Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) immune responses against *Aeromonas salmonicida* subsp. *salmonicida* infection

by

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ABSTRACT

Atlantic cod and Atlantic salmon are among the main species of the Canadian fisheries and aquaculture industries. Populations of both fish species are exposed to different pathogens such as bacteria that cause infectious diseases. *Vibrio anguillarum*, *Francisella noatunensis*, *Renibacterium salmoninarum*, and *Aeromonas salmonicida* subsp. *salmonicida* are the most frequent bacteria that infect Atlantic cod and Atlantic salmon.

Aeromonas salmonicida subsp. *salmonicida*, is one of the oldest known fish pathogens and the causative agent of furunculosis in marine and freshwater fish. This Gram-negative pathogen is having a negative impact on fish health, especially in emergent marine aquaculture in Canada. *A. salmonicida* is also an excellent bacterial model to study fish host-pathogen interactions and assist with analyzing compounds that can be useful to stimulate the immunity of the fish.

Here, I evaluate the effect of *A. salmonicida* on the innate immune response of Atlantic cod and Atlantic salmon primary macrophages utilizing microbiological and molecular biology tools. Additionally, I determine the effect of vitamin D_2 and D_3 as potential immunostimulants against the bacterial infection caused by *A. salmonicida* in Atlantic salmon.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degrees centigrade
µl	Microlitre
AC	Apoptotic bodies
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
AS	*Aeromonas salmonicida*
BPI/LBP	bactericidal permeability-increasing protein/lipopolysaccharide-binding protein
BSA	Bovine Serum Albumin
CAMP	Cathelicidin Antimicrobial Peptide
cat	Catalase
CFU	Colony forming unit
CHSE-214	Chinook salmon embryo cell line
C_T	Threshold cycle
Cu/Zn-Sod	CuZn superoxide dismutase
DBP	Vitamin D binding protein
DHR	Dihydrorhodamine
DMSO	Dimethyl sulfoxide
EF-1a	Elongation factor 1 alpha
Eif3	Eukaryotic initiation factor 3
ER	Endoplasmic reticulum
EV	Endoplasmic vesicles
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FK J223	Formalin-killed *A. salmonicida* J223 strain
g	Grams
GP12	*Psychrobacter* spp. bacterial probiotic
GP21	*Pseudomonas* spp. bacterial probiotic
g-Type Lysozyme	Goose type lysozyme
h	Hour
HAMP	Hepcidin Antimicrobial Peptide
IL-1b	Interleukin 1 beta
IL-8	Interleukin 8
IL-10	Interleukin 10
J223	*Aeromonas salmonicida* J223 strain

JBARB	Dr. Joe Brown Aquatic Research Building
l	Litre
LPS	Lipopolysaccharide
mg	Milligram
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MI	Mitochondria
min	Minutes
ml	Millilitre
Mn-Sod	Mn superoxide dismutase
MOI	Multiplicity of infection
nox1	NADPH oxidase 1
nrf2	Nuclear factor erythroid 2-related factor 2
NU	Nucleus
O.D.	Optical density
OMV	Outer membrane vesicles
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol 12-Myristate 13-Acetate
PS	Pseudopodia
qPCR	Quantitative reverse transcription-polymerase chain reaction
ROS	Reactive oxygen species
SB	Secretion bodies
SEM	Standard error of mean
SHK-1	Atlantic salmon head kidney cell line
TEM	Transmission electron microscopy
TLR	Toll-like receptor
tlrs5	Soluble toll-like receptor 5
tnf-α	Tumor necrosis factor alpha
TSB	Trypticase Soy Broth
U	Units
VDR	Vitamin D receptor

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CO-AUTHORSHIP STATEMENT

The research described in the present thesis was conducted by Manuel Soto Dávila with guidance from Dr. Javier Santander. Manuel Soto Dávila was responsible for conducting the experiments, all the laboratory work, data collection and analysis, and thesis writing. Moreover, the Ph.D. candidates, Ahmed Hossain[1] and Setu Chakraborty[1] collaborate in the experiments of the chapter II. Additionally, Dr. Khalil Eslamloo[2], Nicole Smith[2], and Stephanie Tucker[3] provided technical support in chapter II research. During chapter III research, Dr. Katherine Valderrama[1] was involved in the experiments related with Atlantic salmon primary macrophages viability; meanwhile, Sabrina Inkpen[2], helped to conduct the experiments of phagocytosis, and Jennifer Hall[4], design primer sequences for the evaluation of *IL-8* gene expression in Atlantic salmon.

All the chapters were written by Manuel Soto Dávila, with suggestions, recommendations and edition provided by Dr. Javier Santander. Aditionally, Dr. Matthew Rise provided valuable suggestions in the preparation of the thesis manuscript, as well as the manuscript already published in the journal *Frontiers in Immunology* from the chapter II results (https://doi.org/10.3389/fimmu.2019.01237).

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1. CHAPTER I: GENERAL INTRODUCTION

1.1 Atlantic cod and the stressors in wild environments

The bottom-dwelling fish, Atlantic cod (*Gadus morhua*), is one of the most important commercial fish species for the North Atlantic fisheries [Tørresen et al. 2016]. In accordance with the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) report published in 2010, the cod populations of Laurentian North, Laurentian South, and Newfoundland and Labrador, are endangered, meanwhile, the population of Arctic Lakes presents a status of special concern, due to overfishing. Overfishing has been recognized as the main factor associated with the decline in the populations of the Atlantic cod, however, studies indicate that factors like global warming, changes in the composition of the bottom environments, the decrease in the populations of capelin (*Mallotus villosus*), the principal prey of Atlantic cod, and bacterial infectious diseases are affecting the populations [Harris 1998; Choi et al. 2004; Drinkwater 2005]. The latter factor, bacterial infectious diseases, have been observed in both wild and cultured Atlantic cod [Magnadóttir et al. 2002]. The emergence of diseases is associated with the pressures associated with environmental stressors to which wild fish are generally exposed to, as well as the intensive culture conditions that predispose them to infections [Kaatari and Tripp 1987; Robertson et al. 1987; Portz et al. 2006]. Outbreaks of *Vibrio anguillarum*, *Streptococcus parauberis*, *Francisella noatunensis* and *Aeromonas salmonicida* have been reported in wild and cultured Atlantic cod [Magnadóttir et al. 2002; Bakkemo et al. 2011]. In contrast to other teleosts, Atlantic cod lacks the genes for the major histocompatibility complex class II (MHC-II), the invariant chain/CD74 (Ii), and CD4+ T cell response, normally necessary to mount an effective humoral adaptive immune response [Langefors et al. 2001; Star et al.

2011]. The mechanisms underlying responses to bacterial infections in *G. morhua* are not totally understood.

1.2 Atlantic salmon aquaculture and infectious diseases

Aquaculture has been the world's fastest growing food producing industry during recent decades [FAO 2010; Asche et al. 2013]. According to the Food and Agriculture Organization of the United Nations (FAO), Atlantic salmon (*Salmo salar*) is the highest value commercial aquaculture fish species globally, with a production of ~2.2 million tonnes between 2016 and 2017. Higher production is not only associated with the increase in the economic income of the producing countries, but also with an important impact on the environments and disease outbreaks where the production occurs [Maisey et al. 2016]. Several studies associate the environmental conditions in the industry with episodes of immunosuppression and diseases in cultured fish [Kaatari and Tripp 1987; Robertson et al. 1987; Portz et al. 2006]. Infectious diseases represent the main causes of losses in Atlantic salmon aquaculture [Maisey et al. 2016]. *Piscirickettsia salmonis*, *Renibacterium salmoninarum*, *Vibrio anguillarum*, and *Aeromonas salmonicida* are the most frequent bacterial pathogens of Atlantic salmon [Toranzo et al. 2005; Higuera et al. 2013; Maisey et al. 2016; Valderrama et al. 2017]. The strategy used by the Atlantic salmon industry to prevent or control bacterial outbreaks is the use of antibiotics and vaccines [Maisey et al. 2016; Lozano et al. 2018]. However, antibiotic treatments frequently lead to the emergence of antibiotic-resistant isolates [Martinez et al. 2018]. Immunostimulants or functional feed ingredients are frequently utilized in the diet in conjunction with the current measures (e.g. vaccines, antibiotics) to prevent infectious diseases [Jadhav et al. 2006; Barman et al. 2013;

Wang et al. 2017]. Understanding how novel and current functional feed ingredients (e.g. vitamins) influence fish immunity and contribute to fighting bacterial infections is necessary for their optimal utilization as immunostimulants in aquaculture species against current pathogens.

1.3 *Aeromonas salmonicida* subsp. *salmonicida*: a common bacterial pathogen in Atlantic cod and Atlantic salmon

Members of the genus *Aeromonas* are found worldwide in aquatic environments and has been implicated in the etiology of a large variety of human and animal diseases [Crivelli 2001; Saavedra 2006]. This genus is composed of around 35 different species and there has been a progressive increase in the discovery of new species in the last two decades [Demarta et al. 2008; Beaz-Hidalgo et al. 2009; Alperi et al. 2010; Beaz-Hidalgo et al. 2010]. From these, the species *A. hydrophila*, *A. veronii*, and *A. salmonicida* have been described as the three main pathogens of fish [Janda 2010]. The first two, *A. hydrophila* and *A. veronii*, are the causative agents of *Aeromonas* septicemia (hemorrhagic septicemia) and red sore disease, respectively, in different fish species [Wilcox 1992; Janda 2010]. On the other hand, *A. salmonicida* has been described as the cause of massive mortality and great economic losses in marine and continental aquaculture species [Goldschmidt-Clermont 2009; Beaz-Hidalgo et al. 2010; Austin and Austin 2016]. *A. salmonicida* has five subspecies, *salmonicida*, *achromogenes*, *smithia*, *masoucida*, and *pectinolytica* [Graf 2015]. Specifically, the facultative anaerobic, non-motile, and bacillus-shaped bacterium *Aeromonas salmonicida* subsp. *salmonicida*, one of the oldest known pathogens of fish, is the etiological agent of furunculosis, which represents a recurrent health problem in the

aquaculture industry [Hiney 1994; O´Brien 1994; Cipriano and Bullock 2001; Dallaire-Dufresne 2014; Valderrama et al. 2017]. *A. salmonicida* subsp. *salmonicida* requires specific attention due to the negative impact that it causes to emergent aquaculture species in Canada (e.g. sablefish, lumpfish,), as it can infect different hosts, including Atlantic salmon [Samuelsen et al. 2006].

The immune response against the subsp. *Achromogenes* has been well studied in Atlantic cod, however information about the interaction with the subsp. *salmonicida* remains unavailable [Magnadóttir et al. 2002; Fazio et al. 2015]. In contrast, in Atlantic salmon, studies have focused on the infection caused by the subsp. *salmonicida*, making it one of the most studied bacterial pathogens of fish [Brown and Johnson 2008; Gulla et al. 2016; Novak et al. 2016]. Despite this, much of the pathogenesis of *A. salmonicida* subsp. *salmonicida* remains poorly understood in Atlantic salmon [Magnadottir and Gudmundsdottir 1992; Boyd et al. 2008; Romstad et al. 2013].

1.4 Vitamin D mechanisms and their role in the immune system of Atlantic salmon.

Vitamin D is a secosteroid hormone that plays a crucial role in calcium and phosphorus metabolism, cell growth, and tissue differentiation, among others [Walters 1992; Miller and Gallo 2010; Darias et al. 2011]. Vitamin D can be obtained through diet from plant sources in the form of vitamin D_2, also known as ergocalciferol, and from animal sources in the form of vitamin D_3, also known as cholecalciferol [Rao and Raghuramulu 1996; Darias et al. 2011].

Once having entered the organism, vitamin D_2 and vitamin D_3 travel through the bloodstream to the liver where they are hydroxylated by 25-hydroxylase, converted into

25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively [Haussler et al. 2013; Shuler et al. 2013; Bikle 2014]. After the first hydroxylation, in mammals the hydroxylated forms of vitamin D travel to the kidneys for a second hydroxylation to produce the final active form of vitamin D; however, in fish the second hydroxylation occurs in the liver without the appearance of precursors in the blood plasma [Kobayashi et al. 1991; Takeuchi et al. 1991; Lock et al. 2010; Christakos et al. 2016].

After the active form is produced, either vitamin D_2 active form $1,25(OH)_2D_2$ or vitamin D_3 active form $1,25(OH)_2D_3$, bind to vitamin D binding protein (DBP) to be transported to the target cells [Hay and Watson 1976, Lock et al. 2010]. Then, the active vitamin D metabolites in addition to DBP passes through the cell membrane and interact with the vitamin D receptor (VDR), a transcriptional factor which belongs to the nuclear receptor superfamily [Hay and Watson 1976, Lock et al. 2010; Shin et al. 2010]. This interaction is capable to trigger among other things, the expression of immune-related genes [Mangelsdorf et al. 1995; Lock et al. 2010; Shin et al. 2010]. For instance, several studies have shown the importance of vitamin D forms in mammals to prevent or fight against diseases [Deluca and Cantorna 2001; Adams et al. 2007; Yamshchikov et al. 2009; Hewison 2011; Badenhoop et al. 2012, Téllez-Pérez et al. 2012; Yue et al. 2017]. In contrast, the role of vitamin D in the regulation of the immune response in fish is poorly understood [Bikle 2008; Lock et al. 2010].

Vitamin D is a compound generally present in fish feeds, nonetheless, its utilization has been focused on the prevention of skeletal diseases, controlling calcium and phosphorus homeostasis, and cell differentiation [Lall and Lewis-McCrea 2007; Oliva-Teles 2012]. In mammals, vitamin D’s actions are related to certain physiological mechanisms such as

intestinal calcium absorption, bone homeostasis, or increasing the expression of antimicrobial peptides during infection, and has evolved recently in time compared with the teleosts [Kamen and Tangpricha 2010; Lin et al. 2012; Haussler et al 2013; Bouillon and Suda 2014]. As a result, it is of interest to understand the role that vitamin D_2 and D_3 can also play in the improvement of the immune system of teleosts such as Atlantic salmon [Bikle 2008], to determine the evolutionary differences with terrestrial vertebrates.

1.5 General objectives

The general objective for chapter II was to determine the responses of Atlantic cod primary macrophages to *A. salmonicida* infection.

The general objective for chapter III was to determine the effect of vitamins D_2 and D_3 in Atlantic salmon (*Salmo salar*) primary macrophages' immune response.

1.6 Specific objectives

The specific objectives for chapter II were to: i) quantify the attachment-invasion and cell viability of Atlantic cod macrophages infected with *Aeromonas salmonicida*; ii) evaluate the relative expression of genes involved in the innate immune response of Atlantic cod macrophages infected with *Aeromonas salmonicida*; iii) determine the ability of Atlantic cod macrophages to produce reactive oxygen species (ROS) against *Aeromonas salmonicida* infection; and iv) co-localize *Aeromonas salmonicida* in Atlantic cod macrophages.

In chapter III, the specific objectives were to: i) determine the growth of *Aeromonas salmonicida* at different concentrations of vitamin D_2 and D_3; ii) evaluate the viability of

Atlantic salmon macrophages at different concentrations of D_2 and D_3; iii) quantify the attachment, invasion and viability of Atlantic salmon macrophages treated with D_2 and D_3; and iv) to determine the relative expression of several genes involved in the innate immune response of Atlantic salmon macrophages treated with D_2 and D_3.

1.7 References

Adams, J.S., Liu, P.T., Chun, R., Modlin, R.L., and Hewison, M. (2007). Vitamin D in defense of the human immune response. Ann. NY Acad. Sci. 1117, 94-105.

Alperi, A., Martinez-Murcia, A., Monera, A., Saavedra, M.J., and Figueras, M.J. (2010). *Aeromonas fluvialis* sp. nov., isolated from a Spanish river. Int. J. Syst. Evol. Microbiol. 60, 72-77.

Asche, F., Roll, K., Sandvold, H. Sørvig, A., and Zhang, D. (2013). Salmon aquaculture: Larger companies and increased production. Aquacult. Econ. Manag. 17, 322-339.

Austin B., and Austin D.A. (2016). Aeromonadaceae Representative (*Aeromonas salmonicida*). In: Bacterial Fish Pathogens. Springer.

Badenhoop, K., Kahles, H., and Penna-Martinez, M. (2012). Vitamin D, Immune Tolerance, and Prevention of Type 1 Diabetes. Curr. Diab. Rep. 12, 635-642.

Bakkemo, K.R., Mikkelsen, H., Bordevik, M., Torgersen, J., Winther-Larsen, H.C., Vanberg, C., Olsen, R., Johansen, L.H., and Seppola, M. (2011). Intracellular localisation and innate immune responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages. Fish Shellfish Immunol. 31, 993-1004.

Barman, D., Nen, P., Mandai, S., and Kumar, V. (2013). Immunostimulants for Aquaculture Health Management. J. Mar. Sci. Res. Dev. 3, 1-11.

Beaz-Hidalgo, R., Alperi, A., Buján, N., Romalde, J.L., and Figueras, M.J. (2010). Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Syst. Appl. Microbiol. 33, 149-153.

Beaz-Hidalgo, R., Alperi, A., Figueras, M.J., and Romalde, J.L. (2009). *Aeromonas piscicola* sp. nov., isolated from diseased fish. Syst. Appl. Microbiol. 32, 471-479.

Bikle, D. (2008). Nonclassic actions of vitamin D. J. Clin. Endocrinol. Metab. 94, 26-34.

Bikle, D. (2014). Vitamin D Metabolism, Mechanism of Action, and Clinical Applications. Chem. Biol. 21, 319-329.

Bouillon, R., and Suda, T. (2014). Vitamin D: calcium and bone homeostasis during evolution. Bonekey Rep. 480, 1-10.

Boyd, J.M., Dacanay, A., Knickle, L.C., Touhami, A., Brown, L.L., Jericho, M.H., Johnson, S.C., and Reith, M. (2008). Contribution of Type IV Pili to the virulence of *Aeromonas salmonicida* subsp. *salmonicida* in Atlantic salmon (*Salmo salar* L.). Infect. Immun. 76, 1445-1455.

Brown, L.L., and Johnson, S.C. (2008). Molecular interaction between fish pathogens and host aquatic animals. In: Fisheries for Global Welfare and Environment, 5th World Fish Congress (ed. by Tsukamoto, K., Kawamura, T., Takeuchi, T., Beard, T.D., and Kaiser, M.J.) pp. 277–288. Terrapub, Tokyo.

Choi, J., Frank, K., Leggett, W., and Drinkwater, K. (2004). Transition to an alternate state in a continental shelf ecosystem. Can. J. Fish. Aquat. Sci. 61, 505-510.

Christakos, S., Dhawan, P., Verstuyf, A., Verlinden, L., and Carmeliet, G. (2016). Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. Physiol. Rev. 96, 365-408.

Cipriano, R.C., and Bullock, G. (2001). Furunculosis and other diseases caused by *Aeromonas salmonicida*. Fish disease leaflet 66. USGS/Leetown Science Center Fish Health Branch, Kearneysville, WV.

COSEWIC (Committee on the Status of Endangered Wildlife in Canada) 2010. COSEWIC assessment and status report on the Atlantic salmon Salmo salar in Canada. Available from http://www.sararegistry.gc.ca

Crivelli, C., Demarta, A., and Peduzzi, R. (2001). Intestinal secretory immunoglobulin A (sIgA) response to *Aeromonas* exoproteins in patients with naturally acquired *Aeromonas* diarrhea. FEMS Immunol. Med. Microbiol. 30, 31-35.

Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., and Charette, S.J. (2014). Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. Vet. Microbiol. 169, 1-7.

Darias, M., Mazurais, D., Koumoundouros, G., Cahu, C., and Zambonino-Infante, J. (2011). Overview of vitamin D and C requirements in fish and their influence on the skeletal system. Aquaculture. 315, 49-60.

Deluca, H., and Cantorna, M. (2001). Vitamin D: its role and uses in immunology. FASEB J. 15, 2579- 2585.

Demarta, A., Küpfer, M., Riegel, P., Harf-Monteil, C., Tonolla, M., Peduzzi, R., Monera, A., Saavedra, M.J., and Martinez-Murcia, A. (2008). *Aeromonas tecta* sp. nov.,

isolated from clinical and environmental sources. Syst. Appl. Microbiol. 31, 278-286.

Drinkwater, K. (2005). The response of Atlantic cod (*Gadus morhua*) to future climate change. ICES J. Mar. Sci. 62, 1327-1337.

Fazio, A., Bragason, B. T., Magnadottir, B., Faggio, C., and Gudmundsdottir, S. (2015). The acute phase response of Atlantic cod (*Gadus morhua* L.): Expression of immune response genes after infection with *Aeromonas salmonicida* subsp. *achromogenes*. J. Biol. Res. 88, 12-13.

Food & Agricultural Organization (FAO) (2010). The State of World Fisheries and Aquaculture 2010. FAO, Rome.

Goldschmidt-Clermont, E., Hochwartner, O., Demarta, A., Caminada, A.P., and Frey, J. (2009). Outbreaks of an ulcerative and haemorrhagic disease in Arctic char *Salvelinus alpinus* caused by *Aeromonas salmonicida* subsp. *smithia*. Dis. Aquat. Org. 86, 81-86.

Graf J. (2015). *Aeromonas*. Norfolk: Caister Academic Press.

Gulla, S., Lund, V., Kristoffersen, A.B., Sørum, H., and Colquhoun, D.J. (2016). *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. J. Fish. Dis. 39, 329-342.

Harris, M. (1998). Lament for an Ocean: The collapse of the Atlantic Cod Fishery. Canada. McClelland & Steward Inc.

Haussler, M., Whitfield, G., Kaneko, I., Haussler, C., Hsieh, D., Hsieh, J.C., and Jurutka, P. (2013). Molecular Mechanisms of Vitamin D Action. Calcif. Tissue Int. 92, 77-98.

Hay, A.W.M., and Watson, G. (1976). The plasma transport proteins of 25-hydroxycholecalciferol in fish, amphibians, reptiles and birds. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 53, 167-172.

Hewison, M. (2011). Antibacterial effects of vitamin D. Nat. Rev. Endocrinol. 7, 337-345.

Higuera, G., Bastías, R., Tsertsvadze, G., Romero, J., and Espejo, R. (2013). Recently discovered *Vibrio anguillarum* phages can protect against experimentally induced vibriosis in Atlantic salmon, *Salmo salar*. Aquaculture. 392-395, 128-133.

Hiney, M.P., Kilmartin, J.J., and Smith, P.R. (1994). Detection of *Aeromonas salmonicida* in Atlantic salmon with asymptomatic furunculosis infections. Dis. Aquat. Org. 19, 161-167.

Jadhav, V.S., Khan, S.I., Girkar, M.M., and Gitte, M.J. (2006). The role of immunostimulants in fish and shrimp aquaculture. Aquac. Asia. 3, 24-27.

Janda, J., and Abbott, S. (2010). The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. Clin. Microbiol. Rev. 23, 35-73.

Kaatari, S., and Tripp, R. (1987). Cellular mechanisms of glucocorticoid immunosuppression in salmon. J. Fish Biol. 31, 129-132.

Kamen, D.L., and Tangpricha, V. (2010). Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. J. Mol. Med. 88, 441-450.

Kobayashi, T., Takeuchi, A., and Okano, T. (1991) An evolutionary aspect in vertebrates from the viewpoint of vitamin D_3 metabolism. In: Vitamin D. Gene Regulation, Structure-Function Analysis and Clinical Application (Norman, A.W., Bouillon, R. & Thomasset, M. eds), pp. 678–680. Walter de Gruyter, Berlin.

Lall, S., and Lewis-McCrea, L. (2007). Role of nutrients in skeletal metabolism and pathology in fish - An overview. Aquaculture. 267, 3-19.

Langefors, A., Lohm, J., Grahn, M., Andersen, Ø, and Schantz, T. (2001). Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. Proc. R. Soc. Lond. (Biol.). 268, 479-485.

Lin, C.-H., Su, C.-H., Tseng, D.-Y., Ding, F.-C., and Hwang, P.-P. (2012). Action of Vitamin D and the Receptor, VDRa, in Calcium Handling in Zebrafish (*Danio rerio*). PLoS ONE 7(9): e45650.

Lock, E.J., Waagbø, R., Wendelaar-Bonga, S., and Flick, G. (2010). The significance of vitamin D for fish: a review. Aquac Nutr. 16, 100-116.

Magnadóttir, B., Bambir, S.H., Gudmundsdóttir, B.K., Pilström, L., and Helgason, S. (2002). Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. J. Fish Dis. 25, 583-597.

Magnadóttir, B., and Gudmundsdóttir, B.K. (1992). A comparison of total and specific immunoglobulin levels in healthy Atlantic salmon (*Salmo salar* L.) and in salmon naturally infected with *Aeromonas salmonicida* subsp. *achromogenes*. Vet. Immunol. Immunopathol. 32, 179-189.

Maisey, K., Montero, R., and Christodoulides, M. (2016). Vaccines for Piscirickettsiosis (salmonid rickettsial septicaemia, SRS): The Chile perspective. Expert. Rev. Vaccines. 16, 215-228.

Mangelsdorf, D., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. (1995). The Nuclear Receptor Superfamily: The Second Decade. Cell. 83, 835-839.

Martínez, D., Díaz-Ibarrola, D., Vargas-Lagos, C., Oyarzún, R., Pontigo, J. P., Muñoz, J. L. P., Yáñez, A. J., and Vargas-Chacoff, L. (2018). Immunological response of the Sub-Antarctic Notothenioid fish *Eleginops maclovinus* injected with two strains of *Piscirickettsia salmonis*. Fish Shellfish Immunol. 75, 139-148.

Miller, J., and Gallo, R. (2010). Vitamin D and innate immunity. Dermatol. Ther. 23, 13-22.

