

**The Effect of Corn Starch and Kappa-Carrageenan Probiotic Encapsulation
on Growth and Immune Response in Chinook Salmon
(*Oncorhynchus tshawytscha*)**

by
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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.

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Statement of Contributions

The work presented in this thesis was done by Maureen Latimer under the supervision of Dr. Brian Dixon, and was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant. Contributions made to this work by collaborators are listed below.

Tank set-up and trial execution were performed by co-op students Abigail Eade and Brooklyn Bolstad and technician Earl Heath at Yellow Island Aquaculture Limited.

Co-op student Gillian McDonald helped with RNA extractions from tissue samples.

Primer design and efficiency testing were performed by fellow graduate student Manuel Soto-Dávila, as referenced in the “Materials and Methods” section. Manuel also designed the *tgfb* primer used in this study, while graduate student Laura Rivera tested its efficiency.

No content from this thesis has previously been published elsewhere.

Abstract

As vaccines have limited efficacy in fish, alternative preventative measures are used to combat infectious diseases in aquaculture, including probiotics. To be effective, a probiotic must remain stable during storage, feeding, and transit through the gastrointestinal tract. Thus, preparing probiotic feed requires the use of a coating agent and several different types have been tested, as some have adverse effects. To better understand the effect of corn starch and kappa (κ)-carrageenan as a combined coating agent on growth and immune performance in Chinook salmon, a growth trial was conducted. Three different treatments were used, including regular feed, regular feed with a corn starch and κ -carrageenan coating, and probiotic feed with the same coating. The probiotic feed included a mixture of BioPower® PA (*Pediococcus acidilactici*, strain CNCM MA 18/5M; Lallemand Inc., Canada) and Seed 14 (*Limosilactobacillus reuteri* LRE2; Probiotal SpA, Italy). Sampling timepoints were at 0-, 1-, 3-, 7-, and 28-days post diet-introduction, and at each sampling timepoint, the weight and length of 6 fish per treatment were measured, and the head kidney, spleen, and hindgut were collected. There was an additional timepoint at Day 42 where the weight and length of the remaining fish in each barrel were recorded, but no tissues were collected. To assess immunological effects of the coating agent, qPCR for IL-1 β , IL-8, and TGF- β was performed on spleen, head kidney, and hindgut samples. In the hindgut, additional genes were assessed to determine changes in gut barrier integrity, including E-cadherin, claudin 15, junctional adhesion molecule 1 alpha, mucin 2, tricellulin, villin 1, and zonula occludens-1. No significant differences in weight or length were observed between treatments measured over 42 days. No significant differences were found in the assessed proinflammatory transcript levels in the spleen or head kidney sampled at 0-, 1-, 3-, 7-, and 28-days post diet-introduction. Transient changes in the regulatory cytokine TGF- β were seen in these tissues. In the hindgut, upregulation of all three immune transcripts were seen on Day 7 in both coated treatments, but these changes were transient, with no significant differences seen on Day 28. Transient changes were also seen in gut barrier gene expression, with no significant differences seen between treatments on Day 28. These results suggest that corn starch and κ -carrageenan as a combined coating agent has no short-term negative effects on the growth or immune status of Chinook salmon and that it can be used to deliver probiotics that will improve the health of fish in aquaculture.

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Introduction

1.1. Aquaculture value and impact of disease

Aquaculture is a rapidly growing and globally important industry. Excluding algae, the quantity of aquatic food consumption increased more than 5 times globally between 1961 and 2019. This means that the average annual rate of increase in aquatic food consumption was nearly double that of the population growth rate during this time (FAO, 2022). Increased production is required to meet a growing demand, and while marine capture fisheries have had stable production since the late 1980s, aquaculture has continued to grow in production since that time (FAO, 2022). Therefore, the growth of the aquaculture industry has been crucial in supporting this increased demand. The aquaculture industry had an average annual growth rate of 4.6% in terms of weight output between 2010 and 2020, and in 2020, the global first sale value reached USD \$265 billion (FAO, 2022).

The overall top aquaculture producers in the world in 2021 were China, India, and Indonesia (FAO, 2023b). Although Canada is not a top producer globally, it is an important player in the aquaculture industry, and in 2021, was the 6th largest exporter of salmon in the world (OECD, 2023). The aquaculture industry is economically important to Canada, with an overall value of around \$1.3 billion in 2021, with nearly \$1 billion of that attributed to salmon farming (DFO Canada, 2023). Looking at worldwide production quantity of fish species groups in 2021, salmon, trouts, and smelts ranked 4th after carps and other cyprinids, tilapias and other cichlids, and catfishes (FAO, 2023a). Looking at total aquaculture (including algae), this group ranked 9th and accounted for 3.4% of world production (FAO, 2023a). Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*), and Coho salmon (*Oncorhynchus kisutch*) are farmed in Canada, with Atlantic salmon being the largest export (DFO Canada, 2017). Although

Atlantic salmon is Canada's largest export, farming of other species like Chinook salmon is important, as this species is native to the West Coast, and the province of British Columbia is where most of Canada's aquaculture production occurs (DFO Canada, 2023).

One obstacle faced by the industry is that there are huge losses every year due to disease, estimated at a value of USD \$6 billion (The World Bank, 2014). High stocking density in fish farms creates ideal conditions for the spread of disease, and antibiotic resistance continues to pose a problem. Antibiotic use can cause changes in the microbiomes of the fish themselves and their aquatic environments, selecting for antimicrobial resistance (Cabello et al., 2016). To avoid antibiotic use, preventative measures such as vaccination are used.

1.2. Vaccination and alternative disease management strategies

The principle of vaccination is that a pathogen, or some molecule of a pathogen, is presented to the immune system so that protective memory cells can be made. When the vaccinated individual encounters the actual pathogen, these long-lived memory cells can launch a faster and more tailored response to the pathogen than naïve cells. In mammals, memory B cells have undergone antibody class-switching and affinity maturation, and they also have higher levels of MHC class II and co-stimulatory molecules on their surface, which means they can respond to lower doses of antigen than naïve cells (Murphy et al., 2012). It has been shown in rainbow trout (*Oncorhynchus mykiss*) that a repeat exposure to an antigen can result in a higher and faster peak than a primary response, and that vaccination can increase serum antibody titer for months. This is proposed to be due to an increase in the number of antigen-specific B cells, as no changed proliferative potential in memory cells is seen (Arkoosh & Kaattari, 1991). It has also been shown that affinity maturation of antibodies does not occur in fish as it does in mammals

(Arkoosh & Kaattari, 1991). The memory response in fish is not well understood, and vaccines made to date have shown some efficacy, although limited.

The first commercial vaccine used in aquaculture was licensed in 1976 for enteric redmouth disease, and since then, a variety of different types of vaccines have been licensed (Du et al., 2022; Soto Dávila et al., 2020). Although there are effective vaccines against many pathogens, there are many limitations; common delivery methods such as intraperitoneal injection can be difficult in high numbers, and the efficacy of the vaccine must outweigh the time and money invested. Even if a commercial vaccine for an important pathogen is available, it may not be effective in all affected species, and there is also a lack of vaccines for use against parasites (Du et al., 2022). For these reasons, alternative disease management strategies besides vaccination must be used as well.

Other strategies for disease management include the use of functional feed ingredients, such as prebiotics, probiotics, and vitamins (Soto Dávila et al., 2020). Prebiotics are non-digestible carbohydrates that support the growth of probiotics and can also benefit the host through innate immune stimulation. Vitamins are nutritional factors that have also been shown to have a number of immunostimulatory properties in multiple species including common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), and Atlantic salmon (Soto Dávila et al., 2020). Others functional feed ingredients include phytogenic compounds, enzymes, organic acids, mycotoxin binders, and algae. These feed ingredients have shown beneficial properties such as improved feed efficiency and growth performance, increased survival during pathogen challenge, enhanced immune parameters, and expression of antioxidative enzymes (Bharathi et al., 2019).

Kappa-carrageenan is an example of a seaweed extract used as a food additive that has shown immunostimulatory properties. Cheng and colleagues (2008) measured innate immune

parameters in brown-marbled grouper (*Epinephelus fuscoguttatus*) at 0, 2, 4, 6, and 8 weeks after introducing κ -carrageenan at 5 g/kg of feed. They saw increased leukocyte count, respiratory burst activity, and lysozyme activity at certain time points over 2-8 weeks, whereas phagocytic activity, phagocytic index, and alternative complement activity were increased at every timepoint from 2-8 weeks (Cheng et al., 2008). Probiotics also belong to this large group of functional feed ingredients that have been tested.

1.3. Commonly used probiotics

Probiotics are living microorganisms, including bacteria, that can be used in aquaculture to confer an advantage to the host through competition with pathogens in the gastrointestinal tract. They can also act through other mechanisms such as provision of beneficial metabolites or enzymes, stimulation of the immune response, or improvement of gut barrier integrity (Langlois et al., 2021; Qi et al., 2009). Common probiotics include the lactic acid bacteria (LAB) and *Bacillus* species, as well as photosynthetic bacteria and yeast (Denev et al., 2009; Hlordzi et al., 2020; Qi et al., 2009; Soto Dávila et al., 2020). The definition of probiotics in aquaculture has also expanded to include groups of living organisms that improve the environment of the host, such as nitrifying and denitrifying bacteria, which can improve water quality (Qi et al., 2009; Verschuere et al., 2000). A few tested probiotics include *Pediococcus acidilactici*, strain CNCM MA 18/5M and different strains of *Limosilactobacillus reuteri* (previously known as *Lactobacillus reuteri*), which have shown promising results for improving immune performance and growth (Ferguson et al., 2010; Giannenas et al., 2015; Giri et al., 2018; Hoseinifar et al., 2019; Soto Dávila et al., 2020). Both of these bacterial species are Gram-positive, facultative anaerobes known to inhabit the intestinal tract of a wide range of animals, and certain strains

have been shown to produce antimicrobial compounds effective against known pathogens (Mu et al., 2018; Porto et al., 2017).

1.4. Immunostimulatory properties of probiotics

Probiotics have been shown to have immunostimulatory properties, resulting in an increase in immune functions such as lysozyme activity, complement activity, phagocytosis, respiratory burst activity, antibody production, and cytokine production (Soto Dávila et al., 2020). Among these cytokines are inflammatory cytokines such as interleukin-1beta (IL-1 β), interleukin-8 (IL-8), and tumour necrosis factor alpha (TNF- α) (Soto Dávila et al., 2020). IL-1 β is a proinflammatory cytokine released when a pathogen-associated molecular pattern (PAMP) is recognized by a pattern recognition receptor (PRR), and this causes increased immune cell migration to the affected site and an increase in other proinflammatory cytokines such as interleukin-6 (IL-6), TNF- α , and IL-8 (Zou & Secombes, 2016). IL-8 is another early proinflammatory cytokine that is a chemoattractant for immune cells such as neutrophils, among others (van der Aa et al., 2010). Transforming growth factor beta (TGF- β) is an anti-inflammatory cytokine with regulatory properties (Zou & Secombes, 2016). Anti-inflammatory cytokines such as TGF- β and interleukin-10 (IL-10) have also been shown to be up-regulated alongside inflammatory cytokines in response to certain probiotics (Beck et al., 2016; Mohammadian et al., 2019).

1.5. Effect of probiotics on gut barrier integrity and junctional complexes

Probiotics have also been shown to have an effect on the integrity of the gut barrier, through mechanisms such as upregulation of tight junction genes and mucus production (Langlois et al., 2021). The junctional complexes between epithelial cells are important for forming a physical selective barrier to stop entry of harmful molecules or pathogens into the body. As seen in Fig.

