# The Effects of Soybean Meal on Inflammatory Gene Expression in the Liver and Intestine of Salmonids

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## Abstract

Studies were conducted to investigate the responses in T-cell markers and *S100* gene expression involved in soybean meal (SBM)-induced enteritis (SBMIE) in Rainbow Trout (*Oncorhynchus mykiss*; RBT) and Atlantic Salmon (*Salmo salar L.*). The S100 proteins are calcium-binding proteins, related to inflammatory processes and multiple cellular functions, but they remain poorly characterized in fish. A strain of RBT (CX strain) at the Hagerman Fish Culture Experiment Station has been selected for growth on a plant-based diet that includes SBM for ten generations, and we compared RBT from the CX strain that were age (CXA) and size (CXS) matched to three commonly available commercial strains (RBT1, RBT2, RBT3). We investigated differences in overall growth, oxidative stress and intestinal inflammation between fish fed a fishmeal (FM) or SBM based diet for 12 weeks. Both CX strains of RBT had greater growth rates compared with other groups with similar feed consumption. Expression of *S100I2*, signaling inflammation in the intestine, was initially elevated at 4 weeks, diminished at 8 weeks, then elevated again by 12 weeks in RBT 1. Expression in other strains peaked at 4 weeks and declined by 12 weeks. In a second study, we compared overall growth, T-cell markers and intestinal inflammation in Atlantic Salmon fed either a FM or SBM based diet for 12 weeks. Expression of calcium binding protein S100I2 signaling inflammation in the liver was elevated over time and peaked at 12 weeks. Expression of S100 proteins showed a similar pattern to other inducible T-cell genes. Overall, these results provide further information regarding SBMIE of current commercial strains of RBT and Atlantic Salmon and help improve the utilization of plant protein sources in their diets.

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## Dedication

I would like to thank my friend Yun-Jung Wu, Cheng-Hong Chen, Joanne Chen, Vincent Wu, and my mom for all their love and support. I would like to dedicate this thesis to my dear family and friends.

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# Chapter1: Literature Review

## World Aquaculture

World population is increasing (UN, 2012) and parallels an increasing consumption of fish per capita and demand for fish (FAO, 2018). Aquaculture production provides an important part of animal-based protein for human consumption. The amount of fish consumed per person over the last 50 years has doubled (FAO, 2018). Before the 20$^{th}$ century, wild capture fisheries provided the primary products, but aquaculture has increased rapidly and now represents over half of fisheries production (FAO, 2018). Expansion of aquaculture production has led to increased use of aquafeed much of which has fishmeal (FM) and fish oil (FO) as primary components. Sources of FM and FO include many types of fish, which are primarily wild-caught, marine pelagic species, such as Menhaden, Pout, Mackerel, and others (Jannathulla et al., 2019). However, annual FM production is limited and insufficient to meet increasing demand, resulting in rising costs. Aquafeed accounts for approximately 60 – 70% of production costs in intensive aquaculture operations (Arru et al., 2019; NRC et al., 2011). From an economic and sustainability viewpoint, alternative protein sources to FM and FO are essential and need rigorous development.

## Alternative Diets

A variety of alternative protein sources have been studied such as plant protein ingredients (e.g., soybean, corn, wheat, lupins and peas, etc.), animal by-products (e.g., meat and bone meal, poultry meal), insect and other protein sources to replace FM in aquafeeds (Gasco et al., 2018; Hua et al., 2019). Soybean meal (SBM) is an outstanding alternative diet because of the high protein content, favorable amino acid profile, and low cost, but there are dietary limitations

observed in a few fish species, which are mostly carnivorous fish (Sahlmann et al., 2013; Trushenski et al., 2014; Venold et al., 2012). Previous studies have reported negative effects on fish growth and intestinal health when FM is substituted with plant-based ingredients (Bakke-McKellep et al., 2007b; Daniel, 2018; Hardy, 1996; Krogdahl et al., 2015). In salmonids, replacement of FM with more than 20% SBM will result in notable distal intestine enteritis, referred to as soybean meal-induced enteritis (SBMIE), and inflammation, which is histopathologically characterized by blunting of intestinal folds, decreased numbers of absorptive vacuoles in the enterocytes, and thickened lamina propria and submucosa with increased leukocyte infiltration (Blaufuss et al., 2020; Krogdahl et al., 2003, 2015; Silva et al., 2015). These inflammatory and histological signs begin to appear 2 to 5 days after exposure to the SBM diet with severity escalating over time in the most sensitive of salmonids, namely Atlantic Salmon (Baeverfjord & Krogdahl, 1996; van den Ingh et al., 1991). However, the comprehensive cause, mechanisms, and negative effects related to SBM replacement in fish are not entirely understood. In SBM, various antinutritional factors (ANFs) have been associated with distal intestine enteritis which include lectins, tannins, protease inhibitors, phytosterols, saponins, phytate, and more (Francis et al., 2001; Krogdahl et al., 2010; Venold et al., 2012). Early inflammation response to SBM in histological examination of distal intestine sections in Atlantic Salmon were reported on day 5 by Sahlmann et al., (2013), and revealed the rapid effects of diet with any inclusion of SBM. Various negative effects of ANFs in soybeans have been shown on fish metabolism including increasing gut permeability, interference with lipid uptake, alteration of cholesterol, and decreased protein digestion (Krogdahl et al., 2010). Saponins, which interfere with lipid and protein digestion (Cuadrado et al., 1995), were shown to cause inflammation in the distal intestine of Atlantic Salmon as the level increased in diet,

and have been strongly correlated as a causative agent in distal enteritis (Chikwati et al., 2012; Krogdahl et al., 2015). Soy lectins bind gut cell receptors and alter metabolism (D'Mello & Duffus, 1991), and trypsin inhibitors significantly affect weight gain and cause pancreatitis in RBT (Hart et al., 2010). Moreover, thickened lamina propria of mucosal folds in the distal RBT have been linked to dietary soya saponin and lectin contents (Iwashita et al., 2009). Phytates affect mineral digestion and reduce the availability of phosphorus in monogastric animals (Francis et al., 2001; Krogdahl et al., 2010), and specifically alter protein availability and reduce growth in RBT (Spinelli et al., 1983). Currently unidentified antigens in SBM can elicit an immune response or alter microbiota in the intestine of RBT, Atlantic Cod, and Atlantic Salmon (Blaufuss et al., 2020; Ringø et al., 2006, 2008). Moreover, the shift in microbial community may lead to elevated presence of harmful bacteria thereby indirectly causing inflammation (Mansfield et al., 2010). Even though ANFs can be diminished in part by physical, chemical or biochemical treatments, economic costs of such treatment lead to heat-treated SBM as the major product in aquafeed (Heikkinen et al., 2006). Some ANFs are difficult to eliminate, and some are thought to have some activated anti-nutritional effects after heat treatment (Krogdahl et al., 2010). To effectively use plant-based protein in fish feeds, processing of plant meals especially SBM, must significantly increase and most importantly, these methods must further reduce or eliminate ANFs in alternative protein diets (Gatlin et al., 2007).

**Selection for Plant-based Diets**

Despite annual improvements in alternative feed formulations and processing, using a complete plant-based feed more or less remains an unrealized goal because of less than optional growth performance and survival in RBT (Boucher et al., 2012; Callet et al., 2017; Cheng et al., 2003). However, genetic selection is an alternative approach to improving growth and

utilization of plant-based feeds in carnivorous fish. In RBT, some genotypes have been shown to present increased growth and survival over that of others while fed a plant-based diet (Boucher et al., 2012; Callet et al., 2021). Moreover, a strain of RBT (the CX strain) has been selected for growth on an all-plant protein diet for over two decades (Blaufuss et al., 2020; Overturf et al., 2013). The selected CX strain of RBT shows superior growth performance on plant-based diets, and resistance to and reduction of inflammatory responses to distal intestine enteritis (Blaufuss et al., 2020). Varying gene expression in immunological pathways between strains of RBT fed a plant-based diet may link the genotype and environment (i.e. diet) as a key mechanism in successful growth performance (Blaufuss et al., 2020; Callet et al., 2021). However, further studies are still required to reveal all the complex interactions among strain, diet, and immune responses.

**Immune Response**

In Salmonids, notable inflammation in the distal intestine exists when the diet contains SBM over 20 % (Burrells et al., 1999; Krogdahl et al., 2003). Anti-nutritional factors found in SBM have been linked to oxidative stress and damage to the antioxidant system in fish (Zhang et al., 2013). Oxidative stress is a cellular process balancing antioxidant and oxidant pools, and it can be exacerbated by numerous environmental factors, chemical pollutants, and more (Ighodaro & Akinloye, 2018; Olsvik et al., 2011). Moreover, oxidative stress influences transcription associated with numerous biochemical pathways, such as immune responses, cytokine production, inflammatory responses, and more (Giulio & Hinton, 2008). The three key enzymes involved in reducing oxidative stress in an organism are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Hoseinifar et al., 2021; Ighodaro & Akinloye, 2018; Miller et al., 1993). In tissues, SOD can reduce the superoxide radical or singlet oxygen radical

to hydrogen peroxide ($H_2O_2$) and $O_2$ (Di Giulio et al., 1989). Hydrogen peroxide is toxic to the body when it accumulates. Catalase can break down $H_2O_2$ to water and oxygen, and GPx reduces $H_2O_2$ to water and lipid peroxides to their corresponding alcohols thereby reducing damage to cells (Góth et al., 2004). Any dietary imbalance resulting from alternative feeds may stimulate an increase of oxidative stress in fish fed altered diets (Olsvik et al., 2011). SOD, GPx, and catalase are easily induced by oxidative stress, therefore their expression and enzyme activities have been used as biomarkers to define oxidative stress in cells (van der Oost et al., 2003).

The immune systems in a higher vertebrates contain T-cells and cytotoxic T lymphocytes (Nakanishi et al., 2002). The cluster of differentiation (CD) 4 (CD4) and CD8 are two major subsets of T-cell markers, for helper T-cells (Th) and cytotoxic T-cells respectively, in the immune system that fight against different pathogens and are characterized by specific cytokines (Moore et al., 2009; Westendorf et al., 2010). Activated naive $CD4^+$ T-cells can differentiate into specific subtypes with lymphocyte type 1 helper T-cells (Th1), Th2, Th9, Th17, and induced regulatory-T-cell, each producing distinct cytokines (Ashfaq et al., 2019; Miossec et al., 2009). The immunological response in fish is not completely understood related in part to insufficient identification of immune cells, cytokines, interleukins, interferons, and other factors. Previous studies have identified the immune-related genes linked to SBMIE, such as CD4, CD8β, interleukin (IL-) 1β (*IL-1β*), and *IL-10* in the Atlantic Salmon (Bakke-McKellep et al., 2007; Marjara et al., 2012) and indicated the possibility of T-cell involvement in intestinal inflammation. In higher vertebrates, Th17 cells, a distinct lineage of the $CD4^+$ Th cell, have been recognized to have a key role in host defense against extracellular antigens and autoimmune diseases by secretion of IL-17 (Miossec et al., 2009; Tesmer et al., 2008). Th17

cell subsets differentiate with IL-6 and transforming growth factor beta (TGF-β), and/or IL-23 in higher vertebrates. Moreover, Th17 can be proinflammatory and prevent autoimmune responses as an immunoregulator and also inhibit pathogenic T-cells (Miossec et al., 2009; Singh et al., 2013). Expression of *IL-17* was upregulated in the distal intestine of salmonids fed an SBM diet (Blaufuss et al., 2020; Krogdahl et al., 2015; Marjara et al., 2012). Despite this research, thorough knowledge of anti-inflammatory cytokines and the complex immune response in fish to ANFs remains elusive.

