Non-specific immune response and disease-resistance in Chinook salmon (*Oncorhynchus tshawytscha*) supplemented with probiotics

by

Manuel Alejandro Soto Davila

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Examining Committee Membership

The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

External Examiner	SAMUEL MARTIN Professor, Personal Chair, Biological Sciences, Aberdeen Centre For Environmental Sustainability Marine Alliance for Science and Technology for Scotland (MASTS) Biological Sciences, Scottish Fish Immunology Research Centre, University of Aberdeen.
Supervisor	BRIAN DIXON Professor, Canada Research Chair in Fish and Environmental Immunology, Department of Biology, University of Waterloo
Internal Members	PAUL CRAIG Associate Professor Department of Biology, University of Waterloo
	DANIEL D. HEATH Professor, Great Lakes Institute for Environmental Research, Department of Biology, University of Windsor
Internal-external Member	MAUD GORBET Associate Professor, Systems Design Engineering University of Waterloo

Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

In the case of published chapters, the numbering system of any published chapters has been updated to fit this thesis.

Chapter 1 – The sections 1.6 and 1.7 of this chapter, as well as the figure 1.1. and table 1.1. are part of "Chapter 4 - Enhancing immune function and fish health in aquaculture" published online in 2020 in Benfey, T.J., Farrell, A.P., Brauner, C.J.B.T.-F.P. (Eds.), *Aquaculture.* Academic Press, pp. 123–161. https://doi.org/https://doi.org/10.1016/bs.fp.2020.09.003.

Chapter 2 – T. Rodriguez-Ramos and X. Dang helped completing the subtractive indirect ELISA assay to determine total serum IgM and IgT levels in Chinook salmon unsupplemented or supplemented with Jamieson® probiotic. Rory A. Webb helped carry out experiments related to the gene expression of head kidney tissues as part of his 499 performed at Dr. Brian Dixon Lab. All other experiments, analyses and the final thesis manuscript preparation was completed by M. Soto-Davila. This chapter has been submitted to Aquaculture.

Chapter 3 – *In vitro* results showed in this chapter are a subset of data utilized by Luana Langlois Fiorotto as part of her M.Sc. This data has been utilized for publication purposes, and results section, discussion, and conclusion has been modified from the original source by M. Soto Davila and Luana Langlois Fiorotto. All other experiments, analyses and the final thesis manuscript preparation was completed by M. Soto-Davila. This chapter has been submitted to Frontiers in Immunology, nutritional immunology section.

Chapter 4 – The hindgut gene expression results showed in this chapter are a subset of data utilized by Luana Langlois Fiorotto as part of her M.Sc. thesis. This data has been utilized for publication purposes and results section, discussion, and conclusion has been modified from the original source. All other *in vitro* and *in vivo* experiments, analyses and the final thesis manuscript preparation was completed by M. Soto-Davila. This chapter has been formatted for submission to Journal of Aquaculture Nutrition.

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Abstract

Finfish aquaculture is the fastest-growing food-production sector in the world. Nevertheless, intensive production faces increased disease outbreaks due to high stock densities and environmental effects. Currently, infectious diseases represent the main causes of losses in salmon aquaculture. To prevent this, antibiotics and vaccines have been frequently utilized as a treatment and control methods for infectious agents. However, restrictions on antibiotic use due to antibiotic-resistant strain outbreaks, as well as, the variable protection exerted by vaccines, has made the industry to explore additional treatments. Probiotics are an important non-toxic and non-polluting tool in aquaculture to improve fish growth, stress tolerance and non-specific defense. In finfish aquaculture, many probiotics have been tested, including lactic acid bacteria (LAB; *Lactobacillus* and *Carnobacterium*), *Bacillus*, and *Pediococcus* spp. The role of probiotics within the digestive tract in the two most important salmonids, Atlantic salmon and rainbow trout, has been widely studied, however the effect of probiotic supplementation on the growth, survival, immune response, and gut integrity of Chinook salmon (*Oncorhynchus tshawytscha*) raised under aquaculture conditions has not been widely studied.

Chinook salmon is a native species of the Canadian Pacific coast with potential economic and environmental benefits for aquaculture. Additionally, Chinook salmon farming can play an important role in diversifying current Canadian aquaculture and help protecting the wild stocks. Some issues that keep to date Chinook salmon farming at a low scale are the risk of escapees diluting the genetic diversity of wild populations and the decrease of the flesh quality when they reach sexual maturation. Sterile triploid salmon offer a solution however, they have an a 10-30%

higher disease mortality rate compared to diploid fish. This makes Chinook salmon an ideal candidate to utilize probiotics as an alternative to antibiotics and vaccines.

The contents of this thesis examine the effect of single-strain and multi-strain probiotics either commercially available or generously donated by companies. Also, this thesis focuses in evaluating probiotic supplementation at different production cycles (freshwater and saltwater), fish size (juveniles and adults), and/or supplementation time (4, 10, 14 months). Since *in vivo* experiments were conducted once a year, preliminary studies to determine the safety of the strains were carried out in the lab using the salmonid intestinal epithelial cell line derived from rainbow trout (*Oncorhynchus mykiss*), RTgutGC.

Overall, the results obtained in this thesis demonstrated that probiotic strains utilized in different experiments did not show negative effects in terms of cell viability and/or Chinook salmon tissues (histopathological analysis). Data collected during the *in vitro* trials demonstrated that probiotic stimulation was able to modulate the expression of TJ and immune genes without impacting the cell viability. For the *in vivo* trial, the most interesting results associated to fish survival was observed after multi-strain Jamieson® probiotic supplementation throughout the freshwater and saltwater production cycle (14 months of supplementation), showing a decrease of ~10% in the mortality of Chinook salmon challenged with *V. anguillarum* compared to fish fed regular pellet.

In all the trials conducted for this thesis, it was not possible to obtain an improvement in fish growth parameters (weight and length), as has been observed in previous probiotic supplementation studies. Nevertheless, research conducted for this thesis represents one of few studies that has been able to evaluate probiotic supplementation over a full year of saltwater production in real aquaculture conditions.

The gene expression results suggest that probiotic supplementation can differentially modulate the early inflammatory response and gut barrier function after infection with *V. anguillarum*, however more studies are still needed to fully understand the correlation between gene expression and the survival and growth data collected in this study.

The resulting data collected from the ELISAs of IgM and IgT provided novel functional information regarding protein levels in different tissues of Chinook salmon. It is important to mention that this study represents the first time that IgT of Chinook salmon has been observed in western blots and the protein levels measured in ELISA, therefore, this thesis would be the primary reference for any future studies evaluating this immunoglobulin in the future.

Overall, these studies demonstrate the importance of investigating the mechanism underlying host-microbe interactions to help improve Chinook salmon aquaculture in an environmentally sustainable manner.

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First, I would like to thank my supervisor, Dr. Brian Dixon, for always supporting me and my ideas and giving me the freedom to develop my career the way it went during my time in your lab. I came to Waterloo because of your expertise in the field of fish immunology, and I leave with that and also with a perspective of the future researcher I would love to become. Your kindness, the time put into your students, your understanding during the difficult time (who would think that a pandemic would happen during the PhD?), the conversations about research with a coffee, and more, are the real treasure that students can get from the experience of working with you. Leaving your lab it is a true sad time for me, however, I know that I would keep getting your advice and your support through my future steps. Also, I would like to thank Dr. Paul Craig and Dr. Daniel Heath, committee members of my PhD thesis, who provided valuables advice for the research conducted during my graduate studies, I really appreciate every word and support to make my thesis better.

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During my PhD, I was fortunate to collaborate with doctors John and Ann Heath at Yellow Island Aquaculture Ltd. These four years in which you welcomed me with great affection in your home and your aquaculture business have been some of the most enriching for my career. As I mentioned the first time we meet, YIAL represented my first time in a real sea pen aquaculture,

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I am glad that I have been able to collaborate with a number of amazing graduate students and scientists throughout my research and degree. Dr. John Lumsden (U. of Guelph), Dr. Gregor Reid (U. of Western Ontario), Dr. Christina Semeniuk (U. of Windsor), Cheryl Duxbury, Ivan Cadonic, Luana Langlois Fiorotto.

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Dedication

I dedicate this thesis to my uncle, Jose Reinaldo Bravo Salas, who passed away a few days before submitting this writing.

Thank you for showing me that true love is found in the simplest gestures. You have always been and will continue to be an important part of my dedication to the study of fish.

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List of Abbreviations

18S	18S ribosomal RNA
ACH_{50}	alternative complement pathway
ACTB	beta actin
AMP	antimicrobial peptide
ANOVA	analysis of variance
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3'-indolylphosphate p-toluidine
CAMP	cathelicidin antimicrobial peptide
CAT	catalase
CCAC	Canadian council on animal care
CDH1	e-cadherin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CLDN1	claudin 1
CLDN3	claudin 3
CLDN12	claudin 12
CLDN15	claudin 15
CLDN28B	claudin 28b
CX6C1	cytochrome c oxidase subunit 6C-1
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DPI	days post injection
EF1A	elongation factor 1 alpha
ELISA	enzyme-linked immunosorbent assay
EU	European union
FAO	Food and Agriculture Organization of the United Nations
FBS	fetal bovine serum
FW	freshwater
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GOS	galacto-oligosaccharides
HAMP	hepcidin antimicrobial peptide
HPI	hours post inoculation
HK	heat-killed
HRP	horseradish peroxidase
IFNY	interferon gamma
Ig	immunoglobulin
IgD	immunoglobulin D
IgM	immunoglobulin M
IgT	immunoglobulin T
IgZ	immunoglobulin Z
IL1B	interleukin 1 beta
IL4	interleukin 4
IL6	interleukin 6
IL8	interleukin 8

IL10	interleukin 10
IL12	interleukin 12
IL17A	interleukin 17 a
I.P.	intraperitoneal
IRG	innate immune response genes
ITS	internal transcribed spacer 2 locus
JAM1A	junctional adhesion molecule 1 alpha
LAB	lactic acid bacteria
LPS	lipopolysaccharide
LSZ	lysozyme activity
MARVELD2	tricellulin
MCA	macrophage activity
MOB	multiplicity of bacteria
MPO	myeloperoxidase activity
MRS	Mann, Rogosa, and Sharpe medium
MUC2	mucin 2
MX1	interferon-induced GTP-binding protein 1
MYD88	myeloid differentiation factor 88
NACL	sodium chloride
NBT	nitroblue tetrazolium activity
NRT	no reverse transcriptase
OCLN	occludin
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PRR	pattern recognition receptor
PSU	practical salinity unit
qPCR	quantitative polymerase chain reaction
RAS	recirculating aquaculture systems
RBA	respiratory burst activity
RNA	ribonucleic acid
RT	room temperature
SAP	serum antibody production
SCA	serum complement activity
scFOS	short-chain fructooligosaccharides
SD	standard deviation
SEM	standard error mean
SGR	specific growth rate
SOD	superoxide dismutase
SW	saltwater
TBS	tris-buffered saline
TBS-T	TBS with Tween 80
TEER	transepithelial electrical resistance
TGC	thermal growth coefficient
TGFB	transforming growth factor beta
TJ	tight junction

TLR3	toll-like receptor 3
TMB	3,3',5,5'-tetramethylbenzidine
TNFA	tumor necrosis factor alpha
TSA	trypticase soy agar
TSB	trypticase soy broth
VIL1	villin 1
WBC	white blood cells
WPS	weeks post supplementation
ZO-1	zonula occludens-1

Chapter 1: General Introduction

1.1 Finfish aquaculture

Finfish aquaculture is the fastest-growing food-production sector in the world (FAO, 2018; Gutiérrez et al., 2020; Lem et al., 2014). Currently, finfish aquaculture supplies over 50% of all fish consumed worldwide, and it is expected to double the production by mid-century due to depletion of wild stocks (FAO, 2022; Fazio, 2019; Froehlich et al., 2018). According to the Food and Agriculture Organization of the United Nations (FAO), in 2017 the three main fish groups cultured were Cyprinids (~25% of total production), Cichlids (~5.25% of total production), and Salmonids (3.11% of total production) (Cai et al., 2019). Since 1980, salmon aquaculture has become one of the most successful finfish aquaculture among all cultured species, and its production has been dominated by Atlantic salmon (*Salmo salar*) with a 68%, followed by rainbow trout (*Oncorhynchus mykiss*) with a 23%, and coho salmon (*Oncorhynchus kisutch*) with a 5.2% (Asche et al., 2013; Cai et al., 2019).

Despite the economic success of salmon aquaculture, the industry has experienced increased disease outbreaks and environmental effects (Cadillo-Benalcazar et al., 2020; Maisey et al., 2017; Rana et al., 2009; Soto-Dávila et al., 2020b). The emergence of diseases has been linked to the intensive culture and precarious environmental conditions that fish are exposed to (Kaattari and Tripp, 1987; Maisey et al., 2017; Robertson et al., 1987). To date, infectious diseases represent the main causes of losses in salmon aquaculture and current prophylactic methods are not sufficient (Robertson et al., 1987; Soto-Dávila et al., 2020c). Due to this, understanding how diseases affect cultured salmon species and the discovery of alternative preventive methods to fight aquatic pathogens is necessary for a safe and sustainable aquaculture industry.

1.2 Canadian finfish aquaculture

The Canadian aquaculture industry has increased in the last decade, especially to net pen farming (Canada, 2011; Milewski and Smith, 2019). The dominant species is Atlantic salmon comprising 63% of the total aquaculture production, representing $1.1 billion annually (Fisheries and Oceans Canada, 2018; Milewski and Smith, 2019). In Canada, production is located in the provinces of British Columbia (West coast), New Brunswick, Newfoundland and Labrador, Nova Scotia, and Prince Edward Island (East coast) (Fisheries and Oceans Canada, 2018). From these provinces, near 70% of the total production has been concentrated in British Columbia (Fisheries and Oceans Canada, 2018), however this percentage is expected to change due to the closure of 30% of the sea pens located in British Columbia in 2022.

Meanwhile Atlantic salmon represents a strategically economical product for British Columbia aquaculture, the fact that it is an introduced species creates a major concern for Canadian Pacific coast species. On the Pacific coast, five native salmonid species can be found: i) Chum salmon (*Oncorhynchus keta*); ii) Chinook salmon (*Oncorhynchus tshawytscha*); iii) Coho salmon (*Oncorhynchus kisutch*); iv) Pink salmon (*Oncorhynchus gorbuscha*); and v) Sockeye salmon (*Oncorhynchus nerka*) (Council, 1996; Fisheries and Ocean Canada, 2004; Ohlberger et al., 2018). To date, alternative aquaculture of Pacific salmon has utilized chum and Chinook salmon, however, the production remains in a small scale (e.g. Chinook salmon production represents 1% of total salmon production worldwide) (Araujo et al., 2021; Christensen et al., 2018; Fisheries and Ocean Canada, 2004; Semeniuk et al., 2019; Semple et al., 2022). Nevertheless, the utilization of native species to diversify salmon aquaculture seems promising. For instance, according to Yellow Island Aquaculture (Quadra Island, BC, Canada), Chinook salmon flesh quality can offer a 50-75¢/lb premium over Atlantic salmon, providing the option for its utilization at a greater scale.

Currently, there is a need for strategic planning and prioritization of sustainable aquaculture in Canada, where the use of native species, such as Chinook salmon, can not only lead into the conservation and recovery of wild stocks but also the diversification from species associated with several negative environmental effects, such as Atlantic salmon.

1.3 Environmental challenges in aquaculture

Increases in aquaculture production have been followed by the emergence of several viral, bacterial, and parasitic diseases associated with unpredictable environmental conditions and high stocking densities (Balcázar et al., 2006; Di Cicco et al., 2017; Kaattari and Tripp, 1987; Morton et al., 2017; Portz et al., 2006). The most important viral infections in aquaculture are produced by member of the genus novirhabdovirus, iridovirus, orthomyxovirus, birnaviruses, and alphavirus (Kibenge, 2019; Rimstad, 2011; Robertsen, 2011). On the other hand, bacterial infection outbreaks in salmonid production are caused by *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, *Piscirickettsia salmonis*, *Renibacterium salmoninarum*, *Yersinia ruckeri*, and *Vibrio anguillarum* (Figure 1.1) (Miller et al., 2014; Yuwono et al., 2021).

Figure 1.1. External lesions associated with *Vibrio anguillarum* infection in Chinook salmon juvenile.

To prevent and control the above-mentioned disease outbreaks, current aquaculture strategies rely on the use of vaccines and antimicrobial agents (Reverter et al., 2014; Rico et al., 2013; Soto-Dávila et al., 2020a). Vaccines are used in cultured fishes as a preventive method against certain bacterial diseases, however, their efficacy is variable when used in different regions, for different species, for specific bacterial strains, or even within the same family of fish due to genetic variation (Figueroa et al., 2020; Rodger, 2016; Tafalla et al., 2013). For viruses, a current cause of major economic losses to the aquaculture industry, no available treatments such as vaccines have been developed for most of the main aquaculture pathogens (Bedekar et al., 2020; Rodger, 2016; Sommerset et al., 2005). On the other hand, antibiotics have been widely utilized against the main bacterial pathogens that affect aquaculture. Nevertheless, restrictions on antibiotic use have become more common in the main fish aquaculture countries due to the fact that antimicrobial agents can generate resistant strains in environment and health concerns of the consumers (Alderman and Hastings, 1998; Defoirdt et al., 2011).

In addition to the supposed transmission of fish pathogens to wild fish stocks and the organic and chemical pollution from fish farms to the environment, fish escapes constitutes another threat to wild populations through disease transmission, habitat alteration and increased predation and competition (Asche et al., 2016; Fischer et al., 2016; Gurevitch and Padilla, 2004; He et al., 2018; Manchester and Bullock, 2000; Peeler et al., 2011; Smith et al., 2010). Moreover, hybridisation between cultured fish and wild stocks may end up diluting genetic diversity, leading to native species extinctions and declines (Arismendi et al., 2009; Clavero and García-Berthou, 2005; Cox, 2004; Houde et al., 2015; Lura and Sægrov, 1991).

To prevent this, salmon industry relies in triploidization to inhibit gonad development (sterile fish), preventing fish from reaching sexual maturation in cultured conditions (Lahnsteiner

and Kletzl, 2018; Maxime, 2008). Due to this, the use of triploid fish for market has gained interest. Aquaculture of fish species such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) use this method constantly (Beaumont et al., 2010; Fraser et al., 2012; Piferrer et al., 2009; Powell et al., 2009). Nonetheless, in other salmon species such as Chinook salmon, the impact of triploidization on physiology is not totally understood, becoming an interesting research topic for future studies.

1.4 Triploid fish as a solution to hybridization

Triploidization can be induced by applying physical or chemical stimulus during embryonic development, inhibiting the release of the second polar body from the oocyte (Bi et al., 2020; Malison et al., 2001; Maxime, 2008). The two commonly used methods to induce triploidization are heat and pressure (Benfey, 1999; Bi et al., 2020; Johnson et al., 2007; Piferrer et al., 2009). From both, the most effective method in teleosts is hydrostatic pressure (Imsland et al., 2014; Opstad et al., 2013; Peruzzi et al., 2007; Trippel et al., 2008). Resulting fish contain three sets of chromosomes (Fraser et al., 2012; Leggatt and Iwama, 2003; Maxime, 2008), showing an increased nuclear size and larger cells (Benfey, 1999; Maxime, 2008). Moreover, triploid fish have shown increased growth, differential behaviour, and high-quality flesh compared to diploid fish (Linhart et al., 2001; Shrimpton et al., 2012; Teskeredžić et al., 1993).

Nevertheless, the performance of triploid fish is not exempt of challenges. According to the scientific evidence, triploid fish can show a higher mortality and susceptibility to diseases compare to diploid fish (Budiño et al., 2006). It is hypothesized that reduced immune competence may be attributed to the way triploid fish deal with stress (Ching et al., 2010; Fraser et al., 2012). For instance, triploid Chinook salmon challenged with *Vibrio anguillarum* showed altered gene expression of immunoglobulin (Ig) M, major histocompatibility complex–II, and beta actin,

compared to diploid fish (Ching et al., 2010; Fraser et al., 2012). However, the mechanisms responsible for the apparent performance differences are poorly understood and further comprehension of triploid fish immune system function is necessary.

1.5 Fish immune system

Compared to higher vertebrates, fish lack of immune organs such as bone marrow and lymph nodes (Rombout et al., 2011; Soulliere and Dixon, 2017; Workenhe et al., 2010). In contrast, the anterior portion of the fish kidney, also known as head kidney, is considered as the functional ortholog of mammalian bone marrow (Sunyer, 2013; Zapata and Amemiya, 2000). In fish, the head kidney is the major endocrine and haematopoietic-lymphoid tissue and is thought to be an immunologically responsive organ (Geven and Klaren, 2017; Press and Evensen, 1999; Sunyer, 2013; Zapata and Amemiya, 2000). Teleosts share a similar immune system with higher vertebrates, divided into innate and adaptive immune systems (Press and Evensen, 1999; Rombout et al., 2011; Whyte, 2007). Nevertheless, general cell mechanisms for antigen recognition and presentation, immune cell activation, proliferation and differentiation, and the final effector stage are utilized differently (Press and Evensen, 1999; Rombout et al., 2011; Whyte, 2007).

In fish, the innate immune response plays a key role in a fast and non-specific response since they are continuously exposed to microbes in natural environment (Salinas, 2015). Also, due to the fact that fish are poikilotherms, the effect of temperature on the innate immune response is lower than the observed in physiological processes associated with the adaptive immune response (Abram et al., 2017; Bowden, 2008). For example, antibody production has shown to be highly dependent on temperature, thus, environmental temperature changes can negatively impact fish immunity (Abram et al., 2019, 2017; Van Muiswinkel, 2019). The recognition of pathogens by the innate immune system is extensive and initiated rapidly. Pathogen-associated molecular patterns

(PAMP) on the surface of the pathogen are recognized by pattern recognition receptors (PRR), which are key to the initiation of the innate immune response (Magnadóttir, 2006). After pathogen recognition, the fish innate cell-mediated immune response is activated, triggering the release of macrophages, neutrophils, basophils, eosinophils and mast cells into the site of infection (Correa et al., 2015; Grayfer et al., 2018; Havixbeck and Barreda, 2015; Soto-Dávila et al., 2020c). The innate immune response, divided in physical barriers, cellular and humoral components, includes lysozymes, agglutinins and precipitins (opsonins, primarily lectins), cytokines, chemokines and antibacterial peptides, among others (Akira et al., 2006; Castro and Tafalla, 2015; Ellis, 1999; Soto-Dávila et al., 2020a). Lysozymes, mainly distributed in the head kidney, play a key role in fish neutrophils (Magnadóttir, 2006). The antibacterial activity of lysozymes is through the disruption of the lipopolysaccharide layer in Gram-negative bacterium and hydrolysing β-linked glycoside bonds of bacterial cell wall peptidoglycans in Gram-positive organisms (Magnadóttir, 2006). Cytokines are proteins involved in the regulation of various mechanisms of the fish immune system (Secombes et al., 2001). To date, it has been reported that the major role of cytokines families are associated to the pro-inflammatory response (interleukin-1 family), to regulate differentiation, survival and activation of T cells (interleukin-2 family), hematopoiesis (interleukin-6 family), anti-inflammatory response (interleukin-10 family), autoimmunity and cytokine inducer (interleukin-17 family), viral response (interferon family), and regulation of leukocyte homing, proliferation and migration (TNF family) (Mills, 2023; Sakai et al., 2021; Secombes et al., 2001; Zou and Secombes, 2016). Chemokines are a family of cytokines that regulate immune cell migration during normal conditions and inflammation (Alejo and Tafalla, 2011). These secreted proteins bind to receptors at leukocyte surfaces, resulting in leukocyte migration to sites of injury or infection and differentiation of the recruited cells (Alejo and Tafalla,

2011; Dixon et al., 1998). Antimicrobial peptides (AMPs) are a diverse group of individually unique small peptides classified in five major families based on their structure: β-defensins, cathelicidins, hepcidins, histone-derived peptides, and piscidins (Katzenback, 2015).

As previously mentioned, diploid and triploid fish share similar components of the immune system, however, the immune response of triploid fish against pathogens shows a lower performance (Budiño et al., 2006). In addition to the efforts to understand the immune response in triploid fish, alternative methods to ongoing aquaculture are needed. Among several candidates, immunostimulants and functional feed ingredients seems to be good choices to enhance the immune system of triploid fish, and are expected to provide an alternative to the use of vaccines and antibiotics in the next years.

1.6 The use of immunostimulants and functional feed ingredients in fish aquaculture

In current finfish aquaculture, due to the lack of efficacy of vaccines and the side effects produced by antibiotics used to prevent and control diseases (i.e., variable protection among strains, regions, and species, and restrictions on use), the incorporation of healthy functional constituents into aquafeeds has become an interesting tool to be utilized (Oliva-Teles, 2012; Sakai, 1999a). These methods have not only helped to improve fish growth and stress tolerance, but also to enhance non-specific defense mechanisms against pathogens (Oliva-Teles, 2012; Sakai, 1999a; Soto-Dávila et al., 2020c).

These essential components, known also as immunostimulants and functional feed ingredients, represent non-toxic, non-polluting and efficient biological agents that can activate immune cells directly and/or indirectly to enhance non-specific innate immune responses (Barman et al., 2013; Wang et al., 2017a). Specifically, immunostimulants and nutritional factors can

improve lysozyme activity, complement system, phagocytic activity, among others (Bridle et al., 2005; Cook et al., 2003; Song et al., 2014; Soto-Dávila et al., 2020c). To date, a large number of immunostimulants and functional feed ingredients have been tested for impact on the immune response of fishes, such as prebiotics, vitamins, and probiotics (Figure 1.2).

From these, probiotics have the advantage of having been broadly studied in terrestrial and aquatic animals, and demonstrate commercial availability, accessible prices, and evidence of immune system enhancement in aquaculture species (Balcázar et al., 2006; Gatesoupe, 1999; Irianto and Austin, 2002; Wang et al., 2008). Given this evidence, the application of probiotics to the aquaculture of triploid fish to enhance the immune system looks promising, however, considerable research efforts are still needed.

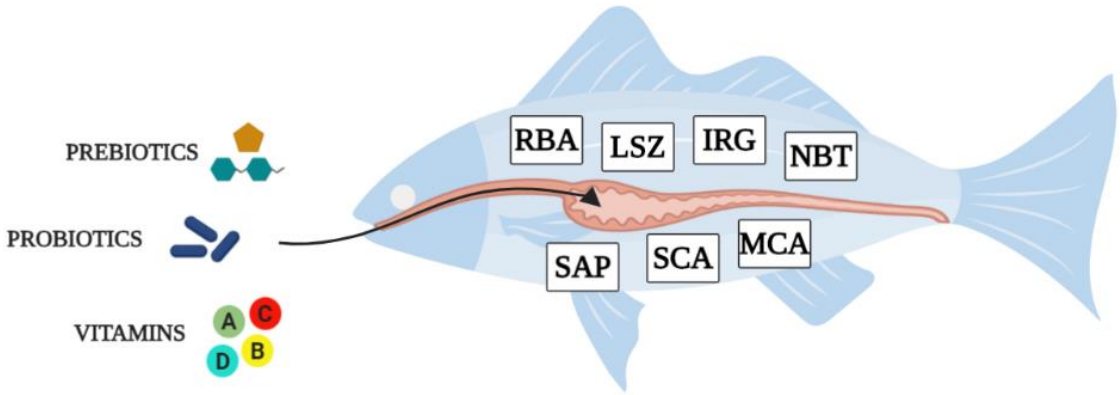


Figure 1.2. Immune parameters enhanced by immunostimulants prebiotics, probiotics, and vitamins. Abbreviations: IRG, innate immune response genes, LSZ, lysozyme activity, MCA, macrophages activity, NBT, nitroblue tetrazolium activity, RBA, respiratory burst activity, SAP, serum antibody production, SCA, serum complement activity.

1.7 Probiotics

The term probiotics, introduced by Parker in 1974, refers to a group of living microorganisms (Gram-positive and Gram-negative bacteria, bacteriophage, microalgae, and yeast) that provide health benefits to the host when they are administered in appropriate amounts (FAO et al., 2002; Parker, 1974). Probiotics can modulate the gastrointestinal microbial community and prevent bacterial diseases either by competing for adhesion sites, nutrients and oxygen, or through the release of repressive molecules (Akhter et al., 2015; Fuller, 1987; Pérez-Sánchez et al., 2014).

In fish aquaculture, a large number of probiotics have been tested, the most commonly utilized are the lactic acid bacteria (LAB; *Lactobacillus* and *Carnobacterium*) and *Bacillus* spp. (Balcázar et al., 2006; Burr et al., 2005; Pérez-Sánchez et al., 2014; Wang et al., 2008). Additionally, the microorganism *Pediococcus acidilactici* (strain CNCM MA 18/5M) has shown promising results in the EU (Al-Hisnawi et al., 2019; Hoseinifar et al., 2019; Jaramillo-Torres et al., 2019; Pérez-Sánchez et al., 2014). The beneficial effects reported in fish have been the enhancement of growth, antioxidant activity, food digestibility, gut microbiota, disease resistance and immune response, among others (Akhter et al., 2015; Dimitroglou et al., 2011; Guerreiro et al., 2018; Merrifield et al., 2010) (Table 1.1).

Due to major economic impact directly attributed to pathogens in Atlantic salmon aquaculture (~20% in 2018 for Norwegian Atlantic salmon industry and 24% in 2019 for the industry in Chile) (Hjeltnes et al. 2019; SERNAPESCA, 2019), the utilization of probiotics represents an interesting and safe option to enhance the non-specific and specific immune response in place of vaccines and antimicrobial agents (Al-Hisnawi et al., 2019; Encarnação, 2016; Merrifield et al., 2010). Current research demonstrates that LAB microorganisms such as

Carnobacterium sp., a bacterium that lives in the intestine of Atlantic salmon, can be effective against infection with *A. salmonicida*, *Vibrio ordalii*, and *Yersinia ruckeri* (Balcázar et al., 2006; Hoseinifar et al., 2018; Kesarcodi-Watson et al., 2008; Ringø and Olsen, 2011; Robertson et al., 2000). Furthermore, *Carnobacterium divergens* and *Lactobacillus delbrueckii* treatment shows diminished damage to epithelial cells, decreased disorganization of the microvilli, and reduced cell debris in the lumen, which are caused by the pathogens *Aeromonas salmonicida* and *Vibrio anguillarum* (Lazado and Caipang, 2014; Ringø et al., 2007; Robertson et al., 2000; Salinas et al., 2008b). Similar to other fish species, such as common carp supplemented with *P. acidilactici* and the prebiotic galactooligosaccharides, the use of a symbiotic diet as a food additive also showed beneficial effects to the Atlantic salmon gut microbial community (Abid et al., 2013). In particular, a mixture of the bacterium *P. acidilactici* and short-chain fructooligosaccharides (scFOS), increases mucosal length, leukocyte infiltration, serum lysozyme activity, and gene expression of the immune-related genes *il1b*, *il8*, *tnfa*, *tlr3*, and *mx1*, in the anterior and posterior intestine compared to control diets (Abid et al., 2013).

Table 1.1. Summary of enhanced immune parameters after supplementation with probiotics in main freshwater and saltwater cultured species.

Fish species	Probiotic	Enhanced immune parameters
Common carp (*Cyprinus carpio)*	*Bacillus* sp., LAB, *A. veronii, A. lentus, F. sasangense*	↑ lysozyme activity; ↑ complement C3 activity; ↑ WBC; ↑ RBA; ↑ *il1b* and *tnfa* relative expression (Chi et al., 2014; Dawood and Koshio, 2016; Gupta et al., 2014; Harikrishnan and Balasundaram, 2010; Soltani et al., 2017; Xu et al., 2014)
	P. acidilactici + GOS	↑ serum IgM; ↑ lysozyme activity; ↑ ACH_{50} serum activity (Modanloo et al., 2017)

Nile tilapia (*Oreochromis niloticus*)	LAB (*E. faecium*, *L. rhamnosus* GG, *L. plantarum*)	↑ RBA; ↑ complement C3 activity; ↑ MPO activity; ↑ *il1b*, *il4*, *il12*, *ifny*, *tnfa* relative expression (Hamdan et al., 2016; Ngamkala et al., 2010; Pirarat et al., 2011; Y.-B. Wang et al., 2008)
	Bacillus sp.	↑ SOD activity; ↑ CAT activity; ↑ lysozyme activity; ↑ MPO activity; ↑ phagocytic cells; ↑ *il1b* and *tnfa* relative expression (Abarike et al., 2018; Aly et al., 2008; Selim and Reda, 2015; Telli et al., 2014; Zhou et al., 2010)
Atlantic salmon (*Salmo salar*)	*P. acidilactici* + scFOS	↑ leukocyte infiltration; ↑ SLA; ↑ *il1b*, *il8*, *tnfa*, *tlr3*, *mx1* relative expression (Abid et al., 2013)

***Abbreviations:** LAB, lactic acid bacteria, GOS, galactooligosaccharides, scFOS, short-chain fructooligosaccharides, RBA, respiratory burst activity, WBC, white blood cell count, ACH_{50}, alternative complement activity, , MPO, myeloperoxidase, SOD, sodium oxide dismutase, CAT, catalase, SLA, serum lysozyme activity, tnfa, tumor necrosis factor alpha, il1b, interleukin 1 beta, il8, interleukin 8, il4, interleukin 4, il12, interleukin 12, , ifny, interferon gamma, tlr3, toll-like receptor 3, mx1, interferon-induced GTP-binding protein MX1.

Despite the beneficial effects of probiotics presented above, more evidence is required before researchers and producers can support the utilization of probiotics as a safe enhancer of fish health (Wang et al., 2008). To date, the main concerns about probiotics are their capability to survive the harsh conditions experienced during feed pellet manufacturing, delivery, and transit to the fish intestine (Burr et al., 2005). Moreover, a lack of knowledge about the modulation mechanisms at the gastrointestinal level where the host/probiotic interaction occurs, do not allow firm conclusions that probiotics are safe for fish (Akhter et al., 2015; Burr et al., 2005; Wang et al., 2008). Finally, the prolonged retention of the beneficial effects in supplemented fish, especially after repeated sub-culturing of strains, is still questionable (Akhter et al., 2015).

1.8 Thesis hypotheses

i. Main hypothesis: Single-strain and multi-strain probiotics can enhance the triploid Chinook salmon survival, growth, immune response, and gut integrity during the challenge with *Vibrio anguillarum*.

ii. Hypothesis chapter 2**:** A multi-strain probiotic supplementation can modulate the immune and tight junction function, leading to an improvement of the survival, growth, and immune response in farmed Chinook salmon

iii. Hypothesis chapter 3: *P. acidilactici* MA18/5M supplementation does not decrease RTgutGC cell viability or cause detrimental effects in the gut barrier function of Chinook salmon.

iv. Hypothesis chapter 4: Lactic acid bacteria (LAB) supplementation in Chinook salmon can enhance the expression of the innate immune response and tight junction molecules, improving fish growth and decreasing mortality after a pathogen challenge with *V. anguillarum*.

1.9 Objectives

i. To evaluate the effect of a multi-strain probiotic in the survival, growth, immune response, TJ expression, and immunoglobulin protein levels of Chinook salmon challenged with *V. anguillarum* **(chapter 2)**.

ii. To determine the impact that production cycle (freshwater, saltwater, or both), as well as time of supplementation can have when fish are supplemented with a multi-strain probiotic **(chapter 2)**.

iii. To investigate the effect of *P. acidilactici* MA18/5M supplementation on barrier function and the expression of tight junction and immune molecules in pacific salmonid models **(chapter 3)**.

iv. To determine the extent to which *V. anguillarum* disrupts barrier function and the protective effects that *P. acidilactici* MA18/5M can exert against pathogen-induced damage, using RTgutGC as an *in vitro* model **(chapter 3)**.

v. To examine the effects of *P. acidilactici* MA18/5M on several physiological parameters of juvenile Chinook salmon **(chapter 3)**.

vi. To assess the effects that LAB supplementation can have on the cell viability, the expression of immune molecules and tight junctions using RTgutGC as an *in vitro* model **(chapter 4)**.

vii. To determine the effect of LAB supplementation on survival, growth (weight), and the expression of tight junction and immune molecules of Chinook salmon challenged with *V. anguillarum* **(chapter 4)**.

Chapter 2: Effect of Dietary Supplementation of Jamieson® Probiotic on Growth, Survival, and Immune Response in Chinook Salmon (*Oncorhynchus tshawytscha*) Challenged with *Vibrio anguillarum*

2.1 Overview

Chinook salmon is a native species of the Canadian Pacific coast with potential economic and environmental benefits for aquaculture. However, its production is jeopardized by low tolerance to high densities and disease susceptibility. Due to this, Chinook salmon represents a great candidate for evaluating the effect of non-polluting alternatives to vaccines and antibiotics, such as probiotics. Preliminary studies have suggested that certain probiotic strains may be able to enhance fish growth, antioxidant activity, food digestibility, modulate the host microbial community in the digestive tract, and prevent bacterial infections. However, there is currently no evidence of its effects in Chinook salmon. Here, we used the commercially available, low-cost multi-strain probiotic used by humans, to determine the impact on Chinook salmon growth, survival, immune response, and gut barrier integrity. Fish were randomly assigned to four netpens and given either regular (control) feed or supplemented with probiotics for 4, 10, or 14 months. After this, fish were transferred to troughs and challenged with *Vibrio anguillarum*. Probiotic supplementation for 14 months decreased mortality by ~10% compared to the control treatment. However, no positive effects of this probiotic supplementation on fish growth occurred. Although there was a weak correlation between the survival curve and the expression of the immune genes *il1b, il8, il10, tnfa, camp, hamp*, and *transferrin* in head kidney, spleen, and hindgut tissues at 4-months, longer supplementation (10 and 14 months) conferred better protection than fish supplemented only in the freshwater cycle. Decreased expression of *cldn1* and *ocln* in fish supplemented for 14 months compared to 4- and 10-months suggested that probiotic treatment during the freshwater and saltwater cycle confers a better protection and intestinal integrity, resulting in an improved survival. In summary, probiotic supplementation modulated the early inflammatory response against *V. anguillarum* infection and helped improve the survival of

Chinook salmon aquaculture in an environmentally sustainable manner when applied in freshwater and up to the transition to saltwater growth phase.