Novak, C.W., Lewis, D.L., Collicutt, B., Verkaik, K., and Barker, D.E. (2016). Investigations on the role of the salmon louse, *Lepeophtheirus salmonis* (Caligidae), as a vector in the transmission of *Aeromonas salmonicida* subsp. *salmonicida*. J. Fish Dis. 39, 1165-1178.

O´Brien, D., Mooney, J., Ryan, D., Powell, E., Hiney, M., Smith, P., and Powell, R. (1994). Detection of *Aeromonas salmonicida*, Causal Agent of Furunculosis in Salmonid Fish, from the Tank Effluent of Hatchery-Reared Atlantic Salmon Smolts. Appl. Environ. Microbiol. 60, 3874-3877.

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. J. Fish. Dis. 35, 83-108.

Portz, D., Woodley, C., and Cech Jr., J. (2006). Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fisher. 16, 125-170.

Rao, D.S., and Raghuramulu, N. (1996). Food Chain as Origin of Vitamin D in Fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 114, 15-19.

Robertson, L., Thomas, P., Arnold, C., and Trant, J. (1987). Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak. Prog. Fish-Cult. 49, 1-12.

Romstad, A.B., Reitan, L.J., Midtlyng, P., Gravningen, K., and Evensen, Ø. (2013). Antibody responses correlate with antigen dose and *in vivo* protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines. Vaccine. 31, 791-796.

Saavedra, M.J., Figueras, M.J., and Martinez-Murcia, A.J. (2006). Updated phylogeny of the genus *Aeromonas*. Int. J. Syst. Evol. Microbiol. 56, 2481-2487.

Samuelsen, O.B., Nerland, A.H., Jorgensen, T., Schroder, M.B., Svasand, T., and Bergh, O. (2006). Viral and bacterial diseases of Atlantic cod *Gadus morhua*, their prophylaxis and treatment: A review. Dis. Aquat. Org. 71, 239-254.

Shin, J.S., Choi, M.Y., Longtine, M.S., and Nelson, D.M. (2010). Vitamin D effects on pregnancy and the placenta. Placenta. 31, 1027-1034.

Shuler, F., Hendrix, J., Hodroge, S., and Short, A. (2013). Antibiotic-like actions of vitamin D. W. V. Med. J. 109, 22-5.

Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., Malmstrøm, M., Gregers, T.F., Rounge, T.B., Paulsen, J., Solbakken, M.H., Sharma, A., Wetten, O.F., Lanzen, A., Winer, R., Knight, J., Vogel, J.H., Aken, B., Andersen, O., Lagesen, K., Tooming-Klunderud, A., Edvardsen, R.B., Tina, K.G., Espelund, M., Nepal, C., Previti, C., Karlsen, B.O., Moum, T., Skage, M., Berg, P.R., Gjoen, T., Kuhl, H., Thorsen, J., Malde, K., Reinhardt, R., Du, L., Johansen, S.D., Searle, S., Lien, S., Nilsen, F.,

Jonassen, I., Omholt, S.W., Stenseth, N.C., and Jakobsen, K.S. (2011). The genome sequence of Atlantic cod reveals a unique immune system. Nature. 477, 207-210.

Takeuchi, A., Okano, T., and Kobayashi, T. (1991) The existence of 25-hydroxyvitamin D3-1a-hydroxylase in the liver of carp and bastard halibut. Life Sci. 48, 275-282.

Téllez-Pérez, A., Alva-Murillo, N., Ochoa-Zarzosa, A., and López-Meza, J. (2012). Cholecalciferol (vitamin D) differentially regulates antimicrobial peptide expression in bovine mammary epithelial cells: Implications during *Staphylococcus aureus* internalization. Vet. Microbiol. 160, 91-98.

Toranzo, A.E., Magariños, B., and Romalde, J.L. (2005). A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

Tørresen, O.K., Rise, M.L., Jin, X., Star, B., µenzie, S., Jakobsen, K.S., Nederbragt, A.J., and Jentoft, S. (2016). An improved version of the Atlantic cod genome and advancements in functional genomics: implications for the future of cod farming. Genomics in Aquaculture. 45-72, Elsevier.

Tsai, H., Hamilton, A., Guy, D.R., and Houston R.D. (2014). Single nucleotide polymorphisms in the insulin-like growth factor 1 (*IGF1*) gene are associated with growth-related traits in farmed Atlantic salmon. Anim. Genet. 45, 709-715.

Valderrama, K., Saravia, M., and Santander, J. (2017). Phenotype of *Aeromonas salmonicida* sp. *salmonicida* cyclic adenosine 3',5'-monophosphate receptor protein (Crp) mutants and its virulence in rainbow trout (*Oncorhynchus mykiss*). J. Fish. Dis. 40, 1849-1856.

Walters, M.R. (1992). Newly identified actions of the vitamin D endocrine system. Endocr. Rev. 13, 719-764.

Wang, L., Xu, H., Wang, Y., Wang, C., Li, J., Zhao, Z., Luo, L., Du, X., and Xu, Q. (2017). Effects of the supplementation of vitamin D_3 on the growth and vitamin D metabolites in juvenile Siberian sturgeon (*Acipenser baerii*). Fish Physiol. Biochem. 43, 901-909.

Wilcox, M.H., Cook, A.M., Thickett, K.J., Eley, A., and Spencer, R.C. (1992). Phenotypic methods for speciating clinical *Aeromonas* isolates. J. Clin. Pathol. 45, 1079-1083.

Yamshchikov, A., Desai, N., Blumberg, H., Ziegler, T., and Tangpricha, V. (2009). Vitamin D for Treatment and Prevention of Infectious Diseases: A Systematic Review of Randomized Controlled Trials. Endocr. Pract. 15, 438-449.

Yue, Y., Hymøller, L., Jensen, S., Lauridsen, C., and Purup, S. (2017). Effects of vitamin D and its metabolites on cell viability and *Staphylococcus aureus* invasion into bovine mammary epithelial cells. Vet. Microbiol. 203, 245-251.

2. CHAPTER II: *Aeromonas salmonicida* subsp. *salmonicida* early infection and immune response of Atlantic cod (*Gadus morhua* L.) primary macrophages

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2.1 Abstract

In contrast to other teleosts, Atlantic cod (*Gadus morhua*) has an expanded repertoire of MHC-I and TLR components, but lacks the MHC-II, the invariant chain/CD74, and a CD4+ T cell response, which are all essential for production of antibodies and prevention of bacterial infectious diseases. The mechanisms by which *G. morhua* fight bacterial infections are not well understood. *Aeromonas salmonicida* subsp. *salmonicida* is a recurrent pathogen in cultured and wild fish, and has been reported in Atlantic cod. Macrophages are some of the first responders to bacterial infection and the link between innate and adaptive immune response. Here, I evaluated the viability, reactive oxygen species (ROS) production, cell morphology, and gene expression of cod primary macrophages in response to *A. salmonicida* infection. I found that *A. salmonicida* infects cod primary macrophages without killing the cells. Likewise, infected Atlantic cod macrophages up-regulated key genes involved in the inflammatory response (e.g., *IL-1β* and *IL-8*) and bacterial recognition (e.g., *BPI/LBP*). Nevertheless, our results showed a down-regulation of genes related to antimicrobial peptide and ROS production, suggesting that *A. salmonicida* utilizes its virulence mechanisms to control and prevent macrophage anti-bacterial activity. Our results also indicate that Atlantic cod has a basal ROS production in non-infected cells, and this was not increased after contact with *A. salmonicida*. Transmission electron microscopy results showed that *A. salmonicida* was able to infect the macrophages in a high number, and release outer membrane vesicles (OMV) during intracellular infection. These results suggest that Atlantic cod macrophage innate immunity is able to detect *A. salmonicida* and trigger an anti-inflammatory response,

however *A. salmonicida* controls the cell immune response to prevent bacterial clearance, during early infection.

2.2 Introduction

Atlantic cod (*Gadus morhua*), one of the most important commercial fish species in the North Atlantic fisheries, has unusual modifications of the immune gene repertoire that set it apart from other teleosts [Malmstrøm et al. 2016]. This Gadiform fish lacks the genes for the major histocompatibility complex class II (MHC-II), the invariant chain/CD74 (Ii), and the CD4+ T cell response, representing an important evolutionary diversification of the adaptive immune system of vertebrates [Star et al. 2011]. The MHC-II binds antigens from extracellular pathogens, and the MHC-II-antigen complex activates helper CD4+ T cells, which play an essential role fighting bacterial infectious diseases [Parham 2016].

The Atlantic cod appears to have compensated for the lack of the MHC-II pathway by expanding the number of MHC-I genes [Malmstrøm et al. 2013]. This expanded MHC-I gene family has been divided into two clades, one maintaining the classical MHC-I functionality, and the other showing a MHC-II-like function [Star et al. 2011]. Indeed, around 80–100 copies of the MHC-I loci are found in the Atlantic cod genome, in contrast to other gadiformes that present only 40 copies [Star et al. 2011; Malmstrøm et al. 2016; Solbakken et al. 2017], or to humans that harbor only ~10 copies [Buonocore and Gerdol 2016]. In addition to the MHC-I diversification, the Atlantic cod has expanded some Toll-like receptor (TLR) families, which have an important role in the innate immune response and pathogen detection [Star et al. 2011; Solbakken et al. 2016a; Solbakken et al. 2016b]. The Atlantic cod lacks TLR1, TLR2, and TLR5 that recognize bacterial surface antigens,

however, this seems to be compensated by an expansion of the TLR7, TLR8, TLR9, and TLR22 families related to nucleic acids recognition [Star et al. 2011; Star and Jentoft 2012; Sundaram et al. 2012; Buonocore and Gerdol 2016].

The Atlantic cod is a very successful teleost species and not particularly susceptible to infectious diseases [Magnadóttir 2014], even though some of the prevalent marine bacterial pathogens such as *Vibrio anguillarum*, *Francisella noatunensis*, and *Aeromonas salmonicida* have been reported in wild and cultured cod [Samuelsen et al. 2006; Bakkemo et al. 2016].

A. salmonicida is found worldwide in aquatic environments and has been implicated in the etiology of a large variety of fish diseases [Graf 2015]. *A. salmonicida* has five subspecies, *salmonicida*, *achromogenes*, *smithia*, *masoucida*, and *pectinolytica* [Graf 2015]. The immune response of Atlantic cod to subsp. *achromogenes* infection has been described [Magnadóttir et al. 2002; Fazio et al. 2015]. In contrast, the immune response of Atlantic cod to subsp. *salmonicida* has not been studied.

Aeromonas salmonicida subsp. *salmonicida* (hereafter *A. salmonicida*) is a causative agent of furunculosis, a recurrent health problem for several marine fish species [Valderrama et al. 2017]. This Gram negative, facultative anaerobic, non-motile, and bacillus shaped bacterium [Janda and Abbott 2010; Dallaire-Dufresne et al. 2014], contains among others, a type-three secretion system that translocates to the eukaryotic cell several effector proteins, which influence immune response, including inflammation [Ebanks et al. 2006; Frey and Origgi 2016].

Inflammation is a protective reaction of the host in response to bacterial infection, involving the migration of leukocytes, including macrophages, to the site of infection

[Suzuky and Lida 1992]. Macrophages, in addition to neutrophils, are the first defense line of vertebrates, including Atlantic cod. Upon infection, macrophages are activated, secreting antimicrobial peptides (AMPs), cytokines, and chemokines, among others immune modulatory molecules [Tort et al. 2003; Whyte 2007; Magnadóttir 2014]. Activated macrophages have an increased phagocytic activity, correlated with an increased production of reactive oxygen species (ROS), and up-regulation of anti-bacterial gene transcription [Secombes and Fletcher 1992; Stafford and Belosevic 2003; Esteban et al. 2015].

How Atlantic cod primary macrophages respond to *A. salmonicida* infection is unknown. Therefore, the aim of this study was to investigate the immune response of Atlantic cod head kidney primary macrophages to *A. salmonicida* infection.

2.3 Material and methods

2.3.1 Aeromonas salmonicida growth conditions

A single colony of *A. salmonicida* J223 [Valderrama et al. 2017] was grown routinely in 3 ml of Trypticase Soy Broth (TSB, Difco, Franklin Lakes, NJ) at 15°C in a 16 mm diameter glass tube and placed in a roller for 24 h. After growth, 300 µl of the overnight culture were added in 30 ml of TSB media using a 250 ml flask and incubated for 24 h at 15°C with aeration (180 rpm). The bacterial growth was monitored spectrophotometrically until O.D. 600 nm ~0.7 (1×10^8 CFU ml^{-1}) using the Genesys 10 UV spectrophotometer (Thermo Spectronic, Thermo Fischer Scientific Inc., Waltham, MA, USA). Then the bacterial culture was centrifuged at 6,000 rpm at room temperature for 10 min. The pellet was washed twice with PBS and centrifuged at 6,000 rpm at room temperature for 5 min,

and finally resuspended in 300 µl of PBS (~5×10^{10} CFU ml^{-1}). The concentrated bacterial inoculum was serial diluted and quantified by plating onto TSA supplemented with Congo red (50 µg ml^{-1}).

2.3.2 Formalin-killed A. salmonicida

A. salmonicida J223 strain was grown in TSB media supplemented with 100 µM 2, 2′-dypyridyl at 15°C with aeration (180 rpm) up to an optical density of O.D. 600 nm ~0.7 (~1×10^{8} CFU ml^{-1}). The bacterial cells were washed three times with PBS and then fixed with 6% formalin for 3 days at room temperature with gentile agitation. Formalin-killed cells were dialyzed (Molecular Weight cut off 3.5 kDa; Spectra/Por, Laguna Hills, CA) in PBS three times and stored at 4°C at the concentration of 6×10^{10} CFU ml^{-1} until utilization.

2.3.3 Fish holding

Adult specimens of Atlantic cod 1.5 ± 0.2 kg (mean ± SE) were obtained from the Dr. Joe Brown Aquatic Research Building (JBARB) at the Department of Ocean Sciences, Memorial University of Newfoundland, Canada. The animals were kept in 21 m^3 tanks, with flow-through (75.l × min^{-1}) of sea water (6°C) and ambient photoperiod. The individuals were fed with commercial dry pellets (Skretting: 50% protein, 18% fat, 1.5% carbohydrate, 3% calcium, 1.4% phosphorus) with a ratio of 1% of body weight 3 days per week. The experiment was performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocols #17-01-JS; #17-02-JS).

2.3.4 Macrophage isolation

Primary macrophages were isolated from Atlantic cod head kidney in accordance to the protocol established by Eslamloo et al. 2018 [Eslamloo et al. 2018] with modifications. Briefly, head kidney tissues from six fish were aseptically removed and individually minced through 100 μm nylon sterile cell strainers (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) in isolation media [(Leibovitz-15 (Gibco®, Gran Island, NY, USA) supplemented with 2 mM L-glutamine, 4.2 mM NaHCO3, 25 mM HEPES, 1.8 mM glucose, 20 U ml^{-1} heparin, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 1% Fetal Bovine Serum (FBS)]. After this period, 3 ml of cell suspension were centrifuged (400 × g at 4°C) for 40 min in a 25/51% Percoll gradient (GE Healthcare, Uppsala, Sweden). Macrophages collected from the macrophage-enriched interface were washed with phosphate buffered saline [PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2)] [Sambrook and Russell 2001] twice and the number and viable cells were determined using the Countness™ cell counter (Invitrogen), and trypan blue stain (Invitrogen). After determining the numbers of cells from each sample, the primary macrophages were seeded in 22 mm 12-well or 35 mm 6-well cell-culture multidishes (Thermo Scientific, Roskilde, Denmark) at a concentration of 1×10^7 cells ml^{-1}. The plates were incubated at 15°C for 24 h in isolation media. After this period the cells were washed with PBS and incubated at 15°C for additional 24 h in 1 ml of culture media [Leibovitz-15 (Gibco®), supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 1% FBS] to allow cell attachment until the infection assay.

2.3.5 Gentamicin exclusion assay

Infections of primary macrophages with *A. salmonicida* were performed according to a previously established protocol [Valderrama et al. 2017]. To remove the antibiotic present in the culture media, the isolated primary macrophages were washed once with 1 ml of PBS, and then inoculated with 1 ml of cultured media without antibiotics. After this, the primary macrophage monolayers were infected with 10 μl of bacterial suspension [~1 $\times 10^6$ cells ml^{-1}; Multiplicity of Infection (MOI) 1:1 (bacteria:macrophage)] and incubated at 15°C. After 1 h post infection, the *A. salmonicida* attached to the Atlantic cod macrophages were quantified. The infected primary macrophage monolayers were washed 3 times with PBS, and lysed with 400 μl of Triton X100 (0.01%; Sigma) during 10 min [Sung et al. 2003] and then 600 μl of PBS were added to complete 1 ml of lysed macrophage suspension. Then the lysed macrophage suspensions were serially diluted (1:10) and plate/counted on TSA plates supplemented with Congo Red to determine the number of viable *A. salmonicida* per monolayer. The plates were incubated at 15°C for 5 days to determine the CFU per well. In addition, samples were taken for cell viability, RNA extraction, and transmission electron microscopy (see below for details).

For the invasion assay, cell monolayers were infected for 1 h, washed 3 times with PBS, followed by the addition of 1 ml of fresh culture media supplemented with gentamicin (10 μg ml^{-1}, a higher concentration than the minimal inhibitory concentration for *A. salmonicida*) [Aravena-Román et al. 2012], and incubated at 15°C. Samples were taken at 2, 3, and 6 h post infection for bacterial count, cell viability, RNA extraction, and transmission electron microscopy. All the macrophages were isolated from three individual fish and triplicates were utilized for each treatment in the assays.

2.3.6 Primary macrophages viability determination

To determine the viability of infected primary macrophages, the cells were seeded in 12 wells plates, infected with *A. salmonicida*, and processed as described in the gentamicin exclusion assay. For each time point post *A. salmonicida* infection, the cells were washed with 1 ml of PBS and then treated with 500 µl of trypsin-EDTA (0.5%; Gibco) for 10 to 15 min. After this period, the trypsin was inactivated with 500 µl of culture media. The cells were stained with trypan blue (0.4%; Invitrogen) in a ratio of 1:1 (10 µl: 10 µl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. The numbers of alive and dead cells were determined at each time point post-infection. All the macrophages were isolated from three individual fish and technical triplicates were utilized in the assays.

2.3.7 RNA extraction and qPCR

To determine the effect of *A. salmonicida* on the innate immune response of Atlantic cod primary macrophages, samples of RNA were isolated from infected cells at 1, 2, and 6 h post *A. salmonicida* infection, using the previously described gentamycin exclusion methodology. Primary macrophages that were either mock infected with PBS or inoculated with 1×10^6 CFU of formalin-killed *A. salmonicida* were utilized as controls. Total RNA was extracted using TRIzol (Invitrogen), and purified using RNeasy (QIAGEN) following manufacturers' instruction [Santander et al. 2014]. RNA samples were treated with TURBO DNA-free™ Kit (Invitrogen) for complete digestion of DNA and removal of remaining

DNase and divalent cations, such as magnesium and calcium. Purified RNA samples were quantified and evaluated for purity using a Nano-quant spectrophotometer (Genway, UK), and evaluated for integrity by 1% agarose gel electrophoresis [Sambrook and Russell 2001]. cDNA was synthetized with the SuperScript™ III First-Strand Synthesis System (Invitrogen) using 500 ng of RNA per reaction and random hexamers according to the manufacturer's instructions.

Primer pair efficiencies were analyzed using a 20 ng μl^{-1} pooled cDNA from each set of samples, which was serially diluted (dilutions starting with 1 (20 ng μl^{-1}), 1:3 (6.67 ng μl^{-1}), 1:9 (2.22 ng μl^{-1}), 1:27 (0.74 ng μl^{-1}), 1:81 (0.25 ng μl^{-1}), 1:243 (0.08 ng μl^{-1}), 1:729 (0.03 ng μl^{-1})). Primer pair efficiencies were calculated using the formula $E = 10^{(-1/slope)}$ [Pfaffl 2001].

All qPCR reactions were done in a final volume of 20 µl, containing 10 µl of 1 × PowerUp-SYBR Master Mix (Applied BioSystems, Foster City, CA, USA), 1 µl (10 µM) of each primer, 6 µl of nuclease free water (Ambion), and 2 µl of cDNA. All samples were amplified and detected in a QuantStudio 3 (Applied BioSystems). The reaction mixtures were incubated for 2 min at 95°C, followed by 40 cycles of 1 s at 95°C, 30 s at 60°C, and finally 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C. Initially, a total of five Atlantic cod genes were tested as reference gene candidates (*EF-1α*, *ß-actin*, *Eif3*, *18S*, *60S*). cDNA from a sub-set of samples (Appendix I) were utilized for evaluation of reference gene stability. The most stable gene for this set of individual samples was determined by using geNorm (M value 0.102) and BestKeeper (Value 0.101) (Appendix I). After this determination, the individual samples were analyzed. The mRNA gene expression was normalized to the Atlantic cod *elongation factor 1 alpha* (*EF-1a*) due to its stability across

different treatments. Gene expression was determined using the comparative $^{-\Delta\Delta Ct}$ method [Livak and Schmittgen 2001].

The primers used in this study are listed in Table 2.1. In all cases, each qPCR was performed with triplicate samples and repeated with six independent fish.

Table 2-1. Primer sequences used in Atlantic cod experiment.

Gene	Forward (5´to 3´)	Reverse (5´to 3´)	Tm ºC	Efficiency (%)	Reference
IL-1b	TGAGGACCTGCTCAACCTCT	TCTTCTGGTGGTCCCTCAAC	55.6	103.7	Perez-Casanova et al. 2010
IL-8	GGTTTGTTCAATGATGGGCTGTT	GACCTTGCCTCCTCATGGTAATACT	56.5	98.4	Seppola et al. 2008
IL-10	CCTATAAAGCCATCGGCGAGTTA	TGAAGTCGTCGTTTTGAACCAAG	56.6	100.1	Seppola et al. 2008
MHC-I	CTAGCGTGGGACCTGAAGAC	CAGAGTGCTCTTCCCGTAGG	56.5	108.4	Perez-Casanova et al. 2010
g-type lysozyme	CATTGACCAAGCCACTGGAATCCT	ATTCGACTCTACCGTCTCCAGTGT	59.3	102.3	Perez-Casanova et al. 2010
BPI/LBP	GACCGTCAACGTGATGGCCCCGGT	CTTTGTTGGCCTCTATGCTGGAGAG	59.4	96.8	Caipang et al. 2008
Cathelicidin (CAMP)	ATTGCAATTTCACCCTGAGC	CCAGACCTGCTCCTTCTCAC	56.4	108.1	Feng et al. 2009
Transferrin	GAGCTCCCATCGACAGCTAC	CAAACCCAGCAGAGGAGAAG	56.7	108.9	Audunsdottir et al. 2012
Hepcidin (HAMP)	CCACAGGCTCCTCTCAAGTC	CTGCAACTGCAATGCTGAAT	56.4	105.1	Feng et al. 2009
nrf2	TCGCAGTAGGAGCTGGATGA	CTCCGGTCTGTCCTTGGAAA	57.0	98.1	Skjærven et al. 2013
nox1	GCCTATATGATTGGCCTGATGAC	GCTGTGCTGAGTGGGTCGTA	55.3	108.6	Skjærven et al. 2013
Mn-Sod	ATGTGGCCTCCTCCATTGAA	GCATCACGCCACCTATGTCA	55.1	109.2	Skjærven et al. 2013
Cu/Zn-Sod	CATGGCTTCCACGTCCATG	CGTTTCCCAGGTCTCCAACAT	56.8	98.0	Skjærven et al. 2013
cat	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	57.3	101.0	Skjærven et al. 2013
EF-1a	GATGCACCACGAGTCTCTGA	GGGTGGTTCAGGATGATGAC	56.2	98.3	Perez-Casanova et al. 2010

2.3.8 Respiratory burst assay

Respiratory burst of primary cod macrophages infected with *A. salmonicida* was determined according to the protocol established by Smith et al. [Smith et al. 2018a; Smith et al. 2018b] with some modifications. Briefly, isolated primary macrophages were infected with 10 µl of bacterial suspension (~1×10^6 cells ml^{-1}; MOI 1:1) and incubated at 15°C for 48 h. Primary macrophages inoculated with PBS were utilized as negative control, and phorbol myristate acetate (PMA 1 mM; Sigma) dissolved in dimethyl sulfoxide (DMSO) was utilized as positive control.

After 1 h post infection, cells were washed and the culture media was replaced with respiratory burst assay buffer (Leibovitz L-15 media supplemented with 1% BSA and 1 mM $CaCl_2$). Then, 1 µl of dihydrorhodamine 123 (DHR, 5 mg/ml; Sigma) was diluted in 1 ml of PBS, and 50 µl of the dilution added to the macrophages for 15 min. Subsequently, 125 µl of PBS for negative control, or 125 µl of PMA (1 mM, final concentration 0.185 µM PMA) were added to the macrophages monolayers for 45 min to stimulate ROS production [Nikoskelainen et al. 2006; Kalgraff et al. 2011]. Finally, the macrophages were detached using 1 ml of trypsin-EDTA (0.5%; Gibco), washed with PBS, centrifuged for 5 min (500 × g at 4°C), and resuspended in fluorescence-activated cell sorting (FACS) buffer (1% FBS in PBS). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer (Becton Dickinson™) and analyzed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). The PBS control cells were used to define the region of ROS negative cells, and based on this gating the FITC positive cells were identified. The mean fluorescence intensity and percentage of FITC-positive cells were determined for

each condition. The experiments were conducted in macrophages isolated from six independent fish, and 10,000 events were measured for each sample.