**S100 Genes**

*S100* genes are calcium-binding proteins with a canonical, twelve amino acid EF-hand motif (Leclerc et al., 2009; Lewit-Bentley & Réty, 2000). Twenty members of the S100 protein family have been discovered in humans, involved in multiple cellular activities with cell cycle regulation, signal transduction, cell differentiation, gene transcription, calcium homeostasis and cell cycle progression (Fritz et al., 2010; Heizmann et al., 2002; Heizmann and Fritz, 2010; Marenholz et al., 2004). Apart from calcium, S100 proteins can bind magnesium, manganese, copper, and zinc to regulate cellular activity (Chazin, 2011; Foell et al., 2007; Heizmann & Cox, 1998). Further, S100 proteins are also involved in host defense against pathogens in extracellular functions (Cole et al., 2001; Moroz et al., 2003) and inflammatory mechanisms, cut signaling and apoptosis in intracellular functions (Foell et al., 2007; Marenholz et al., 2004). S100 proteins have been discovered to be closely associated in humans with inflammatory bowel disease and have been commonly used as biomarkers (Manolakis et al., 2011). Certain types of cancer, as well as inflammatory and autoimmune diseases have also been associated with *S100* genes (Salama et al., 2008).

However, information on the numerous S100 proteins in fish is limited in comparison with what is known in humans and rodents (Kraemer et al., 2008). *S100Z* in zebrafish is similar in structure to *S100A4* in humans (Moroz et al., 2011). S100I protein has been found in abundance in the skin of Zebrafish, Atlantic Salmon, and Channel Catfish (Easy and Ross, 2009; Hsiao et al., 2003; Porta et al., 1996). Members of the S100 protein family (*HORN*, *S10A5*, *S10AD* and *S10I*) are active calcium-sensing proteins in green spotted puffer fish (*Dichotomyctere nigroviridis*)(Pinto et al., 2010). Furthermore, the immunoreactivity of S100 proteins have been used as markers to identify crypt cells in Zebrafish (Ahuja et al., 2013). Previous studies have also shown varying expression among several *S100* genes in RBT in response to dietary SBM (Blaufuss et al., 2019, 2020).

**Summary**

The breadth and severity of SBM-induced inflammatory responses among current growth-selected, commercial lines of RBT is unknown. The mechanisms of how the plant-based diet selected CX strain shows resistance to SBM and whether commercial lines of RBT show similar responses is also unclear, which prompted the first study presented here. The gastrointestinal tract in salmonids is physiologically complete and functions to process food and maintain osmoregulation (Bucking and Wood, 2006; Taylor and Grosell, 2006). The intestine also plays an important role in calcium and magnesium uptake in salmonids (Bucking and Wood, 2007). More importantly, the intestine of marine teleost fishes significantly contributes to total calcium uptake (Gregório and Fuentes, 2018). Physiological relationships between adaptation to a marine environment and calcium-binding protein expression in the gut of Atlantic Salmon remains unclear. Whether *S100* gene expression is affected by a high soy diet fed to Atlantic Salmon raised in freshwater remains unresolved and is pursued in the second study.

## Hypothesis

1. Feeding different strains of Rainbow Trout with fishmeal or plant meal for a period of 12 weeks shows no difference in growth performance or gene expression.
2. Feeding Atlantic Salmon with a fishmeal or plant meal for 12 weeks, shows no differences in growth performance or gene expression.

## Objectives

1. To evaluate the performance of the plant-based diet selected CX strain of Rainbow Trout compared with that of commercial selected strains (RBT1, RBT2, RBT3) fed a high-soy diet (40% SBM). Determine if any observed differences in the distal intestine and liver are associated with changes in the expression of genes related to oxidative stress and *S100* activity.
2. To compare the performance between Atlantic Salmon fed a high-soy diet and a FM diet. Determine if any observed differences in the distal intestine and liver are associated with changes in the expression of genes related to Th17 and *S100* activity.

# Chapter 2: Comparative Analysis of Soybean Meal Effects in Commercial Strains of Rainbow Trout *Oncorhynchus mykiss*

## Introduction

World aquaculture production is growing rapidly and playing an important role in providing animal protein for human consumption (FAO, 2018). Currently, the cost of fishmeal is increasing with rising world demand and it is the primary cost consideration in fish feeds. To help with limited fishmeal quantities, plant protein ingredients have been considered as protein substitutes in fish diets. Plant protein sources such as soybean meal (SBM) provide an abundant, more sustainable and an affordable alternative to fishmeal in aquafeeds. However, there are some concerns with replacing fishmeal (FM) with SBM in carnivorous fish, as it often contains anti-nutritional factors (ANFs) such as saponins resulting in distal intestine inflammation referred to as soybean meal induced enteritis (SBMIE). This inflammatory response is characterized by intestinal fold height shortening, thickening of the lamina propria and submucosa thickening, which is concomitant with infiltration of granulocytes (Baeverfjord & Krogdahl, 1996; Burrells et al., 1999; Knudsen et al., 2007; Romarheim et al., 2011; Silva et al., 2015). In salmonids, replacing fishmeal with plant-based ingredients also raises concerns with carbohydrate metabolism changes because fish are generally considered "glucose intolerant" (Geay et al., 2011; Panserat et al., 2009). Despite soybean meal being a more sustainable and economical protein source and having an advantageous amino-acid profile, the inflammatory responses observed in some aquaculture species such as Atlantic Salmon (*Salmo salar*) and Rainbow Trout (*Oncorhynchus mykiss*; RBT) limit soybean meal's utilization to replace FM as an alternative ingredient (Blaufuss et al., 2019; Collins et al., 2013; Krogdahl et al., 2015; Silva et al., 2015).

Numerous studies have concluded that distal intestine enteritis can be attributed to various levels of ANFs remaining in plant-based meals used in high protein diets for salmonids (Krogdahl et al., 2010; Venold et al., 2012). One strategy to help reduce SBMIE when FM is replaced by plant-based proteins, is to increase overall protein availability thus reducing the portion of plant-based proteins used and minimizing the ANFs in the diet (Gatlin et al., 2007). There is also evidence that exposure of RBT to dietary challenges during early stages affects acceptance and utilization of feeds in later life stages (Balasubramanian et al., 2016; Geurden et al., 2013). Although ANFs have been described as one factor in a plant-based diet affecting growth (Krogdahl et al., 2010), an unbalanced fatty acid profile in plant-based diets has also been shown to affect fish immunity (Turchini et al., 2010). Continually changing alternative feed formulations using plant-based proteins and oils to substantially or totally replace fishmeal and fish oil continues to be problematic while trying to avoid impairing growth performance and survival in rainbow trout (Boucher et al., 2012; Callet et al., 2017; Cheng et al., 2003).

Genetic selection is another approach to help improve growth and utilization of plant-based feed in carnivorous fish. In RBT, some genotypes have been observed to grow and survive better than others while fed a plant-based diet (Boucher et al., 2012; Callet et al., 2021). In addition, one strain of RBT has been specifically selected for growth on an all-plant protein diet for over decade (Blaufuss et al., 2019; Overturf et al., 2013). This selected strain of RBT (the CX strain) shows resistance to SBMIE and a reduction in inflammatory responses in the distal intestine (Blaufuss et al., 2019).

In this study, we compared three current commercial strains of RBT to the CX strain of RBT in a 12-week feeding trial. We aimed to assess how different strains of RBT might show differences in resistance to SBMIE and differences in utilization of plant proteins when

compared to a fishmeal diet. To understand the effect of this dietary alteration on oxidative stress, expression levels of three genes (SOD, superoxide dismutase; glutathione peroxidase, GPx; and catalase) were compared in the liver and distal intestine in each RBT strain during each time point. We also examined calcium-binding protein genes expressed in the intestine (*S100I2*) and in the liver (*S100V2*) as inflammatory markers.

## Material and Methods

### *Diets*

Two experimental diets were formulated to provide 46.7% digestible protein and 18.7% crude lipid containing in fishmeal (FM diet) or 48% digestible protein and 17.6% crude lipid containing in soybean meal (PM diet) as the primary protein source (Table 1). Both diets were balanced for digestible protein and supplemented with essential amino acids to reach or exceed known nutrient requirements (NRC, 2011).

### *Fish Culture and Feeding*

Three, same age, commercial strains of Rainbow Trout, which only select for growth (RBT1, RBT2, RBT3) and size-matched Hagerman selected strain (Blaufuss et al., 2020; Overturf et al., 2013; Venold et al., 2012) Rainbow Trout (CXS; approximately 2 weeks younger) collectively averaging 6.8 ± 0.49 g were stocked separately into 140 L poly tanks along with age-matched Hagerman strain Rainbow Trout (CXA) averaging 7.87 ± 0.14 g. Fish from each strain were randomly assigned to 4 replicate tanks (2 tanks/diet, 30 fish/tank) in a flow-through system with spring water inflow. The water temperature was maintained at 15 °C with a 14:10 light: dark cycle during the experiment. Tanks were randomly assigned the FM or

PM diet and fed twice daily to apparent satiation, 6 days per week, for 12 weeks. All fish were counted and bulk weighed every 4 weeks.

### *Sample Collection*

Initially and at weeks 4, 8, and 12, five fish (n=5/treatment) randomly picked from each treatment tank were euthanized with MS-222 (Western Chemical Co., Ferndale, WA). Samples of distal intestine and liver (~100 mg) were removed from each fish and placed in 1 ml TRIzol® (ThermoFisher Scientific, Waltham, USA) and stored frozen at -80 °C until RNA was extracted for gene expression.

