reference genes in the study was calculated. Further, using the RQ values of the target genes and the geometric values, the relative gene expression was calculated (Equation 5).

Equation 3

$$\Delta Ct = Caliberator\ Ct - sample\ Ct$$

Equation 4

$$RQ = E^{\Delta Ct}$$

Equation 5

$$Relative\ gene\ expression = \frac{RQ\ of\ target\ genes}{Geomean[RQ\ of\ reference\ genes]}$$

2.7 Statistical analysis.

The relative gene expression from the qPCR results were statistically analysed using Graphpad Prism 10.2.1 software (GraphPad Software, La Jolla California USA, www.graphpad.com). The outliers were identified using the Grubb's test (https://www.graphpad.com/quickcalcs/grubbs1/) and were excluded from the data. The Shapiro Wilk's test was used to confirm the normality of the data. The non-normal data was transformed using the log10 function in Graphpad Prism to attain normality. Since the data contains multiple variables, two-way ANOVA was used as the statistical tool to analyse the relative gene expression of the markers used in the study. The Tukey's HSD multiple comparison post hoc test was followed to identify the significant difference between the time points and the treatments. The level of significance was set to $p < 0.05$. A similar approach was used to statistically analyse the gene expression between both the tissues. Heat maps were generated based on means of the data using R studios Version (2024.04.0+735). The graphs of the statistical results were generated through Graphpad Prism software. The scoring data from the histopathological analysis was statistically tested by employing a Chi-square test with a significance level set at $p < 0.05$. Graphpad prism software was used to carry out the statistical analysis.

3. Results

No mortality of the fish was recorded following the infection challenge, however 100% of the fishes were euthanized at the end of the experiment for the tissue sample collection. Only a few species showed mild haemorrhaging on the ventral surface (Figure 5).

3.1 Detection results of *Y. ruckeri* using qPCR

The detection for *Y. ruckeri* was undetermined in the head kidney of almost all the samples. A replicate in the spleen showed a positive detection (Ct = 39.215) in the positive control and all the replicates in the *Y.ruckeri* DNA sample were amplified. In spleen samples, only the replicates with *Y. ruckeri* DNA samples used for reference were amplified, whereas all the samples showed undetermined results.

Figure 5. Sample with mild haemorrhages present on the ventral region under *Y. ruckeri* infection.

3.2 Histopathological analysis.

3.2.1 Pathologies in head kidney.

No severe changes in the tissue were observed. Chi-square test revealed no significant change within the tissue over different treatments and within timepoints ($p = 0.8280$). Typical pathological changes in the tissues were observed during the histopathological analysis under *Y. ruckeri* infection challenge in the samples collected at F2, F4, F5 and F6 timepoints. Pathologies such as early haematopoietic necrosis, fibrous connective tissue surrounding the tubules, presence of melano-macrophages, general cell congestion and necrosis of epithelial cells were observed (Figure 6).

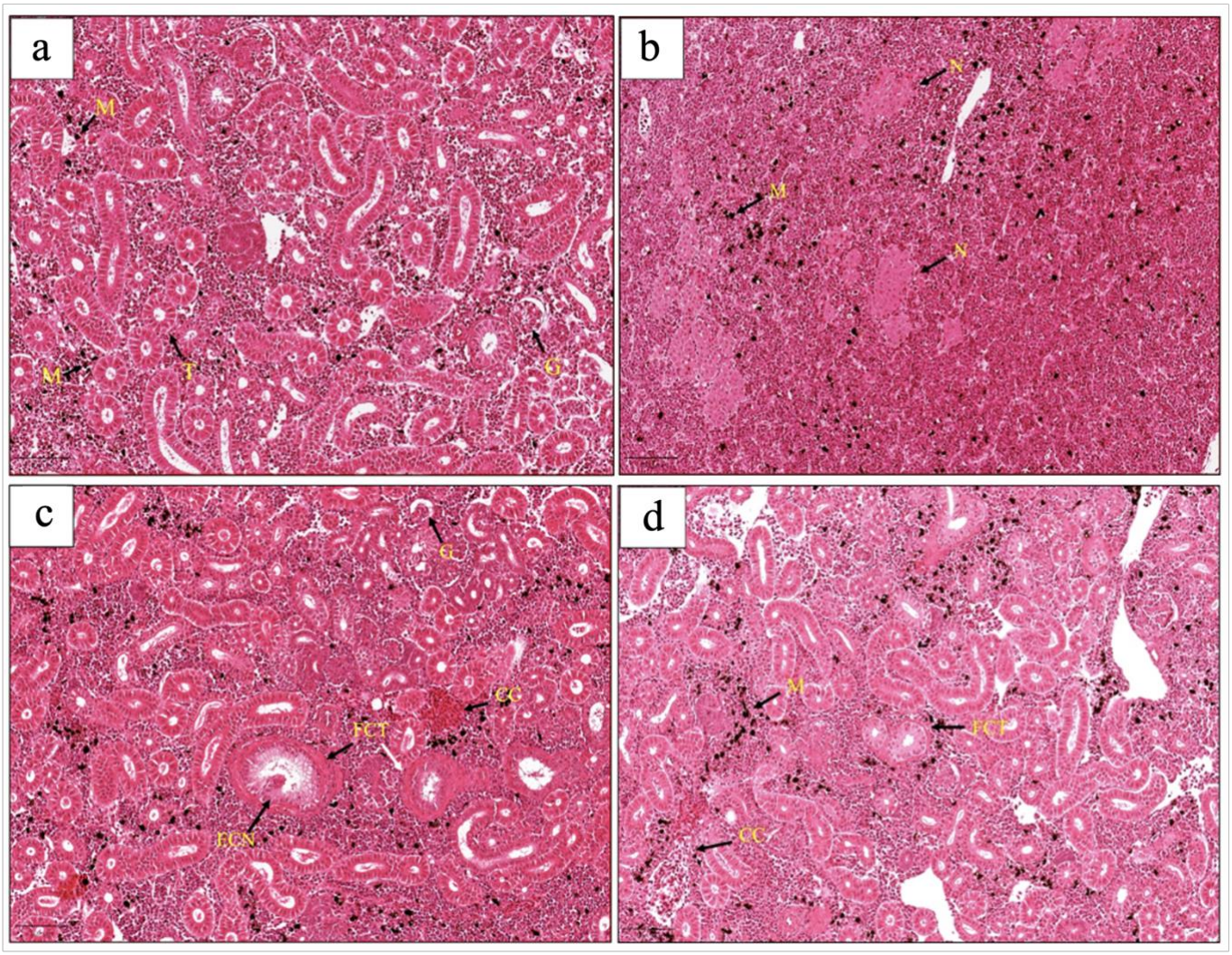

Figure 6. Histopathological assessment of *Y. ruckeri* infection in the head kidney of Atlantic salmon smolt (H&E staining), bar = 100µm. Figure 6a, shows the head kidney sample from T1 (control) at timepoint F1, b and c are samples from T2 at timepoints F2 and F3 respectively and d represents samples from T3 collected at timepoint F4. M = melano-macrophages, T = tubules, G = glomeruli, N = necrosis, FCT = fibrous connective tissues, ECN = epithelial cell necrosis and CC = cell congestion.

No change in the structure of the tissues was seen in the T1 control (Figure 6a). The tubules and glomeruli and collecting duct appeared normal. Figure 6b, which represented the fish sample from T2 at timepoint F2 , showed early signs of necrosis and a mild presence of melano-macrophages surrounding the

necrotic regions within the tissue. Figure 6c shows a presence of fibrous connective tissues surrounding the tubules and cell congestion indication haematopoietic necrosis in T2 fish sample collected at timepoint F3. Epithelial cell degeneration and necrosis in the glomeruli was also observed. Figure 6d shows a mild presence of melano-macrophages, haematopoietic necrosis and fibrous deposition around the tubules in fish samples from T3 collected at timepoint F4. No severe change was seen in the tissues.

3.2.2 Pathologies in spleen.

No severe changes in the tissue were recorded. Chi-square test showed no significant difference in the tissue damage between the treatments and within time points ($p = 0.1046$). Mild changes in the spleen was observed under the infection conditions in samples collected at time points F2, F4, F5 and F6. Typical pathologies such as necrosis of ellipsoids, high inflow of leukocytes in the tissue, melano-macrophages and cell necrosis (Figure 7).