2.2 Introduction

The rise of aquaculture has provided important economic benefits to the producer countries in the last 60 years (Cadillo-Benalcazar et al., 2020; FAO, 2022; Gutiérrez et al., 2020). Nevertheless, outbreaks of viral, bacterial, fungi and parasitic infections due to high stocking densities and precarious environmental conditions represent one of the main causes of losses in the aquaculture industry (Kaattari and Tripp, 1987; Maisey et al., 2017; Robertson et al., 1987; Soto-Dávila et al., 2020b; Tran et al., 2022). Although antibiotics are commonly employed to prevent infectious diseases, the emergence of antibiotic-resistant pathogens have increased the regulations for their use in the industry (Ferri et al., 2022; Hvistendahl, 2012; Liu et al., 2017; Martínez et al., 2018; Schmidt et al., 2000; Sørum, 2005; Soto-Dávila et al., 2020b; Wang and Xiong, 2007). As an alternative to antibiotics, immunostimulants and functional feed ingredients have been assessed for their ability to improve fish growth, stress tolerance, and cellular and humoral immunity (Barman et al., 2013; Langlois et al., 2021; Nikoskelainen et al., 2003; Oliva-Teles, 2012; Sakai, 1999a; Vendrell et al., 2008; Wang et al., 2017a). Several feed additives have been tested in aquaculture, with polysaccharides, vitamins, medicinal plants, prebiotics, and probiotics utilized most in research (Akhter et al., 2015; Dawood and Koshio, 2018; Jamal et al., 2020; Lieke et al., 2019; Song et al., 2014; Wang et al., 2017b).

Probiotic are live microorganisms, that when administered in adequate amounts, confer a health benefit on the host (FAO et al., 2002; Hill et al., 2014; Langlois et al., 2021; Parker, 1974). In finfish aquaculture, a number of candidate probiotics have been tested, mainly lactic acid

bacteria (LAB; *Lactobacillus* and *Carnobacterium*) and *Bacillus* species (Balcázar et al., 2006; Burr et al., 2005; Pérez-Sánchez et al., 2014; Wang et al., 2008). It has been well established that probiotics can enhance fish growth, antioxidant activity, food digestibility, modulate the host microbial community in the digestive tract, and prevent bacterial infections by competing for adhesion sites, nutrients, and oxygen (Dawood et al., 2018; Gatesoupe, 1999; Irianto and Austin, 2002; Jamal et al., 2020; Khati et al., 2018; Langlois et al., 2021; Pérez-Sánchez et al., 2014; Ringø and Olsen, 2011; Salinas et al., 2008b). A potentially important mechanism for probiotic activity is to modulate the expression of tight junction molecules, such as occludins, claudins, and zonula occludens 1 (Patel et al., 2012; Ringø et al., 2007; Ukena et al., 2007; Vasanth et al., 2015; Wang et al., 2018) in the intestine, as well as, upregulate the expression of the mucosal antibody (Al-Hisnawi et al., 2019; Rodriguez-Estrada et al., 2013).

To date, efforts in determining the effects of probiotic supplementation in salmonid species have focused principally on two fish species, Atlantic salmon (*Salmo salar*) (Encarnação, 2016; Hoseinifar et al., 2018; Lazado and Caipang, 2014; Merrifield et al., 2010; Ringø et al., 2007; Ringø et al., 2012; Robertson et al., 2000; Salinas et al., 2008b) and rainbow trout (*Oncorhynchus mykiss*) (Brunt et al., 2007; Burbank et al., 2011; Gram et al., 2001; Kim and Austin, 2006; Korkea-aho et al., 2012; LaPatra et al., 2014; Nikoskelainen et al., 2003; Robertson et al., 2000; Salinas et al., 2008b; Vendrell et al., 2008). However, the benefits to low-scale salmonid aquaculture species such as Chinook salmon (*Oncorhynchus tshawytscha*) have not been explored.

Chinook salmon is the largest and most valued species of Pacific salmon in North America due to their size, resilience, flavor, and nutritional composition (Christensen et al., 2018; Healey, 1991; Ohlberger et al., 2018; Toews et al., 2019). Although Chinook salmon production has sustainably increased in the last 20 years, it remains low compared to Atlantic salmon and rainbow

trout (1% of total salmon production worldwide) (Araujo et al., 2021; Bourret et al., 2016; Christensen et al., 2018; Council, 1996; Healey, 1991; Semeniuk et al., 2019; Semple et al., 2022; Willson and Halupka, 1995). The causes of this are associated with their low tolerance to the high densities commonly used for commercial production and the high mortalities associated with infectious diseases (Olson and Paiya, 2013; Semple et al., 2022). Overcoming these problems would provide a huge opportunity for the aquaculture industry.

Candidate probiotic strains for salmonids come from the following species: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Aeromonas*, *Bacillus*, *Vibrio*, *Micrococcus*, *Carnobacterium*, *Enterococcus*, *Brochothrix*, *Clostridium*, *Shewanella*, *Enterobacter*, *Pseudomonas*, and *Saccharomyces* spp. (Balcázar et al., 2006; Hoseinifar et al., 2018; Jamal et al., 2020; Nayak, 2010; Ringø and Olsen, 2011; Robertson et al., 2000). Unfortunately, strain characteristics suitable for the desired effect (pathogen inhibition, immune modulation, enhanced nutrient uptake) are invariably not the reason for selecting strains. Since strain-to-strain variations are important, the species per se is insufficient. Another approach has been to use synbiotics such as fructooligosaccharides, galactooligosaccharides, or short-chain fructooligosaccharides mixed with *Pediococcus acidilactici* (Abid et al., 2013).

In the present study, a multi-strain probiotic product was chosen for several reasons. Although the product was sold for human use, it was felt that the broad selection of strains offered a better chance of success than a single isolate. The dried nature of the strains was deemed useful for reproducibility and ease of application. For any application to be successfully taken up by the industry, cost is a key component. Thus, the low-priced product we selected provided another advantage. Since probiotics have never been utilized before in Chinook salmon aquaculture, this

study also evaluated supplementation during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months).

2.3 Materials and Methods

2.3.1 Probiotic supplementation experimental design

Chinook salmon (*Oncorhynchus tshawytscha*) juveniles (9 ± 2 g body weight) farmed at Yellow Island Aquaculture (www.yellowislandaquaculture.ca, Quadra Island, British Columbia, Canada) were vaccinated in summer 2019 and kept in freshwater (14 ± 2°C) at a density of 9.0 kg/m^3 using a flow-through system under a photoperiod of 12:12 h dark:light. Chinook salmon (Oncorhynchus tshawytscha) juveniles (9 ± 2 g body weight) farmed at Yellow Island Aquaculture (www.yellowislandaquaculture.ca, Quadra Island, British Columbia, Canada) were vaccinated in summer 2019 and kept in freshwater (14 ± 2°C) at a density of 9.0 kg/m3 using a flow-through system under a photoperiod of 12:12 h dark:light. To evaluate the effect of probiotic supplementation, fish were fed with either regular feed or a probiotic containing 10 billion active cells of *Lactococcus lactis* UALI-08, *Lactobacillus gasseri* UALg-05, *Lactobacillus rhamnosus* UALr-06, *Bifidobacterium animalis* subsp. *lactis* UABIa-12, *Bifidobacterium breve* UABbr-11, *Lactobacillus paracasei* UALpc-04, *Lactobacillus rhamnosus* UALr-18, *Lactobacillus acidophilus* DDS©-1, *Lactobacillus plantarum* UALp-05, *Bifidobacterium longum* subsp. *longum* UABI-14, *Bifidobacterium bifidum* UABb-10, *Lactobacillus casei* UALc-03, *Lactobacillus reuteri* UALre-16, *Bifidobacterium longum* subsp. *infantis* UABi-1. The product also contained NON-GMO Maltodextrin (rice), water-soluble cellulose, inulin (chicory root), vegetable magnesium stearate, silica. NON-GMO, NO soy, starch, gluten, lactose, artificial flavours or preservatives (Jamieson, Canada).

A total of 800 fish were randomly distributed between eight 120 L barrels (four barrels for regular diet and four barrels for probiotic diet, 100 fish per barrel) and kept for four months in freshwater. After this, 200 fish comprising the regular diet treatment (0 months probiotic supplementation) group, were transferred to netpens and fed regular diet for 10 months. For the short-term probiotic supplementation treatment (4 months), 200 fish with probiotic supplemented feed during freshwater phase were switched to regular feed while kept for 10 months in saltwater conditions. The mid-term probiotic supplementation group (10 months corresponded to 200 fish supplemented for 10 months with probiotics feed when moved to the netpens. Finally, the long-term probiotic supplementation treatment (14 months) comprised fish that were supplemented with probiotic for the entire trial (freshwater and saltwater phase) (Figure 2.X).

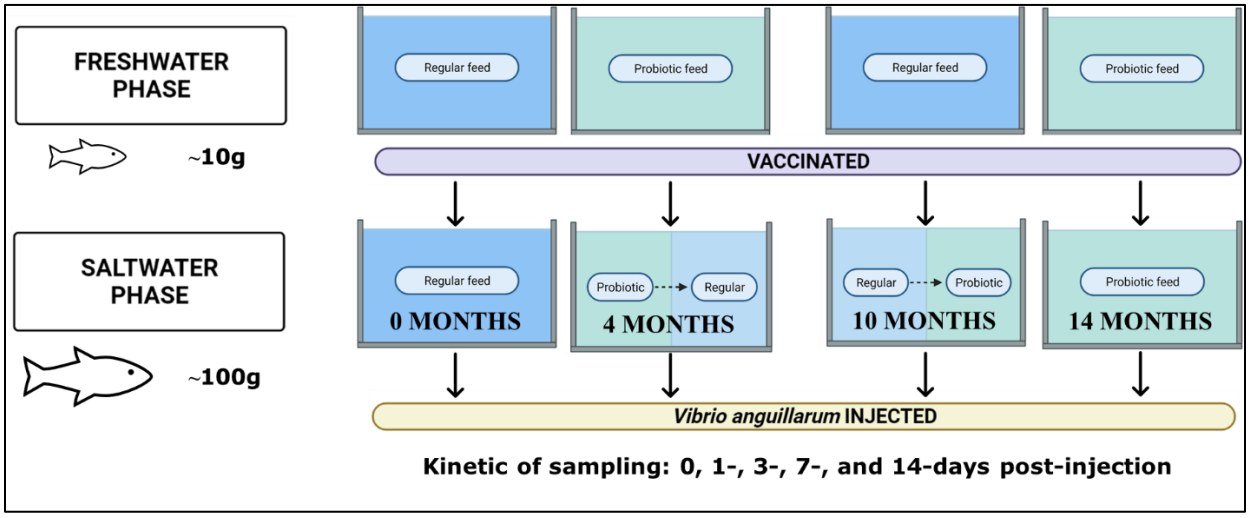


Figure 2.1. Experimental design. Two groups of Chinook salmon juveniles were fed regular pellets, while two groups were supplemented with Jamieson® probiotic. After vaccination, fish were transferred to sea pens for 10 months, at which point one regular feed and one probiotic supplemented group was switched to the opposite diet. On the other hand, one regular feed and one probiotic supplemented group stayed under the same feeding regimen. Fish were transferred back to the hatchery for a *V. anguillarum* challenge.

Only one netpen per group was used as the industrial partner needed the other pens for production. During the trial, fish were fed commercial dry pellets (EWOS Harmony 2 mm: 47% protein, 18% fat, 0.7% fibre, 2.9% calcium, 1.2% phosphorus, and 0.6% sodium) twice a day with a ration of 2% body weight, with probiotic added when appropriate (Please refer to Appendix A1 for formulation details). Although we could not guarantee that each fish received the same amount of pellets, efforts were made to distribute the pellets on the surface to increase the chance of equal opportunity for feeding.

All fish were kept and handled under a permit (ethical protocol #43212) from the University of Waterloo Animal Care Committee according to CCAC guidelines.

2.3.2 Vibrio anguillarum growth conditions

Vibrio anguillarum serotype O1 (J382) isolated from winter Steelhead trout obtained from Little Campbell River (British Columbia, Canada) was utilized for the infection trial (Machimbirike et al., 2023). Briefly, a single colony of *V. anguillarum* was grown in 2.5 mL of trypticase soy broth (TSB) 2% sodium chloride (TSB 2% NaCl; Multicell Wisent, Quebec, Canada) at 20°C in a 16 mm diameter glass tube and placed in a shaker for 24 h at 200 rpm. After growth, 150 µL of the culture were added in 150 mL of TSB 2% NaCl media using a 250 mL flask and incubated for 24 h at 20°C with aeration (200 rpm). Then, the bacterial inoculum was centrifuged at 6,000 rpm at room temperature for 10 min, the pellet was washed three times with PBS and centrifuged at 6,000 rpm at room temperature for 10 min, and finally resuspended in 25 mL of PBS (~8.6×10^8 CFU mL^{-1}). The concentrated bacterial inoculum was serially diluted and quantified by plating onto TSA 2% NaCl for 2 days.

2.3.3 Chinook salmon infection trial with V. anguillarum

After 14 months of diet supplementation, Chinook salmon individuals (100 ± 40 g body weight) were transferred back to the hatchery to four rectangular 2,500 L troughs each specific to the respective diet treatments. Before the infection trial, fish were acclimated for four weeks in sea water (SW, 32 psu, 14 ± 2°C) at a density of 9.6 kg/m^3 under the same 12:12 photoperiod as above. The groups were then inoculated intraperitoneally (I.P.) with 100 µL of live *V. anguillarum* (1×10^5 CFU mL^{-1}). Time 0 (n = 5 fish per treatment) corresponded to fish sampled before the injection. The timing of sampling was 0, 1-, 3-, 7-, and 14-days post-inoculation (dpi) with *V. anguillarum*. For sampling, fish were anesthetized with a non-lethal dose of clove oil, followed by euthanasia by cervical dislocation. Head kidney, spleen, and hindgut tissues were isolated and immediately snap-frozen in liquid N_2 prior to being stored at -80°C until utilization. Blood was collected from the caudal peduncle into 1 mL syringes containing 100 µL of heparin and plasma was obtained by centrifugation of whole blood (5 min, 2,500 × g, 4°C) and stored at -80°C until analysis.

2.3.4 RNA extraction

Total RNA was isolated from Chinook salmon head kidney, spleen, and hindgut samples using 1 mL of TRIZOL reagent following the manufacturers protocol (Invitrogen). After extraction, the RNA was treated with Ambion DNase I (RNase free) (Ambion™ DNase I, Invitrogen) following the manufacturer's instructions to degrade any residual genomic DNA. Briefly, 5 µg of RNA was treated with 2 µL of Ambion DNase I, 4 µL of DNase buffer x10, and DEPC water to complete 40 µL. Then, samples were incubated at 37°C for 30 min, washed twice with wash solution A, centrifuged at 3,500 x g for 1 min and purified in an RNA/Protein

Purification Column. The supernatant containing the RNA was carefully transferred to a new tube. DNase treated RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using a Take3 plate of a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA). Column purified RNA samples had A260/280 ratios between 2.0 and 2.1 and A260/230 ratios between 2.0 and 2.2. A PCR test was conducted using the reference genes' primers elongation factor 1 alpha (*ef1a*), beta actin (*actb*) and the RNA as template to rule out the presence of DNA. All RNA samples did not show presence of DNA.

First-strand cDNA templates for qPCR were synthesized from 250 ng of DNaseI-treated, column-purified total RNA using qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Each sample was incubated at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min. Samples were stored at -20°C until utilization.

2.3.5 qPCR analysis

All qPCR reactions were performed in a final volume of 10 µL, containing 5µL of 2x WISENT ADVANCED™ qPCR master mix (Wisent, Quebec, Canada), 2.5 µL of forward and reverse primer mix (Sigma Aldrich, USA) at a final concentration of 0.25 µM each, and 2.5 µL of cDNA (2.5 ng/µL, 6.25 ng per reaction). All samples were amplified and detected using the LightCycler® 480 II (Roche, USA). The reaction mixtures were pre-incubated for 2 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, annealing for 30 s at 60°C, and finally extension for 8 s at 72°C. The melt curve was completed for each run every 5 s from 65°C to 97°C.

The primer sequences of interleukin 1 beta (*il1b*), interleukin 8 (*il8*), interleukin 10 (*il10*), tumor necrosis factor alpha (*tnfa*), hepcidin antimicrobial peptide (*hamp*), cathelicidin antimicrobial peptide (*camp*), transferrin antimicrobial peptide (*transferrin*), claudin 1 (*cldn1*),

claudin 3 (*cldn3*), claudin 12 (*cldn12*), occludin (*ocln*), and zonula occludens-1 (*zo-1*), used in this study are listed in Table 2.1. Gene discovery, qPCR primer design and initial quality testing were performed as described in Soto-Davila et al. (Soto-Dávila et al., 2020c). Since the reagents, cycling conditions and samples were different in the current study, primer efficiencies (Table 2.1) were reassessed. Briefly, a 7-point 1:3 dilution series starting with cDNA representing 5 ng of input total RNA was generated, and efficiencies then calculated using the formula $E = 10^{(-1/slope)}$ (Pfaffl, 2001).

Table 2.1. Primers used in this study.

Gene name (symbol)	Nucleotide sequence (5'-3')	Accession number	[c]Efficiency (%)	Amplicon size (bp)	Reference
Interleukin 1 beta (*il1b*)	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	AJ223954	100.1	153	(Frenette et al., 2023)
Interleukin 8 (*il8*)	F: ACCAGCGAGATAACAA R: CCAGGAGCACAATGACAA	JX157147	98.2	NR	(Polinski et al., 2014)
Interleukin 10 (*il10*)	F: GCCTTCTCCACCATCAGAGAC R: GATGCTGTCCATAGCGTGAC	NM_001245099	102.2	120	(Semple et al., 2020)
Tumor necrosis factor alpha (*tnfa*)	F: ACCAAGAGCCAAGAGTTTGAAC R: CCACACAGCCTCCATAGCCA	DQ778945	93.6	154	(Bjork et al., 2014)
Hepcidin antimicrobial peptide (*hamp*)	F: GCTTCTGCTGCAAATTCTGAGG R: GTACAAGATTGAGGTTGTGCAG	HQ711993	98.4	NR	This study
Cathelicidin antimicrobial peptide (*camp*)	F: ATGGGAAACGAATGATGTGC R: CGGTCAGTGTTGAGGGTATT	NM_001124463	90.4	NR	(Broekman et al., 2013)
Transferrin antimicrobial peptide (*transferrin*)	F: TCAAGAAGATCATGCGTAAAGAG R: ATGACAGGGACCAGACCACATTT	D89083	96.1	NR	(Aegerter et al., 2005)
Claudin 1 (*cldn1*)	F: GAGGACCAGGAGAAGAAGG R: AGCCCCAACCTACGAAC	BK008768	98.6	186	(Kolosov et al., 2014)
Claudin 3 (*cldn3*)	F: AGGCAACGACGCTACATCAA		100.4	112	

	R: GAAACCCAAGCAATGCGTCA	XM_021587920			(Wang et al., 2019)
Claudin 12 (*cldn12*)	F: ATCATCGCCTTCATCTCCGT R: TAGCAGCCAGAGTAGCCATC	XM_021621241	91.5	161	This study*
Occludin (*ocln*)	F: CAGCTTGCCGTTGTAGAGG R: CAGCCCAGTTCCTCCAGTAG	GQ476574	103.1	346	(Kolosov et al., 2014)
Zonula occludens-1 (*zo-1*)	F: GCTGTTCCTCCTAGACCTT R: TCACCCACATCTGACTCTAC	XM_021607172	98.2	99	(Schug et al., 2019)
[a,b]Elongation factor 1 alpha (*ef1a*)	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	NR	99.1	134	(Semple et al., 2018)
[a,b]βeta actin (*actb*)	F: TGGACTTTGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG	AJ438158	90.2	139	(Ma et al., 2019)
[b]Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	F: GCTGGAATGGGACTCACAC R: GTCAAAACCGTCTCAGTGGG	NR	100.8	NR	(Rajanbabu and Chen, 2011)
[b]18S ribosomal RNA (*18S*)	F: CGTCGTAGTTCCGACCATAAA R: CCACCCACAGAATCGAGAAA	NR	101.4	NR	(Giroux et al., 2019)

[a]Normalizers used in experimental qPCR analyses.
[b]Candidate normalizer genes.
[c]Amplification efficiencies were calculated using a 7-point 1:3 dilution series starting with cDNA representing 50 ng of input total RNA. See methods for details.
[*]Primer designed by Luana Langlois Fiorotto, Ontario Genomics, Canada.
NR: Not reported.

Transcript levels of the genes of interest (*il1b*, *il8*, *il10*, *tnfa*, *hamp*, *camp*, *transferrin*, *cldn1*, *cldn3*, *cldn12*, *ocln*, and *zo-1*) were normalized to two endogenous control genes. Levels of four candidate normalizers [*ef1a*, *actb*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and 18S ribosomal RNA (*18S*),] were assessed in 50% of the samples (i.e., in 3 random samples per treatment) using cDNA representing 2.5 ng (6.25 ng per reaction) of input total RNA. Reference

gene stability was assessed using geNorm, NormFinder, BestKeeper, and Delta Ct comparison, through the bioinformatic open-access portal RefFinder (Soto-Dávila et al., 2022; Xie et al., 2012). Most stable genes assessed were *ef1a* and *actb*.

After normalization testing was completed, transcript levels of the genes of interest were analyzed in the individual study samples, with normalization to both *ef1a* and *actb*. In all cases, levels were assessed (in technical triplicates) in five individuals per treatment per time-point using cDNA representing 2.5 ng of input total RNA. A no reverse transcriptase (NRT) control was included on each plate. Gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3.6 Western blot analysis

To validate IgM and IgT antibodies, a western blot was performed in spleen and plasma samples following the protocol of Sever et al. (Sever et al., 2014) with modifications (Figure 2.6). First, protein concentrations were determined via BCA assay (Pierce™ BCA Protein Assay®, Thermo Fisher Scientific) following the manufacturer's recommendations. Then, 20 µg of protein from representative samples were mixed with 4 × Laemmli sample buffer (BioRad) with 10% β-mercaptoethanol. After boiling for 5 min, sample buffer were stacked and resolved under reducing conditions in 12% acrylamide gel by SDS-PAGE (Laemmli, 1970). Samples and protein standards (PageRuler pre-stained protein ladder, Thermo Fisher Scientific) were electrophoresed for 30 min at 80 V, and then 60 min at 160 V. Proteins were then transferred to 0.45 µm nitrocellulose membranes (BioRad) using a TransBlot Turbo transfer system (BioRad) using the manufacturer's instruction (35 min at 25 V and up to 1A). To ensure that a similar number of proteins were loaded in each sample, nitrocellulose membranes were stained with Ponceau-S [0.1% (w/v) Ponceau S in

5% (v/v) acetic acid] to enable visualization of electrophoresed proteins. Subsequently, blots were blocked with 5% skim milk in tris-buffered saline with Tween-20 (TBS-T; 2 mM Tris, 30 mM NaCl, pH 7.5 with 0.1% Tween-20) for 1 h at room temperature on a platform shaker and probed with monoclonal mouse anti-IgM antibody (1:50 in 5% skim milk TBS-T; F11, Aquatic diagnostics Ltd.), or polyclonal chicken anti-IgT antibody (1 µg/mL) for primary detection of IgM and IgT antibody respectively. Blots were incubated overnight on a rotating shaker at 4°C, then washed 3 times with TBS-T and incubated with alkaline phosphatase-conjugated rabbit anti-mouse (Sigma) or rabbit anti-chicken (Sigma) at room temperature for 1 h, and developed via inclusion of alkaline phosphatase substrates, nitro-blue tetrazolium chloride (NBT, Sigma) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP, Sigma) until visualization of the bands. The enzymatic reaction was stopped by rinsing the membrane thoroughly with distilled water.

2.3.7 Indirect ELISA for IgM and IgT detection

To determine the levels of IgM and IgT in Chinook salmon supplemented with probiotics, plasma, hindgut, spleen, and head kidney samples from fish collected from the netpens were evaluated by indirect ELISA. Before conducting the assay, total proteins were determined from samples by using the commercial kit, Pierce™ BCA Protein Assay® (Thermo Fisher Scientific) following the manufacturer's instructions. Once the total protein concentration was determined, 8 plates (two per tissue, one per immunoglobulin) of Immulon 4HBX 96-well polystyrene flat bottom plate (ThermoFisher) were coated with 100 µL of coating buffer containing 100 µg/well of each sample. The plates were incubated at room temperature for 4 h, then washed four times with 300 µL of tris-buffered saline with 1% Tween 80 (TBS-T) using a plate washer (Biotek 50TS microplate washer). After this, 300 µL of blocking solution (5% skim milk dissolved in TBS-T) was applied and wells incubated for an hour at 37°C. Wells were washed four times followed for

the addition of 100 µL of the monoclonal mouse anti-IgM antibody (1:50 in 5% skim milk TBS-T; F11, Aquatic diagnostics Ltd.), or polyclonal chicken anti-IgT antibody (1 µg/mL) for primary detection of IgM and IgT antibody respectively. Plates were incubated overnight at 4°C, then washed four times and 100 µL of goat anti-mouse conjugated to HRP (1:10000 dilution in 5% skim milk TBS-T; Sigma) or goat anti-chicken conjugated to biotin (1:10000 dilution in 5% skim milk TBS-T; Arigo) were added and plates incubated for 1 h at room temperature in the dark. After removal of the secondary antibody and washing four times, 100 µL of TMB plus2 (Kementec) was added to the IgM wells and incubated at room temperature in the dark for 30 min. In contrast, to determine the presence of IgT, 100 µL of streptavidin conjugated to horse-radish peroxidase (HRP, Biolegend) at a 1:1000 dilution in 5% skim milk TBS-T was added to IgT plates and incubated at room temperature in the dark for 1 h, followed by four washes and 30 min incubation with substrate TMB plus2. After 30 min with the substrate, enzymatic reactions in both IgT and IgM plates were stopped with 100 µL of stop solution (0.2 M H2SO4) and the absorbance was measured at 450 nm using a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA).

2.3.8 Statistical analysis

All data are shown as the mean ± standard error (SEM). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***) All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism 9 (GraphPad Software, La Jolla California USA).

2.4 Results

2.4.1 Survival curve and growth analysis

The experimental diets significantly affected the survival of fish ($p < .05$). The highest survival was observed in fish supplemented with Jamieson® probiotic for 14 months (99.3% average) while the lowest survival was recorded in fish supplemented with probiotic for 4 months (90.6% average) (Figure 2.2). Between treatment groups, fish supplemented for 14 months showed a statistically significant increase in survival compared to fish fed with probiotics for 4 months after 4-, 5-, 6-, 7-, 8-, 9-, and 10-days post-infection (dpi) (11.9%, 12.9%, 12.5%, 14.0%, 14.0%, 13.6%, and 13.6% respectively) (Figure 2.2). Moreover, fish fed for 14 months showed a higher survival compared to fish with a non-probiotic supplemented diet (0 months) after 6-, 7-, 8-, 9-, and 10-dpi (9.7%, 9.8%, 9.8%, 9.4%, and 9.4% respectively) (Figure 2.2). A statistically significant increased survival in fish fed for 10 months with probiotic diet was recorded compared to fish supplemented with probiotic for 4 months after 7-, 8-, 9-, and 10- dpi with live *V. anguillarum* (11.2%, 10.3%, 10.3%, and 9.8% respectively) (Figure 2.2).

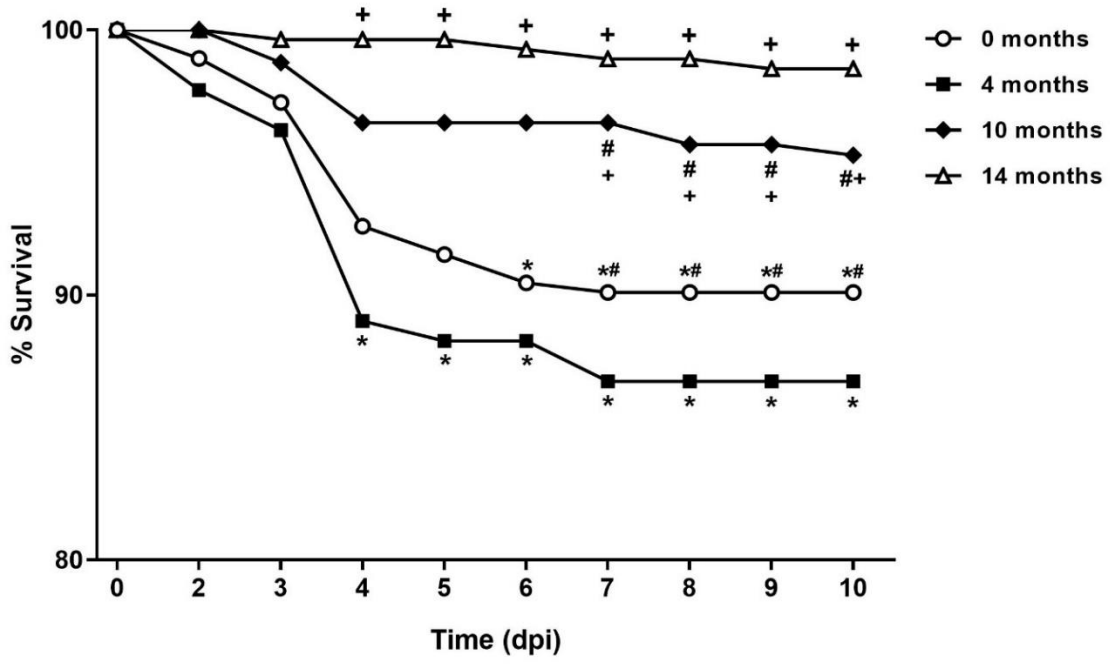


Figure 2.2. Survival of Chinook salmon unsupplemented or supplemented with Jamieson® probiotic during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months) and then challenged with live *Vibrio anguillarum* J382. Two-way ANOVA analysis using one dependent variable % survival (square root conversion for statistical analysis) and the independent variables treatment and time, has shown that survival varied among treatments ($p < 0.0001$), time ($p < 0.0001$), and the interaction between treatments and time ($p < 0.0001$).

After 14 months of Chinook salmon being reared in freshwater (4 months in hatchery) and saltwater (10 months in sea pens), fish weight and length were evaluated before the *V. anguillarum* challenge to determine the effect of probiotic supplementation on growth. The results show that fish weight was not improved after 4 months, 10 months, or 14 months of probiotic supplementation compared to fish fed with regular pellet, the 0 months group (Table 2.2). Similarly, fish length did not show an increase after 4 months, 10 months, or 14 months of Jamieson® supplementation compared to the control group (Table 2.2).

Table 2.2. Effect of Jamieson® probiotic supplementation on body growth (weight and length) in Chinook salmon reared in sea pens (mean ± S.D.).

		Time under probiotic supplementation (months)			
	Days post-injection (dpi)	0	4	10	14
Weight (g)	0	91.6 ± 37.1	140.3 ± 59.6	111.7 ± 35.3	90.0 ± 24.7
Length (cm)	0	22.4 ± 3.0	25.1 ± 3.2	23.2 ± 2.4	22.3 ± 2.2

A one-way ANOVA analysis using the dependent variable growth [weight (g) or length (cm)] and the independent variable treatments, has shown that weight data among treatments ($p < 0.2237$) and length data among treatments ($p < 0.6587$) were not significantly affected.

2.4.2 Head kidney relative gene expression

Transcript levels of innate immune response-related genes were evaluated by qPCR in head kidney samples of fish infected with *V. anguillarum* (Figure 2.3). A significant increase in the transcript expression of *il10* after 14 days of infection was observed in fish supplemented with probiotic for 10 months compared to the control group (Figure 2.3c). The relative expression of antimicrobial peptide (AMP) encoding genes showed different expression patterns. After three days post-infection (dpi), a down-regulation of the *camp* gene in 10 months probiotic-supplemented fish was observed compared to the non-probiotic-fed group (Figure 2.3e). Moreover, *camp* encoding transcripts in 10-month probiotic-supplemented fish showed a down-regulation compared to 4- and 14-month Jamieson®-supplemented groups at 14 dpi (Figure 2.3e). In contrast, an increased relative expression of *hamp* gene expression was observed in fish

supplemented with probiotics for 4 months compared to the control (Figure 2.3f). Transcripts encoding the expression of *il1b*, *il8*, *tnfa*, and *transferrin* did not show statistically significant differences between different probiotic supplementation time points and the non-probiotic fed group (Figure 2.3a, b, d, and g).

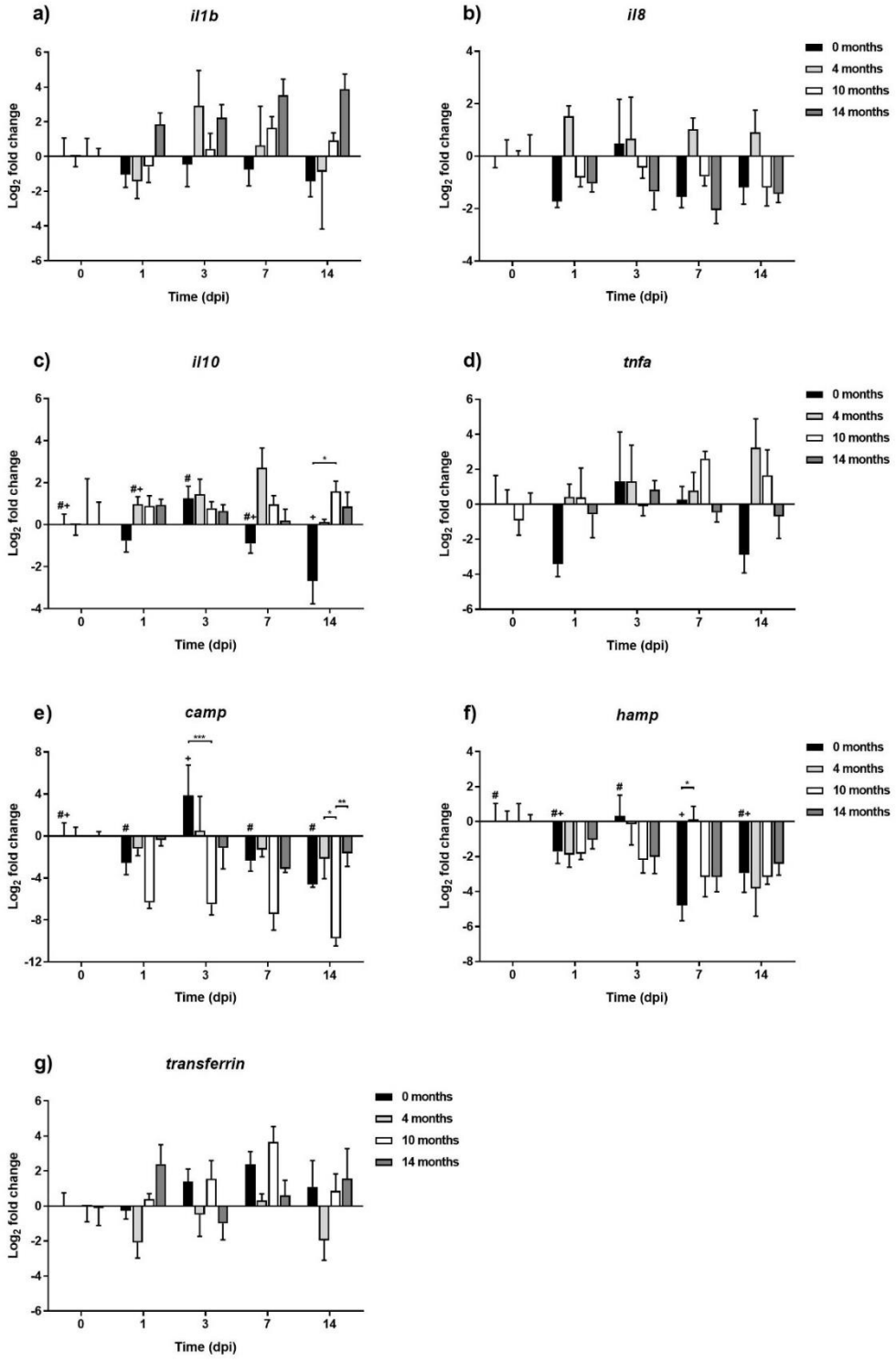

a)
il1b
b)
il8
c)
il10
d)
tnfa
e)
camp
f)
hamp
g)
transferrin
0 months
4 months
10 months
14 months
Log2 fold change
Time (dpi)
0
1
3
7
14

Figure 2.3. Head kidney gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) tumor necrosis factor alpha (*tnfa*), (e) cathelicidin (*camp*), (f) hepcidin (*hamp*), and (g) transferrin, in Chinook salmon unsupplemented or supplemented with Jamieson® probiotic during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months) and then challenged with live *Vibrio anguillarum* J382. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using elongation factor 1 alpha (*ef1a*), beta actin (*actb*) as internal reference genes. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*il10*: $p < 0.0035$; *camp*: $p < 0.0001$) and time (*camp*: $p < 0.0001$; *hamp*: $p < 0.0001$).

2.4.3 Spleen relative gene expression

The relative expression of Chinook salmon immune-related genes was also evaluated by qPCR in the spleen (Figure 2.4). In this tissue, at 3 dpi, fish supplemented for 4 months with probiotics showed a statistically significant increase in the relative expression of *il1b* (Figure 2.4a) and *tnfa* (Figure 2.4d) compared to fish supplemented with Jamieson® probiotic for 10 months (Figure 2.4a and d). The transcripts encoding the expression of *il10* were upregulated in individuals supplemented for 14 months with probiotic compared to 10-months supplemented fish at 14 dpi (Figure 2.4c). Additionally, an up-regulation in the expression of *il10* was observed in 14-month supplemented fish at 7 and 14 dpi compared to fish sampled after 3 dpi (Figure 2.4c). Gene transcription of the AMP *camp* was up-regulated in fish supplemented with Jamieson® probiotic for 14 months at 1 and 7 dpi, compared with the time-matched 10-months and 4-months supplemented groups respectively (Figure 2.4e). In contrast, the transcriptional expression of *camp* showed an increase at 3 dpi in fish supplemented for 4 months with probiotics compared with non-supplemented and 10-month probiotic-supplemented fish at the same time-point (figure 2.4e). Fish supplemented for 4 months with probiotic also showed a significant increase of *camp* expression

at 3 dpi compared to fish from 0, 7, and 14 dpi under the same supplementation treatment (Figure 2.4e). In fish supplemented with probiotics over 4 months, a statistically significant increase after 3 days of infection compared to day 0 was observed in the transcripts encoding *hamp* (Figure 2.4f). Finally, no significant differences in the expression of *il8* (Figure 2.4b) and *transferrin* (Figure 2.4g) were observed (Figure 2.4).