### *RNA Extraction and cDNA Synthesis*

For gene expression analysis, total RNA from sample tissues were extracted in the TRIzol® reagent. The liver or distal intestine tissues were put in 2-mL round-bottom centrifuge tubes and homogenized in a bead mill (MixerMill 200, Retsch GmbH, Hann, Germany). After homogenization, tubes were centrifuged at 12,000 g at 4 °C for ten minutes and the supernatant transferred to new 1.5 mL tubes. Chloroform (200 ul) was added to each tube and vigorously shaken for 15 seconds before incubation at room temperature for ten minutes. Tubes were then centrifuged at 12,000 g at 4 °C for 15 minutes, and the supernatant transferred to a new 1.5 mL tube. Isopropanol (500 ul) was added and mixed well before incubation at -20 °C overnight. RNA was pelleted by centrifuging at 12,000 g at 4 °C for 10 minutes, then washed with cold 75% ethanol, and resuspended in nuclease-free water. RNA quantity and purity were examined spectrophotometrically using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA). RNA sample concentrations were adjusted with nuclease-free water to 100 nM, treated with DNase (DNase I, Invitrogen, Carlsbad, USA), and reverse transcribed into cDNA (High-

Capacity cDNA Reverse Transcription kit, ThermoFisher Scientific, Waltham, USA) using the manufacturer's instructions. Resulting cDNAs were stored at -80 °C for further analysis.

### *Quantitative PCR and Data Analysis*

All samples were run in duplicate using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Fast SYBR™ Green Master Mix (Life Technologies, Carlsbad, USA) was used according to the manufacturer's instructions along with 40nM of each primer and 10 ng of template cDNA. After amplification, a melt curve was used to check the specificity of the qPCR product. A standard curve with a 7-point, fivefold dilution series was used with pooled total RNA from all samples. Expression efficiencies ranged from 97-103%. Primers for reference and target genes are shown in Table 2. Target genes were normalized against the reference gene *RPS15* (Blaufuss et al., 2020) and quantified using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

### *Statistical Analysis*

Fish/treatment was used as replicate unit (n=5) in growth parameters and gene expression and analyzed with one-way and two-way ANOVA using R Studio® version 1.2.1335 (R Studio, Inc, Boston, MA), with "Tukey HSD" *post-hoc* tests when significant differences were observed. Significance was defined at $\alpha < 0.05$ for all statistical tests.

## Results

### *Growth Performance*

On the FM diet, CXA fish averaged the greater final weight (Table 3). On PM diet, both CXA and CXS fish showed greater final weight compared with that for the commercial strains,

and RBT3 showed significantly lower final mass despite a greater feed intake. Although not significant, the CXA fish fed both the FM and PM diets had a lower FCR compared with that of the other strains. The survival rate was between 0.76-0.93 ± 0.02% with no significant difference among strain and diet.

***Gene Expression***

In the distal intestine, overall expression of SOD in both the FM and PM diet fed fish was significantly higher at week 4 and week 12 compared to that at week 8. With the FM diet, expression of SOD in CXS and RBT1 fish was higher than expression observed in CXA, RBT2 and RBT3 strains at 4 weeks but not significantly different among strains by week 12 (Fig. 1A). On the PM diet, expression of SOD was also downregulated at week 8 but elevated again by week 12 (Fig. 1B). The effect of strain was significant on GPx expression in the intestine over time with the FM diet (Fig. 1C). Expression of GPx in the intestine with the PM diet was variable among strains within time points but not significant over time (Fig. 1D). Catalase expression in the intestine increased significantly over time in RBT3 with both FM and PM dietary treatments (Fig. 1E and F). Catalase expression was also elevated in RBT2 over time in the PM diet treatment (Fig. 1F).

In the liver, SOD expression was downregulated among strains on both diets except for RBT3, which showed increased expression by week 12 on the FM diet (Fig. 2A) and week 8 on the PM diet (Fig. 2B). The RBT2 strain also showed increased expression at week 12 on the PM diet (Fig. 2B). The expression of GPx was significantly different over time with all strains on both the FM and PM diets with lower expression again observed at week 8 (Fig. 2C and D). A significant time effect was present with the expression of catalase in both FM and PM diets,

with higher expression at week 4 and expression diminishing to initial levels (time 0) by week 8 in both diets (Fig. 2E and F).

Strain and time effect were significant for expression of *S100I2* in the intestine of fish fed both FM and PM diets. Overall expression was lower by week 8 with significant upregulation of *S100I2* observed in RBT1 in week 12 on both diets (Fig. 3A and B). Both strain and time effect were significant with expression of *S100V2* in the liver of fish fed the FM and PM (Fig. 4A and B). Overall expression was higher at week 4 and diminished over time in all strains by week 12.

**Discussion**

Rainbow Trout selected for increased utilization of plant protein diets (CXS, CXA) showed superior growth compared with other commercial strains (RBT1, RBT2, and RBT3) on both FM and PM diets after twelve weeks (Table 3). Similar results have been observed in previous feeding trials using this selected strain when fishmeal was replaced with up to 40% SBM (Blaufuss et al., 2020; Overturf et al., 2013). Advantages of these selected rainbow trout was first reported by Venold et al., (2012), where they examined the level of fatty acid binding protein and enterocyte proliferation rate to define the different responses with 4$^{th}$ generation CX select line fish and non-selected RBT. Callet et al., (2021) recently showed transcriptome profiles differ along with growth performance within three isogenic lines of RBT with varying sensitivity to SBM suggesting sufficient genetic variation is present in RBT lines to select for further improvement in oral tolerance of SBM.

Maintaining homeostasis with variable generation of reactive oxygen species (ROS) is important for preventing oxidative injury and is maintained by superoxide dismutase (SOD),

glutathione peroxidase (GPx) and catalase as the first line of antioxidant defense reducing the oxidative stress in an organism (Hoseinifar et al., 2021; Livingstone, 2003; Fig. 5). SOD, GPx and catalase are easily induced by oxidative stress; therefore, their expression and enzyme activities have been used as a biomarkers to define oxidative stress in cells (van der Oost et al., 2003). Anti-nutritional factors found in SBM have been linked to damage related to the antioxidant system in fish (Zhang et al., 2013).

In the distal intestine, we observed significant up-regulation in expression of the SOD gene in fish fed with the FM or PM diet (Fig. 1A and B). At week 8, both diet and strains showed lower SOD expression than at other time points. This up and down regulation over time has been previously observed (Blaufuss et al., 2020) and may indicate an adaptive response by the fish to the experimental and control diets which are different than the diet fish were fed prior to the study. The CXA and RBT1 strain showed greater expression of SOD than other strains in week 4 while fed FM diet but this pattern was unresolved by week 12. The expression of GPx in the distal intestine was highly variable and showed overall down-regulation regardless of the diet or strain (Fig. 1C and D). At week 12, CXS and CXA fish on both FM and PM diets showed up-regulation in GPx in the distal intestine. The expression of catalase in the distal intestine was observed a significant effect between strain and up-regulation among all-time points in FM or PM diets. Catalase expression generally increased over time but interestingly, expression differences were significant between CXS and CXA strains on both diets at all time points (Fig. 1 E and F). This suggests that despite these fish being from the same cohort and same strain, significant variability exists between families and expression of catalase can be very time- and age-dependent. Comparison with similar work shows intestinal antioxidant activity was significantly higher in largemouth bass after 8 weeks feeding a 28% SBM diet. Specifically,

bass fed on a high-SBM diet showed about two-fold greater activity of SOD and GPx in the intestine than bass fed an FM diet (Chen et al., 2021). In addition, Blaufuss et al., (2019, 2020) observed up-regulation in other genes related to inflammation in distal intestine of RBT while fed a 40% SBM diet. In that study, upregulation of *IL-17* was related to the commonly observed mucosal inflammatory response in salmonids. RBT fed on a 40% SBM diet showed greater expression on *IL-17A/F2a*, *F2b*, and *F3* than the RBT fed an FM diet at weeks 4, 8, and 12 (Blaufuss et al., 2020).

Expression of SOD in the liver showed downregulation at weeks 4 and 8 among all strains of fish fed the FM diet (Fig. 2A). In general, variable expression of SOD in the liver was only observed in RBT2 and RBT3 of the commercial strains with both CXS and CXA fish remaining unaffected by dietary treatment. In gilthead sea bream, SOD enzyme activity in the liver increased significantly as the FM percentage in the diet was replaced with SBM (Kokou et al., 2015). The expression of SOD in the liver of Atlantic Salmon was downregulated after 2 days and 17 days when the diet was changed from FM to a plant-based diet (Olsvik et al., 2011). With GPx gene expression in the liver, we observed significant differences across all-time points in with fish fed the FM diet. Expression of GPx in fish fed the PM diet was variable but generally downregulated from the initial time point (time 0, Fig. 2C and D). Both the CXS and CXA strains showed modest but significantly increased expression of GPx at week 12 regardless of diet. Previously, GPx upregulation in the liver has not been observed as a hallmark for this strain. Expression of GPx in the liver of Atlantic Salmon was shown to be elevated at 17 days after FM was replaced with a plant-based diet but not on day 0 and 2 (Olsvik et al., 2011). This shortened timeline may also be true for RBT, but we did not sample fish at two weeks.

Catalase expression in the liver was variable with both time and strain and there was a general tendency for upregulation of catalase by week 12, regardless of diet. Catalase expression between the CXA and CXS was significantly different by week 12 again suggesting that age and size differences between these cohorts of the same strain convey significantly different responses to the same diet, as observations of GPx expression did. Our data suggest that regardless the diets and strains, the expression of catalase peak at week 4 (Fig. 2E and F). This expression pattern over time has been previously observed (Blaufuss et al., 2020) and possibly describe that the first 4 week fish responded and altered to the experimental and control diet, witch different from the diet fish were fed before the trial started. In the liver of Atlantic Salmon, increases in expression and enzyme activity of catalase was positively correlated with a plant-based diet after day 17 (Olsvik et al., 2011).

Catalase processes $H_2O_2$ to water, but GPx can also reduce lipid peroxides (Ighodaro and Akinloye, 2018). The patterns of SOD, GPx and catalase expression observed between diets and among strains may indicate variation in changes to metabolism that strains encounter over time as they detoxify $H_2O_2$ or reduce fatty acid peroxides. The time effect has statistical difference with lower at week 8 regardless the diet or strain compared to a previous study (Blaufuss et al., 2020), which may possibility indicate a putative acclimation period from control or experiment diet is around at 8 week in RBT. Most research on SBMIE of salmonids did not observer this pattern due to the overall experimental time of less than 4 weeks. The different gene expression of SOD, GPx and catalase between liver and distal intestine may be caused by cellular preference for a particular pathway to process ROS or alternatively, the cell may have sufficient stored enzymes (in peroxisomes) to respond to ROS. Further research should investigate different metabolic pathways and longer experimental times.

