MASTER THESIS

Course code: BIO5002

Name / Candidate no.: 13
Megan Louise Doxford

Master Thesis in Biology and Aquaculture:

Interactions between Atlantic salmon (*Salmo salar*) intestinal macrophage-like cells and LPS.

Date: 14.05.2019

Total number of pages: 55

NORD University

www.nord.no

Acknowledgements

Thank you to Kiron Viswanath for supervising me on this project. A huge thank you to Youngjin Park for his invaluable assistance and time in the lab. Thank you to Ghana Vasanth for her time and help in running qPCR. To Ingvild Berg, thank you for passing on your skills and knowledge in cell isolation and cultivation. Thank you also to Qirui Zhang for your time and advice.

To Tina Reppe, Caroline Stensø, Kathrine Steinvik, Andrea Bozman and Cesilie Røtnes Amundsen thank you for being the exceptionally inspirational women you are. You have all pushed and inspired me to achieve the best I possibly can achieve.

To my Sku Bare family, thanks for putting up with me and getting me through some of the toughest days!

To my Parents, Lynda and Walter Doxford (and to Dougie). I cannot express my thanks enough for the endless support you have given me in so many ways! Your faith in me has kept me going, even during the times when I had lost it myself. You have always been there for me, every step of the way no matter how challenging it has been, and thanks to your unrelenting support I managed to get the job I always dreamed of!

Thank you to (Grandma) Joan King for always being a life line at the other end of the phone, no matter what time of day! You have always picked me up, dusted me down and helped me get back on track.

And finally, to Ioannis Konstantinidis. Without your knowledge, insight, help, chocolate cake and endless patience I would never have been able to complete my thesis. You have been the light at the end of a what has felt like a very long and dark tunnel. From lab partner to husband, you have given me help and support in every way imaginable. Ευχαριστώ παρα πολύ!

"If you want to improve, be content to be thought foolish and stupid…"

- Epictetus

Index

Index 1
1.0 Abstract 3
2.0 Introduction 4
2.1 Aquaculture 4
2.1.1 Fish Health 4
2.1.2 Antibiotics 4
2.1.3 Functional Diets 5
2.1.4 Vaccination 5
2.1.5 Treatment 5
2.2 The Gut 6
2.2.1 Anatomy and physiology 6
2.3 The Immune System 9
2.3.1 Innate Immunity 9
2.3.2 Adaptive Immunity 12
2.3.3 Humoral Factors 14
2.4 Gram Negative Bacteria 17
2.4.1 Cell Wall and LPS Structure 17
.... 18
2.4.2 LPS Cellular Response 19
2.5 Immune relevant genes selected in this experiment 20
3.0 Materials and Method 22
3.1 Fish 22
3.2 Tissue Extraction 22
3.3 Cell Isolation 22
3.4 RNA Extraction, transcription and qPCR 23
3.5 Flow Cytometry 24
3.6 Solution Preparation 24
4.0 Results 26
4.1 Cell Extraction 26
4.2 Flow Cytometer 26
4.3 Post-Extraction Cell Counts and RNA Concentration 27
4.4 qPCR 30
4.4.1 Distal Intestine qPCR 30
4.4.2 Statistical analysis of qPCR results 31
5.0 Discussion 34
5.1 Cell isolation and identification 34
5.2 Gene Expression 34
5.2.1 MyD88 dependent pathway 34
5.2.2 MyD88-independent pathway 35

5.2.3 TNFα 36
5.2.4 IL-1β 37
5.2.4 IL-10 37
5.2.5 CR3 38
5.2.6 IFNγ 38
5.2.7 Mul1b 39
5.3 Alternative genes and cellular pathways 39
5.3.1 Toll-Like Receptor 2 39
5.3.2 COX-2 40
5.3.3 Macrophage colony stimulating factor (M-CSF) 40
5.3.4 MAPK 40
5.3.5 NFκB 41
5.4 Respiratory Burst/NOS 42
5.5 Protein expression 42
5.6 Type of LPS used 43
6.0 Conclusion 44
References/Bibliography 45

1.0 Abstract

Vaccine use in the salmon farming industry is now common practice, however current vaccines tend to be delivered by intraperitoneal injection. This method is labour intensive and requires handling of fish. An alternative route of vaccination could be through oral vaccines that can be delivered via feed, reducing the need for handling and stressing fish. To develop successful oral vaccines, more knowledge on the immune system in the gastrointestinal tract is required. The aim of this thesis is to help broaden the knowledge of the immune response of macrophage-like cells from the gastrointestinal tract of Atlantic salmon.

In the current thesis, the immune response of macrophage-like cells exposed to lipopolysaccharide (LPS) stimulation was investigated. To achieve this, cells were extracted from the distal intestine and pyloric caeca of Atlantic salmon. After their isolating cells, they were separated into two groups. The first group was treated with a L-15 media containing LPS from *Escherichia coli* (*E. coli*) while the second group was treated with L-15 media alone as a control. After incubating for 6hours, RNA was extracted and gene expression of TNFα, IL-1β, IL-10, MyD88, CR3, Mul1b and IFNγ was quantified. Interestingly, TNFα, IL-10 and IFNγ showed higher expression in cells stimulated with LPS.

2.0 Introduction

2.1 Aquaculture

In 2016, global fish production peaked at 171 million tonnes. Aquaculture represented 47% of said production (FAO, 2016). The demand for fish is on the rise, with human consumption of fish per capita rising by approximately 1.5% each year in the period between 1961 and 2015. In the latter year, 17% of all animal protein consumed by humans was fish, with 20.2kg of it being consumed per person (FAO, 2016). As this trend rises, more fish must be sourced to accommodate the needs of the population. Wild fisheries are a finite source, and can be heavily influenced by numerous uncontrollable factors such as El Niño. Therefore, to meet demands, aquaculture must continue to grow and develop.

Global farmed fin fish production lay at 54.1million tonnes and was worth USD 138.5billion in 2015. Norway is the second largest exporter of fish after China. Atlantic salmon (*Salmo salar*) accounted for 4% of all aquaculture produce in 2016 with Norway contributing 1.7% of all food fish production (FAO, 2016). In 2016, Atlantic salmon accounted for 93% of all aquaculture production in Norway (Statistisk sentralbyrå, 2016) producing a total biomass of 738000tonnes (Fiskehelserapporten, 2016).

2.1.1 Fish Health

Mass production of any livestock however, does not come without issues. Farming is intensive, and under such conditions of high density of a given species in one area, pathogens and their consequential diseases spread easily. Both the cost of treatment and mortalities is high in the salmon farming industry. Sea lice are ectoparasites that feed on salmon, causing stress and lesions to the fish. Farming conditions allow for the rapid spread and growth of this parasite at a phenomenal cost to the industry. To prevent such health issues and to allow for good fish welfare, health management practices must be implemented to reduce losses.

2.1.2 Antibiotics

Antibiotics are used in the treatment of bacterial infection outbreaks. Their use however, is highly undesirable due to a number of reasons. Firstly, increasing antibiotic use also has the potential to increase bacterial resistance to said antibiotics, limiting future treatments. Secondly, many people are against eating fish exposed to antibiotics, and so a reduced usage of antibiotics also pleases the market.

Antibiotic use is on the decline. In 2012, 1591kg of active substance was used in Norwegian salmon farming that had a total biomass of 709000tonnes. This has decreased to 212kg in 2016 despite an increase to 738000tonnes total biomass (Fiskehelserapporten 2016).

2.1.3 Functional Diets

Functional diets refer to feed formulated to give extra beneficial protection to the fish. These feeds may contain prebiotics and/or probiotics. Prebiotics are non-digestable ingredients that positively affect the host, for example the carbohydrate Mannanoligosaccharides (MOS). Probiotics are live organisms that are beneficial for health (Gonçalves et al., 2017). Microbiota in the intestine are able to be modulated by both, which could in turn alter both endocrine and neurocrine responses in the gut (Mayer, E.A., 2015).

2.1.4 Vaccination

Vaccination is used to develop the fish adaptive immune system to be able to respond to specific pathogenic invasions. Vaccines may be used to defend against bacterial or viral diseases. For example, *Salmonid alphavirus* (SAV) causes Pancreas Disease which is fatal in Atlantic salmon and can cause mass mortalities. It is now common practice to vaccinate against SAV which helps to reduce the likelihood of outbreaks and overall mortality caused by the disease, however it is not as efficient as vaccines against bacterial infections (Fiskehelserapporten, 2016).

Vaccination can often lead to the formation of connective tissue between the inner organs and peritoneal walls, as well as melanin deposits within the organs and it may also cause reduced appetite post-vaccination (Fiskehelserapporten, 2016). Therefore there is a growing interest into the development of oral vaccines which would negate these negative side effects.

2.1.5 Treatment

Current treatments within the aquaculture industry are often labour and handling intensive. It is thus more beneficial to develop treatments that would reduce handling stress, production costs and most importantly provide healthy protection to the fish.

One such line of treatment is the development of oral vaccinations. Creating encapsulated antigens for uptake in the gastrointestinal tract. Petrie and Ellis (2006) have shown that soluble antigens are able to be absorbed in the hindgut. Joosten et al. (1996) showed that fractions of the bacteria *Vibrio anguillarum* can be up taken in the hindgut via anal intubation. Interestingly, it has been shown that T-Cell populations in the gut of Atlantic salmon can be modified by dietary changes (Bakke-McKellep et al., 2007). It is also thought microbiota have an influence on modulating the immune response (Nayak at al., 2010). Further to this, Gonçalves et al. (2017) have shown that probiotics are capable of protecting against density stress in farmed Rainbow Trout.

In order to create successful oral vaccines or modified diets, in depth knowledge of the salmon immune system specific to its digestive tract must be developed. The focus of this thesis is to help broaden the knowledge in this field. For the purpose of this thesis, a discussion will take place on the anatomy and physiology pf the gastrointestinal tract and the immune system of Atlantic salmon, as well as its cellular components.

2.2 The Gut

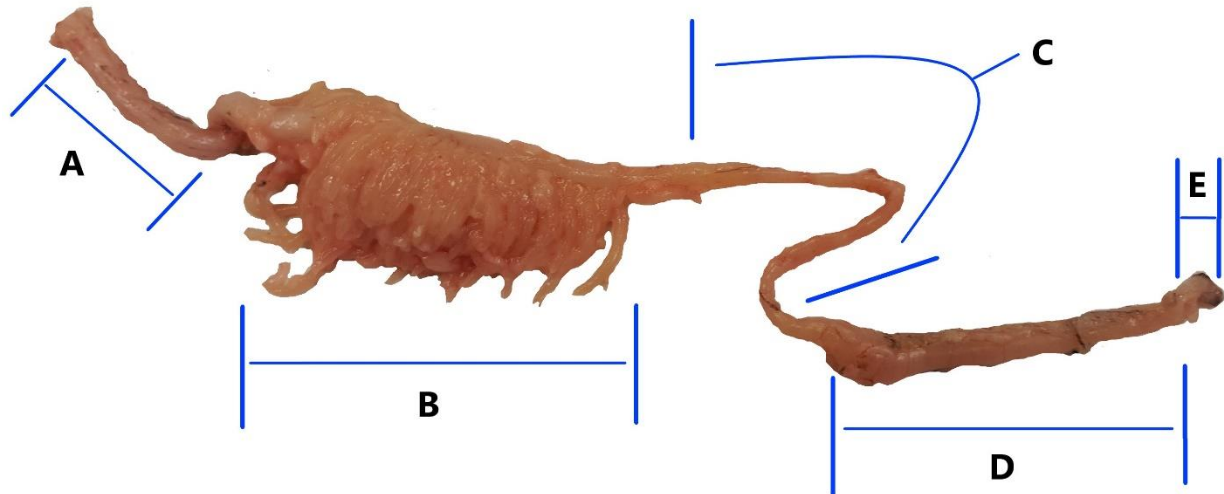


Figure 1*: The salmon gastrointestinal tract. A - oesophagus, B - pyloric caeca, C - mid/anterior intestine, D - distal/posterior intestine, E – Rectum.*

2.2.1 Anatomy and physiology

The gut refers to the gastrointestinal (GI) tract from the mouth to anus as shown in figure 1. It is involved in osmoregulation, digestion of food and nutrient absorption, as well as pathogen defence. It can be divided in to four main regions (Olsson et al., 2011):

- Head gut: mouth and pharynx
- Foregut: oesophagus and stomach
- Midgut: pyloric caeca and anterior/mid-intestine
- Hindgut: distal intestine

The head gut is responsible for ingestion of food. The stomach has a high pH environment to help digest food and has the potential to destroy any entering pathogens. The midgut is where the main nutrient absorption occurs. In this region there is a high concentration of free nutrients, low bacterial load and high paracellular permeability. In contrast, the hindgut has a higher concentration of bacteria, lower free nutrient content and a lower paracellular

permeability but higher water extraction. There is also higher macromolecule endocytic and transport activities in the hindgut, potentially making it an area for high antigen sampling activity (Jutfelt et al., 2011).

The morphology of the teleost gut varies between species and their respective diets. The GI tract tissue is made up of multiple layers; mucosa, lamina propria and muscularis mucosa. The mucosa is a singular layer of epithelial cells that includes goblet cells which produce mucus, endocrine cells which react to lumen contents and enterocytes for nutrient absorption (Olsson et al., 2011).

Jutfelt et al. (2011) describe the intestinal immune barrier as being separated into 3 layers:

- Extrinsic: mucus which acts as a lubricant and as a physical and chemical barrier
- Intrinsic: physical barrier formed by cells connected by tight junctions
- Immunological: innate and adaptive immune system

Epithelial cells form a monolayer with the apical ends joined by proteins. Adjacent cells are then joined via cytoskeletons attached by desososomes and cadherins. This monolayer is highly regulated, with tight junctions being maintained closely connected to reduce permeability between cells (Jutfelt et al., 2011).

The intestinal epithelium is composed of intestinal epithelium cells (IECs), macrophages, intraepithelial lymphocytes (IELs) and goblet cells. IECs form a physical barrier, joining together via tight junctions, preventing pathogen entry into bodily fluids and tissues. Goblet cells produce mucus which lines the inner lumen of the intestine. IELs produce cytokines and chemokines which signal to both the innate and adaptive immune systems (Cain and Swan, 2010). The epithelial immune response within the gut is modulated by cortisol (Niklasson et al., 2014).

Mucus is a lubricant and protects the surface of the intestine from the abrasive mechanisms from food, bacteria and sloughed cells (Neutra and Forstner, 1987). Mucosal surfaces provide both a physical barrier and an immunologically active site for both the innate and adaptive response (Salinas et al., 2011). It is composed of glycoproteins, cytokines, peptides, lysozymes, lipoproteins, complement, lectins, protease and antibodies. Additionally, it has been shown to have antimicrobial activity targeting both bacteria and viral pathogens (Cain and Swan, 2010). Mucus producing cells are found in the foregut, liver, pancreatic tissue and intestines (Krogdahl et al., 2011).

In trout, it has been shown that there is a zonation of metabolic activity along the intestinal tract. The highest activity being in the pyloric caeca, with activity decreasing along the length of the small intestine (Mommsen et al., 2003). The authors argue that this is not only limited to digestive functions, but is also related to nutrient resorption and osmoregulation needs.

The lamina propria is largely populated by macrophages, granulocytes, lymphocytes and plasma cells. Whereas IEL has a more adaptive response with T-cells being the most commonly found immune cell, and B-cells having a lower presence (Salinas et al., 2011). Rombout et al. (1985) provided evidence for enterocytes/large intraepithelial macrophages functioning as antigen sampling cells. Later, Fuglem et al. (2010) brought forward evidence to argue the presence of cells resembling M-cells present in the posterior intestine of salmonids.

The proximal and distal regions of the intestine are both highly permeable. The latter having a lower transepithelial electrical resistance and having a less mucus (Schep et al., 1997). The posterior region uptakes lipids and proteins via phagocytosis, and has a much lower rate of active absorption of glucose and amino acids than the anterior. The anterior intestine is associated with lipid absorption, whereas the distal intestine absorbs macromolecules – proteins which do not undergo hydrolysis in the lumen and protein fragments that pass into epithelial cells via large vacuolar systems (Georgopoulou et al. 1988).