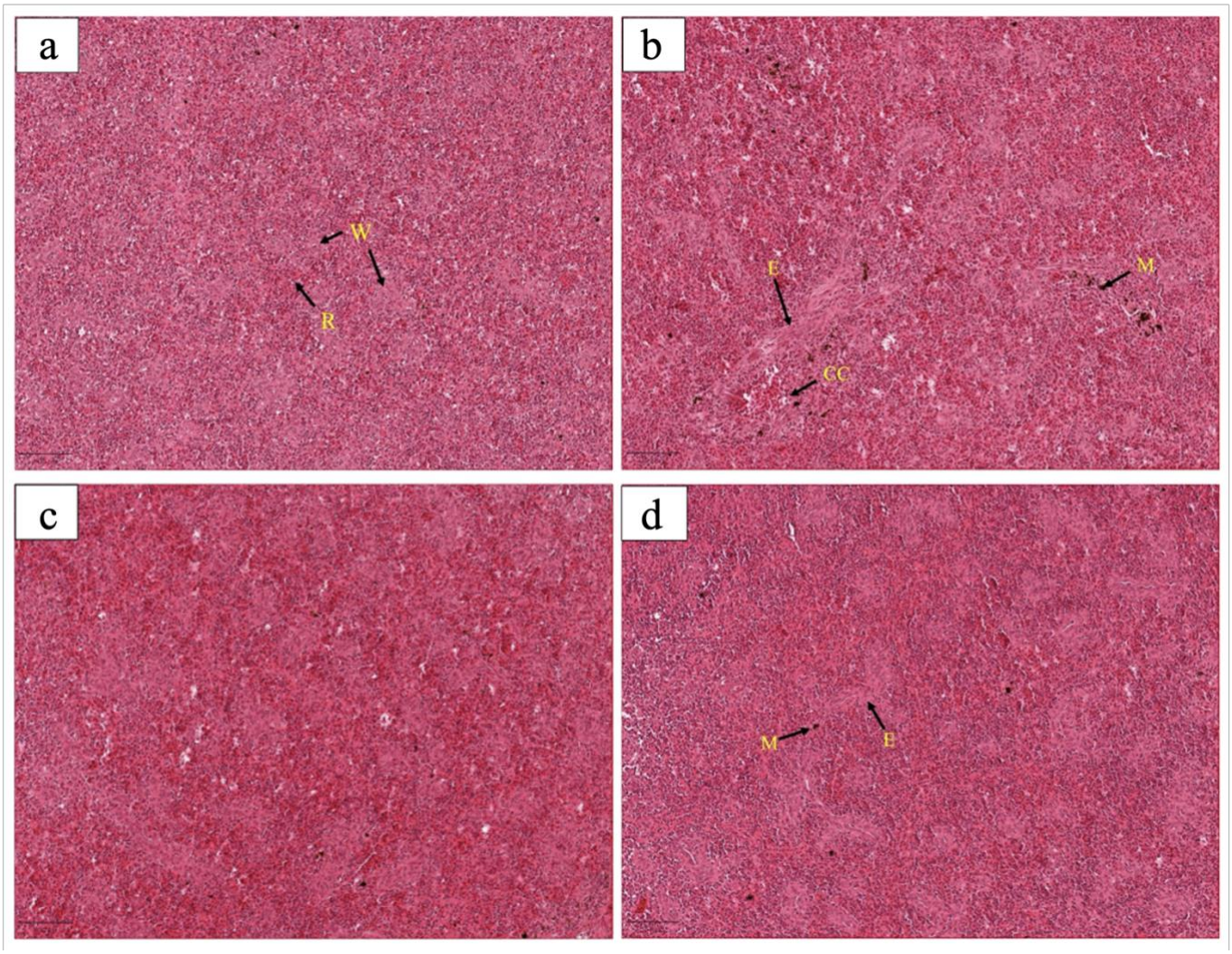

Figure 7. Histopathological assessment of *Y .ruckeri* infection in the spleen of Atlantic salmon smolts (H&E staining), bar = 100µm . Figure 7a, shows spleen sample from T1 (control) at time point F6, b represents sample from T2 at timepoint F4 and Figure 7c and d represents sample from T3 collected at timepoints F2 and F4 . M = melano-macrophages, W = white pulp, R = red pulp, E = ellipsoids and CC = cell congestion.

No damage was observed in the control group T1 collected at timepoint F6. Red pulp and white pulp appeared to be healthy (Figure 7a). A slight change in the tissue in the T2 from timepoint F4, where a mild cell congestion and lymphocytic depletion of the white pulp was seen with the presence of melano-macrophages in the vicinity of the damage (Figure 7b). The Figure 7c showed no changes in the tissue from T3 at time point F2 while some necrotic region was observed in tissue collected at timepoint F4 (Figure 7d) representing sample from T3. These results indicated no severe damage to the tissue due to low concentration pathogen challenge.

3.3 Gene expression response of Atlantic salmon to *Yersinia ruckeri* infection

Quantitative real-time expression of selected genes were evaluated in the head kidney and spleen of Atlantic salmon smolts during *Y. ruckeri* infection. The expression of genes from the samples from three different treatments (T1, T2 and T3) collected across three different timepoints F2 (before infection), F4 (72 hr or 3 dpi) and F5 (168 hr or 7 dpi) was compared.

As the Pfaffl method was used, the primer efficiency for all the markers were analysed. The primer efficiency (E) of the 10 genes used to test gene expression in head kidney, ranged from 90.10% to 107.02%, and the correlation coefficient (R^2), ranged from 0.9111 to 0.9985. The same genes tested for spleen resulted in primer efficiency (E) ranging between 88% to 103.63% and the R^2 values ranging between 0.9659 to 0.9954 (Table 8).

Table 8. Primer efficiency for head kidney

Reference genes	Primer efficiency(%) for head kidney	Primer efficiency(%) for spleen
actb	93.01	95.98
18s rna	90.10	88.38
Target genes		
il-1b	106.07	91.54
il-10	107.02	93.41
il-8	101.13	88.00
cath	103.06	102.33
hep	96.71	92.78
lys	93.33	102.43
mIgM	94.35	97.70
tnf-alpha	96.83	103.63

3.3.1 Gene marker expression in head kidney

A pro-inflammatory cytokine *interleukin-1 beta* (*il-1b*) gene showed a significant difference in *il-1b* expression at both 3 and 7 days post-infection (dpi) compared to pre-infection levels in Treatment 1 (T1), involving salmon fed a stable RAS diet without infection. The gene expression at 3 dpi was significantly higher ($p = 0.0001$) with respect to the pre-infection levels in the same treatment (T1). However, there was no significant difference between 3 dpi and 7 dpi within T1. Treatment 2 (T2) did not show significant changes in *il-1b* expression over time. In contrast, Treatment 3 (T3) displayed significantly lower *il-1b* expression compared to T1 at 3 dpi (mean difference: 0.5099, $p = 0.0376$). Additionally, at 3 dpi, T1 had significantly higher *il-1b* levels than T3 (Figure 8A).

Similarly, *interleukin-8* (*il-8*), a pro-inflammatory cytokine gene showed no significant difference over time in T1, however it was significantly higher ($p = 0.0001$) at pre-infection levels in T2 compared to T1. Additionally, the expression levels were significantly different within the timepoints in T2. The *il-8* levels in pre-infection were significantly higher compared to 3 dpi ($p = 0.0006$) and 7 dpi ($p < 0.0001$). There was no significant change in *il-8* gene expression levels in T3 over time (Figure 8B).

In contrast, an anti-inflammatory cytokine *interleukin-10* (*il-10*) showed no variations in the level of expression in T2 over time. T1 showed significant change in expression levels over time, where pre-infection time points differed from 3 dpi and 7 dpi significantly with $p = 0.0092$ and $p = 0.0341$ respectively. At 7 dpi, T1 and T3 showed significant changes in the expression levels of *il-10* gene ($p = 0.0337$). There was no significant change in the expression levels seen in T3 over time (Figure 8C). An anti-microbial protein encoding gene *cathelicidin* (*cath*) showed no significant difference in the levels of expression within and between the treatments (Figure 8D). *Hepcidin* (*hep*) gene, a key iron regulator anti-microbial peptide showed significant variations in the expression levels within T1. At 7 dpi, the expression of the gene was significantly higher than pre-infection levels ($p = 0.0080$). Similarly, *hep* gene levels significantly differed at 3 dpi when compared to pre-infected levels ($p = 0.0489$). When different treatments were compared, expression levels in T1 showed significant difference compared to T3 at pre-infection time point ($p = 0.0195$). However, *hep* expression levels did change not significantly over time within T2 and T3 (Figure 8E).

Membrane bound immunoglobulin (*mIgM*) expression showed changes under different treatments and over time. At 3 dpi, there is a significant difference in *mIgM* expression in T1 when compared to T2 and T3. An increased expression level of *mIgM* in T1 at 3 dpi is observed compared to expression levels at 3 dpi in T2 ($p = 0.0291$) and at 3 dpi in T3 ($p = 0.0118$). Additionally, *mIgM* levels showed a significant difference at pre-infection compared to 3 dpi ($p = 0.0429$) and to 7 dpi ($p = 0.0081$) within T2. Although no significant difference was observed within T1 and T3 over time (Figure 8F). No significant difference was observed in the gene expression levels of an innate immune gene *lysozyme* (*lys*) within and between treatments (Figure 9G). The expression levels did not change significantly over time.

Another pro-inflammatory cytokine gene *tumour necrosis factor-alpha* (*tnfa*) recorded a significant change in the expression levels at 7 dpi between treatments. A higher expression level was seen at 7 dpi in T1 compared to 7 dpi at T2 ($p = 0.0125$) and T3 ($p = 0.0134$). No significant changes were seen between the timepoints within all the treatments (Figure 9H).

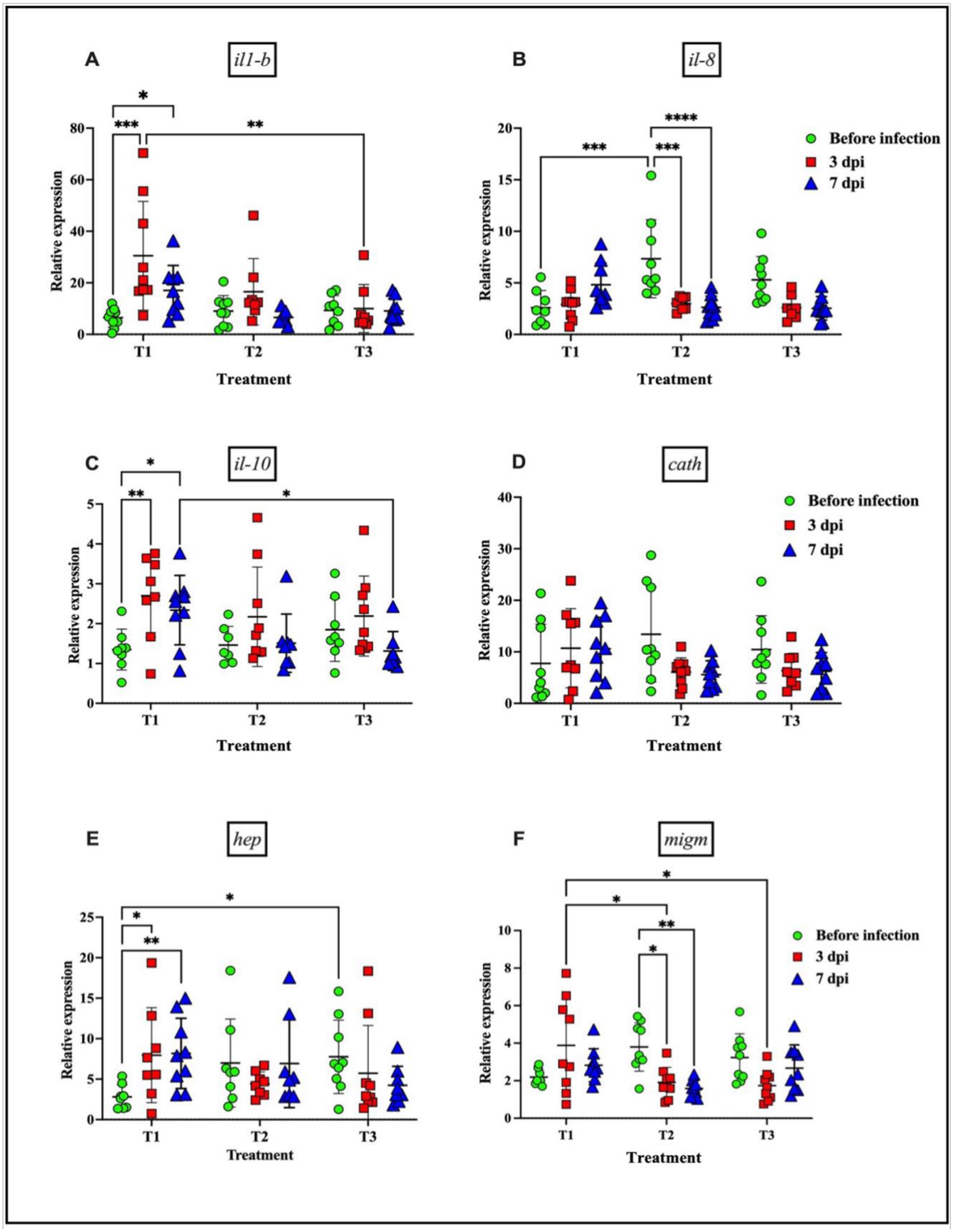

Figure 8. Gene expression in head kidney of Atlantic salmon smolts under *Y. ruckeri* infection indicating (A) *Interleukin-beta 1* (*il-1b*), (B) *Interleukin-8* (*il-8*), (C), *Interleukin-10* (*il-10*), (D) *Cathelicidin* (*cath*), (E) *Hepcidin* (*hep*) and (F) *Membrane bound immunoglobulin* (*mIgM*) under different treatments T1, T2 and T3 at different time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