Information on *S100* gene expression in fish is limited. Calcium-binding proteins are found in the skin and mucus membranes of Atlantic Salmon in both sea lice infected and non-infected fish. One of these proteins, *S100I2*, has been identified as ictacalcin (Easy and Ross, 2009). Channel catfish skin has been shown to have abundant S100-like calcium-binding proteins with ictacalcin comprising up to 5% of these proteins (Karsi et al., 2002). Physiological relationships between adaptation to a marine environment and calcium-binding protein expression in the gut remains unclear. In marine fish, the major area of calcium uptake and homeostasis is in the gut, and thus *S100* gene expression could be important when feeding anadromous RBT or Atlantic Salmon feeds that contain high levels of SBM (Gregório and Fuentes, 2018). Previous work has shown the expression of calcium-binding protein genes (*S100* genes) are affected by high SBM diets in RBT (Blaufuss et al., 2019, 2020). In this study, expression *S100I2* in the distal intestine showed a similar pattern to SOD expression over time and GPx expression over time in the liver, with overall lower expression at week 8 regardless of diet (Fig. 3A and B). Initially at week 4, *S100I2* expression in both CXS and RBT1 strains was significantly upregulated regardless of diet but remained higher only in the commercial RBT1 strain after week 12. The same commercial strain of RBT was also used in a previous study (RBT1) and was observed with elevated *S100I2* expression fed a high-SBM diet in that study (Blaufuss et al., 2020). In the distal intestine, the CXS strain showed greater expression of *S100I2* than CXA strain regardless of diet.

In previous work, *S100V2* gene upregulation was observed in the liver when RBT were fed a high-SBM diet (Blaufuss et al., 2019). In this study, expression of *S100V2* in the liver showed a similar pattern to catalase gene expression with greater expression at week 4 regardless of diet and an overall downregulation at weeks 8 and 12 (Fig. 4 A and B).

## Conclusions

In summary, our results demonstrate and reiterate significant growth differences between the CX strain selected for growth on a plant protein-based diet using 40% SBM and current commercial strains. Callet et al., (2021) also observed different levels of energy production in RBT fed on a plant-based diet with numerous genetic differences between the isogenic lines. Moreover, the CXS and CXA groups were the same strain and same cohort which only differed in spawning age and showed significant differences in gene expression on the same diet at the same time points. Thus, age and timing of sampling needs further study and must be taken into consideration when conducting comparative studies.

# Chapter 3: Effects of Dietary Soybean Meal Inclusion on Calcium-Binding Protein Expression and Inflammatory Gene Markers in Liver and Intestine of Atlantic Salmon (*Salmo salar L.*)

## Introduction

World aquaculture production has increased annually and is negatively correlated to marine fisheries catch (FAO, 2018). Fishmeal (FM) from marine fisheries is the primary protein source for most carnivorous fish diets. To keep up with the requirements of increasing use of FM in the growing aquaculture industry, alternative and sustainable protein sources have been pursued to reduce reliance on FM (Cottrell et al., 2020). Soybean meal (SBM) is an outstanding alternative to FM because of its high protein content, favorable amino acid profile, and low cost. However, research has shown there are dietary limitations on partially replacing FM with SBM when feeding carnivorous fish (Sahlmann et al., 2013; Trushenski et al., 2014; Venold et al., 2012). Several anti-nutritional factors (ANFs) are present in commonly de-hulled, solvent-extracted SBM that include phytates, lectins, saponins, protease inhibitors, oligosaccharides, and more. And their presence has been implicated as the primary factors influencing fish growth performance and health (Francis et al., 2001; Krogdahl et al., 2010). In Atlantic Salmon, SBM has been observed to induce an immune response (Bakke-McKellep et al., 2007) resulting in inflammatory, distal intestine enteritis (Krogdahl et al., 2010, 2015).

The complete mechanisms behind SBM-induced immune responses in trout and salmon are not completely understood. Previous studies have shown upregulated expression of immune-related genes, clusters of differentiation (CD) *CD4* and *CD8b*, in the distal intestine of SBM-fed salmon (Bakke-McKellep et al., 2007; Marjara et al., 2012), and indicated the

possibility of T-cell involvement in intestinal inflammation. Moreover, upregulated expression of Interleukin 17 (*IL-17)* was observed in response to distal intestine enteritis in salmonids fed a diet containing SBM (Blaufuss et al., 2020; Krogdahl et al., 2015; Marjara et al., 2012). In higher vertebrates, Th17 cells have been recognized as playing a key role in defense against the extracellular antigens and autoimmune disease by secretion of IL17 (Miossec et al., 2009; Tesmer et al., 2008).

Generally, information on calcium-binding proteins remains limited in fish (Kraemer et al., 2008) compared to that for humans and rodents despite their range of cellular functions and involvement in $Ca^{2+}$ signaling events (Carafoli et al., 1999). In humans, S100 proteins have been associated with inflammatory bowel disease and a heterodimer of S100 proteins called calprotectin is commonly used as a bio-marker (Manolakis et al., 2011).

In fish, immunoreactivity of S100 proteins have been used as markers to identify crypt cells in Zebrafish (Ahuja et al., 2013). In Rainbow Trout (*Oncorhynchus mykiss*) fish fed a diet containing 40% SBM showed significantly upregulated expression of *S100* genes in the distal intestine (Blaufuss et al., 2019, 2020).

However, the intestine is a main component of calcium homeostasis in marine teleosts (Silva et al., 2015; Wilson & Grosell, 2003). Thus, our interest in *S100* genes relate to whether calcium-binding protein expression in Atlantic Salmon may be affected in the early stages of soy-induced distal enteritis. In this study, we compared Atlantic Salmon fed either a FM-based or SBM-based diet, over a 12-week feeding trial. The effects on expression of immune-related markers including *CD4*, *CD8b*, interleukin 1b (*IL-1b*), *IL-17A*, and interleukin 10 (*IL-10*), and

S100, *S100I2* and *S100V2* were studied in liver and distal intestine samples taken over the course of the feeding trial.

## Material and Methods

### *Diets*

Two experimental diets were prepared with 46.7% digestible protein and 18.7% crude lipid containing in fishmeal (FM) or 48% digestible protein and 17.6% crude lipid containing in plant meal (PM diet; Table 1). Diets were balanced and supplemented with essential amino acids to reach or exceed nutritional requirements for Atlantic Salmon (NRC, 2011).

### *Fish Culture and Feeding*

Triplicate tanks per treatment with twenty-six Atlantic Salmon (71±0.28 g; National Cold Water Marine Aquaculture Center, Orono, ME) per tank were kept in a flow through system with spring water in 140-L poly tanks connected to common bio-filter. The environment was sustained at 14:10 light: dark cycle with 15 °C water temperature during the experiment. Fish were fed twice daily to appetent satiation with FM or PM diets, 6 days per week, for 12 weeks. All fish were counted and weighed every 4 weeks. All work was completed under the guidelines of the University of Idaho Animal Care and Use committee under protocol #2021-16.

### *Sample Collection*

At weeks 4, 8, and 12, five fish from each tank (n=5/tank; n=15/treatment) were euthanized with MS-222 (Western Chemical Co., Ferndale, WA), weighted and tissues sampled for liver and distal intestine ~100 mg. The samples were submerged in cold TRIzol® (ThermoFisher Scientific, Waltham, USA) and stored frozen at -80 °C until processed for gene expression.

### *RNA Extraction and cDNA Synthesis*

Total RNA was extracted from sample tissue using TRIzol®. Liver or intestine samples were put in 2-mL round-bottom centrifuge tubes with 1 mL of TRIzol® and homogenized in a bead mill (MixerMill 200, Retsch GmbH, Hann, Germany). Tubes were centrifuged at 12,000 g at 4 °C for 10 minutes then removed from the supernatant to new 1.5 mL tubes. Two hundred uL of chloroform was added into tubes and vigorously shaken for 15 seconds before incubation at room temperature for 10 minutes. The tubes were again centrifuged at 12,000 g at 4 °C for 15 minutes and the supernatant transferred to a new 1.5 mL tube. Five hundred uL of isopropanol was added, mixed well, and incubated at -20 °C overnight. Total RNA and DNA was pelleted by centrifuging at 12,000 g at 4 °C for 10 minutes, then washed with cold 75% ethanol, and resuspended in nuclease-free water. The quantity and purity of RNA was spectrophotometrically determined (Nanodrop 2000, ThermoFisher Scientific, Waltham, USA). All the RNA samples were adjusted to the same amount with nuclease-free water and treated with DNase (DNase I, Invitrogen, Carlsbad, USA), and reverse transcribed into cDNA (High-Capacity cDNA Reverse Transcription kit, ThermoFisher Scientific, Waltham, USA). Resulting cDNA was stored at -80 °C for further analysis.

### *Quantitative RT-PCR*

Samples were amplified in duplicate on 96-well plate (7500 Fast Real-Time PCR system, Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions (Fast SYBR™ Green Master Mix, Life Technologies, Carlsbad, USA) using a melt curve to check specificity of the product. Expression efficiencies ranged between 97-104% and are listed in Table 4. Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was chosen as the reference

gene to normalize to target sequence expression (Marjara et al., 2012; Vasanth et al., 2015). Changes in threshold (ΔCt) values were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). For each target gene, expression of the initial sample time was set as a baseline to compare relative to each time point.

### *Histological Examination*

Histological analysis of intestine followed as previously described (Blaufuss et al., 2019). Briefly, sections of dissected distal intestine were dehydrated in ethanol, flushed with xylene and paraffin embedded. Tissue from each section was thin sliced and stained with hematoxylin and eosin (H&E). We used a semi-continuous scoring system (ranging from 0 to 5) to evaluate goblet cell number, lamina propria thickness and cellularity, supranuclear vacuolization, mucosal fold height, granulocyte infiltration and sub-epithelial mucosa appearance in the intestine. A score of 0–1 represented normal variation in morphology and scores > 2 indicating morphological changes consistent with increasing severity of intestinal inflammation. Scores above 4 indicate severe disruption of normal histological appearance and significant distal enteritis. Slides were prepared at the Bozeman Fish Health Center and scored at the Hagerman Fish Culture Experiment Station using light microscopy (Zeiss Axioscope A1, Carl Zeiss Ltd, Cambridge, UK). All scoring was blinded to treatment and consisted of combined average scores of 10 fields from 5 random individuals from each treatment (n=50 scored observations / treatment).

### *Statistical Analysis*

The growth performances were using tank as unit and analyzed with one-way ANOVA and gene expression were analyzed with two-way ANOVA using R Studio® version 1.2.1335

(R Studio, Inc, Boston, MA), with Dunnett's *post-hoc* tests when significant differences were observed. Significance was defined at $\alpha < 0.05$ for all statistical tests.

Statistical analysis of gut histology scores used the nonparametric, Mann-Whitney Rank Sum Test, with a Yates continuity correction (Sigmaplot 14.0, Systat Software, Inc. San Jose, CA) to evaluate differences in histological scores with significance assigned at $\alpha < 0.05$.

**Results**

There were no detectable differences between the two experimental groups in final body weight, feed intake, FCR, SGR, and body composition (Table 5). Survival was 100% in both groups.