Schep et al. (1997) describe the proximal intestine as having a 'bulbous shape, annulospiral folding and extensive elongated villi'. The distal having fewer and shorter villi and being lighter in colour. Abundance of goblet cells found in both sections.

Sundh et al. (2011) demonstrate the ability of the infectious pancreatic necrosis virus (IPNV) to move across the intestinal epithelium in Atlantic salmon. The virus was translocated in both proximal and distal regions of the intestine, however the rate of which was greater in the distal intestine. It was also shown that the barrier function of the intestine was affected by this, leading to greater mucus secretion and an increase in the uptake of ^{14}C-mannitol in the distal intestine.

In trout, mitochondrial enzyme activity has been found to be highest in the pyloric caeca with reduced activity along the length of the intestine (Mommsen et al., 2003). This is also true of nutrient absorption (Bakke-McKellep et al., 2009).

Enterocytes actively sample luminal contents via endocytosis. Antigens and particles are presented at the basal membrane for lymphocytes to sample which then promotes the appropriate immune response (Jutfelt et al., 2011).

Intestinal fluid transport is regulated by cortisol. In seawater, uptake of fluids in via a transcellular route utilising aquaporins in enterocyte cell membrane and by changing the fatty acid composition in the lipid bilayer of cells (Sundell and Sundh, 2012).

Larger hydrophilic molecules can be transported across the epithelial membrane via active transcytosis in intercellular spaces. This type of transport is also how bacteria and viruses can cross the initial physical barrier of the gut (Jutfelt et al., 2011).

2.3 The Immune System

Responses are pathogen specific and dependent on whether the pathogen is intra- or extracellular. Intracellular responses are targeted at pathogens that are within host membrane enclosed structures, such as when a virus is present in a host cell. The immune system will seek to remove these via phagocytosis. When a pathogen is too large to be encapsulated via phagocytosis, or it is able to prevent internalization, the immune system will respond by producing anti-microbial products and by inducing inflammation to the area of infection (Rieger and Barreda, 2011).

Chemokines and cytokines are secreted in response to a pathogenic challenge (Salazar-Mather et al., 2000). Their role in the immune response is vast. They are responsible for priming macrophages, directing cells to areas of inflammation and dictating both the inflammation and tissue repair responses (Grayfer and Belosevic, 2012).

2.3.1 Innate Immunity

The innate immune system is the first line of defence in the war on immune challenges. It works due to its wide spectrum of non-specific antimicrobial activity (Noga et al., 2009**).** The innate immune response in teleosts may give greater overall immunity than in mammals. The reason for which is due to the slower, more temperature-dependent response of the adaptive immune system (Cain and Swan, 2010) Cells from the innate immune system have been observed in the gut from first feeding (Rombout et al., 2011).

Monocytes and Macrophages

Monocytes and macrophages are capable of phagolysosome fusion (Rieger et al., 2010). The pathogen is engulfed and internalized by the cell in a phagosome. Next lyzosyme fusion occurs in which the acidic and enzymatic lyzosymal contents is released into the phagosome,

degrading the pathogen engulfed within (Rieger et al., 2011). Rate of phagocytosis is determined by factors such as size, concentration and opsonization of the particle for uptake, with fewer phagocytic cells being present as the particle size decreased (Li et al. 2006). Opsonisation enhances phagocytic uptake and is mediated by ligand-receptor interactions between pathogen and phagocyte (Claire et al., 2002).

Epithelial cells and fibroblasts also possess this ability, however it is to a lesser extent. They lack the diversity and multitude of cell receptors, such as complement C3, to identify foreign bodies present on macrophages and thus are unable to ingest the wide array of particles that lymphocytes can (Li et al., 2006).

Macrophages have numerous pathogen destroying abilities. They are capable of producing nitric oxide, a highly microbicidal compound (MacMicking et al., 1997). After priming with cytokines such as TNFα, IFNγ or IL-1β; monocytes respond with Reactive Oxygen Intermediate (ROI) generation after short stimulation, whereas macrophages respond with ROI generation after a longer stimulation (Grayfer and Belosevic, 2012). Macrophages can be found throughout the body, and within the kidneys there are macrophages present for phagocytosing material in the reticuloendothelial system (Zwollo et al., 2005).

Macrophages may be activated in four ways; innate, classical and alternative activation, as well as regulatory as shown in figure 2, leading to different phenotype formation. Innate activation occurs when a macrophage responds to a pathogen stimulus alone. Classic activation of macrophages is when a microbial stimulus and IFNγ act together to stimulate cell activity. Classic activation leads to higher respiratory burst, increased iNOS expression and greater antigen presentation. It is therefore a highly potent response requiring strict regulation. Alternatively activated macrophages can be described as wound healing. They are activated by T-helper cell cytokines. The fourth type of macrophage activation, regulatory activation, happens upon microbial stimulus in combination with other immune complexes or hormones such as prostaglandin or glucorticoids and leads to IL-10 production (Forlenza et al., 2011).

Melanomacrophages have an additional role in immune complex clearance. They form in clusters in the spleen and kidney known as melanomacrophage centres (MMC) where complement proteins are destroyed (Claire et al., 2002).

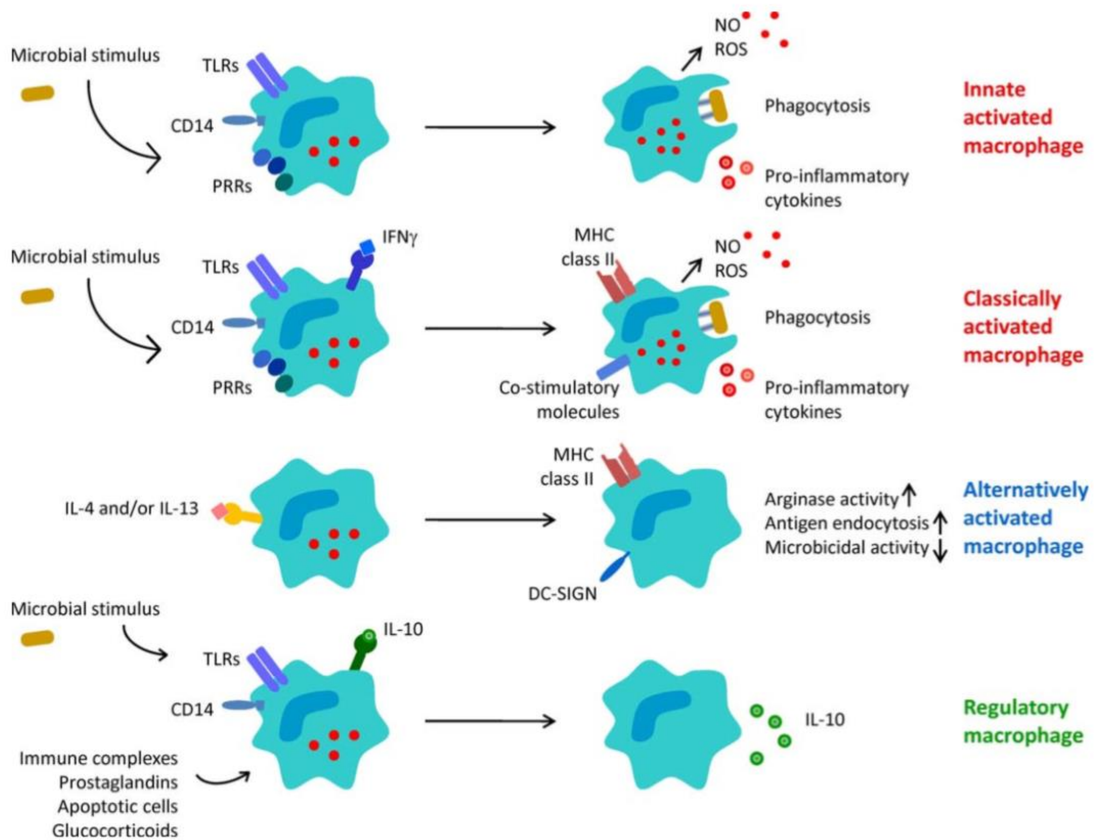


Figure 2: *The four types of macrophage activation; innate, classical, alternative activation and regulatory. Figure taken from Forlenza et al., 2011.*

Granulocytes: Neutrophils, Basophils, Eosinophils

Neutrophils are often responding first to a site of inflammation. They are able to respond with intracellular and extracellular antimicrobial mechanisms (Rieger et al., 2011). Neutrophils release chromatin that forms extracellular fibres in neutrophil extracellular traps (NETs). NETS work by binding bacteria in one area with a high concentration of antimicrobial granule components (Brinkmann et al., 2004).

Basophils contain large granules and mediate inflammatory responses. These cells circulate in the body and are able to infiltrate inflamed tissues. They express cytokines and chemokines and have receptors that are utilized in the complement system as well as in immunoglobulin recognition and toll-like receptors. (Stone et al., 2009). Basophils induce the proliferation and survival of both naïve and activated B-cells, as well as aiding in their differentiation into antibody producing cells (Merluzzi et al., 2014).

As with basophils, eosinophils (also known as mast cells) are involved in the inflammatory response. The latter have their name due to their ability to retain eosin dye. They are derived from hematopoietic stem cells and circulate in an immature state. Eosinophils then mature at the site of infection (Galli et al., 2005). Once activated they contain granules and form large lipid bodies. They release pro-inflammatory mediators, granule-stored cationic proteins, newly synthesized eicosanoids and cytokines (Stone et al., 2009). Eosinophils have great phenotypic plasticity, and can be found within and beneath epithelial layers within the gastrointestinal tract. They behave as effector cells directly interacting with TH2 cells and promoting the innate immune system response against bacterial infection via TLRs (Galli et al., 2005).

Rodlet Cells

Named after the rodlet inclusions within the cell, rodlet cells have been found in epithelial tissues of Atlantic salmon (Bullock, 1963). Immature rodlet cells are oval, with a pale nucleus and their mitochondria elongated and small. As they mature, rodlet cells develop a thin, fibrillate layer forming an encapsulating membrane that thickens with age (Manera et al., 2004). They are associated with the inflammatory response and have been found in the gills and the intestine of salmonids infected by intestinal cestodes and trematodes (Reite et al., 1997). Rodlet cells are believed to be multi-functioning secretory cells involved in osmoregulation, ion transportation and in the innate immune response. They have been found at sites of contagion by metazoan and protozoan parasites as well as sites of viral infection (Manera et al., 2004) and have been found to be present in when fish are in states of stress and toxin responses (Smith et al., 1995).

Natural Cytotoxic Cells (NCCs)

Akin to natural killer cells in mammals, NCCs appear similar to monocytes, express perforin and granulysin and target infected host cells (Roberts, 2012). They are part of the adaptive immune system as they are non-specific.

2.3.2 Adaptive Immunity

The adaptive immune system, also known as the acquired immune system, is more specialized than the innate immune system. It is slower and more specific, and may take up to two weeks to manifest in teleosts (Cain and Swan, 2010).

The role of the adaptive immune system is to create a microenvironment in which to allow the development of immune cells, to select the appropriate response to a pathogen challenge and

to regulate the efficacy of the immune system. Antigens and interferons are responsible for the regulation of the lymphocytes (Scapigalitati, 2013). Lymphocytes include Natural Killer (NK) Cells, B-Cells and T-Cells (Fischer et al., 2013).

In fish, the dexterity of the adaptive response is related to genome duplication (Kasahara et al., 1997).

B-Cells

These cells secrete soluble antigen receptors throughout the body (Scapigalitati, 2013). The main site of haematopoiesis in fish is the anterior region of the kidney, also known as the head kidney. It is here plasma cells and B-cell precursors are produced, with maturation of these cells occurring in the posterior kidney (Zwollo, 2005). During periods in which the fish do not face any immune challenges, the anterior kidney has been shown to store Ig-secreting cells (Zwollo, 2005). The spleen is responsible for identifying antigens within the blood, leading to the activation and differentiation of B-cells (Salinas et al., 2011).

Li et al. (2006) have suggested that B-cells in rainbow trout have phagocytic abilities, leading to the phagolysosome fusion and degradation of pathogens, something that is not seen in higher vertebrates. These results suggest B-Cells evolved from an ancestral phagocytic cell and can offer an understanding between mammalian B-lymphocytes and macrophages.

B Cells in teleost fish resemble mammalian B-1 Cells. They express membrane IgM as a monomer, and secrete tetramer IgM (Miller et al., 1998).

T-Cells

Differing to B-cells, T-cells requires cell to cell contact (Scapigalitati, 2013).

In Salmonids, T-cells are formed in the thymus, spleen and interbranchial lymphoid tissue (Koppang et al. 2010). T-cell producing tissues found in interbranchial lymphoid tissue develop from the lamina propria of the gastrointestinal tract located at the base of the gill arches. This tissue migrates to the underlying mesenchyme during growth. The resulting tissue can be identified as a capsule enclosing lymphatic tissue (Bowden, 2005). T-cells are derived from the thymus, then go on to populate the intestine, followed by the head kidney and spleen (Rombout et al., 2005).

Further to this, memory γδ T-cells within intestinal tissues are believed to have a role in maintaining epithelial integrity, homeostasis and assist in IgA production and induce cytolysis of infected cells (Sheridan et al., 2013).

Genes coding for CD4+ like proteins have have been found in Atlantic salmon (Moore et al., 2009). Maisey et al. (2016) have shown that CD4+ T-Cells are present in the spleen, thymus of Rainbow trout and express CD4-1 transcripts. CD4 binds to the MHC class II complexes, and this interaction initiates T-cell activation. (Davis et al., 2003). They are capable of antigen recognition and secrete effector and regulatory cytokines specific to the pathogen. These cells further differentiate into TH1, TH2 and TH17. TH1 cells provide immunity against intracellular challenges. TH2 defend against parasitic and allergies. TH17 respond to extracellular infections from bacteria, fungi and autoimmune diseases. All of these are regulated by regulatory T-cells known as Treg cells (Maisey et al., 2016).

Fish lymphocytes have been shown to express receptors for serotonin (5-HT3), these receptors have been linked to T-Cell proliferation (Meyniel et al., 1997).

Additionally, there are γδ T-cells. These cells are thought to function as a bridge between the innate and adaptive immune responses (Holtmeier et al., 2005, Buonocore et al. 2012).

Toll Like Receptors (TLRs)

TLRs recognize pathogen-associated molecular patterns (PAMPs), and respond by initiating molecular pathways causing inflammatory and interferon production (Purcell et al., 2006). TLR signaling is key to commensal tolerance and protecting the intestinal epithelial barrier (Cario et al., 2005). TLR2 recognises bacterial lipopeptides and lipoteichoic acids in the cell walls of gram positive bacteria (Matsuguchi et al., 2000). TLR4 responds to LPS in the cell walls of gram negative bacteria (Poltorak et al., 1998).

2.3.3 Humoral Factors

Immunoglobulins

Teleost fish are the most primitive vertebrates to possess immunoglobulins. Immunoglobulins have the ability to neutralize viruses, toxins and bacterial adhesins. They also perform the role of activating the complement system and oponisation of particles (Roberts, 2012). Production of immunoglobulins with secretory function is mainly performed by plasmablasts and plasma cells (Salinas et al., 2011). However, a limited number of classic plasma cells have been found and they have not been readily detected in mucosal tissues (Rombout et al., 2014).

Despite only a thin basement membrane separating the epithelium and lamina propria, the two tissues form distinctive immunological regions (Mowat and Agace, 2014). In trout, it has been shown within the lamina propria there is a preference for IgM+ cells, whereas IELs have an affinity for IgT+ cells. The latter would suggest the recruitment of both T and B cells occur here (Rombout et al., 2014).

The dominant immunoglobulin present in mucosal surfaces is IgT (Zhang et al., 2010). It has been found present in both teleost skin and gill mucosal immune responses (Xu et al, 2013, Xu et al. 2016). Both IgT and IgZ have been found to be highly prevalent in the immune responses of the gut (Salinas et al., 2011).