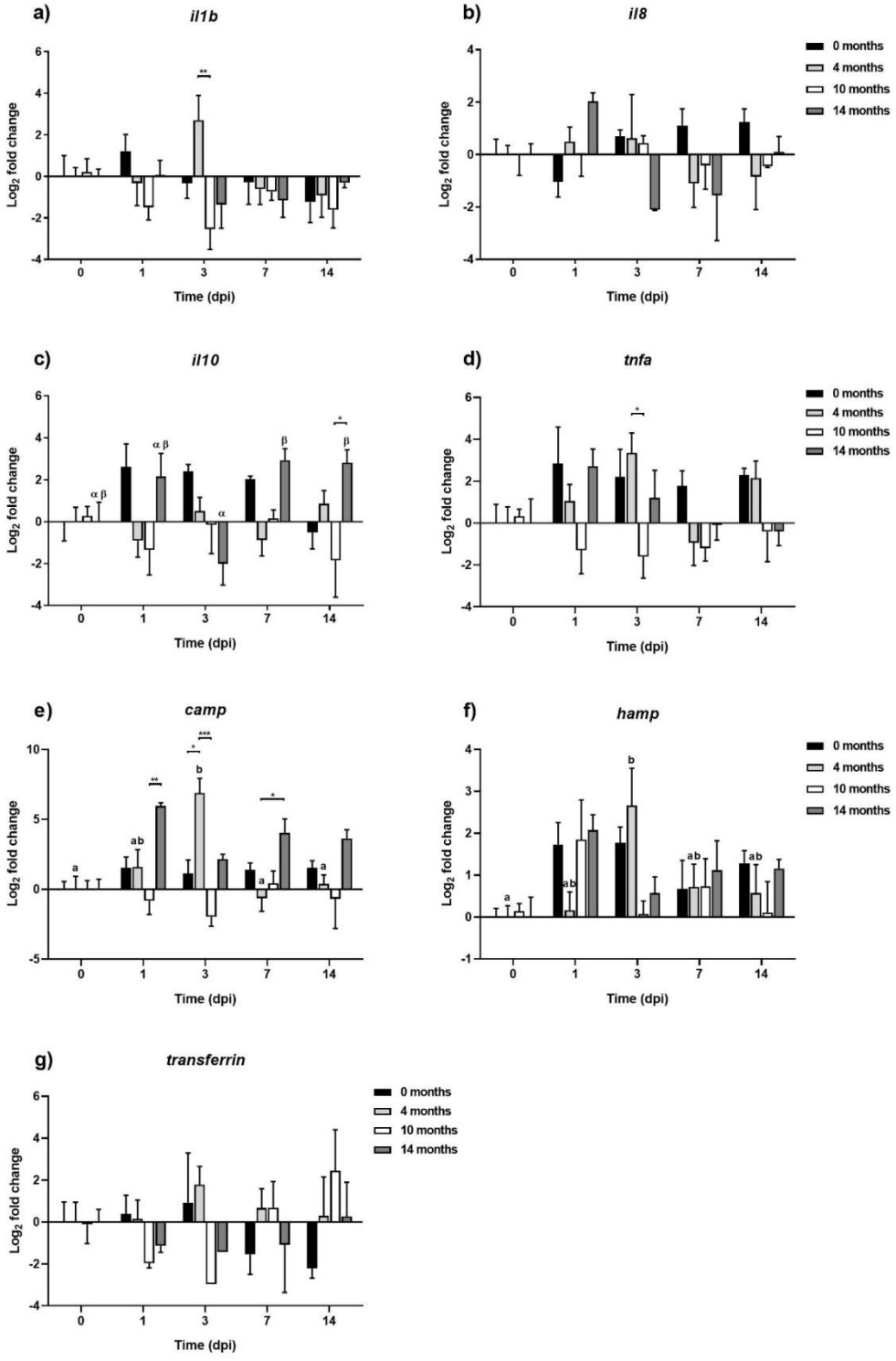


Figure 2.4. Spleen gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) tumor necrosis factor alpha (*tnfa*), (e) cathelicidin (*camp*), (f) hepcidin (*hamp*), and (g) transferrin, in Chinook salmon unsupplemented or supplemented with Jamieson®

probiotic during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months) and then challenged with live *Vibrio anguillarum* J382. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using elongation factor 1 alpha (*ef1a*), beta actin (*actb*) as internal reference genes. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*il10*: $p < 0.0015$; *tnfa*: $p < 0.0004$; *camp*: $p < 0.0001$), time (*camp*: $p < 0.0202$; *hamp*: $p < 0.0037$), and the interaction between treatments and time (*il10*: $p < 0.0006$; *camp*: $p < 0.0005$; *hamp*: $p < 0.0423$).

2.4.4 Hindgut relative gene expression

In the experiments conducted for fish supplemented with Jamieson® probiotic, a significant increase in the hindgut transcript expression of *il1b* was observed in 4-month supplemented individual at 3 dpi compared to time-matched 10-month and 14-month treatments (Figures 2.5a). Additionally, a significant increase of *il1b* was observed in fish fed with probiotics over 4 months after 3 dpi with *V. anguillarum* compared to individuals from the same treatment at 0 and 1 dpi (Figure 2.5a). The relative expression of the pro-inflammatory chemokine *il8* showed a statistically significant increase in 10-month supplemented fish compared to non-probiotic-fed fish at 7 dpi (Figure 2.5b). Also, a significant up-regulation of *il8* in 10-month supplemented individuals was observed in 7 dpi compared to non-injected fish at day 0 (Figure 2.5b). Similarly, an up-regulation of *il8* 7 dpi was observed in fish supplemented for 14 months with Jamieson® probiotic compared to fish sampled at 3 dpi (Figure 2.5b). The individuals supplemented with probiotics did not show significant differences of *tnfa* gene transcription among treatments at the same time point (Figure 2.5d). Nevertheless, transcripts encoding the expression of *tnfa* showed a significant increase in the unsupplemented fish at 14 dpi compared to non-infected fish at the same time point (Figure 2.5d). Also, *tnfa* relative expression in fish supplemented with probiotic for 14 months was

significantly up-regulated compared to individuals under the same supplementation treatment after 1 dpi (Figure 2.5d).

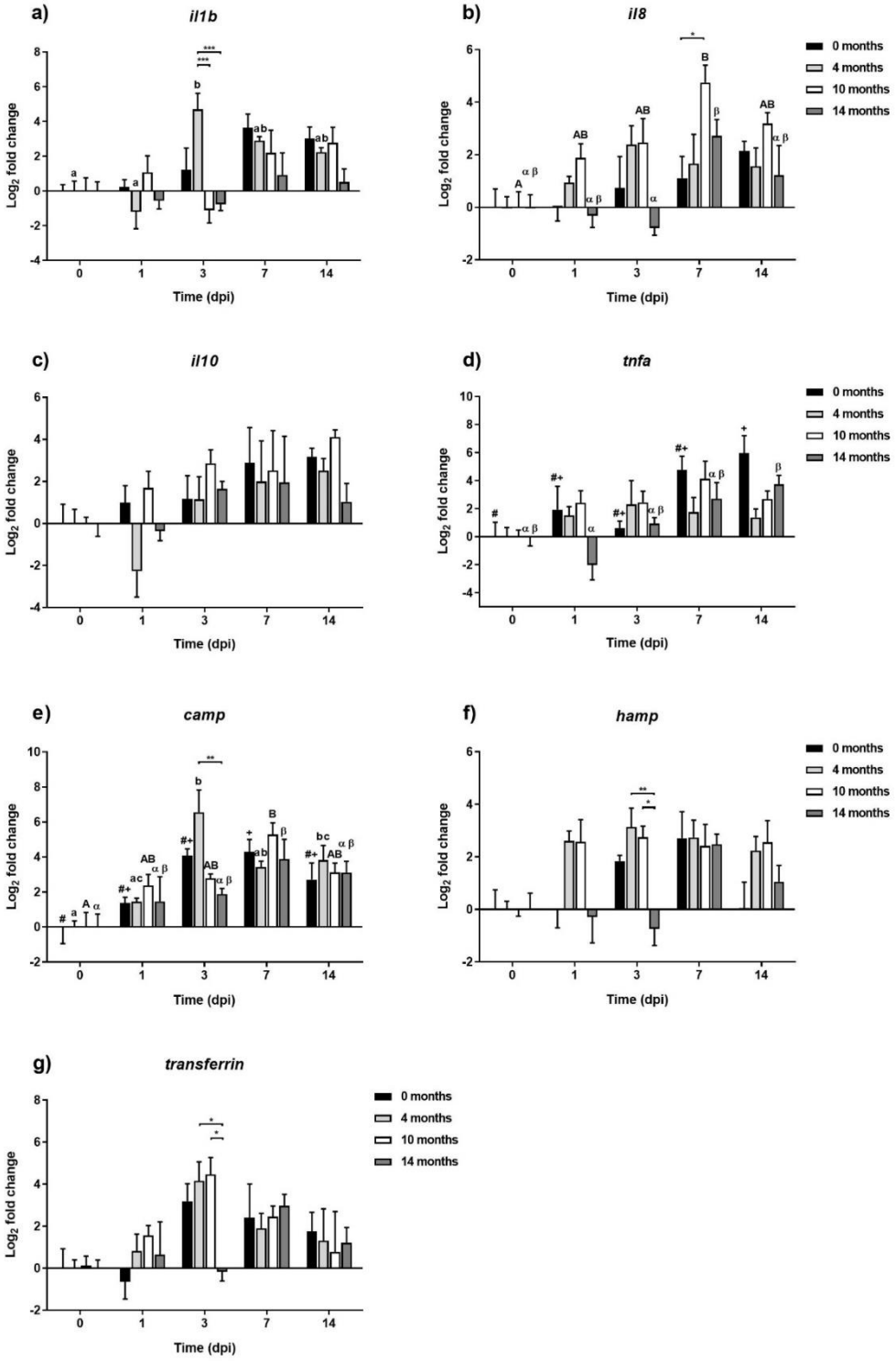


Figure 2.5. Hindgut gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) tumor necrosis factor alpha (*tnfa*), (e) cathelicidin (*camp*), (f) hepcidin (*hamp*), and (g) transferrin, in Chinook salmon unsupplemented or supplemented with Jamieson® probiotic during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months) and then challenged with live *Vibrio anguillarum* J382. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using elongation factor 1 alpha (*ef1a*), beta actin (*actb*) as internal reference genes. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*il1b*: $p < 0.0025$; *il8*: $p < 0.0001$; *tnfa*: $p < 0.0412$; *hamp*: $p < 0.0002$; *transferrin*: $p < 0.0002$), time (*il1b*: $p < 0.0001$; *il8*: $p < 0.0001$; *tnfa*: $p < 0.0001$; *camp*: $p < 0.0001$; *hamp*: $p < 0.0037$), and the interaction between treatments and time (*il1b*: $p < 0.0006$; *tnfa*: $p < 0.0461$; *camp*: $p < 0.0474$).

The AMPs genes evaluated in this study showed a significant up-regulation at 3 dpi between treatments (Figure 2.5e-g). For instance, the transcriptional expression of *camp* was up-regulated in fish supplemented for 4 months with probiotics compared to 14-month supplemented specimens (Figure 2.5e). Moreover, an increase of *hamp* and *transferrin* expression in hindgut was observed after 4 and 10 months of supplementation with probiotic compared to 14-month supplemented fish after 3 dpi (Figure 2.5 f,g). When determining differences in the same treatment at different infection time points, statistically significant differences were only observed in the transcripts encoding the expression of *camp* gene (Figure 2.5e). In fish fed with a regular diet, an up-regulation of *camp* was observed after 7 dpi with *V. anguillarum* compared to day 0 (Figure 2.5e). Similarly, in fish supplemented with probiotics for 10 and 14 months, a significant increase of *camp* gene was determined at 7 dpi compared to day 0 fish (Figure 2.5e). The *camp* gene transcription in 4-month supplemented fish showed an up-regulation at 7 dpi compared to days 0 and 1 (Figure 2.5e).

To evaluate the effect of multi-strain probiotic supplementation in Chinook salmon gut integrity, the expression of tight junction molecules was evaluated (Figure 2.6). Our results showed that fluctuations only occurred in the gene transcription of *cldn1* (Figure 2.6a), *ocln* (Figure 2.6d), and *zo-1* (Figure 2.6e). Transcripts encoding the expression of *cldn1*, a member of the tight junctions, was significantly up-regulated in fish supplemented with probiotics for 10 months at 3 and 7 dpi compared to 14-month supplemented fish. Moreover, 10-month supplemented individuals were up-regulated 7 dpi compared to unsupplemented and 4-month supplemented treatments (Figure 2.6a). Differences in the same treatment at different infection time points showed that 10 months of supplementation with probiotic increased the expression of *cldn1* 7 dpi compared to day 0 and 14 (Figure 2.6a). The individuals supplemented with probiotics for 10 months showed a statistically significant increase in the expression of *ocln* at 1, 3, and 7 dpi compared to 14-month supplemented fish (Figure 2.6d). In contrast, fish fed with probiotic for 4 months showed an up-regulation in the transcriptional expression of *ocln* compared to non-supplemented fish at 14 dpi (Figure 2.6d). Comparisons of the same treatment at different inoculation time points showed that *ocln* was significantly up-regulated after 7 and 14 dpi compared to day 0 in fish supplemented with probiotics for 4 months (Figure 2.6d). On the other hand, fish supplemented with probiotics for 10 months showed a significant increase in the transcripts encoding *ocln* at 3 and 7 dpi compared to day 0 (Figure 2.6d). In this study, the transcriptional expression of the *zo-1* gene was significantly up-regulated in non-probiotic-supplemented fish hindguts after 7 days of infection compared to the day 0 group (Figure 2.6e). Results obtained in this experiment showed that transcripts encoding the expression of *il10* (Figure 2.5c), *cldn3* (Figure 2.6b), and *cldn12* (Figure 2.6c) did not show statistically significant

differences between treatments at the same time point and within each treatment at different times of infection.

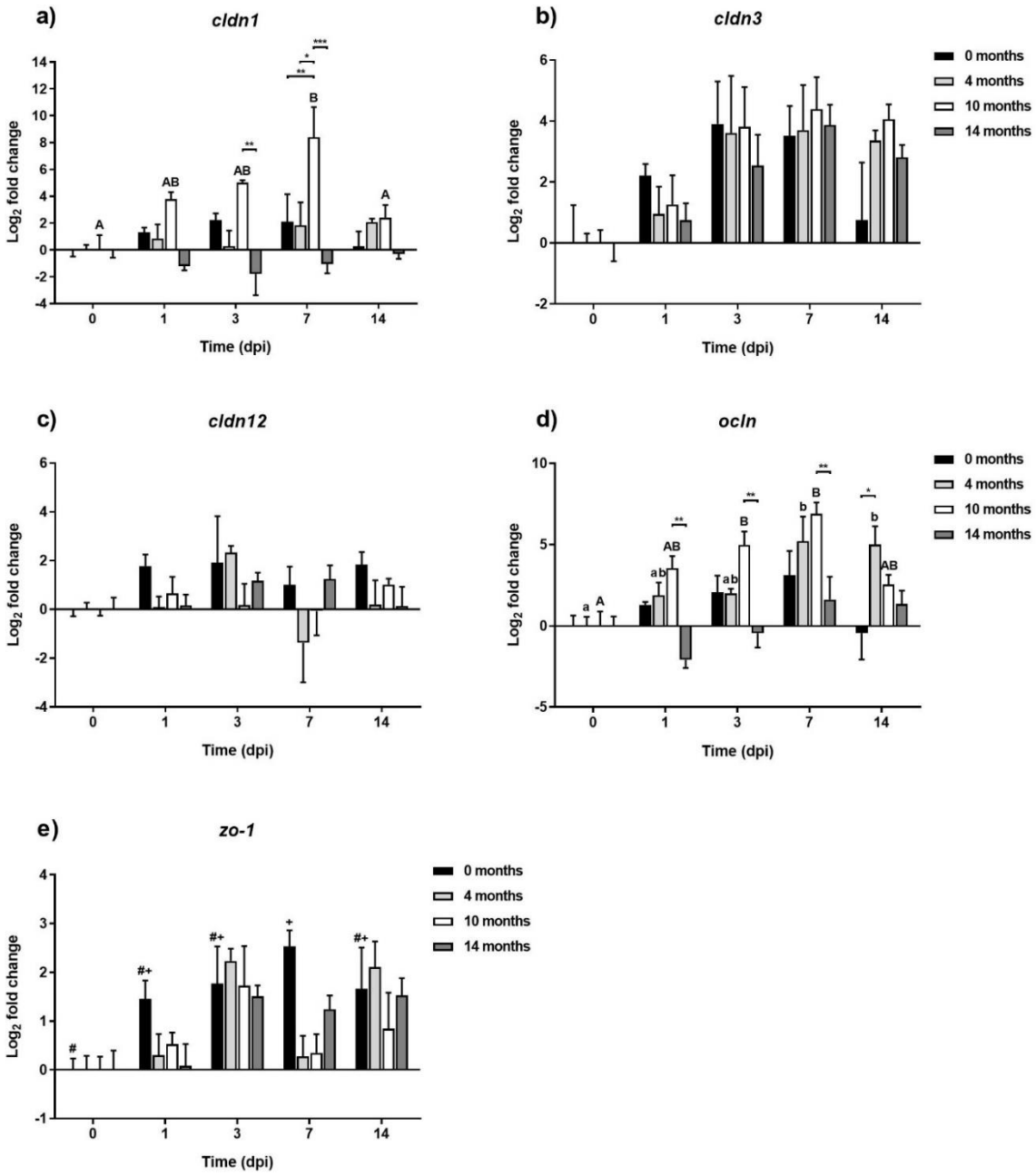


Figure 2.6. Hindgut gene expression of (a) claudin 1 (*cldn1*), (b) claudin 3 (*cldn3*), (c) claudin 12 (*cldn12*), (d) occludin (*ocln*), and (e) zonula occludens 1 (*zo-1*), in Chinook salmon unsupplemented or supplemented with Jamieson® probiotic during hatchery time (freshwater; 4

months), sea pen time (saltwater; 10 months), or both (14 months) and then challenged with live *Vibrio anguillarum* J382. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and Log_2 converted using elongation factor 1 alpha (*ef1a*), beta actin (*actb*) as internal reference genes. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*cldn1*: $p < 0.0001$; *ocln*: $p < 0.0001$), time (*cldn1*: $p < 0.0119$; *ocln*: $p < 0.0001$; *zo-1*: $p < 0.0001$), and the interaction between treatments and time (*cldn1*: $p < 0.0132$; *ocln*: $p < 0.0092$).

2.4.5 IgM and IgT protein levels

To determine the protein levels of IgM and IgT in the plasma, hindgut, spleen, and head kidney of Chinook salmon supplemented for 4, 10, and 14 months with probiotic under aquaculture conditions, a commercial IgM antibody and an in-house (in collaboration with Somru BioScience) IgT antibody were utilized. To determine the specificity of anti-IgT antibody in Chinook salmon tissues, a western blot analysis in plasma and spleen was conducted (Figure 2.7). Western blotting showed that, under reduced conditions in both tissues, a protein with a molecular mass of ~60-65 kDa was present corresponding to the expected size of the IgT heavy chain.

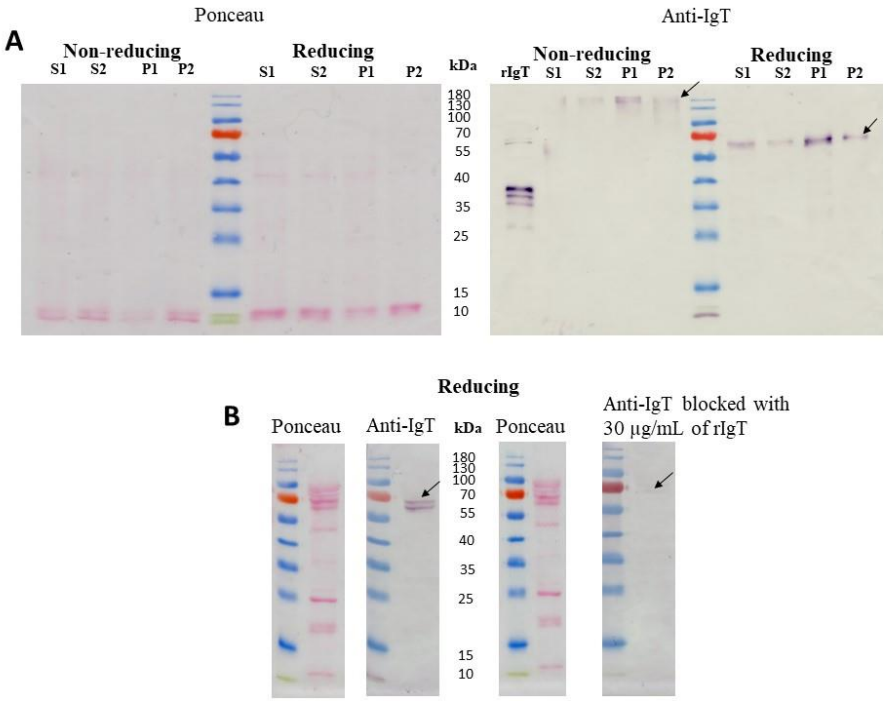


Figure 2.7. The presence of IgT in plasma (P1, P2) and spleen (S1, S2) samples were analyzed by western blot under reducing and non-reducing conditions. A: Twenty micrograms of total proteins from spleen and plasma were run using SDS-PAGE, transferred to a nitrocellulose membrane, stained with Ponceau's stain, and labelled with a polyclonal chicken anti-IgT antibody. Under non-reducing conditions the arrow shows a band with high molecular weight (~180 kDa) consistent with the size of the unreduced form of IgT. Under reducing conditions the arrow shows bands between 55 and 70 kDa corresponding with the expected size of the heavy chain of rainbow trout IgT (60-66 kDa). Recombinant IgT (37 kDa of rainbow trout IgT heavy chain) was used as a positive control. B: To test the specificity of the assay, one sample was run twice and labelled with either the anti-IgT antibody or the anti-IgT antibody mixed with 30 µg/mL of recombinant protein (rIgT). The reactivity of the blocked anti-IgT antibody decreased considerably showing specificity of the antibody to IgT.

The total plasma, hindgut, spleen, and head kidney IgM and IgT levels were measured in all the samples obtained from fish supplemented 14 months with either regular diet- or probiotic (4, 10, or 14 months) (Figure 2.8). As for the total plasma proteins, there were no significant differences in plasma IgM between the non-probiotic-supplemented fish and fish supplemented for 4, 10, and 14 months with probiotic (Figure 2.8a). In contrast, a statistically significant increase in the total levels of IgT was observed in 4-month supplemented fish compared to fish fed for 10

months with probiotics (Figure 2.8b). Total levels of IgM and IgT evaluated in head kidney (Figure 2.8c and 2.8d), spleen (Figure 2.8e and 2.8f), and hindgut (Figure 2.8g and 2.8h) did not show statistically significant differences among treatments. Based on the ODs obtained in the indirect ELISA conducted in this study, we determined that total IgM levels are much higher than total IgT in plasma (Figure 2.8a and 2.8b), and similar in head kidney (Figure 2.8c and 2.8d) and spleen (Figure 2.8e and 2.8f). In contrast, total IgM levels detected in Chinook salmon hindgut were lower than the ones obtained for total IgT (Figure 2.8g and 2.8h).

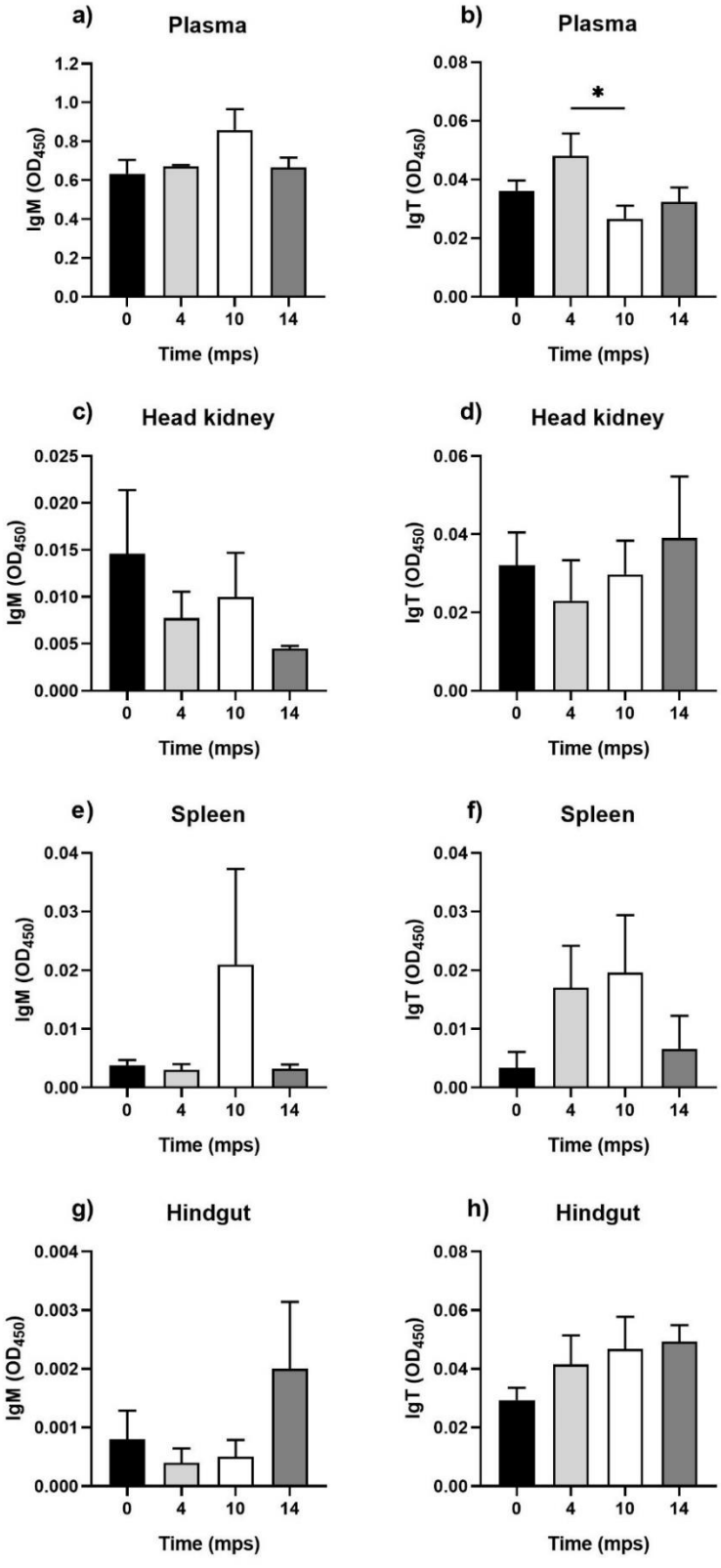

a)
Plasma
IgM (OD450)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
4
10
14
Time (mps)
b)
Plasma
IgT (OD450)
0.08
0.06
0.04
0.02
0.00
*
0
4
10
14
Time (mps)
c)
Head kidney
IgM (OD450)
0.025
0.020
0.015
0.010
0.005
0.000
0
4
10
14
Time (mps)
d)
Head kidney
IgT (OD450)
0.06
0.04
0.02
0.00
0
4
10
14
Time (mps)
e)
Spleen
IgM (OD450)
0.04
0.03
0.02
0.01
0.00
0
4
10
14
Time (mps)
f)
Spleen
IgT (OD450)
0.04
0.03
0.02
0.01
0.00
0
4
10
14
Time (mps)
g)
Hindgut
IgM (OD450)
0.004
0.003
0.002
0.001
0.000
0
4
10
14
Time (mps)
h)
Hindgut
IgT (OD450)
0.08
0.06
0.04
0.02
0.00
0
4
10
14
Time (mps)

Figure 2.8. Indirect ELISA analysis of total IgM and IgT in plasma (a and b), head kidney (c and d), spleen (e and f), and hindgut (g and h), in Chinook salmon unsupplemented or supplemented with Jamieson® probiotic during hatchery time (freshwater; 4 months), sea pen time (saltwater; 10 months), or both (14 months). Each value is the mean ± S.E.M (n = 5). Bars indicate statistically significantly differences in protein concentrations among treatments (*p > 0.05, **p > 0.01, ***p > 0.001). One-way ANOVA analysis using the dependent variable protein level (OD_{450}) was not affected among treatments.

2.5 Discussion

Currently, the use of probiotics has shown potential as an efficient ecologically safe method to improve the growth, immunity, disease resistance, and the diversity of the intestinal microbial community in farmed salmonids species such as Atlantic salmon and rainbow trout (Balcázar et al., 2007; Castro and Tafalla, 2015; Lazado and Caipang, 2014; Nayak, 2010; Nikoskelainen et al., 2003; Oliva-Teles, 2012; Robertson et al., 2000; Salinas et al., 2008a). However, its benefits in low-scale salmonid aquaculture species such as Chinook salmon have not been fully investigated. Due to this, our study evaluated the effect of a multi-strain probiotic, Jamieson®, on growth, survival, and immune response of Chinook salmon raised in an aquaculture facility. Additionally, we investigated how probiotic supplementation at different production stages affects the above-mentioned parameters.

To date, Chinook salmon production continues to face high mortalities associated with disease susceptibility (Olson and Paiya, 2013; Semple et al., 2022). Our results show that fish supplemented with Jamieson® probiotic for 14 months (freshwater and saltwater cycle) significantly decreased ~10% of the mortality caused by *V. anguillarum* compared to fish receiving regular feed (Figure 2.2). Although the reason why the fish survival was higher in the 14-months supplemented fish compared to the control and the 4-months supplemented treatments requires further investigation, the indirect regulation of the structure and function of the gut microbial

community, such as the limitation of mucosal surfaces for pathogen adherence, the direct antagonism through the secretion of antimicrobial molecules, or the nutrient exclusion due to competition, seems to be the most probably causes to explain this finding (Galindo-villegas, 2015; Langlois et al., 2021; Merrifield and Carnevali, 2014; Ringø et al., 2020). On the other hand, fish supplemented for 4 months with the same multi-strain probiotic diet showed an increased mortality compared to the 10- and 14-months supplemented treatments (Figure 2.2). In salmonids aquaculture, the transfer from hatchery (freshwater) to the sea-pens (saltwater) represents a stressful stage in the life cycle, leading to an increased disease susceptibility. Due to this, the utilization of immunostimulants, such as probiotics, can improve the nutrient digestion, absorption, and immunological barrier function of the gut during the transition (Jaramillo-Torres et al., 2019; Langlois et al., 2021; Soto-Dávila et al., 2020a). The evidence obtained in this study supports the need for supplementation with probiotics not only during the hatchery stage of Chinook salmon, but also while, perhaps especially when, the fish are raised in the sea-pens.

In addition to fish survival, growth represents one of the most important variables in aquaculture from a production perspective. Available data has shown that supplementation of salmonids with members of the lactic acid bacteria (LAB), *Bacillus*, and other beneficial microorganisms in closed experimental conditions or RAS systems, can result in a positive influence on growth parameters after 8-10 weeks (Dawood et al., 2018; Nikoskelainen et al., 2001; Niu et al., 2019; Vendrell et al., 2008; Wang et al., 2019). However, our results did not show differences between treatments even though the fish were supplemented for a minimum of 4 months and a maximum of 14 months (Table 2.2). The lack of differences in growth parameters between treatments can be explained by the large size variability of fish sampled in this study. Although the study started with individuals of similar size for every treatment (9 ± 2 g body

weight), factors like feeding uptake, saltwater adaptation, physiological stage of smoltification, predation, and diseases, might have randomly impacted the fish in netpens and have masked the effects of the probiotic supplementation (Fritts et al., 2007; Mahnken and Waknitz, 1979). Although we expected to see an impact of probiotic supplementation in growth, the data collected in this study show an accurate picture of the interaction between the host, the probiotics, and the biotic and abiotic factors during Chinook salmon production.

To determine the effects that Jamieson® probiotic supplementation had on the immune parameters and gut integrity of Chinook salmon infected with *V. anguillarum*, the relative expression profile in head kidney, spleen, and hindgut was assessed by qPCR. In head kidney, a tissue with a major role in the inflammatory response and immune regulation (Jalili et al., 2020; Joerink et al., 2006), no statistically significant differences were observed in the expression of the proinflammatory cytokines *il1b* and *tnfa* (Figure 2.3a and 2.3d), and the chemokine *il8* (Figure 2.3b). Although it has been previously demonstrated that the transcripts encoding *il1b*, *il8*, and *tnfa* in Chinook salmon increases after infection with *V. anguillarum* (Ching et al., 2010; Semple et al., 2022), we were not able to determine a differential modulation of these genes after probiotic supplementation in freshwater, saltwater or both production stages (Figure 2.3a, b, and d). In contrast, the relative expression of the anti-inflammatory cytokine *il10* showed a significant decrease 14 dpi compared to 3 dpi in the regular feed treatment (Figure 2.3c). Despite that a statistically significant difference was not observed in the transcripts encoding *il1b* (Figure 2.3a) and *il8* (Figure 2.3b), the *il10* results seems to be associated with a cease in the anti-inflammatory response after 7 days of infection with *V. anguillarum* in the regular fed group (Ching et al., 2010; Jamal et al., 2020; Lokesh et al., 2012; Magnadóttir, 2006).

Antimicrobial peptide activity was also evaluated in head kidney by measuring the expression of cathelicidin (*camp*) (Figure 2.3e), hepcidin (*hamp*) (Figure 2.3f), and *transferrin* (Figure 2.3g).The results obtained did not show any up-regulation associated with the probiotic supplementation during the production cycle compared to fish infected with *V. anguillarum* that were fed with the regular diet (Figures 2.3e, f, and g). Previous studies have shown an increase in the antimicrobial activity against *V. anguillarum* infection (Álvarez et al., 2016; Broekman et al., 2013; Maier et al., 2008). Therefore, it seems to be that, even though the infection of *V. anguillarum* induced a host response occurred during our study, this was not enhanced by the supplementation with Jamieson® probiotic for 4, 10, or 14 months compared to the diet control group.

Interestingly, in comparison to the response observed in head kidney, the spleen showed some correlations that help to explain the survival curve obtained in this study (Figure 2.4). During the infection trial, fish supplemented for 4 months with the multi-strain probiotic showed a significantly higher mortality than fish supplemented with the same product for 10 and 14 months (Figure 2.2). The statistically significant up-regulation of *il1b* (Figure 2.4a), *tnfa* (Figure 2.4d), and *camp* (Figure 2.4e) in fish supplemented for 4 months compared to 10-months Jamieson® probiotic fed fish, suggest that probiotic supplementation exclusively during the salmonid freshwater cycle may have a negative effect when fish are infected with *V. anguillarum*, resulting in an increased disease susceptibility, inflammation, and innate immune response. Although the dietary probiotic supplementation has mostly been reported as beneficial in salmonids (Nikoskelainen et al., 2001; Ringø and Olsen, 2011; Salinas et al., 2008b, 2008a), the transfer from freshwater to seawater has a substantial impact on the bacterial communities and host homeostasis of the gut, which may increase salmonids' susceptibility to infectious disease (Jaramillo-Torres et al., 2019). To prevent this, our study suggests that fish must be supplemented with Jamieson®

probiotic in both production cycles, emphasizing the supplementation efforts in the saltwater stage. In fish supplemented for 14 months with a multi-strain probiotic, we observed an up-regulation of the transcripts encoding *il10* after 7 and 14 dpi compared to 3 dpi (figure 2.4c). This response observed in the treatment with the best survival suggests that one of the mechanisms that can lead to a lower mortality is associated with the reduction of the inflammatory response in spleen to avoid lethal sepsis (Semple et al., 2022).

Available literature supports the importance of probiotics in the gut immune response of fish, as studies have shown that different immunological parameters can be modulated by probiotic feeding (Hoseinifar et al., 2018; Jamal et al., 2020; Langlois et al., 2021; Patel et al., 2012; Ringø et al., 2007). In the innate immune response, a controlled inflammation of the gut plays a key role in protecting the host from pathogens (Morimoto et al., 2021). In fish supplemented for 4 months with Jamieson® probiotic, we were able to determine an increased inflammatory response compared to 10- and 14-months supplemented fish (Figure 2.55a). Since this group also showed higher mortality among probiotic-supplemented treatments (figure 2.2), we suggest that the use of probiotics in this group did not confer a protection but instead developed a negative interaction while supplementation in freshwater conditions that resulted in an increased and sustained inflammatory response during the infection. A well-regulated inflammatory response is critical for optimal immune responses and survival (Haddad et al., 2023). In addition to this, our study proposes that supplementation with the multi-strain probiotic for 10 months might be able to delay the pathogenesis of *V. anguillarum* since the expression of *il8* was up-regulated after 7 days of infection compared to the control group, as well as an increase in expression compared to day 0 (Figure 2.5b). The transcripts encoding the antimicrobial peptides *camp* (Figure 2.5e), *hamp* (figure 2.5f), and *transferrin* (Figure 2.5g) showed an up-regulation after 3 days of infection with

V. anguillarum in 4-months supplemented fish (*camp*), or 4- and 10-months supplemented fish (*hamp* and *transferrin*) compared to fish supplemented with Jamieson® probiotic for 14 months (Figures 2.5e, f, and g). As mentioned above, these antimicrobial peptides are important during bacterial infection, acting against the structure and function of the microbial cell membranes (Álvarez et al., 2016; Broekman et al., 2013; Ellis, 1999; Soto-Dávila et al., 2019). On the other hand, in the supplementation treatment with the best survival of this trial, the 14-month supplemented fish, we observed that an increased antimicrobial peptide response was not required for survival (Figures 2.5e, f, and g). We propose that even though *camp*, *hamp*, and *transferrin* can play an important role in pathogen inactivation in Chinook salmon, additional defenses needed against *V. anguillarum* can be exerted by the probiotic strains located in the gut of 14-months supplemented fish, as it has been reported that probiotics have antimicrobial activity with the production of bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases, organic acids and volatile short-chain fatty acids in the host (Jamal et al., 2020; Melo-Bolívar et al., 2021). To elucidate this, future studies should focus in determining the number of probiotic strains that could colonize the gut of Chinook salmon after the different supplementation times and water conditions, emphasizing and further understating their antimicrobial activity against *V. anguillarum*.

