Feed-Based Protocols for Mitigating Soybean Meal-Induced Enteritis (SBMIE) in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

As the world population continues to show exponential growth, wild fish stocks are becoming stagnant causing a shortfall in capture fisheries and increasing the price of fish meal as a source of protein. Thus, different combinations of raw materials, including plant-derived ingredients, need to be considered to fulfil the increasing demands of the aquaculture industry and maintain cost-effectiveness. Soybean meal became a preferred source of protein in aquafeeds, however, it has typically encountered a variety of challenges at high inclusion levels, especially in piscivorous and carnivorous species like rainbow trout (*Oncorhynchus mykiss*). Despite the adequate protein content and amino acid profile of SBM, the presence of antinutritional factors such as phytate, saponins, lectins, etc., has historically led to significant repression of growth performance. In addition, the inflammation of the distal intestine, referred to as soybean meal-induced enteritis (SBMIE), is a noteworthy sign of reduced tolerance to SBM, and it is related to poor nutrient absorption in the intestine, ultimately impacting other organs such as the liver. Nowadays, the use of feed additives in fish feed formulations has become a popular approach to enhance feed utilization while providing additional benefits, including affordability of the feed, and boosting immunity. Attenuation of SBMIE will make it possible to increase SBM inclusion levels in practical formulations while maintaining the health status of farmed fish and reduce risk for disease.

The overall goal of this thesis was to gain insight into the evolution of SBMIE in the long-term and attempted to identify biomarkers associated to SBMIE. In addition, this research evaluated the suitability of glutamine, bile acids and vitamin D as dietary supplements in mitigating said pathology in rainbow trout. Three feeding trials were carried out to study each additive over a period of thirty, eighteen, and ten weeks, respectively. This research further aimed to investigate the potential detrimental effects that either a prolonged exposure to dietary bile acids or extra high vitamin D doses (i.e., > 10 mg Kg^{-1}) could have on this species.

Results from this work confirmed the occurrence of SBMIE when feeding SBM at 30% and 40% inclusion levels and that these effects are maintained throughout >18 weeks. In addition, the supplementation of high level SBM diets with either 2.0% glutamine or 1.5% bile acids had the potential to reduce intestinal inflammation. The research detailed herein also demonstrates the capacity of dietary bile acids in reverting hypocholesterolemia derived from 40% level SBM diets. Moreover, no apparent bile-associated cytotoxicity was detected over the long-term, further validating bile acids as an additive for use in aquafeeds. A significant increase in growth performance was observed from the supplementation with 2.0% glutamine in combination with vitamin D. The data

obtained here are, however, insufficient to determine the potential benefits derived solely from dietary vitamin D.

Future research will be required to gain in-depth understanding of molecular markers that can shed light on underlying SBMIE mechanisms, which will allow continuing improvement of mitigation approaches as well as developing monitoring tools that can be used in farm operation.

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Dedication

Gracias a todos aquellos que han estado apoyándome con los retos afrontados durante los últimos cuatro años. Gracias, Papá y Mamá, que me lo habéis dado todo; y Pablo, nunca hubiera conseguido llegar aquí si no fuera porque vosotros siempre habéis creído en mí. Gracias a los cotorros por hacer mi vida en el Palouse algo que recordaré siempre con inmenso cariño. Y gracias a mis amigas: Cristina, Esther, Nuria, Natalie y Laura. Todas habéis sido un apoyo fundamental en las diferentes fases de este capítulo de mi vida.

English translation

I would like to thank all who have been supporting me during the challenges faced over the last four years. Thank you Mum and Dad, because you have given me everything; and Pablo, I would never have gotten to this point without having you by my side, believing in me. I thank my Pullman friends, for making my life in the Palouse something I will be always extremely fond of. And thank you my friends: Cristina, Esther, Nuria, Natalie and Laura. Your support has been fundamental in the different phases of this chapter in my life.