1.1. below, occludins, claudin-15, junctional adhesion molecule 1alpha (JAM-1 α), and zonula occludens-1 (ZO-1) (alongside other proteins in these families) are a few of the proteins involved in forming tight junctions, which are the apical-most junctions in a junctional complex that form between adjacent epithelial cells and regulate what can pass between them (Ulluwishewa et al., 2011). Claudins are important in forming the physical barrier that stops antigens from being able to pass between epithelial cells, and in maintaining cell polarity, forming ion pores that confer ion size and charge selectivity based on which claudin proteins are present in the junction at a particular location (Capaldo, 2023). Claudin-15 is expressed throughout the small intestine and colon of mice, with higher expression seen in the duodenum and jejunum, and lower expression in the ileum and colon (Fujita et al., 2006). It is expressed apically in the lateral membranes throughout the small intestine and colon, but in the colon, appears to be expressed in the crypts only and not at the epithelial surface (Fujita et al., 2006). Claudin-15 forms a cation channel that has been shown to have a critical role in sodium transport (Tamura et al., 2011). Tricellulin is a tetra-span protein located primarily in tricellular tight junctions of epithelial cells but can also be found in bicellular tight junctions (Ikenouchi et al., 2005). Occludins and claudins are also tetra-span, while JAM only crosses the cell membrane once (Ulluwishewa et al., 2011). All of these proteins contribute to forming a selective barrier. ZO-1 is not transmembrane but is a cytoplasmic scaffolding protein that connects the transmembrane junction proteins, including claudin, tricellulin, and JAM-A, to the cytoskeleton (Van Itallie et al., 2009). ZO-1 knockdowns in MDCK II (epithelial dog kidney) cell monolayers show increased permeability to large solutes and abnormal changes in morphology and actin distribution, demonstrating a critical role of ZO-1 in stabilizing the tight junction (Van Itallie et al., 2009). Beneath the tight junction in the junctional complex there is the adherens junction, whose main function is cell adhesion. E-

cadherin is a transmembrane protein involved in this junction and also has an important role in cell signaling (Perez-Moreno & Fuchs, 2006). Both the tight junction and adherens junction associate with the actin cytoskeleton of cells in order to regulate the permeability of the epithelial barrier (Ulluwishewa et al., 2011).

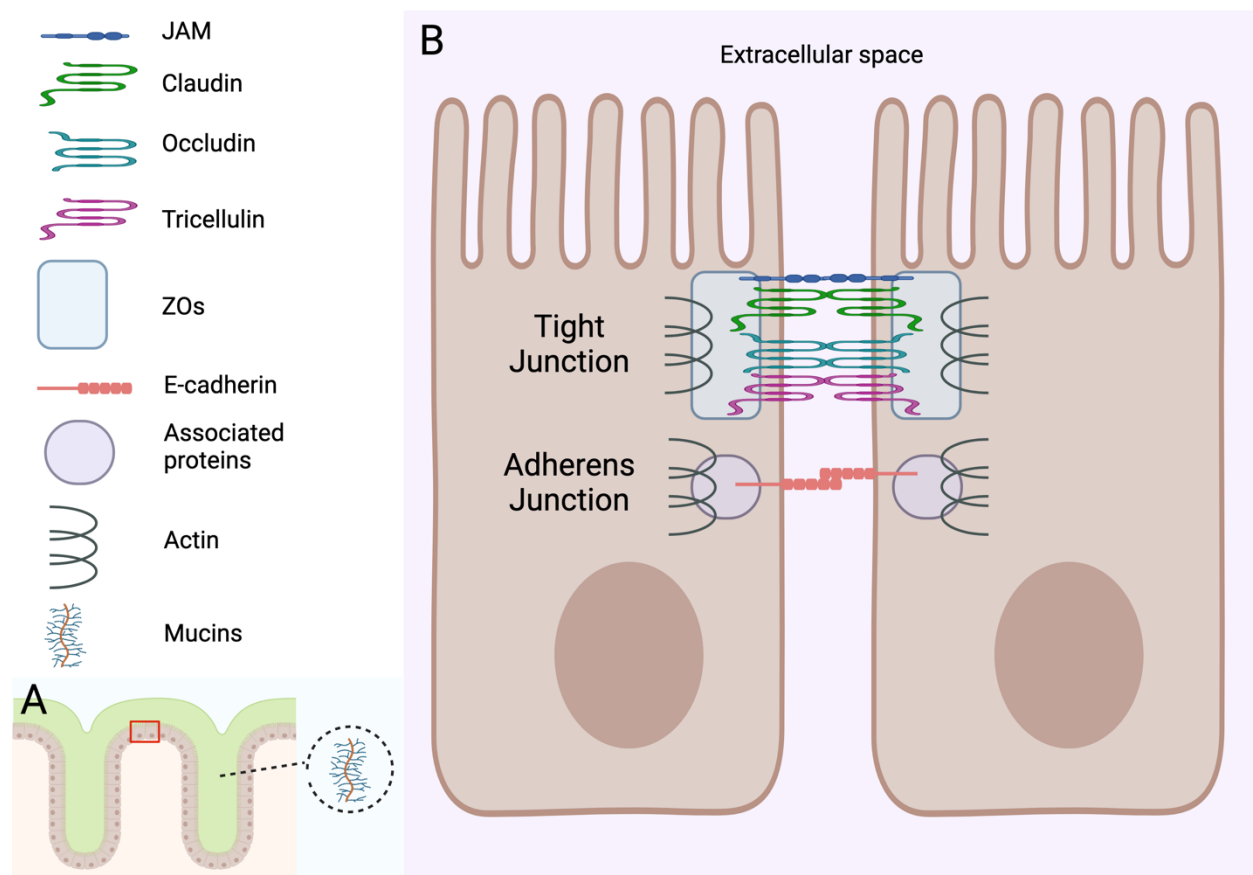


Figure 1.1. Molecules involved in forming the junctional complex between epithelial cells. (A) The gut epithelial layer is covered by a protective layer of mucus, which contains mucins. Boxed in red are two adjacent epithelial cells, between which the tight junction and adherens junction molecules are shown in (B). Modified from Knights and colleagues (2012). Created with BioRender.com.

Two other proteins involved in the intestinal barrier are mucin 2 and villin 1. Mucin 2 is a main component of the mucus that is secreted to form a protective layer over the epithelial cells of the intestine (Y. Liu et al., 2020). Villin 1 is a protein expressed in intestinal epithelial cells that is most strongly expressed at the villus tip and is involved in actin polymerization and

depolymerization (Khurana, 2006). Assessing these transcripts will give an indication of what gut barrier components, if any, may be affected by the introduction of the coating agent and/or the probiotic.

1.6. Encapsulation materials for probiotics

In order to be effective, a probiotic must be resistant to different environmental conditions during storage, feeding, and transit through the stomach to reach the intestine of the fish. The probiotic must be able to withstand temperature changes, low pH, and bile salts, among other harsh factors (Terpou et al., 2019). Many encapsulation strategies have been tested for this purpose, including a variety of polysaccharide and protein-based materials, as previously reviewed (Dong et al., 2013; Langlois et al., 2021). These include materials such as poly-L-lysine, chitosan, starch, gelatin, and dairy, soy, and pea proteins (Dong et al., 2013). One of the most widely tested coating agents has been alginate, an anionic polysaccharide extract of algae, due to its ease of use in forming hydrogels and its biocompatibility, as well as its low toxicity and cost (Dong et al., 2013; Lee & Mooney, 2012). However, there are also drawbacks to this coating material; it is porous and has sensitivity to low pH as well as chelating agents and monovalent ions (Heidebach et al., 2012; Smidsrød & Skjåk-Bræk, 1990). Co-encapsulation using multiple compounds at once, such as polymer-coated alginate beads or protein-coated alginate beads, has been shown to be effective at increasing the viability of a probiotic under low pH conditions compared to uncoated alginate beads (Yucel Falco et al., 2019). Other studies have also shown that creating coating agents out of multiple materials or coating microcapsules in an additional layer has proven to be beneficial for creating more robust protection which could result in more efficient probiotic delivery (Benavent-Gil et al., 2018; Gbassi et al., 2009; Srivastava et al., 2023).

1.7. Kappa-carrageenan and corn starch as encapsulation materials

Kappa-carrageenan is a disaccharide extracted from red algae that is a common food additive and has been used as a coating agent for probiotics (Heidebach et al., 2012; Wu et al., 2022). It is useful for forming hydrogels, but while it has good structural integrity, it is brittle; combining it with other complementary materials such as gellan (along with chitosan) has been shown to produce a mechanically robust coating (Srivastava et al., 2023). Kappa-carrageenan has been shown to have good oxygen resistance, a quality that could make it a good option in a combined coating for oxygen-sensitive probiotics (Phùng et al., 2022). Carrageenan gum has also been shown to be protective in acidic environments and in taurocholic acid, simulating bile salt in the intestines, increasing viability of the tested probiotics compared to the uncoated control (Ding & Shah, 2009).

Corn starch is also a common food additive that has been tested as a probiotic coating. Corn starch and sodium alginate combined in an electrospun nanofiber mat has been shown to provide protection in simulated gastric and intestinal fluid (Atraki & Azizkhani, 2021). A different study found that co-encapsulating *Lactobacillus casei* in 2% Hi-maizeTM starch and alginate improved encapsulation efficiency compared to alginate alone (Sultana et al., 2000). Corn starch has also been used in co-encapsulation studies as a prebiotic for the enclosed bacteria. In a study by Iyer and Kailasapathy (2005), it was determined that co-encapsulation incorporating Hi-maizeTM starch at a concentration of 1% w/v with alginate significantly increased viable counts of the tested *Lactobacillus* spp. after incubation at pH 2 for 3 hours compared to the other tested prebiotics (inulin and oligofructose), which performed better than both free and alginate-coated cells.

Both corn starch and κ -carrageenan have been shown to have some beneficial properties for encapsulating probiotics and have been effective in combination with other materials. They have also been tested for gelling properties when combined together. Many properties of starch and carrageenan interactions have been reported (Huc et al., 2014; You et al., 2021), and it has been shown that the addition of starch to carrageenan dispersions increases gel strength and decreases water loss during storage compared to carrageenan alone (Lin et al., 2016).

1.8. Objective of this study

Although both corn starch and κ -carrageenan have been shown to be an effective coating agent for certain probiotics when in combination with other materials, their effectiveness as a combined coating has not been reported in aquaculture. The efficacy of a certain coating agent would be expected to vary depending on the probiotic encapsulated, the storage conditions, and the target consumer species. The aim of this study is to look at the effects of a corn starch and κ -carrageenan coating on the transcription level of several immune and gut barrier genes to see if this coating agent has a negative impact on the health of Chinook salmon in terms of inflammation or gut barrier integrity. The effect of the coating on growth will also be monitored. This will help determine if the coating agent could be used to deliver probiotics that improve the health of Chinook salmon, and potentially other fish species, in aquaculture.

Materials and Methods

2.1. Experimental design

This trial included three different treatments including regular feed (2mm dry pellets, EWOS Harmony), regular feed with a corn starch and κ -carrageenan coating, and probiotic feed with the same corn starch and κ -carrageenan coating. The probiotic feed included both BioPower® PA (*Pediococcus acidilactici*, strain CNCM MA 18/5M; Lallemand Inc., Canada) and Seed 14 (*Limosilactobacillus reuteri* LRE2; Probiotical SpA, Italy). There were duplicate 120 L freshwater barrels per treatment, each containing 30 triploid Chinook salmon. At the beginning of the trial, the fish had an average weight of 18.45 ± 5.09 grams, based on the fish sampled at timepoint 0 (n =18), before treatment diets were introduced.