In addition to the improvement of gut immunity, the maintenance of the intestinal mucosal barrier integrity is maintained by a superficial mucous layer, epithelial cells, and tight junction proteins (De et al., 2014; Gatesoupe, 1999; Ringø et al., 2012; Ringo and Birkbeck, 1999). Tight junction proteins, such as claudins, occludins, and zonula occludens 1, have shown to be improved by probiotic supplementation, denying pathogens access to the subepithelial cells (Langlois et al., 2021; Patel et al., 2012; Ukena et al., 2007). To date, few studies conducted in fish have focused on the modulation of tight junctions after probiotic supplementation followed by a disease

challenge (Ringø et al., 2007; Vasanth et al., 2015). Moreover, no evidence of the effect of probiotics on tight junctions has been reported in Chinook salmon. The results obtained in this study showed a significant up-regulation of *cldn1* in fish supplemented for 10 months with Jamieson® probiotic compared to the control group, or the 4- and 14-months supplemented fish (Figure 2.6a). Furthermore, *ocln* of 10-months supplemented fish was significantly up-regulated after 1, 3, and 7 dpi with *V. anguillarum* compared to 14-months supplemented fish, meanwhile fish supplemented with Jamieson® probiotic for 4 months up-regulated their expression 14 dpi (Figure 2.6d). Even though the precise mechanism of action remains to be elucidated, the decreased expression in fish supplemented for 14 months compared to 4- and 10-months supplemented fish, suggests that probiotic treatment during both the freshwater and saltwater cycle confers a better protection and intestinal integrity, resulting in a better survival.

In teleost fish, the recognition of the molecular structure of an antigen acquired after first contact with a pathogen, also known as immunological memory, results in T and B cell responses (Castro and Tafalla, 2015; Stosik et al., 2021; Yamaguchi et al., 2019). The latter, B cells, produces immunoglobulins (Igs) and display them on their cell surface (Chen and Cerutti, 2011; Rombout et al., 2014; Soto-Dávila et al., 2020a; Zhang et al., 2010). To date, it has been described that teleost B cells can express 3 different Ig isotypes, IgM, IgD, and IgT (Mashoof and Criscitiello, 2016; Stosik et al., 2021). IgM is the main systemic isotype with a high concentration in fish blood, and it plays a crucial role in pathogen opsonization and neutralization (Cuesta et al., 2004; Nikoskelainen et al., 2003). An increase in serum IgM has been reported in several aquaculture fish species (e.g. common carp, rohu, rainbow trout, catfish) (Hoseinifar et al., 2019; Nayak, 2010; Ringø et al., 2020) following probiotic supplementation. Nevertheless, in our study, no differences between probiotic-supplemented fish and the control treatment were observed in the IgM levels of

plasma (Figure 2.8a), head kidney (Figure 2.8c), spleen (Figure 2.8e), and hindgut (Figure 2.8g). Since fish were raised for 10 months in sea-pens before sampling for IgM detection, previous encounters with different pathogens could have generated variation among fish IgM levels, impacting the determination of differences associated with the probiotic supplementation. Additionally, fish require between 8 to 10 weeks to mount a strong memory response associated to Igs, thus, differences in time when the fish have encountered other pathogens in the natural environment would affect the determination on the Igs level evaluated after probiotic supplementation. Because of this, further research would focus on determining the levels of specific IgM against *V. anguillarum* J382 strain by using the bacterial protein extract and Chinook salmon serum collected after 8- and 10-weeks post-infection.

First reported in 2005 in rainbow trout and zebrafish (Danilova et al., 2005; Hansen et al., 2005), IgT (also known as IgZ in zebrafish) has been described as an immunoglobulin specializing in mucosal immunity (Rombout et al., 2011; Tacchi et al., 2014; Zhang et al., 2017, 2010). Due to this, an increased protein secretion of IgT would have been expected in tissues such as hindgut, due to its high mucus concentration. However, the results obtained in this study showed a low detection of IgT in the four tissues evaluated (Figures 2.8b, d, f, and h). It is important to remember that IgT production assessed in this study comes from fish obtained from sea-pens and without a pathogen challenge, thus, we focused in determining the differential IgT protein secretion that probiotic supplementation can exert. However, new studies conducted by our research group are focusing on stimulating the mucosal immunity of the Chinook salmon gut by delivering *V. anguillarum* through oral gavage, to determine the levels of IgT in fish supplemented with probiotics after a disease challenge. Despite the lack of differences associated with probiotic supplementations, this study represents the first IgT protein measurement in Chinook salmon. In

addition to this, our ability to measure IgT in plasma, head kidney, spleen, and hindgut of this species provides a reliable protocol to determine IgT production by indirect ELISA utilizing an in-house prepared antibody for further studies.

2.6 Conclusions

In conclusion, the results presented in this study demonstrate that Jamieson® probiotic supplementation for 14 months (freshwater and saltwater production cycle) had beneficial effects in terms of survival after *V. anguillarum* infection. Although a clear innate immune and gut barrier correlation of survival and the genes evaluated in head kidney, spleen, and hindgut was not detected in our study, the results observed in the 4-months supplemented fish suggest that an actual up-regulation on the gene expression of immune markers, especially inflammatory ones, might not necessarily be perceived as positive, but in some cases as a lack of protection conferred by probiotic colonization in the gut. Further experiments are necessary to understand the precise mechanism within the immune system and gut barrier improvement of Chinook salmon induced by probiotics. Also, the results presented in this study highlight the importance to consider both, the freshwater and saltwater cycle, during probiotic supplementation, as well as the importance in the utilization of multi-strain probiotics as a first approach to evaluate their immunostimulatory effect in novel species, such as Chinook salmon.

Additionally, this study provides for the first time a reliable methodology to evaluate IgT in Chinook salmon tissues by utilizing indirect ELISA and an in-house made anti-IgT. Finally, we have provided novel sequences to evaluate tight junction genes in Chinook salmon for future research, together with the need of evaluate tight junctions in every study associated with dietary immune stimulation.

Chapter 3: The effects of *Pediococcus acidilactici* MA18/5M on growth performance, gut integrity, and immune response using *in vitro* and *in vivo* Pacific salmonid models

3.1 Overview

Pediococcus acidilactici MA18/5M has shown promising results promoting growth, modulation of the immune response, and diseases resistance in many fishes. However, there no studies about the use of *P. acidilactici* MA18/5M in Pacific salmonid models. The aims of this study were to assess the protective effects of *P. acidilactici* MA18/5M by examining gut barrier function and the expression of tight junction (TJ) and immune genes during *in vitro* and *in vivo* studies. To evaluate this, a preliminary assessment utilizing the gut cell line RTgutGC was conducted. Barrier formation and integrity assessed by TEER measurements in RTgutGC, showed a significant decrease in resistance in cells exposed only to *V. anguillarum* for 24 h, but pre-treatment with *P. acidilactici* MA18/5M for 48 h mitigated these effects. While *P. acidilactici* MA18/5M did not significantly upregulate tight junction and immune molecules, pre-treatment with this strain protected against pathogen-induced insults to the gut barrier. In particular, the expression of *occludin* was significantly induced by *V. anguillarum*, suggesting that this molecule might be implicated in the host response against this pathogen. Increases in the gene expression of RTgutGC *il17a* and *tgfb* suggest a response to secreted virulence factors, while *il8* upregulation might be associated to the response to lipopolysaccharides. After the promising results observed in RTgutGC, the effect of *P. acidilactici* MA18/5M was evaluated in Chinook salmon reared in real aquaculture conditions. Supplementation with *P. acidilactici* MA18/5M had no effect on Chinook salmon growth parameters after 10 weeks. Interestingly, histopathological results did not show alterations associated with *P. acidilactici* MA18/5M supplementation, indicating that this strain is safe to be used in the industry. Finally, the expression pattern of transcripts encoding TJ and immune genes in all the treatments suggest that variation in the expression is more likely to be due to developmental processes rather than *P. acidilactici* MA18/5M supplementation. Overall,

our results showed that *P. acidilactici* MA18/5M is a safe strain to be use in fish production, however, to observe the effects in growth and immune response previously observed in other salmonid species, an assessment in adult fish is needed.

3.2 Introduction

In finfish aquaculture, the incorporation of healthy functional constituents into aquafeeds has become a potential tool to improve fish growth, stress tolerance, and disease resistance (Oliva-Teles, 2012; Sakai, 1999b; Teles et al., 2016). Functional food components have been demonstrated to increase the respiratory burst activity, cytokine activity, complement system, phagocytosis, among others (Bridle et al., 2005; Jamal et al., 2020; Nayak, 2010; Song et al., 2014). To date, several immunostimulants and functional feed ingredients have been tested in aquaculture species, among which prebiotics, probiotics, vitamins, and symbiotics are the most commonly studied groups (Akhter et al., 2015; Barman et al., 2013; Lieke et al., 2019; Nayak, 2010; Wang et al., 2017a). Notably, probiotics have been broadly studied in terrestrial and aquatic species and have shown promising results for the pork, poultry, and aquaculture industry (Balcázar et al., 2006; Dawood and Koshio, 2016; Hoseinifar et al., 2018; Jamal et al., 2020; Langlois et al., 2021). This non-polluting and efficient biological alternative to antibiotics is commercially available at low prices, making them a great alternative for large-scale production (Barman et al., 2013; Hoseinifar et al., 2018; Jamal et al., 2020; Wang et al., 2017a).

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). In fish, probiotic use has been shown to improve fish growth and feed conversion rates, modulate the gastrointestinal microbial community and prevent bacterial diseases by competitive exclusion, and contribute to the digestive and nutritional processes of the host (Balcázar et al., 2006; Pérez-Sánchez et al., 2014; Soto-Dávila

et al., 2020a; Wang et al., 2008). Indeed, the fish gut constitutes the largest interface for host-microbe interactions and harbours the most abundant and diverse microbial community that can modulate overall host physiology (Langlois et al., 2021). Therefore, modulation of host health at this interface may prove advantageous in aquaculture systems where optimal fish health is directly associated with profits in this highly margin-sensitive industry.

Recent reports have pointed at the potential of using *Pediococcus acidilactici* MA18/5M in terrestrial livestock as well as marine and freshwater fish, particularly salmonid species (Jaramillo-Torres et al., 2019). In fact, the European Union has approved the use of the probiotic product for aquaculture applications, but data supporting the use of this probiont in North American species has yet to be recognized by the local regulatory agencies (Hoseinifar et al., 2019; Pérez-Sánchez et al., 2014). Moreover, this year the Canadian Food Inspection Agency (CFIA) conducted consultations to evaluate the use of dehydrated *Pediococcus acidilactici* culture for use in gut modifier products for livestock species, such as fish. In salmonids, the administration of this probiotic strain has been associated with a more robust nonspecific immune response (both at the mucosal interface and at a systemic level); growth performance; microbiota composition; and disease resistance (Abid et al., 2013; Jaramillo-Torres et al., 2019; Merrifield et al., 2011).

Salmonids constitute the most economically important family of finfish. In particular, Chinook salmon (*Oncorhynchus tshawytscha*) is the largest species of this family and presents potential economic and environmental advantages compared to farming of its Atlantic counterpart on the Pacific Northwest (Christensen et al., 2018; Houde et al., 2015; Semeniuk et al., 2019; Toews et al., 2019). However, Chinook salmon farming is hampered by the risk of escapees diluting the genetic diversity of wild populations and their poor tolerance to commercial production conditions (Olson and Paiya, 2013; Semple et al., 2022). Sterile triploid salmon

effectively circumvents escapee issues, however these fish exhibit 10-30% higher disease mortality rate compared to diploid fish (Data provided by Yellow Island Aquaculture Ltd.).

The fish gut is thought to be a primary site for pathogen attachment, proliferation, and entry into the bloodstream (Lee et al., 2021; Olsson et al., 1996). Additionally, the intestine serves as the primary site for digestion and nutrient absorption, immune modulation, osmoregulation, and acts as a barrier against pathobionts in the gut (Dawood, 2021; Lee et al., 2021). Notably, *Vibrio anguillarum* is a common aquatic pathogen that afflicts farmed fish worldwide, causing substantial financial losses to the industry (Lafferty et al., 2015). Therefore, investigating mechanisms that can prevent pathogen expansion, bolster gut barrier function and integrity will potentially prevent infections and sustainably improve productivity. There is currently no evidence for the effect of beneficial microbes in Chinook salmon.

Recent reports on a salmonid intestinal epithelial cell line derived from rainbow trout (*Oncorhynchus mykiss*), RTgutGC, have established the usefulness of this cell line as a model for functional studies on fish feed development based on gut barrier function and immune competence (Kawano et al., 2011; Langan et al., 2017; Minghetti et al., 2017; Wang et al., 2019). Furthermore, by employing semipermeable membrane supports (Transwell®), it is possible to recapitulate the intestinal environment *in vitro* and conduct studies on the permeability and integrity of the cell monolayer (Hubatsch et al., 2007).

Specifically, the objectives of the present study are to i) investigate the effect of *P. acidilactici* MA18/5M supplementation on barrier function and the expression of tight junction and immune molecules, ii) determine the extent to which *V. anguillarum* disrupts barrier function, iii) assess whether *P. acidilactici* MA18/5M can protect monolayer integrity against pathogen-

induced insults to the barrier, and iv) examine the effects of *P. acidilactici* MA18/5M on several physiological parameters of juvenile Chinook salmon.

3.3 Materials and Methods

3.3.1 In vitro Pediococcus acidilactici MA18/5M trials

3.3.1.1 Tissue culture maintenance

RTgutGC was cultured in Leibovitz's 15 media (HyClone, Cytiva), supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Life Technologies), and incubated in plates or T75 flasks (Corning, Millipore Sigma) sealed with Parafilm (Bemis) at 22ºC and atmospheric conditions. The medium was replaced every 3-4 days and cells were passaged when ≥80% confluent in a 1:2 to 1:4 subcultivation ratio, depending on the downstream experimental application. Cells were washed with 4 mL sterile PBS at room temperature, and residual buffer was aspirated with a glass Pasteur pipette (Fisher Scientific) connected to a vacuum line. Four mL of trypsin (0.05% w/v; Thermo Fisher Scientific) was then added and incubated for 10 min at room temperature on a flask vortex to facilitate detachment.

Cells were monitored periodically using a Nikon inverted microscope to ensure detachment from the plastic. Upon detachment, 8 mL of complete culture medium (L-15 + 10% FBS) was added to quench the trypsin protease activity. The suspension was then vigorously pipetted to break up clumps of cells, before the transfer of the cells to a 15 mL sterile conical tube from which a sample was taken for viable counting using a trypan blue (0.04%; Thermo Fisher Scientific) exclusion assay and the automated Countess cell counter (Invitrogen) prior to seeding into the cell culture dishes. The medium renewal was performed 24-48 h following trypsinization and seeding into new culture dishes to remove residual trypsin.

3.3.1.2 Bacterial strains and culture conditions

To determine the probiotic effect on RTgutGC, *P. acidilactici* MA18/5M (BioPower® PA, registration number 982989) was utilized. This probiotic strain was generously provided as a lyophilized powder by Lallemand Animal Nutrition Incorporated. *P. acidilactici* MA18/5M was routinely cultured anaerobically at 37ºC in Mann, Rogosa, and Sharpe (MRS) medium (BD Difco) for each of the trials.

For RTgutGC cell stimulation trials, the Gram-negative pathogen *Vibrio anguillarum* was used. *V. anguillarum* J382 (serotype O1) (Machimbirike et al., 2023) isolated from winter Steelhead trout obtained from Little Campbell River (British Columbia, Canada) was utilized for cell stimulation. Briefly, a single colony of *V. anguillarum* was grown in 2.5 mL of Trypticase Soy Broth 2% sodium chloride (TSB 2% NaCl; Multicell Wisent, Quebec, Canada) at 20°C in a 16 mm diameter glass tube and placed in a shaker for 24 h at 200 rpm. After growth, 150 µL of the overnight culture were added to 150 mL of TSB 2% NaCl media using a 250 mL flask and incubated for 24 h at 20°C with aeration (200 rpm). After the overnight culture, the bacterial inoculum was centrifuged at 6,000 rpm at room temperature for 10 min. The pellet was washed thrice with PBS and centrifuged at 6,000 rpm at room temperature for 10 min, and finally resuspended in 25 mL of PBS (~8.6×10^8 CFU mL^{-1}). The concentrated bacterial inoculum was serial diluted and quantified by plating onto TSA 2% NaCl for 48 h. Heat-killed *V. anguillarum* was prepared by transferring 1 mL of this inoculum to a 1.7 mL tube, which was then centrifuged at 10,000 rpm for 8 min at room temperature. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of sterile PBS and incubated at 100ºC for 30 min. Then, 100 µL of the heat-killed suspension was plated in TSA + 2% NaCl in triplicates to ensure sterility.

3.3.1.3 Coculture experiments

RTgutGC cells were cultured in 6- or 12-well plates (BD Falcon) for at least 3 weeks prior to the experiments to ensure that the cells established the brush border membrane and tight junction complexes. Frozen stocks of *P. acidilactici* MA18/5M and *V. anguillarum* were streaked onto agar plates of the appropriate medium and incubated for 24 h. Single colonies were then re-streaked and incubated for another 24 h. Fresh single colonies were used to inoculate 3 mL of the appropriate growth medium and cultures were incubated for 48 h. Assuming that the concentration per area of cells at confluency is approximately 1.3 x 10^5 cells/cm^2, the *P. acidilactici* MA18/5M was diluted to a final multiplicity of bacteria (MOB) of 1:100 gut cells to bacteria, while *V. anguillarum* was diluted to a final concentration of 2:1 MOB. Heat-killed *V. anguillarum* was diluted in like manner. The bacterial suspensions were mixed in the cell culture growth medium, and the spent cell culture medium was aspirated using a sterile glass Pasteur pipette connected to a vacuum line. The bacteria were then added to the RTgutGC cells and incubated for various durations. Cells were then harvested at specific time-points by aspirating the culture medium and adding 1 or 0.5 mL (for 6- or 12-well plates, respectively) of TRIzol reagent (Invitrogen), and removed by vigorously pipetting the RTgutGC cell lysate, which was then transferred to a 1.7 mL tube and stored at 4ºC until further processing.

3.3.1.4 RTgutGC RNA extraction and cDNA synthesis

To prepare RNA for subsequent gene expression analyses, 0.3 volumes of chloroform per 1 volume of TRIzol were added to the RTgutGC cell lysates. Samples were vortexed for 15 s, incubated at room temperature for 10 min, then centrifuged at 16,000 rpm for 15 min at 4ºC. The aqueous layer was collected and transferred to a new 1.7 mL tube. To improve RNA quality, 0.3 volumes of chloroform per 1 volume of the aqueous layer were again added, and this step was

repeated. Samples were kept on ice henceforward. To the new aqueous fractions, 0.7 volumes of 100% isopropanol per 1 volume of the sample were added, vortexed briefly, and incubated at room temperature for 5 min. Samples were then centrifuged at 16,000 rpm for 15 min at 4ºC. The supernatant was discarded, and residual isopropanol was removed with a pipette. Then, 1 mL of 70% ethanol in nuclease-free water (Invitrogen) was added and samples were centrifuged at 16,000 rpm for 15 min at 4ºC. The ethanol was decanted, and this step was repeated to improve RNA quality and remove contaminants. The residual ethanol was carefully removed, and pellets were air-dried for 15 to 20 min. The RNA was then resuspended in 30 µL of warm (56ºC) nuclease-free water and quantified using a NanoDrop spectrophotometer.

The RNA concentration was consistently between 100-500 ng/µL, depending on the size of the well that was used for the experiments. The cDNA was synthesized from 1 µg of the freshly isolated RNA using a High-Capacity cDNA Reverse Transcription Kit, following the manufacturer's instructions (Applied Biosystems), for a total volume of 40 µL per reaction. The remaining RNA was stored at -80ºC.

3.3.1.5 qPCR analysis of RTgutGC samples

Reverse-transcribed cDNA was diluted 10x and used in qPCR reactions with Power SYBR Green Kit (Thermo Fisher Scientific). The primers used in this study are summarized in Table 3.1. For analyses of gene expression, the gene cytochrome c oxidase subunit 6C-1 (*cx6c1*) was used as the reference because it was identified to be the most stable reference gene of those tested. The PBS vehicle control groups were used as the endogenous control in all qPCR experiments. Each qPCR reaction had a total volume of 10 µL (performed in three technical replicates). Reactions consisted of 4.58 µL of diluted cDNA, 0.42 µL of primers (forward and reverse primer mix; 14.4 µM), and 5 µL of Power SYBR Green 2x Master Mix. The PCR reaction conditions were 50ºC for

2 min, then 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, then 60ºC for 1 min. The melt curve stage consisted of 95ºC for 15 s, then 60ºC for 1 min and 95ºC for 15 s. The qPCR was performed on a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the associated cloud-based Design and Analysis software (Thermo Fisher Scientific; version 2.5.1). Gene expression ($2^{-\Delta\Delta Ct}$) was calculated using fold change (Livak and Schmittgen, 2001). PCR efficiencies were assessed using the LinRegPCR software version 2016.1 and determined to be above 1.80 (Table 3.1).

3.3.1.6 Transepithelial electrical resistance (TEER)

To determine the change in epithelial electrical resistance given by the effect of different treatments on the cell monolayer, a TEER experiment was carried out. Prior to seeding cells onto the Corning Transwell polyester membrane cell culture inserts (6.5 mm and 0.4 μm pore size), baseline resistance was determined to be 107/0.33 cm^2 using a STX2 chopstick electrode connected to a voltmeter. RTgutGC cells (passage number 20-30) were grown in T75 flasks, trypsinized when maximally (>90%) confluent, and cell counts were performed using the trypan blue (0.04%; Thermo Fisher Scientific) exclusion assay and the automated Countess cell counter (Invitrogen) prior to seeding into the cell culture dishes. The cells were seeded on semipermeable Transwell membrane supports (Corning Costar Transwell, Millipore Sigma) at a density of approximately 2.6 x 10^5 cells/cm^2 or a final number of about 8.58 x 10^4 cells per insert (cell growth aera of 0.33 cm^2). The cells were cultured for at least 3 weeks prior to the experiment to ensure that they established the brush border membrane and tight junction complexes. To the apical and basolateral compartments 100 μL or 500 μL, respectively, of L-15 media supplemented with 10% FBS were added, and the medium was replaced every 4-5 days. Periodic inspection of the cell monolayers was carried out using a Nikon inverted light microscope.

Bacterial cultures were prepared as outlined in section 2.1.2. and bacteria containing cell culture growth medium was added to the RTgutGC cells and incubated for 24 h. At the end of the incubation time, culture medium was carefully removed so not to disturb the cell monolayer and the Transwell inserts were transferred to a new 24-well plate containing sterile PBS on the basolateral compartment. To the apical compartment, 100 µL of PBS were added. Cell monolayers were likewise washed two more times, 100 mL of PBS was added to the apical compartment and 500 µL to the base of the electrode, and measurements were recorded using a cup electrode collected to a voltmeter. The baseline reading (membrane only) was subtracted from the measurements and the resistance per cm^2 was determined. Statistical analyses were performed on the resistance values per area.

3.3.2 In vivo Pediococcus acidilactici MA18/5M supplementation trial

3.3.2.1 Probiotic supplementation experimental design

Chinook salmon (*Oncorhynchus tshawytscha*) juveniles (n = 280; 8.168 ± 0.721 g) were obtained from Yellow Island Aquaculture Ltd. (www.yellowislandaquaculture.ca, Quadra Island, British Columbia, Canada) and the experiments were conducted there. Before the infection trial, fish were kept in freshwater (FW, 14 ± 2°C) at a density of 2.3 kg/m^3 using a flow-through system under natural photoperiod (12:12 h dark:light). After this, fish were randomly distributed between eight 120 L barrels (2 barrels per treatment, 35 fish per barrel) one-day prior the starting of feeding trial (figure 3.1). During the transfer, the initial fish weight (g) was recorded (Figure 3.6a). To minimize growth differences related to the initial fish size and not to the diet supplementation, only fish around 8 ± 1 g were selected (Figure 3.6a). The experimental diets (treatments) utilized in this experiment were: i) Control diet, ii) *P. acidilactici* (MA18/5M probiotic strain), iii) Control diet intraperitoneally injected with heat-killed *V. anguillarum*, and iv) *P. acidilactici*

intraperitoneally injected with heat-killed *V. anguillarum* (Please referrer to Appendix A1 for formulation details). During the trial, fish were fed commercial dry pellet (EWOS Harmony 2 mm: 47% protein, 18% fat, 0.7% fiber, 2.9% calcium, 1.2% phosphorus, and 0.6% sodium) twice a day with a ration of 2% body weight.

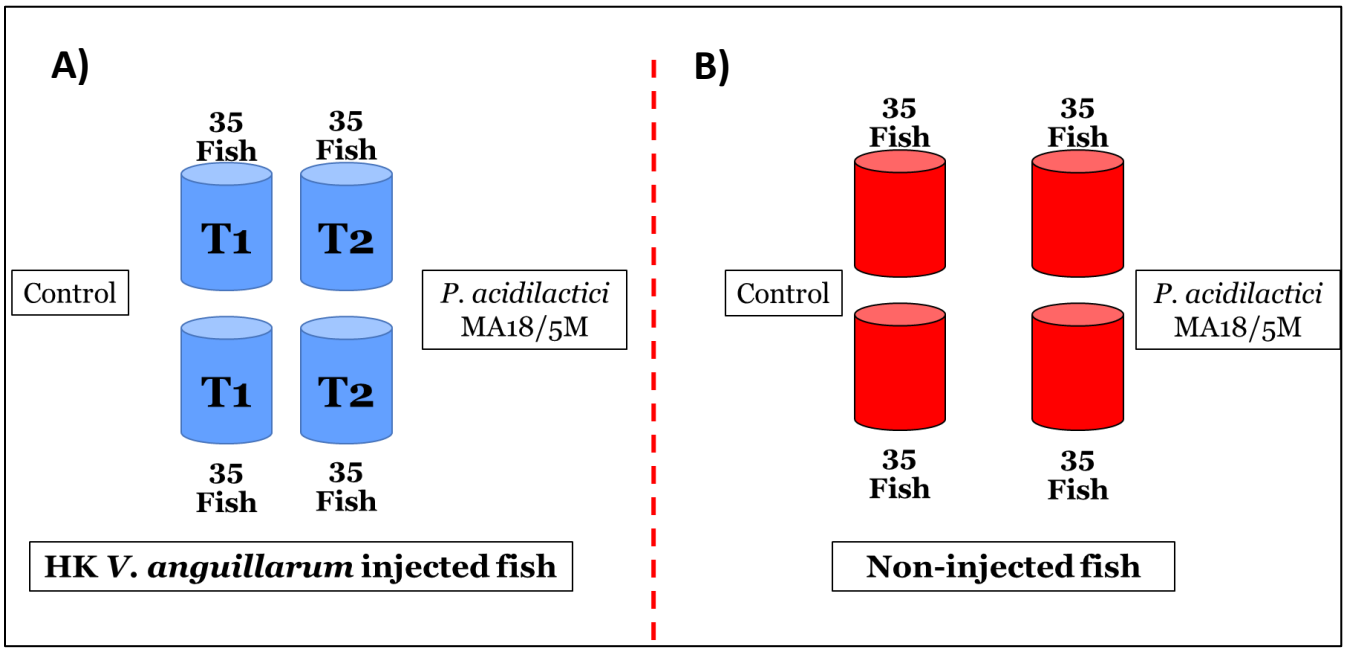


Figure 3.1. Experimental design. A group of 140 fish were divided in four 120 L barrels in duplicate (35 fish per barrel, 70 fish per treatment) to evaluate the effect of a control diet or supplementation with *P. acidilactici* MA18/5M. After four weeks fish were either A) inoculated with heat-killed *V. anguillarum* or B) not inoculated.

3.3.2.2 Chinook salmon probiotic and heat-killed V. anguillarum stimulation trial

To determine the effect of *P. acidilactici* MA18/5M on Chinook salmon juveniles, both Control diet groups and both *P. acidilactici* groups were fed with dry pellet or dry pellet supplemented with the probiotic strain respectively for four weeks. After this, to evaluate the effect of probiotic supplementation after inactivated-bacteria stimulation, one control group and one *P.*

acidilactici group was injected with heat-killed *V. anguillarum* grown as mentioned in section 2.1.2.

The timing of sampling for this experiment was 0-, 2-, 4-, and 6-weeks post-diet supplementation. For sampling, fish were exposed to a non-lethal dose of clove oil, and one half of the hindgut tissue was isolated immediately, placed in RNAlater, and stored at -20°C for RNA extraction and qPCR analysis. The other half of the hindgut was placed in 10% neutral buffered formalin (Azer Scientific, Fisher Scientific) at 4°C for further histopathology analysis. Additionally, weight (g) and length (cm) were also recorded at 8-, and 10-weeks post-diet supplementation.

3.3.2.3 Hindgut RNA extraction and cDNA synthesis

Total RNA was isolated from Chinook Salmon hindgut samples using 1 mL of TRIZOL reagent following the manufacturers protocol (Invitrogen, 2020). After extraction, the RNA was treated with Ambion DNase I (RNase free) (Ambion™ DNase I, Invitrogen) following the manufacturer's instructions to degrade any residual genomic DNA. Briefly, 5 µg of RNA was treated with 2 µL of Ambion DNase I, 4 µL of DNase buffer 10x, and DEPC water complete to 40 µL. Then, samples were incubated at 37°C for 30 min, washed twice with Wash solution A, centrifuged at 3,500 x g for 1 min and purified in an RNA/Protein Purification Column. The supernatant containing the RNA was carefully transferred to a new tube. DNase treated RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using a Take3 plate of a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA). Column purified RNA samples had A260/280 ratios between 1.9 and 2.1 and A260/230 ratios between 1.9 and 2.2.

First-strand cDNA templates for qPCR were synthesized from 250 ng of DNaseI-treated, column-purified total RNA using qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Each sample was incubated at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min. Samples in a concentration of 25 ng/µL were stored at -20°C until utilization.

3.3.2.4 qPCR analysis of chinook salmon hindgut

All qPCR reactions were performed in a 10 µL reaction, 5µL of 2x WISENT ADVANCED™ qPCR master mix (Wisent, Quebec, Canada), 2.5 µL of forward and reverse primer mix (Sigma Aldrich, USA) at a final concentration of 0.5 µM each, and 2.5 µL of cDNA (2.5 ng/µL, 6.25 ng per reaction). All samples were amplified and detected using the LightCycler® 480 II (Roche, USA). The reaction mixtures were pre-incubated for 2 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, annealing for 30 s at 60°C, and finally extension for 8 s at 72°C. The melt curve was completed for each run every 5 s from 65°C to 97°C.

The primer sequences of interleukin 1 beta (*il1b*), interleukin 6 (*il6*), interleukin 8 (*il8*), interleukin 10 (*il10*), interleukin 17a (*il17a*), tumor necrosis factor alpha (*tnfa*), myeloid differentiation factor 88 (*myd88*), transforming growth factor β (*tgfb*), tricellulin (*marveld2*), E-cadherin (*cdh1*), villin 1 (*vil1*), claudin 3 (*cldn3*), claudin 12 (*cldn12*), claudin 15 (*cldn15*), occludin (*ocln*), junctional adhesion molecule 1 alpha (*jam1a*), zonula occludens-1 (*zo-1*), and mucin 2 (*muc2*) used in this study are listed in Table 3.1. Gene nomenclature abbreviations were obtained from the Zebrafish Information Network database (www.zfin.org). Gene discovery, qPCR primer design, and initial quality testing were performed as described in Soto-Davila et al. (Soto-Dávila et al., 2020c). Since the reagents, cycling conditions and samples were different from previous studies, primer efficiencies (Table 3.1) were measured. Briefly, a 7-point 1:3 dilution

series starting with cDNA representing 5 ng/µL (12.5 ng per reaction) of input total RNA was generated, and efficiencies then calculated using the formula $E = 10^{(-1/slope)}$ (Pfaffl, 2001).

Table 3.1. Primers used in this study.

Gene name	Sequence (5'-3')	Accession number	Amplicon size (bp)	Reference
Interleukin 1 beta (*il1b*)	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	AJ223954	153	(Frenette et al., 2023)
Interleukin 6 (*il6*)	F: GTTCTGGGTGAGGTGTCTA R: GGTGTCAACCAGGAAGTTAC	NM_001124657	93	(Schug et al., 2019)
Interleukin 8 (*il8*)	F: ATTGAGACGGAAAGCAGACG R: CGCTGACATCCAGACAAATCT	NM_001140710	136	(Wang et al., 2020)
Interleukin 10 (*il10*)	F: CCATCAGAGACTACTACGAGGC R: TCTGTGTTCTGTTGTTCATGGC	NM_001245099.1	165	(Wang et al., 2020)
Interleukin 17a (*il17a*)	F: TGGTTGTGTGCTGTGTGTCTATGC R: TTTCCCTCTGATTCCTCTGTGGG	GW574233	136	(Wang et al., 2020)
Tumor necrosis factor alpha (*tnfa*)	F: GTGATGCTGAGTCCGAAAT R: GTCTCAGTCCACAGTTTGTC	AJ277604.2	97	(Semple et al., 2018)
Myeloid differentiation factor 88 (*myd88*)	F: GACAAAGTTTGCCCTCAGTCTCT R: CCGTCAGGAACCTCAGGATACT	NM_001136545	110	This study
Transforming growth factor β (*tgfb*)	F: AGTTGCCTTGTGATTGTGGGA R: CTCTTCAGTAGTGGTTTGTCG	EU082211	191	This study
Tricellulin (*marveld2*)	F: TCCAACACAGGCTCATCTCTT R: ATGGGGTTCATGACGGACAC	XM_036977097.1	83	This study
E-cadherin (*cdh1*)	F: ACTACGACGAGGAGGGAGGT R: TGGAGCGATGTCATTACGGA	XM_021585993.2	107	This study
Villin 1 (*vil1*)	F: AAAGTTCAGGTGCTGTAAATCGC R: TGTGGCATGGTGCCAGATTC	XM_021579239.2	148	This study
Claudin 3 (*cldn3*)	F: AGGCAACGACGCTACATCAA R: GAAACCCAAGCAATGCGTCA	XM_021587920	112	(Wang et al., 2019)
Claudin 12 (*cldn12*)	F: ATCATCGCCTTCATCTCCGT R: TAGCAGCCAGAGTAGCCATC	XM_021621241	161	This study
Claudin 15 (*cldn15*)	F: GGCACGTCTGAGAAACAACC R: TAGGAAGTGGCAGCCTGACT	XM_036987534.1	92	This study
Occludin (*ocln*)	F: F: GACAGTGAGTTCCCCACCAT R: AGCTCTCCCTGCAGGTCCTT	XM_021601275.2	101	This study
Junctional adhesion molecule 1 alpha (*jam1a*)	F: TGAGGATGGAAGTCCGCAAC R: GTACCACAGTCCGAAGCACA	XM_021564368.2	98	This study
Zonula occludens-1 (*zo-1*)	F: GCTGTTCCTCCTAGACCTT R: TCACCCACATCTGACTCTAC	XM_021607172.1	99	(Schug et al., 2019)
Mucin 2 (*muc2*)	F: CCAGTGTCAGTGCAAACACG R: ATGTAGCAGGGCTGGGTAGA	XM_042327631.1	122	This study
[a]Cytochrome c oxidase subunit 6C-1 (*cx6c1*)	F: GCCTGCAATGCGAGGACTCC R: TTCCTTGGTTCTGTTACGCCGTAC	FR904651.1	114	This study

[b,c]Elongation factor 1 alpha (*ef1a*)	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	NM_001124339	134	(Semple et al., 2018)
[b,c]Beta actin (*actb*)	F: TGGACTTTGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG	AJ438158.1	139	(Ma et al., 2019)
[b]Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	F: GCTGGAATGGGACTCACAC R: GTCAAAACCGTCTCAGTGGG	NR	NR	(Rajanbabu and Chen, 2011)
[b]18S ribosomal RNA (*18S*)	F: CGTCGTAGTTCCGACCATAAA R: CCACCCACAGAATCGAGAAA	NR	NR	(Giroux et al., 2019)
Internal transcribed spacer 2 locus (*its2*)	F: TCATCAATCGGAACCTCTGG R: AAGGAAGAGCGCACGGG	NR	156	(Eder et al., 2009)

[a]Normalizers used in experimental RTgutGC qPCR analyses.

[b]Normalizers used in experimental Chinook salmon qPCR analyses.

[c]Candidate normalizer genes for *in vivo* trial

Amplification efficiencies were calculated using a 7-point 1:3 dilution series starting with cDNA representing 50 ng of input total RNA. See methods for details.

NR: Not reported.

Transcripts levels of the genes of interest (*il1b*, *il6*, *il8*, *il10*, *il17a*, *tnfa*, *myd88*, *tgfb*, *marveld2*, *cdh1*, *vil1*, *cldn3*, *cldn12*, *cldn15*, *ocln*, *jam1a*, *zo-1*, *and muc2*) were normalized to transcript levels of two endogenous control genes. Levels of five candidate normalizers [elongation factor 1 alpha (*ef1a*), beta actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), 18S ribosomal RNA (*18S*), and internal transcribed spacer 2 locus (*its2*)] were assessed in 50% of the samples (i.e., in 3 random samples per treatment) using cDNA representing 6.25 ng of input total RNA. Reference gene stability was assessed using geNorm, NormFinder, BestKeeper, and Delta Ct comparison, through the bioinformatic open-access portal RefFinder (Andersen et al., 2004; Pfaffl, 2001; Silver et al., 2006; Vandesompele et al., 2002; Xie et al., 2012). Most stable genes assessed were *ef1a* and *actb*.

After normalizer testing was completed, transcript levels of the genes of interest were analyzed in the individual study samples, with normalization to both *ef1a* and *actb*. In all cases, levels were assessed (in triplicate) in 7 individuals for day 0 and five individuals per treatment per time-point for 2-, 4-, and 6-weeks post-diet time-points using cDNA representing 6.25 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).