Table of Contents

ABSTRACT……ii

ACKNOWLEDGMENTS……iv

DEDICATION……v

LIST OF TABLES……xii

LIST OF FIGURES……xiv

LIST OF ABBREVIATIONS……xvi

STATEMENT OF CONTRIBUTION……xix

CHAPTER 1: REVIEW OF LITERATURE……1

SOYBEAN MEAL USE IN AQUAFEED……1

Introduction……1

THE GASTROINTESTINAL TRACT OF FISH……4

Morphology and function……4

Immune function……6

SOYBEAN MEAL-INDUCED ENTERITIS……8

Intestinal inflammatory mechanisms……8

SBMIE……10

FEED ADDITIVES AND FUNCTIONAL FEEDS……13

Introduction……13

Glutamine……15

Bile acids……18

Vitamin D……21

Additive interactions……26

Bile acids, vitamin D and vitamin A……26

CONCLUSION AND OBJECTIVES……28

REFERENCES……29

CHAPTER 2: EVALUATING LONG TERM EFFECTS OF SOYBEAN MEAL-INDUCED ENTERITIS ON GROWTH AND HISTOLOGY OF TWO RAINBOW TROUT (*Oncorhynchus mykiss*) STRAINS AND THE POTENTIAL MITIGATING EFFECTS OF THE NON-ESSENTIAL AMINO ACID GLUTAMINE…….....66

ABSTRACT…….....67

INTRODUCTION…….....68

MATERIALS AND METHODS…….....70

Experimental diets…….....70

Long-term feeding trial and sampling…….....70

Performance indices…….....71

Proximate composition of experimental diets and fish whole-body; and amino-acid composition of diets…….....71

Histopathology of distal intestine…….....72

Statistical analysis…….....73

RESULTS…….....73

Growth performance…….....73

Proximate composition of fillets …….....74

Histopathology of distal intestine…….....75

DISCUSSION…….....76

Growth performance and feed utilization…….....76

Nutritional composition of fish fillet…….....78

Histopathology of distal intestine…….....78

Commercial strains and adaptation to soybean meal…….....80

CONCLUSION…….....81

REFERENCES…….....83

CHAPTER 3: GLUTAMINE SUPPLEMENTATION OF SOYBEAN MEAL DIETS: EFFECTS ON GENE EXPRESSION, ANTIOXIDANT METABOLISM AND AMINO ACID COMPOSITION IN TWO STRAINS OF RAINBOW TROUT (*Oncorhynchus mykiss*) OVER A LONG-TERM FEEDING TRIAL…….....105

ABSTRACT…….....106

INTRODUCTION…….....107

MATERIALS AND METHODS……..…….…..…108

Experimental diets……..……..….....108

Fish rearing conditions and sampling…….…….....109

Glutamate quantification…….……....110

Glutathione peroxidase activity…….…….....110

RNA extraction, cDNA synthesis and real-time PCR…….…....111

Statistical analysis……...…....112

RESULTS……..……...……...112

Glutamate quantification in distal intestine……...….112

Glutathione peroxidase enzymatic activity in distal intestine……..…112

Gene expression of SBMIE markers and SLC1A5…….……..…113

DISCUSSION……..…….....114

Glutamate quantification and glutathione peroxidase activity…….……...114

Cytokine gene expression…….……..…..116

Tight junction, myosin light chain kinase and glutamine transporter gene expression...117

CONCLUSION…….……..…….....120

REFERENCES…….....…..121

CHAPTER 4: THE POTENTIAL ROLE OF BILE ACIDS IN SOYBEAN MEAL-INDUCED ENTERITIS MITIGATION IN RAINBOW TROUT (*Oncorhynchus mykiss*) FED TWO LEVELS SBM DIETS OVER AN 18 - WEEK FEEDING TRIAL……...…137