2.2. Feed formulation

To make the probiotic feed, 0.1 g of Seed 14 and 1 g of BioPower® PA were first added to 4 mL of a 1% w/v starch solution. Next, 4 mL of a 1% w/v κ -carrageenan solution was added. The probiotic solution was mixed thoroughly following the addition of each component. The resulting 8 mL of probiotic solution was then added to 100 g of feed (2mm dry pellets, EWOS Harmony) in small increments, while mixing thoroughly, followed by the addition of 8 mL of 0.1 M CaCl₂ solution in the same manner. The probiotic feed mixture was allowed to sit for at least 15 minutes prior to feeding in order to allow for the total absorption of all liquid, and the final formulation contained each probiotic at a concentration of 10^{11} colony forming units (CFU) per kg of feed. The same coating formulation without probiotics was applied to the feed for the coating-only treatment group.

2.3. Feed trial sampling

Throughout this 6-week trial, fish were fed twice daily with an amount of ~1.6% body weight per fish at each feed. The sampling timepoints were Day 0 (before starting the diets), and Day 1, 3, 7, 28, and 42 post diet-introduction. Three fish per barrel (6 per treatment) were sampled at each timepoint, except Day 42. This included measuring the weight and length of each fish, as well as collecting the head kidney, spleen, and hindgut. Organs were stored in RNAlater at -20°C. On Day 42, no organs were collected, but the weight and length of each of the remaining 15 fish in each barrel was measured.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from head kidney, spleen, and hindgut samples using TRIzol (Invitrogen) according to the manufacturer's instructions, with 50 mg of tissue being added to 1 mL of TRIzol reagent for all samples. RNA yield was estimated using the A260/A280 reading from the Synergy H1 Hybrid Plate Reader (Biotek Instruments, Inc.). The samples were then treated with RNase-free Ambion™ DNase I (Invitrogen) according to the manufacturer's instructions, where 5 µg of RNA was added to 2 µL of DNase, 4 µL of 10x buffer, and molecular grade water up to a final volume of 40 µL, as previously described by Soto-Davila and colleagues (2024). After DNase treatment, 60 µL of molecular grade water and 60 µL of 100% ethanol were added to all samples, which were then loaded onto an RNA/Protein Purification Column (Norgen Biotek Corp.). After passing the samples through the columns, three washes were done by loading 400 µL of Wash Solution A (Norgen Biotek Corp.) onto each column and centrifuging at 3500 x g for 1 minute, for a total of three times. Samples were eluted into clean tubes using 20 µL of molecular grade water and centrifuging at 200 x g for 2 minutes, followed by 14,000 x g for 1 minute. Purified RNA was quantified using the A260/A280 reading, as

previously described. For cDNA synthesis, 250 ng of RNA was added to 2 µL of qScript cDNA Supermix (Quantabio) and molecular grade water up to a total volume of 10 µL. PCR was then carried out according to the manufacturer's instructions. All cDNA samples were then stored at -20°C until use.

2.5. Testing primer efficiency

As cycling conditions and tissue type were the same, primer efficiency was not re-calculated; reported values were previously determined by Soto-Dávila and colleagues (Soto-Dávila, Langlois Fiorotto, et al., 2024; Soto-Dávila, Webb, et al., 2024). Briefly, all cDNA samples were diluted to a concentration of 5 ng/µL using molecular grade water and 50% of all samples were pooled. From these pooled samples, a 7-point 3x dilution series was created. Each PCR reaction contained 5 µL of 2x WISENT Advanced™ qPCR MasterMix (WISENT, Inc.), 2.5 µL of primer mix (at a final concentration of 0.5 µM each), and 2.5 µL of cDNA (for a total of 12.5 ng/reaction at the first dilution point), making up a total reaction volume of 10 µL. Each dilution point was tested in triplicate and primer efficiencies were calculated using the formula $E = 10^{\left(\frac{-1}{slope}\right)}$ (Pfaffl, 2001).

2.6. Selecting a housekeeping gene

All cDNA samples were diluted to a concentration of 1 ng/µL using molecular grade water. There were four candidate housekeeping genes tested, including elongation factor 1 alpha (*efla*), beta actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and 18S ribosomal RNA (*18S*), and reference gene stability was assessed as previously described by Soto-Dávila, Webb, and colleagues (2024) using values from 50% of samples tested in triplicate using 2.5 ng of cDNA per reaction. Based on stability, the *efla* gene was selected for normalization of target genes.

2.7. Quantitative-PCR of target genes

To assess immunological effects of the coating agent, qPCR for interleukin 1 beta (*il-1b*), interleukin 8 (*il-8*), and transforming growth factor β (*tgfb*) was performed on spleen, head kidney, and hindgut samples. Gut barrier genes in the hindgut were also assessed, including E-cadherin (*cdhl*), claudin 15 (*cldn15*), junctional adhesion molecule 1 alpha (*jam1a*), mucin 2 (*muc2*), tricellulin (*marveld2*), villin 1 (*vill*), and zonula occludens-1 (*zo-1*). Primer sequences for all genes (including all candidate housekeeping and target genes) are shown below in Table 2.1. All qPCR reactions contained 5 μ L of 2x WISENT Advanced™ qPCR MasterMix (WISENT, Inc.), 2.5 μ L of primer mix (at a final concentration of 0.5 μ M each), and 2.5 μ L of cDNA (for a total of 2.5 ng/reaction), making up a total reaction volume of 10 μ L. Cycling conditions began with pre-incubation at 95°C for 2 minutes, followed by 40 cycles of amplification (denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 8 seconds). Next was a melting stage with parameters as follows: hold at 95 °C for 1 second, cool to 65°C at a ramp rate of 2.2 °C/s for 1 minute, and heat from 65°C to 97°C at a ramp rate of 0.11 °C/s. While the samples were heating, five data points were collected per °C for melt curve analysis. Finally, there was a cooling stage at 60°C for 10 seconds. All qPCR was performed using the LightCycler® 480 II (Roche, USA). A negative control containing no cDNA template was included on all plates.

2.8. Sample analysis

Gene transcription was determined using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). A two-way ANOVA was performed for each gene in each tissue to determine whether the effect of

the treatment diet, time, or the interaction of these variables was significant ($p \leq 0.05$).

Following this, Tukey's multiple comparisons test was used to determine where significant differences were seen ($p \leq 0.05$). All statistical analyses were done using GraphPad Prism 9.3.1 for MacOS (GraphPad Software, California, USA).

Table 2.1. Primer sequences for genes assessed in this study

Target (gene)	Primer sequence (5' to 3')	Efficiency (%)	Reference
Interleukin 1beta (<i>il-1b</i>)	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	100.1 ^a	(Frenette et al., 2023)
Interleukin 8 (<i>il-8</i>)	F: ACCAGCGAGATAACAA R: CCAGGAGCACAATGACAA	98.2 ^a	(Polinski et al., 2014)
Transforming growth factor beta (<i>tgfb</i>)	F: TGTGGGGAGACAACACAAGG R: AAACCAGCGCCATCAAAAAGG	91.5 ^c	(Soto-Dávila, unpublished)
E cadherin (<i>cdh1</i>)	F: ACTACGACGAGGAGGGAGGT R: TGGAGCGATGTCATTACGGA	96.7 ^b	*
Claudin 15 (<i>cldn15</i>)	F: GGCACGTCTGAGAAACAACC R: TAGGAAGTGGCAGCCTGACT	102.8 ^b	*
Junctional adhesion molecule 1 alpha (<i>jam1a</i>)	F: TGAGGATGGAAGTCCGCAAC R: GTACCACAGTCCGAAGCACA	101.9 ^b	*
Mucin 2 (<i>muc2</i>)	F: CCAGTGTCAAGTGCAAACACG R: ATGTAGCAGGGCTGGGTAGA	100.6 ^b	*
Tricellulin (<i>marveld2</i>)	F: TCCAACACAGGCTCATCTCTT R: ATGGGGTTCATGACGGACAC	99.3 ^b	*
Villin 1 (<i>vill</i>)	F: AAAGTTCAGGTGCTGTAAATCGC R: TGTGGCATGGTGCCAGATTC	105.6 ^b	*
Zonula occludens-1 (<i>zo-1</i>)	F: GCTGTTCCTCCTAGACCTT R: TCACCCACATCTGACTCTAC	98.2 ^a	(Schug et al., 2019)
Elongation factor 1 alpha (<i>efla</i>)	F: CGCACAGTAACACCGAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	99.1 ^a	(Semple et al., 2018)
Beta actin (<i>actb</i>)	F: TGGACTTTGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG	90.2 ^a	(Ma et al., 2019)
Glyceraldehyde-3-phosphate dehydrogenase (<i>gapdh</i>)	F: GCTGGAATGGGACTCACAC R: GTCAAAACCGTCTCAGTGGG	100.8 ^a	(Rajanbabu & Chen, 2011)
18S ribosomal RNA (<i>18S</i>)	F: CGTCGTAGTTCGACCATAAA R: CCACCCACAGAATCGAGAAA	101.4 ^a	(Giroux et al., 2019)

^a Previously reported (Soto-Dávila, Webb, et al., 2024)

^b Previously reported (Soto-Dávila, Langlois Fiorotto, et al., 2024)

^c Efficiency tested by Rivera (unpublished) in rainbow trout cell lines

* (Soto-Dávila, Langlois Fiorotto, et al., 2024)

Results

3.1. Growth results

The growth of the fish on different treatment diets was monitored over 42 days. As seen in Fig. 3.1, there were no significant differences in weight (a) or fork length (b) between treatments at any of the observed time points.

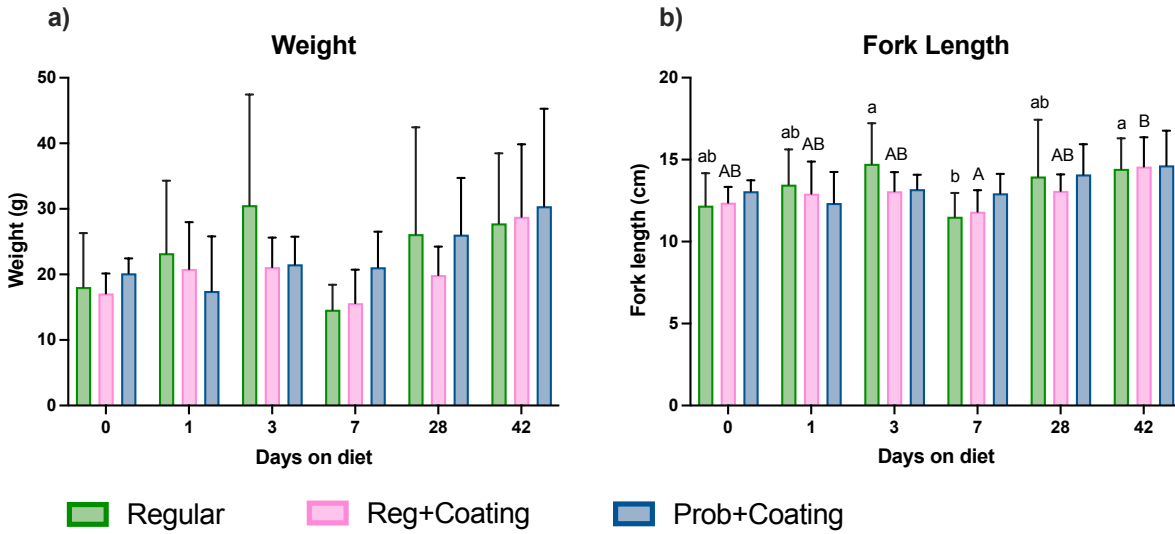


Figure 3.1. Growth measurements of a) weight and b) fork length over 42 days on treatment diets. Plotted values are the mean $\pm$ S.D. At time points 0, 1, 3, 7, and 28, $n=6$. At the final time point, $n=29$ for the regular feed group and $n=30$ for the regular coated and probiotic coated groups. No significant differences between treatment groups were observed at any time points at a level of $p \leq 0.05$. Significant differences within a treatment over time are indicated at a significance level of $p \leq 0.05$ using different letters/symbols.