3.3.2.5 Semi-quantitative and qualitative histological analysis

For Chinook salmon samples collected at 0-, 2-, 4-, and 6-weeks post-diet supplementation, five individuals per treatment were processed further for histology. Each 10% formalin-fixed hindgut sample was cut into 5 equal-sized pieces, dehydrated in an alcohol gradient, cleared in two changes of xylene and sequentially embedded into a single paraffin block. For each fish, 5 µm-thick cross-sections of all five pieces of hindgut were mounted onto a glass slide, stained with haematoxylin and eosin and sealed under a coverslip. All the samples were processed at the Animal Health Laboratory, the University of Guelph (Guelph, ON, Canada).

All analyses were performed using bright field light microscopy on a Leica DMR light microscope (Leica Microsystems Inc., Concord, ON, Canada) equipped with Openlab imaging software (Openlab 5.5, PerkinElmer, Waltham, MA, USA). To visualize the presence of edema, and inflammation of the serosa, submucosa, and lamina propria, samples were observed using a 4x objective (40x magnification). To assess their suitability, the criteria and scales utilized were the following: 0 = none, 1 = mild, 2 = moderate, 3= severe or more extensive than 2. To determine the epithelial vacuolization (vacuolization) and numbers of goblet cells, a 10x objective was used (100x magnification). For epithelial vacuolization, the scores utilized were: 0 = none, 1 = mild vacuolization, 2 = moderate, 3= more extensive than 2 and sometimes involved folding of the

epithelium and distortion of the cells and/or folds. For goblet cells, the criteria utilized were: 0 = non-visible, 1 = small numbers present as a minority of cells in the epithelium, 2 = greater numbers often grouped together, 3= large numbers or more extensive than 2. Finally, to evaluate the mitotic figures (mitoses), epithelial cell death (apoptosis/necrosis), and multifocal inflammation, a 20x objective was employed (200x magnification). To assess mitoses, the numbers were counted for the five intestinal folds.

3.3.3 Statistical analyses

For all RTgutGC *in vitro* trials, data are expressed as mean values ± SEM (n=3). Nonparametric data were statistically compared with a one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test. Experiments with two factors were compared with a two-way ANOVA and Tukey's multiple comparisons test. All statistical analyses were done using GraphPad Prism software v9.0 (GraphPad Software, La Jolla California USA).

For Chinook salmon *in vivo* trials, data are shown as the mean ± SEM (n=5). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different dietary treatments and time-points as factors of variance, followed by a Tukey's multiple comparisons test to identify differences between groups. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism v9.0 (GraphPad Software, La Jolla California USA).

Histopathological data were analyzed using repeated measures using GraphPad Prism v9.0 (GraphPad Software, La Jolla California USA). A Bonferroni correction was applied, and statistical significance was declared at $P \leq .05$ for all dependent variables.

3.4 Results

3.4.1 Effect of coincubation with P. acidilactici MA18/5M on the expression of tight junction and immune molecules in RTgutGC cells

To examine the effect of *P. acidilactici* exposure on the expression of tight junction and immune molecules, an endpoint coincubation experiment was carried out. Moreover, to examine whether *P. acidilactici* could promote gut barrier integrity and modulate immune-related genes in the established RTgutGC *in vitro* model, a 48 h endpoint coincubation experiment was performed using differentiated RTgutGC cells (Pumputis et al., 2018). This strain was chosen based on reports suggesting barrier-promoting properties (Del Piano et al., 2010) and prior use in aquaculture settings (Al-Hisnawi et al., 2019). However, *P. acidilactici* did not cause a significant change in the expression of the tight junction genes assessed (*cldn3*, *cldn12*, and *zo-1*) nor did it induce changes in expression of key proinflammatory cytokines (Figure 3.2).

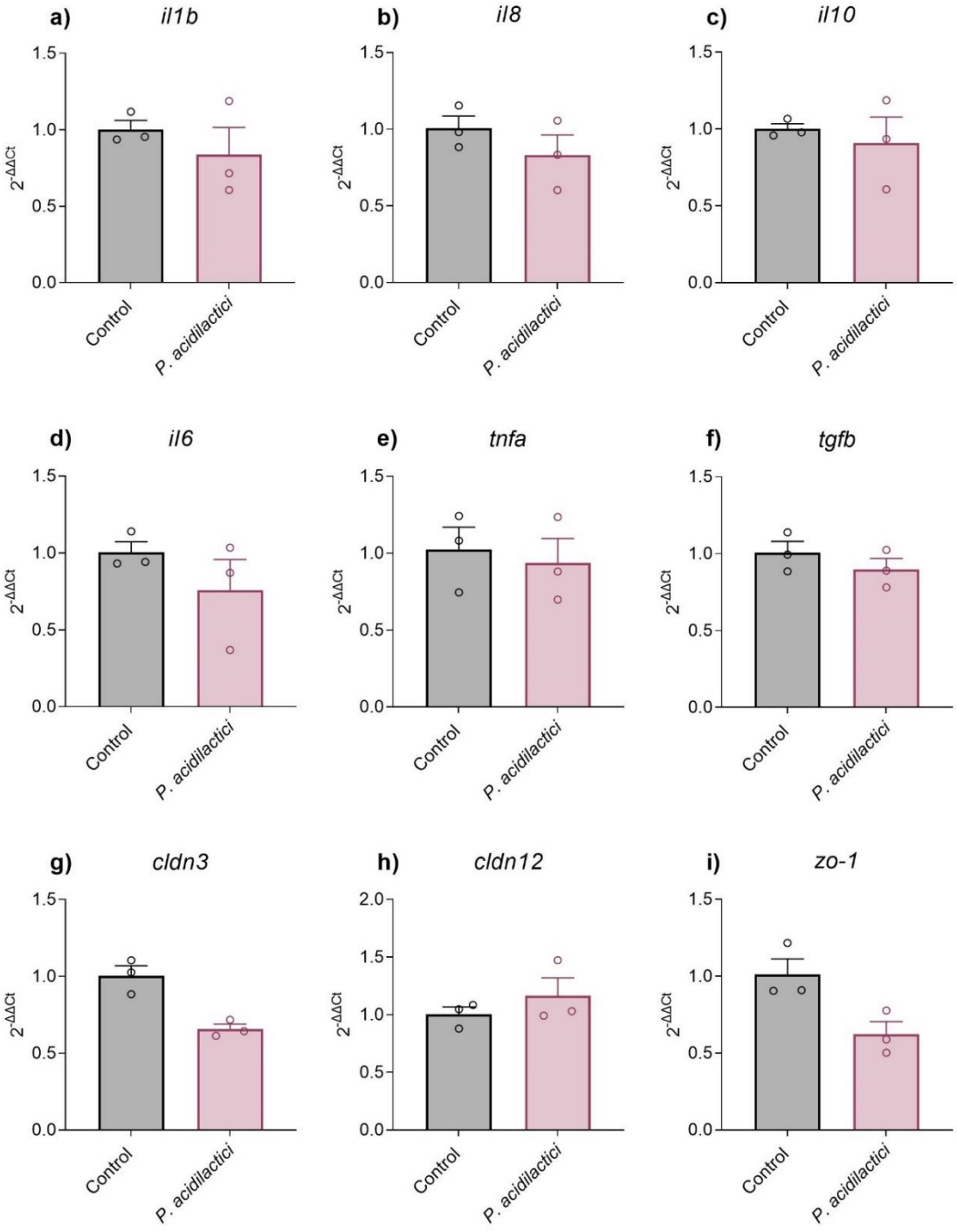


Figure 3.2. Relative gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) interleukin 6 (*il6*), (e) tumor necrosis factor alpha (*tnfa*), (f) transforming growth factor beta (*tgfb*), (g) claudin 3 (*cldn3*), (h) claudin 12 (*cldn12*), and (i) zonula occludens 1 (*zo-1*) in RTgutGC unstimulated (control) or inoculated with *P. acidilactici* MA18/5M for 48 h. All data are expressed as mean values ± SD (n=3). Differences were not statistically significant

(Kruskal-Wallis non-parametric test). One-way ANOVA analysis using the dependent variable relative expression (fold change) was not affected among treatments.

3.4.2 Changes in transepithelial electrical resistance in response to P. acidilactici MA18/5M and V. anguillarum coincubation

To investigate whether the candidate probiotic and/or *V. anguillarum* could modulate the integrity of the epithelial layer, a transepithelial electrical resistance (TEER) assay was performed (Srinivasan et al., 2015). Differentiated RTgutGC cells cultured on semipermeable Transwell polyester membrane supports (pore size 0.4µm) were exposed to suspensions of the LAB (MOB 1:100; ~4 x 10^6 CFU/mL) or *V. anguillarum* (MOB 2:1; ~2 x 10^4 CFU/mL) in L-15 cell culture media for 24h (Figure 3.3). *V. anguillarum*, but not *P. acidilactici*, caused a significant decrease in resistance relative to the vehicle control (Figure 3.3a).

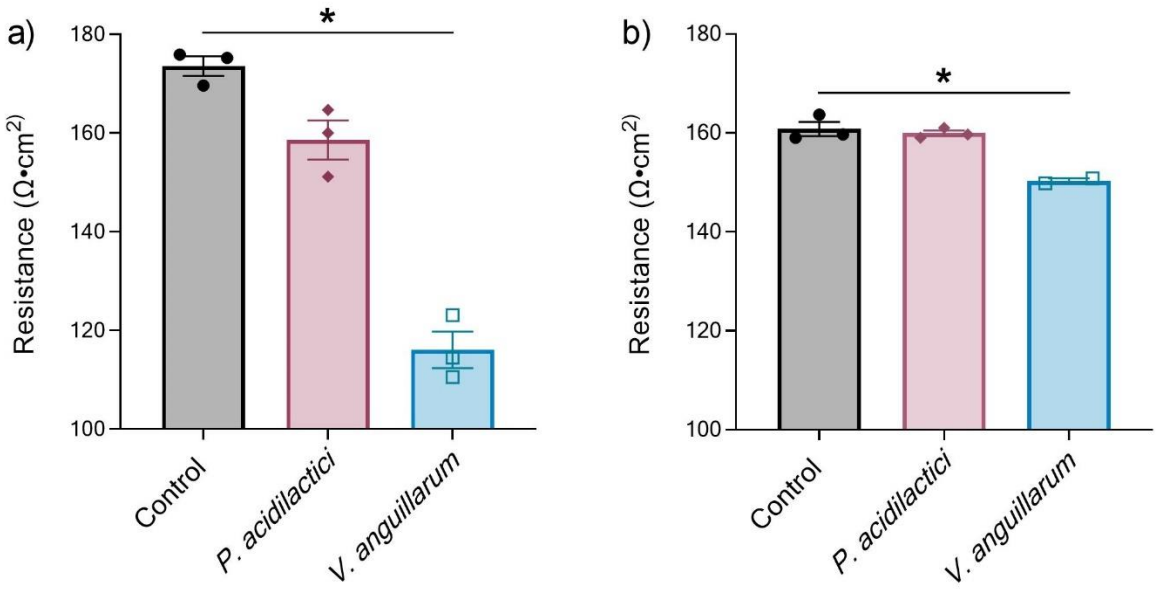


Figure 3.3. Transepithelial electrical resistance in response to exposure to *P. acidilactici* MA18/5M or *V. anguillarum*. RTgutGC cells were seeded on Transwell semipermeable

transmembrane supports and bacteria were added to the apical compartment for 24 h. The vehicle group was not exposed to bacteria at any time, and the *V. anguillarum* group was incubated only with the bacterium for the latter 24 h of the experiment. TEER was determined based on the resistance given by the monolayer per area, normalized to the blank measurement. Bars indicate statistically significantly differences among treatments (*p > 0.05, **p > 0.01, ***p > 0.001). One-way ANOVA analysis using the dependent variable resistance ($\Omega \cdot cm^2$) was significantly different between control and *V. anguillarum* (a) $p < 0.0001$; b) $p < 0.0354$).

To determine whether pre-treatment with the candidate probiotic could protect the cell monolayer against the pathogen-induced damages to the intercellular tight junctions, slight modifications to the aforementioned experimental design were performed. Briefly, the same strain was grown and added to the apical compartment of the membrane inserts in like manner for 48h. Then, *V. anguillarum* (MOB 2:1; ~2 x 10^4 CFU/mL) was added for 24h and TEER measurements were taken at the end of the incubation period (Figure 3.3b). There were no statistically significant differences in resistance in either of the LAB-pretreated group, despite the addition of the pathogen. However, a statistically significant decrease in resistance ($P = 0.0265$) was observed in the group incubated with *V. anguillarum* only (Figure 3.3b).

3.4.3 Effect of exposure to Vibrio anguillarum on the expression of tight junction and immune molecules

To characterize the response of RTgutGC cells to live or heat-killed (HK) *V. anguillarum*, a time-course coincubation experiment was carried out. Samples were collected at 0, 3, 6, and 24 h. Of the TJ-related molecules assessed, there was a significant downregulation of *cdh1* and *jam1a*, but not *ocln*, which had a puzzling upregulation by 24 h (Figure 3.4a, b, and c). Of the cytokines assessed, all exhibited a time-dependent upregulation, which was statistically significant at the 24 h timepoint relative to the 0 h control group (Figure 3.4d, e, and f). In the case of *il8*, but not *il17a* or *tgfb*, the upregulation was observed in cells exposed to both live and HK bacteria (Figure 3.4d,

e, and f). In an independent replicate of this experiment, a similar time-dependent response was observed, in which there was a significant upregulation of *il1b*, *il8*, and *tnfa* at the 24 h timepoint for groups exposed to live bacteria (data not shown).

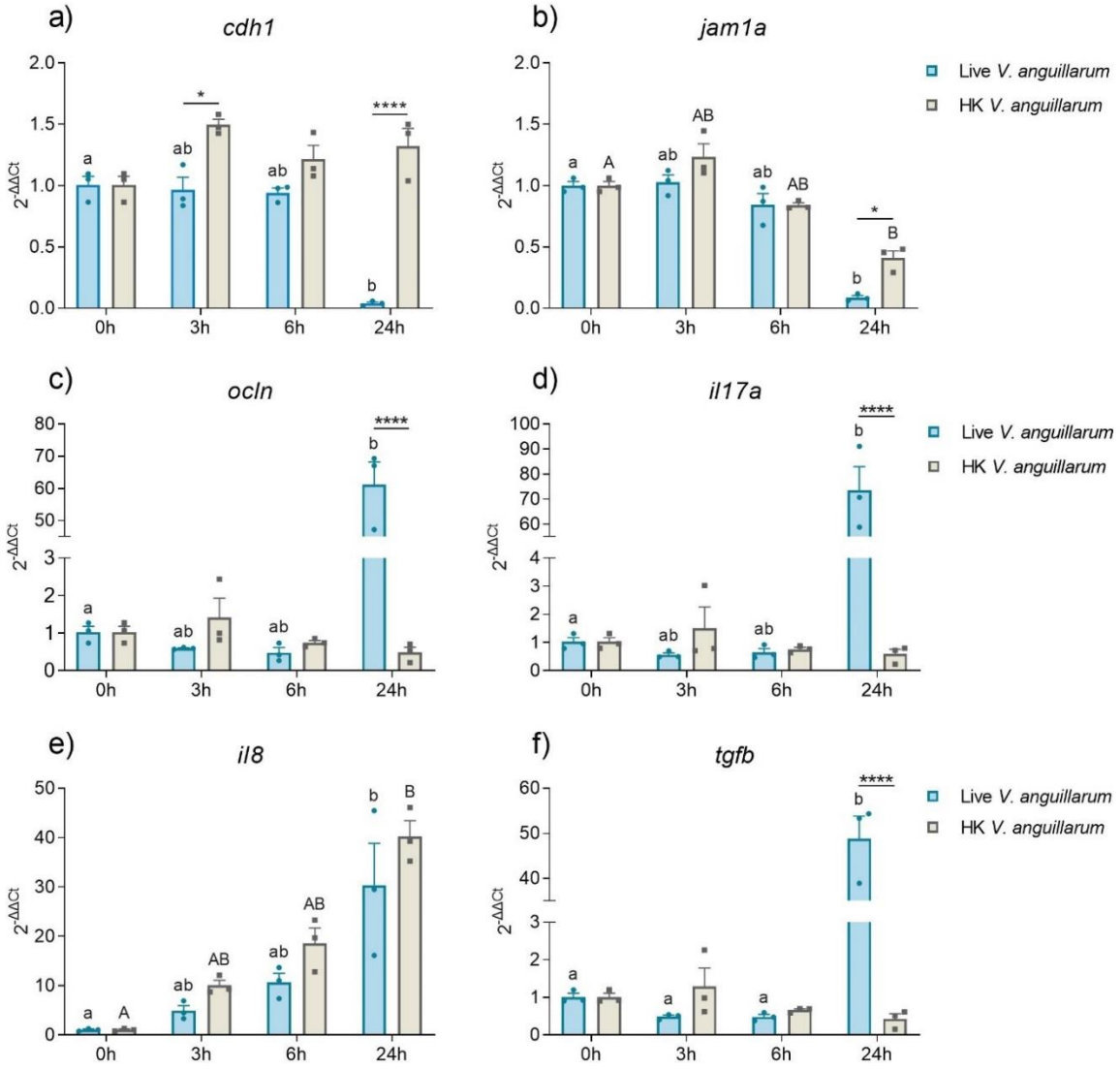


Figure 3.4. Time-course analysis of salmonid intestinal cells to live or heat-killed *V. anguillarum*. RTgutGC cells were exposed to either live or HK bacteria (2:1 MOB at the time of inoculation) and gene expression was measured using RT-qPCR. All data are expressed as mean values ± SD (n=3). Bars represent significant differences between treatments at the same time-points. Different letters (Live bacteria: lower cases; HK bacteria: Upper cases) represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (fold change) and the independent variables

treatment and time, has shown that relative expression varied among treatments (*cdh1*: $p < 0.0159$; *jam1a*: $p < 0.0410$; *ocln*: $p < 0.0001$; *il17a*: $p < 0.0001$; *tgfb*: $p < 0.0001$), time (*cdh1*: $p < 0.0421$; *jam1a*: $p < 0.0002$; *ocln*: $p < 0.0001$; *il17a*: $p < 0.0001$; *il8*: $p < 0.0001$; *tgfb*: $p < 0.0015$), and the interaction between treatments and time (*cdh1*: $p < 0.0204$; *ocln*: $p < 0.0092$; *tgfb*: $p < 0.0196$).

3.4.4 Effect of pre-treatment with LAB and exposure to V. anguillarum on the expression of immune and tight junction molecules

To examine the potential use of LAB as a disease prevention strategy by stimulating immunity and gut barrier function, a time-course coincubation experiment was executed. Briefly, differentiated RTgutGC cells were pre-treated with *P. acidilactici* for 48 h and then exposed to *V. anguillarum*. Samples were taken at 0 h, 3 h, 6 h, and 12 h after infection with the pathogen and the relative expression of key TJ and immune molecules was assessed through RT-qPCR (Figure 3.5).

The expression of *zo-1* was significantly upregulated in the *P. acidilactici* at the 12 h timepoint relative to the expression level at 0, 3, and 6 h (Figure 3.5a). In contrast, a significant upregulation of *cldn3* was observed 6 h post-exposure with *V. anguillarum* compared to 0 and 12 h timepoints (Figure 3.5b). There was a statistically significant difference in the expression of *cdh1* for both treatment groups after 12 hpi compared to the other timepoints (Figure 3.5c). Lastly, there was also a significant downregulation of the *jam1a* molecule 12 hpi in the control and *P. acidilactici* MA18/5M groups compared to 0, 3, and 6 hpi, and 0 hpi respectively (Figure 3.5d). The expression of the pro-inflammatory cytokines *il8* and *il17a* was also assessed. There was an extremely significant upregulation of *il8* at the 12h timepoint relative to the baseline control and the 3 and 6 hpi timepoints (Figure 3.5e). In contrast, no significant differences were observed in the expression of *il17a* between treatments and timepoints (Figure 3.5f).

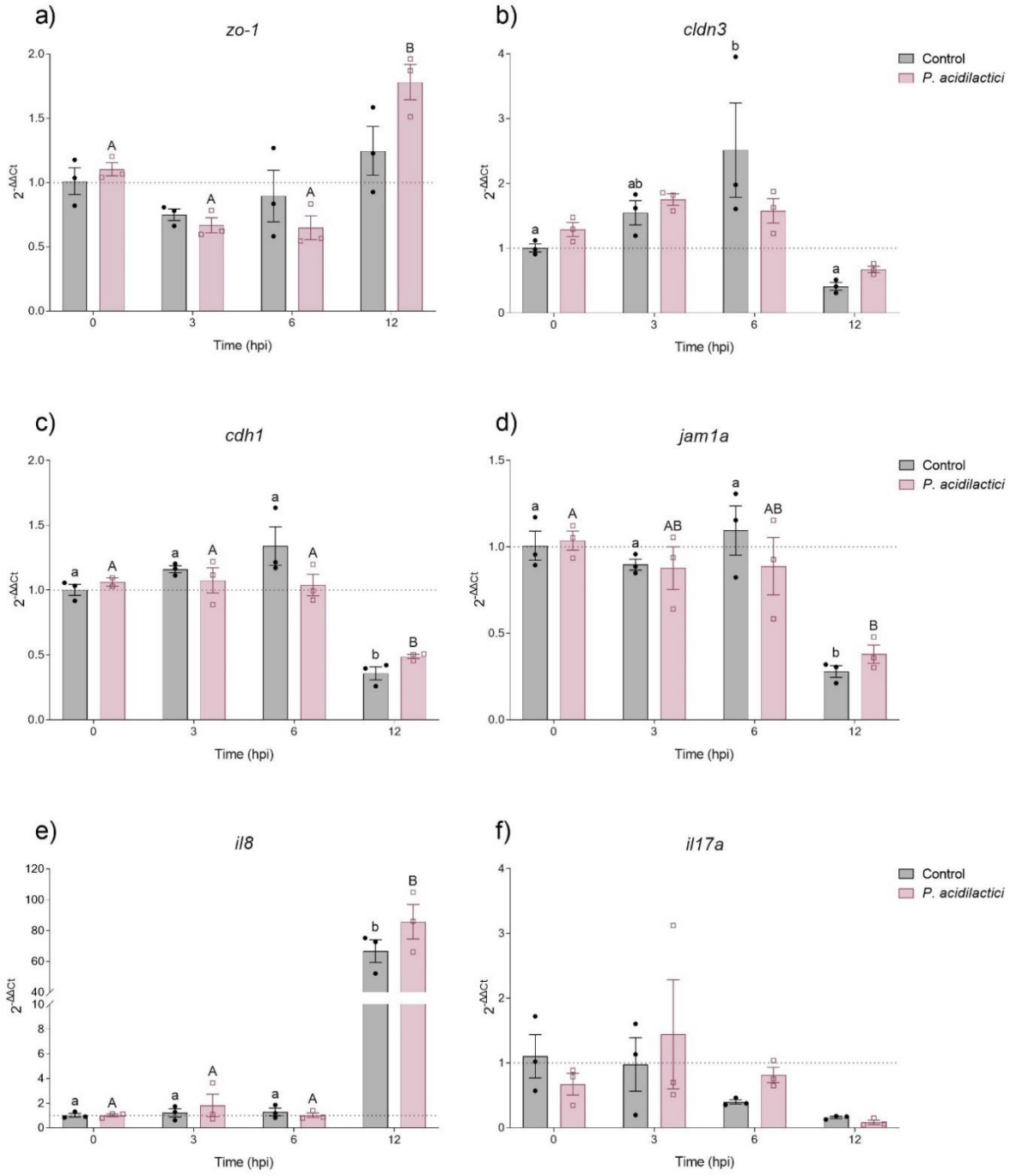


Figure 3.5. Effect of pre-treatment with *P. acidilactici* MA18/5M followed by V. anguillarum inoculation on the expression of key tight junction and immune molecules. RTgutGC intestinal epithelial cells were incubated with *P. acidilactici* MA18/5M for 48 h (MOB 1:100; ~7.5 x 10^8 CFU/mL), then infected with *V. anguillarum* (2:1 MOB at the time of inoculation) and samples were collected at 0, 3, 6, and 12 h post-infection. The control group was not pretreated with *P. acidilactici* MA18/5M at any time and was only exposed to *V. anguillarum* for the latter 12 h of

the experiment. Gene expression was assessed using RT-qPCR. All data are expressed as mean values ± SD (n=3). Different letters (control: lower cases; *P. acidilactici* MA18/5M: Upper cases) represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (fold change) and the independent variables treatment and time, has shown that relative expression varied among time (*zo-1*: $p < 0.0277$; *cldn1*: $p < 0.0158$; *cdh1*: $p < 0.0206$; *jam1a*: $p < 0.0124$; *il8*: $p < 0.0004$).

3.4.5 Growth Analysis

To minimize growth differences related to the initial fish size and not to the diet supplementation, fish weight (g) was recorded during the transfer to each of the treatments (Figure 3.6a). Overall, no statistically significant differences were observed in weight between the treatments tested in this study (Figure 3.6a).

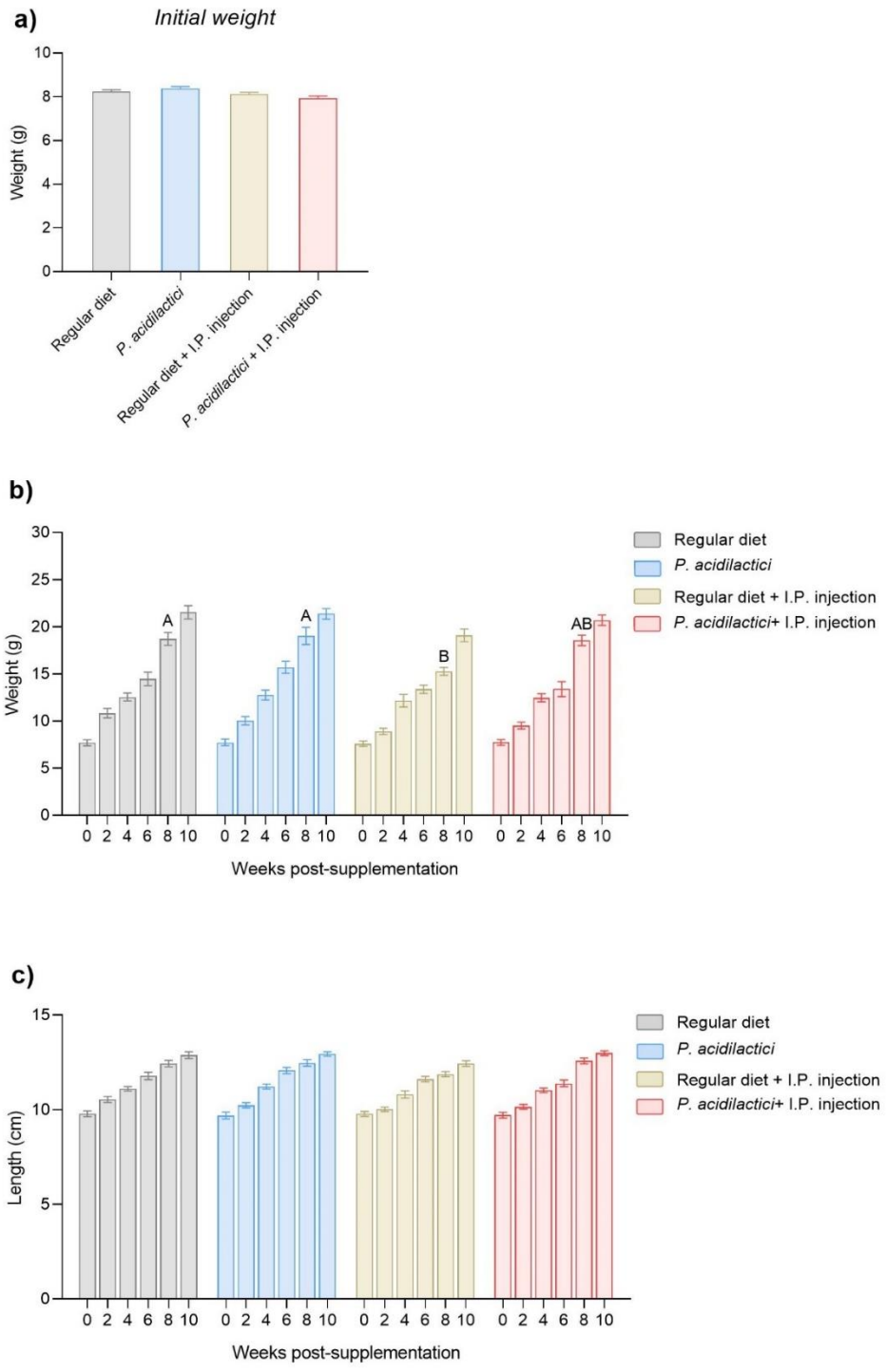


Figure 3.6. (a) Initial weight (g) of fish transferred to test the different supplementation groups. Each column represents an average of 35 fish. (b) Weight (g) increase recorded for a total of 10 weeks post-supplementation. (c) Length (cm) increase recorded for a total of 10 weeks post-supplementation. Each color represents different diets. Each value is the mean ± S.E.M. Different

letters represent significant differences among treatments at the same time (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable growth (g or cm) and the independent variables treatment and time, has shown that relative expression varied among treatments ($p < 0.0450$ in graph b).

Results collected during the trial showed significant differences in weight (g) of fish belonging to the regular diet and *P. acidilactici* treatments compared to the regular diet + I.P. injected treatment 8 wps (Figure 3.6b). In contrast, no significant differences in weight (g) among treatments were observed at 0-, 2-, 4-, 6-, and 10-wps (Figure 3.6b). Length (cm) data collected during the supplementation trial did not show statistically significant differences between treatments (Figure 3.6c).

3.4.6 Chinook Salmon Gut-Specific Relative Expression

Transcript levels of gut-specific genes were evaluated by qPCR in hindgut samples (Figure 3.7). A significant increase in the transcript expression of *marveld2* 6 wps was observed in fish from the regular diet, *P. acidilactici*, and regular diet + I.P. injection treatments compared to their respectively time 0 (Figure 3.7a). An upregulation on the relative expression of *cdh1* gene was observed at 4- and 6 wps in the regular diet + I.P. injection and *P. acidilactici* + I.P. injection treatments compared to their respective control timepoint (Figure 3.7b), meanwhile, an upregulation of *cdh1* was observed in *P. acidilactici* fish at 4 wps compared to time 0 (Figure 3.7b).

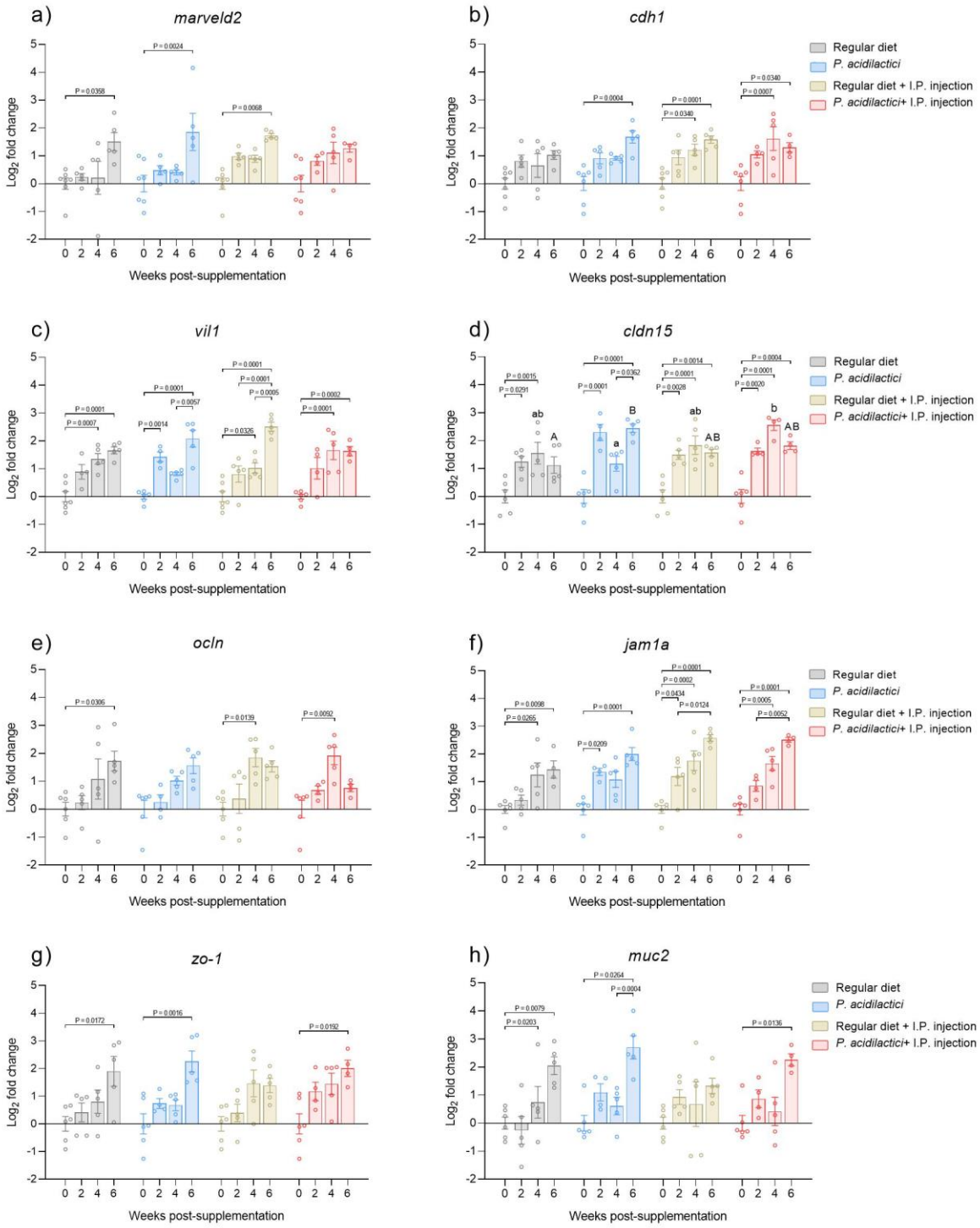


Figure 3.7. Gene expression of (a) tricellulin (*marveld2*), (b) e-cadherin (*cdh1*), (c) villin 1 (*vil1*), (d) claudin 15 (*cldn15*), (e) occludin (*ocln*), (f) junctional adhesion molecule 1 alpha (*jam1a*), (g) zonula occludens-1 (*zo-1*), and (h) mucin 2 (*muc2*) in Chinook salmon juveniles unsupplemented or supplemented with *P. acidilactici* MA18/5M, and challenged with *Vibrio anguillarum* J382.

Each value is the mean ± S.E.M (n = 6). Bars represent significant differences between time-points at the same treatment. Different letters represent significant differences between treatments at the same time-point (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*cldn15*: p < 0.0119; *jam1a*: p < 0.0039), time (*marveld2*: p < 0.0001; *cdh1*: p < 0.0001; *vil1*: p < 0.0001; *cldn15*: p < 0.0001; *ocln*: p < 0.0001; *jam1a*: p < 0.0001; *zo-1*: p < 0.0001; *mucin2*: p < 0.0001), and the interaction between treatments and time (*vil1*: p < 0.0101; *cldn15*: p < 0.0028).

The relative expression of the *vil1* encoding gene showed different upregulation patterns among treatments (Figure 3.7c). After 4- and 6- wps, an increase of *vil1* in the regular diet treatment was observed compared to 0 wps (Figure 3.7c). In *P. acidilactici* treatment, *vil1* encoding transcripts in 2- and 6-wps fish showed an upregulation compared to 0 weeks supplemented fish (Figure 3.7c). Moreover, fish supplemented with *P. acidilactici* for 6 weeks showed an upregulation in the *vil1* gene compared to 4 wps (Figure 3.7c). At 4 wps, regular diet + I.P. injection fish showed an increase in *vil1* compared to the time 0 of supplementation (Figure 3.7c). Moreover, 6 wps fish showed an increase in the *vil1* expression compared to 0-, 2-, and 4 wps (Figure 3.7c). Finally, individuals from the *P. acidilactici* + I.P. injection treatment showed an up-regulation after 4- and 6 wps on the expression of the *vil1* gene compared to time 0 (Figure 3.7c).

An increased relative expression of *cldn15* was observed in fish supplemented for 2- and 4 weeks with a regular diet compared to day 0 (Figure 3.7d). In *P. acidilactici* treated fish, the *cldn15* gene showed an upregulation at 2- and 6-wps compared to 0-weeks supplemented fish (Figure 3.7d), whereas, 6-weeks supplemented fish also showed a statistically significant upregulation compared to 4-weeks *P. acidilactici* supplemented fish (Figure 3.7d). For regular diet + I.P. injection and *P. acidilactici* + I.P. injection treatments, a similar pattern of upregulation of the *cldn15* gene was observed at 2-, 4-, and 6-wps compared to their respective time 0 (Figure 3.7d). Interestingly, *cldn15* was the only gene that showed significant differences between diet

treatments at the same time point. For instance, a statistically significant downregulation of *cldn15* was observed in *P. acidilactici* treatment compared to the *P. acidilactici* + I.P. injection treatment (Figure 3.7d). Moreover, a statistically significant upregulation of *cldn15* was observed after 6 wps with *P. acidilactici* compared to the regular diet treatment (Figure 3.7d).

Transcripts encoding the expression of *ocln* showed statistically significant differences in regular diet treatment between 0- and 6 wps (Figure 3.7e). Also, an upregulation after 4-weeks of diet supplementation in the relative expression of *ocln* was observed in the regular diet + I.P. injection and *P. acidilactici* + I.P. injection treatments compared to 0 wps (Figure 3.7e). The relative expression of *jam1a* in regular diet treated fish was upregulated after 4- and 6 wps compared to time 0 (Figure 3.7f). In *P. acidilactici* treatment, an upregulation at 2- and 6 wps was observed compared to time 0 (Figure 3.7f). For regular diet + I.P. injection treated fish, significant differences were observed in the expression of *jam1a* after 2 wps compared to 0 wps (Figure 3.7f). An increased relative expression of *jam1a* was observed in fish under regular diet + I.P. injection or *P. acidilactici* + I.P. injection treatments for 4- and 6 weeks compared to their respectively time 0 (Figure 3.7f). Additionally, a statistically significant upregulation of this gene after 6 weeks of either regular diet + I.P. injection or *P. acidilactici* + I.P. injection treatments was observed compared to their respectively 2-weeks supplemented fish (Figure 3.7f).