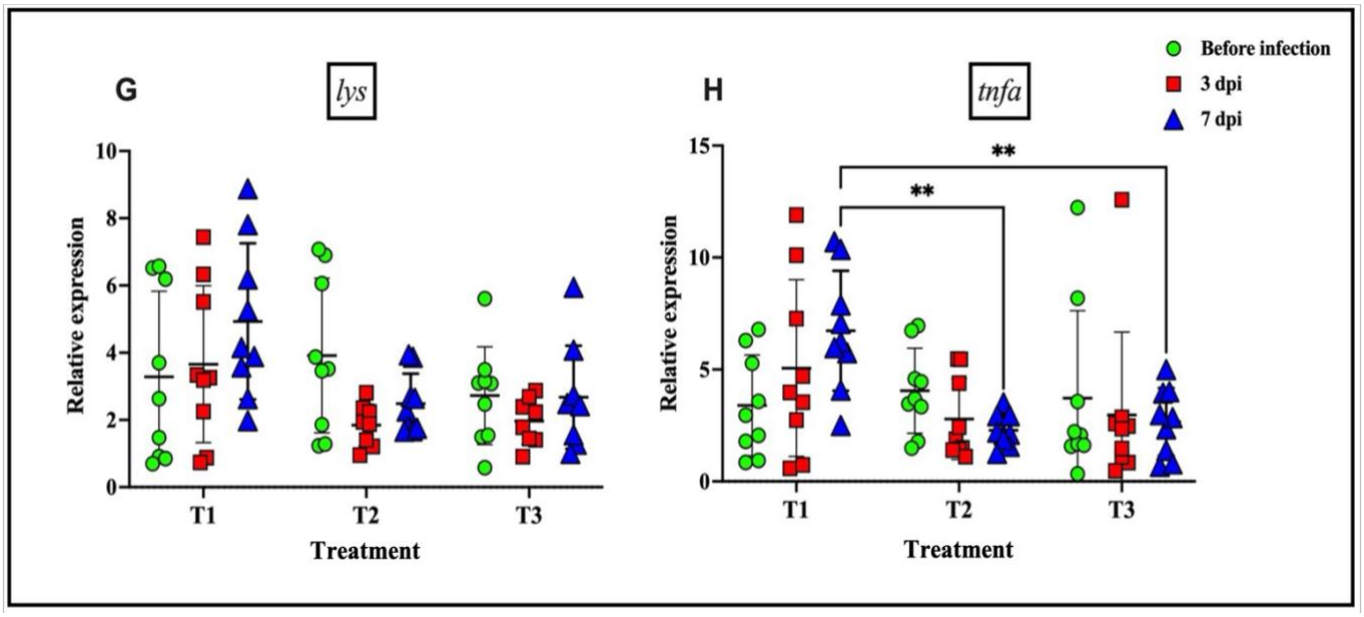

Figure 9. Gene expression in head kidney of Atlantic salmon smolts under *Y. ruckeri* infection indicating (G) *Lysozyme* (*lys*) and (H) *Tumour Necrosis Factor-alpha* (*tnfa*) under different treatments T1, T2 and T3 at different time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

3.3.2 Gene marker expression in spleen

A pro-inflammatory cytokine *il-1b* gene showed a significant difference in *il-1b* expression within T2. The gene expression levels were significantly higher at pre-infection levels compared to 7 dpi with p = 0.0231. No significant changes were observed within the T1 and T3 over time. Also no significant change was observed in the expression levels of *il-1b* gene across the treatments (Figure 10A). In contrast, an anti-inflammatory cytokine *il-10* showed no variations in the level of expression between and within all three treatments (Figure 10B).

Another pro-inflammatory cytokine *il-8* gene showed some significant changes in the treatments. A significantly higher expression level was observed at pre-infection time point compared to 3 dpi in T1(p = 0.0425). A similar change was observed in T2 where the gene expression was significantly higher before infection compared to 3 dpi (p = 0.0072). Although no significant interaction was observed in the gene levels in T3. Also no significant change was observed in the gene expression between the treatments (Figure 10C). *Cathelicidin* (*cath*) gene showed no significant difference in the levels of expression within T1 and T3. However, a significant change was observed in T2 where, the expression of the gene *cath* was significantly different between before infection and at 3 dpi timepoint (p = 0.0121). No significant change in the expression was observed between the treatments (Figure 10D).

Hepcidin showed a significant variation in the interaction across the treatments. A significant difference in the expression level was observed at before infection time point between T1 and T2 (p = 0.0448), where the T2 showed higher expression of *hep* gene than T1 (Figure 10E). However no interaction was reported between the time points in T1. A significant change was observed within T2. The *hep* gene levels during pre-infection were significantly higher than that during 3 dpi (p = 0.0005) and 7 dpi (p = 0.0062). T3 also showed some significant difference in gene levels within the treatment. Gene levels during pre-infection significantly differed compared to 3 dpi (p = 00391).

No significant difference was observed across treatments in the expression levels of *lys* gene. Although, in T2 a significantly higher expression value was seen during pre-infection when compared to 3 dpi (p = 0.0291). However, within treatment interaction in T1 and T3 did not show any significant difference in the expression levels (Figure 10F).

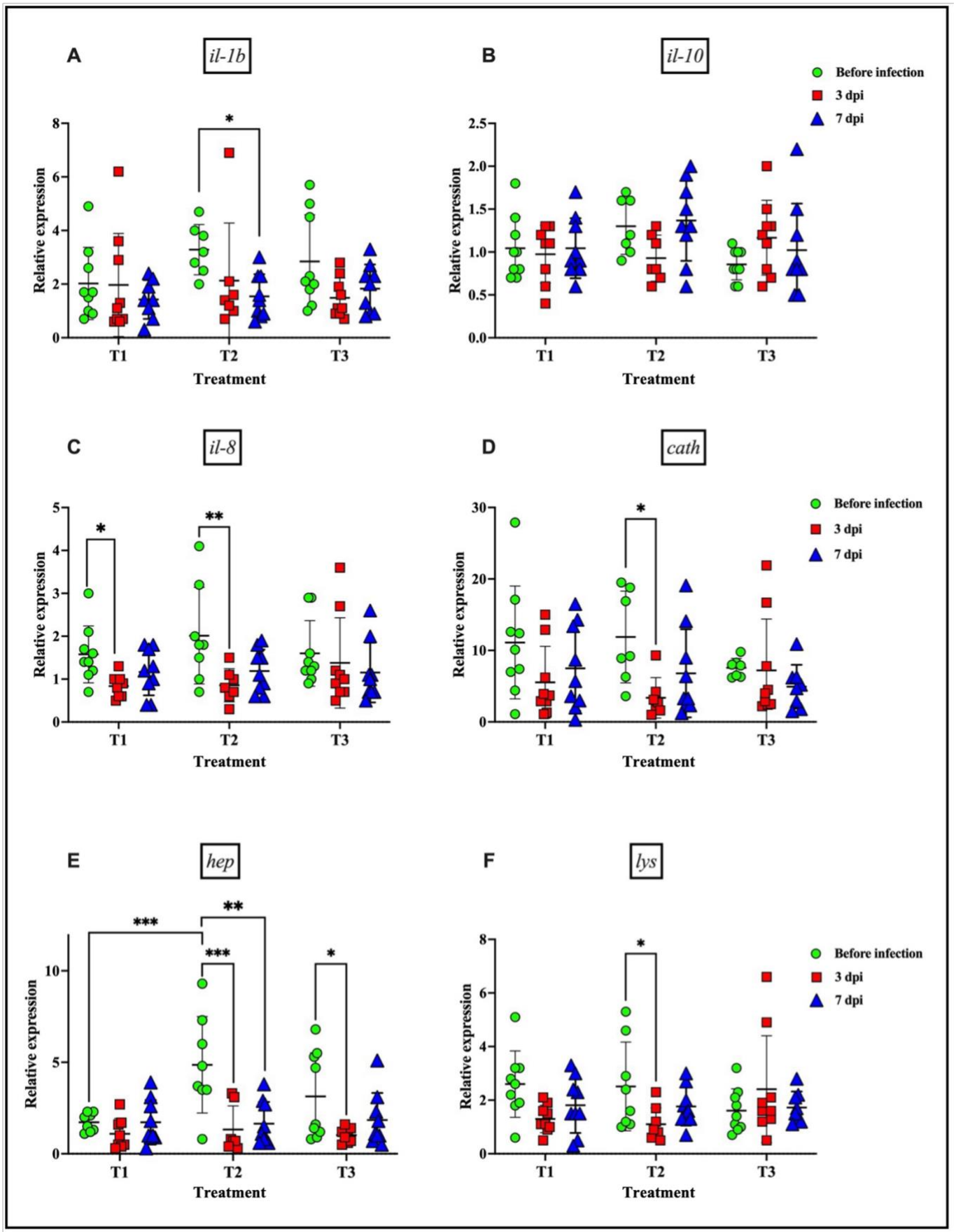

Figure 10. Gene expression in spleen of Atlantic salmon smolts under *Y. ruckeri* infection indicating (A) *Interleukin-beta 1* (*il-1b*), (B) *Interleukin-10* (*il-10*), (C) *Interleukin-8* (*il-8*), (D) *Cathelicidin* (*cath*), (E) *Hepcidin* (*hep*) and (F) *Lysozyme* (*lys*) under different treatments T1, T2 and T3 at different time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