ABSTRACT…….……...……...….138

INTRODUCTION……..…….……..…..139

MATERIALS AND METHODS…….……..…….....141

Experimental diets…….……...…141

Experimental design and sampling…….....……141

Growth, condition and feeding efficiency…….……...…...142

Proximate composition of feed and fish fillet samples…….....….....143

Liver and distal intestine histopathology…….…….....144

Statistical analysis……..……..….....….144

RESULTS……..……..……144

Growth performance……..……..……144

Proximate composition…….……...……145

Histopathology of distal intestine…….……...145

Histopathology of liver…….……...146

DISCUSSION…….…….....147

Growth performance……..…….…….…….....147

Histopathology of distal intestine…….…..…….149

Histopathology of liver…….……..…...…...150

CONCLUSION……..……...……152

REFERENCES……..……...……153

CHAPTER 5: LONG-TERM EFFECTS OF TWO LEVELS SOYBEAN MEAL DIETARY INCLUSION, WITH OR WITHOUT BILE ACID SUPPLEMENTATION, ON PLASMA BILE AND CHOLESTEROL, DIGESTA BILE AND GENE EXPRESSION IN LIVER AND INTESTINE OF RAINBOW TROUT (*Oncorhynchus mykiss*)……..…….170

ABSTRACT…….……..…….171

INTRODUCTION…….....……..……..……..172

MATERIALS AND METHODS…….……..…….174

Experimental diets…….……..…..…174

Fish rearing and sampling conditions…….…...….174

RNA extraction, cDNA synthesis and real-time qPCR……..…..175

Plasma bile and cholesterol levels……..……176

Proximal and distal intestine digesta bile quantification……...…..……...……176

Statistical analysis…….…….....…..…..177

RESULTS…….....….177

Plasma bile and cholesterol levels…….…….….177

Proximal and distal intestine digesta bile quantification…….…..…….....178

Gene expression analysis in distal intestine and liver……...…….178

DISCUSSION……..……..…..179

Bile quantification in plasma and digesta; and cholesterol quantification in plasma….179

Distal intestine gene expression…….……..…….....181

Hepatic gene expression……..…….…….184

CONCLUSION…….…....…..…..185

REFERENCES…….……...……....…..…187

CHAPTER 6: MITIGATION OF SOYBEAN MEAL-INDUCED ENTERITIS IN RAINBOW TROUT (*Oncorhynchus mykiss*) USING VITAMIN D AND GLUTAMINE AND DIETARY ADDITIVES……..….198

ABSTRACT…….…….....199

INTRODUCTION…….…….....200

MATERIALS AND METHODS…….…….……...202

Experimental diets…….…….....202

Experimental design and sampling……..…...…202

Growth performance parameters…….……...…….....203

Proximate composition of whole body and diets; and amino acid composition of diets.203