2.3.9 Transmission electron microscopy (TEM)

Primary macrophages were fixed in anhydrous paraformaldehyde (4%; Electron Microscope Sciences, Hatfield, PA, USA) at 4°C until the samples were processed at the Electron Microscopy/Flow Cytometry Unit at Memorial University of Newfoundland. The cells were pelleted and resuspended in Karnovsky fixative for 20 min [Karnovsky 2003], washed in 0.1 M sodium cacodylate buffer pH 7.4 for 5 min, and post-fixed in 1% Osmium tetroxide during 15 min. After this, the fixed cells were dehydrated in increasing concentrations of ethanol and acetone followed by infiltration with EPON resin (Sigma). Cells were pelletized between incubation steps. Resin blocks were polymerized in BEEM capsules (Electron Microscope Sciences) overnight at 70°C and ultra-thin sections were cut with a diamond knife (Diatome, Hatfield, PA, USA). The ultra-thin sections were mounted on 300 copper mesh grids, stained with uranyl acetate and lead citrate, and examined in a Tecnai™ Spirit TMA with an accelerating voltage of 80 kV. Cells incubated for 3 h with PBS (control), J223 strain, and formalin-killed *A. salmonicida* were observed.

2.3.10 Statistical analysis

All data are shown as the mean ± standard error (SE). Assumptions of normality and homogeneity were tested for the detected variances. A Kruskal-Wallis nonparametric test was performed for gentamicin exclusion assay results. Gene expression and ROS data were analyzed using a repeated measures two-way ANOVA test, followed by Sidak

multiple comparisons *post hoc* test to identify significant differences of each treatment in different times and between treatments in the same time point. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.4 Results

2.4.1 Macrophage viability and Aeromonas salmonicida infection

The viability of Atlantic cod primary macrophages infected with *A. salmonicida* was determined at 1, 2, 4, and 6 h post-infection. The results did not show significant differences between the time points post-infection in the number of live cells and percentage of viability. For instance, after 1, 2, 4, and 6 h post-infection $1.32 \times 10^6 \pm 8.02 \times 10^5$, $1.18 \times 10^6 \pm 7.13 \times 10^5$, $1.17 \times 10^6 \pm 6.64 \times 10^5$, and $7.77 \times 10^5 \pm 4.77 \times 10^5$ cells were quantified, respectively (Fig. 2-1a). The percentage of viability during the infection process also did not show significant differences between time points post-infection. After 1, 2, 4, and 6 h the infected cells showed a viability of 89% ± 4.8, 87% ± 3.1, 80% ± 4.2%, and 92% ± 6%, respectively (Fig. 2-1b).

Although the primary cod macrophage cells seemed to survive the *A. salmonicida* infection, the bacteria infected and invaded the cell monolayers. The Atlantic cod macrophages were infected with a MOI of 1:1 (bacteria: macrophage) with an initial inoculum of 9.6×10^6 CFU. After 1 h post-infection, 7.39% (6.8×10^5 CFU) was attached to the macrophage monolayer, and after 2, 4, and 6 h post-infection, 0.42% (3.87×10^4 CFU), 0.37% (3.42×10^4 CFU), and 0.21% (1.94×10^4 CFU) of *A. salmonicida* were located intracellularly, respectively (Fig. 2-1c).

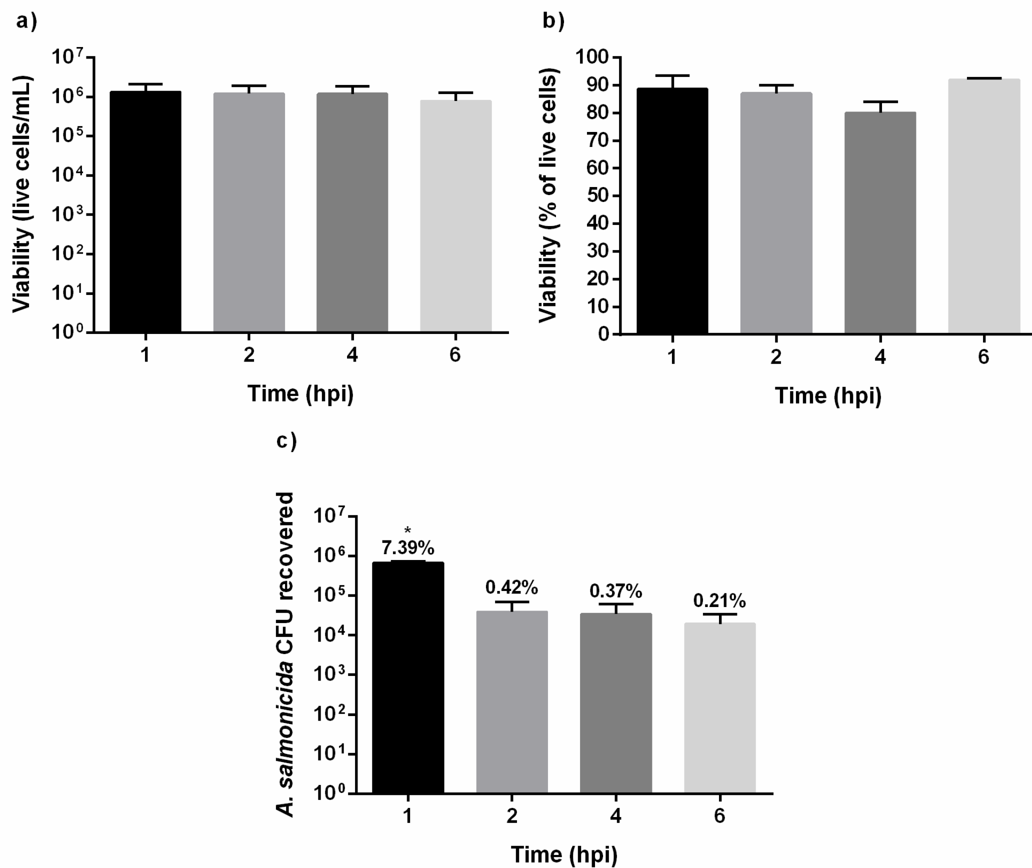


Figure 2-1. Gentamicin exclusion assay in Atlantic cod macrophages infected with *Aeromonas salmonicida* subsp. *salmonicida*. (a) Live cells and (b) percentage of viability, after 1, 2, 4, and 6 h post infection. (c) Colony forming units (CFU) recovered after 1, 2, 4, and 6 h post infection with *A. salmonicida*. The percentage showed above bars indicate the total % of attachment (1 h post infection) and invasion (2, 4, and 6 h post invasion) of *A. salmonicida* in Atlantic cod macrophages, $p < 0.05$. Each value represents the mean ± S.E.M (n = 3). Symbol (*) indicate statistical differences between each time post infection.

2.4.2 Gene expression response of Atlantic cod macrophages to A. salmonicida infection

Quantitative real-time expression of selected genes related to Atlantic cod macrophage immunity was evaluated during *A. salmonicida* infection, and compared with macrophages inoculated with inactivated *A. salmonicida* (formalin-killed), and PBS inoculated controls. Significant increases in the expression of the pro-inflammatory cytokine interleukin 1β (*IL-1β*) gene were observed 1, 2, and 6 h post *A. salmonicida* infection compared to the time-matched PBS controls (Fig. 2-2a). An up-regulation of the pro-inflammatory cytokine interleukin 8 (*IL-8*) gene, was also observed, nonetheless, this up-regulation occurred at 2 h, and 6 h post *A. salmonicida* infection compared to the PBS controls (Fig. 2-2b). In contrast, the macrophages inoculated with inactivated *A. salmonicida* showed a higher expression of *IL-1β* at 2 and 6 h post-inoculation compared to their respective PBS controls (Fig. 2-2a). *IL-8* was up-regulated in cells treated with formalin-killed bacteria after 1 and 2 h post-inoculation (Fig. 2-2b). At 6 h post-inoculation with the inactivated *A. salmonicida*, the expression of *IL-8* in cod macrophages did not show differences compared to the PBS inoculated cells (Fig. 2-2b). The expression of *IL-1b* was significantly up-regulated at 1 and 2 h after *A. salmonicida* infection compared with bacterin-exposed macrophages, whereas for *IL-8* significant up-regulation in infected vs. bacterin-exposed macrophages was only seen at the 6 h time point (Fig. 2-2a, b).

In contrast, the relative expression of the anti-inflammatory cytokine Interleukin 10 (*IL-10*) gene, was significantly down-regulated after 1 and 2 h post *A. salmonicida* infection compared to the non-infected control macrophages (Fig. 2-2c). Cod macrophages inoculated with the inactivated pathogen also showed a significant down-regulation at 1, 2, and 6 h compared with PBS controls (Fig. 2-2c).

Genes involved in antigen recognition and host defense showed different patterns of expression after macrophage exposure to the live or inactivated *A. salmonicida*. In the case of the major histocompatibility complex class I (*MHC-I*) gene, a significant down-regulation was observed after 6 h post live *A. salmonicida* infection and post-inoculation with the formalin-killed bacteria (Fig. 2-2d).

A down-regulation of the relative expression of the Goose-type lysozyme (*g-type lysozyme*) gene was observed at 1 and 2 h post *A. salmonicida* infection, and at 1, 2, and 6 h post inoculation with the formalin-killed pathogen, compared to their respective controls (Fig. 2-2e). At 6 h post-treatment, *g-type lysozyme* expression was significantly different in infected vs. inactivated pathogen exposed macrophages (Fig. 2-2e).

In contrast, the bactericidal permeability-increasing protein/lipopolysaccharide-binding protein (*BPI/LBP*) gene, involved in the antimicrobial defense against Gram negative bacteria, showed a significant up-regulation at 2 and 6 h post-infection only in cells inoculated with the live bacteria compared with the control and formalin-killed inoculated treatments (Fig. 2-2f).

The relative expression of AMPs encoding-genes showed different expression patterns. Cathelicidin (*CAMP*) gene was down-regulated in macrophages infected with *A. salmonicida* and those inoculated with formalin-killed *A. salmonicida* at 1, 2, and 6 h post-infection compared with time-matched PBS controls (Fig. 2-2g). Macrophages infected with *A. salmonicida* showed a significant down-regulation of *CAMP* 2 h post-infection compared to the cells inoculated with inactivated bacteria (Fig. 2-2g).

Transferrin did not show variation in the level of expression of both treatments compared with the time-PBS matched controls during the assays, as well as, between

macrophages inoculated with live and bacterin *A. salmonicida* (Fig. 2-2h). Hepcidin (*HAMP*) expression was significantly up-regulated only at 6 h post-exposure to the formalin-killed *A. salmonicida* (Fig. 2-2i). However, *HAMP* was significantly down-regulated at 2 h post-infection with *A. salmonicida* (Fig. 2-2i).

Expression of genes involved in the synthesis of ROS was also evaluated (Fig. 2-2j, 2-2k, 2-2l, 2-2m, 2-2n). Nuclear factor erythroid 2-related factor 2 (*nrf2*), a transcriptional factor that is translocated into the nucleus under oxidative stress and initiates transcription of antioxidative genes, did not show transcriptional variation in cells inoculated with formalin-killed *A. salmonicida* compared to their respective controls (Fig. 2-2j). In contrast, macrophages infected with *A. salmonicida* showed a significant down-regulation in the expression of *nrf2* at 2 and 6 h post-infection (Figure 2.2j). After 1 h of infection, *nrf2* transcript was significantly lower expressed in pathogen-infected macrophages compared to bacterin-exposed macrophages.

The expression of NADPH oxidase 1 (*nox1*) gene, which encodes a membrane-bound pro-oxidant enzyme that catalyzes superoxide synthesis, was significantly down-regulated 1 h post-infection with *A. salmonicida* compared to the non-treated cells and the bacterin-exposed macrophages (Fig. 2-2k). Furthermore, this gene was significantly lower expressed at 1 h compared with 2 and 6 h post-infection (Fig. 2-2k).

The relative expression of the Mn superoxide dismutase (*Mn-Sod*) and the CuZn superoxide dismutase (*Cu/Zn-Sod*) genes, involved in the transformation of superoxide into H_2O_2 in the mitochondria and the cytosol, respectively, also was evaluated. The *Mn-Sod* was significantly up-regulated at 2 h post-inoculation with inactivated *A. salmonicida*, compared to the controls (Fig. 2-2l). In contrast, macrophages infected with *A. salmonicida*

showed a *Mn-Sod* down-regulation tendency, with a significant down-regulation at 2 and 6 h post-infection compared to the bacterin-exposed macrophages (Fig. 2-2l).

Similar patterns were observed in *Cu/Zn-Sod* and catalase (*cat*) expression, where a higher expression was observed post-inoculation with the formalin-killed *A. salmonicida* and a down-regulation was observed in cells infected with *A. salmonicida* (Fig. 2-2m, n). For instance, *Cu/Zn-Sod* show a significant down-regulation at 6 h post *A. salmonicida* infection compared to the PBS control inoculated cells, and 2 and 6 h post-infection compared to cells treated with the inactivated pathogen (Fig. 2-2m).

The relative expression of *cat*, which encodes for the catalase enzyme that plays an important role in H_2O_2 detoxification, showed a down-regulation at 1, 2, and 6 h in macrophages infected with *A. salmonicida* compared to the control and the cells treated with the inactivated pathogen (Fig. 2-2n). In contrast, macrophages exposed to inactivated *A. salmonicida* did not show significant differential expression of *cat*, compared to their respective controls (Fig. 2-2n).

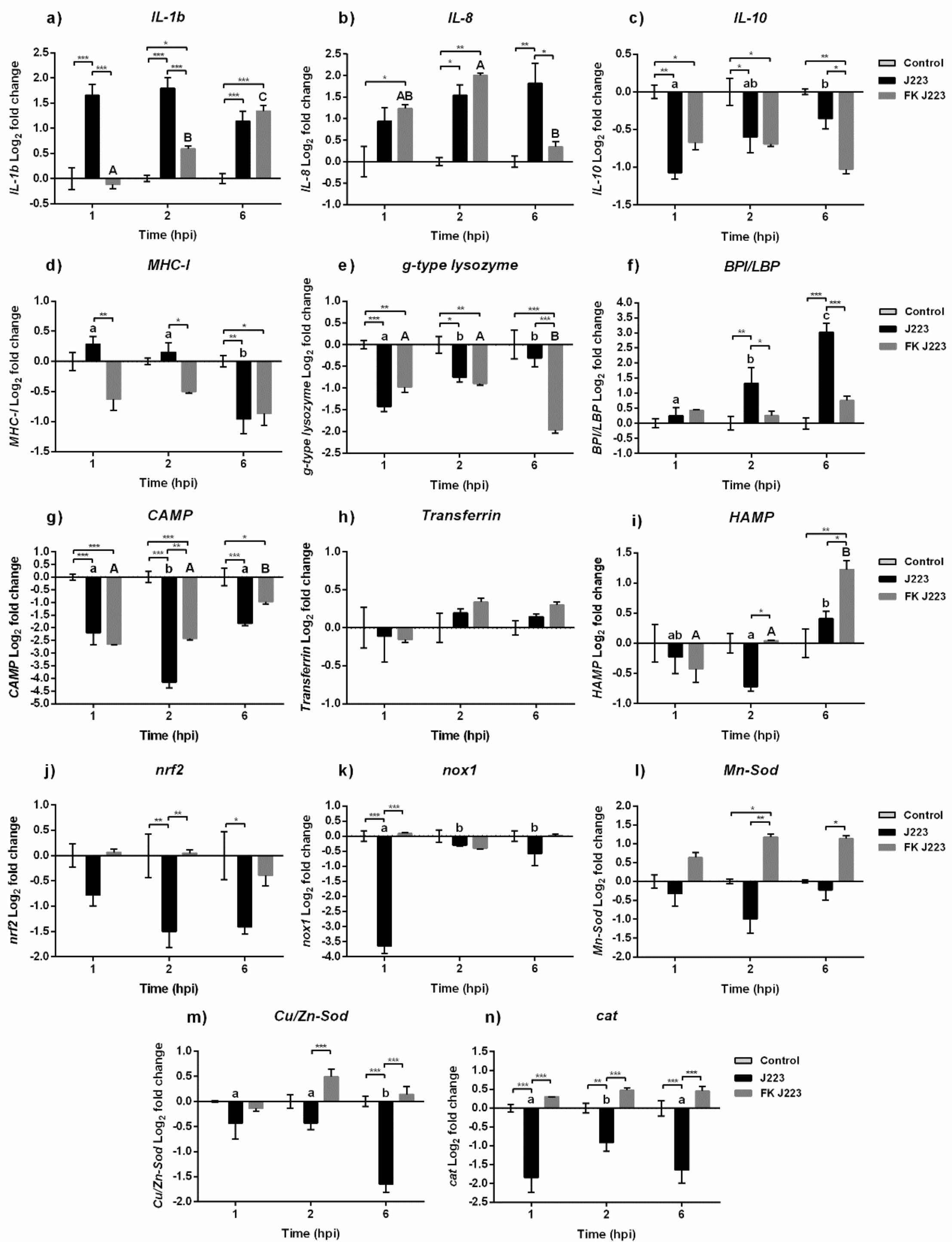


Figure 2-2. Gene expression of (a) Interleukin 1b (*IL-1b*), (b) Interleukin 8 (*IL-8),* (c) Interleukin 10 (*IL-10*), (d) Major histocompatibility complex class 1 (*MHC-I*), (e) Goose

type lysozyme (*g-type lysozyme*), (f) Bactericidal permeability increasing protein / lipopolysaccharide-binding protein (*BPL/LBP*), (g) Cathelicidin (*CAMP*), (h) Transferrin, (i) Hepcidin (*HAMP*), (j) Nuclear factor erythroid 2-related factor 2 (*nrf2*), (k) NADPH oxidase 1 (*nox1*), (l) Mn superoxide dismutase (*Mn-Sod*), (m) CuZn superoxide dismutase (*Cu/Zn-Sod*), and (n) Catalase (*cat*) in Atlantic cod primary macrophages isolated from head kidney and infected with live (J223) and formalin-killed *A. salmonicida* (FK 223) at different times post infection (1, 2, and 6 h). Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using *EF-1a* as internal reference gene. Different letters represent significant differences between primary macrophages infected with J223 strain (lower case) or inoculated with FK J223 (upper case) at different times-points. Asterisks (*) represent significant differences between treatments on each time-point (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Each value is the mean ± S.E.M (n = 6).

2.4.3 Reactive oxygen species (ROS) production

ROS production was determined in Atlantic cod primary macrophages infected with *A. salmonicida*. Macrophages inoculated with PBS or PMA were utilized as negative and positive controls, respectively. ROS production was analyzed at 1 h and 6 h post *A. salmonicida* infection. I did not observe significant differences in ROS production between treatments. Macrophages treated with PBS showed that 85.1% ± 6.1 and 70.4% ± 6.2 were producing ROS after 1 h and 6 h, respectively. Macrophages treated with PMA showed that 75.8% ± 7.0 and 53.6% ± 12.4 were producing ROS after 1 h and 6 h, respectively. Similarly, macrophages treated with PBS and infected with *A. salmonicida* showed that 74.5% ± 6.4 and 52.9% ± 11.9 of the cells were producing ROS after 1h and 6 h post

infection, respectively. Additionally, macrophages treated with PMA and infected with *A. salmonicida* showed that 68.5% ± 9.5 and 53.1% ± 12.4 of the cells were producing ROS after 1 h and 6 h post treatment, respectively (Fig. 2-3a, b).

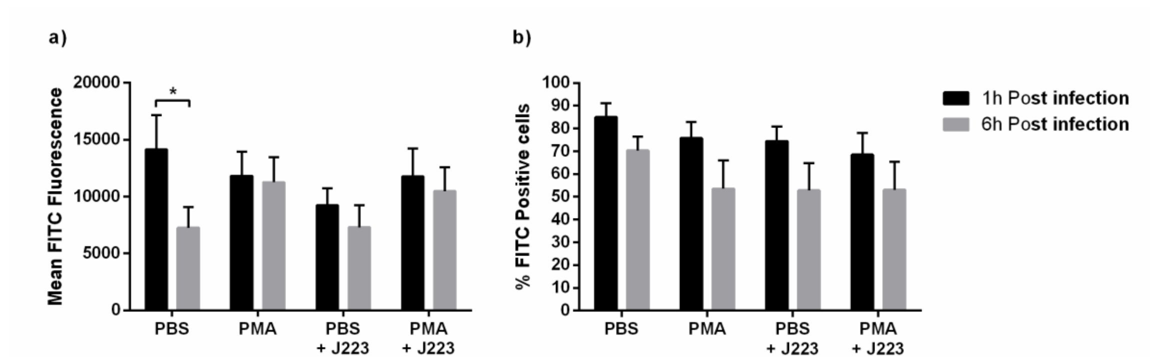


Figure 2-3. Reactive oxygen species (ROS) production in Atlantic cod macrophages after 1 and 6 h post-infection with *A. salmonicida*. (a) Mean FITC fluorescence and (b) percentage of FITC positive cells were obtained by flow cytometry. PBS inoculated cells (PBS) and PMA inoculated cells were utilized as negative and positive controls, respectively. Each value is the mean ± S.E.M (n = 6). Symbol (*) indicate differences on each group at different times of infection, $p < 0.05$.

2.4.4 Transmission electron microscopy (TEM)

Cod primary macrophages infected with *A. salmonicida* were visualized 3 h post infection using TEM. A group of non-infected cells were utilized as reference control (Fig. 2-4a, b). These cells showed a rounded cell morphology, large nucleus, and evident presence of cell organelles (e.g. mitochondria, endoplasmic reticulum, endocytic vesicles) and pseudopodias (Fig. 2-4a, b). In contrast, Atlantic cod macrophages infected with *A.*

salmonicida showed poorly defined nuclei, membrane ruffling, and large vesicles containing *A. salmonicida* cells (average of 2-3 bacterial cells per macrophage, a maximum of 8 bacterial cells per macrophages, and 70-80% macrophages infected) (Fig. 2-4c, 2-4d, 2-4e). Furthermore, secretion of *A. salmonicida* outer membrane vesicles (OMVs) was observed in intracellular bacterial cells (Fig. 2-4f, g).

Macrophages inoculated with the formalin-killed *A. salmonicida* showed a defined nucleus and a large number of secretion bodies within the cytoplasm (Fig. 2-4h). Apoptotic-like bodies were observed in high numbers in the presence of extracellular *A. salmonicida* bacterin (Fig. 2-4i).

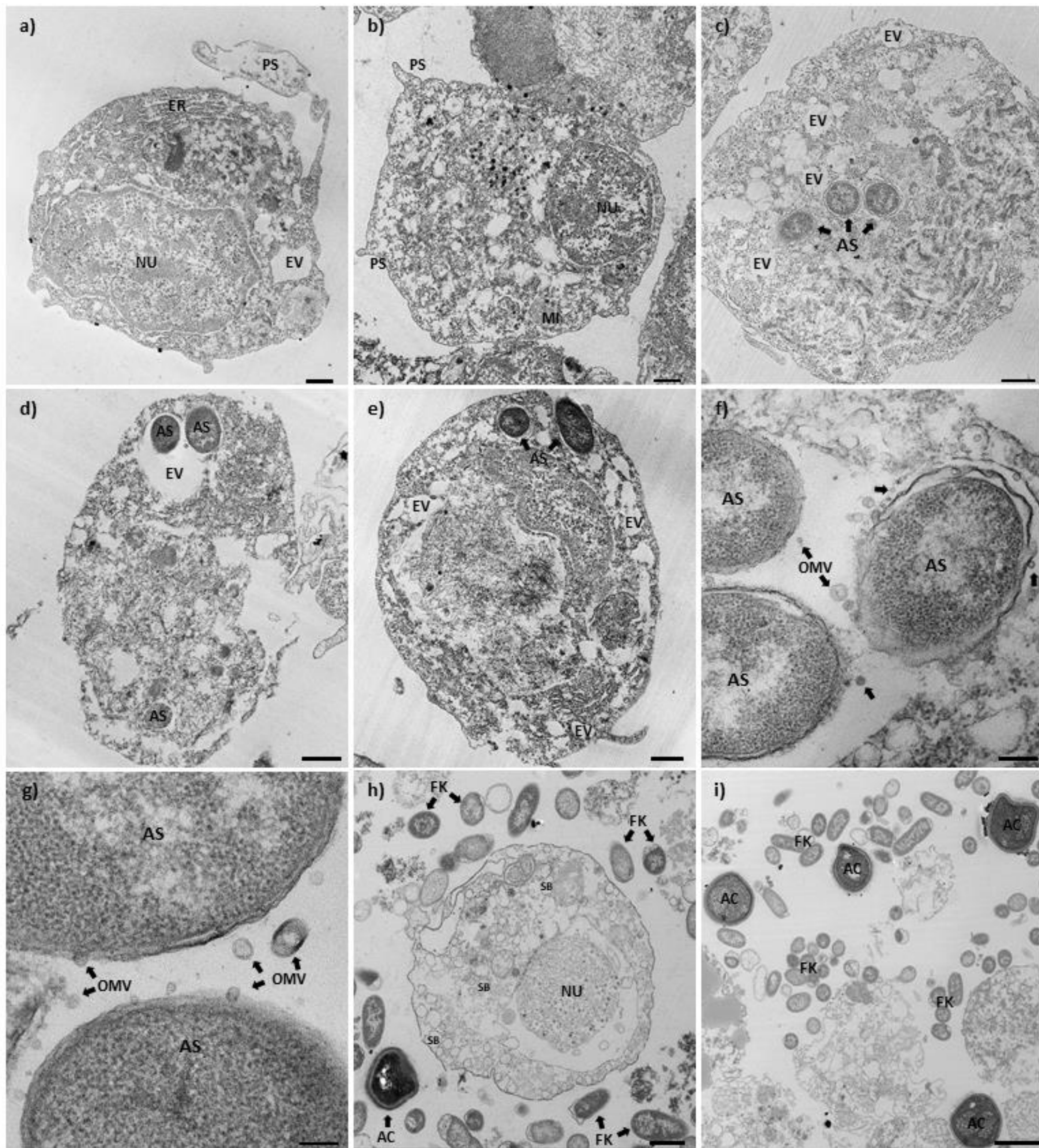


Figure 2-4. Transmission electron microscopy of Atlantic cod macrophages infected with *A. salmonicida*. (a, b) Mock infected Atlantic cod head kidney macrophages (control). (× 2,100, scale bar 1 µm). (c–e) Atlantic cod head kidney macrophages infected with *A. salmonicida*. (c and d: × 2700, scale bar 1 µm; e: × 2,100, scale bar 1 µm). (f, g) Intracellular *A. salmonicida*. (f: × 15,000, scale bar 200 nm; g: × 30,000, scale bar 100 nm). (h, i) Atlantic

cod head kidney macrophages infected with the formalin killed *A. salmonicida*. (h: × 2,700, scale bar 1 μm; i: × 1,650, scale bar 2 μm). NU, Nucleus; PS, Pseudopodia; ER, Endoplasmic reticulum; MI, Mitochondria; AS, *A. salmonicida*; EV, Endoplasmic vesicles; OMV, Outer membrane vesicles (arrows); FK, Formalin killed *A. salmonicida*; SB, Secretion bodies; and AC, Apoptotic bodies.