Specific growth rate (SGR) was not assessed statistically, as fish were not individually tagged for repeated growth measurements, and there were not triplicate tanks for each treatment that would allow calculation of an average SGR for each group. However, an average SGR for each treatment group was determined based on initial and final average weights based on the following calculation suggested by Crane and colleagues (2020), where G is the SGR:

$$g = \frac{\log_e(w_2) - \log_e(w_1)}{\Delta t}$$

$$G = 100(e^g - 1)$$

Table 3.1. Calculating SGR after 42 days on treatment diets

	Regular	Regular + Coating	Probiotic + Coating
W₁	18.09	17.09	20.17
W₂	27.79	28.80	30.42
g	0.0102	0.0124	0.0098
G	1.03	1.25	0.98

W₁ = average weight at Day 0, W₂ = average weight at Day 42

The calculated values in Table 3.1 above show that the treatment group given regular feed with the coating had the highest SGR (1.25%). The probiotic and coating treatment group had the lowest SGR (0.98%), although this was very similar to the control group (1.03%).

3.2. Gut barrier gene expression

The expression level of several different gut barrier gene transcripts was measured over 28 days following the introduction of the treatment diets. Fig. 3.2 below shows that E-cadherin (a) showed significant down regulation at Day 1 in the coated treatment compared to the regular feed. At Day 7, the regular and coated treatments were not different from each other, but the probiotic coated treatment was significantly upregulated compared to both. Significant differences were also seen in claudin 15 (b) expression at Day 7, with upregulation in both the coated and probiotic coated treatments compared to the regular feed. For JAM-1 α (c), downregulation of the coated treatment was seen at Day 1 and Day 7 compared to the regular feed, but this difference was not seen in the probiotic coated treatment. At Day 7, expression of both mucin 2 (d) and tricellulin (e) in the probiotic treatment group was significantly higher than expression in the regular feed group. Villin 1 (f) and zonula occludens-1 (g) only showed significant differences on Day 1, where the probiotic coated treatment showed higher expression than the regular coated treatment for both genes.

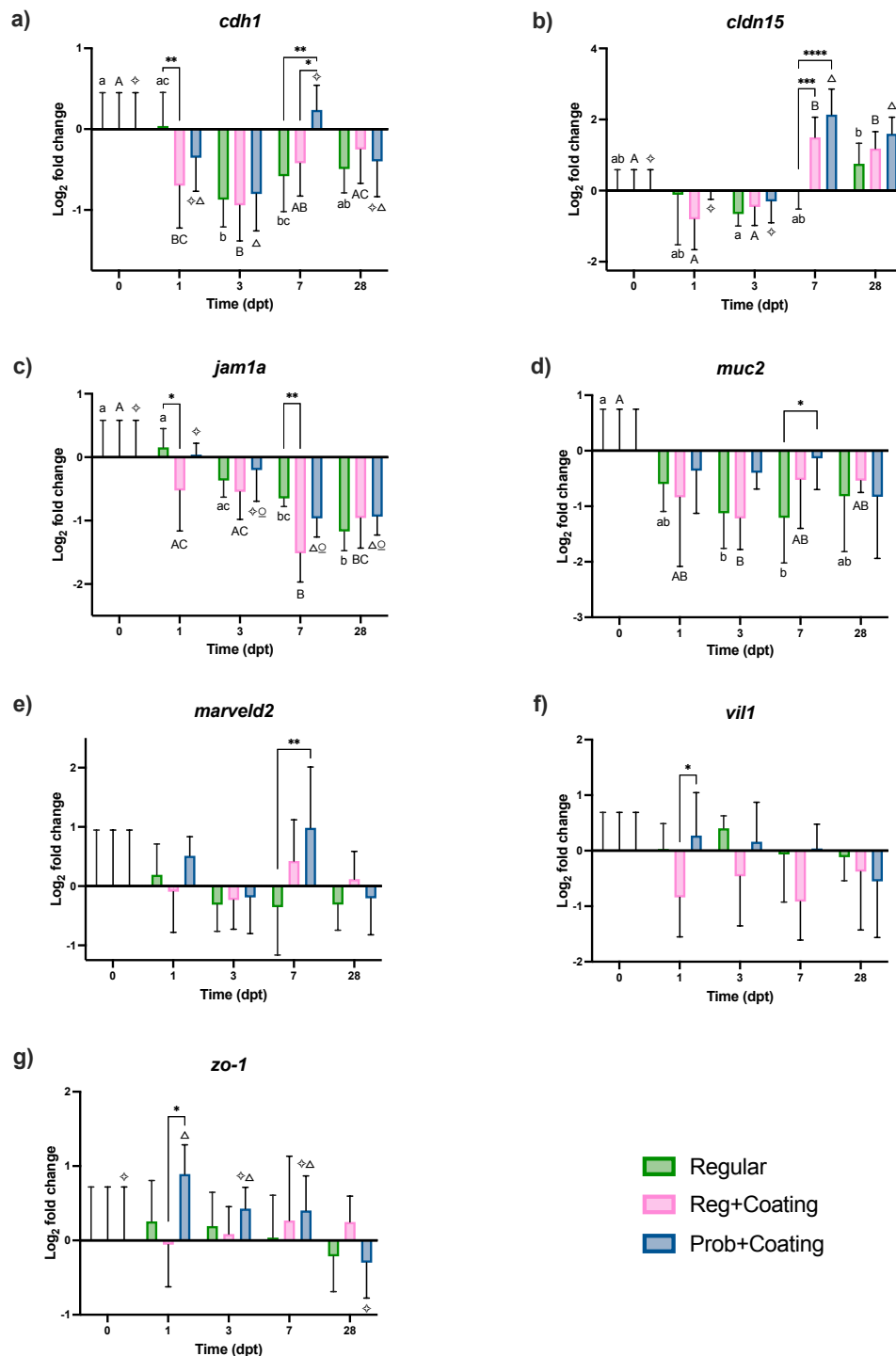


Figure 3.2. Gut barrier gene expression in the hindgut over time on treatment diets. Gene expression of a) E-cadherin (*cdh1*), b) claudin 15 (*cldn15*), c) junctional adhesion molecule 1 alpha (*jam1a*), d) mucin 2 (*muc2*), e) tricellulin (*marveld2*), f) villin 1 (*vil1*), and g) zonula occludens-1 (*zo-1*) are shown. Plotted values are the mean $\pm$ S.D. At time point 0, n=16. At all other time points, n=6. Significant differences between treatments are indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Significant differences within a treatment over time are indicated at a significance level of $p \leq 0.05$ using different letters/symbols.

3.3. Immune gene expression

The expression level of selected immune gene transcripts in the hindgut was also measured over 28 days following the introduction of the treatment diets. Fig. 3.3 shows that significant differences were seen at Day 1 and Day 7 in certain immune genes. Interleukin-1 beta (*il-1b*) (a) was significantly upregulated in the coated and probiotic coated treatments compared to the regular feed at Day 7. Interleukin-8 (*il-8*) (b) showed significantly higher expression in the probiotic coated treatment compared to the regular coated treatment at Day 1. At Day 7, the probiotic coated treatment was significantly upregulated compared to both other treatment groups, and the regular coated and regular feed groups were also significantly different than each other. Transforming growth factor β (*tgfb*) (c) showed significant downregulation in the coated treatment compared to the regular feed and probiotic coated treatment groups at Day 1. At Day 7, the expression of *tgfb* was significantly upregulated in the coated and probiotic coated treatments compared to the regular feed group.

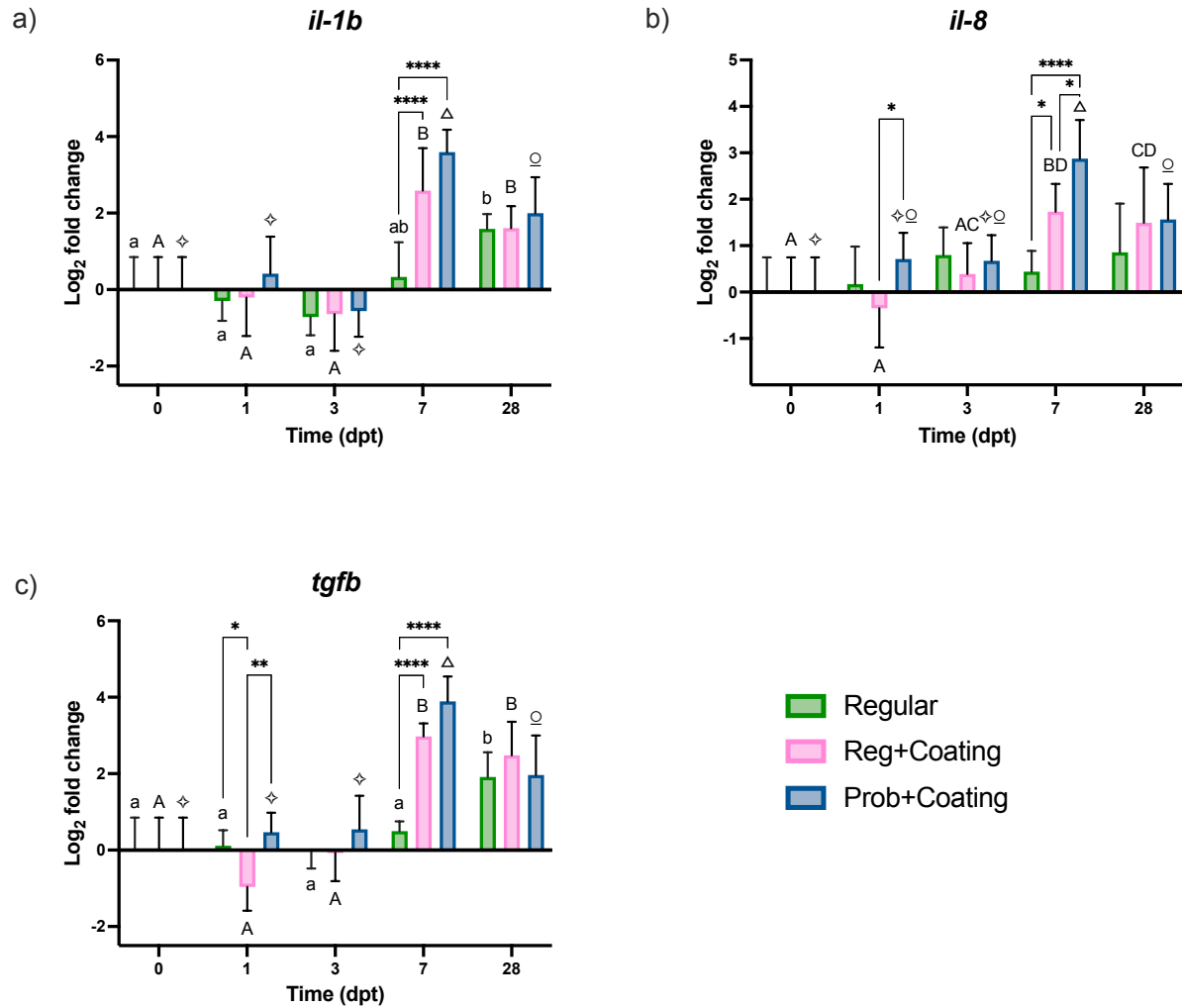


Figure 3.3. Immune gene expression in the hindgut over time on treatment diets. Gene expression of a) interleukin 1 beta (*il-1b*), b) interleukin 8 (*il-8*), and c) transforming growth factor β (*tgfb*) are shown. Plotted values are the mean $\pm$ S.D. At time point 0, n=16. At all other time points, n=6. Significant differences between treatments are indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Significant differences within a treatment over time are indicated at a significance level of $p \leq 0.05$ using different letters/symbols.