Calcium analysis……...……...204

Histopathology analysis of distal intestine……...…..…..205

RNA extraction, cDNA synthesis and real-time qPCR……....205

Statistical analysis…….…….…...…..206

RESULTS…….....206

Growth performance and whole-body proximate composition……206

Calcium analysis…...……...…….……207

Histopathology of distal intestine…….…….....207

Gene expression analysis…….....207

DISCUSSION……...…...208

Growth performance and proximate composition…….……..208

Calcium analysis…….…….…...210

Histopathology of distal intestine…….……...…...211

Gene expression……211

CONCLUSION……215

REFERENCES……216

CHAPTER 7: CONCLUSION……235

SYNTHESIS OF RESEARCH FINDINGS……235

GLUTAMINE EXPERIMENT……235

BILE ACIDS EXPERIMENT……236

VITAMIN D EXPERIMENT……237

GENE EXPRESSION OF SBMIE MARKERS……238

REFERENCES……240

List of Tables

Table 1.1. Crude protein composition and essential amino acid composition on dry matter (g Kg^{-1}) of soybean meal and other plant protein ingredients. Soybean meal and canola meal are solvent extracted; all other ingredients are heat extracted (Kumar 2011). 53
Table 1.2. Antinutritional factors (ANFs) found in soybean meal (SBM), their respective amounts and physiological effect. 53
Table 1.3. Summary of studies that investigated different effects of dietary soybean products and related soybean meal-induced enteritis (SBMIE) symptoms. 55
Table 1.4. Summary of studies that have investigated the effects of different dietary levels glutamine (Gln) for different time periods in commercial fish species. 59
Table 1.5. Summary of studies that have investigated the effects of dietary emulsifiers and taurine in different commercial species fed SBM-based diets. 61
Table 2.1. Dietary formulation and proximate composition of the three experimental diets fed to two commercial strains of rainbow trout for thirty weeks. 92
Table 2.2. Essential and non- essential amino acid (AA) profile of the three experimental diets. 93
Table 2.3. Growth performance and growth metrics. 94
Table 2.4. Muscle proximate composition of two commercial strains of rainbow trout after being fed three experimental diets for thirty weeks. 97
Table 3.1. The primer list and accession numbers of target and reference genes and efficiencies of the PCR reactions. 129
Table 4.1. Ingredients and proximate composition of five experimental diets expressed as percentages (%). 159
Table 4.2. Proximate amino acid composition of each experimental diet. 160
Table 4.3. Growth performance of rainbow trout fed five experimental diets over an eighteen-week feeding trial. 161
Table 4.4. Proximate composition of initial (W0) and final (W18) fillet samples (% wet weight), and nutrient retention (PR: protein retention, %; LR: lipid retention, %). 162
Table 5.1. Primer pair sequences, efficiency for the genes used for qPCR. 192
Table 5.2. Total BA levels in intestinal digesta collected from proximal and distal segments and reduction rates (%). Values presented are as means ± standard deviation (*n* = 12). 193
Table 6.1. Dietary formulation of the experimental diets expressed as percentages (%). 225
Table 6.2. Proximate amino acid composition of each experimental diet (g/100 g feed). 227

Table 6.3. Primer sequences used for each gene of interest in qPCR analyses. 228
Table 6.4. Growth performance parameters calculated at W5 and W10 of the feeding trial. .. 229
Table 6.5. Whole body proximate composition (% wet weight) of rainbow trout fed seven experimental diets at W10. 230
Table 6.6. Calcium (Ca^{2+}) concentrations measured in kidney, vertebra, and muscle (mg g-1 dry weight); and serum (nmol mL-1) from pooled samples from fish sampled at W10 of the feeding trial. 231

List of Figures

Figure 1.1. World capture fisheries and aquaculture (FAO, 2022). 63
Figure 1.2. Global fishmeal consumption by aquaculture sector in 2020. Source: IFFO 2020..... 63
Figure 1.3. Crop feed ingredients used in aquaculture feeds (Troell et al., 2014). 64
Figure 1.4. Schematic representation of enterocyte structure and shape including apical membrane transporters, tight junction proteins and actin cytoskeleton. 64
Figure 1.5. Comparison of endothelial barrier function in healthy enterocytes and loss of integrity in enterocytes in an inflammation context. 65
Figure 2.1. Distal intestine histopathology of rainbow trout after six weeks of feeding trial (W6). 98
Figure 2.2. Distal intestine histopathology of rainbow trout after twelve weeks of feeding trial (W12). 99
Figure 2.3. Distal intestine histopathology of rainbow trout after eighteen weeks of feeding trial (W18). 100
Figure 2.4. Distal intestine histopathology of rainbow trout after twenty-four weeks of feeding trial (W24). 101
Figure 2.5. Distal intestine histopathology of rainbow trout after thirty weeks of feeding trial (W30). 102
Figure 2.6. Cumulative histology scores at each 6, 12, 18, 24 and 30 weeks of experimental trial. 103
Figure 2.7. Villi length and width (µm) at each of 6, 12, 18, 24 and 30 weeks of experimental trial. 104
Figure 3.1. Glutamate concentration levels (µM) found in distal intestine enterocytes at W12, W18 and W30 of the feeding trial. 130
Figure 3.2. Measurement of glutathione peroxidase (GPx) activity (nmol/min/mL) found in distal intestine enterocytes at W6, W18 and W24 of the feeding trial. 131
Figure 3.3. Relative gene expression ratio of barrier integrity makers in distal intestine of rainbow trout strain R (A, C, E) and strain T (B, D, F) fed three experimental diets. 133
Figure 3.4. Relative gene expression ratio of immune makers in distal intestine of rainbow trout strain R (A, C, E, G) and strain T (B, D, F, H) fed three experimental diets. 135
Figure 3.5. Relative gene expression of neutral amino acids transporter in distal intestine of rainbow trout strain R (A, C, E) and strain T (B, D, F) fed three experimental diets. 136