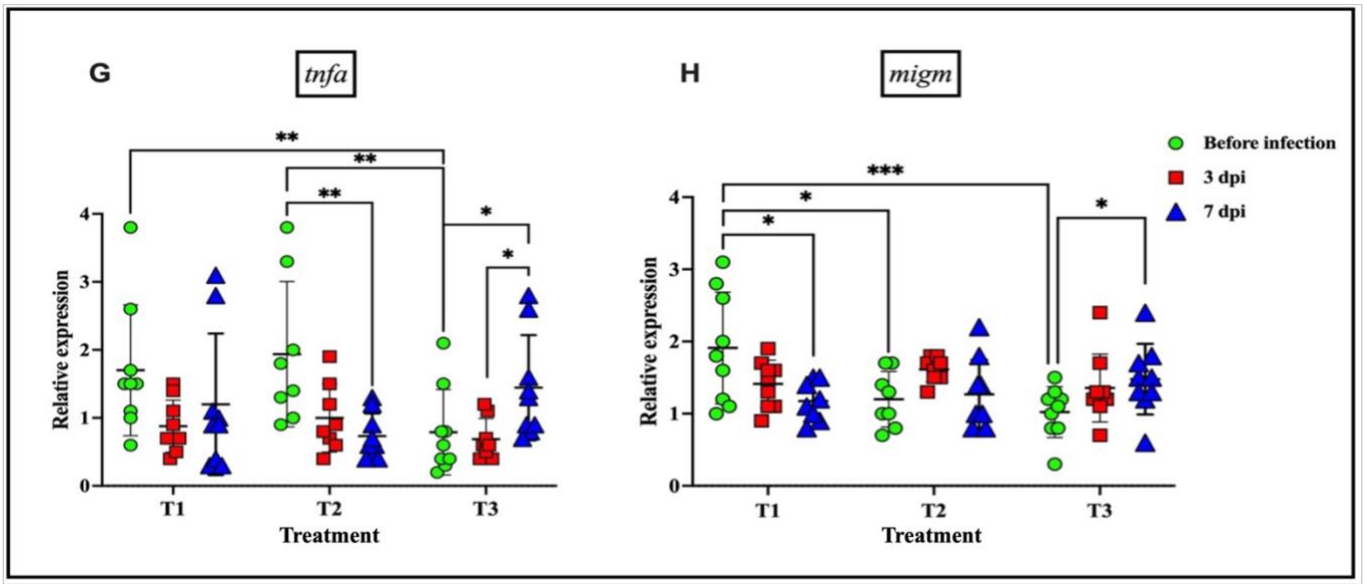

Figure 11. Gene expression in spleen of Atlantic salmon smolts under *Y. ruckeri* infection indicating (G) *Tumour Necrosis Factor-alpha* (*tnfa*) (H) *Membrane bound immunoglobulin* (*mIgM*) under different treatments T1, T2 and T3 at different time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Tumour Necrosis Factor-alpha gene showed significant change in expression levels across all the treatments. The *tnfa* gene expression value was significantly higher during pre-infection in T1 compared to T3 ($p = 0.0042$). Similarly, the expression levels differed significantly at before infection between T2 and T3 ($p = 0.0012$). A variation in the interaction within T2 and T3 was also observed. In T2, a higher gene expression values was seen at pre-infection compare to 7 dpi ($p = 0.0038$). In T3, the expression levels of *tnfa* was significantly higher than that of pre-infection ($p = 0.0203$) and 3 dpi ($p = 0.0367$) time point. However, within treatment interaction was not significant in T1 (Figure 11G).

Membrane bound immunoglobulin (*mIgM*) gene showed significant variations across the treatments. At the pre-infection time point, T1 showed a relatively higher expression of the gene *mIgM* compared to T2 ($p = 0.0274$) and T3 ($p = 0.0006$). It was also observed that in T1 the gene level during pre-infection was significantly different compared to 7 dpi ($p = 0.0283$). However, the expression level was significantly lower during pre-infection when compared to 7 dpi in T3 ($p = 0.0451$). No within treatment interaction was significant in T2 (Figure 11H).

3.3.3 Gene expression comparison between head kidney and spleen

The heatmaps provided below illustrates the means of relative expression levels of eight target genes in two tissues, the head kidney and spleen, under different treatment at three different time points: pre-infection, 3 days post-infection (3 dpi), and 7 days post-infection (7 dpi). Using the hierarchical clustering function for the heatmaps in R- studio, a pattern in the gene expression comparing the tissue was observed in the heatmaps.

Observing the pattern in Figure 12, which illustrates the comparison between the two tissues under T1, there is an upregulation of the gene *cath* in both tissues , while the gene *il-1b* showed a distinctly different expression pattern, being relatively higher in the head kidney than in the spleen. Also genes such as *hep* and *tnf-a* showed a higher expression in head kidney at 3 dpi and 7 dpi compared to spleen. Gene *lys* and *il-8* are clustered together indicating a difference in the expression pattern between the two tissues. While gene *il-10* and *mIgM* show a relatively similar trend in both the tissues.

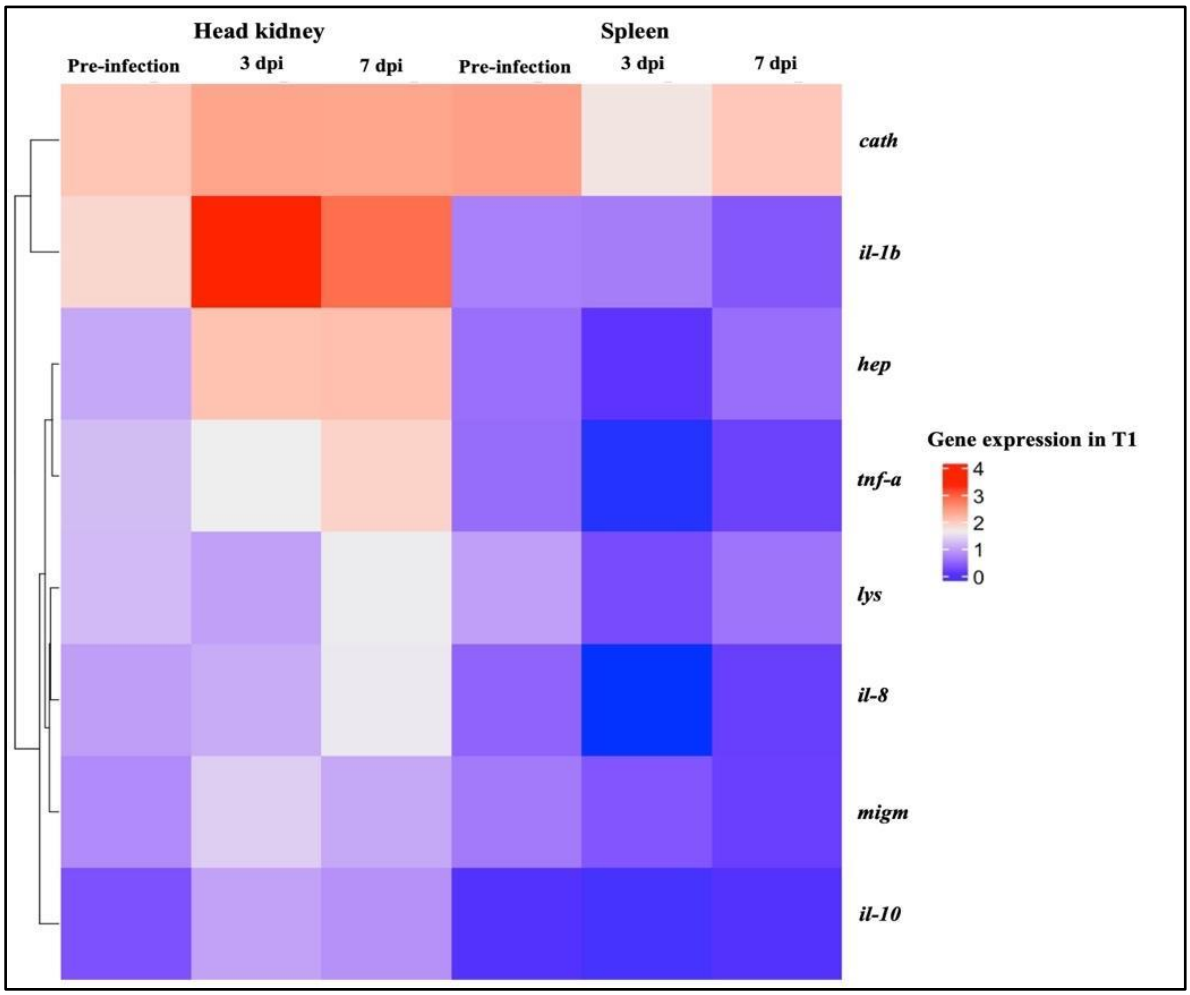

Figure 12. Gene expression comparison between the tissue head kidney and spleen in treatment T1 at particular time points shown in this heatmap created using R Studios. Rows representing the genes *Interleukin-beta 1* (*il-1b*), *Interleukin-10* (*il-10*), *Interleukin-8* (*il-8*), *Cathelicidin* (*cath*), *Hepcidin* (*hep*) and *Lysozyme* (*lys*), *Tumour Necrosis Factor-alpha* (*tnfa*) and *Membrane bound immunoglobulin* (*mIgM*) and columns representing the tissues under different time points; Pre-infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Log transformed means of the relative gene expression values were used for plotting the heatmaps and the log values are coded on red to blue scale (high expression = red and low expression = blue).