The same immune genes assessed in the hindgut were also assessed in the spleen and head kidney over 28 days on the treatment diets. As seen in Fig. 3.4 below, no significant differences between treatments were seen in *il-1b* or *il-8* in the head kidney (a, c) or the spleen (b, d). In the head kidney, *tgfb* (e) in the regular coated treatment was downregulated compared to the regular feed group at Day 1 but was upregulated compared to the regular feed group on Day 3. On Day 7, there was no difference in these two treatments, but there was a significant down-regulation of the probiotic coated treatment compared to the regular feed treatment. In the spleen, the only significant difference in *tgfb* (f) expression between treatments was seen at Day 1, with lower expression in the regular coated treatment compared to the regular feed, similar to the difference seen in the head kidney at this timepoint.

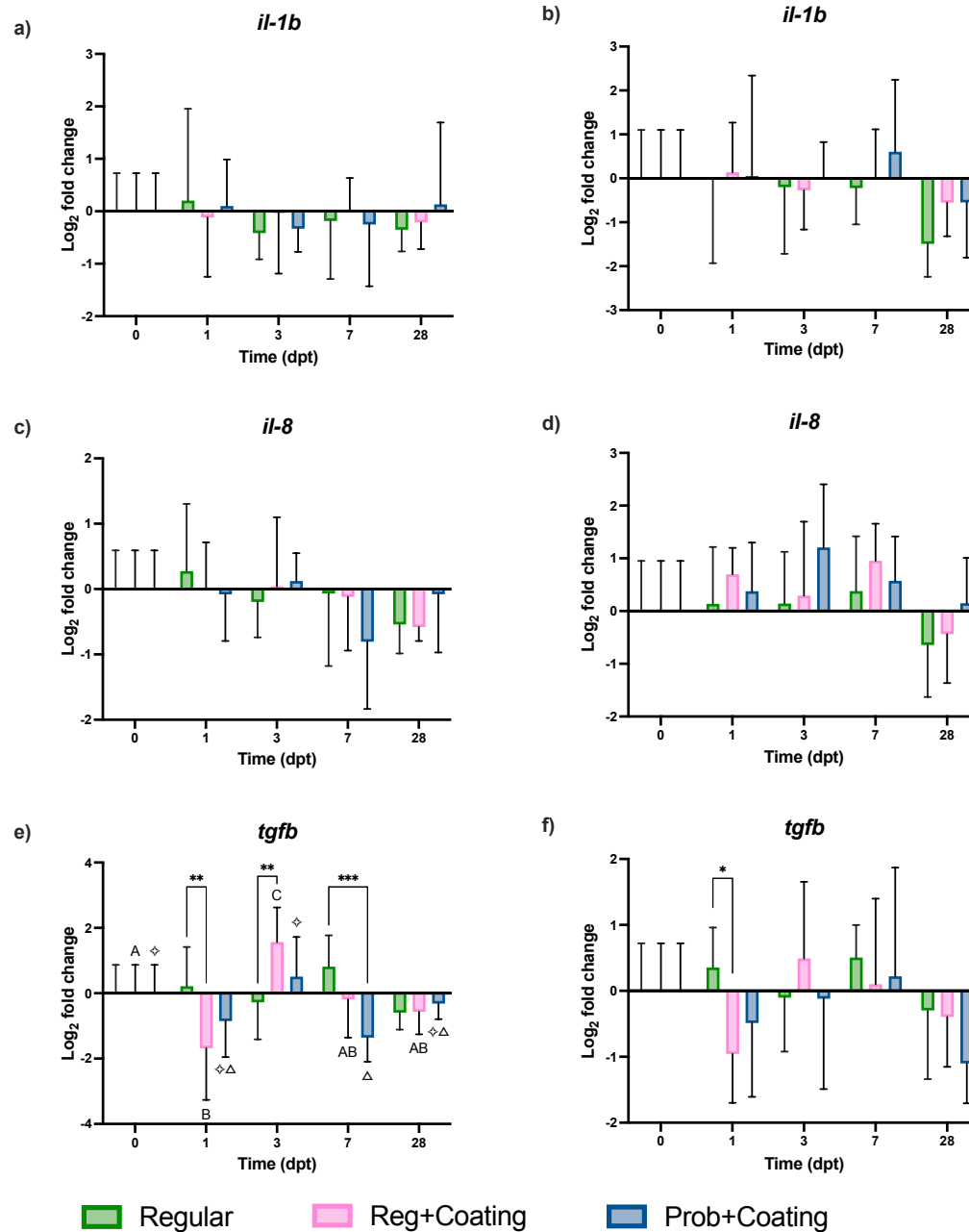


Figure 3.4. Immune gene expression in the head kidney (a, c, e) and spleen (b, d, f) over time on treatment diets. Gene expression of interleukin 1 beta (il-1b), interleukin 8 (il-8), and transforming growth factor β (tgfb) are shown. Plotted values are the mean $\pm$ S.D. At time point 0 in the head kidney, n=18. At all other time points, n=6, except for the probiotic coated group at time point 7 for all genes, and the regular coated group at time point 3 for tgfb, where n=5. At time point 0 in the spleen, n=17. At all other time points, n=6, except for the regular coated group at time point 3 for all genes, and the regular coated group at time point 1 for tgfb, where n=5. Significant differences between treatments are indicated as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Significant differences within a treatment over time are indicated at a significance level of $p \leq 0.05$ using different letters/symbols.

Discussion

4.1. Growth data

In this study, there were no significant differences in weight or fork length between treatments at any of the observed timepoints over 42 days. The probiotic mix used in this study consisted of BioPower® PA (*Pediococcus acidilactici*, strain CNCM MA 18/5M) and Seed 14 (*Limosilactobacillus reuteri* LRE2). A study by Ferguson and colleagues (2010) looking at the effect of the probiotic *P. acidilactici* MA18/5M on red tilapia found no significant improvement in growth performance with the probiotic after 32 days, with no significant differences in SGR, feed conversion ratio, or protein efficiency ratio between the control and probiotic groups. Although no significant differences were seen, it is unknown whether this would change if growth was assessed for a longer period of time. In a study by Standen and colleagues (2013) looking at Nile tilapia, no significant differences in growth parameters were seen in a treatment group receiving *P. acidilactici* compared to the control group after 6 weeks. Jaramillo-Torres and colleagues (2019) did a 12-week feeding trial looking at the effects of *P. acidilactici* on Atlantic salmon and also found no significant differences in SGR or thermal growth coefficient after 6 weeks in fresh water, or after an additional 6 weeks in salt water. This is similar to the results of the current experiment, as no beneficial growth effects were seen after 6 weeks. However, one study looking at this probiotic found significant increases in growth of Asian sea bass after 6 weeks (Ashouri et al., 2020). A significant increase in percent weight gain and SGR using the probiotic *L. reuteri* P16 was also seen in common carp after just 6 weeks (Giri et al., 2018), showing that it is possible to see a significant improvement in this short amount of time.

Although it is possible that significant effects of the coating agent and/or the probiotic might be seen if administered over a longer period of time, this can't be assumed, as there are

cases where no significant difference in specific growth rate is seen even after 8 weeks. One study in common carp found no differences in specific growth rate between any treatments after 8 weeks when testing the effects of *P. acidilactici* and formic acid, either individually or combined (Heshmatfar et al., 2023). However, this study did find significantly more weight gain in groups receiving either formic acid either alone or in combination with the probiotic, with the most significant difference seen in the combined group. A difference in weight gain was not seen in the group receiving the probiotic alone, illustrating that the incorporation of an organic acid with a probiotic can have synergistic effects on growth (Heshmatfar et al., 2023). *P. acidilactici* has been shown to enhance growth parameters over a period of 8 weeks in zebrafish and Nile tilapia (M. Ahmadifar et al., 2023; Arani et al., 2021; Hendam et al., 2023), and after a period of 10 weeks in Atlantic salmon (Dhanasiri et al., 2023). A multi-strain probiotic including *Bacillus subtilis*, *Enterococcus faecium*, *P. acidilactici*, and *L. reuteri* caused a significant increase in weight gain after 8 weeks at a dose of 1 gram (g) of probiotic per kilogram (kg) of feed in rainbow trout (Giannenas et al., 2015). Zixin Yang and colleagues (2023) fermented food for 3 days with the bacteria *Lactobacillus acidophilus*, *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* before feeding it to fish. When looking at the nutritional composition, the fermented feed had a significantly higher percentage of crude protein compared to the regular feed, and the group receiving this fermented feed was found to have significantly lower food intake, and significantly higher feed and protein efficiency compared to the control group. After 8 weeks, there was an increase in final body weight and specific growth rate seen in the group receiving fermented feed, although the difference wasn't significant (Z. Yang et al., 2023). It is unknown whether this difference would have reached significance if diets were administered over a longer period of time, or whether similar results would be seen if the feed

supplemented with these probiotics was given directly to the fish without the initial fermentation period. Regardless, both *P. acidilactici* and *L. reuteri* have both been used successfully, either individually or in combination, to enhance growth parameters in fish within a period of 8 weeks. The probiotic and coating agent used in this study could be tested for a minimum of 8 weeks to see if a beneficial effect is seen. However, as different growth results are seen across both 6- and 8-week studies, it is clear that there are other factors to consider when assessing the effect of a probiotic or coating agent.

In addition to time, an important factor in determining whether a probiotic will be effective is the dose administered. Giannenas and colleagues (2015) observed that a multi-strain probiotic caused a significant increase in weight gain of rainbow trout when administered at a dose of 1 g/kg of feed, but not when the dose was increased to 5 g/kg. Although physical growth was not measured, relative expression of the growth-related genes insulin-like growth factor 1 (IGF-1) and growth hormone 1 (GH) were significantly higher after 8 weeks in a group of zebrafish fed 10^7 CFU/g of *P. acidilactici*, but not in groups fed either 10^8 or 10^9 CFU/g (E. Ahmadifar et al., 2020). When testing 4 different doses of *P. acidilactici* in zebrafish, all probiotic treatment groups had significantly more weight gain and a higher food consumption efficiency than the control group, with 4×10^6 CFU/g and 8×10^6 CFU/g groups significantly higher than lower doses. Weight gain, food consumption efficiency, and survival rate increased with increasing *P. acidilactici* dose up to 4×10^6 CFU/g, and then decreased in the 8×10^6 CFU/g group (Arani et al., 2021). These studies indicate that a higher dose of a probiotic is not always better, and that it's important to test for the maximum beneficial dose. Although no significant effects on growth were seen in this experiment, a different dose of the probiotics could be tested.

Both probiotics were incorporated into the feed at a concentration of 10^8 CFU/g in this study, but given the results above, perhaps a lower dose in the range of 10^6 to 10^7 CFU/g should be tested.

In a 60-day feed trial looking at growth performance of common carp fed the prebiotic raffinose and the probiotic *P. acidilactici*, either individually or combined, a significantly lower specific growth rate and higher feed conversion ratio was seen in the probiotic group than all other groups, including the prebiotic and synbiotic groups (Hoseinifar et al., 2019). In this case, it would appear that the incorporation of the prebiotic raffinose was beneficial in mitigating the negative growth effects seen using the probiotic alone. However, the addition of a prebiotic is not always beneficial to growth. A study by Ashouri and colleagues (2020) looked at the effects of *P. acidilactici* and low molecular weight sodium alginate (LMWSA) on Asian sea bass, both individually at different doses, and combined. All treatment groups had a significantly higher specific growth rate than the control group, but the most significant difference was seen in the group fed the probiotic alone, followed by the diet containing the probiotic with LMWSA at 5 g/kg of feed. In this case, the inclusion of a prebiotic did not increase growth performance compared to the probiotic alone. The diet containing just the LMWSA at 5 g/kg of feed had significantly more weight gain than the control group, but both diets containing LMWSA at 10 g/kg of feed (alone and with the probiotic) did not (Ashouri et al., 2020). This study shows two things. First, similar to probiotics, prebiotics can be useful for enhancing growth, although a higher dose is not always better. Second, even if a prebiotic shows evidence of enhanced growth when used alone, this does not always translate into an improved result when combined with a certain probiotic, compared to that probiotic alone. Therefore, it is difficult to predict what the effects of a certain mix of prebiotics and probiotics will be, even if the effects of each individual

component have been studied separately. Perhaps the combined coating agents and probiotics used in this study could be tested in different ratios to see if a different growth effect is seen.