2.5 Discussion

Atlantic cod lacks the genes for MHC-II, the invariant chain/CD74 (Ii), and CD4+ T cell responses, representing a paradigm in the context of adaptive immunity against bacterial infectious diseases [Star et al. 2011; Parham 2016]. Additionally, Atlantic cod lacks TLR1, TLR2, and TLR5, which recognize bacterial surface antigens [Star et al. 2011; Star & Jentoft 2012; Sundaram et al. 2012; Buonocore & Gerdol 2016]. However, this absence seems to be compensated for by an expansion of the MHC-I [Malmstrøm et al. 2013; Malmstrøm et al. 2016; Solbakken et al. 2017] and the TLR7, TLR8, TLR9, and TLR22 families [Star et al. 2011; Solbakken et al. 2016a]. Nevertheless, how Atlantic cod fight bacterial infections is unknown. Here, I evaluated the early response of Atlantic cod primary macrophages to *A. salmonicida* infection.

To evaluate the early response of Atlantic cod primary macrophages to *A. salmonicida* infection, a gentamicin exclusion assay was conducted. The macrophage viability results obtained 1 h post infection (attachment) and 2, 4, and 6 h post-infection (invasion) showed similar viability, with 89% ± 4.8, 87% ± 3.1, 80% ± 4.2%, and 92% ± 6%, respectively (Fig. 2-1b). In contrast, Atlantic salmon primary macrophages isolated and infected under similar conditions, showed 3 h post *A. salmonicida* infection a viability

around ~40% (see Appendix II). Similar reduction in viability was reported in non-phagocytic Chinook salmon embryo cell line (CHSE-214) infected with the *A. salmonicida* J223 strain, showing a viability of ~40% at 2 and 4 h post-infection [Valderrama et al. 2017]. These results reveal that Atlantic cod macrophages are more resistant to *A. salmonicida* infection compared with Atlantic salmon macrophages and CHSE-214 cells.

The attachment of *A. salmonicida* in cod primary macrophages was 7.39%, and only 0.42, 0.37, and 0.21% was able to invade 2, 4, and 6 h post-infection, respectively (Fig. 2-1c). A study conducted in CHSE-214 embryo cell line infected with *A. salmonicida* J223 strain showed an attachment of ~60% at 1 h post-infection, and an invasion of 0.47% and 0.29% after 2 and 4 h post-infection, respectively [Valderrama et al. 2017]. Moreover, *A. hydrophila* isolated from ornamental fish, showed an attachment between 75 and 80% in the mammalian cell line CaCo-2 (cells from human colon adenocarcinoma) at 1 h post-infection [Saidi et al. 2011]. In contrast, the attachment of *A. salmonicida* J223 in Atlantic salmon primary macrophages was 10.7% at 1 h post-infection, and the invasion was 0.42, 0.26, and 0.28% after 2, 3, and 4 h post-infection, respectively (Appendix II). These results suggest that primary macrophages are less susceptible to be infected by *A. salmonicida* than non-phagocytic cells, even when the intracellular *A. salmonicida* recovered in non-phagocytic and phagocytic cells have been shown to be relatively similar.

The transcriptional profiles of cytokine genes, antibacterial genes, antimicrobial peptide genes, and ROS related genes were determined by using qPCR. The observed up-regulation of *IL-1β* and *IL-8* after infection with *A. salmonicida* or inoculation with formalin killed *A. salmonicida* (Fig. 2-2a, b) indicates a canonical macrophage innate immune response [Secombes et al. 2001; Tort el al. 2003]. Coincident with the up-

regulation of the pro-inflammatory *IL-1β* and *IL-8* genes (Fig. 2-2a, b), the anti-inflammatory *IL-10* gene was down-regulated in macrophages infected with *A. salmonicida* or inoculated with the formalin killed pathogen (Fig. 2-2c).

Similar up-regulation of the pro-inflammatory cytokine *IL-1β* was observed in Atlantic cod intramuscular injected with *A. salmonicida* subsp. *achromogenes*, meanwhile an up-regulation in both, *IL-1β* and *IL-8*, has been reported in Atlantic cod gill epithelial cells infected with *Vibrio anguillarum* and *A. salmonicida* [Caipang et al. 2010], and in Atlantic cod macrophages infected with *Francisella noatunensis* [Bakkemo et al. 2011], reinforcing the importance of these canonical interleukins against Gram-negative pathogens during the first hours of infection.

Antigen recognition and host defense genes, like *MHC-I*, *g-type lysozyme*, and *BPI/LBP* were also evaluated. As mentioned previously, *BPI/LBP* participates in the recognition of lipopolysaccharide (LPS) [Kono & Sakai 2003; Stenvik et al. 2004], the major component of Gram negative bacterial outer membrane [Raetz et al. 2007], *MHC-I* participate in the recognition of intracellular pathogens, like viruses or cytoplasmic invader bacteria [Parham 2016], and *g-type lysozyme* is related to both Gram positive and Gram negative antibacterial activity [Larsen et al. 2009]. Interestingly, *BPI/LBP* was up-regulated only after infection with live *A. salmonicida* but not in presence of formalin-killed *A. salmonicida* (Fig. 2-2f). *BPI/LBP* up-regulation has been observed in Atlantic cod vaccinated with inactivated *V. anguillarum* and *A. salmonicida* [Stenvik et al. 2004; Magnadottir 2014], and in Atlantic cod intestinal epithelial cells after exposure with the bacterial probiotics GP21 (*Pseudomonas* spp.) and GP12 (*Psychrobacter* spp.) isolated

from the intestinal tract of an adult Atlantic cod [Lazado et al. 2010; Lazado & Caipang 2014].

The AMP-encoding genes *CAMP* and *transferrin*, both involved in the iron homeostasis, showed unexpected transcriptional profiles (Fig. 2-2g, h), meanwhile *HAMP*, also related to the iron ion homeostasis, was the only AMP-encoding gene that showed an up-regulation after inoculation with the inactivated bacteria (Fig. 2-2i). *CAMP* was down-regulated after infection with the live bacteria or inoculation with the formalin-killed pathogen (Fig. 2-2g), meanwhile transferrin did not show variations compared to the controls (Fig. 2-2h, i). In the case of *HAMP*, up-regulation was observed only at 6 h post-inoculation with the inactivated pathogen (Fig. 2-2i). As previously mentioned, it has been reported that *CAMP*, *transferrin*, and *HAMP* AMPs are key during bacterial infection, and in general these genes are expressed in several immune tissues of Atlantic cod after bacterial (e.g., *Mycobacterium chelonei*, *Aeromonas salmonicida* subsp. *salmonicida*, and *Aeromonas salmonicida* subsp. *achromogenes*) and viral (infectious pancreatic necrosis virus) infection, or viral mimic [poly (I:C)] stimulation [Seppola et al. 2008; Solstad et al. 2008; Magnadottir 2014]. However, our results show that these genes were either lower expressed (*CAMP* and *HAMP*) or not affected (*transferrin*) in Atlantic cod macrophages compared with formalin-killed *A. salmonicida* stimulation (Fig. 2-2g, 2-2h, 2-2i). A study in Atlantic cod intramuscular infected with *A. salmonicida* subsp. *achromogenes* showed an up-regulation in the expression of *transferrin* and *HAMP* [Fazio et al. 2015]. In contrast, I found that in Atlantic cod primary macrophages, the infection with *A. salmonicida* subsp. *salmonicida* down-regulated the expression of *CAMP* and *HAMP*. This can suggest a different mechanism of infection between *A. salmonicida* subsps.

The Atlantic cod *CAMP* has a potent antimicrobial activity against Gram-negative bacteria and fungi [Broekman et al. 2011]. Nonetheless, some bacterial pathogens, like *V. anguillarum*, *A. salmonicida* subsp. *achromogenes* and *A. hydrophila* are able to evade the action of *CAMP* [Broekman et al. 2011], and this can be the case for *A. salmonicida* J223 in Atlantic cod macrophages.

Atlantic cod injected with turpentine oil, an inducer of acute immune response that involves inflammation and other biological processes (e.g., hemostasis), showed an increase in the relative expression of these AMP genes after 24 h of injection [Audunsdottir et al. 2012], and similar results were observed in intestinal epithelial cells after probiotic exposure to the probiotics GP21 and GP12 [Lazado & Caipang 2014]. Moreover, a study conducted in Atlantic cod stimulated with formalin-killed *A. salmonicida* showed lower levels of expression in the transcripts encoding *CAMP* and *HAMP* in head kidney and spleen, compared with PBS control samples [Feng et al. 2009]. In this study, only a peak was observed after 24 h of stimulation with the inactivated pathogen [Feng et al. 2009]. Therefore, our results suggest that likely more time is required for Atlantic cod macrophages to up-regulate the *CAMP*, *transferrin*, and *HAMP* genes after inactivated *A. salmonicida* exposition.

A study conducted in the Gram-negative bacteria, *Pseudomonas syringae*, showed that outer membrane vesicles (OMVs) can potentially suppress the action of AMPs [Kulkarni et al. 2014]. OMVs bind and sequester AMPs to prevent bacterial cell damage, and also induce the release of peptidases, proteases, and other lytic enzymes to degrade the host AMPs [Chua et al. 2013; Kulkarni et al. 2014]. Combining this reported evidence with our results, where a high number of OMVs were released by *A. salmonicida* during

intracellular infection (Fig. 2-4f, g), I hypothesize that the presence of OMVs during intracellular infection might be related to a mechanism by which the bacterium tolerates the action of host AMPs or translocate virulence factors to the host cell. This is in addition to unknown *A. salmonicida* mechanism involving the down-regulation of AMP-related genes.

Typically, macrophages phagocytize the invading bacteria, assemble the lysosome, and eliminate them through the action of several enzymes and production of ROS [Skjærven et al. 2013]. Here, I evaluated the expression of *nrf2*, *nox1*, *Mn-Sod*, *Cu/Zn-Sod*, and *cat* genes that are part of the redox system and antioxidant enzymes related to ROS synthesis [Kansanen et al. 2012; Skjærven et al. 2013]. Relative expression levels of *nrf2*, *nox1*, *Mn-Sod*, *Cu/Zn-Sod*, and *cat* genes in cod primary macrophages were down-regulated after infection with *A. salmonicida* (Fig. 2-2j, 2-2k, 2-2l, 2-2m, 2-2n). In contrast, macrophages inoculated with the formalin-killed *A. salmonicida*, showed an up-regulation in the expression of the *Mn-Sod* and *Cu/Zn-Sod* genes (Fig. 2-2l, 2-2m, 2-2n).

The macrophage gene expression of *nrf2*, *nox1*, *Mn-Sod*, *Cu/Zn-Sod*, and *cat* after *A. salmonicida* infection, together with the ROS flow cytometry results, shows that Atlantic cod macrophages do not increase ROS levels after exposure to *A. salmonicida* (Fig. 2-3a, b).

High basal levels of ROS production have been observed in non-induced Atlantic cod blood phagocytes [Nikoskelainen et al. 2006], suggesting that ROS synthesis in Atlantic cod cells is related to the *in vitro* culture conditions [Nikoskelainen et al. 2006]. However, I hypothesize that high basal production of ROS could be normal in *G. morhua*, and the lack of ROS production, above the basal levels after *A. salmonicida* infection, could

be associated to mechanisms used by *A. salmonicida* to control the macrophage immune response, as described previously in other pathogens such as *Mycobacterium leprae*, *R. salmoninarum*, *Salmonella* spp, and *Edwardsiella tarda* [Holzer et al. 1986; Bandin et al. 1993; Foster & Spector 1995; Rao et al. 2001].

Macrophages are usually highly efficient killers of bacteria, however pathogenic bacteria, like *A. salmonicida*, have evolved multiple strategies to infect, avoid enzymatic digestion, and trigger immunosuppression of the host [Bakkemo et al. 2011]. Our TEM results showed that *A. salmonicida* was localized intracellularly in bacteria containing vesicles (Fig. 2-4c, e). Also, I observed that the cytoskeleton of the infected cells was rearranged, and several structures, like the nuclei, pseudopodia, and endoplasmic reticulum, were not observed in infected macrophages, in contrast to non-infected cells (Fig. 2-4a, 2-4b, 2-4c, 2-4d, 2-4e). Additionally, the TEM images of infected macrophages showed a significant number of intracellular bacteria at 3 h post-infection (Fig. 2-4c, 2-4d, 2-4e). Not all Gram-negative pathogens gain access to macrophages in high numbers. For instance, *F. noatunensis* and *F. tularensis* invade in lower numbers, even 24 h post-infection with high infectious doses. Thus, few *F. tularensis* cells are required to cause fatal diseases [Anthony et al. 1991; Fortier et al. 1995; Golovliov et al. 2003; Kirimanjeswara et al. 2008; Bakkemo et al. 2011]. In contrast to these previous studies, our results indicate that *A. salmonicida* required a higher number of infecting bacterial cells, compared to other bacterial pathogens, to have a productive infection (Fig. 2-4c, 2-4d, 2-4e), Additionally, a single macrophage is infected with a significant number of bacterial cells (maximum of 8 bacterial cells per macrophage was founded, 2–3 average) suggesting that these are the target cells of *A. salmonicida*.

An interesting finding of our study was the presence of several OMVs produced by *A. salmonicida* during intracellular infection (Fig. 2-4f, g). The OMVs are virulence factors released by mammalian pathogens like *Neisseria meningitides*, *Escherichia coli*, *Vibrio* spp., *Brucella* spp., among others [Avila-Calderón et al. 2015]. These OMVs play an important role during pathogenesis, delivering toxins and immunomodulatory proteins to the host cell [Avila-Calderón et al. 2015]. Also, OMVs have been observed and described previously in marine pathogens such as *F. noatunensis* subsp. *orientalis*, *Edwardsiella anguillarum*, and *Piscirickettsia salmonis* [Oliver et al. 2016; Shahin et al. 2018; LiHua et al. 2019]. Our results suggest that *A. salmonicida* release the OMVs during intracellular infection in order to control the immune response of the Atlantic cod primary macrophages, and perhaps neutralizing antimicrobial peptides to avoid lysis.

Phagocytosis is a highly efficient mechanism for bacterial elimination used by macrophages. However, Atlantic cod macrophages exposed to formalin-killed *A. salmonicida* for 3 h showed that most of the formalin-killed bacteria were localized in the extracellular milieu, with a reduced number of bacterial cells in phagocytic vesicles (Fig. 2-4h, i). These results suggest that *A. salmonicida* promote macrophage phagocytosis, in contrast to inactivated bacteria.

Also, I observed that Atlantic cod macrophages exposed to formalin-killed *A. salmonicida* produced a large amount of apoptotic-like bodies (Fig. 2-4h, i). Programmed cell death (i.e., apoptosis), is a highly regulated process and an important mechanism used by the host to prevent infectious diseases [Jacobson et al. 1997]. Cells undergoing apoptosis maintain membrane integrity until very late in the process, unlike cells undergoing necrosis, but produce several morphological and biochemical changes inside the cells, including

chromatin condensation, nuclear segmentation, internucleosomal DNA fragmentation, and cytoplasmic vacuolization [Häcker 2000; do Vale et al. 2003]. Similar morphological changes were observed in Atlantic cod macrophages inoculated with formalin-killed *A. salmonicida* (Fig. 2-4h, i). This suggest that *A. salmonicida* is displaying pathogenesis mechanisms to avoid not only phagocytosis but also to prevent apoptosis.

2.6 Conclusion

In this study I evaluated the infection of Atlantic cod macrophages with *A. salmonicida* J223 strain. *A. salmonicida* infects and invades Atlantic cod primary macrophages. I found between 2 to 8 *A. salmonicida* cells per infected macrophage. The infected Atlantic cod macrophages survived during the first 6 h of *A. salmonicida* infection. Nevertheless, TEM observations showed that *A. salmonicida* remained in *A. salmonicida*-containing vesicles. Gene expression results from infected macrophages suggest that *A. salmonicida* modulate the expression of several genes involved in the innate immune response. For instance, relative expression of *HAMP*, *nrf2*, *nox1*, *Mn-Sod*, *Cu/Zn-Sod*, and *cat* genes were down-regulated and *BPI/LBP* was up-regulated after *A. salmonicida* infection. Additionally, I observed that *A. salmonicida* secrete OMVs during intracellular infection. These results suggest that *A. salmonicida* has immune suppressive mechanisms to control cod macrophage immune response, where OMVs could play an essential role.

In contrast, macrophages inoculated with formalin-killed *A. salmonicida*, showed a canonical innate immune response, where most of the evaluated genes were up-regulated or not induced, like the relative expression of *HAMP*, *nrf2*, *nox1*, *Mn-Sod*, *Cu/Zn-Sod*, and *cat* genes. Additionally, I observed that macrophages inoculated with inactivated *A.*

salmonicida did not phagocytize the formalin-killed pathogen, and post-exposure to the bacterin, several apoptotic-like bodies were presented. These results suggest that inactivate *A. salmonicida* trigger a potent innate immunity modulated by the macrophage.

Previously, the effects caused by *A. salmonicida* subsp. *achromogenes* have been reported in Atlantic cod, therefore, the results obtained in this chapter represents one of the first evidence on the effects of *A. salmonicida* subsp. *salmonicida* on the innate immune response of Atlantic cod primary macrophages. Moreover, Atlantic cod exhibit a markedly different immunological model compared to vertebrate standard and other fish species studied, therefore, the results showed in this chapter can be utilized for comparative studies with other fish families commonly infected by *A. salmonicida* subsp. *salmonicida* such as salmonids. Finally, this model showed that during early infection of fish macrophages *A. salmonicida* controls the cellular machinery and can be utilized to study fundamental aspects for bacterial pathogenesis in fish host.

2.7 References

Anthony, L.D., Burke, R.D., and Nano, F.E. (1991). Growth of *Francisella* spp. in rodent macrophages. Infect. Immun. 59, 3291-3296.

Aravena-Román, M., Inglis, T.J.J., Henderson, B., Riley, T.V., and Changa, B.J. (2012). Antimicrobial Susceptibilities of *Aeromonas* Strains Isolated from Clinical and Environmental Sources to 26 Antimicrobial Agents. Antimicrob. Agents Chemother. 56, 1110-1112.

Audunsdottir, S.S., Magnadottir, B., Gisladottir, B., Jonsson, Z.O., and Bragason, B.T. (2012). The acute phase response of cod (*Gadus morhua* L.): expression of immune response genes. Fish Shellfish Immunol. 32, 360-367.

Avila-Calderón, E.D., Araiza-Villanueva, M.G., Cancino-Diaz, J.C., López-Villegas, E.O., Sriranganathan, N., Boyle, S.M., and Contreras-Rodríguez, A. (2015). Roles of bacterial membrane vesicles. Arch. Microbiol. 197, 1-10.

Bakkemo, K.R., Mikkelsen, H., Bordevik, M., Torgersen, J., Winther-Larsen, H.C., Vanberg, C., Olsen, R., Johansen, L.H., and Seppola, M. (2011). Intracellular localisation and innate immune responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages. Fish Shellfish Immunol. 31, 993-1004.

Bakkemo, K.R., Mikkelsen, H., Johansen, A., Robertsen, B., and Seppola, M. (2016). *Francisella noatunensis* subsp. *noatunensis* invades, survives and replicates in Atlantic cod cells. Dis. Aquat. Organ. 121, 149-159.

Bandin, I., Ellis, A.E., Barja, J.L., and Secombes, C.J. (1993). Interaction between rainbow trout macrophages and *Renibacterium salmoninarum in vitro*. Fish & Shellfish Immunol. 3, 25-33.

Broekman, D., Zenz, A., Gudmundsdottir, B., Lohner, K., Maier, B.H., and Gudmundsson, G. (2011). Functional characterization of codCath, the mature cathelicidin antimicrobial peptide from Atlantic cod (*Gadus morhua*). Peptides 32, 2044-2051.

Buonocore, F., and Gerdol, M. (2016). Alternative adaptive immunity strategies: coelacanth, cod and shark immunity. Mol. Immunol. 69, 157-169.

Caipang, C.M., Hynes, N., Puangkaew, J., Brinchmann, M.F., and Kiron, V. (2008). Intraperitoneal vaccination of Atlantic cod, *Gadus morhua* with heat-killed *Listonella anguillarum* enhances serum antibacterial activity and expression of immune response genes. Fish Shellfish Immunol. 24, 314-322

Caipang, C.M., Lazado, C.C., Brinchmann, M.F., and Kiron, V. (2010). Infection-induced changes in expression of antibacterial and cytokine genes in the gill epithelial cells of Atlantic cod, *Gadus morhua* during incubation with bacterial pathogens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 156, 319-325.

Chua, S.L., Tan, S.Y., Rybtke, M.T., Chen, Y., Rice, S.A., Kjelleberg, S., Tolker-Nielsen, T., Yang, L., and Givskov, M. (2013). Bis-(3'-5')-cyclic dimeric GMP regulates antimicrobial peptide resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 57, 2066-2075.

Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., and Charette, S.J. (2014). Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. Vet. Microbiol. 169, 1-7.

do Vale, A., Marques, F., and Silva, M.T. (2003). Apoptosis of sea bass (*Dicentrarchus labrax* L.) neutrophils and macrophages induced by experimental infection with *Photobacterium damselae* subsp. *piscicida*. Fish Shellfish Immunol. 15, 129-144.

Ebanks, R.O., Knickle, L.C., Goguen, M., Boyd, J.M., Pinto, D.M., Reith, M., and Ross, N.W. (2006). Expression of and secretion through the *Aeromonas salmonicida* type III secretion system. Microbiology 152, 1275-1286.

Eslamloo, K., Xue, X., Booman, M., Smith, N.C., and Rise, M.L. (2016). Transcriptome profiling of the antiviral immune response in Atlantic cod macrophages. Dev. Comp. Immunol. 63, 187-205.

Esteban, M.A., Cuesta, A., Chaves-Pozo, E., and Meseguer, J. (2015). Phagocytosis in Teleosts. Implications of the New Cells Involved. Biology 4, 907-22.

Fazio, A., Bragason, B. T., Magnadottir, B., Faggio, C., and Gudmundsdottir, S. (2015). The acute phase response of Atlantic cod (*Gadus morhua* L.): Expression of immune response genes after infection with *Aeromonas salmonicida* subsp. *achromogenes*. J. Biol. Res. 88, 12-13.

Feng, C.Y., Johnson, S.C., Hori, T.S., Rise, M., Hall, J.R., Gamperl, A.K., Hubert, S., Kimball, J., Bowman, S., and Rise, M.L. (2009). Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*. Physiol. Genomics 37, 149-63.

Fortier, A.H., Leiby, D.A., Narayanan, R.B., Asafoadjei, E., Crawford, R.M., Nacy, C.A., and Meltzer, M.S. (1995). Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect. Immun. 63, 1478-1483.

Foster, J.W., and Spector, M.P. (1995). How Salmonella Survive Against the Odds. Annu. Rev. Microbiol. 49, 145-174.

Frey, J., and Origgi, F.C. (2016). Type III Secretion System of *Aeromonas salmonicida* Undermining the Host's Immune Response. Front. Mar. Sci. 3, 130.

Graf J. (2015). *Aeromonas*. Norfolk: Caister Academic Press.

Golovliov, I., Baranov, V., Krocova, Z., Kovarova, H., and Sjöstedt, A. (2003). An Attenuated Strain of the Facultative Intracellular Bacterium *Francisella tularensis* Can Escape the Phagosome of Monocytic Cells. Infect. Immun. 71, 5940-5950.

Häcker, G. (2000). The morphology of apoptosis. Cell Tissue Res. 301, 5-17.

Holzer, T.J., Nelson, K.E., Crispen, R.G., and Andersen, B.R. (1986). *Mycobacterium leprae* fails to stimulate phagocytic cell superoxide anion generation. Infect. Immun. 51, 514.

Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed Cell Death in Animal Development. Cell 88, 347-354.

Janda, J.M., and Abbott, S.L. (2010). The Genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin. Microbiol. Rev. 23, 35-73.

Kalgraff, C.A., Wergeland, H.I., and Pettersen E.F. (2011). Flow cytometry assays of respiratory burst in Atlantic salmon (*Salmo salar* L.) and in Atlantic cod (*Gadus morhua* L.) leucocytes. Fish Shellfish Immunol. 31, 381-388.

Kansanen, E., Jyrkkänen, H.-K., and Levonen, A.-L. (2012). Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. Free Radic. Biol. Med. 52, 973-982.

Karnovsky, M.J., A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27, 137A.