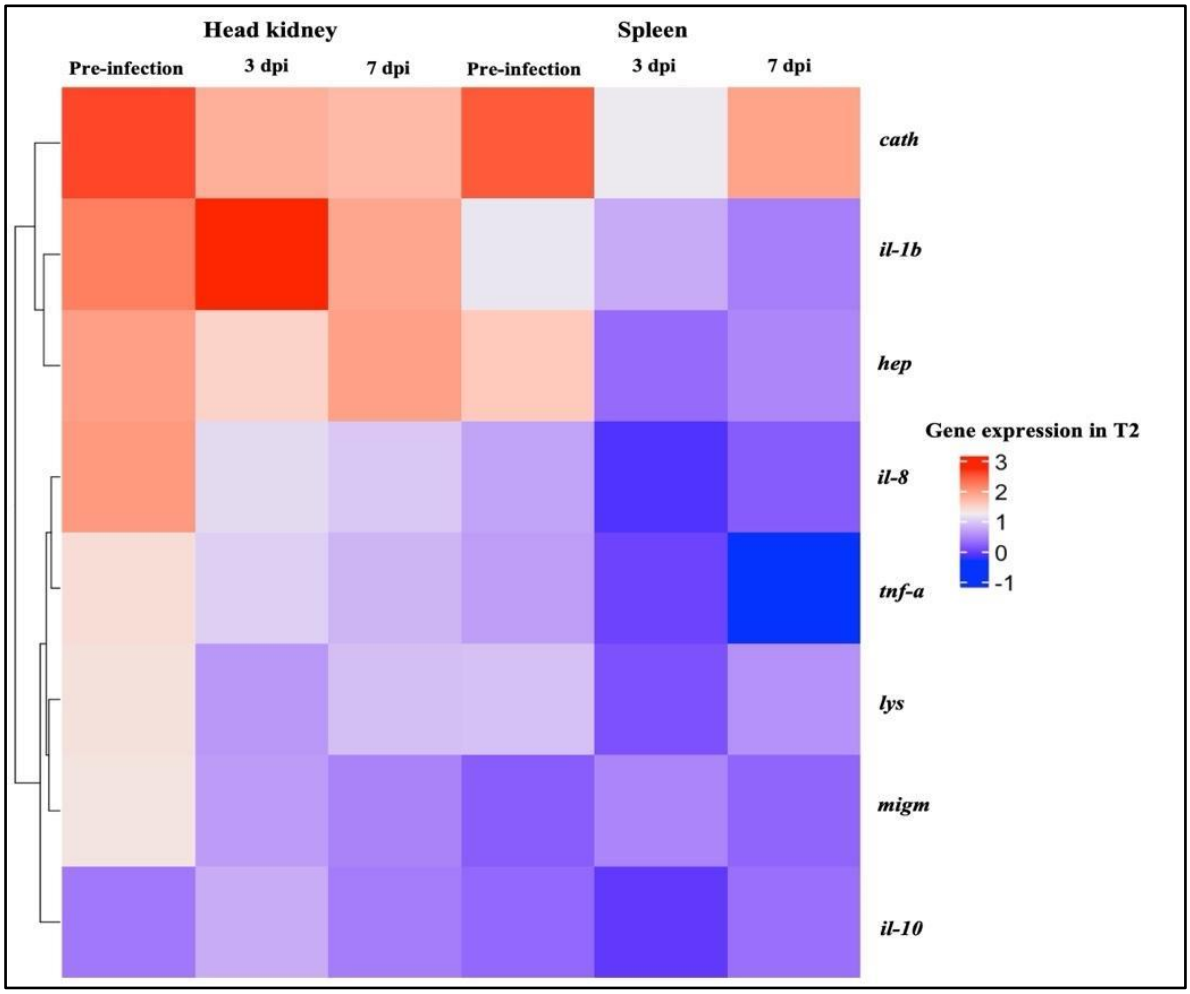

Figure 13. Gene expression comparison between the tissue head kidney and spleen in treatment T2 at particular time points shown in this heatmap created using R Studios. Rows representing the genes *Interleukin-beta 1* (*il-1b*), *Interleukin-10* (*il-10*), *Interleukin-8* (*il-8*), *Cathelicidin* (*cath*), *Hepcidin* (*hep*) and *Lysozyme* (*lys*), *Tumour Necrosis Factor-alpha* (*tnfa*) and *Membrane bound immunoglobulin* (*mIgM*) and columns representing the tissues under different time points; Pre-infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Log transformed means of the relative gene expression values were used for plotting the heatmaps and the log values are coded on red to blue scale (high expression = red and low expression = blue).

As far as the T2 is concerned (Figure 13), gene *cath* showed an upregulation in both the tissue, while both the genes, *il-1b* and *hep* show a dissimilar trend of higher expression and lower expression in head kidney and spleen respectively. A similar trend was seen in a gene cluster with *il-8* and *hep* showed higher expression values in head kidney compared to spleen. An elevated expression of genes *tnf-a, lys* and *mIgM* was observed at pre-infection timepoint in head kidney compared to spleen. Gene *il-10* seems to lie in the same spectrum of gene expression in both the tissues.

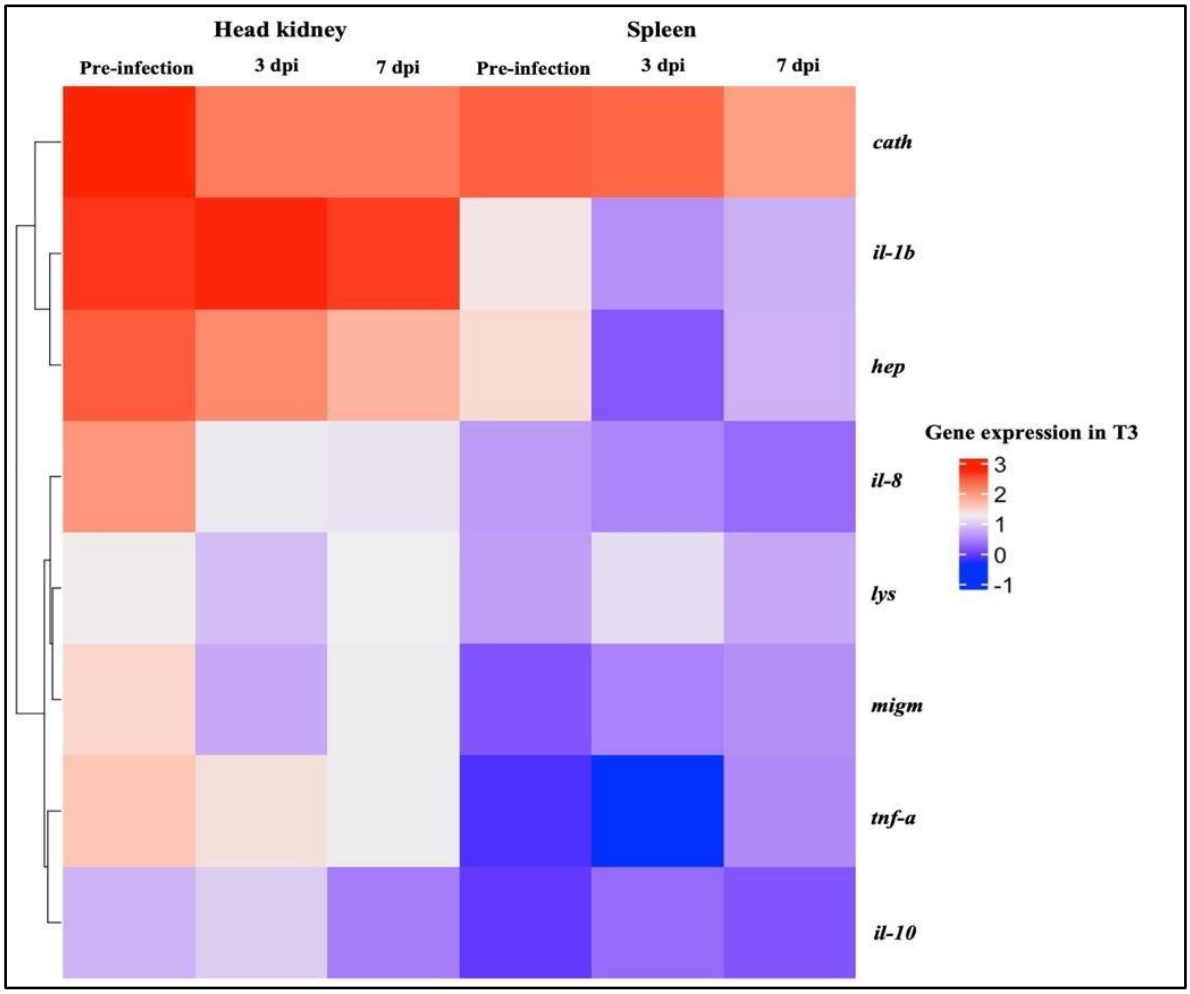

Figure 14. Gene expression comparison between the tissue head kidney and spleen in treatment T3 at particular time points shown in this heatmap created using R Studios. Rows representing the genes *Interleukin-beta 1* (*il-1b*), *Interleukin-10* (*il-10*), *Interleukin-8* (*il-8*), *Cathelicidin* (*cath*), *Hepcidin* (*hep*) and *Lysozyme* (*lys*), *Tumour Necrosis Factor-alpha* (*tnfa*) and *Membrane bound immunoglobulin* (*mIgM*) and columns representing the tissues under different time points; Pre-infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Log transformed means of the relative gene expression values were used for plotting the heatmaps and the log values are coded on red to blue scale (high expression = red and low expression = blue).

In T3, gene *cath* more or less have a similar expression in both the tissues. In case of *il-1b* gene a different trend was observed as a higher expression value in the head kidney was seen, whereas it appears to be down regulated in the spleen. A similar trend was seen in gene *hep.* A cluster of genes *il-8, lys* and *mIgM,* were differentially expressed in both the tissues, showed an elevated expression in head kidney compared to spleen. Gene *tnfa* and *il-10* lie in the same spectrum on gene expression in both the tissues (Figure 14).

As a trend of differential expression of genes was observed between the tissues under different treatments, a statistical analysis was carried out to understand the trend of gene expression within the treatments. Figure 15, Figure 16 and Figure 17 below illustrates the gene expression trend between the head kidney and spleen. A comparison was made between the time points within the treatments for head kidney and spleen.

Figure 15A depicts the comparison of expression of gene *il-1b* between the tissue head kidney and spleen. T1 showed a higher expression of the gene in the head kidney at pre-infection time point ($p = 0.0171$), as well as at 3 dpi ($p < 0.0001$) and 7 dpi ($p < 0.0001$) compared to spleen. In T2, no significant difference was observed at pre-infection between the tissues, however, there was a significantly higher expression of the gene in head kidney at 3 dpi ($p < 0.001$) and at 7 dpi ($p = 0.002$) when compared to spleen. T3 showed a significant difference in the expression of gene *il-1b* at all the time points between the two tissues, where head kidney showed an elevated expression than spleen at pre-infection ($p = 0.0121$), 3 dpi ($p < 0.001$) and 7 dpi ($p < 0.001$).

Figure 15B compares the *il-10* gene expression between the tissues. No significant difference was observed in the gene expression values between the tissues. Though a variation pattern was seen in the graph but was not statistically significant. Figure 13C shows a variation in gene *il-8* expressed between the two tissues. In T1, a significantly higher expression of the gene is seen at 3 dpi ($p < 0.001$) in head kidney compared to spleen. Similarly, higher gene expression values were seen at 7 dpi ($p < 0.001$) in former than latter. However, no significant change was seen at pre-infection time points between the tissues. In T2, head kidney resulted in higher expression of *il-8* gene ($p < 0.0001$) compared to spleen during pre-infection. However, at 3 dpi a downregulation of the gene in the spleen was observed compared to head kidney ($p < 0.001$). At 7 dpi, a significantly higher expression was seen in the head kidney ($p = 0.0096$) than the spleen. In T3, the head kidney showed significantly elevated values of gene *il-8* at all the three time points, before infection ($p < 0.001$), 3 dpi ($p = 0.0216$) and 7 dpi ($p = 0.0067$) compared to spleen.

Similar to gene *il-10,* no significant difference was observed in the expression levels of gene *cath* (Figure 16D). The expression values remained relatively similar between the time points within the treatments of both tissues. However, a different trend was observed in the expression of *hep* gene between the tissues (Figure 16E). An increased expression of the gene was seen in head kidney at 3 dpi ($p < 0.0001$) and 7 dpi ($p < 0.0001$) in T1 compared to spleen which showed a downregulation of gene at 3 dpi. However, no significant change was observed during the pre-infection time point. In T2, a downregulation was observed in the spleen at 3 dpi with a significantly higher expression of the gene in the head kidney ($p = 0.0007$). It was also significantly different at 7 dpi in the head kidney compared to spleen ($p = 0.0008$). However, no change was observed at the pre-infection interval. T3 had an elevated expression of the gene at pre-infection ($p = 0.0340$) and 3 dpi ($p = 0.0009$) in head kidney compared to spleen, whereas no significant difference was observed at 7 dpi. Figure 16F shows the *lys* gene expression comparison between the tissues. A significantly elevated expression of the gene was seen at 3 dpi ($p = 0.0298$) and 7 dpi ($p = 0.0016$) in head kidney when compared to spleen in T1. However, no significant differences were observed in T2 and T3 over time.