The relative expression of *zo-1* encoding gene was upregulated after 6 weeks of either regular diet, *P. acidilactici*, or *P. acidilactici* + I.P. injection treatment compared to their correspondingly time 0 (Figure 3.7g). Similarly, an upregulation in the transcripts encoding *muc2* was observed in fish from the regular diet, *P. acidilactici*, and *P. acidilactici* + I.P. injection treatments (Figure 3.7h). In the regular diet treatment, *muc2* relative expression was upregulated after 4- and 6 wps compared to time 0 (Figure 3.7h). For *P. acidilactici* -treated fish, this gene

showed a statistically significant increase at 6 wps compared to 0- and 4 wps (Figure 3.7h). Finally, a significant increase in the expression of *muc2* was determined in fish from the *P. acidilactici* + I.P. injection treatment after 6 weeks compared to 0-weeks treated fish (Figure 3.7h).

3.4.7 Chinook Salmon Gut Immune Relative Expression

To determine the effect of the *Pediococcus acidilactici* MA18/5M strain and posteriorly inactivated pathogen stimulation, Chinook salmon immune genes were evaluated by qPCR (Figure 3.8). In our study, an upregulation of the pro-inflammatory cytokine *il1b* was only observed in the regular diet + I.P. injection treatment at 2 wps compared to time 0 (Figure 3.8a). In contrast, a statistically significant increase in the expression of the pro-inflammatory chemokine *il8* was observed in each treatment (Figure 3.8b). For instance, an upregulation in *il8* was observed after 4- and 6 wps compared to 0-weeks of regular diet treatment (Figure 3.8b). Also, a significant increase in the expression of *il8* was determined after 2-, 4-, and 6 wps with *P. acidilactici* compared to day 0 (Figure 3.8b). In fish sampled from the regular diet + I.P. injection and *P. acidilactici* + I.P. injection treatments, a similar upregulation was observed after 4 wps compared to their respectively time 0 (Figure 3.8b). In contrast, the relative expression of *tnfa* and *il10* was not modulated by the treatments utilized in this study (Figure 3.8c and d).

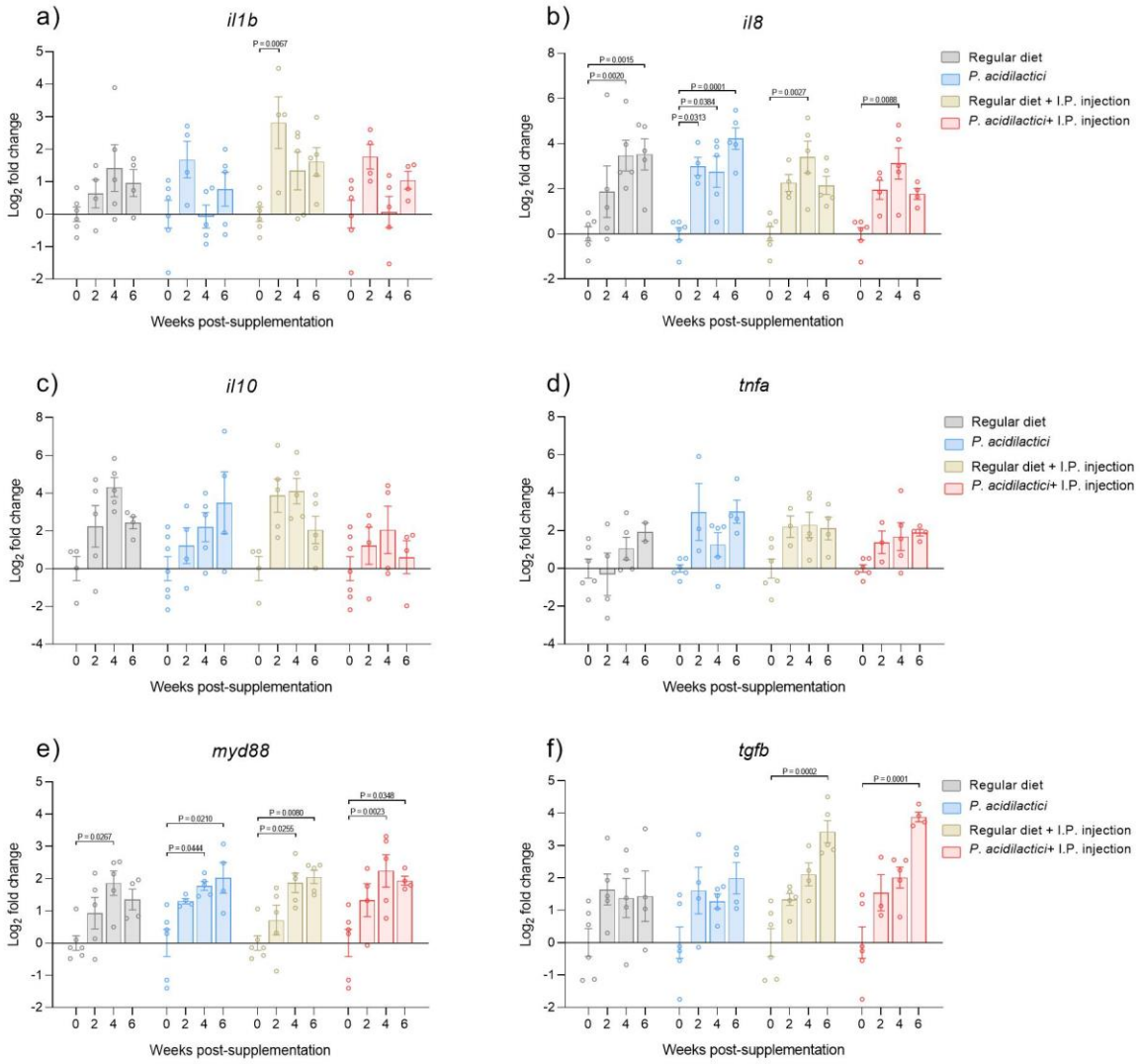


Figure 3.8. Gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) tumor necrosis factor alpha (*tnfa*), (e) myeloid differentiation factor 88 (*myd88*), and (f) transforming growth factor β (*tgfb*) in Chinook salmon juveniles unsupplemented or supplemented with *P. acidilactici* MA18/5M, and challenged with *Vibrio anguillarum* J382. Each value is the mean ± S.E.M (n = 6). Bars represent significant differences between time-points at the same treatment. Different letters represent significant differences between treatments at the same time-point (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among times (*il1b*: $p < 0.0001$; *il8*: $p < 0.0001$; *myd88*: $p < 0.0001$; *tgfb*: $p < 0.0001$).

Transcript levels of *myd88* were upregulated in all conditions after 4 wps compared to time 0 in each treatment (Figure 3.8e). Moreover, an upregulation in the expression of *myd88* compared to time 0 was observed in the *P. acidilactici*, regular diet + I.P. injection, and *P. acidilactici* + I.P. injection treatments (Figure 3.8e). Finally, a statistically significant increase in the expression of *tgfb* was observed in fish sampled from the regular diet + I.P. injection and *P. acidilactici* + I.P. injection treatments at 6 wps compared to time 0 (Figure 3.8f).

3.4.8 Histopathological Effects After P. acidilactici Supplementation and Heat-killed pathogen stimulation

To examine the effect of *P. acidilactici* supplementation on Chinook salmon hindgut integrity, a histopathological analysis was conducted. Moreover, to determine whether *P. acidilactici* could promote hindgut integrity in presence of an immunomodulator, fish from both feeding treatments were I.P. injected with heat-killed *V. anguillarum*.

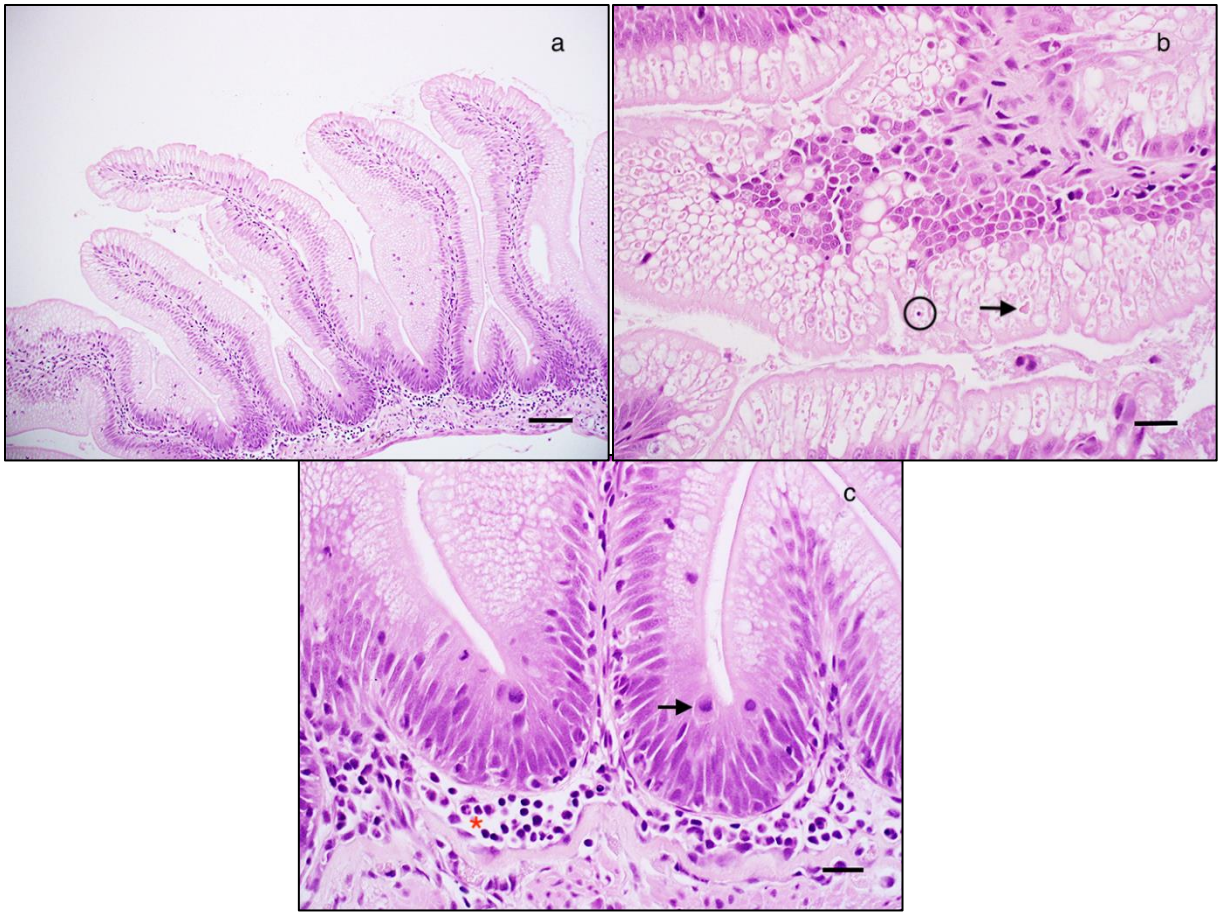


Figure 3.9. Chinook salmon intestine. (a) Simple intestinal folds with epithelial cells filled with absorptive vacuoles. Bar = 120 mm. (b) Atypical intestinal fold with eosinophilic material (arrow) in absorptive vacuoles along with an example of epithelial apoptosis/necrosis, which was uncommon. Bar = 30 mm. (c) Detail of a, with numerous mitotic figures in the epithelium (arrow) and a mild increase in inflammatory cells in the submucosa (Asterix). Bar = 30 mm

Our results showed that fish feed with the regular diet or *P. acidilactici* that were or not stimulated with heat-killed *V. anguillarum*, did not show evidence of edema, inflammation of the serosa, submucosa and lamina propria, and multifocal inflammation after 0, 4, and 6 weeks (Table 3.2). In the case of epithelial vacuolization, although scores associated with moderate (up to half of the cell is filled with absorptive vacuoles) and extensive (involved folding of the epithelium and distortion of the cells and/or folds) vacuolization were reported, no significant differences were observed among treatments and time points (Table 3.2). Small numbers of goblet cells were noted

in the epithelium and no statistically significant differences were associated to the treatments and time points variables, as well as the interaction of both (Table 3.2). In this analysis, the numbers of mitotic figures counted in the five intestinal folds did not show differences among the regular diet, *P. acidilactici*, regular diet + I.P. injection, and *P. acidilactici* + I.P. injection after 0, 4, and 6 weeks of study (Table 3.2). Finally, our results showed evidence of epithelial cell death (apoptosis/necrosis) in Chinook salmon hindgut, however, this was not associated to probiotic supplementation and/or stimulation with heat-killed *V. anguillarum* (Table 3.2).

Table 3.2. Histopathological analysis of Chinook salmon hindgut (n = 5 per treatment per time-point) during six weeks of dietary and/or stimulation treatment.

Time (wps)		Treatments			
		Regular diet	*P. acidilactici*	Regular diet + I.P injection	*P. acidilactici* + I.P injection
0 weeks	Edema	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation serosa	0.00 ± 0.00	0.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
	Inflammation submucosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.50
	Inflammation lamina propria	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.50
	Vacuolization	3.00 ± 0.00	2.50 ± 0.50	3.00 ± 0.00	2.50 ± 0.50
	Goblet cells	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	Mitoses	5.55 ± 0.50	4.00 ± 2.00	3.00 ± 0.00	5.55 ± 4.50
	Necrosis/apoptosis	3.00 ± 0.00	4.00 ± 2.00	2.00 ± 0.00	2.00 ± 1.00
	Multifocal inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4 weeks	Edema	0.00 ± 0.00	0.20 ± 0.40	0.00 ± 0.00	0.00 ± 0.00
	Inflammation serosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation submucosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation lamina propria	0.20 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Vacuolization	2.60 ± 0.80	2.40 ± 0.49	2.60 ± 0.49	2.00 ± 1.27
	Goblet cells	1.40 ± 0.80	1.40 ± 0.49	1.20 ± 0.40	1.80 ± 0.75
	Mitoses	5.60 ± 4.03	3.20 ± 2.04	2.40 ± 3.83	2.60 ± 2.42
	Necrosis/apoptosis	2.00 ± 1.10	2.20 ± 1.94	0.60 ± 0.49	1.60 ± 1.36
	Multifocal inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

6 weeks	Edema	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation serosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation submucosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Inflammation lamina propria	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Vacuolization	3.00 ± 0.00	3.00 ± 0.00	2.40 ± 0.80	3.00 ± 0.00
	Goblet cells	1.00 ± 0.00	1.00 ± 0.00	1.80 ± 0.75	1.00 ± 0.00
	Mitoses	5.33 ± 4.19	6.00 ± 2.83	3.20 ± 2.48	2.00 ± 1.00
	Necrosis/apoptosis	1.33 ± 0.47	1.67 ± 0.94	0.80 ± 0.75	2.00 ± 0.00
	Multifocal inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

***wps:** weeks post-supplementation

3.5 Discussion

Pediococcus acidilactici MA18/5M is a homofermentative Gram-positive coccus, member of the lactic acid bacteria. This strain has shown promising results promoting growth performance, modulation of both innate and adaptive immunity, improved survival rates, and resistance to infection in fish (Dawood and Koshio, 2016; Hoseinifar et al., 2018; Wang et al., 2017a). To date, available research has demonstrated that this strain of *P. acidilactici* increases growth, blood leucocyte levels, immunoglobulin (Ig) levels, serum lysozyme activity, and transcripts encoding *il1b*, *il8*, *il10*, and *tnfa*, among others, in rainbow trout (*Oncorhynchus mykiss*) (Al-Hisnawi et al., 2019; Ferguson et al., 2010; Hoseinifar et al., 2017; Merrifield et al., 2011) and Atlantic salmon (*Salmo salar*) (Abid et al., 2013; Jaramillo-Torres et al., 2019; Langlois et al., 2021; Vasanth et al., 2015). Due to these promising findings, in 2023, a consultation has been conducted by the CFIA to approve the use of *P. acidilactici* in animal production across Canada. This represents a great opportunity to evaluate its effect in a non-traditional aquaculture fish species, providing new insight for a future decision.

Host-microbe interactions are central to fish health and aquaculture's profitability. However, studies to date are predominantly disease-centric and lack the resolution necessary for elucidating mechanistic links between microbes (beneficial or otherwise) and their aquatic hosts. The pressing need for novel sustainable solutions for mitigating infections makes the use of models appealing as a rapid and cost-effective tool for screening candidate beneficial microbes and for investigating underlying mechanisms. This knowledge is useful because it can inform *in vivo* studies and can provide actionable insights for targeted solutions.

Studies to date have focused on the characterization of the pathogenesis and virulence factors of *V. anguillarum* (Machimbirike et al., 2023), but emphasis should also be devoted to understanding the mechanisms underlying the host response to pathogenic threats. It is thought that *V. anguillarum* induces changes in the barrier function by producing proteases and toxins to invade the lamina propria (Olsson et al., 1996). Following a time-course coincubation experiment with live or heat-killed *V. anguillarum*, it was found that *e-cadherin* and *jam-1a* were significantly downregulated by 24 h post-infection in the live group relative to the heat killed group and the baseline control. Interestingly, the expression of *occludin* was extremely significantly increased in the live group at the 24 h timepoint. These data seem to suggest that *V. anguillarum* not only impairs the barrier integrity, but the pathogen can also inhibit the expression of key barrier-forming TJ molecules.

The role of *occludin* in fish is not well understood, but studies in other organisms suggest that this protein is not only an integral component of tight junctions in various tissues, but that it can also participate in tight junction remodeling in response to cytokines (Sawada, 2013; Van Itallie et al., 2010). High levels of proinflammatory cytokines, such as *tnfa* and *ifny*, promote the endocytosis of *occludin* in the tight junction complexes, which coincides with increases in tight

junction permeability (Yu and Turner, 2008). Moreover, cytokine-induced changes in TEER and flux are directly proportional to *occludin* levels (Van Itallie et al., 2010). Paradoxically, the results in the present study appear to be at odds with the observations previously reported, in which the increased expression of *occludin* given by live *V. anguillarum* exposure is associated with a decrease in resistance.

Tight junctions have complex regulatory networks that dynamically respond to physiological stimuli (Sawada, 2013). Therefore, post-transcriptional and post-translational modifications can impact the biological function of the junctions. Analyses that consider not only the molecular phenomena impacting barrier function, but also the dynamic nature of these intercellular junctions, would be instrumental in understanding how the gut epithelium responds to threats and activates immune defence mechanisms.

There was a robust upregulation of *il17a* and *tgfb* assessed by the 24 h timepoint for cells incubated with live *V. anguillarum*. Additionally, there was a time-dependent increase in the expression of *il8* for both live and heat-killed groups, and these levels were significantly higher by 24 h. These results seem to indicate that *il17a* and *tgfb* are involved in the response to secreted virulence factors, whereas *il8* might be more involved in the response to cell wall components such as lipopolysaccharides (LPS). These results are in line with the proposed mechanism of *il8* induction given by LPS in other organisms (Yan et al., 2017). The role of *il17a* secreted by intestinal epithelial cells is less clear. In mammals, *il17a* is produced by a subset of T helper cells that induce the production of antimicrobial peptides, among other proinflammatory molecules (Iwakura et al., 2008). Host stimulation by LPS, peptidoglycans, and other antigens through pattern recognition receptors enables antigen-presenting cells to activate naïve T cells that mediate the adaptive immune response to the threat (Iwakura et al., 2008). Increased expression of *il17a* is also

related to increased permeability of the blood brain barrier and small intestinal epithelial barrier (Rahman et al., 2018). In the context of the present study, pathogen-induced upregulation of *il17a* can potentially enhance the damage to the epithelial barrier and thus contribute to the establishment of the infection.

Barrier formation and integrity was assessed by TEER measurements, which were in line with levels reported previously (Geppert et al., 2016; Minghetti et al., 2017; Wang et al., 2019). A significant decrease in resistance was observed in cells exposed only to *V. anguillarum* for 24 h, but pre-treatment with *P. acidilactici* MA18/5M for 48 h prior to exposure to the pathogen mitigated these effects. These results might indicate that *P. acidilactici* MA18/5M can protect the epithelial barrier against pathogen-induced insults. Improvements in barrier function have been reportedly associated with increased levels of related tight junction gene (*cldn3* and *cdh1*) and protein (Claudin-3) levels (Geppert et al., 2016; Minghetti et al., 2017; Wang et al., 2019). Although the data in the present study is apparently in contrast with these findings, the effect observed is modest and incubation with the probiotic strain protected against pathogen-induced damage but did not increase resistance after a 48-h incubation period.

The sole interaction between *P. acidilactici* MA18/5M and RTgutGC cells demonstrated that inoculation with this strain does not affect the modulation of immune (*il1b*, *il8*, *il10*, *il6*, *tnfa*, and *tgfb*) and TJ genes (*cldn3*, *cldn12*, *zo-1*). Interestingly, our results demonstrate that pre-stimulation with *P. acidilactici* MA18/5M decreases the observed upregulation of *il17a* that occurred in presence of *V. anguillarum*, suggesting that *P. acidilactici* MA18/5M is a good candidate to evaluate in Chinook salmon. Due to this, Chinook salmon reared under aquaculture conditions were supplemented for four months to determine the effect of this strain at physiological and immunological level.

For the purpose of determining the effect of *P. acidilactici* MA18/5M on fish growth, a key variable for the aquaculture industry, it was imperative to start the study with similar size fish for every single treatment. No differences associated to the probiotic supplementation were seen during the trial. A previous *P. acidilactici* MA18/5M supplementation study performed in Atlantic salmon, showed that a 12 week supplementation with *P. acidilactici* MA18/5M did not improve the growth, specific growth rate (SGR), and thermal growth coefficient (TGC) (Jaramillo-Torres et al., 2019). This, in addition to our findings in Chinook salmon, suggest that a longer supplementation time might be required to positively impact the fish growth desired for production.

In the past, inflammation associated to the presence of antinutritional factors in plant ingredients used to feed salmonids (e.g. soybean meal products, soy protein concentrate) has increased the focus in evaluating possible inflammatory processes induced by external products in the fish intestine (Djordjevic et al., 2021; Krogdahl et al., 2023; Król et al., 2016). Although it is unknown whether enteritis can be induced by probiotic supplementation, a histopathological analysis is of a great importance to determine the safety of *P. acidilactici* MA18/5M supplementation in Chinook salmon. When *P. acidilactici* MA18/5M was added to the diet, there were no histological alterations, indicating that probiotic supplementation did not change the gut morphology in comparison to the regular pellet. Overall, in addition to the *in vitro* data indicating that *P. acidilactici* MA18/5M pre-treatment can protect the epithelial barrier against pathogen-induced damage, our study demonstrated that *P. acidilactici* MA18/5M supplementation does not affect the gut epithelial barrier, making it a great candidate for future supplementation in Canadian aquaculture.

The fish pathogen, *V. anguillarum*, is well known for inducing immune transcripts of the inflammatory response when infecting salmonids (Johnson et al., 1982; Semple et al., 2022). Therefore, it is not surprising to see an increase in the expression of *il1b*, *il8*, and *myd88* after inoculation with heat-killed *V. anguillarum*. Following this, upregulation of *tgfb*, a suppressor of the activation, proliferation, and function of T-cells to protect the organisms from inflammation is expected (Zhang et al., 2023). However, we observed increases in the expression of these genes in non-injected fish. Even though more evidence is needed to confirm the obtained results, we hypothesize that variation in the transcript encoding the genes seen here, is more likely to be associated with an ontogenic process during the parr/smolt transition, instead of the treatments. A study conducted in coho salmon (*Oncorhynchus kisutch*) proposed that variations in the hepatic gene expression profiles observed in smolts and adults might be associated to complex physiological transformations as the fish start preparing to migrate towards seawater (Gallagher et al., 2008). Also, Johansen et al. (Johansen et al., 2016) obtained differential expression of chemokines and antiviral genes in uninfected Atlantic salmon parr and smolts.

Similar to the immune genes evaluated in this study, TJ genes *marveld2*, *cdh1*, *vil1*, *cldn15*, *ocln*, *jam1a*, *zo-1*, and *muc2* did not show a modulation pattern associated with probiotic supplementation, the heat-killed *V. anguillarum* stimulation, or both. As hypothesized above, the parr/smolt transition represents an important physiological change in Chinook salmon, therefore, for genes with a complex regulatory network, such as TJ, variations can be extensive during this process. Since parr/smolt transformation represents a stressful stage that affects the intestinal homoeostasis of salmonids (Jaramillo-Torres et al., 2019; Wang et al., 2020), expected effects on gut barrier function after *P. acidilactici* MA18/5M supplementation might be masked by ontogenic

changes. To avoid this, future research should focus in determining the effect of *P. acidilactici* in adult Chinook salmon already transferred to sea pens.

3.6 Conclusion

Overall, these data suggest that the exogenous *P. acidilactici* MA18/5M strain tested, which has been isolated from terrestrial organisms, might be ill-suited to thrive in and promote host health in distantly related and physiologically distinct organisms. Second, the *in vitro* coculture system is a powerful and cost-effective tool for the investigation of host-microbe interactions. The advent of the Transwell system physiologically mimics the intestinal epithelial environment, in which the apical and basolateral compartments recapitulate the intestinal lumen and portal blood, respectively. Third, the present study is the first of its kind to employ a tissue culture of the salmonid intestine for investigating host-microbe interactions and to evaluate the potential of *P. acidilactici* MA18/5M as a candidate fish probiotic to offer protection against pathogen insults to the epithelial barrier. Lastly, gene expression of immune and TJ genes and histopathological analysis supported the findings obtained in RTgutGC, showing that *P. acidilactici* MA18/5M supplementation does not negatively impact Chinook salmon homeostasis. Future research would do well to continue to build up on the observations discussed here and use iterations of the RTgutGC model system as a high-throughput tool to identify suitable beneficial strains that can prevent pathogen attachment and pathogenesis. Additionally, future immunostimulant research should focus in evaluating an increased number of TJ genes in addition to canonical immune response transcripts, since during probiotic supplementation, gut plays a primary role in the host-microbe interaction. Since intraperitoneal infection does not simulate what occurs in natural environments, following studies would benefit from using a different delivery method for infection, such as bath immersion, to compare the results obtained by our group.

<u>Chapter 4</u>: Supplementation of lactic acid bacteria strains has differential effects in RTgutGC cells, and the immune response and gut integrity of triploid Chinook Salmon (*Oncorhynchus tshawytscha*) challenged with *Vibrio anguillarum*

4.1 Overview

Lactic acid bacteria (LAB) are well known for their potential as probiotics, improving fish growth, stress tolerance, and immune system. Hence, the long-term supplementation of LAB strains in salmonids used for aquaculture has been proven to confer beneficial effects. However, whether these strains could function as immune modulators and enhancers of the gut barrier integrity has not yet been investigated in non-traditional cultured salmonids, such as Chinook salmon (*Oncorhynchus tshawytscha*). Thus, in this study, we have performed a series of experiments in the intestinal epithelial cell line RTgutGC, and Chinook salmon juveniles aimed at establishing the potential of *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, and a strain mixture consisting of *Limosilactobacillus reuteri* RC-14 and *Limosilactobacillus rhamnosus* GR-1, as oral immunostimulants. Initially, the cytotoxicity of three LAB strains was evaluated by using the alamarBlue viability assay. *L. reuteri* LRE2, *L. reuteri* 830, and the mixture *L. reuteri* RC-14/*L. rhamnosus* GR-1 did not affect RTgutGC viability after exposure to 10^3, 10^5, and 10^7 concentrations. After this, RTgutGC was incubated with the three LAB strains for 24 h, and gene expressions of *il1b*, *il6*, *tnfa*, and *il10* were evaluated. Transcripts encoding *il1b* and *il6* in RTgutGC cells incubated with *L. reuteri* LRE2 were significantly downregulated compared to cells incubated with *L. reuteri* 830 or the mixture *L. reuteri* RC-14/*L. rhamnosus* GR-1, respectively. In contrast, *tnfa* relative expression was significantly downregulated in *L. reuteri* 830 stimulated RTgutGC cells compared to the time 0 control. Following the experiments conducted in RTgutGC, an *in vivo* study in Chinook salmon juveniles was conducted. Fish were supplemented with the above-mentioned LAB strains, and a regular feed control and a sodium alginate coating control were evaluated. Following probiotic supplementation, fish were challenged with *Vibrio anguillarum* to evaluate the effect of probiotic supplementation. After four months of LAB

supplementation, no changes in fish weight among treatments were observed. Moreover, LAB supplementation did not improve the survival of Chinook salmon IP injected with *V. anguillarum*. A significant increase in the expression of *il8* was observed in *L. reuteri* 830 or the mixture *L. reuteri* RC-14/*L. rhamnosus* GR-1 supplemented fish at 7 dpi. In contrast, no significant differences were observed in tight junction genes among treatments. Overall, our results provide valuable information regarding how members of the LAB modulate the immune response of RTgutGC and Chinook salmon.

4.2 Introduction

One major area of concern in the aquaculture of salmonids is their high susceptibility to diseases, generally attributed to unpredictable environmental conditions and high stocking densities (Figueroa et al., 2020; Peeler et al., 2011; Soto-Dávila et al., 2020a). To prevent this, antibiotics and vaccines have been frequently used as a treatment and control methods for infectious agents (Amin et al., 2017; Plant and LaPatra, 2011). Although antibiotics have been shown to improve fish survival when a disease outbreak occurs, government restrictions on antibiotic use in aquaculture have become more common since they can alter the intestinal microbiota, induce outbreaks of resistant strains, and have unpredictable long-term effects on the consumers (Miranda and Zemelman, 2002). On the other hand, vaccines are utilized as a preventive method for certain bacterial diseases. Nevertheless, since fish secondary responses do not elicit the logarithmic increases in antibody titer or affinity that are often seen in mammals after vaccination, they often lack efficacy (Arkoosh and Kaattari, 1991; Gao et al., 2014; Soto-Dávila et al., 2020a) and this problem is exacerbated by several other factors such as host species genetics and vaccine design (Figueroa et al., 2022, 2020; Valenzuela-Aviles et al., 2022). Thus, there is a need for alternative methods for protecting salmonids against infectious diseases.

As an alternative to antibiotics and vaccines, the incorporation of probiotics into aquafeeds has become a promising tool to improve fish growth, stress tolerance, and enhance non-specific defense mechanisms against pathogens (Oliva-Teles, 2012; Sakai, 1999a; Soto-Dávila et al., 2020a). In finfish aquaculture, several probiotics have been tested, and the most commonly utilized are lactic acid bacteria (LAB; *Lactobacillus* and *Carnobacterium*) (Ringø and Gatesoupe, 1998). Members of the LAB are non-motile, non-sporulating, Gram-positive, and produce lactic acid as a major or sole product of fermentative metabolism (Ringø and Gatesoupe, 1998).

Currently, it is known that supplementation with *Lacticaseibacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactococcus lactis* can modulate non-specific humoral responses, lysozyme activity, complement and plasma immunoglobulin levels in rainbow trout (*Oncorhynchus mykiss*) (Balcázar et al., 2007; Jamal et al., 2020; Vendrell et al., 2008). Moreover, *Carnobacterium divergens* and *Lactobacillus delbrueckii* sp. *lactis* has improved fish growth, lysozyme activity, and innate antiviral response in Atlantic salmon by upregulating the expression of toll-like receptor 3 and interferon-alpha (Abid et al., 2013; E Ringø et al., 2007; Salinas et al., 2008b). While the role of LAB within the digestive tract of these two salmonids has been widely studied, to date no research has been performed on the effect of supplementation with individual LAB strains on the immune response of Chinook salmon (*Oncorhynchus tshawytscha*) raised under aquaculture conditions.

Chinook salmon (also known as King salmon) is an anadromous species native to the sub-arctic North Pacific Ocean (Ohlberger et al., 2018). The fish's size and quality of flesh makes this species of particular interest to Indigenous, sport and commercial fisheries (Christensen et al., 2018; Semeniuk et al., 2019; Welch et al., 2021). However, this has led to a decrease in the number of wild stocks in the last decade (Ohlberger et al., 2018; Welch et al., 2021), so currently,

aquaculture production of Chinook salmon represents a great alternative from a commercial and conservation perspective. However, since this species is semelparous, their flesh quality decreases when the fish prepare for reproduction. To prevent this, Chinook salmon farms rely on the utilization of sterile triploid fish, preventing decreased flesh quality and extending the time available for harvesting (Ching et al., 2010; Johnson et al., 2004). Nevertheless, triploid Chinook salmon have shown an increased disease susceptibility, and have 10-30% greater mortality rates compared to diploid members of the same family (personal communication Yellow Island Aquaculture Ltd.). This high susceptibility to diseases makes triploid Chinook salmon a great candidate for probiotic supplementation.

To date, LAB supplementation in salmonids has shown promising results in laboratory conditions when using primary cell cultures (Aly et al., 2008; Amin et al., 2017; Salinas et al., 2008b). For instance, Salinas et al. (Salinas et al., 2008b) reported that *in vitro* stimulation of Atlantic salmon foregut with *Lactobacillus delbrueckii* subsp. *lactis* can help prevent cellular damage caused by *Aeromonas salmonicida* subsp. *salmonicida*. However, a lack of consistency in both the modulatory mechanisms observed during *in vitro* studies and when the host/probiotic interaction occurs, do not allow firm conclusions that probiotics are safe for fish and can exert their beneficial effects in intensive aquaculture systems (Akhter et al., 2015; Burr et al., 2005; Y. B. Wang et al., 2008). Understanding how supplementation with members of the LAB interacts with the digestive tract, using both in vitro and in vivo models is critical if probiotics are to be used for commercial aquaculture.

To do this, the present study evaluated three lyophilized products containing LAB strains *Limosilactobacillus reuteri* LRE2 (SEED 14), *Limosilactobacillus reuteri* 830 (SEED 19), and a strain mixture consisting of *Limosilactobacillus reuteri* RC-14 and *Limosilactobacillus rhamnosus*

GR-1 (Pro-B, RepHresh™) coated on feed pellets and fed to Chinook salmon over a period of 4 months. Since this study represents the first time these strains have been tested in Chinook salmon, an initial *in vitro* evaluation was conducted using the salmonid intestinal epithelial cell line derived from rainbow trout, RTgutGC. After assessing the effects of probiotic treatment on RTgutGC viability and immune gene expression, this study then investigated the effects of the LAB probiotic strains on Chinook salmon survival, growth, and the expression of tight junction and immune molecules after infection with a bacterial pathogen.

4.3 Materials and Methods

4.3.1 In vitro evaluation of the inoculation with LAB in RTgutGC

4.3.1.1 Tissue culture maintenance

The rainbow trout intestinal epithelial cell line RTgutGC was cultured as described by Kawano et al. (Kawano et al., 2011) with modifications. Briefly, cells were grown in a 75 cm^2 culture flask with Leibovitz's 15 media (HyClone, Cytiva), supplemented with 10% heat-inactivated FBS and incubated in plates or flasks sealed with Parafilm (Bemis) at 22ºC with atmospheric conditions. Cells were sub-cultured every 2-3 weeks.

To prepare plates for the viability and gene expression studies, cells were washed with 4 mL sterile PBS at room temperature (RT), and residual buffer was aspirated. Then, 4 mL of trypsin (0.05% w/v; Thermo Fisher Scientific) was added and cells were incubated for 10 min at RT in a flask to facilitate detachment. Upon detachment, 8 mL of complete culture medium (L-15 + 10% FBS) was added to quench the trypsin protease activity. The suspension was then vigorously pipetted to break up clumps of cells, and cells were seeded into 96-well plates (cell viability) and 12-well plates (RNA extraction) for following experiments.

4.3.1.2 Bacterial strains and culture conditions

The bacterial strains *Limosilactobacillus reuteri* LRE2 and *Limosilactobacillus reuteri 830* were generously provided by Seed Health, Inc. The strain mixture consisted in *Limosilactobacillus reuteri* RC-14 and *Limosilactobacillus rhamnosus* GR-1 was commercially available (Pro-B, RepHresh™). The above-mentioned strains were routinely cultured anaerobically at 37ºC in Mann, Rogosa, and Sharpe (MRS) medium (BD Difco).

4.3.1.3 Cell viability assay in RTgutGC cells exposed to LAB

RTgutGC cell line viability was quantified after exposure to *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, or *Limosilactobacillus reuteri* RC-14 + *Limosilactobacillus rhamnosus* GR-1 using alamarBlue™ cell viability reagents (Thermo Fisher Scientific). AlamarBlue™ was used to monitor the reduction of resazurin to resorufin by metabolically active cells. RTgutGC cells were seeded at 1 x 10^6 cells/well on a 96-well plate and incubated overnight at 22°C in L-15 + 10% FBS. The medium was aspirated, and the cells were treated with three different concentrations of each probiotic treatment (10^3, 10^5, and 10^7). After 24 h of incubation, cells were washed with PBS and the medium was replaced with 100 µL of alamarBlue™ and incubated 1-4 h according to the manufacturer's instructions. The quantification of fluorescence was carried out using a Take3 plate on a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA) at excitation wavelength of 560nm and emission wavelength of 590nm. RTgutGC viability for each treatment/concentration was calculated using the time 0 as a reference for 100% viability.

4.3.1.4 RNA extraction in RTgutGC cells exposed to LAB

RNA samples were obtained from RTgutGC exposed to either *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, or *Limosilactobacillus reuteri* RC-14 + *Limosilactobacillus rhamnosus* GR-1 at a concentration of 1 x 10^7 CFU/mL. Cells were treated with the respective concentration for 24 h prior RNA extraction.

Total RNA was extracted using 1 ml of TRIzol Reagent (Invitrogen) and purified using Ambion DNase I (RNase free) (Ambion™ DNase I, Invitrogen) following the manufacturer's instructions. Briefly, 5 µg of RNA was treated with 2 µL of Ambion DNase I, 4 µL of DNase buffer x10, and DEPC water to complete 40 µL. Then, samples were incubated at 37°C for 30 min, washed twice with wash solution A, centrifuged at 3,500 x g for 1 min and purified in an RNA/Protein Purification Column. DNase treated RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using the Take3 plate of a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA). Column purified RNA samples had A260/280 ratios between 1.9 and 2.1 and A260/230 ratios between 2.0 and 2.2. A PCR test was conducted using the reference gene elongation factor 1 alpha (*ef1a*) and the RNA as template to rule out the presence of DNA. All RNA samples did not show presence of DNA.

First-strand cDNA templates for qPCR were synthesized from 250 ng of DNaseI-treated, column-purified total RNA using qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Each sample was incubated at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min. Samples were stored at -20°C until utilization.