Kirimanjeswara, G.S., Olmos, S., Bakshi, C.S., and Metzger, D.W. (2008). Humoral and cell-mediated immunity to the intracellular pathogen *Francisella tularensis*. Immunol. Rev. 225, 244-255.

Kono, T., and Sakai, M. (2003). Molecular cloning of a novel bactericidal permeability-increasing protein/lipopolysaccharide-binding protein (BPI/LBP) from common carp *Cyprinus carpio* L. and its expression. Mol. Immunol. 40, 269-278.

Kulkarni, H.M., Swamy, Ch.V., and Jagannadham, M.V. (2014). Molecular characterization and functional analysis of outer membrane vesicles from the antarctic bacterium *Pseudomonas syringae* suggest a possible response to environmental conditions. J. Proteome Res. 13, 1345-1358.

Larsen, A.N., Solstad, T., Svineng, G., Seppola, M., and Jorgensen, T.O. (2009). Molecular characterisation of a goose-type lysozyme gene in Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 26, 122-132.

Lazado, C.C., and Caipang, C.M. (2014). Bacterial viability differentially influences the immunomodulatory capabilities of potential host-derived probiotics in the intestinal epithelial cells of Atlantic cod *Gadus morhua*. J. Appl. Microbiol. 116, 990-998.

Lazado, C.C., Caipang, C.M., Gallage, S., Brinchmann, M.F., and Kiron, V. (2010). Expression profiles of genes associated with immune response and oxidative stress in Atlantic cod, *Gadus morhua* head kidney leukocytes modulated by live and heat-inactivated intestinal bacteria. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 155, 249-255.

LiHua, D., JianJun, F., Peng, L., SongLin, G., Le, H., and YiQun, X. (2019). Evaluation of an outer membrane protein as a vaccine against *Edwardsiella anguillarum* in Japanese eels (*Anguilla japonica*). Aquaculture 498, 143-150.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25, 402-408.

Magnadóttir, B. (2014). The immune response of Atlantic cod, *Gadus morhua* L. Icel. Agric. Sci. 27, 41-61.

Magnadóttir, B., Bambir, S.H., Gudmundsdóttir, B.K., Pilström, L., and Helgason, S. (2002). Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. J. Fish Dis. 25, 583-597.

Malmstrøm, M., Jentoft, S., Gregers, T.F., and Jakobsen, K.S. (2013). Unraveling the evolution of the Atlantic cod's (*Gadus morhua* L.) alternative immune strategy. PLoS ONE. 8(9): e74004.

Malmstrøm, M., Matschiner, M., Torresen, O.K., Star, B., Snipen, L.G., Hansen, T.F., Baalsrud, H.T., Nederbragt, A.J., Hanel, R., Salzburger, W., Stenseth, N.C., Jakobsen, K.S., and Jentoft, S. (2016). Evolution of the immune system influences speciation rates in teleost fishes. Nat. Genet. 48, 1204-1210.

Nikoskelainen, S., Kjellsen, O., Lilius, E.M, and Schroder, M.B. (2006). Respiratory burst activity of Atlantic cod (*Gadus morhua* L.) blood phagocytes differs markedly from that of rainbow trout. Fish Shellfish Immunol. 21, 199-208.

Oliver, C., Valenzuela, K., Hernández, M., Sandoval, R., Haro, R.E., Avendaño-Herrera, R., Cárcamo, J.G., Villar, M.T., Artigues, A., Garduño, R., and Yáñez, A.J. (2016). Characterization and pathogenic role of outer membrane vesicles produced by the fish pathogen *Piscirickettsia salmonis* under *in vitro* conditions. Vet. Microbiol. 184, 94-101.

Parham, P. (2016). How the codfish changed its immune system. Nat. Genet. 48, 1103-1104.

Perez-Casanova, J.C., Hamoutene, D., Samuelson, S., Burt, K., King, T.L., and Lee, K. (2010). The immune response of juvenile Atlantic cod (*Gadus morhua* L.) to chronic exposure to produced water. Mar. Environ. Res. 70, 26-34.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45.

Raetz, C.R., Reynolds, C.M., Trent, M.S., and Bishop R.E. (2007). Lipid A modification systems in gram-negative bacteria. Annu. Rev. Biochem. 76, 295-329.

Rao, P.S.S., Lim, T.M., and Leung, K.Y. (2001). Opsonized Virulent *Edwardsiella tarda* Strains Are Able To Adhere to and Survive and Replicate within Fish Phagocytes but Fail To Stimulate Reactive Oxygen Intermediates. Infect. Immun. 69, 5689-5697.

Saidi, N., Snoussi, M., Usai, D., Zanetti, S., and Bakhrouf, A. (2011). Adhesive properties of *Aeromonas hydrophila* strains isolated from Tunisian aquatic biotopes. Afr. J. Microbiol. Res. 5, 5644-5655.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuelsen, O.B., Nerland, A.H., Jorgensen, T., Schroder, M.B., Svasand, T., and Bergh, O. (2006). Viral and bacterial diseases of Atlantic cod *Gadus morhua*, their prophylaxis and treatment: A review. Dis. Aquat. Org. 71, 239-254.

Santander, J., Kilbourne, J., Park, J.Y., Martin, T., Loh, A., Diaz, I., Rojas, R., Segovia, C., DeNardo, D., and Curtiss 3rd, R. (2014). Inflammatory effects of *Edwardsiella ictaluri* lipopolysaccharide modifications in catfish gut. Infect. Immun. 82, 3394-3404.

Secombes, C.J., and Fletcher, T.C. (1992). The role of phagocytes in the protective mechanisms of fish. Annu. Rev. Fish Dis. 2, 53-71.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., and Zou, J. (2001). Cytokines and innate immunity of fish. Dev. Comp. Immunol. 25, 713-723.

Seppola, M., Larsen, A.N., Steiro, K., Robertsen, B., and Jensen, I. (2008). Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). Mol. Immunol. 45, 887-897.

Shahin, K., Thompson, K.D., Inglis, N.F., McLean, K., Ramirez-Paredes, J.G., Monaghan, S.J., Hoare, R., Fontaine, M., Metselaar, M., and Adams, A. (2018). Characterization of the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*. J. Appl. Microbiol. 125, 686-699.

Skjærven, K.H., Penglase, S., Olsvik, P.A., and Hamre, K. (2013). Redox regulation in Atlantic cod (*Gadus morhua*) embryos developing under normal and heat-stressed conditions. Free Radic. Biol. Med. 57, 29-38.

Smith, N.C., Christian, S.L., Taylor, R.G., Santander, J., and Rise, M.L. (2018a). Corrigendum to "Immune modulatory properties of 6-gingerol and resveratrol in Atlantic salmon macrophages" [Mol. Immunol. 95 (2018) 10-19]. Mol. Immunol. 104 (2018) 139.

Smith, N.C., Christian, S.L., Taylor, R.G., Santander, J., and Rise, M.L. (2018b). Immune modulatory properties of 6-gingerol and resveratrol in Atlantic salmon macrophages. Mol. Immunol. 95, 10-19.

Solbakken, M.H., Rise, M.L., Jakobsen, K.S., and Jentoft, S. (2016a). Successive Losses of Central Immune Genes Characterize the Gadiformes' Alternate Immunity. Genome Biol. Evol. 8, 3508-3515.

Solbakken, M.H., Torresen, O.K., Nederbragt, A.J., Seppola, M., Gregers, T.F., Jakobsen, K.S., and Jentoft, S. (2016b). Evolutionary redesign of the Atlantic cod (*Gadus morhua* L.) Toll-like receptor repertoire by gene losses and expansions. Sci. Rep. 6, 25211.

Solbakken, M.H., Voje, K.L., Jakobsen, K.S., and Jentoft, S. (2017). Linking species habitat and past palaeoclimatic events to evolution of the teleost innate immune system. Proc. Biol. Sci. 284.

Solstad, T., Larsen, A.N., Seppola, M., and Jørgensen T.Ø. (2008). Identification, cloning and expression analysis of a hepcidin cDNA of the Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 25, 298-310.

Stafford, J., and Belosevic, M. (2003). Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev. Comp. Immunol. 27, 539-554.

Star, B., and Jentoft, S. (2012). Why does the immune system of Atlantic cod lack MHC II?. BioEssays 34, 648-651.

Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., Malmstrøm, M., Gregers, T.F., Rounge, T.B., Paulsen, J., Solbakken, M.H., Sharma, A., Wetten, O.F., Lanzen, A., Winer, R., Knight, J., Vogel, J.H., Aken, B., Andersen, O., Lagesen, K., Tooming-Klunderud, A., Edvardsen, R.B., Tina, K.G., Espelund, M., Nepal, C., Previti, C., Karlsen, B.O., Moum, T., Skage, M., Berg, P.R., Gjoen, T., Kuhl, H., Thorsen, J.,

Malde, K., Reinhardt, R., Du, L., Johansen, S.D., Searle, S., Lien, S., Nilsen, F., Jonassen, I., Omholt, S.W., Stenseth, N.C., and Jakobsen, K.S. (2011). The genome sequence of Atlantic cod reveals a unique immune system. Nature 477, 207-210.

Stenvik, J., Solstad, T., Strand, C., Leiros, I., and Jørgensen T.Ø. (2004). Cloning and analyses of a BPI/LBP cDNA of the Atlantic cod (*Gadus morhua* L.). Dev. Comp. Immunol. 28, 307-323.

Sundaram, A.Y., Kiron, V., Dopazo, J., and Fernandes, J.M. (2012). Diversification of the expanded teleost-specific toll-like receptor family in Atlantic cod, *Gadus morhua*. BMC Evol. Biol. 12, 256.

Sung, K., Khan, S.A., Nawaz, M.S., and Khan A.A. (2003). A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. FEMS Microbiol. Lett. 229, 97-101.

Suzuky, Y., and Lida, T. (1992). Fish granulocytes in the process of inflammation. Annu. Rev. Fish Dis. 2, 149-160.

Tort, L., Balasch, J.C., and Mackenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. Inmunología 22, 277-286.

Valderrama, K., Saravia, M., and Santander, J. (2017). Phenotype of *Aeromonas salmonicida* sp. *salmonicida* cyclic adenosine 3',5'-monophosphate receptor protein (Crp) mutants and its virulence in rainbow trout (*Oncorhynchus mykiss*). J. Fish. Dis. 40, 1849-1856.

Whyte, S.K. (2007). The innate immune response of finfish - a review of current knowledge. Fish Shellfish Immunol. 23, 1127-1151.

3. <u>CHAPTER III</u>: Effects of Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) on Atlantic salmon (*Salmo salar*) primary macrophages immune response to *Aeromonas salmonicida* subsp. *salmonicida* infection.

The research described in Chapter III has been submitted to Frontiers in Immunology as:

Soto-Davila, M., Valderrama, K., Inkpen, S., Hall, J., Rise, M.L., and Santander, J. Effects of Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) on Atlantic salmon (*Salmo salar*) primary macrophage immune response to *Aeromonas salmonicida* subsp. *salmonicida* infection.

3.1 Abstract

Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are fat-soluble secosteroid hormones obtained from plant and animal sources, respectively. Fish incorporate vitamin D_2 and D_3 through their diet since they lack the mechanisms to produce them through the photochemical pathway. In mammals, vitamin D forms are involved in mineral metabolism, cell growth, tissue differentiation, and antibacterial immune response. Vitamin D is an essential nutrient in aquafeeds for finfish. However, the influence of vitamin D on fish cell immunity has not yet been explored. Here, I examined the effects of ergocalciferol and cholecalciferol on *Salmon salar* primary macrophage immune response after *A. salmonicida* subspecies *salmonicida* infection. I determined that high concentrations of vitamin D_2 and D_3 affect the growth of *A. salmonicida* and decreases the viability of *S. salar* primary macrophages. I determined that primary macrophages pre-treated with a biologically relevant concentration of vitamin D_3 for 24 h had decrease *A. salmonicida* infection compared with non-vitamin D_3 pre-treated cells. In contrast, vitamin D_2 did not influence the antibacterial activity of the *S. salar* macrophages infected with *A. salmonicida*. Vitamin D_2 and D_3 did not influence the expression of canonical genes related to innate immune response (e.g. *il-1b*, *il-8*, *tnf-α*, and *tlr5s*). In contrast, *A. salmonicida* stimulated the expression of several canonical genes (e.g. *il-1b*, *il-8*, *tnf-α*, and *tlr5s*) and suppressed the expression of *lect-2*, involved in neutrophil recruitment. Primary macrophages pre-treated for 24 h with vitamin D_3 counteracted this immune suppression and up-regulated the transcription of *lect-2*. I conclude that vitamin D_3 affects *A. salmonicida* attachment to the *S. salar* primary macrophages, and as a consequence the *A. salmonicida* invasion decreased. The positive effects of D_3 on fish cell immunity seems to

be related to the *lect-2* innate immunity pathways. I did not identify effects of D_2 on the fish cell immunity. Vitamin D_3 induced anti-bacterial innate immunity pathways, confirming its utilization as component of a healthy aquafeed diet for the Atlantic salmon.

3.2 Introduction

Vitamin D is a fat-soluble secosteroid hormone that plays a crucial role in calcium and phosphorus homeostasis, cardiovascular physiology, cell proliferation and differentiation, among other functions [Zittermann 2003; Grant 2006; Lips 2006; Lock et al. 2010; Borges et al. 2011; Wang et al 2017]. In fish, vitamin D is involved in the endocrine control of calcium and phosphorus homeostasis, similar to mammals [Lock et al. 2010]. Also, it has been shown that vitamin D can act as an immunomodulatory agent in mammals [Miller and Gallo 2010; Téllez-Pérez et al. 2012].

In contrast to terrestrial vertebrates, fish are not able to obtain vitamin D through the photochemical pathway, thus fish must ingest vitamin D from dietary sources [Rao and Raghuramulu 1996]. In wild freshwater and marine environments, the main diet source of vitamin D is the phytoplankton and zooplankton [Rao and Raghuramulu 1996]. The phytoplankton provide the fish with vitamin D_2 (ergocalciferol), while the zooplankton provide the fish with vitamin D_3 (cholecalciferol) [Darias et al. 2011].

The beneficial stimulatory effects of vitamins D_2 and D_3 on innate immunity have been described in humans and other mammals [Mora et al. 2008; Prentice et al. 2008; Borges et al. 2011; Téllez-Pérez et al. 2012; Alva-Murillo et al. 2014; Yue et al. 2017]. The beneficial effects of vitamins in fish are well established, and currently vitamin D_2 and D_3 are essential component of aquafeed diets [Lock et al. 2010]. Additionally, vitamin D is

utilized as an adjuvant in aqua-vaccine preparation [Sadarangani et al. 2015]. However, the role of vitamin D in fish physiology is still enigmatic, and the immune stimulant mechanisms against infectious diseases are unknown.

Atlantic salmon (*Salmo salar*) is a high-value cultured finfish species, and the main species cultured in Canada, Chile, UK, and Norway [Asche et al. 2013; Liu et al. 2016; FAO 2018]. Infectious diseases, including *Renibacterium salmoninarum*, *Piscirickettsia salmonis*, *Vibrio anguillarum*, and *Aeromonas salmonicida* subsp. *salmonicida* [Fryer and Sanders 1981; Cvitanich et al. 1991; Toranzo et al. 2005; Higuera et al. 2013; Maisey et al. 2016; Valderrama et al. 2017] have affected this industry since its origin [Kaatari & Tripp 1987; Robertson et al. 1987]. Currently, several measures are utilized to prevent infectious diseases in the Atlantic salmon aquaculture industry, including a healthy diet that includes immunostimulants [Siwicki et al. 1994; Murray et al. 2003; Dawood et al. 2017].

Functional constituents like essential nutrients in healthy diets used in aquaculture (such as probiotics, prebiotics and immunostimulants) are currently considered to improve not only fish growth and stress tolerance, but also resistance to diseases by enhancing non-specific defense mechanisms [Sakai 1999; Olivia-Teles 2012]. These essential nutrients are able to directly activate immune mechanisms, such as phagocytic activity (i.e. macrophages and neutrophils), complement system, lysozyme activity, and others [Cook et al. 2003; Bridle et al. 2005; Song et al. 2014]. Phagocytosis is an active host defense mechanism, involving the action of monocytes, dendritic cells, neutrophils and macrophages [Secombes et al. 1992; Song et al. 2014; Esteban et al. 2015]. From these phagocytic leukocytes, the macrophages play an important role linking the innate and adaptive immune response, and previous studies have shown that immunostimulants (i.e. Fructooligosaccharides,

mannanoligosaccharides) can successfully enhance their phagocytic activity [Torrecillas et al. 2011; Vogt et al. 2013; Song et al. 2014].

Immunostimulants are natural compounds that have shown to be safe and efficient for fish [Sakai 1999; Bridle et al. 2005; Smith et al. 2018]. In humans, vitamin D plays an important role in the suppression of pro-inflammatory cytokines like IL-17, IL-1β, and TNF-α in individuals affected by type 2 diabetes and autoimmune diseases, preventing chronic inflammation [Szodoray et al. 2008; Chagas et al. 2013]. Additionally, vitamin D significantly reduces the infection of *Staphylococcus aureus* in pre-treated bovine mammary epithelial cells and modulates the expression of innate immune related genes [Téllez-Pérez et al. 2012; Alva-Murillo et al. 2014; Yue et al. 2017]. This evidence suggests that vitamin D could trigger a similar protective effects in fish. Here, I evaluate the effects of vitamins D_2 and D_3 on the innate immunity responses of Atlantic salmon primary macrophages to *A. salmonicida* infection.

3.3 Material and methods

3.3.1 Aeromonas salmonicida growth conditions

A. salmonicida was grown in accordance to the protocol used by Soto-Dávila et al. (2019). Briefly, a single colony of *A. salmonicida* J223 [Valderrama et al. 2017] was grown in 3 ml of Trypticase Soy Broth (TSB, Difco, Franklin Lakes, NJ) at 15ºC in a 16 mm diameter glass tube and placed in a roller for 24 h. After growth, 300 µl of the overnight culture were added in 30 ml of TSB media using a 250 ml flask and incubated for 24 h at 15ºC with aeration (180 rpm). After bacteria reached an O.D. 600 nm ~0.7 (1×10^8 CFU

ml^{-1}), the bacterial culture was centrifuged at 6,000 rpm at room temperature for 10 min. The pellet was washed twice with phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.5 mM KH2PO4 (pH 7.2)) and centrifuged at 6,000 rpm at room temperature for 5 min, and finally resuspended in 300 µl of PBS (~5 x 10^{10} CFU ml^{-1}). The concentrated bacterial inoculum was serial diluted and quantified by plating onto TSA supplemented with Congo red (50 µg ml^{-1}) for4 days.

3.3.2 Vitamin D_2 and D_3 inhibitory effects in A. salmonicida growth

To determine whether ergocalciferol and cholecalciferol have inhibitory effects on *A. salmonicida* growth, 30 µl of the overnight growth bacteria were placed in 3 ml of TBS containing different concentrations of ergocalciferol (10; 100; 1,000; 10,000; and 100,000 ng/ml) or cholecalciferol (10; 100; 1,000; and 10,000 ng/ml). Bacterial growth was measured by O.D. 600 nm until 48 h. Each concentration was measured in triplicate and a blank containing the respective vitamin D concentration was utilized as a control.

3.3.3 Fish holding

Adult specimens of Atlantic salmon 4.0 ± 0.1 kg (mean ± SE)) were obtained from the Dr. Joe Brown Aquatic Research Building (JBARB) at the Department of Ocean Sciences, Memorial University of Newfoundland, Canada. The animals were kept in 37 m^3 tanks, with flow-through (100 l x min^{-1}) seawater (6.5 ºC) and ambient photoperiod. The individuals were fed twice per day with commercial salmonid dry pellets (Skretting optiline microbalance 3000 ep, 12.0 mm pellets: 38% protein, 33% fat, 1.6% calcium, 1.5% fiber,

1% phosphorus) with a ration of 0.5% of body weight per day. The experiment was performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocols #17-01-JS; #17-02-JS).

3.3.4 Macrophage isolation

Primary macrophages were isolated from Atlantic salmon head kidney. Tissues from 6 fish were aseptically removed and individually minced through 100 µm nylon sterile cell strainers (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) in isolation media ((Leibovitz-15 (Gibco®, Gran Island, NY, USA) supplemented with 250 µg ml^{-1} heparin, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 0.1% Fetal Bovine Serum (FBS)). After this period, 4 ml of cell suspension were centrifuged (1,000 x g at 4ºC) for 30 min in a 34/51% Percoll gradient (GE Healthcare, Uppsala, Sweden). Macrophages collected from the macrophage-enriched interface were washed with PBS [Sambrook and Russell 2001] twice and the number and viable cells were determined using the Countness™ cell counter (Invitrogen), and trypan blue stain (Invitrogen). After determining the cell concentration (number of cells per ml) of each sample, the primary macrophages were seeded in 22 mm 12-well or 35 mm 6-well cell-culture multidishes (Thermo Scientific, Roskilde, Denmark) at a concentration of 1 x 10^7 cells ml^{-1}. The plates were incubated at 15ºC for 24 h in isolation media. After this period the cells were washed with PBS and incubated at 15ºC for an additional 4 days in 1 ml of culture media (Leibovitz-

15 (Gibco®), supplemented with 0.1% 2-Mercaptoethanol, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 5% FBS) to allow cell attachment until the assays were performed.

3.3.5 Vitamin D_2 and D_3 toxicity in Atlantic salmon primary macrophages

Atlantic salmon primary macrophages seeded in 12 well cell-culture multidishes in a concentration of 1 x 10^7 cells ml^{-1} were utilized. After 4 days of isolation the culture media was removed, cells washed with PBS, and 1 ml of culture media containing different concentrations of ergocalciferol (10; 100; 1,000; 10,000; and 100,000 ng/ml) or cholecalciferol (10; 100; 1,000; and 10,000 ng/ml) was added. Twenty-four hours and 48 h post-vitamin treatment, cells were treated with 500 µl of trypsin-EDTA (0.5%; Gibco) for 10 min, and then trypsin was inactivated with 500 µl of culture media. The cells were stained with trypan blue (0,4%; Invitrogen) in a ratio of 1:1 (10 µl: 10 µl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. Viability of cells was determined for each vitamin D_2 and D_3 concentration and the control group. All samples were taken from 6 individual fish.

3.3.6 Gentamicin exclusion assay

Infections of primary macrophages with *A. salmonicida* were performed according to the protocol used by Soto-Dávila et al. (2019) with modifications. Briefly, after 4 days, cells were washed with 1 ml of PBS and inoculated with 1 ml of culture media without antibiotics containing either 100 ng/ml of vitamin D_2 and D_3 for 24 h. After this period,

media was removed, cells washed with 1 ml of PBS, and pre-treated primary macrophage monolayers were infected with 10 µl of bacterial suspension (~1 x 10^7 cells ml^{-1}; Multiplicity of Infection (MOI) 1:1 (bacteria:macrophage)) and incubated at 15°C. After 1 h (attachment), and 2 h, 3 h, and 4 h (invasion) of infection, *A. salmonicida* was quantified. Infected primary macrophage monolayers in each well were washed twice with PBS and then lysed using 400 µl of Triton X100 (0.01%; Sigma) for 10 min [Sung et al. 2003]. After this, 600 µl of PBS were added to make a total of 1 ml of lysed macrophage suspension. Lysed macrophage suspensions were serially diluted (1:10) and plate/counted on TSA plates supplemented with Congo Red to determine the number of viable *A. salmonicida* per monolayer. The plates were incubated at 15°C for 5 days to determine the CFU per well. All samples were taken from 6 individual fish.

3.3.7 Vitamin D_2 and D_3 pre-treated primary macrophage viability against A. salmonicida

To determine the viability of infected primary macrophages, the cells were seeded in 12 wells plates, pre-treated with 100 ng/ml of ergocalciferol or cholecalciferol, infected with *A. salmonicida*, and processed following the method used during the gentamicin exclusion assay. Cells were washed with 1 ml of PBS and then treated with 500 µl of trypsin-EDTA (0.5%; Gibco) for 10 min. After this period, the trypsin was inactivated with 500 µl of culture media. The primary macrophages were stained using trypan blue (0,4%; Invitrogen) in a ratio of 1:1 (10 µl: 10 µl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen)

according to the manufacturer's instructions. The numbers of alive and dead cells were determined at each time point post-infection. All the primary macrophages were isolated from 6 individual fish and technical triplicates were utilized.

3.3.8 RNA extraction and qPCR

RNA samples were obtained from head kidney primary macrophages inoculated with either PBS; live *A. salmonicida* (J223); formalin-killed *A. salmonicida* (FK J223); 100 ng ml^{-1} vitamin D_2 or D_3; 1,000 ng ml^{-1} vitamin D_2 or D_3, or 100 ng ml^{-1} vitamin D_2 or D_3 + live *A. salmonicida* (J223). The treatments that include vitamin D (D_2 or D_3) were pre-treated with the respective concentration 24 h before the challenge, meanwhile treatments without vitamin D were pre-treated only with the control vehicle 24 h before the challenge. Each sample was obtained 3 h post-inoculation.

Total RNA from Atlantic salmon primary macrophages was extracted using 1 ml of TRIzol Reagent (Invitrogen), and purified using the RNeasy® Mini Kit (QIAGEN) following the manufacturer's instructions [Santander et al. 2014]. RNA samples were treated with 2 U of TURBO DNase (TURBO DNA-free™ Kit, Invitrogen) following the manufacturer's instructions to degrade any residual genomic DNA. Briefly, samples were incubated at 37°C for 30 min, 2.5 µl of DNase Inactivation Reagent was added, and samples incubated 5 min at room temperature. Then, samples were centrifuged at 10,000 x g for 1.5 min and the supernatant containing the RNA carefully transferred to a new tube. Purified RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using a Nano-quant spectrophotometer (Genway, UK), and evaluated for integrity using 1% agarose gel electrophoresis [Sambrook and Russell 2001]. Column purified RNA

samples had A260/280 ratios between 1.9 and 2.1 and A260/230 ratios between 1.9 and 2.2. A PCR test was conducted using the reference genes primers (*60S ribosomal protein* and *β-actin*) and the RNA as template to discard presence of DNA. All RNA samples did not showed presence of DNA.