In the distal intestine, expression of the *S100I2* gene showed no detectable difference between treatments at all time points, but week 8 showed significant differences by time (Fig. 6A). Expression of *S100V2* in the intestine was significant at weeks 8 and 12 by diet, and at week 4 by time (Fig. 6B). Expression of *CD8β* and *IL-17A* were observed with significant upregulation in fish intestine fed the PM diet compared to that for fish fed the FM diet at weeks 4 but not 8 or 12 (Fig. 6D and F). Over time, *IL-1β* expression showed upregulation, peaking at week 4 (Fig. 6E). There were no detectable differences in expression of *CD4* in the intestine in fish fed PM or FM diets among all time points (Fig. 6C).

In the liver, expression of *S100I2* was elevated over time in both FM and PM diets and expression at week 12 was significantly higher, about 200%, more than that at week 4 (Fig. 7A). On the contrary, expression of *S100V2* was downregulated in the liver with both the FM and PM diet fed fish over time with significantly lowest at week 12 (Fig. 7B). In the liver, expression of *CD4* was upregulated in both the FM and PM diet fed fish among all time points, but *CD8β*

expression was downregulated (Fig. 7C and D). Expression of *IL-10* in the liver was upregulated at weeks 8 and 12 with both FM and PM diets (Fig. 7E). The diet effect was only significant in the *IL-17A* expression at week 4, nevertheless, the expression of *IL-17A* was upregulated from 2.6 to 5-fold among all time points in both FM and PM diet fed fish (Fig. 7F).

Comparison of histological scores of distal intestines between the FM and SBM groups indicated evidence of moderate distal enteritis at 12 weeks in the SBM diet group. Combined semiquantitative scores in the FM dietary group averaged 1.1 ± 0.2 compared to 2.9 ± 0.4 in the SBM diet group ($P<0.001$, $U = 879.3$; Table 6).

**Discussion**

S100 proteins are members of the calcium-binding, cytosolic protein family involved in the regulation of cell apoptosis, proliferation, differentiation, migration, calcium balance, protein phosphorylation, and inflammation (Potts et al., 1995; Xia et al., 2018). Besides calcium, some S100 proteins also bind copper and zinc to regulate cellular activity (Chazin, 2011; Foell et al., 2007). S100 proteins are also involved in the anti-infectious host defense in extracellular functions (Cole et al., 2001; Moroz et al., 2003) and inflammatory responses in intracellular functions (Foell et al., 2007; Marenholz et al., 2004).

Previous studies have found S100 proteins in the skin and mucus membranes of Atlantic Salmon and have identified the S100I2 protein as ictacalcin (Easy and Ross, 2009). The *S100I2* gene was upregulated in the distal intestine of rainbow trout affected by a high SBM diet (Blaufuss et al., 2019, 2020). In this study, the expression of *S100I2* and *CD4* in the distal intestine of Atlantic Salmon showed no detectable between FM and PM diets over time; however, difference on expression of *S100I2* was observed over time (Fig. 6A and C). However,

*S100V2* expression in fish fed a PM diet showed higher expression in the distal intestine at weeks 4 and 8 than fish fed with PM, and fish showed significantly lower expression by week 12 (Fig. 6B). Expression of *S100V2* was also observed elevated in the distal intestine of RBT when fed a high-SBM diet (Blaufuss et al., 2019).

The expression of *CD4* in the distal intestine was downregulated in both FM and PM diets at three sampling time points (Fig. 6C). On the contrary, Bakke-McKellep et al., (2007a) found the expression of *CD4* elevated at week 3 in salmon after being fed on an SBM diet. Moreover, expression of *CD4* was increased overtime in the first to third week after salmon were fed a SBM diet (Marjara et al., 2012). Expression of *CD8β* was significantly higher in fish fed on a PM than FM diet at week 4 in the distal intestine (Fig. 6D), and is consistent with previous studies in SBM-fed salmon (Marjara et al., 2012). By contrast, Lilleeng et al., (2009) found the expression of *CD8β* was lower at the third day after salmon fed with SBM, which might have been related to the depletion or damage of epithelial and T-cells with exfoliation of intestine folds (Bakke-McKellep et al., 2007b). The inflammatory reaction may use activation of native T-cells in the intestine or recruit from other immune tissues through blood instead of proliferation of the cells that might result in low transcriptional changes in *CD4* and *CD8β* (Marjara et al., 2012). In the distal intestine, expression of *IL-1β* was downregulated (Fig. 6E) with a similar pattern observed in a previous study where salmon reacted at 7 day after being fed an SBM diet (Lilleeng et al., 2009). *IL-17A* gene expression showed significant in the distal intestine of salmon fed on PM at week 4, but not in week 8 and 12 (Fig. 6F). On the contrary, Marjara et al., (2012) observed significantly elevated expression of *IL-17A* gene in the first to third week after salmon fed an SBM diet. On expression of *IL17A* showed non-significant or downregulation at weeks 8 and 12, this may due to the adaptation and oral tolerance even the

size or strain different, which our salmon showed less sensitive to SBM. The *IL-10* gene expression was downregulated (Fig. 6G) and similar to a previous study by Marjara et al., (2012) when salmon were fed for 3 weeks with a diet containing SBM.

In the liver, expression of *S100I2* was elevated over time and peaked at week 12 in both FM and PM diets (Fig. 7A). On the contrary, the expression of *S100V2* was downregulated over time in both FM and PM diets and significantly lower at week 12 (Fig. 7B). In previous studies, Rainbow trout fed for 12 weeks with diets containing SBM showed more elevated expression of *S100I2* in the distal intestine and more elevated expression of *S100V2* in the liver (Blaufuss et al., 2019, 2020). In the liver, *CD4* and *IL-17A* expression was upregulated among all time and diets but not *CD8β* expression (Fig. 7C and D). The liver in salmon reacting to a SBM-rich diet showed downregulation of metabolic functions, specific to lipid metabolism (De Santis et al., 2015; Skugor et al., 2011), and lower expression of immune related gense but higher cell proliferation and apoptosis processes (Tacchi et al., 2012). The complex mechanisms of T-cell activation and reaction to SBM-induced inflammation in salmonids needs further investigation.

In this experiment, we observed an inflammatory response in Atlantic Salmon induced by a 40% SBM diet in fresh water. Overall *S100* gene expression was similar in part to the pattern observed in RBT in the gut (Blaufuss et al., 2019; 2020). Expression of T-cell gene markers in the distal intestine of Atlantic Salmon, *CD8β* and *IL-10* showed similarity with previous studies (Bakke-McKellep et al., 2007; Lilleeng et al., 2009; Marjara et al., 2012). In contrast, the T-cell gene markers, *IL-1β*, *IL-17A* and *CD4* had different responses than previously observed in the in distal intestine (Bakke-McKellep et al., 2007; Krogdahl et al., 2015; Marjara et al., 2012). These previous studies cultured Atlantic Salmon in seawater with initial weights ranging from 172g to 600g. Thus, these studies differ not only from the freshwater environment used in our

study but also the use of larger, older fish. In RBT, exposure to plant-based diets affected metabolism, acceptance and utilization at later life stages (Balasubramanian et al., 2016; Geurden et al., 2013). Therefore, the period of time exposed to dietary SBM might also result in differing responses in salmonids of differing age, but more evidence is needed to confirm this.

The function of the gastrointestinal tract in fish is to not only process food but also osmoregulation (Bucking and Wood, 2006; Taylor and Grosell, 2006). Another important function is calcium and magnesium uptake in salmonids (Bucking and Wood, 2007). Moreover, the intestine of salmonids grown in seawater fish contributes highly to total calcium uptake from drinking and dietary intake (Gregório and Fuentes, 2018). Salinity has been shown to negatively affect nutrient digestibility in Nile tilapia, *Oreochromis niloticus* (Tran-Ngoc et al., 2017), and affect osmoregulation and the ratio of intestinal nutrient transporters to total nutrient absorption in salmonids (Nordrum et al., 2000). The S100 proteins might also be affected by salinity because of their relevance to calcium sequestration and transport. However, the interaction between SBM and salinity in salmonids remains unclear. The mechanism of ion-relative transportation related to functions such as nutrient acquisition and osmoregulation needs further research to characterize responses to salinity and dietary changes.

**Conclusions**

To summarize, we examined expression of *S100* and inflammatory-related genes in the liver and distal intestine of Atlantic Salmon fed for 12 weeks on either a FM or PM diet. The expression of *S100I2* in liver was more highly expressed than expression of *S100V2*. In contrast, *S100V2* expression in the distal intestine was higher in fish fed PM diet. Both *S100I2* and

*S100V2* may be interesting targets for further study. In general. these gene expression data provide further information on SBM inflammatory responses and S100 proteins in salmonids.

## Chapter 4: Future Research

In the first study, responses from strains of RBT were surprisingly different. Genetic selection of the CX strain on a plant-based diet showed superior growth performance over RBT1, RBT2 and RBT3, which were only selected for growth. Even though the CXS and CXA groups were the same strain and same cohort, the CXS and CXA showed significant differences in gene expression on the same diet at the same time points which indicates care should be taken when comparing groups or strains of fish that have significantly different growth rates. The commercial strain, RBT1, RBT2, and RBT3, showed no difference in growth performance but did show differences in gene expression on the same diet at the same time points. Further research will need to investigate feeding trial time and multiple time point sampling on different strains of RBT response to an SBM diet with other inflammation-related gene markers and ROS pathway gene expression. The relationship between selected strain genetics and mechanisms preventing or reducing inflammation requires much more research to fully understand. In general, the reasons for reduced inflammation to dietary SBM in any selected RBT are worthy of further investigation. Moreover, the age of fish and timing of sampling need further study and must always be taken into consideration when doing comparative studies.

With Atlantic Salmon, expression in immune-related genes showed a similar pattern to previous studies. Still, further research is needed to fully characterize the immune-relevant genes linked to inflammation due to dietary SBM. As noted, previous studies of *S100* genes in fish were limited. These studies observed *S100* gene expression in both RBT and Atlantic Salmon in response to an SBM diet. These data provide further evidence for the involvement of *S100* genes related to SBM-induced intestinal inflammation in salmonids in freshwater. However, how calcium-binding proteins in salmonids react in a marine environment when

challenged with a diet containing a high inclusion rate of SBM needs further study. We have a basic understanding of SBM replacement in the salmonid diet but have no information regarding potentially adverse effects on cellular calcium-binding when calcium and other minerals are being actively taken up in the gut in a saltwater environment.

For the overall sustainability of aquaculture in the future, additional studies are necessary to investigate different, alternative proteins sources to replace or reduce FM use in the diets of intensively fed fish.