The coating components tested in this experiment were κ -carrageenan and corn starch. Kappa-carrageenan caused no significant difference in the SGR of Nile tilapia after 30 days when incorporated into feed at 3 different doses ranging from 2.5 to 10 g/kg of feed (Villamil et al., 2019). A study by Cheng and colleagues (2008) on brown-marbled grouper looked at the effect of incorporating κ -carrageenan at a dose of 5, 10, or 20 g/kg of feed and found that percentage weight gain was not significantly different over 14 weeks. Although the study was not looking at corn starch as part of a probiotic formulation, Enes and colleagues (2008) performed a 12-week growth trial to determine the effects of two different types of corn starch at different concentrations on gilthead sea bream. No significant difference in final body weight, SGR, feed efficiency, or protein efficiency ratio was found when native or waxy corn starch was incorporated into feed at levels of 10 or 20 percent (Enes et al., 2008). Although performed in other species, and therefore not directly comparable, these studies suggest that κ -carrageenan and corn starch as a combined coating agent would not be expected to significantly affect growth parameters in Chinook salmon. The effect these coating agents have on growth when used in combination in aquaculture has not been reported. Although they cannot be compared statistically, the calculated SGR value of the coating-only treatment group (1.25) in this experiment was the highest, followed by regular feed group (1.03) and the probiotic group (0.98), which had similar values. These values, along with the lack of significant differences found in both weight and length after 42 days suggest that the combination of κ -carrageenan and corn starch does not have any negative impact on the growth of Chinook salmon when

administered alone, or when used as a coating agent for the probiotic mix of *P. acidilactici* and *L. reuteri*.

4.2. Immune gene expression

IL-1 β is an early proinflammatory cytokine expressed when pathogen-associated-molecular-patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs) on immune cells, and is secreted by monocytes and macrophages, among other cell types (Garlanda & Jaillon, 2016). This cytokine has a cytosolic inactive precursor that must be cleaved into its active form (Lopez-Castejon & Brough, 2011). This has also been shown in teleosts, as well as the proinflammatory functions of the mature peptide (Secombes, 2022). Intraperitoneal injection of a synthetic peptide of an IL-1 β functional domain in rainbow trout caused an increase in expression of *il-8* in the head kidney, lysozyme in the gill, and *il-1b* in both organs (Hong & Secombes, 2009). Recombinant IL-1 β has been shown to increase leukocytes at the site of injection, increase expression of certain immune genes, and enhance phagocytic activity in rainbow trout (Hong et al., 2001; Peddie et al., 2003).

IL-8 in teleosts has also kept its proinflammatory and chemotactic properties. In grass carp, recombinant IL-8 significantly increased gene expression of *il-1b*, *tnf-a*, and *il-8* from head kidney leukocytes, and also increased nitric oxide products (Zhao et al., 2022). Another study on grass carp showed that recombinant IL-8 significantly enhanced chemotactic migration of neutrophils and mononuclear leukocytes in vitro, and intramuscular injection caused infiltration of inflammatory cells to the site (T.-T. Wang et al., 2013). A study on Siberian sturgeon also found that recombinant IL-8 caused chemotactic activity of peripheral blood leukocytes (PBLs) and spleen cells, but the effect was greater in PBLs, and the main cell types attracted were lymphocytes and monocytes (X. Wang et al., 2019).

Across multiple fish species, both *il-1b* and *il-8* have been shown to be upregulated in response to PAMPs such as LPS and poly I:C, and pathogens such as viral haemorrhagic septicemia virus (VHSV), *Streptococcus iniae*, and *Vibrio anguillarum* (Herath et al., 2016; Z. Li et al., 2018; Seppola et al., 2008; Tafalla et al., 2005; X. Wang et al., 2019; Zhou et al., 2020). The role of these cytokines in early inflammation has been well established in teleosts.

This study looked at the expression levels of the proinflammatory cytokines *il-1b* and *il-8*, and the regulatory cytokine *tgfb* in the hindgut, head kidney, and spleen. The expression of *il-1b* and *il-8* were not significantly different between treatments at any of the studied timepoints in the head kidney or the spleen. In teleosts, the head kidney is the site of hematopoiesis, and it is also a secondary immune organ, as is the spleen (Dixon et al., 2016). The lack of proinflammatory cytokine expression in these organs suggests that neither the coating agent nor the probiotic mix induced a systemic inflammatory response.

Other studies have looked at the effect of the probiotics *P. acidilactici* or *L. reuteri* on the expression of either one or both of these proinflammatory cytokines. A range of responses have been seen. Modanloo and colleagues (2017) saw a decrease in expression of *il-1b* in the intestine of common carp in response to *P. acidilactici*. Wei Li and colleagues (2024) also saw a decrease in *il-1b* expression in the intestine in Nile tilapia in response to *L. reuteri*, while no difference was seen in the liver. Some studies show no significant difference in expression of these inflammatory genes. Giri and colleagues (2018) found no significant difference in *il-1b* expression in the head kidney of common carp treated with *L. reuteri*, while Ahmadifar and colleagues (2020) found no significant difference in either *il-1b* or *il-8* in zebrafish treated with *P. acidilactici*. A study testing the effect of selected probiotics on isolated intestinal epithelial cells of rainbow trout found no significant differences in *il-8*, or other proinflammatory genes

like *tnf-a* or *il-6* in response to either *P. acidilactici* or *L. reuteri* (Pillinger et al., 2022).

Interestingly, upon addition of a pathogen, both of these probiotic treatment groups showed significantly lower expression of *il-8* and *il-6* compared to the control group, and an ELISA showed lower IL-8 protein levels in both groups as well (Pillinger et al., 2022).

Many papers do show an increase in expression of these cytokines in response to probiotics. A study by Heshmatfar and colleagues (2023) in common carp found an increase in *il-1b* in response to *P. acidilactici*, and another study in Atlantic salmon found an increase in *il-1b* in the distal intestine in response to this probiotic in fresh water (but not salt water) (Jaramillo-Torres et al., 2019). Studies in zebrafish (M. Ahmadifar et al., 2023) and Nile tilapia (Hendam et al., 2023) have also shown significantly increased expression of both *il-1b* and *il-8* in the liver in response to *P. acidilactici*. It is clear that the response to these probiotics in terms of inflammatory markers varies between studies, perhaps due to differences such as probiotic dose, host species, and measurement timepoints.

Although no difference in proinflammatory cytokines was seen in the head kidney or spleen, there were differences seen in *il-1b* and *il-8* expression in the hindgut. On Day 1, the probiotic treatment group had significantly higher expression of *il-8* compared to the coating-only treatment group, while neither showed a significant difference compared to the control group. An increase in this chemotactic cytokine is logical given that there is bacteria (and therefore more PAMPs) present in the probiotic group. Interestingly, no difference in *il-1b* was seen, although it is possible that an initial increase in this gene was missed given that the first timepoint after diet-introduction was measured at 24 hours. For example, a study on Black rockfish found that after stimulation with poly I:C, spleen cells already showed significant

upregulation of both *il-1b* and *il-8* at 3 hours, showing peak expression at 6 hours. *Il-8* was still upregulated after 12 hours, while *il-1b* was not (Herath et al., 2016).

An increase in both proinflammatory cytokines was seen at Day 7 in the hindgut in both the coating-only and the probiotic treatment group. This shows that the corn starch and κ -carrageenan coating itself could induce expression of these genes. A study by Campos-Sánchez, Mayor-Lafuente and colleagues (2021) in gilthead seabream found that subcutaneous injection of carrageenan caused an increase in expression of cell markers for macrophages, antigen-presenting cells, and acidophilic granulocytes in skin sampled from the injection site at certain timepoints between 1.5 and 6 hours. The same study found an increase in certain pro-inflammatory genes including *il-1b*, *tnf-a*, *il6*, and *il8*. A different study found that subcutaneous injection of λ/κ -carrageenan caused acute skin inflammation, as an increase in superoxide dismutase, peroxidase, lysozyme activity, total immunoglobulin (Ig), and bactericidal activity were found to be increased in the skin mucus at certain time points between 1.5 and 6 hours (Campos-Sánchez, Guardiola, et al., 2021). However, no systemic immune response was seen, as there was no difference in serum total Ig levels or peroxidase activity, and head kidney leukocytes showed no difference in respiratory burst activity or phagocytic ability at any timepoints. In this study, *il-1b* was upregulated in the head kidney at 1.5 hours, but not at 3- or 6-hours post-injection (Campos-Sánchez, Guardiola, et al., 2021). This is similar to what was seen in this experiment, as the coating agents (and the probiotic mix) appeared to induce expression of proinflammatory genes locally in the hindgut, but not systemically. Carrageenan has been shown to bind Toll-like receptor 4 (a PRR) on human colonic epithelial cells and mouse macrophages and has been shown to stimulate innate immune pathways (Bhattacharyya et al., 2008). Whether the mechanism of immune stimulation is similar in Chinook salmon remains to be determined,

however, stimulation of innate immunity by carrageenan is consistent with the findings in the current experiment.

In this study, it can be seen that the expression profile of *tgfb* in the hindgut looks similar to the changes seen in the expression of both proinflammatory cytokines. TGF- β is a regulatory cytokine that acts on all cells of the immune system, and has important roles in maintaining immune homeostasis, as well as initiating and resolving immune responses (M. O. Li et al., 2006). TGF- β has been shown to stimulate chemotaxis of monocytes and granulocytes, maintain homeostasis in cell types like NK cells and regulatory T cells, and has a large role in inhibiting proliferation of B and T lymphocytes and inhibiting differentiation of T helper cells, as previously reviewed (M. O. Li et al., 2006). The same 3 isomers of TGF- β found in tetrapods have been found in teleosts, plus an additional isomer, with TGF- β 1 considered to be the most immunologically active (Secombes, 2022).

As shown in Fig. 3.3 and Fig. 3.4, the same trend in *tgfb* expression is seen on Day 1 in the head kidney, spleen, and the hindgut, where lower expression is seen in the coating-only treatment compared to the control, but this is not seen in the probiotic treatment group. In fact, in the hindgut, the coating-only treatment also has significantly lower expression of *tgfb* compared to the probiotic treatment group on Day 1. It is known that TGF- β is a molecule that can autoregulate expression of its own mRNA (Hariyanto et al., 2021). It has also been shown that carrageenan is capable of blocking the binding of TGF- β to certain cell types such as Swiss 3T3 mouse fibroblast cells (Hoffman, 1993). The author suggests that this may be due to the similarity between polysulphated carbohydrates and heparin, causing the heparin binding domain on the tested fibroblast growth factors to be blocked (Hoffman, 1993). TGF- β is not translated directly into an active form but is rather produced as a precursor molecule with an N-terminal

signal peptide and a latency-associated polypeptide (LAP), followed by the mature peptide at the C-terminal. Processing, involving dimerization and proteolytic cleavage, forms a latent TGF- β complex, which can associate with extracellular matrix (ECM) proteins until activation occurs (Kubiczkova et al., 2012). TGF- β 1 is known to bind heparin and heparan-sulphate, and binding to cell surface or ECM heparan-sulphate proteoglycans has been shown to be important in controlling the diffusion of certain TGF- β superfamily members and maintaining high localized concentrations where necessary (Rider & Mulloy, 2017). It could be speculated that the decrease in *tgfb* seen on Day 1 in the hindgut could be due to a direct interaction of the carrageenan component of the coating with this cytokine. However, as this decrease is also seen in the head kidney and spleen, this would suggest that the decrease in expression is through a more indirect mechanism.