First-strand cDNA templates for qPCR were synthesized from 500 ng of DNaseI-treated, column-purified total RNA using SuperScript™ IV VILO™ Master Mix (Invitrogen) following the manufacturer's instructions. Each sample was incubated at 25°C for 10 min, at 50°C for 10 min, and at 85°C for 5 min.

All qPCR reactions were performed in a 20 µl reaction, containing 1× PowerUp SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA), 500 nM (final concentration) of both the forward and reverse primer and the indicated cDNA quantity. All samples were amplified and detected using the QuantStudio 3 Real Time PCR System (Applied BioSystems). The reaction mixtures were incubated for 2 min at 50°C, then 2 min at 95°C, followed by 40 cycles of 1 s at 95°C, 30 s at 60°C, and finally 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C.

Gene paralogue discovery, qPCR primer design and quality testing was performed as described in Caballero-Solares et al. (2017). Since the reagents, cycling conditions and samples were different in the current study, primer efficiencies (Table 3.1) were reassessed. Briefly, a 7-point 1:3 dilution series starting with cDNA representing 40 ng of input total RNA was generated, and efficiencies then calculated using the formula $E=10^{(-1/slope)}$ [Pfaffl 2001].

Transcripts levels of the genes of interest (*il-1b*, *il-8*, *tnf-α*, *stlr5*, and *lect-2*) were normalized to transcript levels of two endogenous control genes. Levels of five candidate

normalizers (*60S ribosomal protein 32*; *β-actin*, *18S*, *elongation factor 1 alpha*, and *hypoxanthine phosphoribosyl transferase 1*) were assessed in 50% of the samples (i.e. in 3 random samples per treatment) using cDNA representing 40 ng of input total RNA. Reference gene stability was then analyzed using both geNorm and BestKeeper. Both analyses identified *β-actin* (geNorm M = 0.592; BestKeeper value = 0.263) and *60S ribosomal protein 32* (geNorm M = 0.592; BestKeeper value = 0.364) and as the most stably expressed genes.

After normalizer testing was completed, transcript levels of the genes of interest were analyzed in the individual study samples, with normalization to both *β-actin* and *60S ribosomal protein 32*. In all cases, levels were assessed (in triplicate) in six individuals per treatment using cDNA representing 40 ng of input total RNA. On each gene a no RT control was included. Gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen 2001].

Table 3-1. Primer sequences used in Atlantic salmon experiment.

Gene	Forward (5´to 3´)	Reverse (5´to 3´)	Efficiency (%)	Reference
IL-1β	GTATCCCATCACCCCATCAC	TTGAGCAGGTCCTTGTCCTT	99.7	This study*
IL-8	GAAAGCAGACGAATTGGTAGAC	GCTGTTGCTCAGAGTTGCAAT	100.7	This study*
tnf-α	GGATGGAATGGAGCATCAGC	TGCACGGTGTTAGCGGTAAG	106.4	Smith et al. 2018
lect-2	CAGATGGGGACAAGGACACT	GCCTTCTTCGGGTCTGTGTA	101.1	Smith et al. 2018
tlr5s	ATCGCCCTGCAGATTTTATG	GAGCCCTCAGCGAGTTAAAG	94.1	Smith et al. 2018
β-actin	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	104.4	Xue et al. 2015
rpl32	AGGCGGTTTAAGGGTCAGAT	TCGAGCTCCTTGATGTTGTG	100.7	Xue et al. 2015

***Pair of primer sequences designed by Jennifer Hall and provided by Dr. Matthew L. Rise.**

3.3.9 Phagocytosis assay

The phagocytosis assay was performed following the protocol used by Smith et al. (2018) with modifications. Cells were incubated for 3 days and inoculated with vehicle control (2 µl of ethanol in 1 ml of culture media without antibiotics), 100; 1,000; or 10,000 ng/ml of either vitamin D_2 or D_3 for 24 h. After this time, cells were washed twice with PBS, and inoculated with 1 µm of Fluoresbrite YG microspheres at a ratio of approximately 1:30 macrophage:microsphere (Polysciences, Warrington, PA, USA) [Overland et al. 2010; Smith et al. 2018]. Twenty-four hours after microsphere addition, primary macrophages were washed with PBS, removed, and cells treated with trypsin-EDTA (0.5%; Gibco) for 10 min. Then, cells were resuspended in 500 µl of FACS buffer (PBS + 1% FBS). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analyzed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). The control pre-treated macrophages were used to compared the FITC positive cells in vitamin D pre-treated cells. Percentage of FITC positive cells were determined for each condition. The experiments were conducted in macrophages isolated from 3 independent fish.

3.3.10 Statistical analysis

All data are shown as the mean ± standard error (SE). Assumptions of normality and homogeneity were tested for the detected variances. A Kruskal-Wallis nonparametric test was performed for *A. salmonicida* growth curve and gene expression results. Macrophage viability, gentamicin exclusion assay, and phagocytosis assay data were analyzed using a repeated measures two-way ANOVA test, followed by Sidak multiple comparisons *post hoc* test to identify significant differences of each treatment in different

times or concentrations and between treatments in the same time point. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

3.4 Results

3.4.1 Inhibitory effects of vitamin D_2 and D_3 in A. salmonicida growth

Growth of *A. salmonicida* at different concentrations of vitamin D_2 and D_3 was determined by O.D. 600 nm at different time points until 48 h. Bacteria growth was not affected by concentrations of 10, 100, 1,000, and 10,000 ng/ml of vitamin D_2 (Fig. 3-1A). In contrast, *A. salmonicida* growth was significantly reduced in the presence of 100,000 ng/ml of vitamin D_2 (Fig. 3-1A). *A. salmonicida* growth was not affected by 10 and 100 ng/ml of vitamin D_3 (Fig. 3-1B). However, *A. salmonicida* growth was significantly affected by 1,000 and 10,000 ng/ml of the vitamin D_3 (Fig. 3-1B).

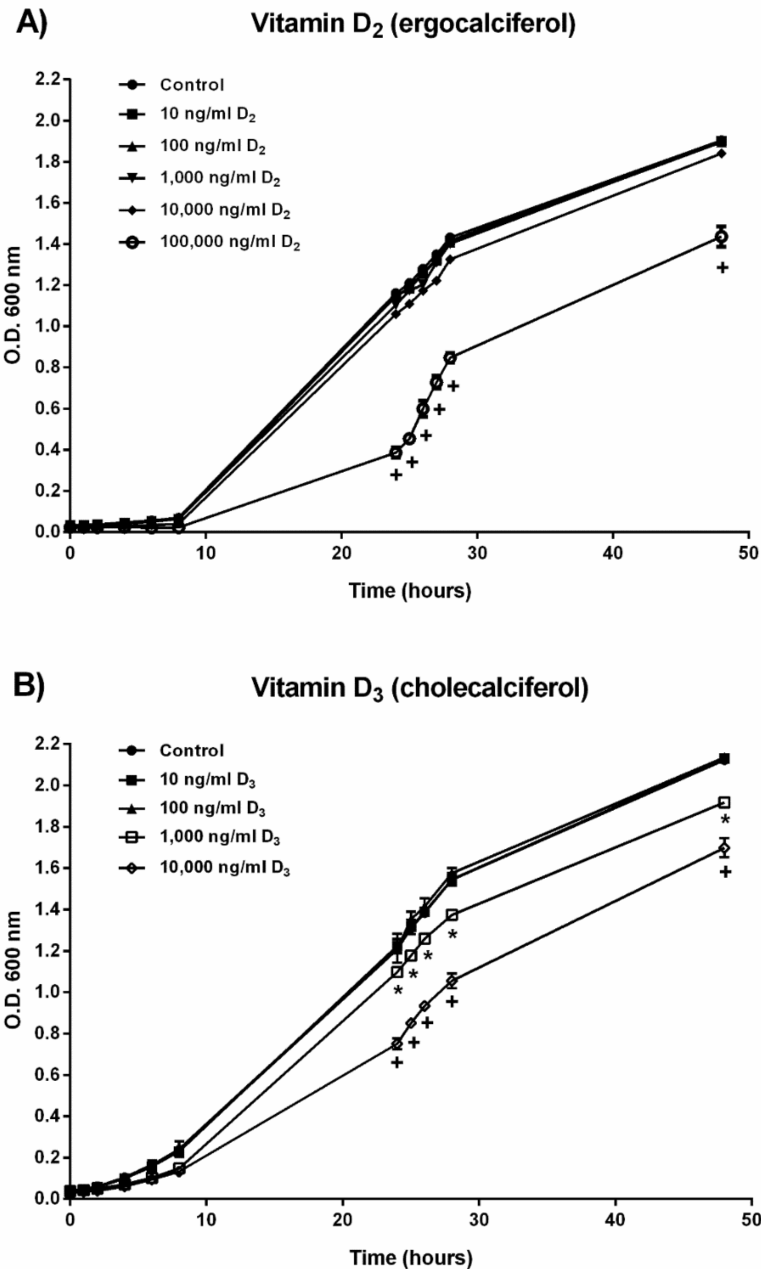


Figure 3-1. *Aeromonas salmonicida* subsp. *salmonicida* growth curve in TSB media supplemented with (A) 10, 100, 1,000, 10,000, and 100,000 ng/ml of vitamin D_2 and (B) 10, 100, 1,000, and 10,000 ng/ml of vitamin D_3. Growth was determined by reading O.D. 600 nm at different time points until 48 h. Each value is the mean ± S.E.M (n = 3). Symbols (*, +) indicate differences between each group at different time points of measure, $p < 0.05$.

3.4.2 Evaluation of the toxicity of vitamin D_2 and D_3 in Atlantic salmon primary macrophages

Atlantic salmon primary macrophage viability was determined after 24 h and 48 h of exposure to concentrations of 0, 10, 100, 1,000, 10,000, and 100,000 ng/ml of vitamin D_2. Results obtained did not show significant differences between the control group ($2.17 \times 10^5 \pm 6.49 \times 10^4$ and $2.10 \times 10^5 \pm 7.77 \times 10^4$ after 24 h and 48 h, respectively), compared with the cells treated with 10 ng/ml ($3.57 \times 10^5 \pm 7.36 \times 10^4$ and $2.33 \times 10^5 \pm 8.51 \times 10^4$ after 24 h and 48 h, respectively), 100 ng/ml ($2.10 \times 10^5 \pm 4.00 \times 10^4$ and $2.37 \times 10^5 \pm 6.33 \times 10^4$ after 24 h and 48 h, respectively), 1,000 ng/ml ($1.77 \times 10^5 \pm 2.03 \times 10^4$ and $2.47 \times 10^5 \pm 5.90 \times 10^4$ after 24 h and 48 h, respectively), and 10,000 ng/ml ($1.43 \times 10^5 \pm 4.67 \times 10^4$ and $2.23 \times 10^5 \pm 7.36 \times 10^4$ after 24 h and 48 h, respectively) of vitamin D_2. However, a significant difference was observed in cells inoculated with the media containing a concentration of 100,000 ng/ml of vitamin D_2 after 24 h ($1.67 \times 10^4 \pm 6.67 \times 10^3$) and 48 h ($6.67 \times 10^3 \pm 6.67 \times 10^3$) (Fig. 3-2A).

The percentage of viability did not show significant differences between the control group (65.67% ± 1.76% and 66.00% ± 5.13% after 24 h and 48 h, respectively) and the primary macrophages treated with 10 ng/ml (77.00% ± 3.51% and 66.67% ± 5.93% after 24 h and 48 h, respectively), 100 ng/ml (75.67% ± 1.76% and 71.33% ± 4.84% after 24 h and 48 h, respectively), 1,000 ng/ml (67.67% ± 5.17% and 63.67% ± 5.24% after 24 h and 48 h, respectively), and 10,000 ng/ml (64.33% ± 0.88% and 67.33% ± 2.91% after 24 h and 48 h, respectively) of vitamin D_2. Nevertheless, a highly significant decrease in macrophage

viability was observed at a concentration of 100,000 ng/ml of ergocalciferol after 24 h (13.33 ± 4.48) and 48 h (5.67 ± 5.67) (Fig. 3-2B).

The viability of Atlantic salmon primary macrophages was also determined at 24 h and 48 h post-treatment with vitamin D_3 in concentrations of 10, 100, 1,000, and 10,000 ng/ml. The number of live cells per ml did not show significant differences in primary macrophages treated with 10 ($4.45 \times 10^5 \pm 8.66 \times 10^3$ and $4.65 \times 10^5 \pm 2.60 \times 10^4$ after 24 h and 48 h, respectively), 100 ($5.70 \times 10^5 \pm 8.08 \times 10^4$ and $4.70 \times 10^5 \pm 1.15 \times 10^4$ after 24 h and 48 h, respectively), and 1,000 ng/ml ($5.85 \times 10^5 \pm 9.53 \times 10^4$ and $5.60 \times 10^5 \pm 2.89 \times 10^4$ after 24 h and 48 h, respectively) (Fig. 3-2C). Moreover, no significant differences were observed in the viability of primary macrophages treated with 10,000 ng/ml of vitamin D_3 after 24 h ($4.25 \times 10^5 \pm 8.95 \times 10^4$) compared with the control (Fig. 3-2C). Nevertheless, in cells exposed to 10,000 ng/ml of vitamin D_3, a significant decrease in the viability was observed after 48 h of treatment ($2.85 \times 10^5 \pm 1.44 \times 10^4$) (Fig. 3-2C).

The percentage of viability in cholecalciferol treated cells did not show significant differences after 24 h of exposure to concentrations of 10 ng/ml (73.67% ± 2.60%), 100 ng/ml (74.67% ± 0.33%), 1,000 ng/ml (69.00% ± 4.04%), and 10,000 ng/ml (69.00% ± 2.31%) compared with the control group (70.33% ± 0.33%) (Fig. 3-2D). Also, no significant differences were observed in Atlantic salmon macrophages treated during 48 h with vitamin D_3 in a concentration of 10 ng/ml (70.67% ± 1.45%), 100 ng/ml (79.67% ± 0.88%), and 1,000 ng/ml (76.00% ± 3.46%) compared with the control (Fig. 3-2D). However, a significant decrease was observed in cells incubated with media and a concentration of 10,000 ng/ml of vitamin D_3 after 48 h (59.00% ± 1.15%) (Fig. 3-2D). Moreover, in the group incubated at a concentration of 1,000 ng/ml of vitamin D_3, a

significant higher percentage of viability was observed in cells treated for 48 h compared with the 24 h group (Fig. 3-2D). Also, in the primary macrophages treated with 10,000 ng/ml of vitamin D_3, a significant difference was observed at different times, showing a decrease in the viability of cells treated for 48 h with vitamin D_3 compared with the cells incubated for only 24 h (Fig 3-2D).

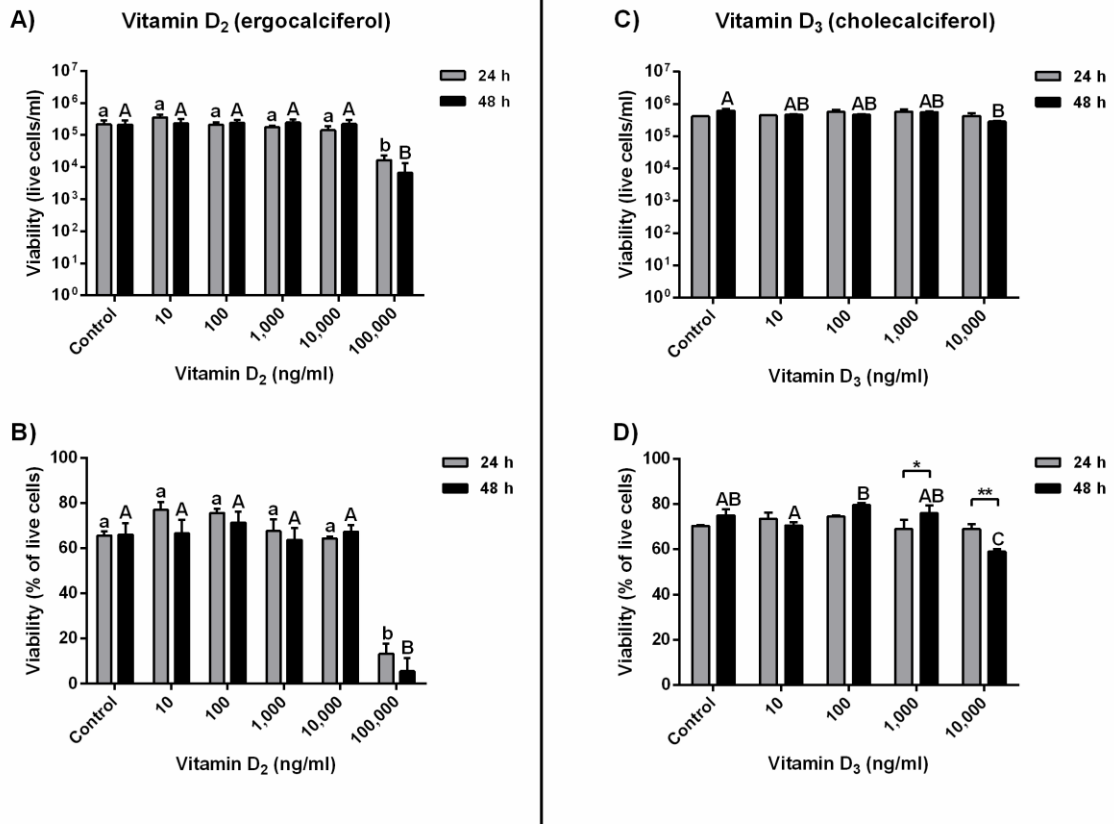


Figure 3-2. Atlantic salmon primary macrophages treated with vitamin D_2 or D_3. (A) Live cells and (B) percentage of viability of primary macrophages treated with 10, 100, 1,000, 10,000, and 100,000 of vitamin D_2, were measured after 24 h and 48 h of treatment. (C) Live cells and (D) percentage of viability of primary macrophages treated with 10, 100, 1,000, and 10,000 ng/ml of vitamin D_3 were measured after 24 h and 48 h of treatment. Each value represents the mean ± S.E.M (n = 6). Lower case letters (a, b) show differences between treatments after 24 h. Upper case letters (A, B, C) show differences between treatments after 48 h, $p < 0.05$.

3.4.3 Gentamicin exclusion assay in vitamin D_2 and D_3 pre-treated cells infected with *A. salmonicida*

The effects of vitamins D_2 and D_3 on the growth of *A. salmonicida* (Fig. 3-1) and the effects on the viability of primary macrophages (Fig. 3-2) were used to determine the concentration to be utilized in the gentamicin exclusion assays. Based on these results, the primary macrophages were pre-treated with 100 ng/ml (vitamin D_2 or D_3) for the gentamicin exclusion assay.

Cells pre-treated with 100 ng/ml of vitamin D_2 and posteriorly infected with *A. salmonicida*, did not show significant differences in cell numbers at 1, 2, 3, and 4 h post-infection ($7.27 \times 10^5 \pm 8.11 \times 10^4$; $6.07 \times 10^5 \pm 1.37 \times 10^5$; $5.27 \times 10^5 \pm 9.94 \times 10^4$; and $5.33 \times 10^5 \pm 1.83 \times 10^{5,}$ respectively) compared with the control group ($6.33 \times 10^5 \pm 1.92 \times 10^5$; $6.9 \times 10^5 \pm 1.31 \times 10^5$; $6.13 \times 10^5 \pm 9.53 \times 10^4$; and $6.27 \times 10^5 \pm 1.46 \times 10^5$, respectively) (Fig. 3-3A). Also, no significant differences were observed in the viability of primary macrophages pre-treated and then infected with *A. salmonicida* at 1, 2, 3 and 4 h post-infection (62% ± 6.81%; 61% ± 5.13%; 62.67% ± 1.86%; and 61.67% ± 2.33%, respectively) compared with the control group (64% ± 5.51%; 62.67% ± 6.96%; 58% ± 3.21%; and 59.33% ± 3.28%, respectively) (Fig. 3-3B).

The primary macrophages were infected with a total of 4.3×10^6 CFU per ml at a MOI of 1. The percentage of *A. salmonicida* attached was significantly higher in primary macrophages pre-treated with vitamin D_2 (49.09% ± 2.76%) compared with the control group (34.39% ± 2.12%) (Fig. 3-3D). At invasion time-points, after 2 h of infection no significant was observed in cells pre-treated with ergocalciferol (4.48% ± 0.68%) compared with the control group (2.84% ± 0.40%) at the same time (Fig. 3-3D). Moreover, no

significant differences were observed either in the control group (2.86% ± 0.57%) and the vitamin D_2 pre-treated primary macrophages (2.49% ± 0.21%) after 3 h of infection with *A. salmonicida* (Fig. 3-3D). No significant differences were also found between the control (2.06% ± 0.42%) and the vitamin D_2 treatment (1.94% ± 0.38%) 4 h post-infection. However, a significant decrease in bacterial invasion was observed between 2 h and 4 h in the primary macrophages pre-treated with vitamin D_2 (Fig. 3-3D).

A similar response was obtained in cells pre-treated for 24 h with vitamin D_3 and then infected with *A. salmonicida*. For instance, no significant differences were found in the percentage of viability between the control group after 1 h, 2 h, 3 h, and 4 h of infection (48.67% ± 4.98%; 48.66% ± 2.03%; 55.67% ± 4.33%; and 58.67% ± 6.74%, respectively) and the cholecalciferol pre-treated macrophages at 1 h, 2 h, 3 h, and 4 h (54% ± 2.31%; 58.67% ± 1.45%; 64% ± 5.77%; and 61% ± 1.73%, respectively) (Fig. 3-3F).

Atlantic salmon primary macrophages pre-treated with vitamin D_3 were infected with a total of 2.56 x 10^6 bacterial cells per ml (Fig. 3-3G). The percentage of attachment (1 h post-infection) was significantly lower in the primary macrophages that were pre-treated for 24 h with cholecalciferol (10.61% ± 0.97%) compared with the control group (51.56% ± 17.12%) (Fig. 3-3H). In contrast, even when a tendency of lower invasion is observed, no significant differences were obtained between the control group after 2 h, 3 h, and 4 h of infection (2.58% ± 0.65%; 2.23% ± 0.54%; and 1.82% ± 0.64%, respectively) compared with the fish cells pre-treated with vitamin D_3 (1.32% ± 0.65%; 1.43% ± 0.26%; and 0.91% ± 0.28%, respectively) (Fig. 3-3H).

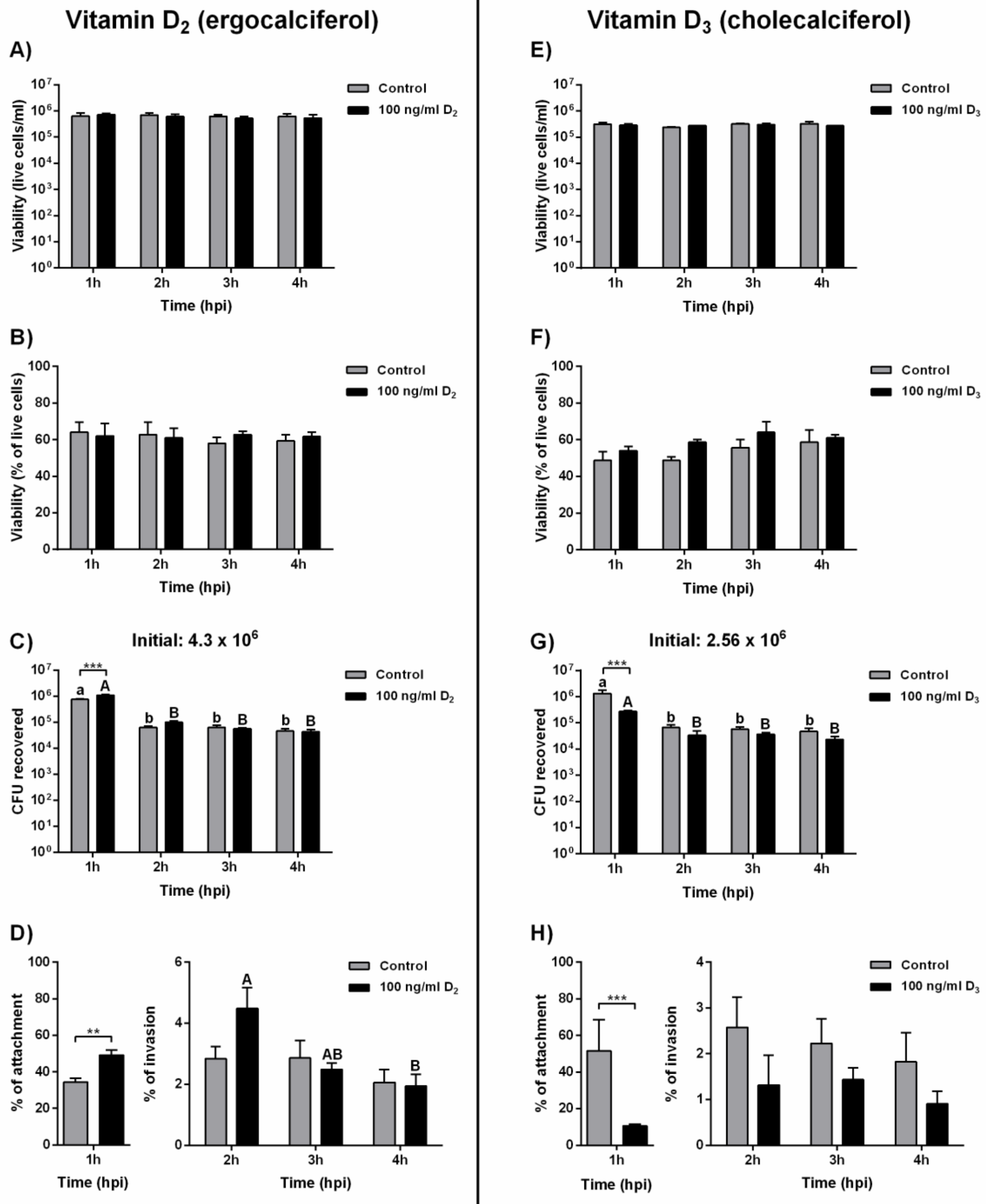


Figure 3-3. Gentamicin exclusion assay in Atlantic salmon primary macrophages pre-treated for 24 h with either control or 100 ng/ml of vitamin D_2 or vitamin D_3, and then infected with *Aeromonas salmonicida* subsp. *salmonicida*. Live cells of primary

macrophages pre-treated with vitamin D_2 (A), or vitamin D_3 (E); percentage of viability of primary macrophages pre-treated with vitamin D_2 (B), or vitamin D_3 (F), Colony forming unit (CFU) of *A. salmonicida* in Atlantic salmon primary macrophages pre-treated with vitamin D_2 (C), or vitamin D_3 (G); and percentage of attachment and invasion of *A. salmonicida* in Atlantic salmon primary macrophages pre-treated with vitamin D_2 (D), or vitamin D_3 (H), were measured 1 h, 2 h, 3 h, and 4 h post-infection. Initial *A. salmonicida* inoculum calculated in TSA Congo red plates are shown above the CFU figures. Each value represents the mean ± S.E.M (n = 6). Asterisks (*) represent significant differences between treatments on each time-point (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Lower case letters (a, b) show differences in the control at different time points post-infection. Upper case letters (A, B) show differences in vitamin D_2 or D_3 pre-treated cells in different time points post-infection.