## Tables

**Table 1**. Feed formulations (g/100g dry weight) and proximate composition of control (0% soybean meal) and experimental diets (40% soybean meal) used in 12 week, comparative feeding trial of Rainbow Trout strains and Atlantic Salmon.

| Ingredient | Fishmeal(FM) | Plant meal(PM) |
|---|---|---|
| Menhaden Fish Meal[a] | 30.00 | 0.00 |
| Soybean meal[b] | 0.00 | 40.00 |
| Chicken meal[c] | 11.50 | 11.50 |
| Corn protein concentrate[d] | 11.50 | 11.50 |
| Menhaden fish oil[e] | 14.72 | 16.35 |
| Wheat gluten meal | 0.16 | 1.30 |
| Wheat flour | 23.29 | 5.54 |
| Lecithin | 1.00 | 1.00 |
| Stay-C 35[f] | 0.15 | 0.15 |
| Vitamin premix ARS 702[g] | 1.00 | 1.00 |
| TM ARS 640[h] | 0.10 | 0.10 |
| NaCl | 0.28 | 0.28 |
| Magnesium Oxide | 0.06 | 0.06 |
| Potassium chloride | 0.56 | 0.56 |
| Monocalcium phosphate | 0.60 | 4.80 |
| Choline chloride 50% | 1.00 | 1.00 |
| DL-Methionine | 0.33 | 0.63 |
| Lysine HCl | 2.17 | 2.56 |
| Threonine | 0.40 | 0.49 |
| Taurine | 1.00 | 1.00 |
| Yttrium oxide | 0.10 | 0.10 |
| Astaxanthin | 0.08 | 0.08 |
| TOTAL | 100.00 | 100.00 |

| Proximate analysis (analyzed) | | |
|---|---|---|
| Protein (%DM) | 46.7 | 48.0 |
| Lipid (%DM) | 18.7 | 17.6 |
| Energy (cal/g DM) | 5268.1 | 5280.9 |

[a] Menhaden Special Select, Omega Proteins, Reedville, VA, USA.

[b] Archer Daniels Midland Co., St. Louis, MO, USA.

[c] American Dehydrated Foods, Springfield, MO, USA.

[d] Cargill, Inc., Empyreal 75, Blair, NE, USA.

[e] Omega Proteins, Inc., Virginia Prime, Reedville, VA, USA.

[f] DSM Nutritional Products, Basel, Switzerland.

[g] Provides per kg diet before processing: vitamin A, 9560 IU; vitamin D, 6600 IU; vitamin E, 132 IU; vitamin K3, 1.1 mg; thiamin mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate DL-calcium, 46.5 mg; cyanocobalamin, 0.03 mg; nicotinic acid, 21.8 mg; biotin 0.34 mg; folic acid, 2.5 mg; inositol, 600mg.

[h] Contributed in mg $kg^{-1}$ of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4.

**Table 2.** Primer sequences used for RT-qPCR gene expression in a 12 week, comparative feeding trial of Rainbow Trout strains.

| Gene | Accession Number* | Primer Sequence |
|---|---|---|
| *SOD*[1] | NM_001124329 | *f. GGC ACG AGG GCA AGT AGG A* |
| | | *r. GCC TTT GAG CAC GCA AAC A* |
| *GPx*[2] | NC_048571.1 | *f. CGC CCA CCC ACT GTT TGT* |
| | | *r. GCT CGT CGC TTG GGA ATG* |
| *S100I2*[3] | XM_021598338.1 | *f. GCT TGG AGA GAT CAT GGG GAA AA* |
| | | *r. GCC ATC TGA GTT AGC GTC CA* |
| *S100V2*[4] | XM_021572132.1 | *f. TTA CGA CTG GAG CGT CAG A* |
| | | *r. CCT CCA GAA GTG ATT GAA GGT G* |
| *Catalase* | NC_048566.1 | *f. GGC TTT GCA GTT AAG TTC TAC* |
| | | *r. AGC ATT GCG TCC CTG ATA AA* |
| *RPS-15*[5] | NM_001165174.2 | *f. ACA GAG GTG TGG ACC TGG AC* |
| | | *r. AGG CCA CGG TTA AGT CTC CT* |

* NCBI Database; [1] superoxide dismutase; [2] glutathione peroxidase; [3] Calcium binding protein S100I2; [4] Calcium binding protein S100V2; [5] Ribosomal protein S-15

**Table 3.** Growth and feeding performance of Rainbow Trout strains fed a fishmeal or plant meal diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different superscripts indicate significant differences among strains within diets.

| Diet | Strain | Initial Mass (g) | Final Mass (g) | Weight Gain (%) | SGR[1] | Feed Intake[2] | FCR[3] |
|---|---|---|---|---|---|---|---|
| Fishmeal | CXS | 7.47 | $128.83^{b}$ | $1624^{b}$ | $3.37^{b}$ | 1.71 | 0.96 |
| | CXA | 7.87 | $194.95^{a}$ | $2377^{a}$ | $3.80^{a}$ | 1.46 | 0.76 |
| | RBT1 | 4.88 | $131.50^{b}$ | $2592^{a}$ | $3.91^{a}$ | 1.70 | 0.80 |
| | RBT2 | 7.25 | $137.66^{b}$ | $1798^{b}$ | $3.50^{b}$ | 1.61 | 0.86 |
| | RBT3 | 7.27 | $137.54^{b}$ | $1790^{b}$ | $3.49^{b}$ | 1.74 | 0.85 |
| Plant meal | CXS | 7.47 | $152.38^{x}$ | $1939^{xy}$ | $3.58^{x}$ | $1.73^{xy}$ | 0.91 |
| | CXA | 7.87 | $155.09^{x}$ | $1870^{yz}$ | $3.54^{xy}$ | $1.52^{xy}$ | 0.76 |
| | RBT1 | 4.88 | $120.28^{y}$ | $2362^{x}$ | $3.80^{x}$ | $1.64^{xy}$ | 0.88 |
| | RBT2 | 7.25 | $116.28^{y}$ | $1503^{yz}$ | $3.28^{y}$ | $1.48^{y}$ | 1.05 |
| | RBT3 | 7.27 | $112.74^{y}$ | $1449^{z}$ | $3.24^{y}$ | $1.92^{x}$ | 0.99 |
| Pooled SEM | | 0.53 | 7.66 | 123.5 | 0.07 | 0.04 | 0.03 |
| *P* | Strain | —— | <0.001 | <0.001 | <0.001 | 0.006 | 0.270 |
| | Diet | —— | 0.005 | 0.007 | 0.008 | 0.780 | 0.250 |
| | S × D | —— | 0.004 | 0.014 | 0.007 | 0.570 | 0.720 |

[1] Specific Growth Rate, = $100 \times (\ln W_f - \ln W_i) / t$; $W_f$: final fish body weight, $W_i$: initial fish body weight, and t: total number of days of feeding

[2] Feed Intake, = g dry feed consumed / average fish mass (g) / culture days

[3] Feed Conversion Ratio, = g dry feed consumed / g wet weight gained

**Table 4.** Primer sequences used for RT-qPCR gene expression in a 12 week, comparative feeding trial of Atlantic Salmon.

| Gene | Accession Number* | Primer Sequence |
|---|---|---|
| *CD4* [1] | NM_001146408 | *f. GAG TAC ACC TGC GCT GTG GAA T* |
| | | *r. GGT TGA CCT CCT GAC CTA CAA AGG* |
| *CD8β* [2] | AY693394 | *f. CGC ACA CAC CTC AAC AAC TC* |
| | | *r. ATT GAT GCG CAG TGT GAA AG* |
| *S100I2* [3] | XM_021598338.1 | *f. GCT TGG AGA GAT CAT GGG GAA AA* |
| | | *r. GCC ATC TGA GTT AGC GTC CA* |
| *S100V2* [4] | XM_021572132.1 | *f. TTA CGA CTG GAG CGT CAG A* |
| | | *r. CCT CCA GAA GTG ATT GAA GGT G* |
| *IL-1b* [5] | AY617117 | *f. GCT GGA GAG TGC TGT GGA AGA* |
| | | *r. TGC TTC CCT CCT GCT CGT AG* |
| *IL-10* [6] | EF165028 | *f. CGC TAT GGA CAG CAT CCT* |
| | | *r. AAG TGG TTG TTC TGC GTT* |
| *IL-17A* [7] | KJ921980.1 | *f. TGG TTG TGT GCT GTG TGT CTA TGC* |
| | | *r. TTT CCC TCT GAT TCC TCT GTG GG* |
| *Hprt* [8] | BT043501 | *f. CCG CCT CAA GAG CTA CTG TAA T* |
| | | *r. GTC TGG AAC CTC AAA CCC TAT G* |

* NCBI database; [1] cluster of differentiation 4; [2] cluster of differentiation 8β; [3] Calcium binding protein S100I2; [4] Calcium binding protein S100V2; [5] Interleukin 1β; [6] Interleukin 10; [7] Interleukin 17A; [8] Hypoxanthine guanine phosphoribosyl transferase

**Table 5.** Growth, feeding performance, and proximate composition of Atlantic Salmon after being fed 12 weeks on a fishmeal (FM) or plant meal (PM) diet (Table 1).

| | Initial Mass (g) | Final Mass (g) | Feed Intake (g/d) | FCR[1] | SGR[2] |
|---|---|---|---|---|---|
| FM | 71.35 ± 0.87 | 205.39 ± 23.77 | 0.20 ± 0.02 | 1.05 ± 0.08 | 1.25 ± 0.12 |
| PM | 71.15 ± 0.64 | 211.12 ± 14.02 | 0.18 ± 0.01 | 0.98 ± 0.05 | 1.29 ± 0.08 |
| | Moisture (%) | Fat (%) | Protein (%) | Energy (cal/g) | HSI[3] |
| FM | 71.09 ± 0.22 | 28.94 ± 0.58 | 63.41 ± 0.57 | 6298 ± 25.09 | 0.77 ± 0.02 |
| PM | 70.78 ± 0.59 | 29.77 ± 0.80 | 62.68 ± 0.88 | 6301 ± 24.52 | 0.82 ± 0.09 |

[1]Feed conversion ratio; [2]Specific Growth Rate, = $100 \times (\ln W_f - \ln W_i) / t$; $W_f$: final fish body weight, $W_i$: initial fish body weight, and t: total number of days of feeding; [3]Hepatosomatic Index, = liver mass x 100 / fish mass

**Table 6.** Histology of combined semiquantitative scores with Standard Error of the Means (SEM) in the Atlantic Salmon after 12 weeks fed on fishmeal or plant meal diet. Asterisks mark significant differences ($P < 0.001$, $U = 879.3$) between diet treatment groups.

| | Fishmeal | Plant meal |
|---|---|---|
| Semiquantitative scores[1] | 1.1 ± 0.2 | 2.9 ± 0.4* |

[1] Observation of goblet cell number, lamina propria thickness and cellularity, supranuclear vacuolization, mucosal fold height, granulocyte infiltration and cub-epithelial mucosal appearance in the intestine from (Blaufuss et al., 2019).