In a study looking at gene expression of gilthead seabream head-kidney leukocytes, incubation with 1000 ug/mL of l-carrageenan caused significant upregulation of *il-1b* and significant downregulation of the anti-inflammatory cytokine *il-10* after 24 hours. The expression of *tgfb* was also lower after 24 hours, although not significantly (Campos-Sánchez et al., 2024). This is consistent with the downregulation of *tgfb* seen at Day 1 in the current experiment.

On Day 3, there are no differences in the expression of *tgfb* in the hindgut, but upregulation is seen in the head kidney in the coating-only treatment compared to the control. On Day 7 in the head kidney, there is a significant downregulation seen in the probiotic treatment group, but not in the coating-only treatment. These differences are seen in the head kidney, but not in the spleen. A study of TGF- β 1 in grass carp found that the cytokine had opposite effects on different cell types, with recombinant TGF- β 1 increasing expression of *tnf-a* and *ifn-g* and

increasing viability of peripheral blood leukocytes (PBLs), while having the opposite effect on head kidney leukocytes (M. Yang et al., 2012). It is clear that this cytokine does not have a strictly pro- or anti-inflammatory role, but rather a regulatory one depending on other environmental factors. Due to the context-dependent effects of the cytokine, it is difficult to know from the expression level alone what immune role this cytokine is playing without also looking at changes in possible immune targets. TGF- β plays a large role in regulating the effector function and chemotaxis of many different types of immune cells (M. O. Li et al., 2006). As previously mentioned, the head kidney is the site of hematopoiesis in fish (Dixon et al., 2016), and as such, it is not unexpected to see changes in expression of this cytokine in the head kidney that are not seen in the spleen.

In grass carp head kidney leukocytes, *tgfb1* and its ALK5 receptor have been shown to be upregulated alongside *il-1b* in response to recombinant IL-1 β (X. Yang et al., 2014). The results of the current study make sense in the context of these findings, as an increase in *tgfb* can be seen in the hindgut at the same timepoint as the increase in *il-1b* at Day 7, with higher expression of *tgfb* correlating with higher expression of *il-1b*. Xiao Yang and colleagues (2014) also found that TGF- β 1 neutralizing antibody prolonged *il-1b* expression and that recombinant TGF- β 1 dampened expression of *il-1b* and its receptor signaling molecules, showing an inhibitory effect of TGF- β 1 on IL-1 β , and suggesting a mechanism of control over the inflammatory response that keeps it from becoming overblown. Given this information, the upregulation of *tgfb* at Day 7 and the absence of inflammation at Day 28 could indicate that a tolerance response to the probiotic and coating has developed.

4.3. Gut barrier gene expression

In this experiment, the effect of the probiotic mix and coating agents on gut barrier integrity were assessed by measuring expression of several genes involved in barrier function in the hindgut, including E-cadherin, claudin 15, junctional adhesion molecule 1 alpha, mucin 2, tricellulin, villin 1, and zonula occludens-1.

Different strains of *L. reuteri* have been shown to have a strengthening effect on the gut barrier of different species. In a rat model of dextran sodium sulfate (DSS)-induced colitis, pre-treatment with a mix of 4 strains of *L. reuteri* significantly decreased translocation of bacteria to mesenteric lymph nodes from the gut. The probiotic mix could not prevent the increased bacterial load in the mucus caused by DSS or the change in the mucus microbiota, but the decreased bacterial translocation indicated that this probiotic mix was capable of increasing barrier integrity through other mechanisms (Dicksved et al., 2012). In a study by Ahl and colleagues (2016), a rat-derived and a human-derived strain of *L. reuteri* were given to mice by oral gavage for 14 days, with DSS added for the last 7 days to induce colitis. Both strains caused a decrease in disease severity and inflammatory markers compared to the group treated with DSS only. Both strains were also shown to significantly increase the thickness of the firmly adherent layer of colonic mucus, but not the loosely adherent layer, compared to the control under both healthy and DSS-treated conditions. Under healthy conditions, the rat-derived strain (but not the human) significantly increased occludin and ZO-1 protein expression in the bottom half of the colonic crypts. Although JAM-A was also increased, this difference didn't reach significance (Ahl et al., 2016). In another study, *L. reuteri* P43-HUV did not significantly inhibit enterotoxigenic *Escherichia coli* (ETEC) adhesion to IPEC-J2 porcine jejunal epithelial cells, but there was significantly higher transepithelial electrical resistance (TEER) with addition

of the probiotic compared to ETEC alone, showing evidence of increased barrier integrity (H. Y. Liu et al., 2015). As shown with anti-ZO-1 and DAPI staining, ETEC caused loss of cell contact, opening of tight junctions, and cell death, while the addition of *L. reuteri* prevented this from occurring, providing further evidence of barrier reinforcement (H. Y. Liu et al., 2015). There is also evidence that *L. reuteri* is capable of strengthening barrier function in fish. In a study on Nile tilapia, treatment groups receiving different doses of *L. reuteri* ATCC53608 showed increased villous height in the hindgut and increased mucus secretion throughout the intestine compared to the control group after 8 weeks. The gene expression of ZO-1 and occludin, proteins associated with the tight junction, were both significantly upregulated throughout the gut in a dose-dependent manner, with the largest increase seen in the foregut (W. Li et al., 2022).

Different strains of *P. acidilactici* have also been shown to affect gut barrier integrity. Selenium-enriched *Pediococcus acidilactici* MRS-7 given to mice for 21 days was able to counteract the detrimental effect of the mycotoxin patulin on the jejunum, preventing the decrease in mucus-secreting goblet cells seen in the group treated with patulin only. The enriched probiotic was also able to significantly increase the levels of claudin 1, occludin, and ZO-1 protein and decrease levels of inflammatory markers compared to this group (Chen et al., 2022). Human-derived *Pediococcus acidilactici* CGMCC NO.17,943 was found to increase colon length, decrease disease severity and colonic damage, and reduce markers of inflammation in a mouse model of DSS-induced colitis (Dai et al., 2024). After oral administration, there was less FITC-dextran found in the serum, and increased gene expression of ZO-1 and JAM-A, as well as increased levels of JAM-A and occludin protein in the colon compared to mice treated with DSS only. When mice were pre-treated with broad-spectrum antibiotics before colitis-induction and probiotic administration, no improvement in disease severity or barrier function

was seen for any of the tested probiotics, leading to the conclusion that the probiotics provided a benefit through influencing the endogenous gut microbiota and their metabolites (Dai et al., 2024). Interestingly, the gene expression of mucin 2 in the colon was significantly reduced by the addition of this probiotic (as well as some others tested, including *L. reuteri*) compared to DSS alone, leading the authors to postulate that direct contact between epithelial cells and the bacteria could benefit barrier function in some way (Dai et al., 2024). However, it could be interpreted that as the epithelial junctions were not compromised to the level seen in the DSS-only group, an excessive increase in mucin production was not necessary in the groups additionally treated with a probiotic, as they did not need to rely more heavily on the protective mucus layer. It is worth noting that a number of probiotic studies have found increased levels of mucin 2 at the gene and protein level, as previously reviewed (La Fata et al., 2018). The effect of *P. acidilactici* on the gut barrier has also been studied in fish. After 48 hours of co-incubation, there was no significant change in expression of immune genes or selected gut barrier genes in intestinal epithelial cells from rainbow trout (RTgutGC) treated with *P. acidilactici* MA18/5M. However, while cells incubated with *V. anguillarum* had a significantly lower TEER value than the control after 48 hours, showing decreased barrier integrity, the addition of the probiotic *P. acidilactici* could prevent this decrease (Soto-Dávila, Langlois Fiorotto, et al., 2024). Therefore, even though the probiotic did not appear to provide a benefit under normal conditions, it showed a benefit when a stressor was added. In an *in-vivo* trial with Chinook salmon, *P. acidilactici* incorporated at 10^8 CFU/g of feed did not cause a significant difference in the gene expression of tricellulin, E-cadherin, villin 1, jam1a, zo-1, or mucin 2 in the hindgut at any timepoints over 6 weeks, and histopathological analysis of the hindgut also showed no differences between treatments (Soto-Dávila, Langlois Fiorotto, et al., 2024). One gene that did show a difference in

expression was claudin 15, which was significantly higher in the probiotic group at the 6-week timepoint compared to the control (Soto-Dávila, Langlois Fiorotto, et al., 2024).

Claudin 15 forms a cation channel that has been shown to have a critical role in regulating sodium permeability and homeostasis in the small intestine, and it is also necessary for efficient glucose absorption (Tamura et al., 2011). The role of this claudin in paracellular ion transport has also been shown in zebrafish (Bagnat et al., 2007). Claudin 15 is under control of the transcriptional regulator hepatocyte nuclear factor 4 alpha (HNF4a) which upregulates its expression, and mutant mice with an epithelial HNF4a deletion show chronic intestinal inflammation with an early down-regulation of claudin 15 in the colon and significant decrease of ion transport, but not paracellular permeability (Darsigny et al., 2009). Given that claudin 15 has a role in ion transport and glucose absorption, but not paracellular permeability, the difference in claudin 15 expression in the study by Soto-Davila, Langlois Fiorotto, and colleagues (2024) could indicate that although *P. acidilactici* did not appear to directly impact the integrity of the junctional complex or mucus layer, it could possibly have an effect on ion homeostasis or nutrient absorption in the gut that was not assessed in the study. In the current experiment, a transient increase in the expression of claudin 15 was seen on Day 7 in the coated treatments, with a more significant increase in the probiotic group, but this was not seen on Day 28. Perhaps differences in expression of this gene would be seen after a longer treatment period, although this remains to be tested.

Villin is an actin-modifying protein exclusively found in epithelial cells that has been shown to have important roles including but not limited to the regulation of cell morphology, migration, and invasion. It is very versatile as it can sever, cap, nucleate, and bundle actin filaments, and it has been shown to be important in the formation of microvilli, which have actin

filaments at their core (Khurana & George, 2008). Studies on the effect of *P. acidilactici* in tilapia and Atlantic salmon showed no significant difference in intestinal microvilli measurements (Jaramillo-Torres et al., 2019; Standen et al., 2013). Other studies using this probiotic in zebrafish and Asian sea bass found an increase in villi length and width in certain treatment groups (Arani et al., 2021; Ashouri et al., 2020). A group of largemouth bass receiving food fermented for 3 days with *Lactobacillus acidophilus*, *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* showed increased villi height, but no difference in villi width or muscle thickness compared to the control group (Z. Yang et al., 2023). As mentioned above, *L. reuteri* increased height of the villi in the hindgut of Nile tilapia (W. Li et al., 2022). It varies across studies, but it's clear that the species of probiotics used in the current study have the potential to modify surface area for absorption in the intestine by modifying villi measurements. As changes in microvilli structure can be observed in certain studies, it would not be surprising to find changes in expression of villin in response to probiotic administration, given its role in actin organization.