A significant difference was observed in the expression levels of gene *mIgM* at 3 dpi ($p = 0.0006$) and 7 dpi ($p = 0.0003$) in T1, where head kidney showed an upregulation of the gene compared to spleen

(Figure 17G). No changes were observed at the pre-infection timepoint. In T2, contrary to T1, a significant difference in the expression of the gene was observed at pre-infection time point with head kidney showing an elevated level of gene ($p > 0.0001$) than spleen. However, the expression of the gene did not change significantly at 3 dpi and 7 dpi between the tissues. T3 showed higher expression levels of the gene in head kidney at pre-infection ($p < 0.0001$) compared to spleen, also significantly higher levels were expressed at 7 dpi in head kidney than in spleen ($p = 0.0458$).

Figure 17H illustrates the comparison of expression of gene *tnf-a* between the tissues. No significant changes in the gene levels in T1 over time, however some variation was observed in T2 and T3. In T2, a significantly higher expression was seen in head kidney at 3 dpi ($p = 0.0467$) and 7 dpi ($p = 0.0042$) as well compared to spleen. In T3, a significant downregulation of the gene was seen in the spleen at pre-infection ($p = 0.0005$) and at 3 dpi ($p = 0.0126$) compared to the head kidney which showed higher gene expression values.

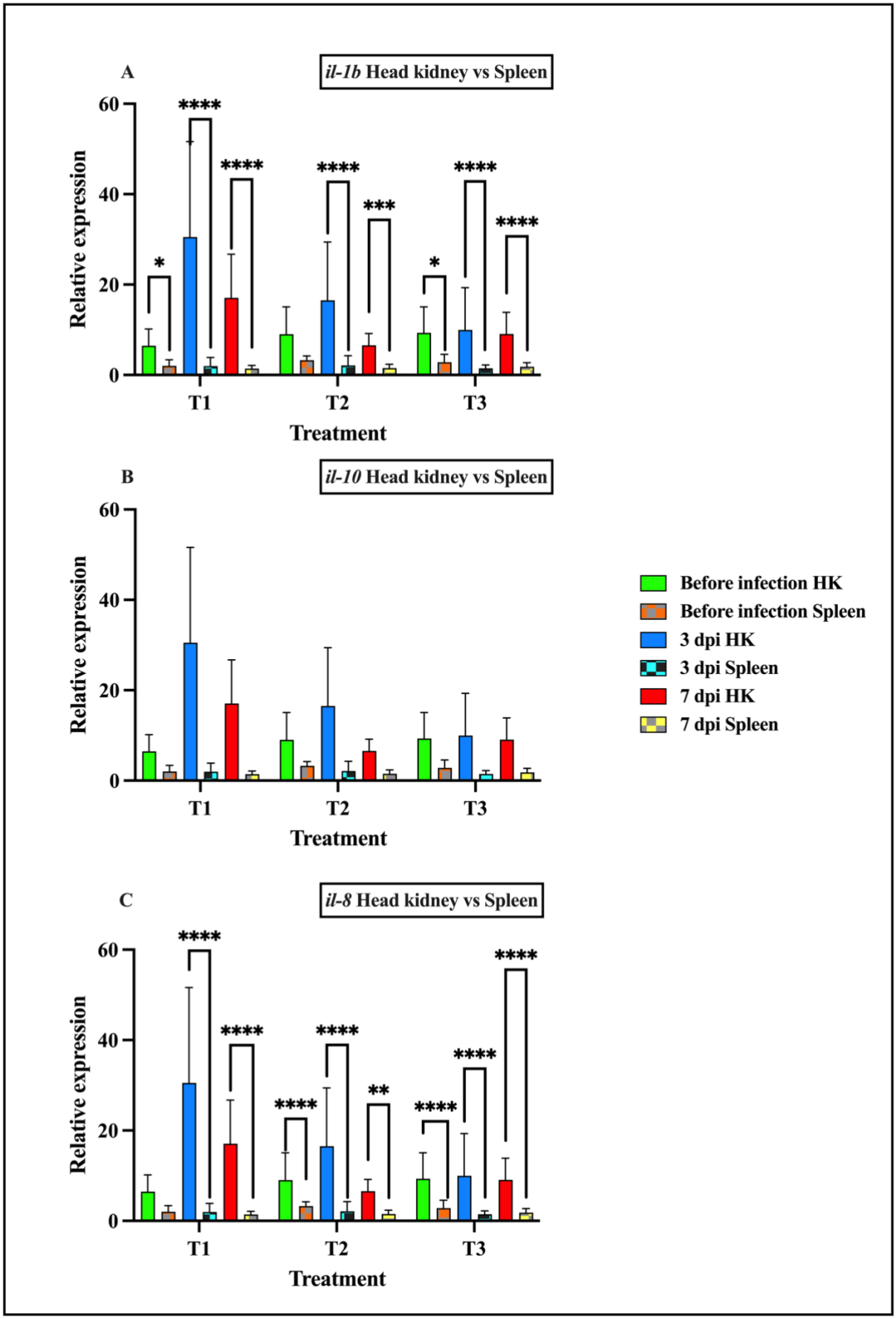

Figure 15. Gene expression comparison between head kidney (HK) and spleen of Atlantic salmon smolts under *Y. ruckeri* infection indicating (A) *Interleukin-beta 1* (*il-1b*), (B) *Interleukin-10* (*il-10*) and (C) *Interleukin-8* (*il-8*) under different treatments T1, T2 and T3 at particular time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

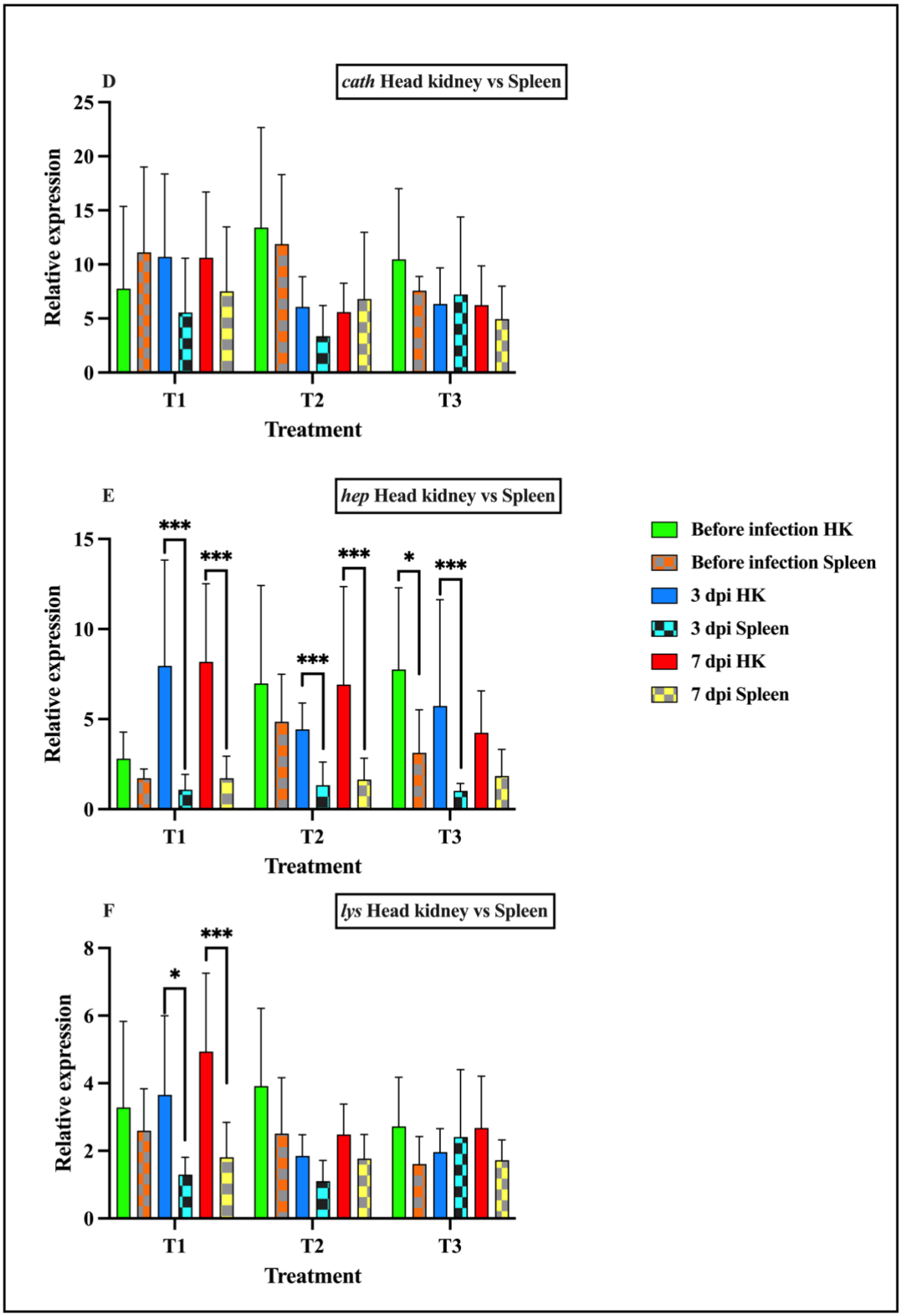

Figure 16. Gene expression comparison between head kidney and spleen of Atlantic salmon smolts under *Y. ruckeri* infection indicating (D) *Cathelicidin* (*cath*), (E) *Hepcidin* (*hep*) and (F) *Lysozyme* (*lys*) under different treatments T1, T2 and T3 at specific time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