## Figures

**Figure 1.** Comparative fold change of gene expression in distal intestine of Rainbow Trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different superscripts indicate significant differences among strains within diets. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).

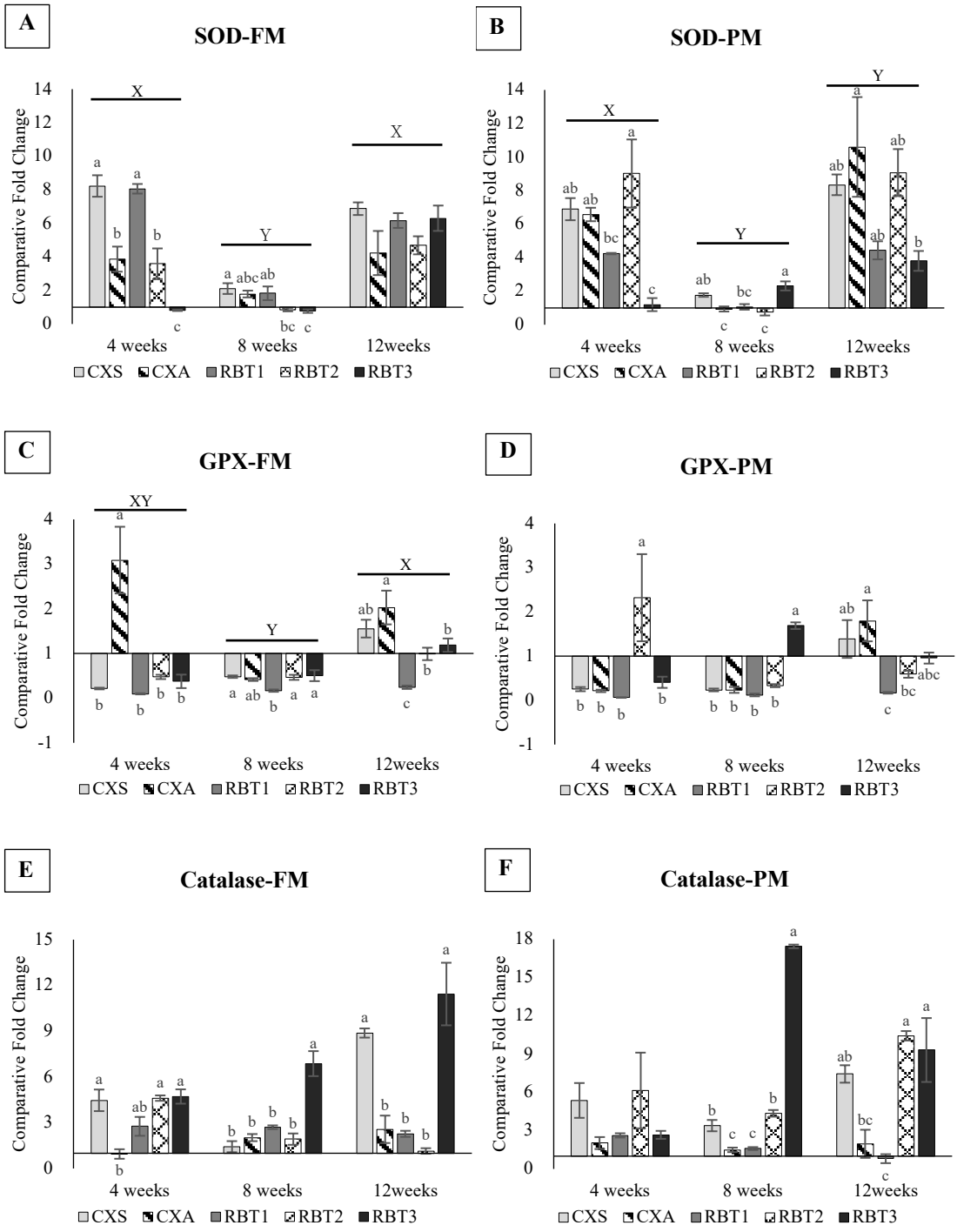


**Figure 2.** Comparative fold change of gene expression in the liver of Rainbow Trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different superscripts indicate significant differences among strains within diets. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).

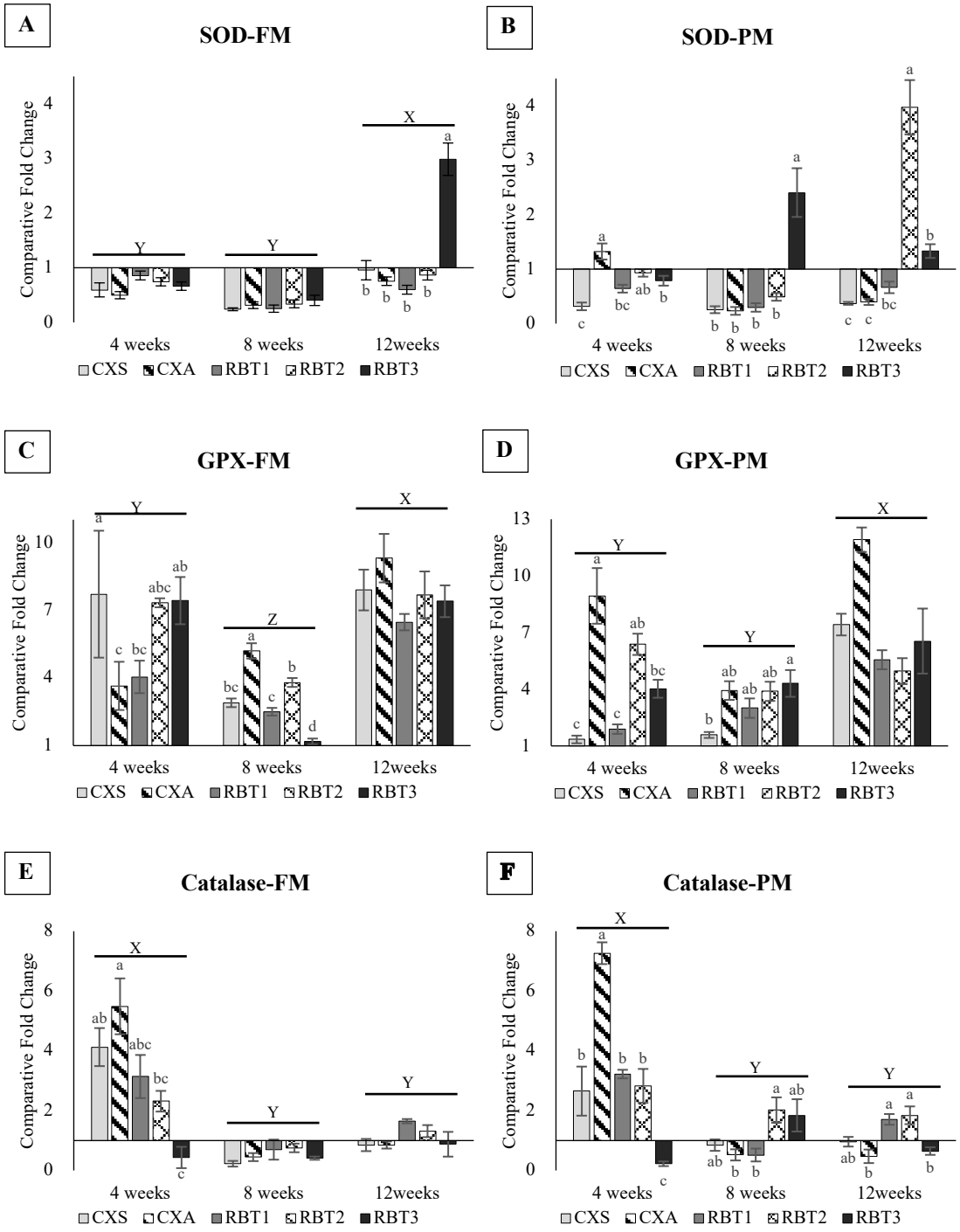


**Figure 3.** Comparative fold change of *S100I2* gene expression in the intestine of Rainbow Trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different superscripts indicate significant differences among strains within diets. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).

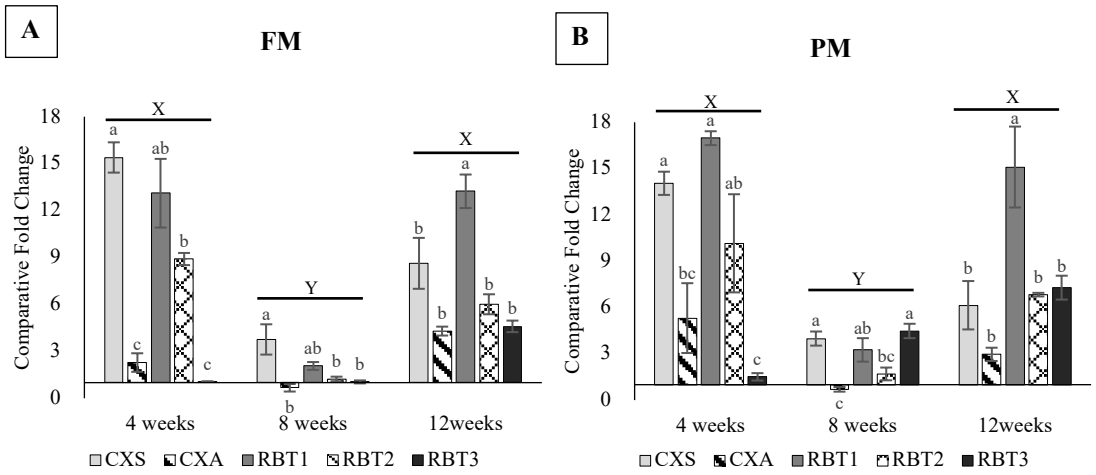


**Figure 4.** Comparative fold change of *S100V2* gene expression in the liver of Rainbow Trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different superscripts indicate significant differences among strains within diets. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).