Figure 4.1. Villi length (µm) (A) and width (µm) (B) of rainbow trout fed each of five experimental diets. 163
Figure 4.2. Cumulative histological scoring in distal intestine (A) and liver (B) of rainbow trout fed each of five experimental diets. 163
Figure 4.3. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after six weeks dietary exposure (W6). .. 164
Figure 4.4. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after twelve weeks dietary exposure (W12). 165
Figure 4.5. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after eighteen weeks dietary exposure (W18). 166
Figure 4.6. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after six weeks dietary exposure (W6). 167
Figure 4.7. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after twelve weeks dietary exposure (W12). 168
Figure 4.8. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after eighteen weeks dietary exposure (W18). 169
Figure 5.1. Plasma cholesterol (A) and BA (B) concentrations at each of three sampling points (W6, W12 and W18). 194
Figure 5.2. Distal intestine gene expression of the cytokines Tnfa, Il8, and Il10. Values are presented as means ± standard deviation (*n* = 12). 195
Figure 5.3. Distal intestine gene expression of the tight junction proteins Cldn12 and Ocln; the enzyme Mylk; and the bile acid transporter Slc10a2. Values are presented as means ± standard deviation (*n* = 12). 196
Figure 5.4. Hepatic gene expression of cholesterol and bile acid synthetic enzymes Hmgc and Cyp7a1. 197
Figure 6.1. Distal intestine relative gene expression of the cytokines Tnfa, Il1b, and Il10 of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9). 232
Figure 6.2. Distal intestine relative gene expression of the tight junction proteins Cldn12 and Ocln of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9). 233
Figure 6.3. Distal intestine relative gene expression of Mylk and Vdr of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9). 234