3.4.4 Atlantic salmon primary macrophage gene expression

Innate immune response related genes were evaluated in Atlantic salmon primary macrophages after 3 h of each treatment previously mentioned.

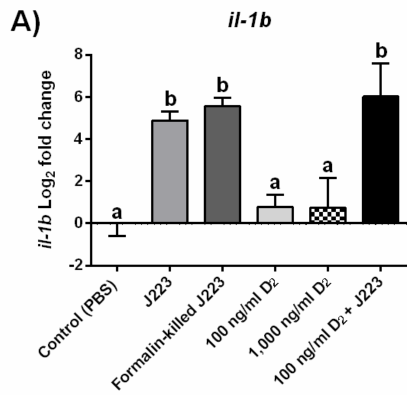
In the experiments conducted for both vitamins D_2 and D_3, a significant increase in the expression of *interleukin 1 beta* (*il-1b*) (Fig. 3-4A, F), *interleukin 8* (*il-8*) (Fig. 3-4B, G), *tumor necrosis factor alpha* (*tnf-α*) (Fig. 3-4A, H), and *soluble toll-like receptor 5* (s*tlr5*) (Fig. 3-4E, J) was observed in the cells inoculated with the live *A. salmonicida*, the formalin-killed *A. salmonicida*, and the cells pre-treated with either vitamin D_2 or D_3 and subsequently infected, compared to the PBS inoculated primary macrophages. In contrast, no differences in the expression of *il-1b*, *il-8*, *tnf-α* and s*tlr5* were observed in Atlantic

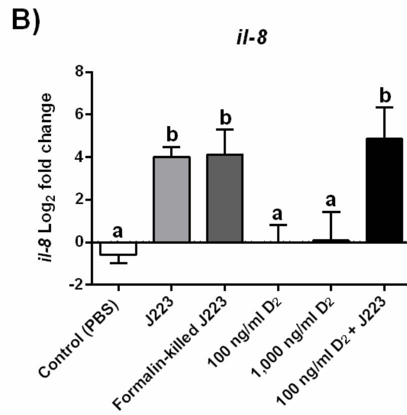
salmon non-infected cells inoculated only with 100 ng/ml or 1,000 ng/ml of each vitamin D forms compared with the control (Fig. 3-4A, 3-4B, 3-4C, 3-4E, 3-4F, 3-4G, 3-4H, and 3-4J).

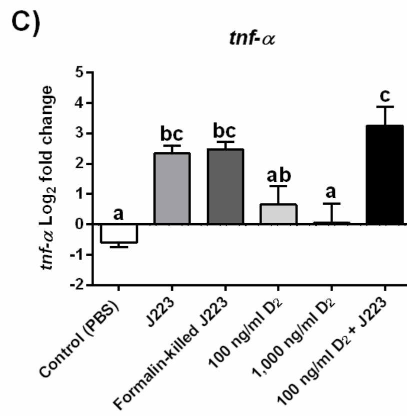
A differential pattern was observed in the transcript encoding for the expression of *leukocyte-derived chemotaxin 2* (*lect-2*) between both assays. For instance, in the vitamin D_2 experiment, no significant differences were observed in the expression of *lect-2* in any of the treatments compared with the PBS inoculated primary macrophages (Fig. 3-4D). In contrast, *lect-2* was significantly up-regulated compared with the control in primary macrophages pre-treated with vitamin D_3 and then challenged with *A. salmonicida* (Fig. 3-4I). The primary macrophages treated with live *A. salmonicida*, formalin-killed *A. salmonicida*, 100 ng/ml of vitamin D_3, and 1,000 ng/ml of vitamin D_3 did not show significant differences in the expression of *lect-2* compared with the control (Fig. 3-4I).

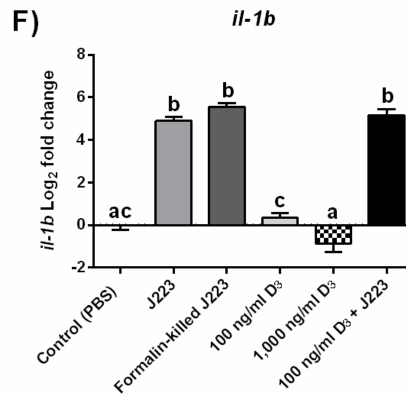
Vitamin D_2 (ergocalciferol)

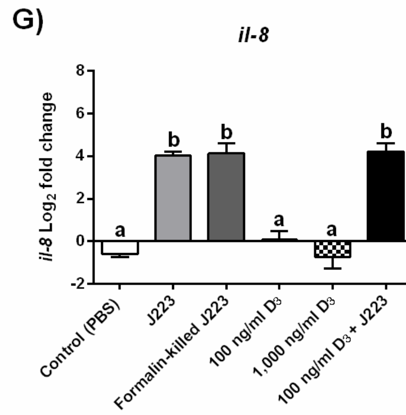
Vitamin D_3 (cholecalciferol)

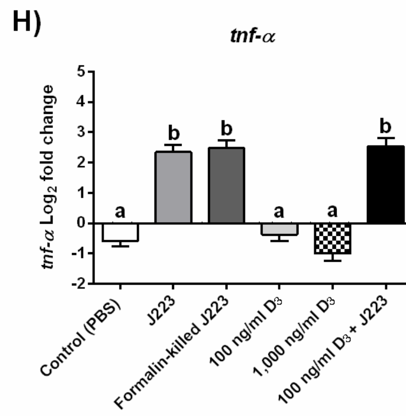












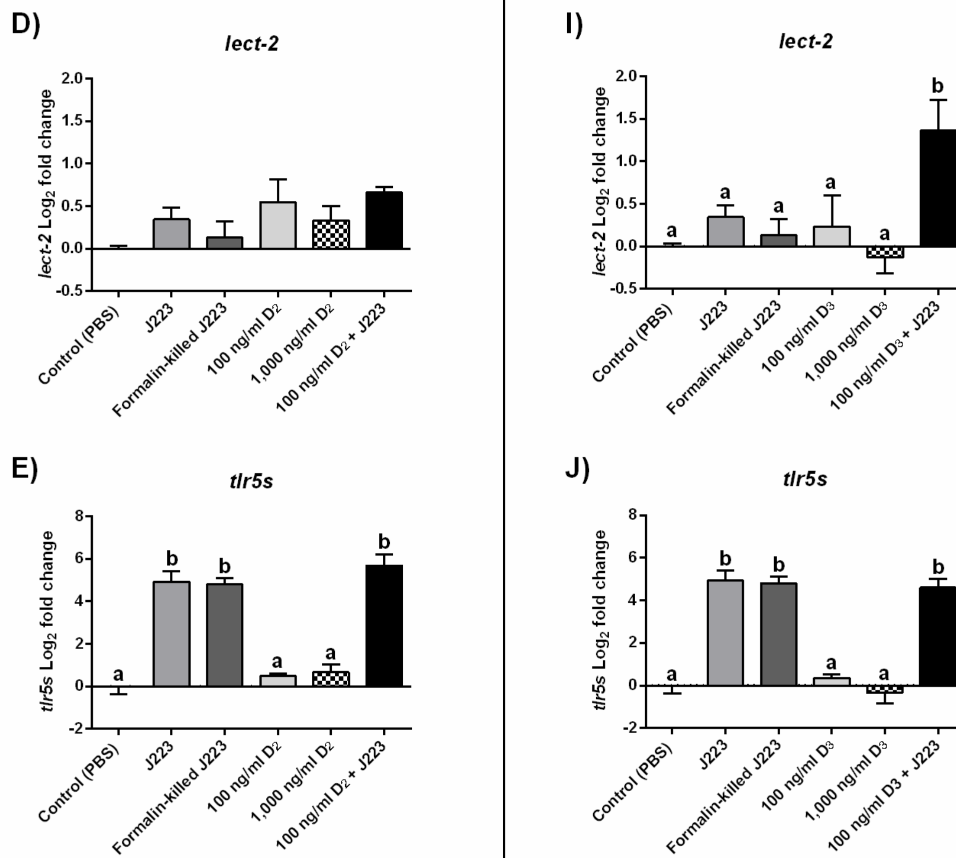


Figure 3-4. Gene expression of (A, E) *Interleukin 1b* (*il-1b*), (B, G) *Interleukin 8* (*il-8*), (C, H) *Tumor necrosis factor alpha* (*tnf-α*), (D, I) *Leukocyte-derived chemotaxin 2* (*lect-2*), and (E, J) *soluble toll-like receptor 5* (s*tlr5*) in Atlantic salmon primary macrophages isolated from head kidney pre-treated 24 h with either the control, vitamin D_2 (100 and 1,000 ng/ml) or vitamin D_3 (100 and 1,000 ng/ml), and then inoculated with PBS (control) or infected with live (J223) or formalin-killed *A. salmonicida* for 3 h. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using *β-actin* and *60S ribosomal protein 32* (*rpl32*) as internal reference genes. Each value is the mean ± S.E.M (n = 6). Different letters represent significant differences between treatments, $p < 0.05$.

3.4.5 Phagocytosis assay

Atlantic salmon primary macrophages did not show significant differences in phagocytosis after 24 h of treatment with 100 ng/ml of vitamin D_2 (4.80% ± 1.62%) and vitamin D_3 (4.93% ± 1.56%), 1,000 ng/ml of vitamin D_2 (3.33% ± 0.67%) and vitamin D_3 (2.67% ± 0.41%), and 10,000 ng/ml of vitamin D_2 (0.97% ± 0.20%) and vitamin D_3 (0.87% ± 0.09%) compared with the control cells (4.80% ± 1.08%) (Fig. 3-5).

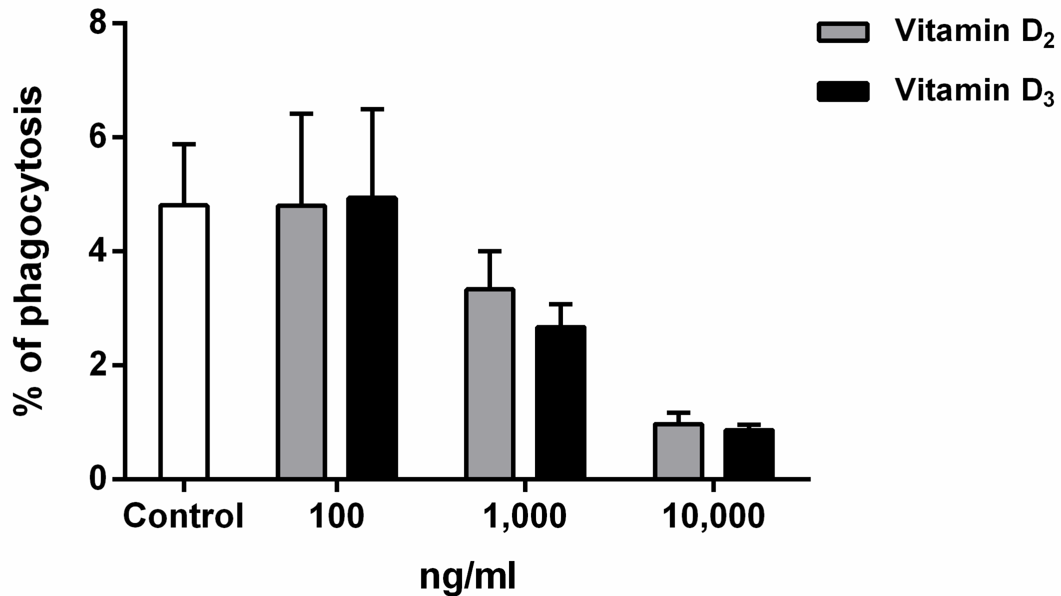


Figure 3-5. Effect of vitamin D_2 and vitamin D_3 in Atlantic salmon primary macrophage phagocytosis. Macrophages were pre-treated with 100, 1,000, and 10,000 ng/ml of vitamin D_2 or vitamin D_3 for 24 h, and then inoculated with 1 μm of Fluoresbrite YG microspheres. A non pre-treated control was utilized to determine the percentage of FITC-positive positive cells. Phagocytosis was determined by flow cytometry. Each value represents the mean ± S.E.M (n = 3), $p < 0.05$.

3.5 Discussion

Vitamin D is involved in important processes including mineral metabolism, cell growth, and cardiovascular physiology, among others [Zittermann 2003; Grant 2006; Lips 2006; Lock et al. 2010; Borges et al. 2011; Wang et al 2017]. Moreover, it has been observed that vitamin D can stimulate the antibacterial immune response in mammals [Miller and Gallo 2010; Téllez-Pérez et al. 2012]. However, these mechanisms have not been explored in fish cells.

The two major sources of vitamin D in natural environments are vitamin D_2 and D_3, obtained by fish after the ingestion of phytoplankton and zooplankton, respectively [Rao and Raghuramulu 1996; Darias et al. 2011]. Even when it was thought that both vitamin D forms have the same impact on physiological mechanisms, previous studies have shown that vitamin D_3 is much more potent compared with vitamin D_2 [Trang et al. 1998; Ostermeyer and Schmidt 2006], suggesting a differential modulation of the innate immune system of fish in the presence of the specific vitamin D form. Also, the effect that vitamin D forms can have over the growth of *A. salmonicida* has not been described.

To evaluate if *A. salmonicida* is able to grow in the presence of vitamin D_2 and D_3, a growth curve experiment was conducted in the presence of different concentrations of vitamin D_2 and vitamin D_3 for 48 h. Our results showed that only high concentrations of vitamin D_2 and D_3 reduced the growth of *A. salmonicida* after 48 h (Fig. 3-1A, B). Normally, *A. salmonicida* is able to reach stationary growth in approximately 36 h [Cipriano and Bullock 2001; Valderrama et al. 2017; Connors et al. 2019]. I observed a similar pattern of growth previously observed in *A. salmonicida* J223 strain [Valderrama et al. 2017] in culture media containing low concentrations of vitamin D_3 (100 and 1,000

ng/ml). Nevertheless, *A. salmonicida* seems to better tolerate higher concentrations of vitamin D_2 since only the highest concentration (100,000 ng/ml) reduced its growth rate. A previous study showed that high doses of vitamin C (128, 512, and 2048 mg/ml) can inhibit the growth of *Helicobacter pylori*, a risk factor for gastric carcinoma in mammals, during *in vitro* and *in vivo* experiments after 12 h [Zhang et al. 1997]. Additionally, it has been reported that high concentrations of vitamin C (90 μM) can also inhibit the growth of *S. aureus* in *in vitro* conditions [Kallio et al. 2012]. High concentrations of vitamin D_2 and D_3 decreased the growth rate of *S. aureus* strain A1 after 24 h [Aarestrup et al. 1994; Yue et al. 2017]. Nevertheless, no significant differences were observed in the growth rate of *S. aureus* subsp. *aureus* (ATCC 27543) in the presence of different concentrations of vitamin D_3 after 48 h [Gutierrez-Barroso et al. 2008; Téllez-Pérez et al. 2012]. These results indicate that in the bacterial strains studied, vitamin D can inhibit the growth when utilized in concentrations over 1,000 ng/ml, affecting both Gram negative and Gram positive bacteria.

The primary *S. salar* macrophage viability decreased after 24 h and 48 h of exposure to 100,000 ng/ml of vitamin D_2, and a lower viability compared with the control was observed after 48 h with 10,000 ng/ml of vitamin D_3 (Fig. 3-2). Similar to our results, it has been observed that low concentrations of vitamin D_3 (1, 10, and 50 nM) did not affect the viability of bovine mammary epithelial cells at 24 h [Téllez-Pérez et al. 2012]. However, a decrease in the viability of bovine mammary epithelial cells occurs in the presence of high concentrations of vitamin D_2 (6,000, 8,000, 10,000, 12,000, and 14,000 ng/ml) and D_3 (8,000, 10,000, 12,000, and 14,000 ng/ml) after 24 h of exposure, suggesting that vitamin D can induce cell cycle arrest, apoptosis, or both [Samuel and Sitrin 2008; Yue et al. 2017].

The values obtained in the *A. salmonicida* growth curve and Atlantic salmon primary macrophages exposed to different vitamin D_2 and D_3 concentrations (Fig. 3-1 and 3-2), were utilized to finally utilized 100 ng/ml for further infection assays.

The number of live cells and percentage of viability of Atlantic salmon primary macrophages after 1 h, 2 h, 3 h, and 4 h of infection did not show significant differences between the control and the vitamin D_2 or D_3 pre-treated cells (Fig. 3-3A, 3-2B, 3-2E, and 3-2F). Our finding agrees with a previous infection assay using *A. salmonicida* J223 strain in Atlantic cod primary macrophages, where no significant differences were observed in macrophages viability after 6 h of infection [Soto-Dávila et al. 2019]. This indicated that Atlantic salmon and Atlantic cod primary macrophages were not killed during this period by *A. salmonicida* J223. Soto-Dávila et al. (2019) suggested that *A. salmonicida* controls the macrophages machinery and prevents cell apoptosis. As mentioned previously, vitamin D_3 in high concentration (8,000, 10,000, 12,000, and 14,000 ng/ml) could induce apoptosis [Samuel and Sitrin 2008; Yue et al. 2017], contrary to the suggested prevention of apoptosis produced by *A. salmonicida* by Soto-Dávila et al. (2019). These results agree with the lower attachment and infection rates of *A. salmonicida* in cell pre-treated with D_3, where vitamin D_3 might interfere with the infection.

One of the most interesting findings in our results is related to the bacterial attachment and invasion. For instance, an unexpected significant increase of *A. salmonicida* infections was observed at 1 h post-infection in primary macrophages pre-treated with vitamin D_2 compared with the control (Fig. 3-3C and D). In contrast, a significant decrease in *A. salmonicida* attachment at 1 h was observed in cells pre-treated with vitamin D_3 compared with the control (Fig. 3-3G and 3-3H). Previous studies indicate that vitamin D_3

has a stronger activity compared to vitamin D_2 in terrestrial mammals [Trang et al. 1998; Ostermeyer and Schmidt 2006]. This agrees with our results where pre-treatment of primary macrophages with vitamin D_3 decreased *A. salmonicida* infection, meanwhile, the vitamin D_2 did not reduce the bacterial infection, in contrast, increased the infection. Our findings agree with the beneficial utilization of vitamin D in aquafeeds [Barnett et al. 1979; Barnett et al. 1982; Lock et al. 2010] and seems to have a broad positive effect in all vertebrates, including fish.

To complement our results showed above, I explored the immune mechanism behind the beneficial effects of vitamin D by profiling the expression of specific innate immune genes using qPCR. An up-regulation of *il-1b*, *il-8*, *tnf-α*, and s*tlr5* occurred in primary macrophages inoculated with either the live or formalin-killed *A. salmonicida* (Fig. 3-4A, 3-4B, 3-4C, 3-4E, 3-4F, 3-4G, 3-4H, and 3-4J). These results were expected, since cytokines, chemokines, and anti-bacterial and inflammatory proteins, such as *il-1b*, *il-8*, *tnf-α*, and s*tlr5*, play essential roles controlling both acute and chronic inflammation in fish tissues mediated by macrophages [Smith et al. 2018]. In Atlantic salmon, the evidence shows that this canonical macrophage innate immune response can be triggered rapidly by either a bacterial pathogen-associated molecular pattern (PAMP), such as lipopolysaccharide (LPS), or pathogens like *Yersinia ruckeri*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Flavobacterium psychrophilum*, among others [Martin et al. 2006; Bridle et al. 2011; Santana et al. 2018; Smith et al. 2018; Hoare et al. 2019].

Some viruses, bacteria, and parasites can modify the expression of genes related with the host immune response as part of a mechanism of evading its defense mechanisms [Finlay and McFadden 2006]. In humans, agents of three important infectious diseases,

such as HIV, tuberculosis, and malaria have developed highly effective mechanisms to subvert the immune response [Finlay and McFadden 2006], making it difficult to control the diseases and develop effective vaccines. Further examples of this are *Yersinia pseudotuberculosis* and *Y. enterocolitica* which are able to control human macrophage immune response and induce apoptosis after the translocation of effector molecules through the type III secretion system [Monack et al. 1998; Schesser et al. 1998; Gao and Kwaik 2000].

In fish head kidney, a modulation of the expression of *il-1b* and the *major histocompatibility complex class 1* (*mhc-I*) has been observed in Atlantic salmon after being infested by the sea louse *Lepeophtheirus salmonis* [Fast et al. 2006]. Moreover, Lewis et al. 2014 [Lewis et al. 2014] show that *L. salmonis* produces substances than modify the expression of genes encoding inflammatory mediators in the Atlantic salmon head kidney (SHK-1) cell line. Here, our results obtained during the infection with live *A. salmonicida* in Atlantic salmon primary macrophages showed no significant differences in the expression of *leukocyte-derived chemotaxin 2* (*lect-2)* compared with the control (Fig 3-4D, I). When the fish cells were pre-treated with vitamin D_2 and then infected with the live bacteria, the gene expression also did not increase, suggesting that *lect-2* is not involved in the first line of defense against *A. salmonicida* in Atlantic salmon. However, the expression of *lect-2* in Atlantic salmon macrophages pre-treated with vitamin D_3 and challenged with live *A. salmonicida* was significantly up-regulated compared to the control. *lect-2* is a chemotactic factor involved in the recruitment of neutrophils to the site of infection [Yamagoe et al. 1996; Smith et al. 2018]. In the study conducted by Smith et al. 2018, an up-regulation of *lect-2* was observed only in treatments with LPS, confirming that its role

in presence of external pathogenic agents is active in Atlantic salmon. Comparing the expression of *lect-2* in primary macrophages treated with the live *A. salmonicida* and the samples pre-treated with vitamin D_3 and then inoculated with *A. salmonicida*, our result suggest that *A. salmonicida* avoid the transcriptional expression of *lect-2*, perhaps to prevent neutrophil recruitment during infection. Our results suggest that pre-treatments with vitamin D_3 can counteract the effect of *A. salmonicida* in this particular gene, and up-regulate the expression of *lect-2* during *A. salmonicida* infection (Fig. 3-4I).

To determine if vitamin D_2 and D_3 can also exert an effect in the phagocytosis of the Atlantic salmon primary macrophages, the phagocytic activity was tested using Fluorescent latex beads (Fig. 3-5). Phagocytosis is used by organisms to eliminate external agents such as bacteria in a highly efficient way [Rabinovitch 1995; Neuman et al. 2001; Øverland et al. 2010; Soto-Dávila et al. 2019]. The effect of vitamins over macrophage phagocytic activity has been previously tested in Atlantic salmon, however, no significant variations were obtained after treatments with vitamin C or vitamin E [Hardie et al. 1990; Hardie et al. 1991]. I found similar results in Atlantic salmon primary macrophages after 24 h pre-treatments with either vitamin D_2 or D_3 suggesting that, independent of the vitamin used, the phagocytic activity of Atlantic salmon is not modulated by its action.

3.6 Conclusion

In this study I evaluated the effects of vitamin D_2 and D_3 over *A. salmonicida* growth, Atlantic salmon primary macrophage viability, and the fish cells' immune response. I determined that only high concentrations of vitamin D_2 (100,000 ng/ml) and vitamin D_3 (1,000 and 10,000 ng/ml) decreased the growth rate of *A. salmonicida*.

Moreover, I determined that 100,000 ng/ml of vitamin D_2 and 10,000 ng/ml decreased the viability of Atlantic salmon primary macrophages after 24 and 48 h. These results suggest that high doses of D_2 and D_3 are toxic for the bacterial and the eukaryotic cells.

Pre-treatment with 100 ng/ml of either vitamin D_2 or D_3 did not have altered primary macrophages viability. Nevertheless, one of the remarkable findings of our study was that pre-treatment with vitamin D_3 reduced *A. salmonicida* attachment, meanwhile, pre-treatment with vitamin D_2 increased attachment, and as a consequence also increased bacterial invasion.