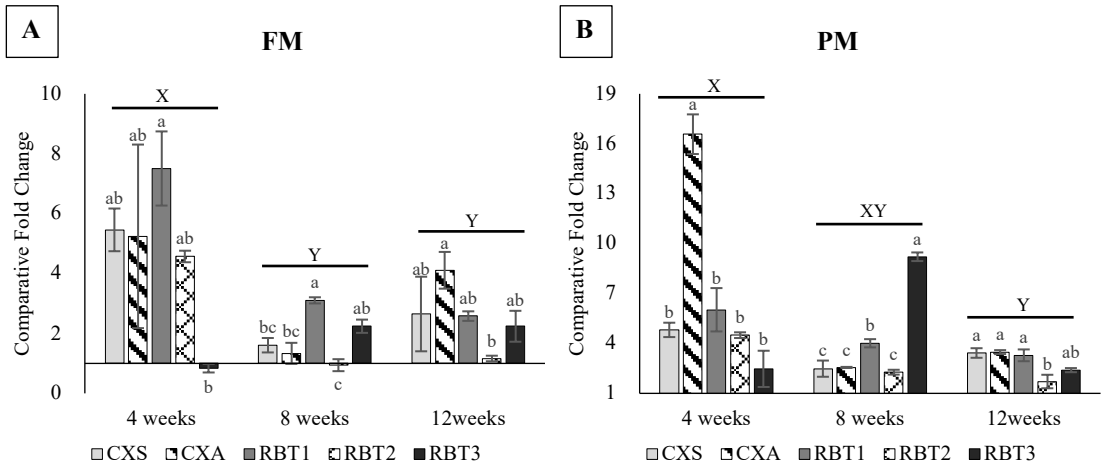


**Figure 5.** Cellular antioxidant defense against reactive oxygen species. [1] SOD: superoxide dismutase; [2] GPx: glutathione peroxidase; [3] CAT: catalase,

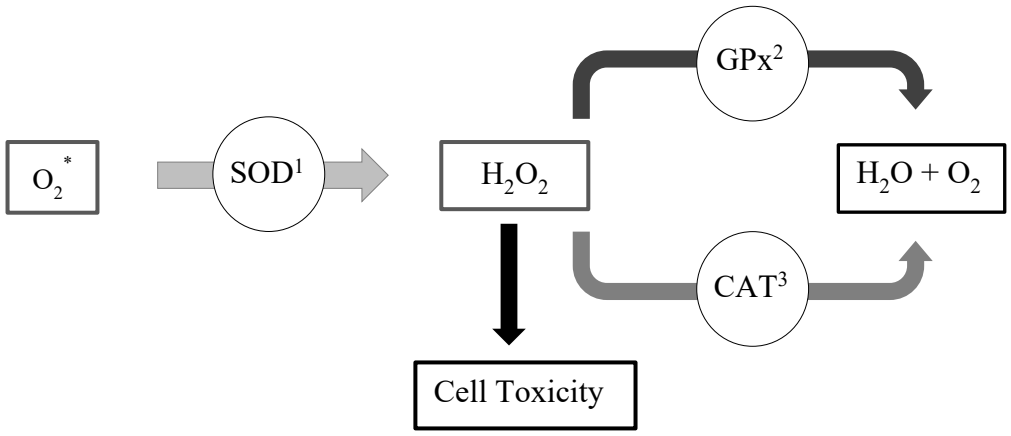


**Figure 6.** Fold change of gene expression in the distal intestine of Atlantic Salmon after being fed on Fishmeal or Plant meal for 12 weeks. Asterisks mark significant differences ($\alpha < 0.05$) between diet treatment groups. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).

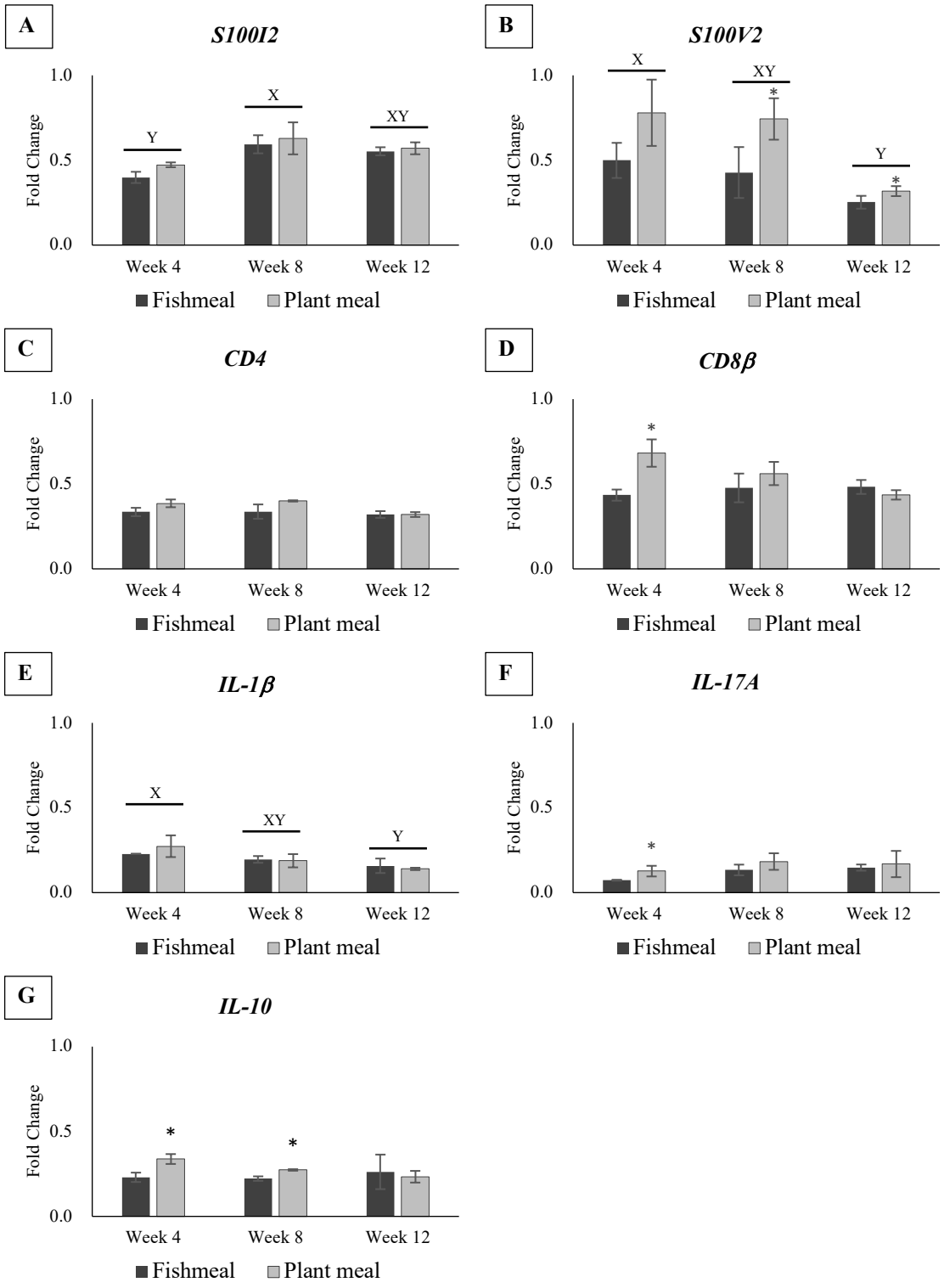
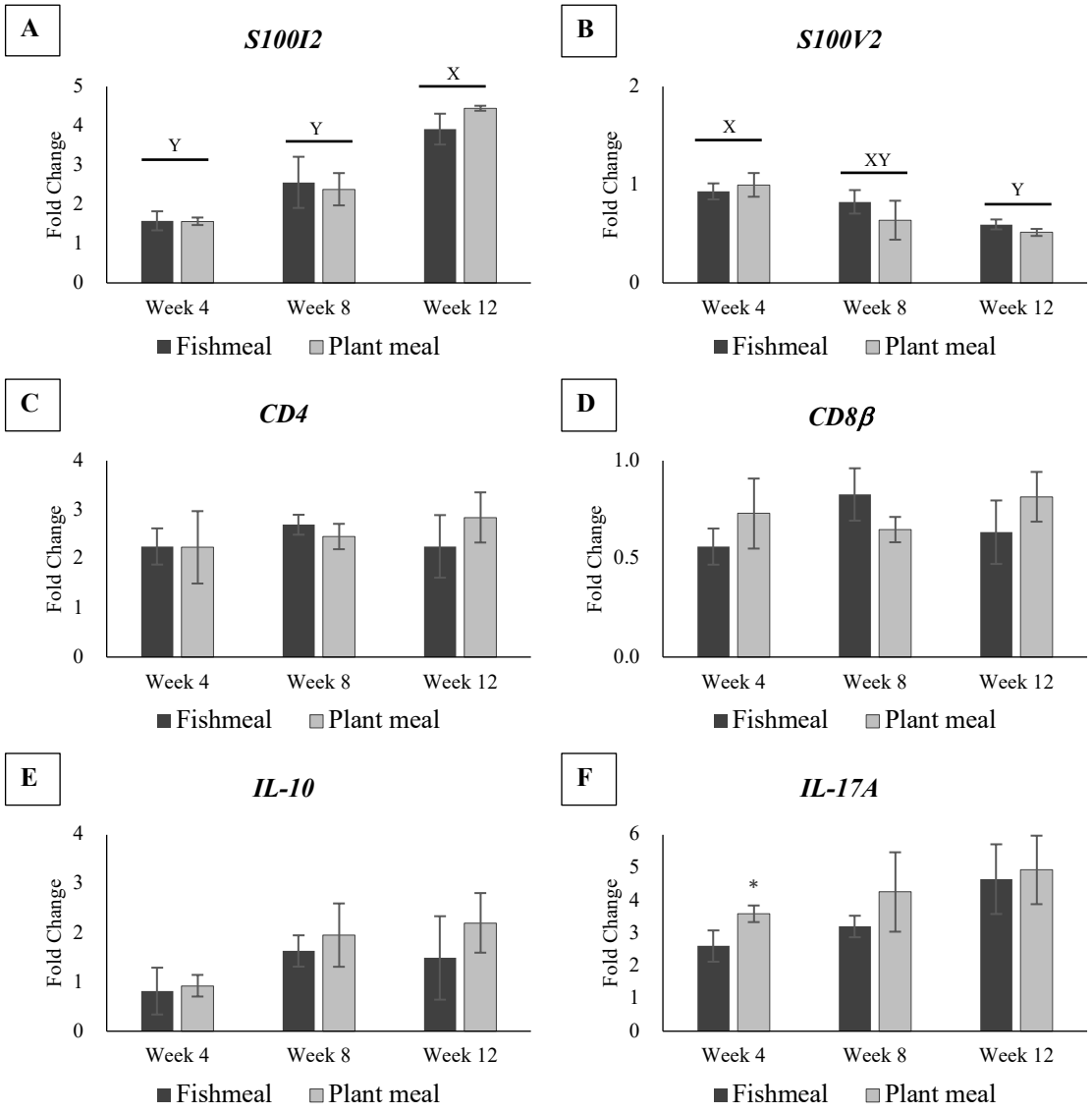


**Figure 7.** Fold change of gene expression in the liver of Atlantic Salmon after being fed on Fishmeal or Plant meal for 12 weeks. Asterisks mark significant differences ($\alpha < 0.05$) between diet treatment groups. Horizontal bars indicate significant differences between time points. Vertical bars indicate Standard Error of the Means (SEM).



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