Zhang at al. (2010) show the presence of IgM in tetrameric form in both the gut and serum of Rainbow trout, however it was seen to be polymeric in the gut and monomeric when present in the serum. This would suggest the function of IgT to be specialised dependent on where it is present. Zhang et al. (2010) also found IgM in serum when an intestinal parasite was present and IgT was found to coat most of the intestinal bacteria.

The transport of immunoglobulins from the mucosal epithelium into the lumen of the gut is assisted by polymeric immunoglobulin receptors (pIgR) (Zhang et al., 2010).

The mRNA transcripts for IgM are several folds greater than those for IgT and IgD in trout, indicating IgM to be the dominate immunoglobulin in plasma (Li et al. 2006).

It is thought that immunoglobulin isotypes specialized into serum and mucosal antibodies during the tetrapod evolution (Scapigalitati, 2013).

Cytokines, Chemokines and Interferons

Cytokines act as a messaging system for the immune system and coordinate the immune response. They are released from leukocytes. Many are proinflammatory such as TNFα and IL-1β. Chemokines are chemoattractant cytokines, and in mammals they can be subdivided into four groups: CXC (α), CC (β), C and CX_3C. However, it has been difficult to find true orthologues between fish and mammalian chemokines due to extensive genome duplication events in teleosts and how quickly chemokines are able to evolve (Alejo and Tafalla, 2011). In Atlantic salmon, there have been thirty different chemokine genes identified (Peatman and Liu, 2007).

Interferons are antiviral proteins (Cain and Swan, 2010). They can by subtyped into type I and type II. Type I interferons, IFNα and IFNβ, are present as the first line of defence for viral

infections. Type II interferons, such as IFNγ, is produced by T-helper cells in response to mitogens and antigens, it is a key to the adaptive cell response as it activates phagocytes (Robertsen, 2006). Chemokines such as IL-12 and IL-18 stimulate IFNγ production (Boehm et al., 1997). In turn, IFNγ combined with TLR ligation will lead to macrophages being primed to respond (Bundschuh et al., 1997, Jurkovich et al., 1991).

Antimicrobial Peptides (AMPs)

Liver-express antimicrobial peptides (LEAPS), defensins, piscidins and cathelicidins have been found across many teleost species. LEAPs are present during pathogenic challenges and have been found to regulate iron. Defensins are both antibacterial and antiviral. Piscidins are found in evolutionary advanced teleosts and are thought to have antibacterial, antiviral, antifungal and antiparasitic properties. Cathelicidins have been found in salmonids and gadoids, and have been found when there is an infection in the fish (Roberts, 2012).

Transferrin

Transferrin binds to iron limiting its availability for bacteria. By restricting iron resources, bacterial and fungal growth is restricted, preventing further development of pathogens (Cain and Swan, 2010). Transferrin is polymorphic, most likely due to it having to be capable of adapting to pathogen competing iron binding molecules such as siderophores (Roberts, 2012). Different transferrin genotypes have been linked to fish susceptibility to bacterial kidney disease in some Coho salmon strains, but not in others (Ellis, 1999).

Lysozymes

The peptidoglycans present in the cell walls of bacteria are degraded by lysozyme, leading to lysis of the bacterial cell. High levels of lysozymes can be found in mucus, serum and lymphoid tissues (Cain and Swan, 2010). Fish lysozyme is thought to be more active and kill a wider variation of bacteria than in higher vertebrates (Yousif et al., 1994).

The Complement System

The complement system is so called due to its role in assisting in the destruction of foreign bodies. It is a biology cascade that is vital to the innate immune response, but which may also activate the adaptive immune system also (Cain and Swan, 2010). It does this by the use of activated protein fragments. Usually, these proteins are synthesized as inactive precursors, requiring enzymes or binding proteins to activate them. Once active, these protein fragments have microbial killing properties, they can opsonise foreign objects for phagocytosis, are

involved in inflammatory reactions and antibody production, and also in the clearance of immune complexes (Claire et al., 2002).

Due to variations between the location and amounts of specific antibodies produced systemically and in the mucosal tissues of fish, it would strongly suggest different antibodies are specific to each (Lobb and Clem, 1981).

The complement system can be activated by three pathways (Claire et al., 2002):

i. Classic Complement activation Pathway (CCP): this pathway is dependent on antibodies bonding to a cell surface and acute-phase proteins. Viruses, bacteria and virus-infected cells triggered this pathway.
ii. Alternative Complement Pathway (ACP): does not require antibodies, instead is activated directly by a virus, bacteria, fungi or tumour cells.
iii. Lectin Complement Pathway (LCP): as with the ACP, the LCP is independent of antibodies. Instead it is initiated by the binding of a protein complex

35 soluble and membrane-bound proteins of the complement system are able to destroy pathogens by creating pores in their surface membranes. This process is activated by microorganisms themselves, or by the formation of antibody-antigen complexes (Claire et al., 2002). Such protein interactions are important in the activation of the adaptive immune response. The complement system in fish has been recognized as having C3 and C5, two compounds which can lead to vasorelaxation and attract neutrophils (Roberts, 2012).

Lectins

Lectins are proteins that bind to sugars and may be implicated in the activation of the complement system (Cain and Swan, 2010).

2.4 Gram Negative Bacteria

2.4.1 Cell Wall and LPS Structure

Gram-negative bacteria are characterized by their outer membrane. The classification arises due to gram-negative bacteria being colourless when gram stained. Although its purpose is structural, it has high toxicity to mammals. The cell wall of gram-negative bacteria is composed of the cytoplasmic membrane, a periplasm layer and an outer membrane. This outer membrane is composed of phospholipids, proteins, lipoproteins, lipopolysaccharide

(LPS). The periplasmic space contains peptidoglycans (PGN), periplasmic proteins and modification of toxins such as penicillin. The cytoplasmic membrane is mainly phospholipids and proteins. (Boltaña et al., 2011). Examples of gram-negative bacteria include species of *Salmonella* and *Escherichia*. Non-virulent, gram-negative bacteria are highly susceptible to cytolysis due to their membranes (Claire et al., 2002).

It is the lipopolysaccharide (LPS) component of the outer membrane of these bacteria that makes them so toxic. LPS is composed of three different parts; O-antigen outer region composed of a polymer of oligosaccharides, lipid A which is a hydrophobic membrane anchor and an oligosaccharide linker core region as shown in figure 3 (Anwar and Choi, 2004). LPS comes in the form of rough and smooth depending on the O-antigen region. Rough type LPS lacks the O-polysaccharide chain found in smooth LPS, due to the non-functionality of the O-antigen gene cluster caused by frame shift mutations (Madigan et al., 2010). The most potent immune stimulating region of LPS is lipid A, a highly conserved structure with antigenic cross-reactivity (Rietschel et al. 1998). The type of inflammatory response elicited by LPS is dependent on the LPS composition and cell type. The O-antigen region activates the humoral response, lipid A stimulates inflammation, with modifications to lipid A allowing a pathogen to avoid causing host response (Loppnow et al., 1990).

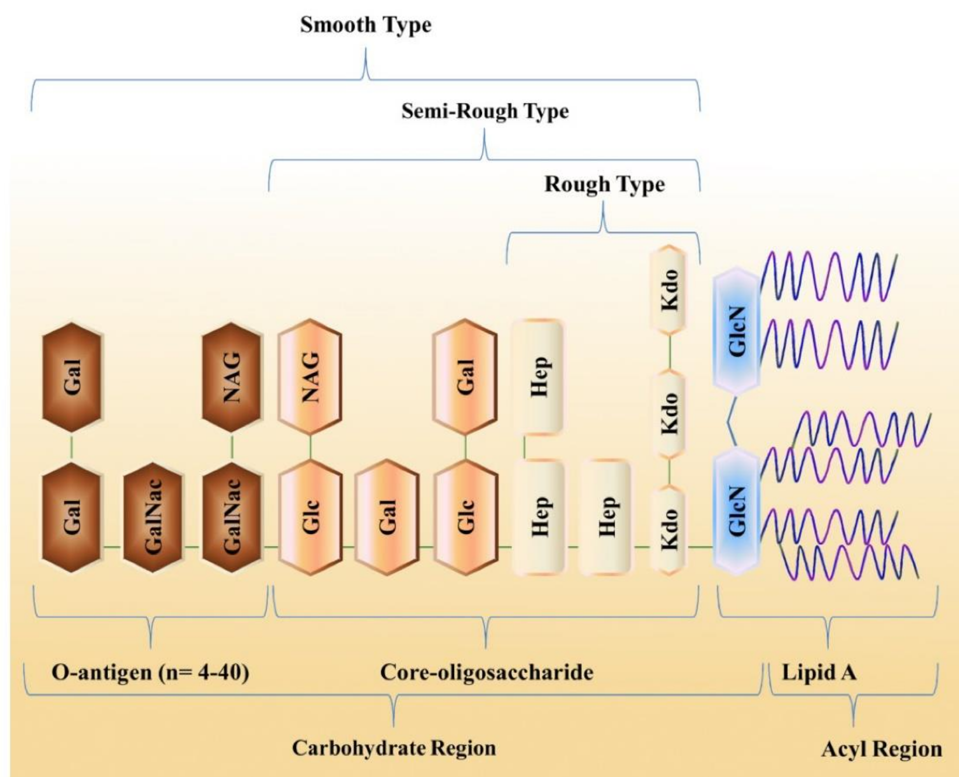


Figure 3: *Overview of LPS Structure taken from Anwar and Choi (2014).*

2.4.2 LPS Cellular Response

The macrophage response to immune challenges is dependent on the signaling pathway from the cell membrane to cell nucleus, leading to a change in gene expression allowing for the cells defence systems to go into production. Once LPS interacts with a TLR on the cell membrane, it triggers the activation of cytoplasmic transcription factors which then translocate to the cell nucleus and alter gene expression (Aderum and Ulevitch, 2000). The cell signaling pathway induced by LPS interacting can lead to either MyD88-dependent or MyD88-independent pathways being triggered as shown in figure 4.

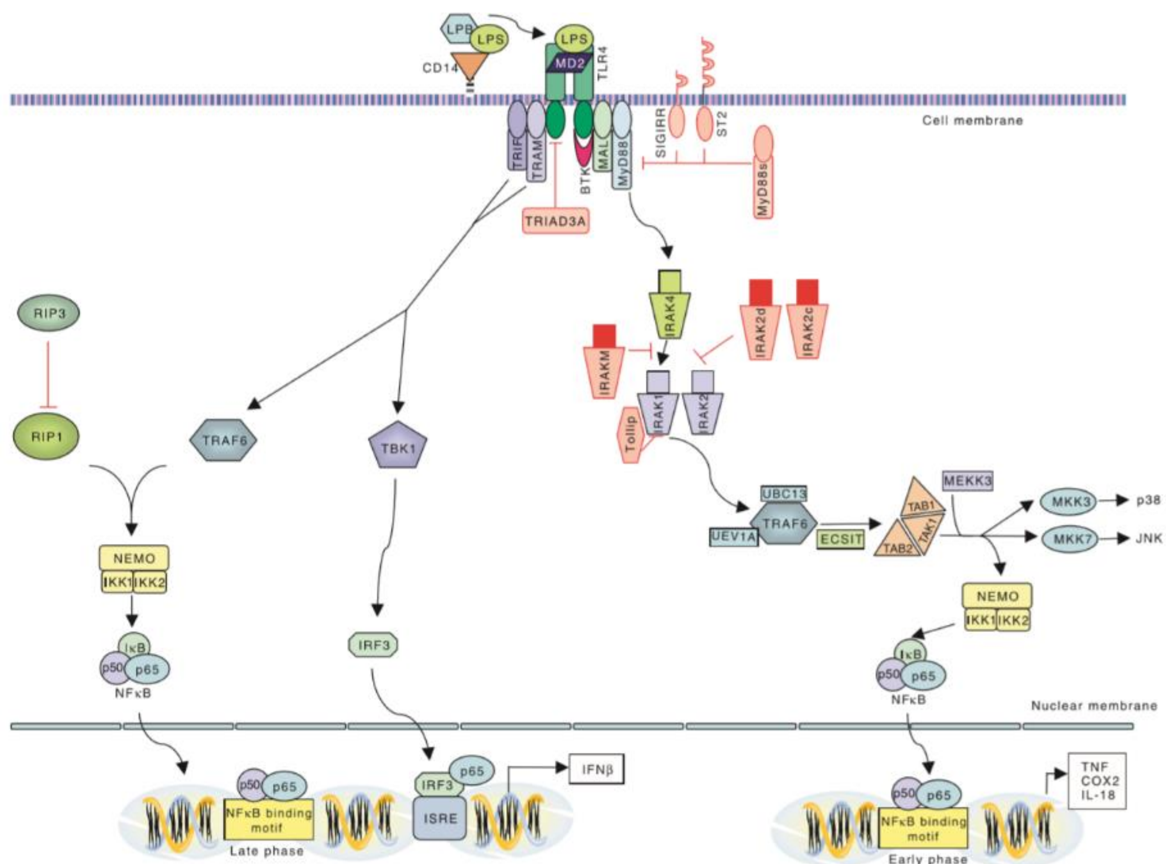


Figure 4*: LPS induced TLR4 signaling of MyD88 dependent and MyD88 independent pathways. Taken from Pålsson-MacDermott, E.M. and O'Neill, L.A.J (2004)*

MyD88-dependent

Myeloid Differentiation protein 88 (MyD88) is a signal adaptor protein used for pro-inflammatory cytokine production. It is activated when a TLR is engaged with an agonist, such as LPS, it acts as a structural platform for kinase and other downstream effector

molecules (Bjorkbacka et al., 2004). The full extent of TLR4 response to LPS stimulation is restricted by the availability of the accessory molecules CD 14 and MD-2 (Wright et al. 1990, Shimazu et al., 1999).

MyD88-independent

LPS is capable of creating signal transduction events in the absence of MyD88, leading to the expression of Type I interferons and interferon-stimulated genes (Kawai et al., 2001). This pathway is also known as the TRIF pathway. It causes an immune response by activating the NFκB pathway. Vogel et al. (2003) demonstrated the requirement of both the MyD88-dependent and MyD88-independent pathways are required in order for full signal transduction.

2.5 Immune relevant genes selected in this experiment

This thesis examines the immune response of Atlantic salmon macrophage-like cells through the analysis of gene expression related to cytokines found to be produced during a cellular immune response. The gene expression for the cytokines outlined in Table 1 were tested for using qPCR.

Table 1: Genes examined in this experiment and their role in the immune response

Gene	Role
MyD88	Myeloid Differentiation protein 88 is a signal adaptor protein used for pro-inflammatory cytokine production (Bjorkbacka et al., 2004).
TNFα	TNFα has multiple biological functions. It is involved in macrophage activation and differentiation, neutrophils migration, phagocytosis and proliferation of lymphocytes. It works across the innate and adaptive immune systems (Qin et al., 2000).
Mul1b	mitochondrial ubiquitin ligase activator of NFκB1 (mul1b) activates NF-κB pathway which has both a pro-inflammatory and anti-inflammatory response (Lawrence, 2009).
IL-1β	Increases phagocytosis, leukocyte mobility, proliferation of macrophage COX-2 expression (Roberts, 2012).
IL-10	Anti-inflammatory cytokine down regulating the inflammatory response (Grayfer and Beloseric, 2013).

IFNγ	Pro-inflammatory cytokine produced in response to intracellular pathogens (Goerdt et al., 1999).
CR3	CR3 is an integrin and has non-specific antimicrobial properties and activates leukocytes (Ehlers, 2000).

House Keeping Genes

These genes were selected as references whose expression should not be altered between groups.

Table 2: Housekeeping genes examined in this experiment and their role within cells

Gene	**Role**
β-actin	Component of protein scaffold determining cell shape (Olsvik et al., 2006).
Ubiq	Ubiquitin alters protein location and activity in cells.
Rpl13	A ribosomal protein subunit.

By understanding the gene expression and relationship between the cytokines examined, we can begin to establish some knowledge into the cellular pathway triggered by LPS. It is only by knowing these pathways we can then begin to influence them in a way that can best benefit the survival of the animal.