4.3.1.5 qPCR in RTgutGC cells exposed to LAB

All qPCR reactions were performed in a final volume of 10 µL, containing 5µL of 2x WISENT ADVANCED™ qPCR master mix (Wisent, Quebec, Canada), 2.5 µL of forward and reverse primer mix (Sigma Aldrich, USA) at a final concentration of 0.25 µM each, and 2.5 µL of cDNA (2.5 ng/µL, 6.25 ng per reaction). All samples were amplified and detected using the LightCycler® 480 II (Roche, USA). The reaction mixtures were pre-incubated for 2 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, annealing for 30 s at 60°C, and finally extension for 8 s at 72°C. The melt curve was completed for each run every 5 s from 65°C to 97°C.

The primer sequences of interleukin 8 (*il8*), interleukin 6 (*il6*), tumor necrosis factor alpha (*tnfa*), and interleukin 10 (*il10*), used in this study are listed in Table 4.1. Gene discovery, qPCR primer design, and initial quality testing were performed as described in Soto-Davila et al. (Soto-Dávila et al., 2022). Since the reagents, cycling conditions and samples were different in the current study, primer efficiencies were reassessed. Briefly, a 7-point 1:3 dilution series starting with cDNA representing 5 ng of input total RNA was generated, and efficiencies then calculated using the formula $E = 10^{(-1/slope)}$ (Pfaffl, 2001).

Table 4.1. Primers used in this study.

Gene name	Sequence (5'-3')	Accession number	Amplicon size (bp)	Reference
Interleukin 1 beta (*il1b*)	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	AJ223954	73	(Frenette et al., 2023)
Interleukin 6 (*il6*)	F: GTTCTGGGTGAGGTGTCTA R: GGTGTCAACCAGGAAGTTAC	NM_001124657	93	(Schug et al., 2019)
Interleukin 8 (*il8*)	F: ATTGAGACGGAAAGCAGACG R: CGCTGACATCCAGACAAATCT	NM_001140710	136	(Wang et al., 2020)
Interleukin 10 (*il10*)	F: CCATCAGAGACTACTACGAGGC R: TCTGTGTTCTGTTGTTCATGGC	NM_001245099.1	165	(Wang et al., 2020)
Interleukin 17a (*il17a*)	F: TGGTTGTGTGCTGTGTGTCTATGC R: TTTCCCTCTGATTCCTCTGTGGG	GW574233	136	(Wang et al., 2020)

Tumor necrosis factor alpha (*tnfa*)	F: GTGATGCTGAGTCCGAAAT R: GTCTCAGTCCACAGTTTGTC	AJ277604.2	97	(Semple et al., 2018)
Myeloid differentiation factor 88 (*myd88*)	F: GACAAAGTTTGCCCTCAGTCTCT R: CCGTCAGGAACCTCAGGATACT	NM_001136545	110	This study
Transforming growth factor β (*tgfb*)	F: AGTTGCCTTGTGATTGTGGGA R: CTCTTCAGTAGTGGTTTGTCG	EU082211	191	This study
Tricellulin (*marveld2*)	F: TCCAACACAGGCTCATCTCTT R: ATGGGGTTCATGACGGACAC	XM_036977097.1	83	This study
E-cadherin (*cdh1*)	F: ACTACGACGAGGAGGGAGGT R: TGGAGCGATGTCATTACGGA	XM_021585993.2	107	This study
Villin 1 (*vil1*)	F: AAAGTTCAGGTGCTGTAAATCGC R: TGTGGCATGGTGCCAGATTC	XM_021579239.2	148	This study
Claudin 28b (*cldn28b*)	F: CTCACTCTACATCGGCTGGG R: CACAGAACTAGCAGCCTTGGA	NM_001195160.1	161	This study
Claudin 15 (*cldn15*)	F: GGCACGTCTGAGAAACAACC R: TAGGAAGTGGCAGCCTGACT	XM_036987534.1	92	This study
Occludin (*ocln*)	F: F: GACAGTGAGTTCCCCACCAT R: AGCTCTCCCTGCAGGTCCTT	XM_021601275.2	101	This study
Junctional adhesion molecule 1 alpha (*jam1a*)	F: TGAGGATGGAAGTCCGCAAC R: GTACCACAGTCCGAAGCACA	XM_021564368.2	98	This study
Zonula occludens-1 (*zo-1*)	F: GCTGTTCCTCCTAGACCTT R: TCACCCACATCTGACTCTAC	XM_021607172.1	99	(Schug et al., 2019)
Mucin 2 (*muc2*)	F: CCAGTGTCAGTGCAAACACG R: ATGTAGCAGGGCTGGGTAGA	XM_042327631.1	122	This study
[a,b,c]Elongation factor 1 alpha (*ef1a*)	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	NM_001124339	134	(Semple et al., 2018)
[b,c]Beta actin (*actb*)	F: TGGACTTTGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG	AJ438158.1	139	(Ma et al., 2019)
[b]Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	F: GCTGGAATGGGACTCACAC R: GTCAAAACCGTCTCAGTGGG	NR	NR	(Rajanbabu and Chen, 2011)
[b]18S ribosomal RNA (*18S*)	F: CGTCGTAGTTCCGACCATAAA R: CCACCCACAGAATCGAGAAA	NR	NR	(Giroux et al., 2019)
Internal transcribed spacer 2 locus (*its2*)	F: TCATCAATCGGAACCTCTGG R: AAGGAAGAGCGCACGGG	NR	156	(Eder et al., 2009)

[a]Normalizer used in experimental RTgutGC qPCR analyses.

[b]Normalizers used in experimental Chinook salmon qPCR analyses.

[c]Candidate normalizer genes for *in vivo* trial

Amplification efficiencies were calculated using a 7-point 1:3 dilution series starting with cDNA representing 50 ng of input total RNA. See methods for details.

NR: Not reported.

After the testing was completed, transcript levels of the genes of interest were analyzed in each of the individual study samples, with normalization to *ef1a*. In all cases, levels were assessed

(in technical triplicates) in three independent wells per treatment using cDNA representing 2.5 ng of input total RNA. A NRT control was included on each plate. Gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.3.2 In vivo evaluation of the inoculation with LAB in Chinook salmon

4.3.2.1 Fish holding

Chinook salmon, *Oncorhynchus tshawytscha* (10 ± 2 g) were obtained from Yellow Island Aquaculture Ltd. (www.yellowislandaquaculture.ca, Quadra Island, British Columbia, Canada), and maintained in 12 Powell River troughs (500 L) in freshwater (FW, 14 ± 2°C) at a density of ~8 kg/m^3 using a flow-through system under natural photoperiod (12:12 h dark:light). Five treatments were conducted to evaluate the effect of LAB probiotics administered to Chinook salmon, each treatment in duplicates, with 150 fish per tank (300 fish per treatment, 1500 fish in total). Four months before the infection trial, the fish were fed commercial dry pellet (EWOS Harmony 2 mm: 47% protein, 18% fat, 0.7% fiber, 2.9% calcium, 1.2% phosphorus, and 0.6% sodium) twice a day with a ration of 2% body weight, in addition to their respective dietary treatments.

All fish were kept and handled under a permit (ethical protocol #43212) from the University of Waterloo Animal Care Committee according to CCAC guidelines.

4.3.2.2 Feeding and Probiotic Supplement Preparation

Commercial Chinook salmon dry food (EWOS Harmony 2 mm) was used as a control diet for the supplementation of probiotic. Additionally, a sodium alginate control, the component utilized to maintain the probiotic stick to the pellet, was tested. The probiotic treatments used in

this experiment were the following: i) *Limosilactobacillus reuteri* LRE2; ii) *Limosilactobacillus reuteri* 830; iii) *Limosilactobacillus reuteri* RC-14/*Limosilactobacillus rhamnosus* GR-1.

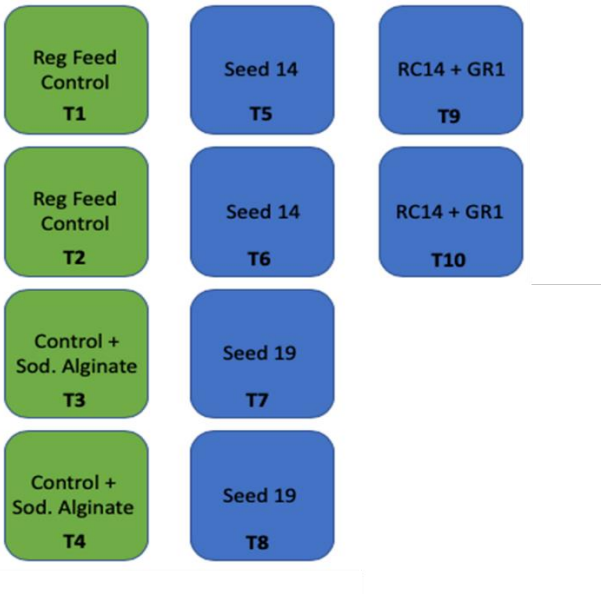


Figure 4.1. Experimental design. A total of 1500 Chinook salmon juveniles were transferred in duplicate into 5 different treatments (150 fish per tank): i) Regular feed control; ii) Control + Sod. Alginate; iii) *Limosilactobacillus reuteri* LRE2 (Seed 14); iv) *Limosilactobacillus reuteri* 830 (Seed 19); v) *Limosilactobacillus reuteri* RC-14/*Limosilactobacillus rhamnosus* GR-1 (RC14/GR-1.

4.3.2.3 Vibrio anguillarum Growth Conditions

Vibrio anguillarum serotype O1 (J382) (Machimbirike et al., 2023) isolated from winter Steelhead trout obtained from Little Campbell River (British Columbia, Canada) was utilized for the infection trial. Briefly, a single colony of *V. anguillarum* from a TSA 2% NaCl (Trypticase Soy Agar 2% Sodium Chloride) stock was grown in 2.5 mL of Trypticase Soy Broth 2% sodium chloride (TSB 2% NaCl; Multicell Wisent, Quebec, Canada) at 20°C in a 16 mm diameter glass tube and placed in a shaker for 24 h at 200 rpm. After growth, 150 µL of the overnight culture were added in 150 mL of TSB 2% NaCl media using a 250 mL flask and incubated for 24 h at

20°C with aeration (200 rpm). After overnight culture, the bacterial inoculum was centrifuged at 6,000 rpm at RT for 10 min. The pellet was washed three times with PBS and centrifuged at 6,000 rpm at RT for 10 min, and finally resuspended in 25 mL of PBS (~7.8×10^8 CFU mL^{-1}). The concentrated bacterial inoculum was serially diluted and quantified by plating onto TSA 2% NaCl for 2 days.

4.3.2.4 LAB supplemented Chinook salmon challenged with V. anguillarum

After 4 months under control diet, control coating diet, or LAB supplementation (*Limosilactobacillus reuteri* LRE2; *Limosilactobacillus reuteri* 830; *Limosilactobacillus reuteri* RC-14/*Limosilactobacillus rhamnosus* GR-1), Chinook salmon juveniles were intraperitoneally (I.P.) injected with 100 µL of live *V. anguillarum* (1×10^5 CFU mL^{-1}). Time 0 (n = 5 fish per tank per treatment) corresponds to fish sampled before the injection. Fish survival was recorded for 18 days. The sampling schedule was 0, 1-, 3-, 7-, and 14-days post-inoculation (dpi). For sampling, fish were anesthetized with a non-lethal dose of clove oil, followed by euthanasia by cervical dislocation. Growth data was collected, and then spleen and hindgut tissues were isolated and immediately snap-frozen in liquid N_2 prior to being stored at -80°C until utilization.

4.3.2.5 RNA Extraction

To prepare RNA for subsequent gene expression analyses, spleen and hindgut samples were homogenized using 1 mL of TRIzol following the manufacturers protocol (Invitrogen). Samples were vortexed for 15 s, incubated at RT for 10 min, then centrifuged at 16,000 rpm for 15 min at 4ºC. The aqueous layer was collected and transferred to a new 1.7mL tube. Samples were kept on ice henceforward. One volume of 100% isopropanol per 1 volume of sample was added to the new aqueous fractions, samples were vortexed briefly, and incubated at RT for 5 min.

Samples were then centrifuged at 16,000 rpm for 5 min at 4ºC, and supernatant was discarded. After washing the RNA thrice with 70% ethanol, the residual ethanol was carefully removed, and pellets were air-dried for 15 min. The RNA was then resuspended in 40 µL of nuclease-free water and allowed to dissolve in a water bath at 55ºC for 15 min.

After extraction, the RNA was treated with Ambion DNase I (RNase free) (Ambion™ DNase I, Invitrogen) following the manufacturer's instructions to degrade any residual genomic DNA. RNA samples were quantified and evaluated for purity (A260/280 and A260/230 ratios) using a Take3 plate of a Synergy H1 Hybrid plate Reader (Biotek Instruments, Inc., USA). Column purified RNA samples had A260/280 ratios between 2.0 and 2.1 and A260/230 ratios between 2.0 and 2.2.

First-strand cDNA templates for qPCR were synthesized from 250 ng of DNaseI-treated, column-purified total RNA using qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Each sample was incubated at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min. Samples were stored at -20°C until utilization.

4.3.2.6 qPCR analysis

All qPCR reactions were performed in a final volume of 10 µL, containing 5µL of 2x WISENT ADVANCED™ qPCR master mix (Wisent, Quebec, Canada), 2.5 µL of forward and reverse primer mix (Sigma Aldrich, USA) at a final concentration of 0.25 µM each, and 2.5 µL of cDNA (2.5 ng/µL, 6.25 ng per reaction). All samples were amplified and detected using the LightCycler® 480 II (Roche, USA). The reaction mixtures were pre-incubated for 2 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, annealing for 30 s at 60°C, and finally extension for 8 s at 72°C. The melt curve was completed for each run every 5 s from 65°C to 97°C.

The primer sequences of interleukin 1 beta (*il1b*), interleukin 8 (*il8*), interleukin 10 (*il10*), interleukin 17a (*il17a*), tumor necrosis factor alpha (*tnfa*), transforming growth factor-beta (*tgfb*), Myeloid differentiation primary response 88 (*myd88*), claudin 15 (*cldn15*), claudin 28b (*cldn28b*), occludin (*ocln*), e-cadherin (*cdh1*), zonula occludens-1 (*zo-1*), junction adhesion molecule 1 alpha (*jam1a*), tricellulin (*marveld2*), mucin 2 (*muc2*), and villin 2 (*vil2*) used in this study are listed in Table 4.1. Gene discovery, qPCR primer design and initial quality testing were performed as described in Soto-Davila et al. (Soto-Dávila et al., 2020c). Since the reagents, cycling conditions and samples were different in the current study, primer efficiencies were reassessed. Briefly, a 7-point 1:3 dilution series starting with cDNA representing 5 ng of input total RNA was generated, and efficiencies then calculated using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001).

Transcript levels of the target genes were normalized to transcript levels of three endogenous control genes. Levels of five candidate normalizers [*ef1a*, beta actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), 18S ribosomal RNA (*18S*), and internal transcribed spacer 2 (*its2*)] were assessed in 50% of the samples (i.e., in 3 random samples per treatment) using cDNA representing 2.5 ng (6.25 ng per reaction) of input total RNA. Reference gene stability was assessed using geNorm, NormFinder, BestKeeper, and Delta Ct comparison, through the bioinformatic open-access portal RefFinder (Soto-Dávila et al., 2022; Xie et al., 2012). Most stable genes assessed were *its2*, *gapdh* and *actb*.

After normalization testing was completed, transcript levels of the genes of interest were analyzed in the individual study samples, with normalization to the average of the three endogenous genes selected as described in Riedel et al. (Riedel et al., 2014). In all cases, levels were assessed (in technical triplicates) in five individuals per treatment per time-point using cDNA

representing 2.5 ng of input total RNA. A no RT control was included on each plate. Gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.3.2.7 Statistical Analyses

Cell viability and RTgutGC gene expression data are shown as the mean ± standard error (SE). Assumptions of normality and homoscedasticity were tested for the detected variances. RTgutGC cell viability data was analyzed using a 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. For qPCR data, a Kruskal-Wallis non-parametric test was performed. Differences were considered significant at $P < 0.05$.

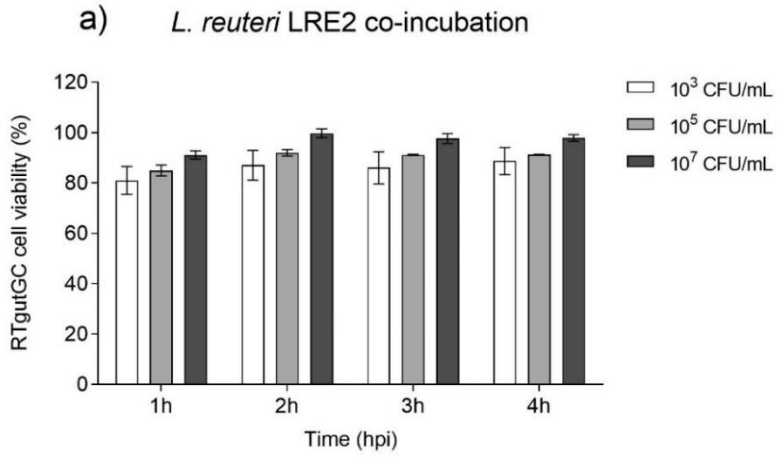
Fish survival, growth, and gene expression data are shown as the mean ± standard error (SEM). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***).

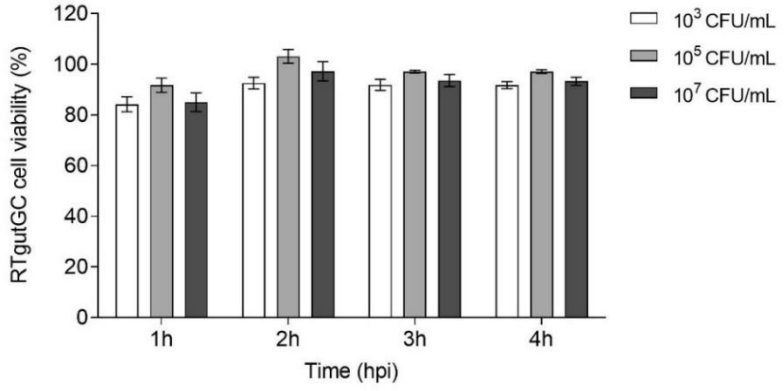
All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism 8 (GraphPad Software, La Jolla California USA).

4.4 Results

4.4.1 Evaluation of the Toxicity of LAB in RTgutGC

Salmonid intestinal epithelial cell line (RTgutGC) viability was determined after 4 h of exposure to the concentrations 1 x 10^3, 1 x 10^5, and 1 x 10^7 CFU/mL^{-1} of *L. reuteri* LRE2 (Figure 4.1a), *L. reuteri* 830 (Figure 4.1b), and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 (Figure 4.1c). Results obtained in this study did not show significant differences in RTgutGC viability exposed to any LAB strains at different concentrations after 1 h, 2 h, 3 h, and 4 h (Figure 4.1a, b, and c).


a)
L. reuteri LRE2 co-incubation
RTgutGC cell viability (%)
120
100
80
60
40
20
0
1h
2h
3h
4h
Time (hpi)
10^3 CFU/mL
10^5 CFU/mL
10^7 CFU/mL


b)
L. reuteri 830 co-incubation
RTgutGC cell viability (%)
120
100
80
60
40
20
0
1h
2h
3h
4h
Time (hpi)
10^3 CFU/mL
10^5 CFU/mL
10^7 CFU/mL

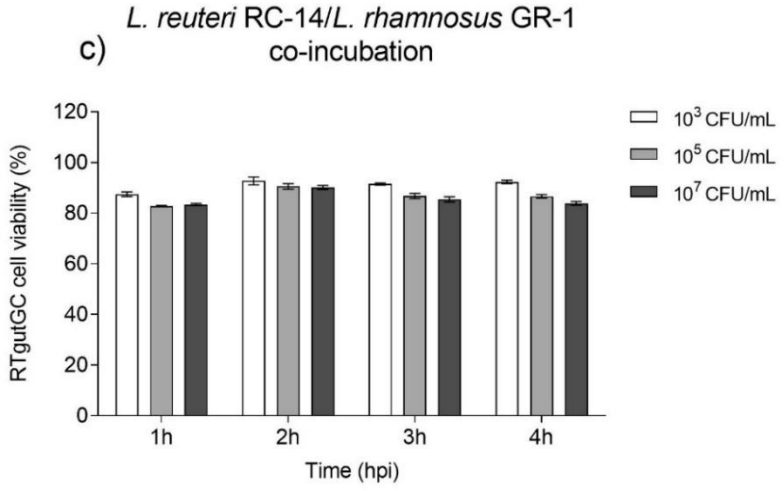

c)
L. reuteri RC-14/L. rhamnosus GR-1
co-incubation
RTgutGC cell viability (%)
120
100
80
60
40
20
0
1h
2h
3h
4h
Time (hpi)
10^3 CFU/mL
10^5 CFU/mL
10^7 CFU/mL

Figure 4.2. Cell viability of RTgutGC treated cells with 10^3, 10^5, and 10^7 CFU/mL of (a) *L. reuteri* LRE2 (b) *L. reuteri 830*, and (c) *L. reuteri* RC-14/*L. rhamnosus* GR-1 for 1, 2, 3, and 4 h. Each value represents the mean ± S.E.M (n = 3). Differences were not statistically significant (Sidak *post-hoc* test). Two-way ANOVA analysis using one dependent variable cell viability (percentage converted with square root) and the independent variables treatment and time, do not varied among treatments, times, and the interaction between treatments and time.

4.4.2 RTgutGC relative expression after LAB inoculation

Transcript levels of the innate immune response-related genes *il1b, il6, tnfa,* and *il10* (Figures 4.2a, b, c, and d) were evaluated by qPCR in RTgutGC after 24 h of stimulation with the previously described treatments.

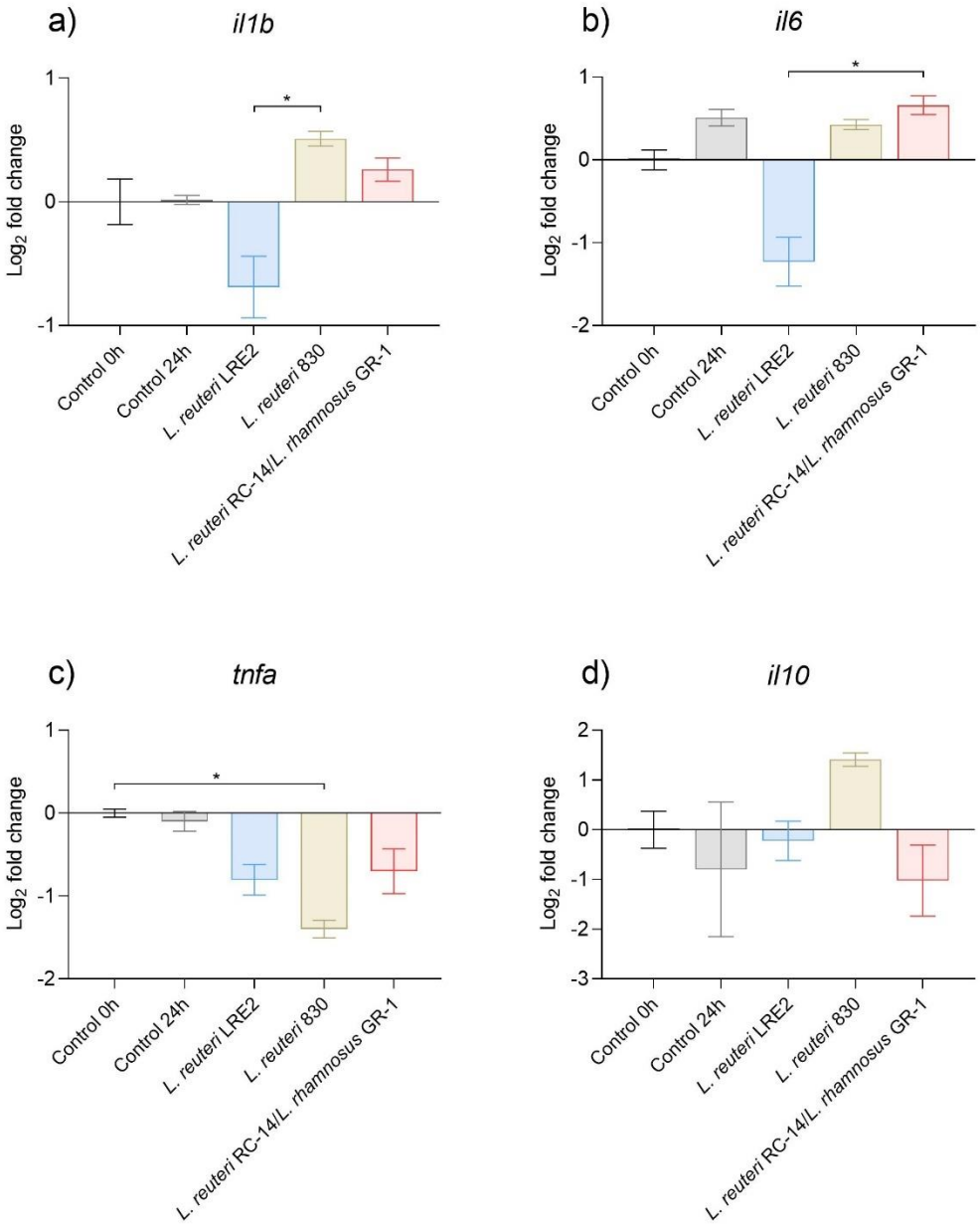


Figure 4.3. Relative gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 6 (*il6*), (c) tumor necrosis factor alpha (*tnfa*), and (d) interleukin 10 (*il10*) in RTgutGC unstimulated (control) or inoculated with *L. reuteri* LRE2, *L. reuteri* 830, or *L. reuteri*RC-14/*L. rhamnosus* GR-1 for 24 h. All data are expressed as mean values ± SEM (n=3). Bars indicate statistically significantly differences among treatments (*p > 0.05, **p > 0.01, ***p > 0.001). One-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variable treatments, has shown that relative expression varied among treatments (*il1b*: $p < 0.0004$; *il6*: $p < 0.0007$; *tnfa*: $p < 0.0009$).

Gene transcription of the pro-inflammatory cytokine *il1b* was significantly up-regulated in RTgutGC inoculated with *L. reuteri* 830 compared to cells inoculated with *L. reuteri* LRE2 (Figure 4.2a). In contrast, *il6* showed a statistically significant increase in cells inoculated with *L. reuteri* RC-14/ *L. rhamnosus* GR-1 compared with the *L. reuteri* LRE2 treatment (Figure 4.2b). Transcripts encoding the expression of *tnfa* in RTgutGC were significantly downregulated in *L. reuteri* 830 stimulated cells compared to the control group without sham stimulation (0 h) (Figure 4.2c). Finally, no significant differences were observed in the expression of *il10* (Figure 4.2d).

4.4.3 Triploid Chinook salmon survival and growth after LAB supplementation

Our results did not show statistically significant differences in fish supplemented for four months with the three experimental probiotic diets (*L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1) and then challenged with *V. anguillarum*, compared to fish supplemented with either a regular control diet or regular control diet + sodium alginate (Figure 4.3).

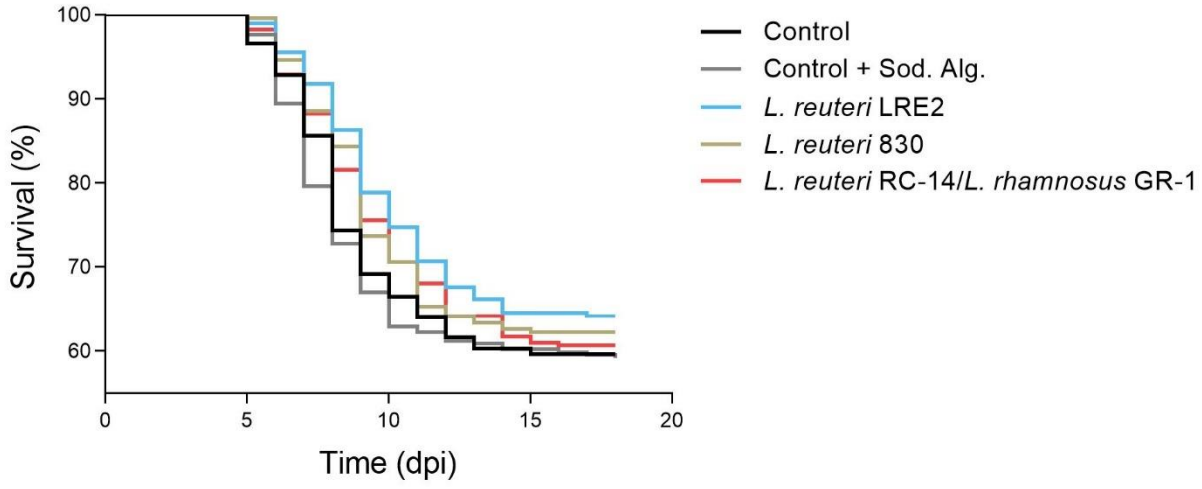


Figure 4.4. Survival of Chinook salmon supplemented with LAB strains to a *Vibrio anguillarum* challenge. Differences were not statistically significant (Mantel-Cox test).

Similarly, no significant differences in the weight (g) of fish supplemented with three different LAB diets and then challenged with *V. anguillarum* were recorded after 14 dpi compared to the control and the control + sod. alg. treatments (Figure 4.4).

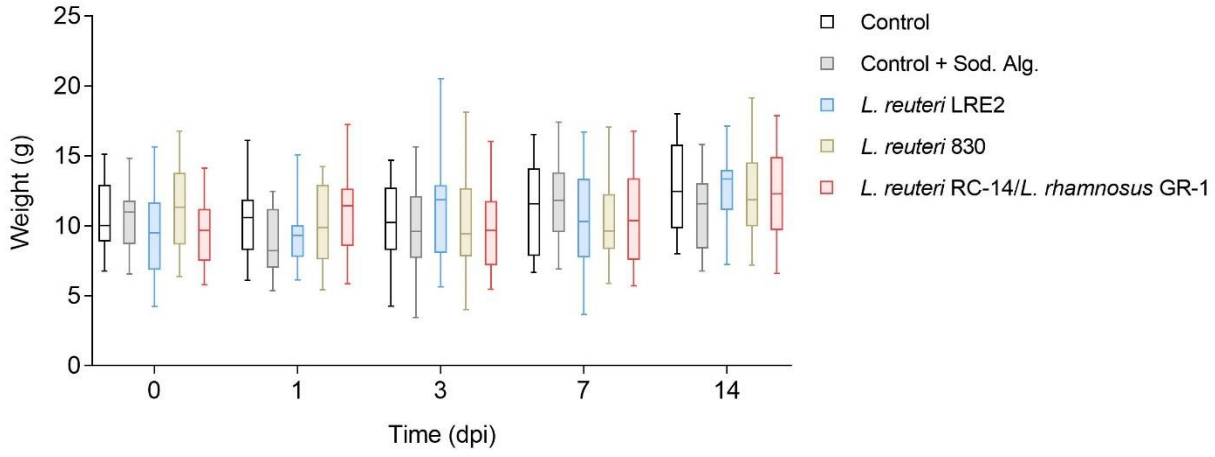


Figure 4.5. Weight (g) increase in Chinook salmon juveniles unsupplemented or supplemented with LAB, and challenged with *V. anguillarum*. Each value is the mean ± S.E.M. Differences were not statistically significant (Tukey *post hoc* test). Two-way ANOVA analysis using one dependent variable weight (g) and the independent variables treatment and time, has shown that relative expression did not variy among treatments, time, or the interaction between treatments and time.

4.4.4 Spleen gene expression of triploid Chinook salmon supplemented with LAB and then challenged with V. anguillarum

Quantitative real-time transcription of genes involved in the innate immune response of Chinook salmon fed with sodium alginate or supplemented with *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 was evaluated after inoculation with *V. anguillarum*

(Figure 4.5). The levels of expression at different timepoints after injection were normalized against the values obtained in the supplementation control (Figure 4.5).

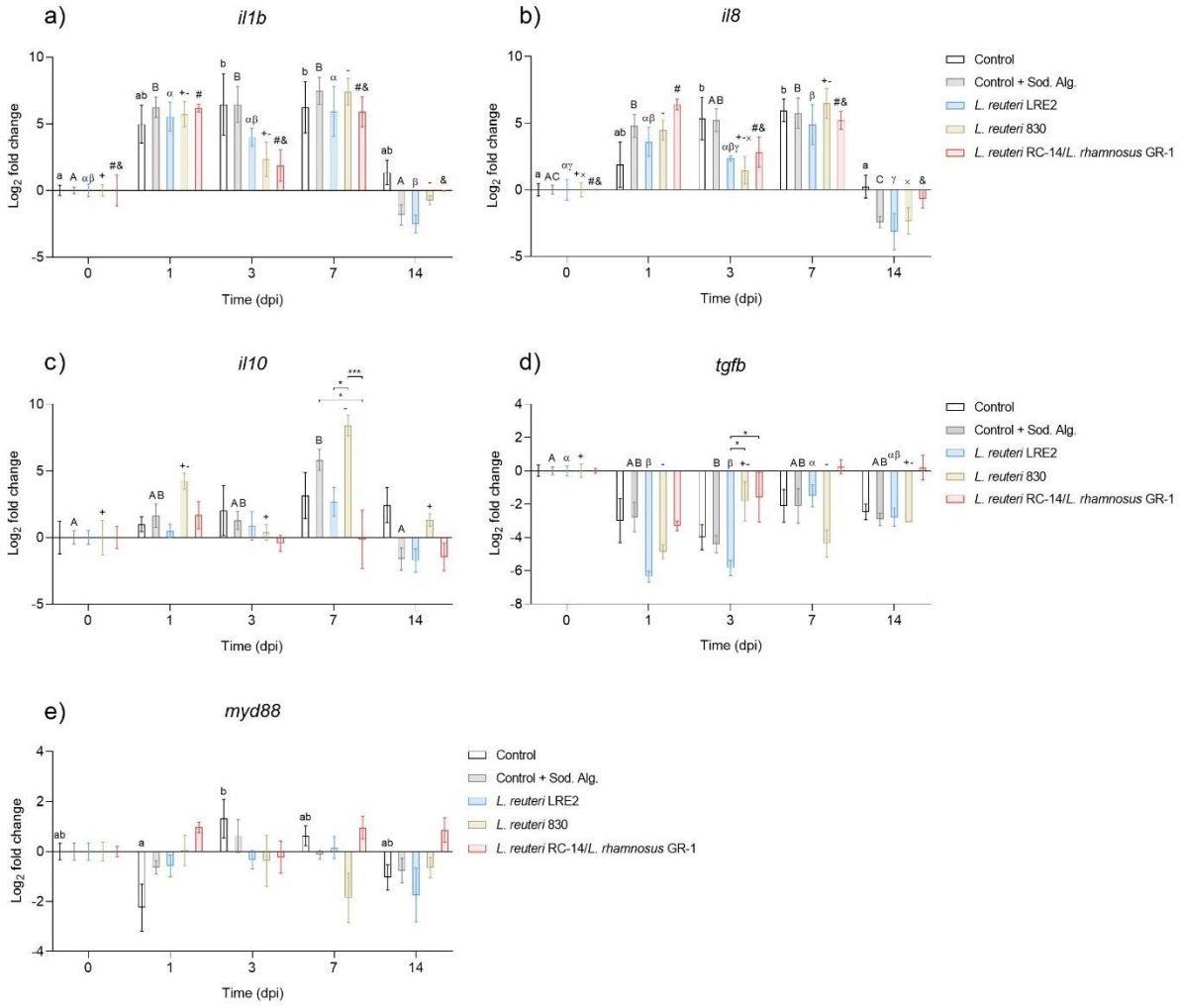


Figure 4.6. Spleen relative gene expression of (a) interleukin 1b (*il1b*), (b) interleukin 8 (*il8*), (c) interleukin 10 (*il10*), (d) transforming growth factor β (*tgfb*), and (e) myeloid differentiation factor 88 (*myd88*) in Chinook salmon supplemented with *L. reuteri* LRE2, *L. reuteri* 830, or *L. reuteri* RC-14/*L. rhamnosus*. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*il10*: $p < 0.0004$; *tnfa*: $p < 0.0027$), time (*il1b*: $p < 0.0001$; *il8*: $p < 0.0001$; *il10*: $p < 0.0001$; *tnfa*: $p < 0.0001$), and the interaction between treatments and time (*il8*: $p < 0.0461$; *il10*: $p < 0.0081$; *tnfa*: $p < 0.0080$; *myd88*: $p < 0.0097$).

The transcripts encoding the expression of the pro-inflammatory cytokine *il1b* and *il8*, a chemotactic factor that attracts neutrophils, basophils, and T-cells during the inflammatory process, showed a similar pattern of upregulation en each treatment at different timepoints (Figures 4.5a and b). Despite some minor differences, all the treatments showed a significant upregulation of *il1b* and *il8* between 1 and 7 dpi with *V. anguillarum* compared to the expression observed 0 and 14 dpi (Figures 4.5a and b). The antagonist response from the anti-inflammatory cytokine *il10* showed that 7 dpi fish fed with the coating control that includes sodium alginate was significantly upregulated compared to the *L. reuteri* RC-14/ *L. rhamnosus* GR-1 treatment (Figure 4.5c). additionally, an upregulation of *il10* was observed in fish supplemented with *L. reuteri* 830 compared to the other two probiotic treatments *L. reuteri* LRE2 and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 (Figure 4.5c).

In contrast to the gene upregulation observed in *il1b*, *il8* and *il10* (Figures 4.5a, b, and c), the expression of *tgfb*, and anti-inflammatory multifunctional cytokine, was sustainedly downregulated after the infection (Figure 4.5d). Specifically, *tgfb* was downregulated 3 dpi in fish supplemented with *L. reuteri* LRE2 compared to *L. reuteri* 830 or *L. reuteri* RC-14/ *L. rhamnosus* GR-1 supplemented fish (Figure 4.5d). In addition, the relative expression of *tgfb* was significantly downregulated among days 1, 3 and 7 post infection in individual obtained from the control + Sod. Alg., *L. reuteri* LRE2, and *L. reuteri* 830 treatments compared to fish collected from their respective groups before the infection (Figure 4.5d). Finally, the expression of *myd88*, a key adapter protein that activates innate effector mechanisms in fish, showed a significant increase in the expression of fish supplemented with the control diet at 3 dpi compared to 1 dpi fish from the same treatment (Figure 4.5e).