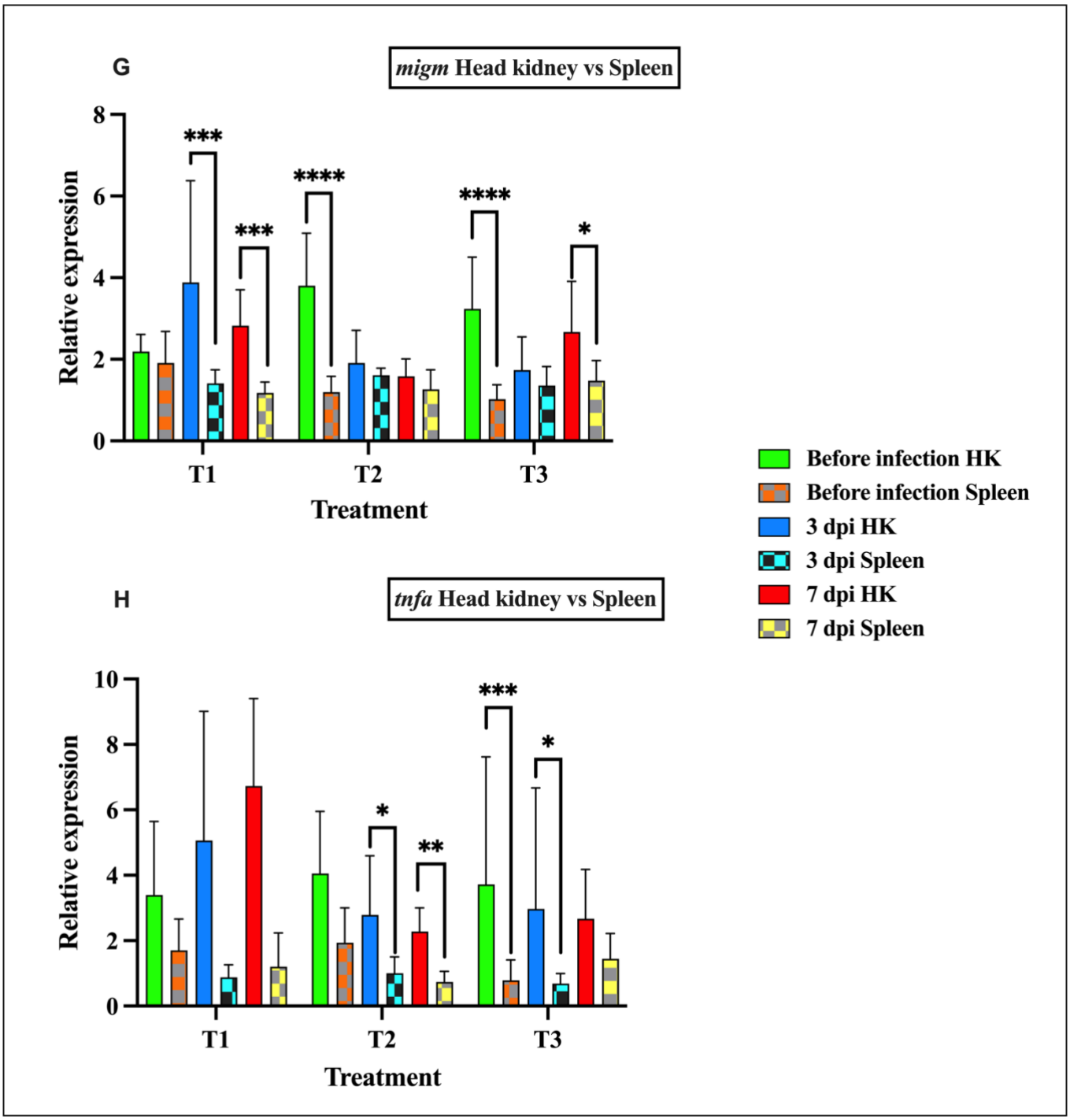

Figure 17. Gene expression comparison in head kidney (HK) and spleen of Atlantic salmon smolts under *Y. ruckeri* infection indicating (G) *Membrane bound immunoglobulin* (*mIgM*) and (H) *Tumour Necrosis Factor-alpha* (*tnfa*) under different treatments T1, T2 and T3 at specific time points, before infection, at 3 days post infection (3 dpi) and 7 days post infection (7 dpi). Asterisks indicate significant differences among groups at different time points as analysed by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

4. Discussion

This study explores the immune response of Atlantic salmon smolts following a simulated biosecurity breach in RAS using the pathogen *Yersinia ruckeri* as a model. In addition, it aims to understand the effects of a more stable RAS diet on the health of the salmon and its influence on the kinetics of response to infection.

RAS systems have gained more attention in the last couple of decades as it allows controlled production of the fish and a better bio-security compared to conventional flow through systems. This increase in the production capacity is achieved by higher stocking densities and controlling the environmental parameters optimal to the target species, despite a superior biosecurity of the RAS, numerous commercial RAS systems have faced infection outbreaks resulting in the high fish mortalities and economical losses. To understand the prevalence of the pathogen, it is important to study the immune response of the fish under the infection condition over a series of time intervals under different treatment conditions. Using the qPCR technology, eight important biomarkers related to the immune response in salmonids were studied in two different tissues, head kidney and spleen over a period of time. The results obtained from the study showed variable expression of these biomarkers in the tissues at different time points and under different treatment conditions. This study also analysed the histopathological changes in the tissues to correlate the change in the tissue based on the infection levels of the pathogen *Y. ruckeri*.

4.1 Undetermined results of *Y. ruckeri* in qPCR detection

No pathogen was reported in both, head kidney and spleen by the qPCR methods. This could be attributed to various different factors. Although, replicate showed a positive result but a higher Ct value above 35 suggesting a lower concentration the possibility of the pathogen present into the system cannot be undermined. As mentioned in the paragraph 3.1, a of the pathogen present. Pathogen was detected in kidney at 3 dpi but gave a negative result at 7 dpi due to increase in melano-macrophages and spleen showed no presence of the pathogen until 7 dpi, was reported in a *Y. ruckeri* challenge to rainbow trout study conducted by Ohtani et al. (2014). Higher pathological changes in the spleen such as splenic congestion in low exchange RAS systems indicated a higher degree of immune system challenge, suggesting a higher load of pathogen (Good et al., 2009). Unlike the current study, which was conducted with a pathogen challenge at 10^8 cfu/ml, no major changes were seen in the spleen indicating a low pathogenic load. A less virulent strain of *Y. ruckeri* is able to infect rainbow trout but is unable to survive inside the host for a long period of time, thus unable to result in ERM (Tobback et al., 2010). Biofiltration system of the RAS has also known to reduce the pathogen from the system. A recent study conducted by Hofstad (2023) demonstrated a non-establishment of 4 opportunistic bacterial strain in the non-disinfected biofilter carrier compared to a party disinfected biofilter carrier when challenged in Atlantic salmon fry RAS. The study also reported more stable RAS microbial community. In the current study biofiltration unit could be an attribute in reducing *Yersinia ruckeri* in the system.

Another possible attribute to undetermined results in the pathogen detection could be a target tissue in the current study. Numerous studies have established the first organ to be infected by *Yersinia ruckeri* to be gills from which it enters the blood and becomes systemic thereafter (Khimmakthong et al., 2013; M. Ohtani, 2019; Ohtani et al., 2014; Tobback et al., 2010). Most studies have also reported the presence of the pathogen in the intestine shortly after the infection (Khimmakthong et al., 2013; M. Ohtani, 2019; Sibinga & Marquis, 2021). In the present study, the target tissue of study were head kidney and spleen, which resulted in low to no presence of the pathogen. A further study by testing other organs that are potentially affected by *Y.ruckeri* could provide a comprehensive comparison between the tissue and its potential pathologies following infection.

4.2 No severe change in the tissue under infection of *Y. ruckeri*

Typical pathological changes to *Yersinia ruckeri* infection in both tissues was observed. Changes revealed in the study were mild to moderate of melano-macrophages present in the head kidney, with some degeneration/mild necrosis within the tubules and glomeruli. In this experiment all the fish survived the pathogen challenge following the biosecurity breach. The histopathological results in head kidney were more or less similar to the study conducted by Tobback et al. (2009) where 4 different strain of *Yersinia ruckeri* were used in challenge study against Rainbow trout (*Oncorhynchus mykiss*), but resulted in maximum fish mortality with *Y. ruckeri* infection. Spleen also showed similar results to this study under *Yersinia* infection with necrotic regions within the tissue and cell congestion. The spleen is a critical organ for both blood cell production and immune function in Atlantic salmon. Microscopically, it lacks distinct features but is primarily composed of various stages of blood cell development and dispersed melano-macrophages (Mousavi, 2023). The spleen has a thin capsule and a fine network of reticular cells that supports erythrocyte production in the red pulp and leukocyte production in the white pulp. Although there is limited literature on the impact of Enteric Red Mouth (ERM) disease on the spleen, two studies on *Yersinia ruckeri* infection in rainbow trout reported necrosis in the spleens of both deceased and acutely ill fish (Tobback et al., 2009). In the current study melano-macrophages in the head kidney were randomly distributed throughout the tissues while in spleen they were more concentrated near the necrotic region which was a similar finding in the previous by Tobback et al. (2009). This suggests a different immune response pattern in head kidney and spleen. Additionally, mild haemorrhages subcutaneously on the ventral surface was found in a few of the samples in this study which is also a typical pathological indicator of *Yersinia* infection, also confirmed in the previous study conducted by Kumar et al. (2015). A cycle of 30-40 days of intestinal shedding of ERM bacterium followed by re-appearance of gross philological changes and mortalities of the fish by 3-5 days was reported by the Busch and Lingg (1975) and reported a variation in the cycle of infection and mortalities based on the water temperature, loading factors, natural immunity of the population and other stressors. However, at different time points in the present study, there was no

significant change in the head kidney and spleen, which indicated that the tissues under this study were not significantly affected by the pathogen.

4.3 Biological relevance of the genes

In the present study, immune response of the Atlantic salmon smolts following a simulated biosecurity breach of *Y. ruckeri* infection was analysed using the 8 important immunoregulatory genes. It is crucial to understand the modulation of pro-inflammatory cytokines. Several studies shed light on how *Yersinia* interacts with the host immune system to evade or trigger inflammatory responses. *Yersinia* infection has been shown to inhibit the activation of pro-inflammatory cytokines like *Tnf-α* through various mechanisms (Philip et al., 2016; Schesser et al., 1998; Yao et al., 1999). Results from the present study indicates a differential expression in pro-inflammatory cytokines *il-1b, il-8* and *tnfa* genes in the head kidney, showed a lower expressions than the control group, which could linked to the immune-suppressive or evading mechanism of the pathogen. Spleen showed a similar trend in *il-1b* and *il-8* genes, but an elevated expression of *tnfa* gene in infected treatment T3 at 7 dpi indicates an active immune response of the cytokine against the pathogen. The downregulation or inactivation of the *il-8* gene in head kidney and spleen could further support the results for the undetermined results of *Y. ruckeri* within the tissue indication effective elimination of the pathogen either by immune response or by the disinfection unit of the RAS system. The role of *cathelicidins* as antimicrobial peptides that play a crucial part in the innate immune response of fish against bacterial infections is well-documented in the scientific literature (Broekman et al., 2013; Chang et al., 2006). Their ability to modulate the immune response by inducing the expression of pro-inflammatory cytokines such as *il-8* has been reported by Bridle et al. (2011). In the present study the insignificant change in the gene expression of *cathelicidins* in the head kidney could potentially mean the presence of a different factor that could suppress the expression of *il-8* gene in the system in T2. A study conducted by Chettri et al. (2012), reported an upregulation in the gene *il-10* at 3 dpi (6 x10^8 cfu/mL) of *Y. ruckeri* infection challenge. On the contrary, in the present study, no upregulation in the gene *il-10* was recorded in the head kidney and spleen against *Yersinia* (10^8 cfu/mL) following the biosecurity breach.