3.0 Materials and Method

The aim of this work was to isolate immune cells from tissues collected from the pyloric caeca and distal intestine of Atlantic salmon. These cells would then either be exposed to LPS or kept in sterile media for an equal length of time. Cells were then harvested, RNA was extracted, followed by qPCR to establish the levels of cytokine gene expression.

3.1 Fish

Fish were of AquaGen strain and stocked from Cermaq, Hopen. They were hatched in February 2017 and smoltified using photoperiod L:D 12:12 for 5 weeks, otherwise 24:00. Water temperature remained between 5.8 – 7.7°C at a salinity of 33ppt since September 6th 2017. All sampling occurred in June 2018.

3.2 Tissue Extraction

The pyloric caeca and distal intestine were removed from four fish. Each tissue type was then cut using a scalpel and mixed with the other samples of the same tissue to create a homogenous pooled sample from the four fish.

3.3 Cell Isolation

The cell isolation protocol was adapted from Salinas et al. (2007).

The samples were then added to 15ml of 1xPBS (Sigma) solution with 0.36mg/ml of collagenase from Clostridium histolyticum (Sigma, C0130-1G) in a 50ml centrifuge tube, kept on ice and shaken on a Heidolph Unimax 1010 platform for 1hour 30minutes. After shaking, samples were then strained through a 100µm sieve (VWR, 734-2762) using a syringe plunger to gently tease the tissue through the sieve into a petri dish. The cellular solution that had been collected post sieving was then transferred into a 15ml centrifuge tube with L-15 (Sigma, L4386-1L) washing solution. Next, the samples were centrifuged at 3200rpm, for 10 minutes at 4°C. After centrifugation, the supernatant was removed and the remaining pellet suspended in 5ml of L-15 washing solution. This was repeated a total of 3 times.

A Percoll (Sigma) gradient of 34% to 51% was then prepared as shown in table 4. First, 3ml of 34% Percoll was added to a 15ml centrifuge tube. A glass Pasteur pipette was then place in the tube and raised very slightly from the bottom. The 51% Percoll was then pipetted into the tube via the glass Pasteur pipette to create the gradient.

Once the samples had undergone the final centrifugation of the washing phase, the pellets were once again resuspended in 3ml of L-15 washing solution. Samples were then layered onto the top of the Percoll gradient. Three distinct layers could be observed. They were then centrifuged at 1800rpm, for 30minutes at 4°C.

Post centrifugation, the layer between the 34% and 51% Percoll interface was removed, placed in a new 15ml centrifugation tube and mixed with 5ml of L-15 Free solution. Samples were centrifuged at 3200rpm, for 5 minutes at 4°C. This was repeated a total of 3 times.

After the last centrifugation, the supernatant was removed and cells were then suspended in 1ml of 1xPBS to be counted using a Scepter handheld automated cell counter (Millipore). Cells were then diluted to $1x10^{6}$cells/ml and plated into a petri dish in 3ml of L-15 II solution or L-15 II solution with 100mg/ml LPS isolated from *Escherichia coli* (Sigma) and incubated for 6 hours at 12°C in an incubator.

After 6 hours of incubation, the samples were pipetted from the petri dish into a 15ml centrifuge tube and petri dishes washed with 1xPBS to collect all cells. Samples were then centrifuged at 3200rpm for 5minutes at 4°C. The supernatant was then removed and the remaining pellet was suspended in 1ml of 1xPBS. This suspension was then used for qPCR with a sample taken for flow cytometry.

All samples and solutions were kept on ice during the procedure.

3.4 RNA Extraction, transcription and qPCR

The remaining sample suspended in 1xPBS was pelleted by centrifuging at 3200RPM for 5minutes at 4°C. Next RNA extraction was performed using the RNeasy Micro Kit from Qiagen, following manufacture's instructions. Once RNA had been extracted, samples were then frozen at -80°C.

Once all samples had been collected and stored at -80°C, they were then left on ice to defrost and the RNA concentration was measured using High Sensitivity RNA Screentapes on the TapeStation (Agilent Technologies) following manufactures' instructions.

Next, the samples were diluted to 150ng of RNA per sample and prepared for transcription using the Qiagen QuantiTect Reverse Transcription Kit according to manufacturer's instructions for RTqPCR.

The primers as shown in table 3 were then added to the cDNA and RTqPCR was performed on a Lightcycler.

Gene name	Forward Primer 5'-3'	Reverse Primer 3'-5'	Reference
TNFα	GCTTGTCTCTTGTTGCCACCA	TGTGTGGGATGAGGATTTGGTT	GenBank: EF079662.1
IL-1β	AGGACAAGGACCTGCTCAACT	CCGACTCCAACTCCAACACTA	GenBank: AY617117.1
IL-10	GGGTGTCACGCTATGGACAG	TGTTTCCGATGGAGTCGATG	GenBank: EF165028.1
CR3	ATGACATGGACTACCCATCTGTT	TCTGACAATACTCCCACCTCA	GenBank: BT058776.1
MyD88	GACAAAGTTTGCCCTCAGTCTCT	CCGTCAGGAACCTCAGGATACT	GenBank: EF672332.1
Mul1b	CCAGAACGACCAACAGGAAGG	GTGAACTCTCTCCAGGAACCAGC	GenBank: JF933931.1
IFNγ	CTAAAGAAGGACAACCGCAG	CACCGTTAGAGGGAGAAATG	GenBank: AY795563.1
β-actin	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	GenBank: AF012125.1
Ubiq	AGCTGGCCCAGAAGTACAACTGTG	CCACAAAAAGCACCAAGCCAAC	GenBank: AB036060.1
Rpl13	CGCTCCAAGCTCATCCTCTTCCC	CCATCTTGAGTTCCTCCTCAGTGC	GenBank: BT048949.1

Table 3: *Primers used for qPCR analysis*

3.5 Flow Cytometry

Cells were prepared for flow cytometry using the pHrodo BioParticles Phagocytosis Kit by life technologies in accordance with manufacturer's guidelines. The Amnis ImageStream Mark II Imaging Flow Cytometer (Millipore Sigma) was used to produce images of cells.

3.6 Solution Preparation

All items and solutions used were autoclaved prior to use. All solutions were prepared to have a final osmolarity of 370mOsm.

L-15 stock solution was adapted in accordance with Haugeland et al. (2012) by the addition of reagents in table 4 to a create a 1L solution of L-15. The solution was then filtered through a Millipore Stericup 1000ml Durapore with a 0.45µm filter.

Table 4: Reagents and quantities required for L-15 stock solution

Reagent	Amount
0,41M NaCL	15ml
0,33M $NaHCO_3$	15ml
D-Glucose	1,98g
HEPEs	3.75g

L-15 solutions specific to each stage were made in batches of 50ml in accordance to table 5. L-15 I was used in the initial washing stages, L-15 II for storage and L-15 Free for the final washing stages.

Table 5:Reagents and quantities required to make 50ml of L-15 I, L-15 II and L-15 Free solutions

Reagents	L-15 I	L-15 II	L-15 Free
L-15 (ml)	48,725	48,75	49,75
Heparin (µl)	25	-	-
Penicillin / Streptomycin (µl)	250	250	250
FBS (ml)	1	1	-

Percoll solutions of 34% and 51% were created as described in table 6.

Table 6:Reagents and quantities required to make 50ml of Percoll at concentrations of 34% and 51%

Reagents	34% Percoll	51% Percoll
Percoll (ml)	17,00	25,50
10xPBS (ml)	5,00	5,00
Distilled Water (ml)	28,00	19,50

4.0 Results

4.1 Cell Extraction

Microscopic Examination

Once cells were isolated as previously described, they were examined under a light microscope to visually assess which cells types were present. Erythrocytes, monocytes, neutrophils were able to be identified (see figures 5 and 6). A higher cell mass could be seen in distal intestine samples, this was further confirmed when cell counts were performed.

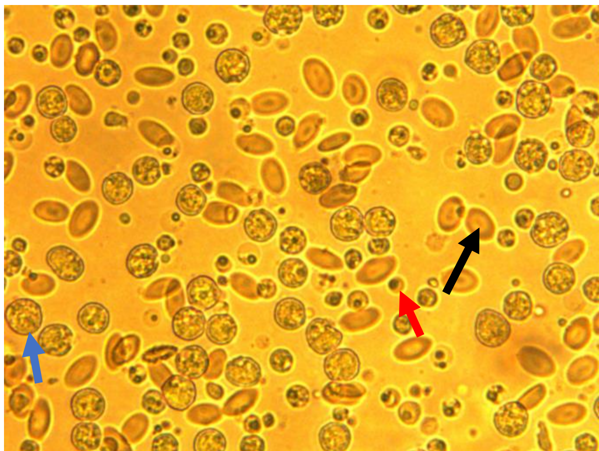


Figure 5:Cells from distal intestine extraction. Black arrow indicates erythrocyets, blue arrow shows neutrophil cells and the red arrow indicates monocyte like cells

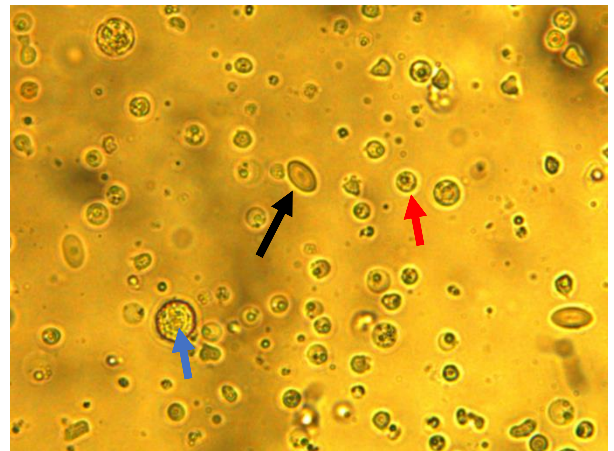


Figure 6:Cells from pyloric caeca extraction. Black arrow indicates erythrocyets, blue arrow shows neutrophil cells and the red arrow indicates monocyte like cells

4.2 Flow Cytometer

Results showed bio-particle uptake by cells sampled from both control and LPS treated cells from the distal intestine and pyloric caeca (see figures 7, 8, 9, 10). This shows that cells sampled were viable, phagocytic cells.

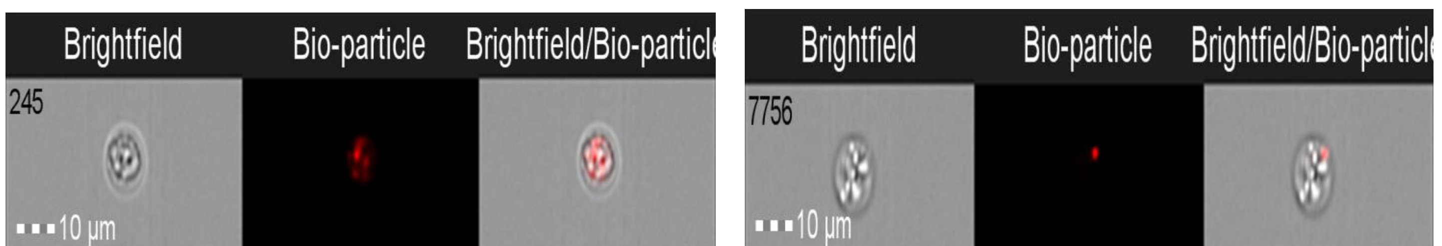
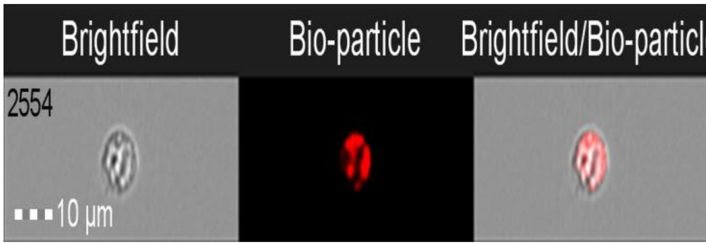


Figure 7: *Distal Intestine Cells from the Control Group*



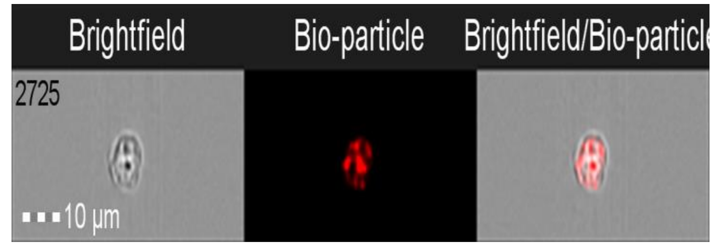
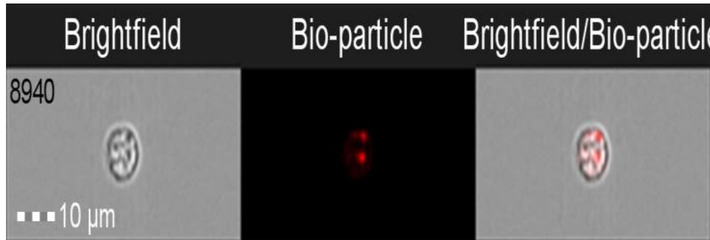


Figure 8: *Distal Intestine LPS Stimulated Cells*



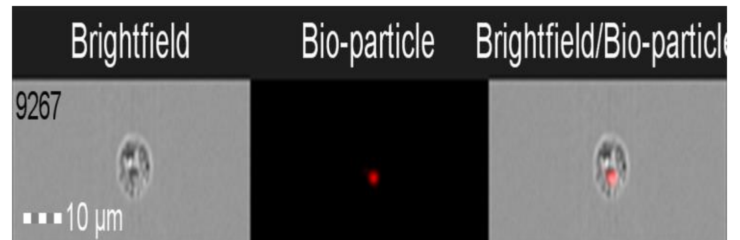
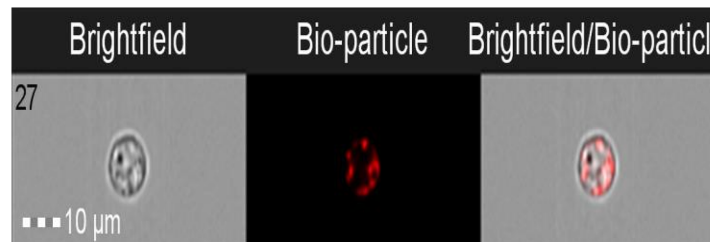


Figure 9*: Pyloric Caeca Cells from the Control Group*



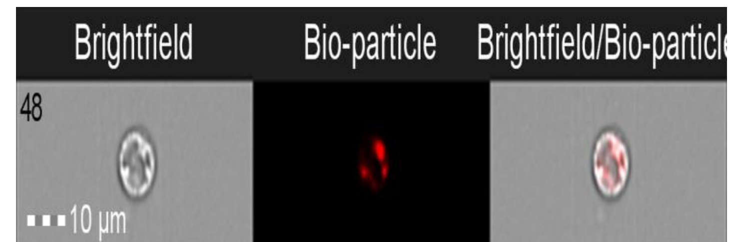


Figure 10*: Pyloric Caeca LPS Stimulated Cells*

4.3 Post-Extraction Cell Counts and RNA Concentration

From 32 tissue samples, only 20 provided a high enough cell count for further testing. This resulted in 14 samples from the distal intestine and 7 samples from the pyloric caeca. Samples that did not produce enough cell mass for both LPS treated and control groups were excluded from further analysis.

Table 7:Distal intestine samples tissue weight, cell count and RNA concentrations. Samples highlighted red did not have a high enough cell count to continue further down the workflow and consequently have not undergone qPCR.