4.4.5 Hindgut immune gene modulation in triploid Chinook salmon supplemented with LAB and then challenged with V. anguillarum

The expression of several immune markers was assessed to investigate the effect LAB supplementation on pro- and anti-inflammatory molecules during the *V. anguillarum* challenge (figure 4.6). Among all the immune genes evaluated in hindgut, only *il8* and *il10* showed significant differences (Figures 4.6a and d). The expression of *il8* was significantly increased 7 dpi in the group supplemented with *L. reuteri* 830 compared to the control + Sod. Alg. and the *L. reuteri* LRE2 treatments (Figure 4.6a). Moreover, an upregulation of *il8* in *L. reuteri* RC-14/ *L. rhamnosus* GR-1 supplemented fish occurred 7 dpi compared to the *L. reuteri* LRE2 treatment (Figure 4.6a).

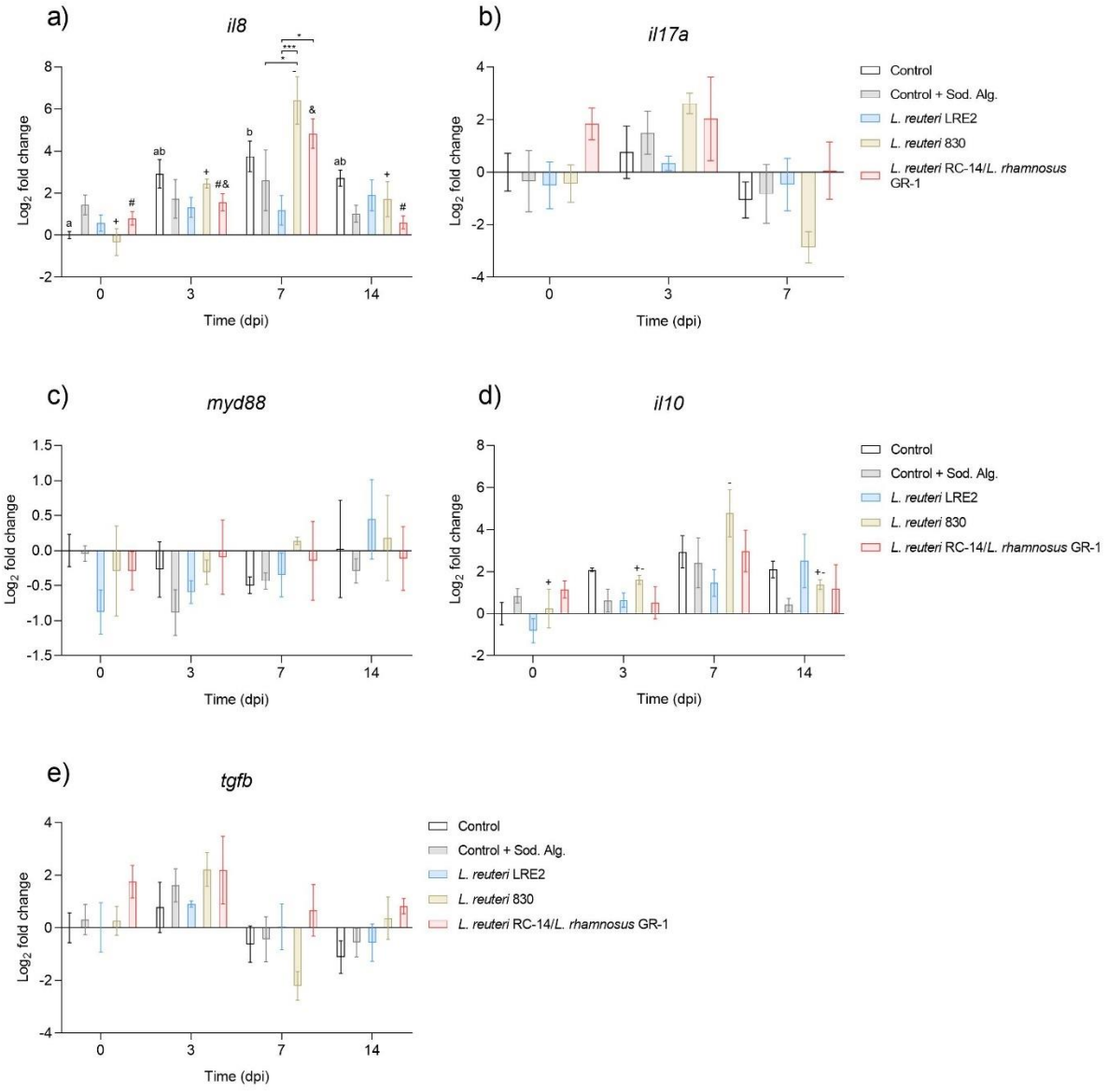


Figure 4.7. Hindgut relative gene expression of (a) interleukin 8 (*il8*), (b) interleukin 17a (*il17a*), (c) myeloid differentiation factor 88 (*myd88*), (d) interleukin 10 (*il10*), and (e) transforming growth factor β (*tgfb*) in Chinook salmon supplemented with *L. reuteri* LRE2, *L. reuteri* 830, or *L. reuteri* RC-14/*L. rhamnosus*. Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*il8*: $p < 0.0008$), time (*il8*: $p < 0.0001$; *il8*: $p < 0.0110$), and the interaction between treatments and time (*il8*: $p < 0.0002$).

The expression of *il10* was significantly increased in the group supplemented with *L. reuteri* 830 after 7 days of disease challenge compared to time 0 (Figure 4.6d). The expression of the pro-inflammatory molecules *il17a*, *tgfb*, and *myd88* were likewise unchanged between and within groups (Figures 4.6b, c, and e).

4.4.6 Expression of hindgut tight junction genes in triploid Chinook salmon supplemented with LAB and then challenged with V. anguillarum

To investigate whether the LAB strain supplementation could modulate gut barrier function transcript level, the expression of tight junction molecules was assessed through qPCR (Figure 4.7). Hindguts of individuals sampled on 0, 3, 7, and 14 dpi were included in the study to assess the short term (3 dpi) and medium- to long-term (7 and 14 dpi, respectively) effects of infection on tight junction markers relative to the day 0 baseline of fish supplemented with LAB. The expression of the pore-forming molecules *cldn15* and *cldn28b* (Figures 4.7a and b), the barrier forming molecules *ocln*, *cdh1*, *zo-1*, and *marveld2* (Figures 4.7c, d, e, and g), as well as the major gel-forming molecule in the intestinal mucus, *muc2* (Figure 4.7h), was not significantly changed throughout the infection challenge. In contrast, the barrier molecule *jam1a*, was significantly upregulated in fish supplemented with *L. reuteri* LRE2 strain 7 dpi compared to day 0 individuals supplemented with the same probiotic (Figure 4.7f). Finally, the transcripts encoding *vil1* gene expression, a molecule that regulates intestinal epithelial morphology, was significantly higher 3 dpi compared to 7 dpi in the control group (Figure 4.7i).

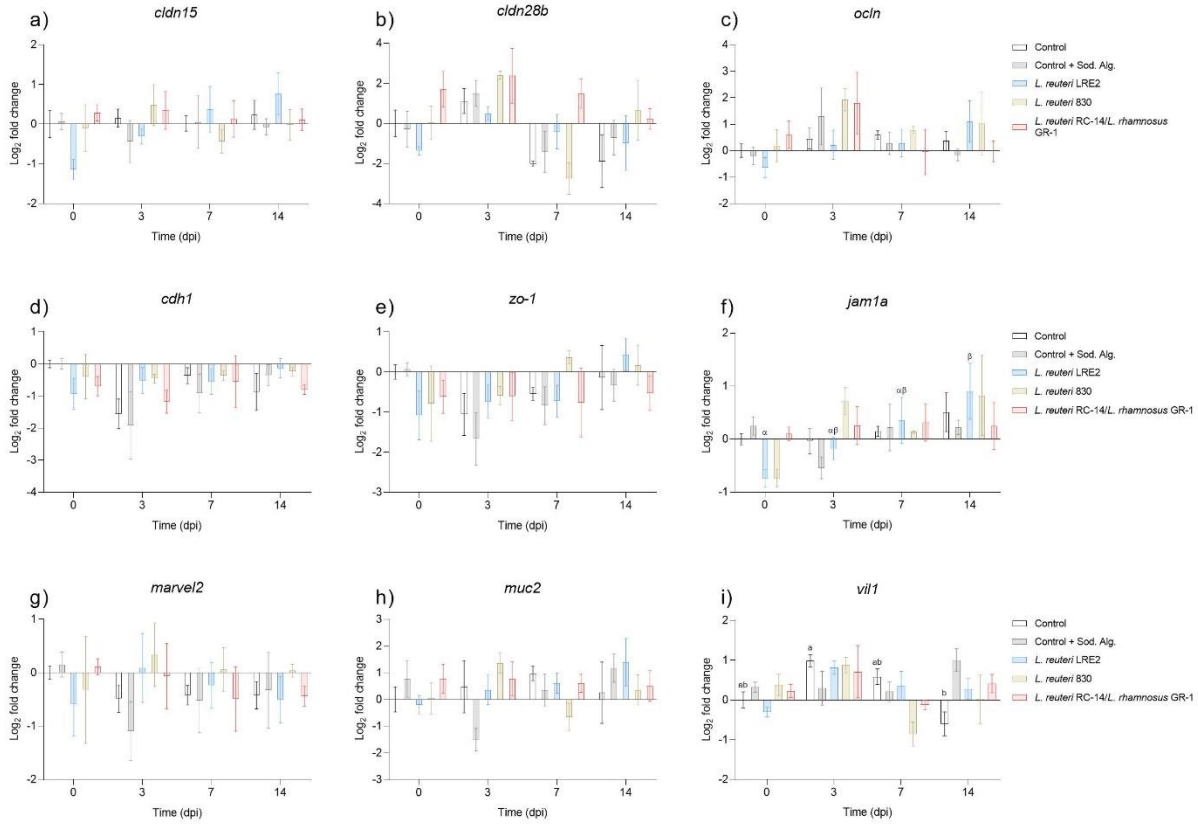


Figure 4.8. Hindgut relative TJ gene expression of (a) claudin 15 (*cldn15*), (b) claudin 28 b (*cldn28b*), (c) occludin (*ocln*), (d) e-cadherin (*cdh1*), (e) zonula occludens 1 (*zo-1*), (f) junctional adhesion molecule 1 alpha (*jam1a*), (g) tricellulin (*marveld2*), (h) mucin 2 (*muc2*), and villin 1 (*vil1*) in Chinook salmon supplemented with *L. reuteri* LRE2, *L. reuteri* 830, or *L. reuteri* RC-14/*L. rhamnosus.* Each value is the mean ± S.E.M (n = 5). Bars represent significant differences between treatments at the same time-points. Different symbols/letters represent significant differences in each treatment at different times (*p > 0.05, **p > 0.01, ***p > 0.001). Two-way ANOVA analysis using one dependent variable relative expression (Log_2 fold change) and the independent variables treatment and time, has shown that relative expression varied among treatments (*jam1a*: $p < 0.0322$; *vil1*: $p < 0.0289$).

4.5 Discussion

The mechanisms by which LAB produces local and systemic effects in Chinook salmon are not understood, but it is thought that after the gut colonization, these beneficial microbes would stimulate commensal bacteria from the host and stimulate the immune cells associated with the

mucosa, and these, in turn, would amplify the effect when translocated into immune organs (Balcázar et al., 2006; Khati et al., 2018; Langlois et al., 2021). To assess this, potential probiotic strains and concentrations should be evaluated in Chinook salmon specimens, however, initial *in vitro* evaluation of the above-mentioned parameters is key to avoid undesirable effects in fish. Due to this, a viability test to determine the safe concentration for *L. reuteri* LRE2 , *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 was conducted using RTgutGC. The evidence obtained in this study showed that even high concentrations (10^7) of the three strains selected for further fish supplementation did not impact RTgutGC viability. Similar to our results, it has been previously observed that large concentrations of probiotics did not affect the viability of fish intestinal mucus and intestinal epithelial cells of *Oreochromis niloticus* and *Miichthys miiuy* respectively (Aly et al., 2008; Pan et al., 2008). These data suggest that *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 should not have negative effects while used as feed s in Chinook salmon and, therefore, can be utilized during the *in vivo* studies.

In addition to determining the safety of selected LAB strains by assessing RTgutGC viability, modulation of immune parameters is required to confirm that probiotic supplementation could produce a positive impact in farm raised Chinook salmon. Currently, it has been demonstrated that RTgutGC can increase the expression of immune canonical genes such as *il1b*, *il6*, and *tnfa* after stimulation with the functional feed ingredients mannanoligosaccharides and beta-glucan (Wang et al., 2019). Interestingly, our results show a differential expression of *il1b* and *il6* expression in *L. reuteri* LRE2 stimulated cells compared to *L. reuteri* 830 and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 treatments. Additionally, RTgutGC cells incubated with *L. reuteri* 830 were the only group to show a downregulation of *tnfa* compared to the time 0 control. Although an increase of the immune genes was expected based on previous evidence, the differential

response observed among LAB strains makes them ideal candidates for an *in vivo* trial. To date, previous researchers have proposed that probiotic formulations containing a single probiotic strain have a low chance of successfully colonizing the host gut (Timmerman et al., 2004). In contrast, multi-strain probiotics have a higher chance of synergetic effects, can control multi-factorial diseases and increase their chances of colonizing the fish gastrointestinal tract (Melo-Bolívar et al., 2021; Timmerman et al., 2004). Whether or not the immune-modulatory effects induced by *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 in RTgutGC can be replicated in Chinook salmon juveniles was unknown, however, our results proved that LAB strains selected were safe for live fish since no sign of an inflammatory and an anti-inflammatory response, was observed during the *in vitro* tests, in addition to the lack of effect on RTgutGC viability.

The effectiveness of probiotic supplementation as protection against infectious pathogens has been widely studied in salmonids. LAB strains, such as *L. sakei, L. lactis*, *L. mesenteroides*, Bacillus sp., and *Carnobacterium* sp., delivered either by diet or bioencapsulation, have helped to increase the survival rate of *S. salar* and *O. mykiss* after challenge with the main fish pathogens *A. salmonicida*, *V. ordalii*, *Y. ruckeri*, and *V. anguillarum* (Balcázar et al., 2007; Brunt et al., 2007; Robertson et al., 2000). Nevertheless, fish supplemented with *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 did not show a differential increase in survival when challenged with *V. anguillarum*. Even though it has been reported that probiotic supplementation may not necessarily lead to significant protection against diseases (Gildberg et al., 1995; Nayak, 2010), we hypothesized that the single strain probiotics used in this study might not being successful colonizing Chinook salmon gut, resulting in a lack of protection against *V. anguillarum*.

In Chinook salmon juveniles, LAB supplementation did not improve weight gain after four months of supplementation and during the *V. anguillarum* challenge. Although scientific literature has shown that LAB supplementation can promote growth in salmonid species (Nikoskelainen et al., 2001; Vendrell et al., 2008), there is also evidence that shows LAB supplementation did not affected growth parameters (Gildberg et al., 1995; Nayak, 2010; Zokaeifar et al., 2012). Currently, the mechanisms associated to growth promotion from probiotic supplementation are poorly understood, however it has been hypothesized that LAB enhances nutrient absorption (Langlois et al., 2021). The results obtained in this study suggest that *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 might lack the metabolic repertoire necessary to facilitate the digestion necessary to affect Chinook salmon weight over four months. Due to this, further studies should focus on increasing the LAB supplementation up to at least 10 months to determine if the strains selected in this study has the potential to improve Chinook salmon weight.

The spleen works as a secondary lymphoid organ in fish, playing a key role in haematopoiesis, antigen degradation and antibody production (Rauta et al., 2012). Over the course of infection, an inflammatory insult will result in a cascade whereby *il1b* would increase, followed by *il8* upregulation (Secombes et al., 2001). In spleen tissues collected in this study, a sustained inflammatory response was observed after one day of infection with *V. anguillarum* and lasted up to 7 dpi. Nevertheless, this inflammatory response was not differentially expressed between *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 supplemented fish, indicating the host was responding to the infection but there was no additive probiotic effect. Additionally, our data suggest that the anti-inflammatory response in Chinook salmon was mediated by *il10*, a key and fast regulator of the immune system, instead of *tgfb*, which plays an important role as an immune modulator of T cell activity (Sanjabi et al., 2009).

In fish, the intestine plays a key role in physiological processes such as digestion, absorption of nutrients, osmoregulation, and immune response (Wang et al., 2019). Probiotic supplementation has been shown to improve these parameters, especially upregulating cellular and humoral immune response at the intestinal mucosal interface (Hoseinifar et al., 2018; Ringø et al., 2012). For instance, Atlantic salmon and rainbow trout supplemented with *P. acidilactici* 18MA/5M, *Lactiplantibacillus plantarum* subsp. *plantarum* CLFP 3, or *Lacticaseibacillus rhamnosus* GG have been shown to significantly upregulate the expression of the proinflammatory markers *il1b*, *il8*, and *tnfa* (Abid et al., 2013; Al-Hisnawi et al., 2019; Jaramillo-Torres et al., 2019; Nikoskelainen et al., 2001). Immune gene upregulation, in addition to significantly lower mortality rates, are needed to determine that probiotic-mediated modulation of immune parameters might be a mechanism for improvements in survival during bacterial infections (Pérez-Sánchez et al., 2014). However, both parameters, survival and immune response modulation were not improved by LAB supplementation in this study. Chinook salmon supplemented with *L. reuteri* 830 were indeed the only ones to have a significant increase in the expression of *il8* compared to the sodium alginate control treatment, however, this did not lead into differences in survival and growth. These findings confirm that either the short-term supplementation (4 months), or the supplementation with single strain probiotics was not sufficient to improve the immune response of Chinook salmon challenged with *V. anguillarum*. Due to this, further studies would benefit from long-term probiotic supplementation as well as multi-strain or multi-species probiotic supplementation.

The complex milieu of the salmonid gut is composed of several inter-dependent factors. These include a rich microbial community, mucosal epithelial cells, secreted antimicrobial peptides plus antibodies, mucus, microbial metabolites, and resident host immune cells, which together greatly impact organismal health (Merrifield et al., 2010). Probiotic-mediated

improvement of gut barrier function has been positively associated with health benefits in several vertebrate species (Bron et al., 2017). Nonetheless, their interaction with TJ molecules and mechanism of improving gut health in salmonids remains unknown. Tight junctions consist of large, dynamic protein complexes that form the circumferential seal between adjacent epithelial cells. Some of the main protein families found in TJs are claudins, occludins, junction-associated membrane proteins (JAM), and zonula occludens (ZO-1) (Sundell and Sundh, 2012). Due to this, we selected TJ molecules representative of four main characteristics of the intestinal epithelium. The first TJ molecules, *zo-1*, *jam1a*, *ocln*, *cdh1*, and *marveld2*, are implicated in the barrier-forming cell-cell connections (Schug et al., 2019). The second molecules, *cldn15* and *cldn28b*, has pore-forming abilities that enables the selectively permeable transport of compounds across the epithelial barrier (Bagnat et al., 2007; Tipsmark et al., 2010). The third is mucus secretion, represented by *muc2*, which is a key structural component of the colonic mucus layer (Van der Sluis et al., 2006). And finally, *vil1*, is a Ca_2+-dependent actin binding protein involved in the structural remodelling and nucleation of microvilli (Ubelmann et al., 2013). After four months of LAB supplementation and 14 days of infection with *V. anguillarum*, there was no evidence that TJ molecules evaluated in this study were modulated differentially from the guy of animals regular fed fish. For probiotics to exert a positive effect in fish gut, colonization and interaction within the commensal bacteria and the host intestinal barrier is needed, nevertheless, no evidence that *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 were successful in accomplishing this was observed through the study. The lack of modulation in presence of *V. anguillarum* might be associated with the IP injection utilized in this study, therefore, the interaction host-pathogen in this case might have occurred in other organs such as spleen, head kidney, or blood, instead of intestine. To assess the real impact of LAB supplementation in TJ

molecules during infection, not only a prolongated period of supplementation is needed, but also a different pathogen delivery method, such as oral gavage.

Although this study collected evidence that suggest *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 can have the potential to be used safely in Chinook salmon production, the limitations of this study should be considered. For instance, it is important to consider that the observed effects are only descriptive of the host and microbe species in which these studies were conducted. However, the reach and gut colonization of each LAB strain used in this study was not assessed. Furthermore, a more comprehensive analysis including a larger pool of sampled individuals can enhance the power and inference ability of these analyses. Also, the investigation of the innate and adaptive immune response in other relevant immune organs in teleosts (such as head kidney, gills, and skin) would provide a clearer picture of the effects of LAB supplementation in Chinook salmon during infection. In considering why our study did not show the effects reported by others, the delivery method comes into question. The transit time for food and the bacteria embedded in the pellet through the intestine of the salmon is approximately 12 h, meaning that the dried organisms must hydrate, become metabolically active and exits their sodium alginate covering to make an impact via the microbiota and epithelial layer before being excreted (Hoseinifar et al., 2017). Future studies should examine whether the increase of probiotic supplementation time, as well as synergetic diets containing two or more strain would benefit Chinook salmon survival, growth, immune response, and gut barrier integrity.

4.6 Conclusion

The present study has provided novel information on how three LAB strains interact with RTgutGC, and Chinook salmon reared in aquaculture conditions. The *in vitro* results indicated that *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 do not have cytotoxic

effects at different concentrations, at the same time demonstrated that supplementation with these microbes can differentially modulate immune parameters. The current supplementation model utilizing single-strain probiotics did not ideally achieved the correct colonization of Chinook salmon intestine, as there was a lack of improvements in survival, growth, immune response, and gut barrier function, compared to regular feed. Due to this, further research may require development of a new multi-strain probiotic product combining the strains here utilized, which might be crucial to enhance growth and survival. Overall, our study demonstrates the suitability of *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14/ *L. rhamnosus* GR-1 for further fish feeding experiments to improve Chinook salmon health and following improvements needed to obtain the required results in Chinook salmon aquaculture.

Chapter 5: General Discussion and Future Directions

5.1 Beneficial effects of probiotic supplementation in Chinook salmon aquaculture

Diseases are the main cause of losses in aquaculture industry, but the chemotherapeutants applied to control this problem have been widely criticized for their negative impacts. Therefore, finfish producers have tried a number of alternatives to build up environmentally friendly aquaculture, such as probiotics (Akhter et al., 2015; Khati et al., 2018). In finfish aquaculture, incorporation of probiotics can help to modulate mucosal surfaces such as intestines, gills, and skin. After reaching, and becoming part of, the mucosal microbiota, probiotics can improve fish growth, survival and immune response of cultured fish (Khati et al., 2018; Wang et al., 2017). Nevertheless, utilization of probiotics to improve Chinook salmon physiology has been poorly explored. Currently, the study conducted by Sadeghiet et al. (Sadeghi et al., 2023) has been the only one to evaluate the effect that a multi-strain probiotic diet has on Chinook salmon. In that study, authors determined that probiotic supplementation does not modify the bacterial community, however, it did show an increase in the frequency of beneficial gut bacteria such as *Lactobacillaceae*, *Bifidobacteriaceae*, and *Streptococcaceae* (Sadeghi et al., 2023). Additionally, Sadeghi et al. (Sadeghi et al., 2023) showed that the multi-strain probiotic Jamieson®, was able to modulate the expression of several immune genes. This information, in addition to the data obtained in chapter 2, chapter 3, and chapter 4 of this thesis, demonstrate that probiotic supplementation has the potential to improve Chinook salmon reared in aquaculture.

As a first approach to improve growth, survival, and immune parameters of Chinook salmon, determining the best strain or combination of beneficial microbes is essential for further utilization during the production cycle. Probiotic selection is key to benefit the target host. A viable probiotic candidate should have few specific characteristics to exert positive impacts. For instance,

a good strain should be capable of producing a beneficial effect on the host animal, usually increased growth and survival (Dawood et al., 2018; Khati et al., 2018). Also, probiotics should not have side effects in the host species, in surrounding aquatic animals, and for future human consumers. Since probiotic intake occurs in either freshwater or saltwater, the strains should remain viable after interaction with the aquatic environment until the host is able to engulf the pellet and the probiotic reaches the fish intestine. Once this occurs, probiotics should be capable of surviving in the low pH encountered in the gut environment, multiply, adhere to the intestine walls, and exert a strong antagonistic activity against pathogenic microorganisms without impacting the commensal communities (Jamal et al., 2020; Langlois et al., 2021). Additionally, from an aquaculture perspective, additional supplements utilized during feeding must be cost-efficient to avoid an increase in the fish production and keep the price market low.

Since the research conducted in this thesis represents one of the first probiotic supplementation studies in Chinook salmon, the most difficult step selecting the best probiotic strain. Scientific evidence suggests that multi-strain supplementation is best tested in fish species where no prior experimentation has been reported, such as Chinook salmon (Niu et al., 2019). Multi-strain probiotics have a high potential to improve the host health through positive complementation among strains (e.g. metabolites exchange, biofilm formation) (Kwoji et al., 2021; Puvanasundram et al., 2022; Salinas et al., 2008a). Due to this, the initial selection was the human multi-strain probiotic Jamieson®. After this, following studies focused in identifying the effects associated to single-strain probiotics.

Currently, most of the studies on probiotic supplementation are performed under controlled laboratory conditions, while only few reports have explored the effect of probiotics in field conditions. One of the advantages that can be found in this thesis (specifically chapter 3), is

determining the effect of *P. acidilactici* in Chinook salmon reared in actual aquaculture conditions for up to 14 months. Because of this, several of the findings discussed in this study represent a true view of the impact of probiotic supplementation on host-microbe-environment interaction. Moreover, in this study we develop an accessible and cost-effective protocol to adhere and deliver different probiotic strains to the dry pellets for Chinook salmon supplementation, showing that its implementation in the aquaculture industry is possible.

5.1.1 Growth parameters in supplemented Chinook salmon

Growth performance is one of the improvements expected from probiotic supplementation in animal production. Currently, studies have suggested that nutritional improvements due to probiotic supplementation are associated with synthesis of proteases, amylases, lipases, vitamins, fatty acids, and amino acids, that beneficially affects the nutrient absorption and digestive processes (Balcázar et al., 2006; Jamal et al., 2020). Several studies have shown that probiotic supplementation have positive effects on growth performance of salmonid species. For instance, supplementation between 30 – 120 days with *L. rhamnosus*, *P. acidilactici*, or *B. subtilis* strains mixed in feed resulted in increased final weight and specific growth rate than the non-supplemented fish (Abid et al., 2013; Hoseinifar et al., 2019; Jamal et al., 2020). However, none of the probiotic diets utilized in this thesis (Jamieson® multi-strain probiotic, *Pediococcus acidilactici* MA18/5M, *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, *Limosilactobacillus reuteri* RC-14 and *Limosilactobacillus rhamnosus* GR-1) improved Chinook salmon growth parameters. Although an opposite response was expected, this study represented the first approach of improving Chinook salmon growth using probiotics.

5.1.2 Immune response in supplemented Chinook salmon

In addition to the production of inhibitory compounds towards and the competition with potential pathogens, the modulation of the immune system has become an area of interest in fish production. To date, the available literature has demonstrated that feed supplementation of several LAB members, such as *L. lactis*, *L. rhamnosus*, *Bacillus* sp., and *L. delbrueckii,* to rainbow trout and Atlantic salmon enhanced the resistance of fish to *Aeromonas salmonicida* and *V. anguillarum*, by increasing the serum lysozyme, phagocytic activity, and respiratory burst activity (Balcázar et al., 2007; Brunt et al., 2007; Salinas et al., 2008a). Moreover, oral supplementation of *L. plantarum*, *L. lactis*, and *L. mesenteroides* have shown to stimulate the expression of canonical immune genes such as *il1b*, *tnfa*, and *il10* in rainbow trout, suggesting that these strains can stimulate the immune response of this salmonid species (Pérez-Sánchez et al., 2014). Since current Chinook salmon aquaculture in British Columbia shows high disease susceptibility (~10-30% mortality), understanding the disease physiological response and resistance, and mitigating risk factors related to disease occurrence through probiotic supplementation was among the aims of this thesis. Due to this, a large selection of genes associated to the innate immune response (proinflammatory response, anti-inflammatory regulation, antimicrobial peptide activity) were evaluated among the chapters presented in this thesis using *in vitro* and *in vivo* models.

When evaluating the immune response in probiotic supplemented RTgutGC, no significant increases were observed in the expression of immune genes when stimulating with *P. acidilactici* MA18/5M, *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14 and *L. rhamnosus* GR-1. In the studies conducted in Chinook salmon, only Jamieson® probiotic was able to modulate the expression of the inflammatory response and the antimicrobial peptide activity in spleen and hindgut tissues, though it was not possible to correlate these results to the decrease in mortality of

fish supplemented for 4, 10, and 14 months. On the contrary, supplementation with *P. acidilactici* MA18/5M, *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14 and *L. rhamnosus* GR-1 did not show any significant results in terms of modulating the expression of the immune response. As was suggested in the data chapters, this is most likely to be associated to the fact that each of the strains were not able to colonize the gut and exert the expected modulatory function, however, utilization of a different supplementation method, a different infection delivery strategy, and an increased supplementation time can help to determine if these finding are expected when using these probiotic strains, or if the effects were missed due to the methodology implemented during the trials.

A significant humoral immune factor in fish is the use of immunoglobulins (Igs). Also known as antibodies, immunoglobulins play a vital role in recognizing a large variety of antigens, such as viruses, bacteria, and parasites, to further recruit immune cells and molecules to destroy these pathogens (Mashoof and Criscitiello, 2016; Rauta et al., 2012; Zhang et al., 2010). Currently, three types of immunoglobulins have been discovered and characterized in teleost fish: IgM, IgD, and IgT/Z (Mashoof and Criscitiello, 2016). Among these, the available research has shown that *L. plantarum*, *L. lactis*, and *L. mesenteroides* supplementation was able to improve the innate and specific immune response of rainbow trout by increasing the total production of IgT (Pérez-Sánchez et al., 2011). Further, rainbow trout supplemented with *Enterococcus casseliflavus* showed increased levels of IgM against *Streptococcus iniae* when infected with this pathogen (Safari et al., 2016). Overall, the total IgM and IgT protein levels presented in this study were not affected by probiotic supplementation. Nevertheless, this study represents the first Western blot evidence of IgT in Chinook salmon, as well as the first reliable protocol to measure IgT in Chinook salmon plasma, head kidney, spleen, and hindgut samples. Currently, no commercial IgT antibody

is available to be utilize in Chinook salmon Western blots and ELISA. Therefore, it is expected that the study conducted in chapter 2, as well as the in-house prepared antibody will become the main reference for following studies in search of evaluating this key mucosal immunoglobulin.

5.1.3 Gut barrier integrity and TJ relative expression in supplemented Chinook salmon

Beneficial microorganisms have significant effects in the intestine of fish, including development, maturation, and modulation of the intestine and host microbiota (Jaramillo-Torres et al., 2019; Langlois et al., 2021). Also, colonization of the gut by probiotic supplementation helps to prevent the colonization of pathogenic bacteria using several mechanisms, such as nutrient and site competition, and secretion of antimicrobial compounds (Hoseinifar et al., 2019; Langlois et al., 2021). Although the way probiotics modulate the intestinal health is poorly understood in fish, it is hypothesised, based on mammalian models, that probiotic supplementation can promote the expression of tight junction (TJ) molecules (Patel et al., 2012; Ringø et al., 2007; Ukena et al., 2007). To date, few studies have assessed the beneficial impacts of probiotics administration on the intestinal microbial community of fish (Jaramillo-Torres et al., 2019; Sadeghi et al., 2023). However, the studies conducted in this thesis represents the first of its kind in terms of the large amount of TJ genes evaluated in fish during probiotic supplementation. When Chinook salmon were supplemented with Jamieson® probiotic, the pore-forming molecule claudin 1 and the barrier-forming cell-cell connection molecule occludin were modulated mainly in fish supplemented for 10 months. Even though this correlated with fish that showed an increased survival when challenged with *V. anguillarum*, an expansion of the evaluated TJ genes would further explain the mechanisms associated to the increased survival of 14 months-supplemented Chinook salmon, which was not possible to be explained by the TJ transcriptional profile. Interestingly, in Chapter 3, although *P. acidilactici* MA18/5M did not differentially impact the expression of TJ genes

compared to regular fed fish and/or heat-killed stimulated Chinook salmon, it was possible to demonstrate the ontogenic transformation that affects the hindgut barrier molecules during the transition from Parr to smolt. This evidence is key to understand further studies conducted in small Chinook salmon specimens, and demonstrate the need to not only consider the developmental variations that can interfere in supplementation studies, but also to re-evaluate the life cycle that this kind of studies must be conducted. Finally, the supplementation with *L. reuteri* LRE2, *L. reuteri* 830, and *L. reuteri* RC-14 and *L. rhamnosus* GR-1 demonstrated that, for probiotic strains to exert a beneficial effect in the gut barrier integrity, they must be able to colonize the host and interact not only with commensal bacteria, but also the host cell population, which more likely was not possible due to the evidence obtained in Chapter 4.

5.2 The importance of time in probiotic supplementation

The prolonged beneficial effects of probiotics in supplemented fish, especially after repeated exposure with the strains, remains questionable. Due to this, it has been proposed that maintenance of a supplementation frequency for several weeks without alterations is key to get the expected and full effectiveness of probiotics (Khati et al., 2018). Available research has indicated that the time interval for probiotic supplementation can range from hours to as long as 8-10 months, depending on the microorganism utilized (Aly et al., 2008; Balcázar et al., 2006; Jamal et al., 2020). However, no consensus for Chinook salmon supplementation, nor for the strains utilized in this research have been explored previously. Due to this, Chapter 2 focused not only on determining the effect that Jamieson® probiotic can have on the physiology of Chinook salmon, but also how the amount of time supplemented would differentially affect the properties exerted by the same multi-strain product. Overall, the results obtained in Chapter 2 not only demonstrated a time-effect associated with probiotic supplementation, but also provided strong evidence that

Chinook salmon supplementation with Jamieson® probiotic should be of at least 10 months. Moreover, even though a similar study was not carried out with the single-strain probiotics used in Chapters 3 and 4, the lack of physiological improvements suggests that, in Chinook salmon, probiotic supplementation for four months is not enough to positively affect the fish.

5.3 Probiotic supplementation over freshwater and saltwater conditions

To date, the main concerns about probiotics are their capability to survive the harsh conditions experienced during feed pellet manufacturing, delivery, and transit to the fish intestine (Soto-Dávila et al., 2020a). Due to this, the first step was to demonstrate that all the strains utilized in this study were able to survive in both freshwater and saltwater conditions (data not shown as has been collected by Dr. Nadeem Akhter, postdoc associated to this grant between 2019-2020). With the confirmation that the probiotic strains were able to survive in both environmental conditions used in Chinook salmon production, several studies have been conducted in freshwater and saltwater using the same probiotic treatments. From the ones presented in this research, data from fish supplemented with Jamieson® probiotic suggest that probiotic supplementation exclusively during the freshwater stage negatively affects the disease-resistance of Chinook salmon after *V. anguillarum* exposure. When fish were supplemented with Jamieson® probiotic exclusively in saltwater, or both (freshwater and saltwater) for 10 and 14 months, respectively, a significant increased survival was obtained after infection with *V. anguillarum*. Altogether, the data collected in Chapter 2 suggest that in Chinook salmon, the probiotic supplementation must contain a sea-pen stage to exert the expected beneficial effect. Although it is not presented in this thesis, recently 2022 processed results from saltwater supplementation with a symbiotic diet containing *P. acidilactici* MA18/5M, *L.* and *reuteri* LRE2 support the idea that, for Chinook salmon, a short supplementation period in freshwater, followed by a year-round saltwater supplementation might

be the best setup to improve fish growth, survival, and enhance the immune and gut barrier function.

5.4 Future directions

In near future, probiotic supplementation will gain more acceptance in salmonids aquaculture, being expected that its application will expand rapidly, specially in non-traditional cultured species. Due to this, there is an increased need to evaluate different probiotic strains to fulfil the specific requirement for aquaculture production. To date, probiotic supplementation in salmonids such as Atlantic salmon and rainbow trout has revealed positive impacts on fish health, suggesting that similar strains can be utilized in Chinook salmon. Among the benefits, improvement of fish growth and disease-resistance against fish pathogens are among the most desirables effects from an industrial perspective. However, despite the increasing evidence that probiotic supplementation positively affects salmonid species, more evidence is required before researchers, producers, and governmental agencies can approved and support the utilization of probiotics as a safe enhancer of fish health.

The results obtained in this thesis provide evidence that probiotic supplementation has the potential to improve Chinook salmon physiology and prevent mortalities when challenged with *V. anguillarum* in a safe manner. Although immune markers and gut-related molecules partially explain the findings observed in these studies, future research should not just focus on repeating these experiments with longer supplementation times as the ones used in chapter 2, but also, expanding the measurements to obtain an overall response at the transcriptional and translational level of the markers here presented. To do this, a development of antibodies for immune genes and tight junctions would benefit future research to properly understand the effect of probiotics during infection, as to date, commercial antibodies for most of the immune and TJ molecules used in this

thesis are not available. Additionally, studies conducted in freshwater should be repeated in saltwater to evaluate if supplementation with *P. acidilactici* MA18/5M, *L. reuteri* LRE2, *L. reuteri 830*, and *L. reuteri* RC-14/*L. rhamnosus* GR-1 produce a similar effect than the one observed in the data here obtained. Similar studies to be conducted in the future would also benefit from the incorporation of new techniques, such as microbiome high-throughput sequencing, to correlate the results obtained with the successful colonization of the gut. Finally, in order to properly assess if probiotic supplementation enhance the immunoglobulin production, long-term supplementation followed by long-term bacterial challenge should be implemented as long as the logistic allows it.

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Appendices

Appendix A1: EWOS harmony 2 mm feed formulation.

Feed ingredient	Restriction	Amount of total feed
Crude Protein	Minimum	47%
Crude Fat	Minimum	18%
Crude Fibre	Maximum	0.7%
Vitamin A	Min. IU/kg	12,000
Vitamin D_3	Min. IU/kg	4,500
Vitamin E	Min. IU/kg	450
Calcium	Actual	2.9%
Phosphorus	Actual	1.2%
Sodium	Actual	0.6%
Selenium		0.1 mg/kg

*Manufactured by: EWOS Canada Limited 7721 – 132nd street, Surrey, British Columbia, Canada, V3W 4M8.