As a dietary component, carrageenan has also been tested for its effects on the gut barrier. Carrageenan is thought to cause inflammation either through changing the microbiota of the intestine, in turn leading to damage of the mucosal layer, or more directly by activating the NF- κ B pathway through binding epithelial cell receptors, as previously reviewed (Guo et al., 2023). Either way, it has been shown to disrupt barrier function. HCT-8 human intestinal epithelial cells showed increased levels of NF- κ B and early growth response gene 1 (EGR-1) in response to carrageenan, and a significant increase in IL-8 gene and protein expression, proven to be mediated by NF- κ B. Carrageenan also caused decreased TEER values in the cell monolayer, as well as a decrease in ZO-1 density and organization, and these negative effects were exacerbated

when expression of either of these transcription factors was blocked, proving their importance in regulating the effect of carrageenan on the barrier (Choi et al., 2012). A study by Wu and colleagues (2021) looking at the effect of λ -carrageenan on induced infectious colitis in a mouse model provided strong evidence that changes in microbiota were responsible for the increased disease severity. This group administered a 90-day treatment with low, medium, or high concentrations of λ -carrageenan in mice followed by oral gavage with 10^9 CFU/mouse of *C. rodentium* to induce colitis. Mice in carrageenan groups showed increased disease severity in a dose-dependent manner, with significantly lower body weight, increased percentage with diarrhea, decreased colon length, and increased inflammatory cytokine levels and pathohistological scores compared to the *C. rodentium* control group. When colitis was induced in germ-free mice, no significant differences were seen in these disease severity indicators between groups that had received high dietary carrageenan vs the control diet. However, germ-free mice that received a fecal transplant from the (non-germ-free) mice that had a high-carrageenan diet showed exacerbated symptoms after *C. rodentium* administration compared to the group that got a fecal transplant from the mice on the control diet. Further, high dietary carrageenan caused a significant decrease in thickness of the mucosal layer and increase in intestinal permeability when directly administered and in fecal transplant recipients compared to their respective controls, suggesting that the decrease in barrier integrity carrageenan caused was due to changes in the microbiota (Wu et al., 2021).

It is important to note that different isomers of carrageenan do not all have the same effect in any given system. A significant increase in the permeability of a Caco-2 monolayer, and a significant increase in gene expression of the IL-8 receptor CXCR1, was seen after treatment with either physiologically digested lambda- or iota-carrageenan, but not kappa-carrageenan.

However, structural changes were seen in ZO-1 and F-actin in response to all 3 isomers, with discontinuity of ZO-1 observed and a decrease in membrane localization (Fahoum et al., 2017). Kappa-carrageenan was the isomer used in this study. A study by Jiang and colleagues (2013) found that when co-cultured with differentiated THP-1 macrophage cells, the barrier integrity of a Caco-2 monolayer significantly decreased, and this effect was significantly amplified with the addition of κ -carrageenan. Kappa-carrageenan also significantly increased levels of apoptosis of the Caco-2 cells, and increased the levels of TNF- α , IL-6, IL-1 β , and IL-8 in the co-culture in a dose-dependent manner, although TNF- α production from THP-1 cells was the only cytokine that showed a significant increase in response to the lowest tested dose of carrageenan. Adding TNF- α to the co-culture significantly increased levels of apoptosis in the Caco-2 monolayer, but pre-treatment with anti-TNFR 1 antibody significantly reduced this effect, and the antibody also significantly decreased levels of IL-6 and IL-1 β . This led the authors to conclude that damage to the epithelial monolayer caused by carrageenan was mediated through TNF- α (Jiang et al., 2013).

Looking at the results of the current experiment, the gene expression of *vill* and *zo-1* on Day 1 in the treated groups are not significantly different than the control group, but they are significantly different from each other, with expression being higher in the probiotic group compared to the coating-only group. These results make sense as probiotics, including the ones used in this study, have been shown to cause changes in villi structure, including increased length, as discussed above. The cores of microvilli are made of actin, and villin is an actin-modifying protein with important organizational roles (Khurana & George, 2008). On the other hand, abnormal structural changes of actin have been seen in response to carrageenan treatment (Fahoum et al., 2017), suggesting abnormal regulation or a lack of organization. Similarly for

ZO-1, *L. reuteri* and *P. acidilactici* have both been shown to increase its expression (Ahl et al., 2016; Chen et al., 2022; Dai et al., 2024; W. Li et al., 2022), while carrageenan has been shown to cause a decrease in density, organization, and membrane localization (Choi et al., 2012; Fahoum et al., 2017).

Tricellulin (*marveld2*) and mucin 2 (*muc2*) were both significantly higher in the probiotic group compared to the control on Day 7, but this was not seen in the coating-only group. Tricellulin has a critical role in barrier function, as tricellulin knockdown in mouse Eph4 epithelial cells causes structural changes in both bicellular and tricellular junctions and significant increase in permeability to 4 kD FITC-dextran (Ikenouchi et al., 2005). Tricellulin therefore is important in controlling the paracellular passage of macromolecules through the gut epithelial layer. Although the studies reviewed above do not mention tricellulin, many of these studies show an increase in other barrier-sealing genes. Different studies using strains of *L. reuteri* have been shown to upregulate the expression of claudin 1, -3, -5, -7, -8, and -9, as previously reviewed (Gou et al., 2022). In the studies mentioned above, *P. acidilactici* was shown to increase expression of claudin 1 (Chen et al., 2022), and both *L. reuteri* and *P. acidilactici* were shown to increase levels of occludin (Ahl et al., 2016; Chen et al., 2022; Dai et al., 2024; W. Li et al., 2022). As these claudins and occludin have a similar function to tricellulin in that they seal the paracellular barrier, an increase in expression of *marveld2* aligns with the literature results. As discussed above, the species of probiotics used in this experiment have been shown to improve mucus parameters such as thickness, secretion, or number of mucus-secreting cells in the intestine (Ahl et al., 2016; Chen et al., 2022; W. Li et al., 2022). On the contrary, carrageenan has the potential to damage the mucosal layer (Wu et al., 2021). This could explain

why *muc2* expression in the probiotic group was significantly upregulated compared to the control, but this was not seen in the coating-only group.

Another gene that was upregulated at Day 7 was claudin 15 (*cldn15*), as mentioned previously. Many studies looking at the effect of probiotics on gut barrier integrity measure expression of claudins that seal the tight junction and decrease permeability, which applies to a large portion of the claudin family, as opposed to those that are pore-forming, such as claudin-2, -10, or -15 (Lechuga & Ivanov, 2017). A study in a celiac disease mouse model showed that the gluten component gliadin caused increased gene expression of the pore-forming proteins claudin 2 and claudin 15, while two different probiotic mixes were able to decrease expression back to levels comparable to the control (Ferrari et al., 2021). A rat model of food allergy showed that ovalbumin-sensitized mice had decreased gene expression levels of claudin 15, while the addition of a probiotic treatment caused an increase back to levels that were not significantly different than the control group (Tulyeu et al., 2019). We can see that the effect of the probiotics in terms of increasing or decreasing expression of claudin 15 was actually opposite in these studies, but in both cases, they returned the expression of this protein to control levels from an altered state, suggesting a beneficial role of probiotics in regulation of this claudin. After observing that there were lower levels of claudin 15 mRNA in ulcerative colitis patients, Chatterjee and colleagues (2021) showed that overexpression of the vitamin D receptor was protective against colitis as it upregulated claudin 15, which was associated with increased resistance to DSS- or *Salmonella*-induced colitis. The results of the current experiment showed a significant upregulation of *cldn15* in both the probiotic and the coating-only group on Day 7, although a more significant difference was seen in the probiotic group. It is not possible to know the mechanism causing this change, but the study by Chatterjee and colleagues (2021) suggests

that this effect could be beneficial in terms of preventing inflammation and dysregulation in the gut.

JAM-A, also known as JAM-1, is involved in formation of epithelial tight junctions, but also has a role in regulating proliferation and migration in epithelial and endothelial layers, and migration of a number of immune cells, as previously reviewed (J. Wang & Liu, 2022). In a study by Laukoetter and colleagues (2007), an increased number of polymorphonuclear leukocytes and lymphoid aggregates were found in the colon of JAM-A deficient mice, which proved to be lymphoid follicles containing large numbers of B and CD4⁺ T lymphocytes. There were also submucosal lymphoid follicles that were not present in the control mice which had high numbers of B cells and dendritic cells. This, along with increased colonic permeability to 4-kD FITC-dextran and increased susceptibility to DSS-induced colitis, was evidence that barrier integrity was compromised (Laukoetter et al., 2007). In the current experiment, as seen in Fig. 3.2c, the expression of *jam1a* was significantly downregulated in the coating-only treatment group compared to the control on both Day 1 and Day 7, but this was not seen in the probiotic group. As previously discussed, there is evidence that carrageenan could negatively affect gut barrier function, which would align with the downregulation of this gene. It is also notable that the expression level of *jam1a* in the probiotic group is at a level between that of the control and the coating-only treatment groups on these days, and the species of probiotics used in this study have been shown to increase expression levels of JAM-A (Ahl et al., 2016; Dai et al., 2024). It is possible that the probiotics and coating agent are having opposing effects on the expression of this gene, but the effect of the probiotic is not enough to counteract the effect of the carrageenan component of the coating.

As seen in Fig. 3.2a, the gene expression of E-cadherin (*cdh1*) is significantly downregulated in the coating-only treatment group on Day 1 compared to the control, whereas on Day 7, the probiotic group is significantly upregulated compared to both the coating-only and control group, with the difference between the probiotic and control group being the most significant. As part of the adherens junction in epithelial layers, E-cadherin plays a critical role in maintaining cell-cell contact and communication, as previously reviewed (Bhatt et al., 2013). A 14-day feeding trial looking at the effects of carrageenan in rats showed through immunostaining that there was a significant decrease in the level of E-cadherin in the epithelial lining of the colon, but an increase of E-cadherin in the stromal cells, which the authors suggested could be due to the process of epithelial to mesenchymal transition (Onishchenko et al., 2022). A significant increase in the number of T cells and pan-monocytic/macrophage cells was seen in the lamina propria of the carrageenan-treated group, as determined using CD3 and CD68 cell markers (Onishchenko et al., 2022). Interestingly, E-cadherin ligands are expressed on certain types of immune cells such as dendritic and T cell subsets, and macrophages and dendritic cells have also been shown to express E-cadherin (Van den Bossche et al., 2012). The results of the current experiment show a decrease in the coating-only group compared to the control on Day 1, but this group is not significantly different than the control group on Day 7. Although no differences are seen on Day 7, it would have been interesting to test whether the localization of E-cadherin expression had changed, or whether there was a difference in the proportions of cell types expressing it. On the other hand, pre-treatment of IPEC-J2 intestinal epithelial cells with two different strains of *L. reuteri* before ETEC inoculation showed that they both increased the protein level of E-cadherin significantly compared to cells that were treated with ETEC only

(Karimi et al., 2018). This could explain the increase seen in *cdhl* expression in the probiotic group on Day 7.

It is important to note that although transient changes in the expression of all assessed gut barrier genes were seen, no significant differences in any genes were seen on Day 28. Therefore, this study showed no sustained response to either the coating agents or probiotics with regard to integrity of the gut barrier.

Conclusions and suggestions for future research

This experiment showed no significant changes in growth and transient changes in immune and gut barrier gene expression that lasted less than 4 weeks. Therefore, the use of corn starch and κ -carrageenan as a probiotic coating showed no short-term negative consequences. Although there was no detrimental effect, no benefit was seen either. It is possible that the coating, more specifically carrageenan, was negating any benefits of the probiotic, as they have been shown to have opposite effects on the gut barrier through their influence on the gut microbiota (Dai et al., 2024; Wu et al., 2021). There are plenty of studies showing beneficial changes in barrier genes using different strains of the probiotics used in this experiment, although not in Chinook salmon. Since this study showed they were not effective at the chosen dose within 4 weeks, perhaps the coating agent was not harmful either at this level but could be if given at a different dose or over a longer time, which needs to be studied. It is also important to note that, the same way a probiotic may not show improvement under baseline conditions but can be beneficial in the face of a stressor, the coating agent may not cause a problem under normal conditions, but perhaps could exacerbate the inflammation and damage caused by a pathogen. Therefore, the effect of these coating agents should be tested with a stressor. Finally, it would be useful to look at changes in the protein expression of inflammatory and gut barrier markers as opposed to gene transcription, as these changes don't always align. However, there are limited antibody markers available for use in Chinook salmon, and these tools still need to be developed. Overall, corn starch and κ -carrageenan as a combined coating agent has the potential to be used in aquaculture for the delivery of probiotics, although further testing is needed to ensure safety. The development and testing of new probiotics and coating agents is crucial to improve preventative measures available to combat diseases in a growing aquaculture industry.

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