Sample Name	Treatment	Tissue Weight (g)	Cell Count (x10^6)	RNA Concentration (ng)
D1A	L-15 100mg/ml	4,76	1,259	26,4
D1B	LPS	6,6	1,197	39,7
D2A	L-15 100mg/ml	3,84	1,61	58,6
D2B	LPS	3,3	1,709	50,4
D3A	L-15 100mg/ml	7,07	1,743	81,1
D3B	LPS	6,46	1,892	109
D4A	L-15 100mg/ml	3,45	2,002	53,8
D4B	LPS	3,97	1,634	21,5
D5A	L-15 100mg/ml	9,01	1,136	94,6
D5B	LPS	8,4	1,231	19,5
D6A	L-15 100mg/ml	5,36	1,202	74,3
D6B	LPS	5,17	1,677	29,1
D7A	L-15 100mg/ml	7,88	1,013	44,6
D7B	LPS	8,11	1	21,5
D8A	L-15 100mg/ml	5,29	0,9006	0
D8B	LPS	6,81	0,9598	0

Table 8: Pyloric caeca samples tissue weight, cell count and RNA concentrations. Samples highlighted red did not have a high enough cell count to continue further down the workflow and consequently have not undergone qPCR.

Sample Name	Treatment	Tissue Weight (g)	Cell Count (x10^6)	RNA Concentration (ng)
PC1A	L-15	4,93	1,912	111
PC1B	100mg/ml LPS	8,62	2,254	0
PC2A	L-15	10,06	0,596	15,6
PC2B	100mg/ml LPS	10,58	1,119	30,9
PC3A	L-15	7,97	1,126	34,3
PC3B	100mg/ml LPS	9,47	1,635	42,5
PC4A	L-15	10,76	0,176	0
PC4B	100mg/ml LPS	9,43	0,765	0
PC5A	L-15	6,85	0,2771	0
PC5B	100mg/ml LPS	7,02	0,6208	0
PC6A	L-15	5,23	1,67	45
PC6B	100mg/ml LPS	4,53	1,86	107
PC7A	L-15	7,84	0,1608	0
PC7B	100mg/ml LPS	4,87	1,834	0
PC8A	L-15	5,11	0,1796	0
PC8B	100mg/ml LPS	6,74	0,7938	0

4.4 qPCR

4.4.1 Distal Intestine qPCR

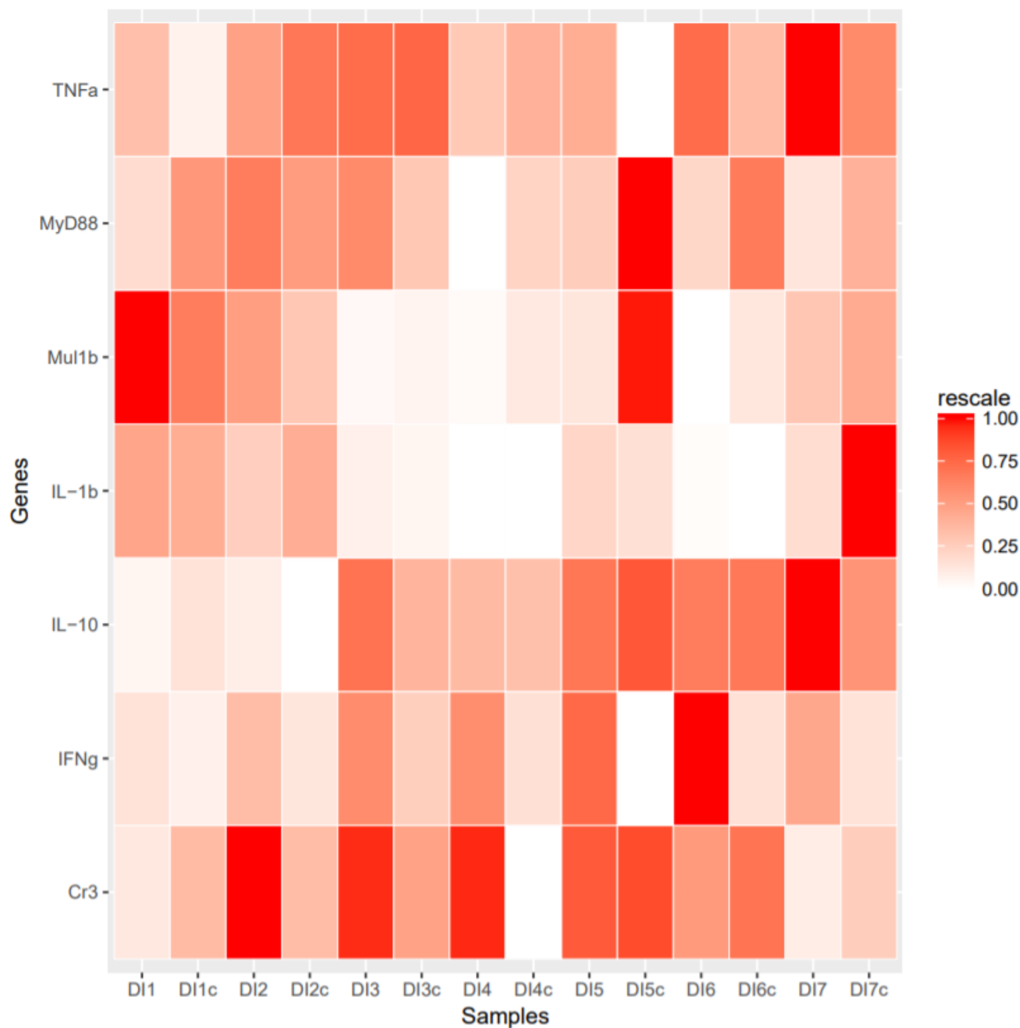


***Figure 11**: Heatmap showing gene expression in distal intestine samples. Samples named with 'c' indicate the control samples, not treated with LPS.*

Samples from the distal intestine show higher IFNγ gene expression in all LPS stimulated samples compared to control samples. Five out of seven samples showed higher expression in LPS stimulated cells for MyD88. In four out of seven samples, higher gene expression for IL-1β, TNFα and IL10 was seen in LPS stimulated cells compared to their controls. Cr3 showed higher expression in the control cells in 4 out of 7 samples. Mul1b showed higher expression in the control samples in 5 out of 7 samples.

Pyloric Caeca qPCR

TNFα had a higher gene expression in cells stimulated with LPS in all samples. Mul1b showed lower gene expression in all samples stimulated with LPS. IFNγ had a higher expression in all samples exposed to LPS.

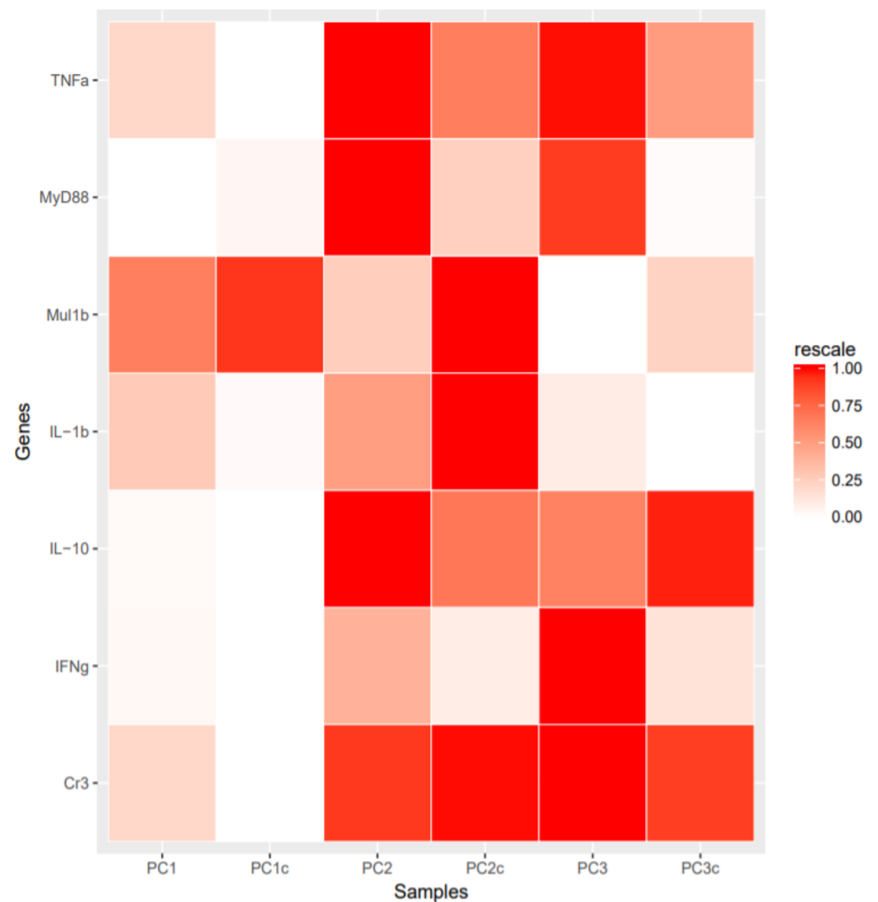


***Figure 12**: Heatmap showing gene expression in pyloric caeca samples. Samples named with 'c' indicate the control samples, not treated with LPS.*

4.4.2 Statistical analysis of qPCR results

Distal Intestine Samples

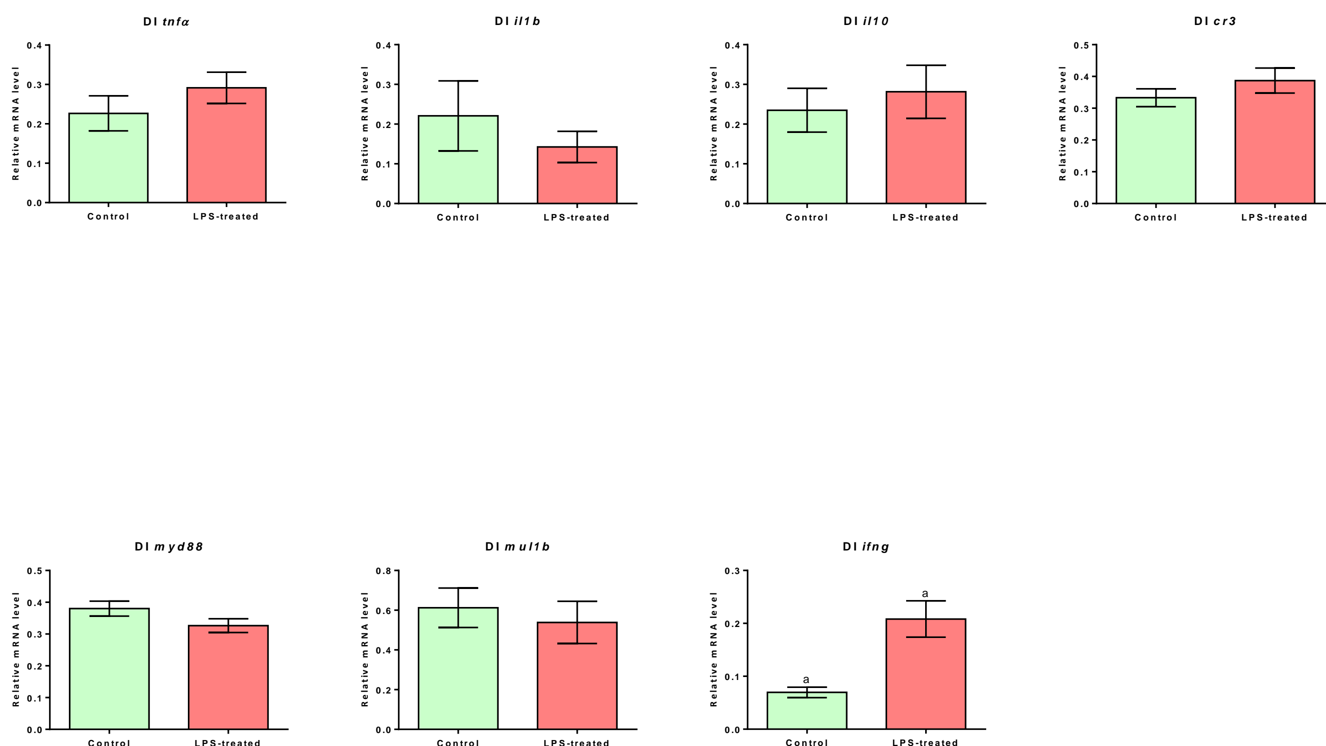
Overall, the expression levels were not statistically significant except for IFNγ which had a p value of 0.00111. MyD88 had almost borderline significant results at 0.059, however this is still above the 0.05 threshold.

Table 9: Distal intestine gene expression p Values

Gene	P Value
TNFα	0.14914
IL-1β	0.21711
IL-10	0.30160
CR3	0.14207
MyD88	0.05965
Mul1b	0.31022
IFNγ	0.00111

In LPS treated cells, the results showed greater mRNA expression for TNFα, IL-10, CR3 and IFNγ than in the control groups. Whereas IL-1β, MyD88 and mul1b expression was lower in LPS treated cells.

***Figure 13**: Gene expression levels in distal intestine*



Pyloric Caeca Samples

Only 3 samples for each group were able to be used for qPCR, and due to this we are unable to perform meaningful statistical analysis as the power is low.

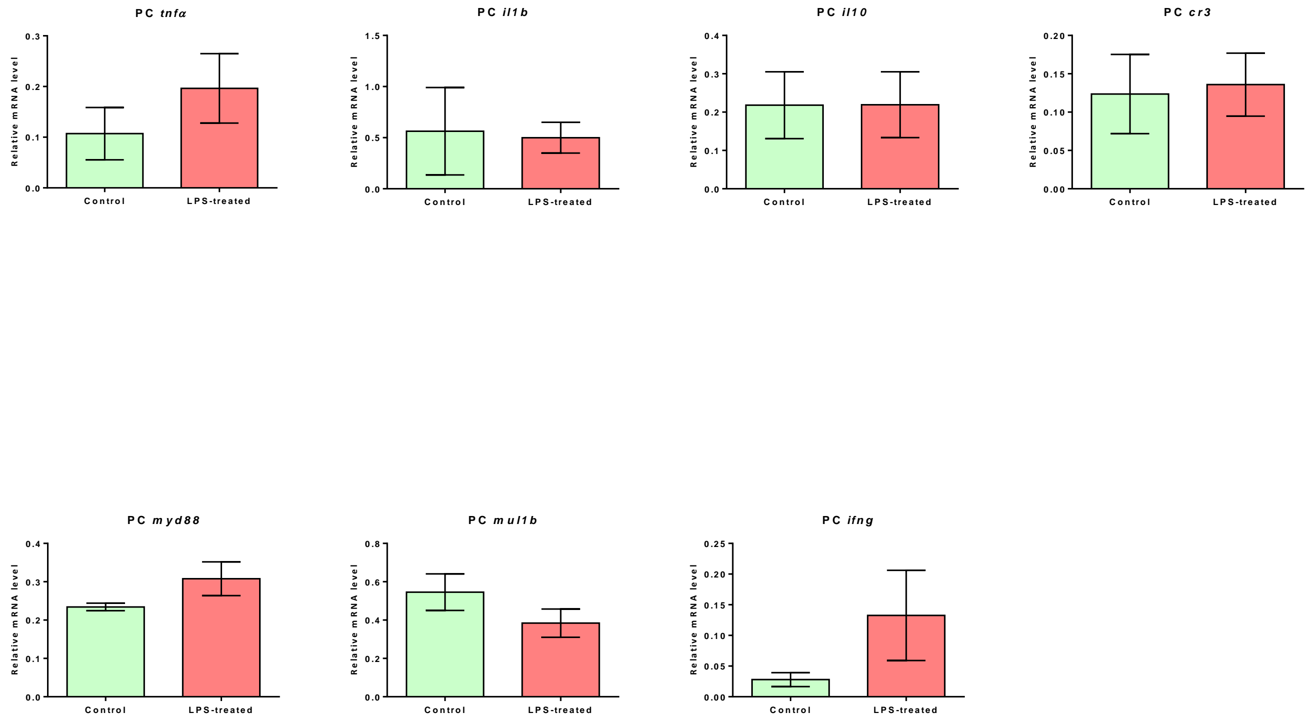


***Figure 14**: Gene expression levels in pyloric caeca*

5.0 Discussion

5.1 Cell isolation and identification

Microscopic examination of cells isolated from the distal intestine and pyloric caeca tissue suggest immune relevant cells, such as neutrophil-like and monocytic-like cells, were present in the samples. The results from flow cytometry showed phagocytic cells had successfully engulfed bioparticles. This indicates the cells isolated using this method included phagocytic cells which remained functional after extraction.