Hepcidin, a key regulator of iron metabolism, plays a crucial role in the host defense against infectious diseases. It controls iron stores by binding to ferroportin, thereby inhibiting the export of iron from cells. In mammals, the production of *hepcidin* is significantly increased during inflammation (Falzacappa & Muckenthaler, 2005; Ganz, 2003; Nemeth et al., 2004; Rivera et al., 2005). Expression of *hep* did not change following the breach.. The production of *hepcidin* is induced by a pro-inflammatory cytokine *interleukin-6* (*il-6*) (Nemeth et al., 2004). Since no major changes were seen in the pro-inflammatory genes in the present study, could indicate no activation of an iron-regulatory gene *hepcidin* in the head kidney against the pathogen. But a downregulation of the gene was identified in the spleen in the treatment T2 compared to the control at 3dpi and 7 dpi, which could be related to hypoxic condition and increased erythropoietic demand created by iron deficiency (Collins et al., 2008) in the diet since, the T2 received a

less stable commercial diet and experienced a higher organic load compared to other treatments (T1 and T3).

Membrane bound immunoglobulin is a type of immunoglobulins also known as B cell receptor which when binds with a specific antigen, triggers the signalling pathway within B cells, resulting in activation of immune response (Ma et al., 2022; Yang & Reth, 2010). In current study, no activation of the *mIgM* gene was seen in the head kidney, but an upregulation of the gene was observed in the spleen, suggesting spleen as an important immune organs in fish that contain large number of lymphocytes that can secrete antibodies against pathogens, which was also reported in a study by Cao et al. (2023) in Black rockfish (*Sebastes schlegelii*) challenged by two pathogen, *E. piscicida* and *V. anguillus*. In comparison of the two tissues, pro-inflammatory genes such as *il-1b, il-8* and *tnfa* had a significantly higher expression in head kidney than spleen. *Hepcidin* gene was significantly higher in head kidney when compared to spleen in all the treatments. This showed a more active immune response by the head kidney compared to spleen. Though a further study is required to understand the reason for a higher response of head kidney in Atlantic salmon smolts. The difference in the gene expression between the treatments 2 and treatment 3 in the present can also be a result of different diets incorporated in the treatments. Treatment 2 in both the tissues showed a significant variation between the time point as compared to treatment 3. This could be linked to a stress response in the fish related to higher microbial load in the treatment 2 as it received a less water stable plant based diet. Suspended micro-particles are capable of inducing stress response of aquatic animals, affect the respiration of gills and reduce the resistance to disease of fish (Bos et al., 2007; Michaud et al., 2009; Pedersen et al., 2017; Schumann & Brinker, 2020). A better relatively better immune response was seen in the group that receive RAS feed (T3), showed a higher expression of gene *tnfa* and *mIgM,* indicating an active inflammatory response including macrophages, T cells, and natural killer cells, in response to infection or injury. An increase in antiviral genes (*irf7b, isg15a, mmp-9, mxb*) related to interferon system against salmon lice and co-infection of *L. salmonis* and salmon *Orthomyxovirus* was reported in Atlantic salmon in RAS when feed with specialised diets containing different levels of EPA/DHA (Carvalho et al., 2024).

4.4 Conclusion and future prospects

In the current study, we studied the histopathological effects and gene expressions in head kidney and spleen tissue of Atlantic salmon smolts exposed to *Yersinia ruckeri* as a simulated biosecurity breach in RAS. The histopathological changes recorded within the tissue were found to be typical pathologies encountered during *Yersinia* infections, following the biosecurity breach. However, the study resulted in lack of differential gene expression of various genes in both the tissue, while gene *tnfa* and *mIgM* was upregulated in spleen, which was in similarity with the results in previous literature. Several other genes showed a down-regulation or no significant change in the expression when compared to the control treatment. This lack in differential expression could be attributed to the fact that the timepoints used in the

study, as some authors have reported the detection of *Yersinia ruckeri,* as early as 30 minutes post infection (mpi) in the intestine lumen and 7 dpi in spleen, liver and heart in Rainbow Trout (*Oncorhynchus mykiss*) (Ohtani et al., 2014) and as early as 0hr to 2.5hr in head kidney Rainbow Trout (*Oncorhynchus mykiss*) (Tobback et al., 2009). Presence of mild to moderate melano-macrophages in head kidney indicated an activated immune response of the head kidney could have resulted in lower expression of immunoregulatory genes in the later time points. A variation in the gene expression in treatment 2 receiving a less water stable plant based diet for both the tissues was observed which could be further studied to understand the effects of different diet formulation on the health of Atlantic salmon smolt under *Yersinia ruckeri* infection.

The complexity of infection dynamics in Recirculating Aquaculture Systems (RAS) can be attributed to various factors, including the intricate interplay between the aquatic environment, the fish species being cultured, and the microbial community within the system. The potential challenges in understanding and managing infection dynamics in RAS are multifaceted and can be influenced by factors such as water quality fluctuations, the presence of pathogens, the immune status of the cultured species, and the overall system design. Microorganisms are introduced into Recirculating Aquaculture Systems (RAS) through multiple pathways: makeup water, air, animal and insect exposure, feed, stocked fish, contaminated equipment, and staff or visitors (Sharrer et al., 2005). Asymptotic carrier is one of the characteristics of *Yersinia ruckeri* surviving in dormant state in a host (Rucker, 1966). These are various vectors through which can result in a biosecurity breach in RAS. Busch and Lingg (1975) recovered the pathogen from various organs of the carried trout population thus making it a systemic pathogen that can impact various organs. Studies have revealed high genetic diversity and epidemic population structures for *Yersinia ruckeri*, indicating the presence of multiple lineages and recent evolution under host selection. This genetic diversity can influence the virulence and adaptability of different strains of *Yersinia ruckeri*, complicating infection dynamics in RAS (Bastardo et al., 2012). *Yersinia ruckeri* produces various virulence factors that contribute to its pathogenicity and ability to establish infections in fish. The presence of adhesins, siderophores, and other virulence factors enhances the bacterium's ability to colonize and cause disease in fish populations (Menanteau-Ledouble et al., 2020; Wobeser, 1973). *Yersinia ruckeri* has the ability to form biofilms, which are structured microbial communities embedded in a self-produced matrix. Biofilms can protect the bacteria from environmental stresses, antimicrobial agents, and host immune responses, contributing to the persistence and resistance of *Yersinia ruckeri* within RAS (Fernández et al., 2004). These are some important factors that make *Yersinia* an important pathogen to study and understand its pathogenicity and impact on the health of the fish. The change in the immune response of the fish gives a better understanding of the immunoregulatory genes involved in activation of the defence mechanism of the fish. It is also noteworthy to study the potential hotspots of the RAS system that can harbour the pathogen which could induce infection into the system.

Due to lack of differential gene expression values in the tissue in the present study, the results for the gene expression study are inconclusive and further research with a high infection levels of *Yersinia ruckeri* along with a wide range of time point of sample collection and investigating different tissue types can provide a more comprehensive dataset to understand dynamics of *Yersinia* infection in Atlantic salmon.

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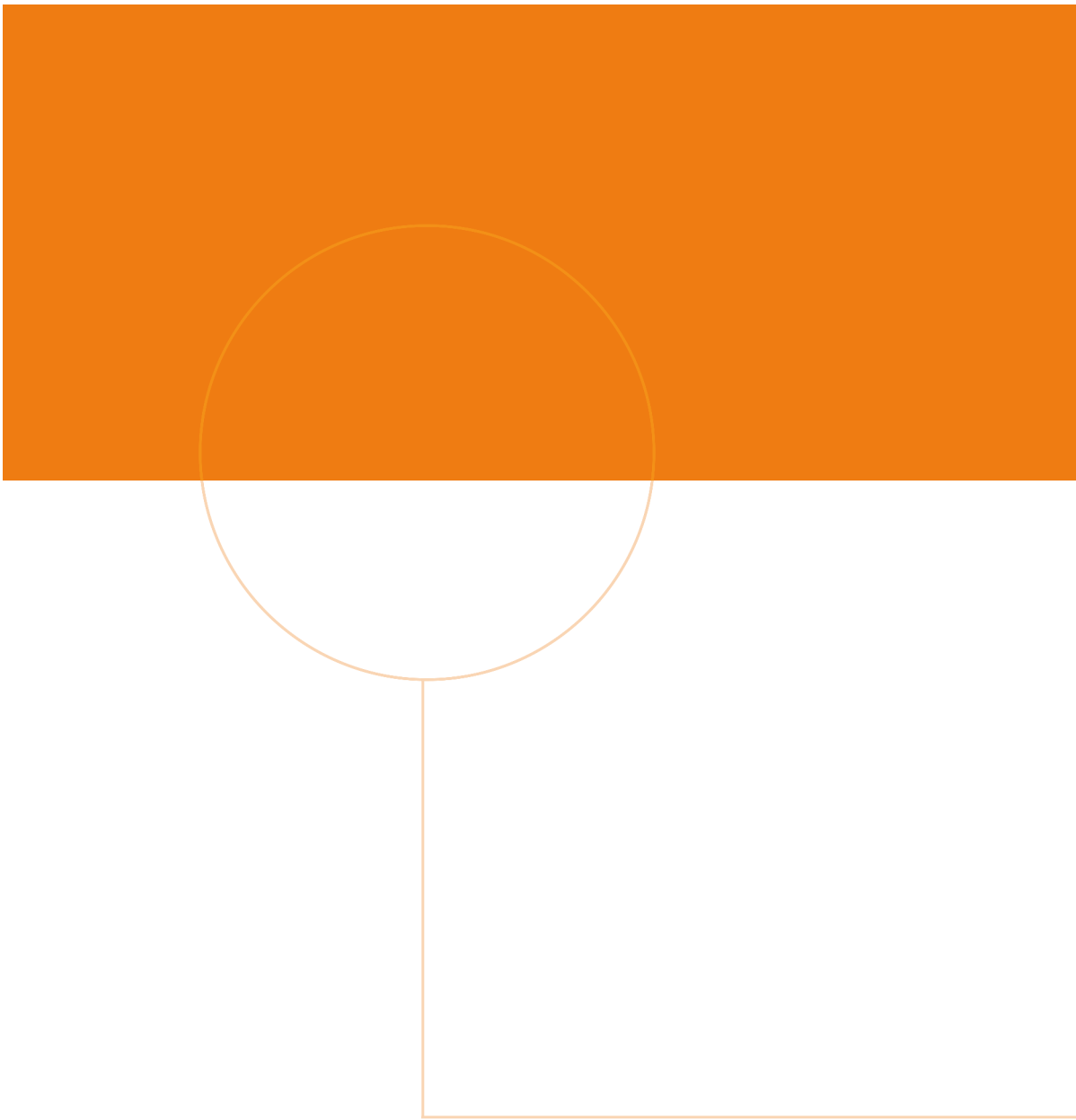

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