Gene expression of *il-1b*, *il-8*, *tnf-α*, and s*tlr5* was up-regulated during *A. salmonicida* infection, agreeing with a canonical non-specific immune response. However, our results showed that *A. salmonicida* was able to suppress the expression of *lect-2*, a gene involved in neutrophil recruitment, key in the fight against pathogen clearance. After the addition of vitamin D_2, no variation in the transcriptional expression of this gene was observed. However, cells pre-treated with vitamin D_3 and then inoculated with live *A. salmonicida*, showed an up-regulation of *lect-2*, suggesting that vitamin D_3 can be useful to counteract the suppression triggered by the pathogen.

Altogether, our results show that vitamin D_3 seems to be a good candidate to be used as an immunostimulant in Atlantic salmon against *A. salmonicida* infection. In contrast, vitamin D_2 did not show to have an effect in the modulation of the immune system of Atlantic salmon, suggesting that vitamin D_2 does not play an important role in fish non-specific immunity.

3.7 References

Aarestrup, F.M., Scott, N.L., and Sordillo, L.M. (1994). Ability of *Staphylococcus aureus* coagulase genotypes to resist neutrophil bactericidal activity and phagocytosis. Infect. Immun. 62, 5679-5682.

Alva-Murillo, N., Téllez-Pérez, A., Medina-Estrada, I., Álvarez-Aguilar, C., Ochoa-Zarzosa, A., and López-Meza, J. (2014). Modulation of the inflammatory response of bovine mammary epithelial cells by cholecalciferol (vitamin D) during *Staphylococcus aureus* internalization. Microb. Pathog. 77, 24-30.

Asche, F., Roll, K., Sandvold, H. Sørvig, A., and Zhang, D. (2013). Salmon aquaculture: Larger companies and increased production. Aquacult. Econ. Manage. 17, 322-339.

Barnett, B.J., Cho, C.Y., and Slinger, S.J. (1979). The essentiality of cholecalciferol in the diets of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 63, 291-297.

Barnett, B.J., Cho, C.Y., and Slinger, S.J. (1982). Relative biopotency of dietary ergocalciferol and cholecalciferol and the role of and requirement for vitamin D in rainbow trout (*Salmo gairdneri*). J. Nutr. 112, 2011-2019.

Bayne, C., and Gerwick, L. (2001). The acute phase response and innate immunity of fish. Dev. Comp. Immunol. 25, 725-743.

Borges, M., Martini, L., and Rogero, M. (2011). Current perspectives on vitamin D, immune system, and chronic diseases. Nutrition. 27, 399-404.

Bridle, A., Carter, C., Morrison, R., and Nowak, B. (2005). The effect of β-glucan administration on macrophage respiratory burst activity and Atlantic salmon, *Salmo*

salar L., challenged with amoebic gill disease – evidence of inherent resistance. J. Fish Dis. 28, 347-356.

Bridle, A., Nosworthy, E., Polinski, M., and Nowak, B. (2011). Evidence of an antimicrobial-immunomodulatory role of Atlantic salmon cathelicidins during infection with *Yersinia ruckeri*. PLoS ONE 6(8): e23417.

Caballero-Solares, A., Hall, J.R., Xue, X., Eslamloo, K., Taylor, R.G., Parrish, C.C., and Rise, M.L. (2017). The dietary replacement of marine ingredients by terrestrial animal and plant alternatives modulates the antiviral immune response of Atlantic salmon (*Salmo salar*). Fish Shellfish Immunol. 64, 24-38.

Chagas, C., Borges, M.C., Martini, L., and Rogero, M. (2012). Focus on Vitamin D, Inflammation and Type 2 Diabetes. Nutrients. 4, 52-67.

Cipriano, R.C., and Bullock, G. (2001). Furunculosis and other diseases caused by *Aeromonas salmonicida*. Fish disease leaflet 66. USGS/Leetown Science Center Fish Health Branch, Kearneysville, WV.

Connors, E., Soto-Dávila, M., Hossain, A., Vasquez, I., Gnanagobal, H., and Santander, J. (2019). Identification and validation of reliable *Aeromonas salmonicida* subspecies *salmonicida* reference genes for differential gene expression analyses. Infect. Genet. Evol. 73, 314-321.

Cook, M.T., Hayball, P.J., Hutchinson, W., Nowak, B.F., and Hayball, J.D. (2003). Administration of a commercial immunostimulant preparation, EcoActiva™ as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter. Fish Shellfish Immun. 14, 333-345.

Cvitanich, J.D., Gárate, O., and Smith, C.E. (1991). The isolation of a Rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. J. Fish Dis. 14, 121-145.

Darias, M., Mazurais, D., Koumoundouros, G., Cahu, C., and Zambonino-Infante, J. (2011). Overview of vitamin D and C requirements in fish and their influence on the skeletal system. Aquaculture. 315, 49-60.

Dawood, M.A., Koshio, S., and Esteban, M.A. (2017). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. Rev. Aquacult. 10, 950-974.

Esteban, M.A., Cuesta, A., Chaves-Pozo, E., and Meseguer, J. (2015). Phagocytosis in Teleosts. Implications of the New Cells Involved. Biology 4, 907-22.

FAO (2018). The State of World Fisheries and Aquaculture (SOFIA) - Meeting the Sustainable Development Goals, Food and Agriculture Organization, Rome, Italy.

Fast, M.D., Muise, D.M., Easy, R.E., Ross, N.W., and Johnson, S.C. (2006). The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*). Fish Shellfish Immunol. 21, 228-241.

Finlay, B., and McFadden, G. (2006). Anti-Immunology: Evasion of the Host Immune System by Bacterial and Viral Pathogens. Cell. 124, 767-782.

Fryer J.L., and Sanders, J.E. (1981). Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiol. 35, 273-298.

Gao, L.Y., and Kwaik, Y.A. (2000). The modulation of host cell apoptosis by intracellular bacterial pathogens. Trends Microbiol. 8, 306-313.

Grant, W. (2006). Epidemiology of disease risks in relation to vitamin D insufficiency. Prog. Biophys. Mol. Bio. 92, 65-79.

Gutiérrez-Barroso, A., Anaya-Lopez, J.L., Lara-Zárate, L., Loeza-Lara, P.D., López-Meza, J.E., and Ochoa-Zarzosa, A. (2008). Prolactin stimulates the internalization of *Staphylococcus aureus* and modulates the expression of inflammatory response genes in bovine mammary epithelial cells. Vet. Immunol. Immunopathol. 121, 113-122.

Hardie, L.J., Fletcher, T.C., and Secombes, C.J. (1990). The Effect of Vitamin E on the Immune Response of the Atlantic Salmon (*Salmo salar* L.). Aquaculture. 87, 1-13.

Hardie, L.J., Fletcher, T.C., and Secombes, C.J. (1991). The effect of dietary vitamin C on the immune response of the Atlantic salmon (*Salmo salar* L.). Aquaculture. 95, 201-214.

Higuera, G., Bastías, R., Tsertsvadze, G., Romero, J., and Espejo, R., (2013). Recently discovered *Vibrio anguillarum* phages can protect against experimentally induced vibriosis in Atlantic salmon, *Salmo salar*. Aquaculture. 392-395, 128-133.

Hoare, R., Jung, S., Ngo, T.P.H., Bartie, K., Bailey, J., Thompson, K.D., and Adams, A. (2019). Efficacy and safety of a non-mineral oil adjuvanted injectable vaccine for the protection of Atlantic salmon (*Salmo salar* L.) against *Flavobacterium psychrophilum*. Fish Shellfish Immun. 85, 44-51.

Johansen, K.A., and Overturf, K. (2006). Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 144B, 119-127.

Kaatari, S., and Tripp, R. (1987). Cellular mechanisms of glucocorticoid immunosuppression in salmon. J. Fish Biol. 31, 129-132.

Kallio, J., Jaakkola, M., Mäki, M., Kilpeläinen, P., and Virtanen, V. (2012). Vitamin C inhibits *Staphylococcus aureus* growth and enhances the inhibitory effect of quercetin on growth of *Escherichia coli in vitro*. Planta Med. 78, 1824-1830.

Lewis, D.L., Barker, D.E., and McKinley, R.S. (2014). Modulation of cellular innate immunity by *Lepeophtheirus salmonis* secretory products. Fish Shellfish Immun. 38, 175-183.

Lips, P. (2006). Vitamin D physiology. Prog. Biophys. Mol. Bio. 92, 4-8.

Liu, Y., Rosten, T., Henriksen, K., Hognes, E., Summerfelt, S., and Vinci, B. (2016). Comparative economic performance and carbon footprint of two farming models for producing Atlantic salmon (*Salmo salar*): Land-based closed containment system in freshwater and open net pen in seawater. Aquacult. Eng. 71, 1-12.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25, 402-408.

Lock, E.J., Waagbø, R., Wendelaar-Bonga, S., and Flick, G. (2010). The significance of vitamin D for fish: a review. Aquac Nutr. 16, 100-116.

Magnadóttir, B. (2014). The immune response of Atlantic cod, *Gadus morhua* L. Icel. Agric. Sci. 27, 41-61.

Maisey, K., Montero, R., and Christodoulides, M. (2016). Vaccines for Piscirickettsiosis (salmonid rickettsial septicaemia, SRS): The Chile perspective. Expert. Rev. Vaccines. 16, 215-228.

Martin, S., Blaney, S., Houlihan, D., and Secombes, C.J. (2006). Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*). Mol. Immunol. 43, 1900-1911.

Miller, J., and Gallo, R. (2010). Vitamin D and innate immunity. Dermatol. Ther. 23, 13-22.

Monack, D., Mecsas, J., Bouley, D., and Falkow, S. (1998). Yersinia-induced apoptosis *in vivo* aids in the establishment of a systemic infection of mice. J. Exp. Med. 216, 2127-2137.

Mora, J.R., Iwata, M., and von Andrian, U. (2008). Vitamin effects on the immune system: vitamins A and D take centre stage. Nat. Rev. Immunol. 8, 685-698.

Murray, A.L., Pascho, R.J., Alcorn, S.W., Fairgrieve, W.T., Shearer, K.D., and Roley, D. (2003). Effects of various feed supplements containing fish protein hydrolysate or fish processing by-products on the innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*). Aquaculture. 220, 643-653.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., and Belosevic, M. (2001). Antimicrobial mechanisms of fish phagocytes and their role in host defence. Dev. Comp. Immunol. 25, 807-825.

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. J. Fish. Dis. 35, 83-108.

Ostermeyer, U., and Schmidt, T. (2006). Vitamin D and provitamin D in fish. Eur. Food Res. Technol. 222, 403-413.

Øverland, H., Pettersen, E.F., Rønneseth, A., and Wergeland, H.I. (2010). Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 28, 193-204.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45.

Prentice, A., Goldberg, G., and Schoenmakers, I. (2008). Vitamin D across the lifecycle: physiology and biomarkers. Am. J. Clin. Nutr. 88, 500S-506S.

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 5, 85-87.

Rao, D.S., and Raghuramulu, N. (1996). Food Chain as Origin of Vitamin D in Fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 114, 15-19.

Robertson, L., Thomas, P., Arnold, C., and Trant, J. (1987). Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak. Prog. Fish-Cult. 49, 1-12.

Sadarangani, S., Whitaker, J., and Poland, G. (2015). "Let There Be Light": The role of Vitamin D in the immune response to vaccines. Expert Rev. Vaccines. 14, 1427-1440.

Sakai, M. (1999). Current research status of fish immunostimulants. Aquaculture. 172, 63-92.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuel, S., and Sitrin, M.D. (2008). Vitamin D's role in cell proliferation and differentiation. Nutr. Rev. 66, 116-124.

Santana, A., Salinas, N., Álvarez, C., Mercado, L., Guzmán, F. (2018). Alpha-helical domain from IL-8 of salmonids: Mechanism of action and identification of a novel antimicrobial function. Biochem. Biophys. Res. Commun. 498, 803-809.

Santander, J., Kilbourne, J., Park, J.Y., Martin, T., Loh, A., Diaz, I., Rojas, R., Segovia, C., DeNardo, D., and Curtiss 3rd, R. (2014). Inflammatory effects of *Edwardsiella*

ictaluri lipopolysaccharide modifications in catfish gut. Infect. Immun. 82, 3394-3404.

Schesser, K., Splik, A., Dukuzumuremyi, J., Neurath, M., Petterson, S., and Wolf-Watz, H. (1998). The *yopJ* locus is required for Yersinia-mediated inhibition of NF-kB activation and cytokine expression: *YopJ* contains a eukaryotic SH2- like domain that is essential for its repressive activity. Mol. Microbiol. 28, 1067-1079.

Secombes, C.J., and Fletcher, T.C. (1992). The role of phagocytes in the protective mechanisms of fish. Annu. Rev. Fish Dis. 2, 53-71.

Siwicki, A.K, Anderson, D.P., and Rumsey, G.L. (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet. Immunol. Immunopathol. 41, 125-139.

Smith, N.C., Christian, S.L., Taylor, R.G., Santander, J., and Rise, M.L. (2018). Immune modulatory properties of 6-gingerol and resveratrol in Atlantic salmon macrophages. Mol. Immunol. 95, 10-19.

Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., and Ringø, E. (2014). Prebiotics as immunostimulants in aquaculture: A review. Fish Shellfish Immunol. 40, 40-48.

Soto-Dávila, M., Hossain, A., Chakraborty, S., Rise, M.L., and Santander, J. (2019). *Aeromonas salmonicida* subsp. *salmonicida* early infection and immune response of Atlantic cod (*Gadus morhua* L.) primary macrophages. Front. Immunol. 10:1237. doi: 10.3389/fimmu.2019.01237

Sung, K., Khan, S.A., Nawaz, M.S., and Khan A.A. (2003). A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. FEMS Microbiol. Lett. 229, 97-101.

Szodoray, P., Nakken, B., Gaal, J., Jonsson, R., Szegedi, A., Zold, E., Szegedi, G., Brun, J.G., Gesztelyi, R., Zeher, M., and Bodolay, E. (2008). The Complex Role of Vitamin D in Autoimmune Diseases. Scand. J. Immunol. 68, 261-269.

Téllez-Pérez, A., Alva-Murillo, N., Ochoa-Zarzosa, A., and López-Meza, J. (2012). Cholecalciferol (vitamin D) differentially regulates antimicrobial peptide expression in bovine mammary epithelial cells: Implications during *Staphylococcus aureus* internalization. Vet. Microbiol. 160, 91-98.

Toranzo, A.E., Magariños, B., and Romalde, J.L. (2005). A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

Torrecillas, S., Makol, A., Benítez-Santana, T., Caballero, M.J., Montero, D., Sweetman, J., and Izquierdo, M. (2011). Reduced gut bacterial translocation in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). Fish Shellfish Immunol. 30, 674-681.

Tort, L., Balasch, J.C., and Mackenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. Inmunología 22, 277-286.

Trang, H.M., Cole, D.E., Rubin, L.A., Pierratos, A., Siu, S., and Vieth, R. (1998). Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. Am. J. Clin. Nutr. 68, 854-858.

Valderrama, K., Saravia, M., and Santander, J. (2017). Phenotype of *Aeromonas salmonicida* sp. *salmonicida* cyclic adenosine 3',5'-monophosphate receptor protein

(Crp) mutants and its virulence in rainbow trout (*Oncorhynchus mykiss*). J. Fish. Dis. 40, 1849-1856.

Vogt, L., Ramasamy, U., Meyer, D., Pullens, G., Venema, K., Faas, M.M., Schols, H.A., and de Vos, P. (2013). Immune Modulation by Different Types of β2→1-Fructans Is Toll-Like Receptor Dependent. PLoS ONE 8 (7): e68367. https://doi.org/10.1371/journal.pone.0068367

Wang, L., Xu, H., Wang, Y., Wang, C., Li, J., Zhao, Z., Luo, L., Du, X., and Xu, Q. (2017). Effects of the supplementation of vitamin D_3 on the growth and vitamin D metabolites in juvenile Siberian sturgeon (*Acipenser baerii*). Fish Physiol. Biochem. 43, 901-909.

Whyte, S.K. (2007). The innate immune response of finfish - a review of current knowledge. Fish Shellfish Immunol. 23, 1127-1151.

Woodward, B. (1994). Dietary vitamin requirements of cultured young fish, with emphasis on quantitative estimates for salmonids. Aquaculture 124, 133-168.

Yamagoe, S., Yamakawa, Y., Matsuo, Y., Minowada, J., Mizuno, S., and Suzuki, K. (1996). Purification and primary amino acid sequence of a novel neutrophil chemotactic factor LECT2. Immunol. Lett. 51, 9-13.

Yue, Y., Hymøller, L., Jensen, S., Lauridsen, C., and Purup, S. (2017). Effects of vitamin D and its metabolites on cell viability and *Staphylococcus aureus* invasion into bovine mammary epithelial cells. Vet. Microbiol. 203, 245-251.

Zhang, H., Wakisaka, N., Maeda, O., and Yamamoto, T. (1997). Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*. Cancer. 80, 1897-1903.

Zittermann, A. (2003). Vitamin D in preventive medicine: are we ignoring the evidence?. Br. J. Nutr. 89, 552-572.

4. SUMMARY

In this thesis, the main objective was to evaluate the impact of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis, on the innate immune response of Atlantic cod and Atlantic salmon primary macrophages. Additionally, this study determined the role of vitamin D_2 and D_3 as immunostimulants in the aquaculture of Atlantic salmon.

In chapter II, the findings provided for first time, to our knowledge, the mechanisms utilized by *A. salmonicida* subsp. *salmonicida* during the infection of Atlantic cod utilizing *in vitro* experiments. I observed that this Gram-negative bacterium is able to suppress the gene expression of transcripts related with the canonical immune response in fish such as AMPs and the production of ROS. Additionally, it was found that *A. salmonicida*, similar to other pathogens (i.e., *Neisseria meningitides*, *Escherichia coli*, *Vibrio* spp., *Brucella* spp.), is able to secrete OMVs, capable of neutralizing the production of AMPs to avoid lysis. Moreover, ROS results obtained in this thesis, correlate with previous findings showing that Atlantic cod has a basal ROS production in non-infected and infected cells. Altogether, these results suggest that Atlantic cod primary macrophages are able to recognize and trigger the immune response against *A. salmonicida*, nonetheless, the mechanisms utilized by the bacterial pathogen have the ability to avoid the host defense, prevent clearance, and invade the primary macrophages during the early infection.

Chapter III of this thesis showed that *A. salmonicida* utilized a different strategy to develop an infection. Results obtained represent a novel evidence that the main mechanism used by *A. salmonicida* during the infection of Atlantic salmon primary macrophages is to

immunosuppress the expression of *lect-2*, an important gene related with the recruitment of neutrophils. In addition, here I evaluate the immunostimulant properties of Vitamin D_2 and D_3 during pre-treatments of 24 h in Atlantic salmon primary macrophages. Vitamin D has been shown to play an important role in mineral metabolism, cell growth, and tissue differentiation. Also, it has been observed in mammals that vitamin D can enhance the antibacterial immune response. Nevertheless, even when vitamin D is an essential nutrient in aquafeeds, its influence in fish immune response is not understood. The results of this study show for first time, that 24 h pre-treatments with a biological concentration of vitamin D_3 (100 ng/ml), can decrease the infection with *A. salmonicida*. Interestingly, I found that 24 h pre-treatment with vitamin D_3 can also counteract the immunosuppression of *lect-2* produced by *A. salmonicida*. In contrast, results obtained show that vitamin D_2, obtained by plant sources, does not produced a similar effect in infected Atlantic salmon primary macrophages, suggesting that should not be utilized as an immunostimulant in Atlantic salmon aquaculture.

In conclusion, I have provided novel information about the mechanisms of *A. salmonicida* during the early infection of Atlantic cod and Atlantic salmon primary macrophages, as well as, the role of vitamin D_3 as an immunostimulant during the infection of Atlantic salmon primary macrophages with *A. salmonicida*. Since vitamin D_3 can be obtained in the market at a low-price, from an industrial and an economical point of view, the results obtained in chapter III provides an environmentally friendly and accessible alternative to the antibiotics in the aquaculture industry. Also, we develop a model to understand bacterial infection mechanisms in fish macrophages.

5. APPENDICES

Appendix I. Ct values Atlantic cod reference gene evaluation after live or formalin-killed *A. salmonicida* inoculation.

Treatment	Fish	*EF-1α*	*B-actin*	*18S*	*Eif3*	*60S*
Control	**1**	19.905	22.737	10.707	28.288	22.231
	2	19.634	22.177	10.443	28.450	20.122
	3	19.923	20.152	12.632	29.816	23.232
1 h post infection live *A. salmonicida*	**1**	19.749	23.261	10.932	28.177	22.509
	2	19.741	22.781	11.556	29.256	22.349
	3	19.614	20.569	13.036	30.889	21.359
2 h post infection live *A. salmonicida*	**1**	19.931	20.379	13.739	31.145	20.234
	2	19.750	20.454	11.559	27.890	22.163
	3	19.626	22.328	10.408	28.798	23.054
6 h post infection live *A. salmonicida*	**1**	19.640	23.561	11.511	27.839	21.445
	2	19.591	21.368	12.849	29.609	19.352
	3	19.677	19.400	14.910	30.732	19.543
1 h post inoculation formalin killed *A. salmonicida*	**1**	19.996	22.001	10.098	31.350	19.123
	2	19.691	21.833	12.936	32.590	21.961
	3	19.827	21.987	9.911		18.932
	1	19.675	19.843	9.886	32.896	18.953

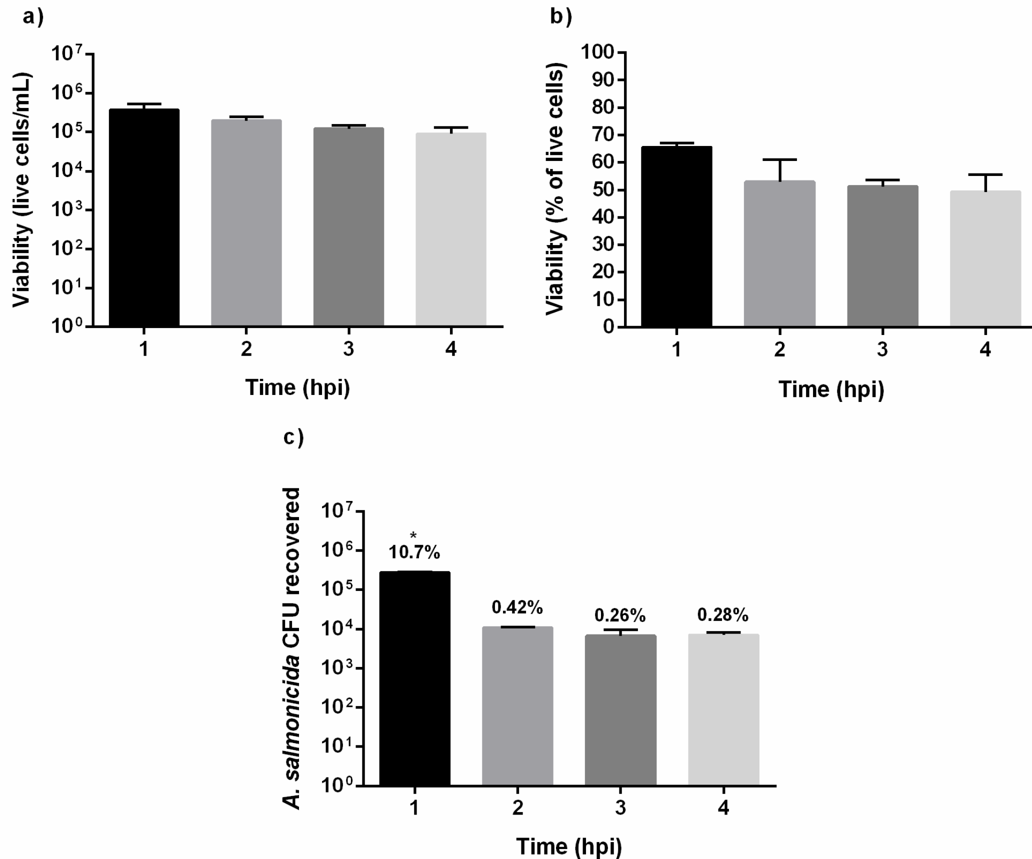
2 h post inoculation formalin killed *A. salmonicida*	**2**	19.549	20.802	10.794	30.432	20.150
	3	19.768	21.018	8.894	31.192	18.034
6 h post inoculation formalin killed *A. salmonicida*	**1**	19.655	24.400	8.497	29.268	17.589
	2	19.751	22.957	12.092	33.135	21.163
	3	19.648	23.715	10.145	30.989	21.267

*Each value represents the mean of technical replicates (n=3).

**geNorm M values were: 0.102 (*EF-1α*), 0.112 (*Eif3*), 0.138 (*60S*), 0.147 (*β-actin*), 0.190 (*18S*); M < 0.15 for most stable genes.

***BestKeeper values were: 0.101 (*EF-1α*), 1 (*Efi3*), 1.124 (*60S*), 1.175 (*18S*), 1.21 (*β-actin*); M < 1 for most stable genes.

Appendix II.



Gentamicin exclusion assay in Atlantic salmon macrophages infected with *Aeromonas salmonicida* subsp. *salmonicida*. The figures show the number of live cells (a) and the percentage of viability (b), after 1, 2, 3 and 4 h post-infection. The figure also shows the colony forming unit (c) recovered from cells during each time post-infection. Each value is the mean ± S.E.M (n=3). Symbol (*) indicate statistical differences between each time post infection. Percentage show above bars indicate the total % of attach (1 h post infection) and invasion (2, 3 and 4 h post invasion) of *A. salmonicida* to Atlantic salmon macrophages, $P < 0.005$.