Pyloric caeca tissue samples provided lower quantities of cells than samples taken from distal intestine tissues. The distal intestine is seen as an immune-relevant tissue and has been examined for its potential use as a targeted area for vaccination (Vandenberg, 2004), whereas the pyloric caeca has not received as much focus. The lower number of cells extracted from the pyloric caeca may be due to the pyloric caeca not being an active immune site for healthy salmon, rather an area for digestive enzymes to breakdown nutrients. In comparision, the distal intestine is thought to be an active immune site, regularly sampling intestinal contents for pathogens (Olsson et al., 2011).

Given the low cell numbers able to be extracted from the pyloric caeca, only three samples were able to go on for further gene expression analysis. This meant that there were not enough samples produced to provide statistically meaningful results.

5.2 Gene Expression

LPS is known to stimulate the immune response in salmonids (MacKenzie et al. 2006). However, the cellular pathway in which it does this is not fully understood.

5.2.1 MyD88 dependent pathway

MyD88 is the best-characterized macrophage response to LPS, but it is not essential (Bjorkbacka et al., 2004). The MyD88 pathway is believed to be stimulated by TLR4 on the cell membrane of macrophages. TLR4 appears to be lacking in salmonids, meaning that TLR4 mediated endotoxin shock does not occur when fish are exposed to LPS (Berczi et al., 1966, Sepulcre et al., 2009). They also lack CD14, TICAM2 and LPS binding proteins in their serum required to illicit a response (Iliev et al., 2005).

Genes associated with the MyD88 pathway include MyD88 itself, TNFα, COX-1 and IL-1β (Pålsson-MacDermott et al., 2004). MyD88 and IL-1β expression was lower in distal intestine cells exposed to LPS. This is the opposite of what one would expect if the MyD88 pathway

was stimulated due to the presence of LPS. TNFα had a higher expression in the LPS stimulated samples compared to the control group. However, TNFα can be expressed as the result of other pathways as well as the MyD88 pathway.

Previous studies have suggested immune relevant gene expression in cultures exposed to LPS may be due to contaminants present. MacKenzie et al. (2010), Iliev et al. (2005) and Purcell at al. (2006) all showed reduced gene expression of proinflammatory and antiviral genes in cells treated with ultrapure LPS (LPS treated to remove contaminants) compared to crude LPS. Crude LPS may have contaminants present such as peptidoglycans (PGN), nucleic acids and lipoproteins. These contaminants may be responsible for eliciting a cellular response.

PGNs are present in the periplasmic layer on gram-negative cell walls (Boltaña et al., 2011). They have been proven to induce immune responses in fish such as expression and activity of PGN recognition proteins. These proteins play an essential role in bacterial defence mechanisms (Li et al., 2006). *E. coli* PGNs have a strong inductive effect on IL-1β and IL-6, which have been shown to be more potent than crude LPS (MacKenzie at al., 2010). Another study showed no gene expression changes to TNFα, IL-1β and IL-6 when cells were stimulated with ultrapure LPS (Roher et al., 2009).

Commercial LPS is often contaminated PGNs, which have been shown to elicit a TLR2 response (Roher et al., 2009). A reduction in IL-1β and IL-6 gene expression has been seen in cells exposed to crude LPS which had been previously treated with lysosyme to degrade PGNs. Due to the strong stimulation caused by PGNs, it is possible that macrophages recognise the PGNs on gram-negative bacteria cell walls leading to an increase in immune relevant gene expression. PGNs have been shown to stimulate the pro-inflammatory cytokines IL-1β, IL-6 and COX-2 in macrophage cells from Rainbow trout (MacKenzie et al., 2010).

Due to PGNs being linked to TLR2 expression and Atlantic salmon lacking TLR4, it could be beneficial to examine gene expression of those cytokines linked to the TLR2 response, as opposed to those connected TLR4.

5.2.2 MyD88-independent pathway

TRAF and TRIM pathways are activated leading to the expression of Type I interferons and interferon-stimulated genes (Kawai et al., 2001). IFNβ is produced at the end of the MyD88-

independent pathway (Pålsson-MacDermott et al., 2004). In mice, cells deficient in MyD88 responded to a LPS challenge by activating IFN-regulatory factor 3. Genes containing IFN-stimulating regulatory elements such as IL-10 have also been seen to have increased expression due to MyD88-independent pathway activation (Kawai et al., 2001). Stimulation of this pathway could lead to an increase in TNFα and IL-10, as was seen in LPS treated cells from the distal intestine in this experiment.

5.2.3 TNFα

Macrophages produce the pro-inflammatory cytokine TNFα in response to antigen exposure. It facilitates lipid metabolism, immunological regulation and apoptosis (MacKenzie et al., 2003). Preadipocyte cells of Atlantic salmon exposed to LPS have been shown to have reduced expression of genes involved in lipid metabolism when TNFα and other TNFα-dependent genes showed upregulation (Škugor et al., 2010).

In this experiment, TNFα was upregulated in LPS treated cells from both the pyloric caeca and distal intestine samples, compared to the control samples. LPS is known to stimulate TNFα in mammals, the presence of TNFα in fish macrophages exposed to LPS could therefore suggests conservation of LPS-induced pathways. It has been shown that LPS induced TNFα mRNA in both trout monocytes and macrophages, with a significant accumulation observed in macrophages 6hours after incubation with LPS (MacKenzie et al., 2003).

LPS also altered the appearance of trout macrophages in vitro, with increased phagocytic abilities and increased TNFα expression than that of freshly isolate monocytes (MacKenzie et al., 2003). Further examination of bioparticle uptake using the flow cytometer would have proven to be useful in assessing phagocytic abilities in relation to LPS exposure and TNFα expression. In addition to this, Roher et al., (2009) has also shown TNFα expression detected after 3hours exposure to LPS, however the highest expression was seen after 12hours of exposure. From this, LPS stimulation may have given more pronounced results if left for a longer period of time compared to the 6 hours the samples in this experiment were exposed for.

Interestingly, ultrapure LPS has been shown to cause an increase in the secretion of TNFα but not gene expression. It is hypothesized this is due to a preformed intracellular pool of TNFα being released upon the challenge from ultrapure LPS (Roher et al., 2009). It would therefore be beneficial to examine the presence of cytokine TNFα and not only its transcript.

5.2.4 IL-1β

The results presented in this thesis showed IL-1β to be expressed lower in LPS treated samples than in control samples. IL-1β had a lower expression than TNFα in LPS treated samples. This is contradictory to other studies showing an increase in IL-1β expression when exposed to LPS (Inoue et al., 2005).

Laing et al. (2002) argued that IL-1β may potentially modulate TNFα via a signalling pathway different to that of LPS. They showed that TNFα had a higher expression when both IL-1β and LPS were present suggesting multiple pathways being active. Therefore, it could be argued the lower expression of IL-1β in comparison to TNFα is due to its activity being linked to an alternative pathway, one that it is not responding to LPS by increased gene expression of IL-1β.

However, enterocyte exposure to LPS has been shown to lead to an increase in the mRNA expression of TNFα, IL-1β and IL-6 (Jiang et al., 2014). MacKenzie et al. (2006) also showed that macrophage cells of *Oncorhynchus mykiss* stimulated with LPS showed increase in IL-6 and TNFα.

In vivo studies have shown Atlantic salmon injected with LPS showed upregulation of IL-1RLP (Receptor Like Protein), and suggested that the balance of IL-1β, IL-1RI (receptor type 1), and IL-1RII dictated the overall magnitude of the LPS response (Subramaniam et al., 2002). To further understand the cellular pathway response, it would therefore be appropriate to examine the expression of proteins such as IL-1RLP in addition to IL-1β.

5.2.4 IL-10

IL-10 in mammals is an immunosuppressant cytokine, however it has been suggested it has an inflammatory role in fish. This is due to its rapid upregulation after LPS stimulation, as seen in other inflammatory cytokines such as IL-1β (Inoue et al., 2005). Distal intestine samples stimulated with LPS showed a higher expression of IL-10 than the control group. The results of this experiment can neither confirm nor dispute the role of IL-10 as an inflammatory cytokine in fish. It’s increase in LPS exposed cells may be due to it leading an inflammatory response, or it’s role as a regulatory cytokine in the presence of other inflammatory cytokines.

5.2.5 CR3

CR3 gene expression was statistically higher in LPS treated cells. This could potentially be explained by their role in MAPK activation pathways. CR3 is active in the innate immune system. It is a receptor present on phagocytes which binds to ligands from microbial molecules, such as the lipid A region of LPS. It is also responsible for linking phagocytes to the extracellular matrix. It mediates in cell migration and signals for both kinase cascades and cytoskeletal rearrangements. CR3 has been shown to linked to the activation of MAPK pathways (Ehlers, 2000). This has been further examined by MacKenzie et al. (2006) that showed cells exposed to LPS for 12hours had increased expression of MAPK and iNF-κB genes responsible for macrophage activation. The results from this experiment show that it's upregulation during cell stimulation could be due to it's role in the activation of immune relevant pathways.

5.2.6 IFNγ

In this experiment, IFNγ had higher gene expression in cells treated with LPS. It is also the only cytokine to provide any statistically significant result. IFNγ is a cytokine produced exclusively by immune cells and belongs to the group of cytokines known as interferon II. Its role is to activate the innate immune system and notify cells in the neighbouring area of pathogenic attack. In macrophages, its presence causes cells to increase antigen processing abilities, antigen presentation and apoptosis modulation (Schroder et al., 2004). IFNγ regulates the expression of TLR signalling accessory molecules and adaptors, and is thus able to promotes TLR signalling. Its stimulation leads to a lower concentration of TLR agonist being required to then go on and affect gene regulation (Costelloe et al., 1999). IFNγ is able to protect against viral infections (Robertsen, 2006). It also attracts circulating monocytes to the site of inflammation (Schroder et al., 2006). It is therefore multifunctional in the immune response, and its increase in gene expression in LPS treated cells suggests the cells isolated have responded to a pathogenic challenge.

LPS requires co-molecule CD14 (Wright et al., 1990) and MD-2 accessory molecule (Shimazu et al., 1999) to fully induce the MyD88-dependent or MyD88-independent pathways, such as the TRAM and TRIF pathways (Vogel et al., 2003). It has been suggested that the CD14 co-molecule and MD-2 accessory molecule are IFNγ-dependent (Schroder et al., 2006). Therefore, the increase in IFNγ expression may indicate a response to LPS. The presence of IFNγ in this experiment does not dispute nor support arguments for LPS stimulating a MyD88-dependent pathway, however it does show the cells producing an

immune relevant response. Further examination of the expression of cytokines further down stream would help to evaluate which cellular pathway is triggered by IFNγ.

IFNγ has been shown to be produced by cells responsible for the innate and the adaptive immune responses. For example, PHA is a mitogen capable of inducing cytotoxic functions in mammals. When trout CD8+ T-cells were exposed to PHA, there was an increase in IFNγ upregulation as well as perforin expression (Toda et al., 2011). Innate immune cells, such as macrophages, have shown that STAT1 regulates IFNγ signalling (Meraz et al., 1996). Testing for STAT1 in addition to IFNγ could potentially widen our understanding of how developed the IFNγ response to LPS is.

5.2.7 Mul1b

The results from this experiment showed lower expression of Mul1b in LPS stimulated cells compared to the control group. This is unexpected as Mul1b activates the NFκB pathway in species such as zebrafish, producing an inflammatory response. However, the use of Mul1b in the study of Atlantic salmon gene expression is questionable. Mitochondrial Ubiquitin Ligase Activator of NFκB (MULAN) is central to NFκB activation. It regulates mitochondria and cell apoptosis. In rainbow trout, MULAN expression has been seen to increase in cells treated with LPS and recombinant IL-1β for 4hours (Tacci et al., 2011). The same paper suggest that the MULAN gene activates NFκB during immune responses. However, it did not find Mul1b gene to give any significant hits in Atlantic salmon genome. It could therefore be more appropriate to examine MULAN expression in Atlantic salmon cells as opposed to Mul1b.

The results presented in this thesis have not been able to give a clear understanding of the cellular response produced when immune cells from the gastrointestinal tract are stimulated with LPS *in vitro*. A larger sample size would help to produce give more power to the statistical analysis results. However, given the data present shows an increase in the expression of the immune relevant genes TNFα, IFNγ and IL-10 in LPS treated cells, it could be argued that cellular pathways have been activated by the presence of LPS. To further understand which pathways are responsible for this response, alternative genes could be examined.

5.3 Alternative genes and cellular pathways

5.3.1 Toll-Like Receptor 2

Macrophages taken from mouse spleens have been shown to have increased TLR2 gene expression in response to in vitro treatment with LPS, not TLR4 (Matsuguchi et al., 2000). In

addition to LPS increasing TLR2 gene expression, Matsuguchi et al. (2000) also noted that synthetic lipid A, IL-1β, TNFα and IFNγ caused an increase in TLR2 gene expression. Given that TLR4 has not currently been confirmed as present in Atlantic salmon macrophages, the cellular response to LPS stimulation may occur via the TLR2 signalling pathway.

However, TLR2 has also been shown to be involved in the regulation of genes in response to peptidoglycans, lipoproteins and lipoteichoic acid (Matsuguchi et al., 2000, Roher et al., 2009). Therefore, it could be possible that contaminants within the LPS could be stimulating cytokine gene expression via the TLR2 signalling pathway and not LPS itself. The use of Ultrapure LPS in cell stimulation would bring clarity to the results by removing other potential stimulants.

5.3.2 COX-2

COX-2 has been shown to mediate prostaglandin production during an inflammatory response in LPS challenged macrophages from Rainbow trout (MacKenzie et al., 2010). This has been further shown by Holen et al. (2010) when Atlantic salmon leukocytes stimulated with LPS showed an increase in COX-2 gene expression. COX-2 is also linked to the MyD88-dependent pathway (Pålsson-MacDermott et al., 2004).

5.3.3 Macrophage colony stimulating factor (M-CSF)

M-CSF has been shown to promote growth and increase chemokine expression in head kidney cells to produce monocytes and macrophages. It also increases proinflammatory gene expression, free radical production in macrophages and phagocytosis (Roberts, 2012). It signals through the MAPK pathway (Curry et al., 2008).

5.3.4 MAPK

Mitogen activated protein kinases (MAPK) transmit signals from cell surface receptors. They mediate cytokine response and are intrinsically linked to the innate immune response to PAMPs (Hansen and Jørgensen, 2007). p38 is a class of MAPK. LPS activation of p38 has been shown to upregulate IL-1β, TNFα and COX-2 (Holen et al., 2011). Iliev et al. (2013) showed LPS induced genes were p38 dependent. Cells pretreated with p38 inhibitor blocked PAMP induced p38 activity, and consequently reduced LPS induced transcripts such as TNFα and IL-1β, demonstrating the importance of p38 in the salmon phagocyte response (Iliev et al., 2013, Hansen and Jørgensen, 2007, Holen et al., 2011). Quantifying p38 expression could potentially demonstrate whether the response to LPS is MAPK dependent.

Iliev et al. (2013) suggest that MAPK activity could be highly influenced by the differentiation status of mononuclear phagocytes. Monocytes have a less developed cytokine response than that of mature macrophages (MacKenzie et al., 2003). In a fresh cell culture, there may be a lower immune response if cells present are still immature. Using flow cytometry to assess the cell identity and stage would allow for the results to indicate if the pathways used are relevant to monocytes or more mature macrophages.

5.3.5 NFκB

NFκB is a transcription factor which regulates numerous genes responding to bacterial and viral infections. Inhibitory proteins (IkBs) prevent NFκB signaling in non-stimulated cells. When a cell is stimulated by a pathogen, IkBs are degraded by ubiquitin proteasome pathways which leads to the translocation of NFκB to the nucleus where it is then capable of regulating gene expression (Tacci et al., 2011).

NFκB has been linked to both IFNγ and MyD88 pathways. IFNγ stimulation affects NF-κB activation (Schroder et al., 2006). IFNγ leads to the activation of the cytoplasmic transcription pathways utlizing NF-κB. which translocates to the nucleus where it alters gene expression (Aderum and Ulevitch, 2000).

In humans, after stimulation with LPS, IFNγ primed cells resulted in super activation of NF-κB, suggesting that IFNγ decreased the DNA-binding activity of AP-1 (de Wit et al.., 1996**).** Given the increase in IFNγ mRNA expression found in distal intestine samples stimulated with LPS, further examination of the NFκB pathway and cytokine expression related to it would be able to demonstrate its role in the cellular response to LPS stimulation.

NFκB leads to the expression of proinflammatory genes IL-2, IL-6, TNFα and IFNγ (Tacci et al., 2011). It could be expected that along with an increase in TNFα and IFNγ expression, if cytokine stimulation was occurring due to the NFκB pathway, we would also find an increase in IL-2 and IL-6. To confirm this, IL-2 and IL-6 gene expression could also be quantified via qPCR.

NFκB has been seen to be essential in LPS mediated TLR2 induction in macrophages. Cytokines found to have increased expression of TLR2 are also active in the NFκB pathway (Matsuguchi et al., 2000). Additionally, LPS mediated TLR2 response has been shown to be reduced by pre-treatment with curcumin, inhibiting NFκB production. This would suggest NFκB is vital to the TLR2 gene expression (Matsuguchi et al., 2000).

Therefore, by examining gene expression of TLR2 along with NFκB and its associated cytokines, we could potentially establish evidence to suggest LPS stimulation in salmon macrophage-like cells from the distal intestine results in activation of the NFκB pathway via TLR2 receptor.

Further to gene expression, identification and quantification of proteins, as well as measuring cellular activity would help to provide a greater understanding of the cellular pathways activated and its products. For example, respiratory burst is a well-known response by macrophages to a pathogen challenge.

5.4 Respiratory Burst/NOS

LPS induces respiratory burst in Atlantic salmon cells via PAMP activation (Sepulcre et al. 2007). By measuring respiratory burst in cells, it is possible to assess cellular activity. Respiratory burst is associated with phagocytosis, linked to an increased oxygen consumption.

Nitric oxide (NO) induces vasorelaxation, inhibits cell proliferation and is involved in intracellular signalling. From an immune perspective, it also acts as a potent toxin (Rieger et al., 2011). Borroso et al., (2000) found inducible NOS gene expression and activity in teleost phagocytes. IFNγ, TNFα, colony stimulation factor 1, IL-6 induce iNOS expression and contribute to NO production. NO is not pathogen specific and can damage the host in high concentration (Rieger et al. 2011). Qin et al. (2001), showed an increase in respiratory burst function when Rainbow trout macrophages were exposed to LPS at 20µg/ml. TNFα has been shown to induce respiratory burst (Rieger et al., 2011).

By measuring only mRNA we do not get a full picture of the cellular response. mRNA may not be translated into the end product which is responsible for the response to the pathogenic challenge. Examining the levels of respiratory burst and NO in the samples could demonstrate the outcome of a cellular pathway, as well as its functionality.

5.5 Protein expression

Physiological alterations are caused by changes in mRNA expression and consequent translation. The variances in expression of mRNA leads to a variation in protein synthesis

(Oleksiak and Crawford, 2005). When mRNA expression is increased, this may not necessarily correlate with an increase protein synthesis. Therefore, to fully understand the outcome of the cellular response to LPS stimulation, studying protein production (ie. cytokines) would provide insight to the product of the activated cell pathway. As previously mentioned, ultrapure LPS has been shown to cause an increase in the secretion of TNFα but not gene expression (Roher et al., 2009). By measuring only transcripts we do not see the whole picture.

5.6 Type of LPS used

In this experiment, the LPS used was not ultrapure. To demonstrate the variations in gene expression were due to LPS stimulation and not of other contaminants, Ultrapure LPS should be used.

The region of LPS responsible for stimulation tends to be the lipid A component (Rietschel et al. 1998). Alterations in the lipid A region can allow a pathogen to avoid detection (Loppnow et al., 1990). Additionally, the inflammatory response to LPS has been shown to be dependent upon its composition. (Tanamoto et al., 2000) showed lipid A from *E. coli* initiated an inflammatory response from both human and mice cells, however lipid A from *salmonella* only caused an immune response in the mice cells. Therefore, variations in LPS sources could provide differing PAMP recognition and pathway activation. Examining the response from alternative LPS sources, not only LPS from *E. coli*, could lead to a stronger immune response and therefore be a better model for studying immune pathways.

Matsuguchi et al. (2000) hypothesized that TLR2 and TLR4 may respond to different types of LPS. This could be further explored in Atlantic salmon, for example, by using LPS from *Aeromonas salmonicida* in comparison *E. coli*. The two types of LPS could potentially lead to different cellular pathways being triggered, or the stimulation induced could vary in intensity.

By examining the gene expression caused by different ultrapure LPS sources, we could establish if salmon macrophages respond to all LPS challenges in the same way, or if the response is more specific for each type of LPS exposed to the cell.

6.0 Conclusion

The outset of this thesis was to examine the cellular pathway induced by LPS stimulation of macrophage-like cells. The results showed TNFα, IL-10 and IFNγ had higher gene expression in cells stimulated with LPS, however only IFNγ in the distal intestine provided any statistically significant results. This project could be taken further by taking into consideration areas addressed in the previous discussion.

Detailed functional studies into the teleost immune system is progressing, but knowledge is still lacking. Pattern recognition receptors (PRRs) potentially involved in gram-negative recognition have been identified in numerous species, however uncertainty still remains around the functionality of these systems (Boltaña et al., 2011).

The increased expression of IFNγ, TNFα and CR3 in cells exposed to LPS can potentially direct further investigations towards the TLR2 and NFκB signaling pathways after stimulation. Additional transcripts should be examined to provide a clearer understanding of cellular pathway activation. For example, genes related to the NFκB and TLR2 pathway such as COX-2, IL-2 and IL-6. The MyD88-independent pathway could also be investigated by measuring TRIF/TRAM expression.

Cellular activity could also be measured via protein analysis and by assessing respiratory burst and NOS capabilities.

In addition to this, the type of LPS used to stimulate cell cultures must be taken into consideration, with ultrapure LPS perhaps being the best choice when attempting to identify specific cell pathways.

References/Bibliography

Aderem, A., Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. Nature 406: 782–787.

Alejo, A., Tafalla, C. (2011) Chemokines in teleost fish species. Developmental and Comparative Immunology 35: 1215 – 1222

Anderson, M.K., et al. (2004) Evolutionary origins of lymphocytes: ensembles of T cell and B cell transcriptional regulators in cartilaginous fish. Journal of Immunology 172(10): 5851-5860.

Anwar, M. and Choi, S. (2014) Gram-negative marine bacteria: structural features of lipopolysaccharides and their relevance for economically important diseases. *Marine drugs*, *12*(5): 2485-2514.

Bakke-McKellep, A.M., Nordrum, S., Krogdahl, A., Buddington, R.K. (2000) Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). Fish Physiol Biochem 22: 33–44.

Bakke-McKellep, A.M., Frøystad, M.K., Lilleeng, E., Dapra, F., Refstie, S., Krogdahl, A., Landsverk, T. (2007) Response to soy: T-Cell-like reactivity in the intestine of Atlantic Salmon, *Salmo salar*. L.. J. Fish Dis. 30: 13-25.

Barroso, J.B., Carreras, A., Esteban, F.J., Peinado, M.A., Martinez-Lara, E., Valderrama,R., Jimenez, A., Rodrigo, J., Lupianez, J.A. (2000) Molecular and kinetic characterization and cell type location of inducible nitric oxide synthase in fish. Am. J.Physiol. Regul. Integr. Comp. Physiol. 279: R650–R656.

Berczi, I., Bertok, L., Bereznai, T. (1966) Comparative studies on the toxicity of Escherichia coli lipopolysaccharide endotoxin in various animal species. Can. J. Microbiol. 12: 1070–1071

Bjorkbacka, H., Fitzgerald, K.A., Huet, F., Li, X., Gregory, J.A., Lee, M.A., Ordija, C.M., Dowley, N.E., Golenbock, D.T., Freeman, M.W. (2004) The induction of macrophage gene expression by LPS predominantly utilizes MyD88-independent signaling cascades. Physiological Genomics. 19(3): 319-330

Boehm, U., Klamp, T., Groot, M. and Howard, J.C. (1997) Cellular responses to interferon-γ. Annual review of immunology, 15(1): 749-795.

Boltaña, S., Roher, N., Goetz, F.W. and MacKenzie, S.A. (2011) PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish. Developmental & Comparative Immunology 35(12): 1195-1203.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria.Science 303: 1532–1535.

Bullock, W. L. (1963). Intestinal histology of some salmonid fishes with particular reference to the histopathology of acanthocephalan infections. Journal of Morphology 112, 23–35

Bundschuh, D.S., Barsig, J., Hartung, T., Randow, F., Docke, W.D., Volk, H.D., Wendel, A. (1997) Granulocyte-macrophage colony-stimulating factor and IFN-gamma restore the systemic TNF-alpha response to endotoxin in lipopolysaccharide-desensitized mice. J. Immunol. 158: 2862–2871.

Buonocore, F., Castro, R., Randelli, E., Lefranic, M.P., Six, A., Kuhl, H, Reinhardt, R., Boudinot, P., Scapigliati, G. (2012) Diversity, molecular characterization and expression of T-cell receptor γ in a teleost fish, the sea bass (*Dicentrarchus labrax*, L) PLoS One 7:e57957

Cario, E. (2005) Bacterial Interactions with Cells of the Intestinal mucosa: toll like receptors and NOD2. Gut 54: 1182-1193.

Chu, Q., Sun, Y., Cui, J., Xu, T. (2017) MicroRNA-3570 Modulates the NF-κB Pathway in Teleost Fish by Targeting MyD88. The Journal of Immunology. doi:10.4049/jimmunol.1602064

Claire, M., Holland, H., Lambris, J.D. (2002) The Complement System in Teleosts. Fish and Shellfish Immunology 12: 399-420.

Costelloe, E.O., Stacey, K.J., Antalis, T.M., Hume, D.A. (1999) Regulation of the plasminogen activator inhibitor-2 (PAI-2) gene in murine macrophages. Demonstration of a novel pattern of responsiveness to bacterial endotoxin. J. Leukoc. Biol. 66: 172–182.

Curry, J.M., Eubank, T.D., Roberts, R.D., Wang, Y., Pore, N., Maity, A. Marsh, C.B. (2008) M-CSF signals through the MAPK/ERK pathway via Sp1 to induce VEGF production and induces angiogenesis in vivo. PloS one, *3*(10): p.e3405.

de Wit, H., Hoogstraten, D., Halie, R.M., Vellenga, E. (1996) Interferon-gamma modulates the lipopolysaccharide-induced expression of AP-1 and NF-kappa B at the mRNA and protein level in human monocytes. Exp. Hematol. 24: 228–235.

Ehlers, M.R.W. (2000) CR3: a general purpose adhesion-recognition essential for innate immunity. Microbes and Infection 2: 289-294

Ellis, A.E. (1999) Immunity to bacteria in fish. Fish and shellfish Immunology 9: 291-308.

Oleksiak, M.F., Crawford, D.L. (2005) Chapter 15: Functional Genomics in Fishes: Insights into Physiological Complexity. The Physiology of Fishes. Evans, D.H.,Claiborne, J.B. Third Edition. CRC Press

FAO. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.

Fischer, U., Koppand, E.O., Nakanish, T. (2013) Teleost T and NK Cell Immunity. Fish and Shellfish Immunology 35: 1215-1222

Forlenza, M., Fink, I.R., Raes, G., Wiegertjes, G.F. (2011) Heterogeneity of macrophage activation in fish. Developmental and Comparative Immunology 35: 1246-1255

Fuglem, B., Jirillo, E., Bjerkas, I., Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U., Koppang, E.O. (2010) Antigen-sampling cells in the salmonid intestinal epithelium. Dev Comp Immunol. 34:768– 774

Gallay, P., Heumann, D., Le Roy, D., Barras, C., Glauser, M.P. (1993) Lipopolysaccharide-binding protein as a major plasma protein responsible for endotoxemic shock. PNAS 90 (21): 9935-9938

Galli, S.J., Nakae, S., Tsai, M. (2005) Mast cells in the development of adapative immune responses. Nature Immunology 6: 135-142.

Georgopoulou, U., Dabrowski, K., Sire, M.F., Vernier, J.M. (1988) Absorption of intact proteins by the intestinal epithelium of trout, *Salmo gairdneri*. A luminescence enzyme immunoassay and cytochemical study. Cell Tissue Research 251: 145-152.

Goerdt, S., Politz, O., Schledzewski, K., Birk, R., Gratchev, A., Guillot, P., Hakiy, N., Klemke, C.D., Dippel, E., Kodelja, V., Orfanos, C.E. (1999) Alternative versus classical activation of macrophages. Pathobiology 67, 222–226.

Gräns, A., Olsson, C. (2011) The Gut: Integrated Function and Control of the Gut: Gut Motility. Elsevier Inc. University of Gothenburg, Gothenburg, Sweden. 1292 -1301.

Grayfer, L., Belosevic, M. (2009) Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma. Dev. Comp. Immunol. 33 (2): 235–246.

Gonçalves, A.T., Valenzuela-Muñoz, V. and Gallardo-Escárate, C. (2017) Intestinal transcriptome modulation by functional diets in rainbow trout: A high-throughput sequencing appraisal to highlight GALT immunomodulation. Fish and shellfish immunology 64: 325-338.

Grayfer, L., Belosevic, M., (2012) Cytokine regulation of the teleost inflammatory responses. New Advances and Contributions to Fish Biology. Turker, H., Ed.; InTech: Rijeka, Croatia. 59-96

Cain, K. And Swan, C (2010) Chapter 3: Barrier Function and Immunology. Fish physiology: the multifunctional gut of fish (Vol. 30). Grosell, M., Farrell, A.P. and Brauner, C.J.Academic Press.

Hansen, T.E., Jørgensen J.B. (2007) Cloning and characterization of p38 MAP kinase from Atlantic salmon. A kinase important for regulating salmon TNF-2 and IL-1β expression. Molecular Immunology 44: 3137-3146.

Holen, E., Winterthen, S., Du, Z.-Y., Krøvel, A.V. (2011) Inhibition of p38 MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (*Salmo salar*) fed soy bean oil or fish oil based diets. Fish and Shellfish Immunology 20: 397-405.

Holtmeier, W. Kabelitz, D. (2005) Gammadelta T cells link innate and adaptive immune responses. Chem Immunol Allergy 86: 151-183

Davis, S.J., Ikemizu, S., Evans, E.J., Fugger, L., Bakker, T.R. and van der Merwe, P.A. (2003) The nature of molecular recognition by T cells. *Nature immunology*, *4*(3): 217.

Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V., Goetz, F.W. (2005) Endotoxin recognition: in fish or not in fish? FEBS Lett. 579: 6519–6528.

Iliev, D.B., Hansen, T., Jørgensen, S.M., Krasnov, A., Jørgensen J.B. (2013) CpG- and LPS-activated MAPK signaling in *in vitro* cultured salmon (*Salmo salar*) mononuclear phagocytes. Fish and Shellfish Immunology 35: 1079-1085.

Ingerslev, H.-C., Rønneseth, A., Pettersen, E.F., Wergeland, H.I. (2008) Differential Expression of Immune Genes in Atlantic Salmon (*Salmo salar L.*) Challenged Intraperitoneally or by Cohabitation with IPNV. Scandinavian Journal of Immunology. 69: 90-98.

Inoue, Y., Kamota, S., Itoa, K. Yoshiura, Y., Ototake, M., Moritomo, T., Nakanishi, T. (2005) Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. Fish and Shellfish Immunology 18:335–44.

Jiang, J., Shi, D., Zhou, X.Q., Hu, Y., Feng, L., Liu, Y., Jiang, W.D. and Zhao, Y. (2015) In vitro and in vivo protective effect of arginine against lipopolysaccharide induced inflammatory response in the intestine of juvenile Jian carp (Cyprinus carpio var. Jian). Fish and Shellfish Immunology 42(2):.457-464.

Joosten, P.H.M., Kuijer, W.J., Rombout, J.H.W.M. (1996) Anal immunization of carp and rainbow trout with different fractions of a *Vibrio anguillarum* bacterin. Fish and Shellfish Immunology 6: 541-551.

Jurkovich, G.J., Mileski, W.J., Maier, R.V., Winn, R.K., Rice, C.L. (1991) Interferon gamma increases sensitivity to endotoxin. J. Surg. Res. 51: 197–203.

Jutfelt, F. (2011) Barrier function of the gut. Encyclopedia of fish physiology: from genome to environment. Academic Press, San Diego, 1322-1331

Kasahara, M., Nakaya, J., Satta, Y., Takahata, N. (1997) Chromosomal duplication and the emergence of the adaptive immune system. Trends Genet. 13: 90-92.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., Hoshino, K., Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. The Journal of Immunology 167: 5887-5894.

Koppang, E. O., U. Fischer, L. Moore, M. A. Tranulis, J. M. Dijkstra, B. Ko ¨llner, L. Aune, E. Jirillo, and I. Hordvik. (2010) Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. J. Anat. 217: 728– 739

Krogdahl, Å., Sundby, A., Bakke, A. M. (2011). Integrated Function And Control Of The Gut: Gut Secretion and Digestion. 1301-1310.

Laing, K.J., Wang, T., Zoe, J., Holland, J., Hong, S., Bols, N., Hirono, I., Aoki, T., Secombes, C.J. (2002) Cloning and expression analysis of rainbow trout Oncorhynchus mykiss tumour necrosis factor-α. European Journal of Biochemistry 268 (5): 1315-1322.

Li, J., Barreda, D.R., Zhang, Y., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., Sunyer, J.O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nature Immunology 7 (10): 1116-1124

Lawrence, T. (2009) The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harbour Perspectives in Biolology;1:a001651

Lobb, C.J., Clem, L.W. (1981) Phylogeny of immunoglobulin structure and function. XI. Secretory immunoglobulins in the cutaneous mucus of the sheepshead, *Archosrgus probatocephalus*. Dev Comp Immunology 5: 587-596.

Loppnow, H.; Libby, P.; Freudenberg, M.; Krauss, J.H.; Weckesser, J.; Mayer, H. (1990) Cytokine induction by lipopolysaccharide (LPS) corresponds to lethal toxicity and is inhibited by nontoxic rhodobacter capsulatus LPS. Infect. Immun. 58: 3743–3750.

MacKenzie, S., Planas, J.V. Goetz, F.W. (2003) LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. Developmental & Comparative Immunology, 27(5): 393-400.

MacKenzie, S.A, Iliev, D., Liarte, C., Koskinen, H., Planas, J.V., Goetz, F.W., Mölsä, H., Krasnov, A. Tort, L. (2006) Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. Molecular immunology, 43(9): 1340-1348.

MacKenzie, S.A., Roher, N., Boltaña, N., Goetz, F.W. (2010) Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS. Molecular Immunology 47: 1450-1457

MacMicking, J., Xie, Q.W., Nathan, C. (1997) Nitric oxide and macrophage function. Annu. Rev. Immunol. 15: 323–350.

Madigan, M.T., Clark, D.P., Stahl, D. and Martinko, J.M., (2010) Brock biology of microorganisms 13th edition. Benjamin Cummings.

Maisey, K., Montero, R., Corripio-Miyar, Y., Toro-Ascury, D., Valenzuela, B., Reyes-Cerpa, S., Sandino, A.M., Zou, J., Wang, T., Secombes, C.J., Imarai, M. (2016) Isolation and Characterization of Salmonid CD4+ T cells. The Journal of Immunology 196: 4150-4163.

Manera, M., Dezfuli, B.S. (2004) Rodlet Cells in teleosts: a new insight into their nature and functions. Journal of Fish Biology 65: 597-619

Matsuguchi, T., Musikacharoen, T., Ogawa, T., Yoshikai, Y. (2000) Gene Expressions of Toll-Like Receptor 2, but not Toll-Like Receptor 4, is induced by LPS and Inflammatory Cytokines in Mouse Macrophages. The Journal of Immunology 165: 5767-5772.

Mayer, E.A., Tillisch, K., Gupta, A. (2015) Gut/Brain axis and the microbiota. The Journal of Clinical Investigation 125(3): 926-938.

Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., Carver-Moore, K., DuBois, R.N., Clark, R., Aguet, M., Schreiber, R.D. (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 84: 431–442.

Merluzzi, S., Elena, B., Ceccaroni, A.A., Magris, R., Giunta, M., Mion, F. (2015) Mast cells, basophils and B cell connection network. Molecular Immunology 63: 94-103.

Meyniel, J.P., Khan, N.A., Ferrière, F., Deschaux, P. (1997) Identification of lymphocyte 5-HT3 receptor subtype and its implication in fish T-cell proliferation. Immunol. Lett. 55: 151-160.

Miller, N. et al. (1998) Functional and molecular characterization of teleost leukocytes. Immunology Rev. 166: 187 – 197

Mommsen, T.P., Osachoff, H.L., Elliot, M.E. (2003) Metabolic zonation in teleost gastrointestinal tract. Effects of fasting and cortisol in tilapia. J Comp Physiol B. 173: 409-418.

Moore, L. J., J. M. Dijkstra, E. O. Koppang, and I. Hordvik. (2009) CD4 homologues in Atlantic salmon. Fish Shellfish Immunol. 26: 10–18.

Mowat, A.M., Agace, W.W. (2014) Regional specialization within the intestinal immune system. Nat. Rev. Immunol. 14: 667-685.

Nayak, S.K., (2010) Probiotics and immunity: a fish perspective. Fish Shellfish Immunol. 29: 2–14

Neutra, M.R., Forstner, J.F. (1987) Gastrointestinal mucus: synthesis, secretion and function. In: Johnson LR (editor) Physiology of the gastrointestinal tract. Raven Press, New York, pp 975-1009

Niklasson L, Sundh H, Olsen R-E, Jutfelt F, Skjødt K, et al. (2014) Effects of Cortisol on the Intestinal Mucosal Immune Response during Cohabitant Challenge with IPNV in Atlantic Salmon (Salmo salar). PLoS ONE 9(5): e94288. doi:10.1371/journal.pone.0094288

Noga, E.J., Silphaduang, U., Park, N.G., Seo, J.K., Stephenson, J., Kozlowicz, S. (2009) Piscidin 4, a novel member of the piscidin family of antimicrobial peptides. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 152: 299–305.

Olsen, R.E., Sundell, K., Hansen, T., Hemre, G.I., Myklebust, R., Mayhew, T.M., Ringø, E. (2002) Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. Fish Physiology and Biochemistry 26: 211–221.

Olsson, C. (2011) The Gut: Gut Anatomy and Morphology. Elsevier Inc. University of Gothenburg, Gothenburg, Sweden. 1268 – 1275.

Olsvik, P.A., Lie, K.K., Jordal, A-E.O., Nilsen, T.O., Hordvik, I. (2006) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Molecular Biology, 6:21

Peatman, E., Liu, Z. (2007) Evolution of CC chemokines in teleost fish: a case study in gene duplication and implications for immune diversity. Immunogenetics 59 (8): 613–623.

Petrie, A.G., Ellis, A.E. (2006) Evidence of particulate uptake by the gut of Atlantic salmon (Salmo salar L.). Fish and Shellfish Immunology. 20: 660-664.

Poltorak, A., He, X., Smirnova, I. et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 282: 2085-2088.

Purcell, M.K., Smith, K.D., Aderem, A., Hood, L., Winton, J.R., Roach, J.C (2006) Conservation of Toll-like receptor signaling pathways in teleost fish. Comparative Biochemistry and Physiology, Part D 1: 77-88.

Pålsson-McDermott, E.M., O'Neill, L.A.J. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113: 153-162.

Qin, Q. W., Ototake, M., Noguchi, K., Soma, G. I., Yokomizo, Y., Nakanishi, T. (2001) Tumor necrosis factor alpha (TNFα)-like factor produced by macrophages in rainbow trout, *Oncorhynchus mykiss*. Fish & shellfish immunology 11(3): 245-256.

Rieger, A.M., Hall, B.E., Barreda, D.R. (2010) Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms. Dev. Comp. Immunol. 34: 1144–1159.

Rieger, A.M., Barreda, D.R., (2011) Antimicrobial mechanisms of fish leukocytes. Developmental and Comparative Immunology 35(12): 1238-1245.

Reite, O. B. (1997). Mast cells/eosinophilic granule cells of salmonids: staining properties and responses to noxious agents. Fish & Shellfish Immunology 7, 567–584.

Reite, O.B., Eversen, Ø. (2006) Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish and Shellfish Immunology 20: 192-208.

Rietschel, E.T., Schletter, J., Weidemann, B., El-Samalouti, V., Mattern, T., Zähringer, U., Seydel, U., Brade, H., FLAD, H.D., Kusumoto, S. Gupta, D. (1998) Lipopolysaccharide and peptidoglycan: CD14-dependent bacterial inducers of inflammation. Microb. Drug Resist. 4 (1): 37–44.

Ridaura, V., Belkaid, Y. (2015) Gut Microbiota: The Link to Your Second Brain. Cell. 161: 193-194.

Roberts, R.J. (2012) Fish Pathology. Fourth Edition. Wiley-Blackwell Publishing Ltd. Wesy Sussex, UK.

Robertsen, B. (2006) The Interferon system of teleost fish. Fish and Shellfish Immunology 20: 172-191.

Rodriguez, M.F., Wiens, G.D., Purcell, M.K., Palti, Y. (2005) Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 57: 510-519.

Roher, N., Callol, A., Planas, J.V., Goetz, F.W., MacKenzie, S.A. (2009) Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. Innate Immunity 17(1): 16-28.

Rombout, J.H.W.M., Lamers, C.H., Helfrich, M.H., Dekker, A., Taverne-Thiele, J.J. (1985) Uptake and transport of intact macromolecules in the intestinal epithelium of carp (Cyprinus carpio L.) and the possible immunological implications. Cell Tissue Res. 239: 519–530.

Rombout, J.H.W.M., Huttenhuis, H.B., Picchietti, S., Scapigliati, G. (2005) Phylogeny and ontogeny of fish leucocytes. Fish and Shellfish Immunology 19; 441-455.

Rombout, J.H.W.M., Abelli, L., Picchietti, S., Scapigliati, G., Viswanath, K. (2011) Teleost Intestinal Immunology. Fish and Shellfish Immunology 31: 616-626.

Rombout, J.H.W.M., Guiwen, Y., Viswanath, K. (2014) Adaptive immune responses at mucosal surfaces of teleost fish. Fish and shellfish Immunology 40: 634-643.

Salinas, I., Meseguer, J. and Esteban, M.Á. (2007) Assessment of different protocols for the isolation and purification of gut associated lymphoid cells from the gilthead seabream (Sparus aurata L.). Biological procedures online *9*(1): 43-55.

Salinas, I., Zhang, Y.A., Sunyer, J.O. (2011) Mucosal immunoglobulins and B cells of Teleost fish. Dev Comp Immunol. 35:1346-1365.

Salazar-Mather, T.P., Hamilton, T.A. and Biron, C.A. (2000) A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. The Journal of clinical investigation, 105(7): 985-993.

Scapigliati, G. (2013) Functional aspects of fish lymphocytes. Developmental and Comparative Immunology 41: 200-208.

Schep, L.J., Tucker, I.G., Young, G., Butt, A.G. (1997) Regional permeability differences between the proximal and distal portions of the isolated salmonid posterior intestine. J Comp Physiol B. 167: 370-377.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A. (2004) Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol. 75: 163–189.

Schroder, K., Sweet, M.J., Hume, D.A. (2006) Signal Integration between IFNγ and TLR signaling pathways in macrophages. Immunobiology 211: 551-524.

Sepulcre, M.P., Lopez-Castejon, G., Meseguer, J., Mulero, V. (2007) The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish. Mol.Immunol. 44: 2009–2016.

Sepulcre, M. P., Alcaraz-Pérez, F., López-Muñoz, A., Roca, F. J., Meseguer, J., Cayuela, M. L., Mulero, V. (2009) Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J. Immunol. 182 (4): 1836–1845.

Sheridan, B.S., Romagnoli, P.A., Pham, Q.M., Fu, H.H., Alonzo 3rd F., Schubert, W.D., Freitag N.E., Lefrançois, L. (2013) γδ T-cells exhibit multifunctional and protective memory in intestinal tissues. Immunity 39: 184-195.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J. Exp. Med. 189: 1777–1782.

Škugor, S., Škugor, A., Todorčević, M., Torgersen, J., Ruyter, B., Krasnov, A. (2010) Exposure to lipopolysaccharide induces immune genes in cultured preadipocytes of Atlantic salmon. Fish & Shellfish Immunology. 29: 817-824.

Smith, S. A., Caceci, T. & Robertson, J. L. (1995). Occurrence of rodlet cells and associated lesions in the vascular system of freshwater angelfish. Journal of Aquatic Animal Health 7: 63–69.

Statistisk sentralbyrå 2016: https://www.ssb.no/en/fiskeoppdrett as of 22/11/2017

Stone, K.D., Prussin, C., Metcalfe, D.D. (2009) IgE, mast cells, basophils and eosinophils. American Academy of Allergy, Asthma and Immunology. 125 (2): S73-S80.

Subramaniam, S., Olsen, L., Zou, J., Secombes, C.J., Cunningham C. (2002) Cloning of Salmo salar interleukin-1 receptor-like cDNA. Developmental Comparative Immunology 26(5): 415-431.

Sundell, K.S., Sundh, H. (2012) Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins. Frontiers in Physiology. 3: Article 388

Sundh, H., Calabrese, S., Jutfelt, F., Niklasson, L., Olsen, R.E., Sundell, K. (2011) Translocation of infectious pancreatic necrosis virus across the intestinal epithelium of Atlantic salmon (*Salmo salar* L.). Aquaculture. 321: 85-92.

Tacci, L., Casadei, E., Bickerdike, R., Secombes, C.J., Martin, S.A.M. (2011) Cloning and expression analysis of the Mitochondrial Ubiquitin Ligase Activator of NF-κB (MULAN) in Atlantic salmon (*Salmo salar*). Molecular Immunology 49: 558-565

Tanamoto, K.I. and Azumi, S. (2000) Salmonella-type heptaacylated lipid A is inactive and acts as an antagonist of lipopolysaccharide action on human line cells. The Journal of Immunology, 164(6): 3149-3156.

Toda, H., Araki, K., Moritomo, T. and Nakanishi, T. (2011) Perforin-dependent cytotoxic mechanism in killing by CD8 positive T cells in ginbuna crucian carp, Carassius auratus langsdorfii. Developmental and Comparative Immunology, 35(1): 88-93.

Vandenberg, G.W. (2004) Oral vaccines for finfish: academic theory or commercial reality?. Animal Health Research Reviews, 5(2): 301-304.

Vogel, S.N., Fitzgerald, K.A., Fenton, M.J. (2003) TLRs: Differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. Mol. Intervent. 3: 466–477.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431–1433.

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y.A., Jørgensen, L.V.G., Heinecke, R.D., Buchmann, K., LaPatra, S., Sunyer, J.O. (2013) Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc. Natl. Acad. Sco. USA. 110: 13097 – 13102.

Xu., Z., Takizawa, F., Parra, D., Gomez, D., Jørgensen, L.V.G., Lapatra, S., Sunyer, J.O. (2016) Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. Nat. Commun. 7: 10728.

Yousif, A.N., Albright, L.J., Evelyn, T.P.T. (1994) In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Disease Aquatic Organisms 19: 15-19

Zhang, Y., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., Sunyer, J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nature Immunology. 11: 827-835.

Zwollo, P., Cole, S., Bromage, E., Kaattari, S. (2005) B Cell Heterogeneity in the Teleost Kidney: Evidence for a Maturation Gradient from Anterior to Posterior Kidney. The Journal of Immunology. 174: 6608-6616.