List of Abbreviations

ANF Antinutritional factor

ASBT Apical sodium dependent bile acid transporter

BA Bile acid

BPSBM Bioprocessed soybean meal

BSEP Bile salt export pump

CA Cholic acid

CDCA Chenodeoxycholic acid

Cldn12 Claudin twelve

Cypa Cyclophilin A

Cyp7a Cholesterol seven alpha hydroxylase

DCA Deoxycholic acid

DPB Vitamin D binding protein

Ef1a Elongation factor one alpha

Fabp2 Fatty acid binding protein two

FCR Feed conversion ratio

FI Feed intake

FGF Fibroblast growth factor

FM Fish meal

FSBM Fermented soybean meal

Fxr Farnesoid X receptor

GIT Gastrointestinal tract

Gln Glutamine

Glu Glutamate

GPx glutathione peroxidase

Hmgc Hydroxy methylglutaryl coenzyme A

HNnf4a Hepatocyte nuclear factor alpha

HSI Hepatosomatic index

IBD Inflammatory bowel disease

Ikb Inhibitor kappa B

Ikk Inhibitor kappa B kinase

Il8 Interleukin eight

Il10 Interleukin ten

Il1b Interleukin one beta

K Condition factor

LCA Lithocholic acid

LPS Lipopolisaccharides

LR Lipid retention

MAMP Microbial associated molecular pattern

MAPK Mitogen activated protein kinase

MLC Myosin light chain

Mylk Myosin light chain kinase

MLCP Myosin light chain phosphatase

Nfkb Nuclear factor kappa B

OATP Organic anion transporting peptides

Ocln Occludin

OST Organic solute transporter

PAMP Pathogen associated molecular pattern

PKC Protein kinase C

PR Protein retention

PRR Pattern recognition receptor

PTH Parathyroid horomne

Rar Retinoic acid receptor

Rxr Retinoid X receptor

SGR Specific growth rate

SHP Small heterodimer partner

SBM Soybean meal

SBMIE Soybean meal-induced enteritis

Slc1a5 Solute carrier family one member five

Slc10a2 Solute carrier family ten member two

SPC Soy protein concentrate

TGR5 Takeda G protein receptor five

TJ Tight junction

TLR Toll like receptor

Tnfa Tumor necrosis factor alpha

VD Vitamin D

VDR Vitamin D receptor

WG Weight gain

$25D_3$ Calcidiol

$1,25D_3$ Calcitriol

Statement of Contribution

The following people and institutions contributed to publications as part of this dissertation:

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- Performed the experiment: Candidate
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- Wrote the manuscript: Candidate

Chapter 1:

Review of Literature

Soybean Meal Use in Aquafeed

Introduction

Aquaculture is one of the most ancient food production systems, believed to exist in China since the first millennium BC and earlier (Costa-Pierce, 2022). Today, aquaculture is well established and is defined as the "breeding, rearing, and harvesting of fish, shellfish and algae and other organisms in all types of water environments" (National Oceanic and Atmospheric Administration, NOAA). As reported by the Food and Agriculture Organization of the United Nations (FAO), aquaculture continues to grow exponentially over the years, especially as the world population is projected to reach 9 billion by the year 2050, with efforts being centered to feed the world in the years to come. In this context, aquaculture, showing a 6.7% growth rate per year between 1990 and 2020 (FAO 2022 – SOFIA REPORT), is positioned to play a pivotal role. The high efficiency of aquaculture sets a solid basis for its productive system to continue to grow sustainably. Feed efficiency for aquaculture is 1:1 which means that for every kg feed, 1 kg growth outcome is produced (Naylor et al., 2021; Shepherd and Jackson, 2013; Tacon and Metian, 2008). In turn, capture fisheries have become static, with a 5.1% decrease in global catches from 2019 (FAO, 2022) while facing the issue of overfishing, threatening species diversity, and endangering marine ecosystems (Maire et al., 2021; Naylor et al., 2000; Srinivasan et al., 2010); thus demanding a shift in production to aquaculture which becomes increasingly patent (Fig 1.1). Today, about 56% of aquatic food available for human consumption comes from aquaculture, while 44% comes from capture fisheries (FAO, 2022). Global aquaculture production of aquatic animals for human consumption registered 87.5 million tonnes in 2020, reaching another record high, 6% higher than in 2018 (FAO, 2022). In North America (Canada, Mexico and United States), aquaculture is growing slower as compared to other regions, with a total production of 898 thousand tonnes in 2020, with the production in the Americas representing a 5% of the world total (FAO, 2022). However, in the United States, a 22.3% growth is predicted towards the year 2030, predicting 1,088 thousand tonnes in aquaculture production in North America by that year.

There are about thirty-five aquatic species that are cultured for consumption and represent 90% of the global production (Troell et al., 2014) of which finfish represent the vast majority (FAO, 2022). Global aquaculture production is also higher for freshwater species comprising 60% of production and expected to continue to increase through 2030, with species like salmonids

and trout maintaining a growing trend (FAO, 2022). Rainbow trout (*Oncorhynchus mykiss*) which is native to the United States Pacific Northwest (Crawford and Muir, 2008) is one of the major species produced in world aquaculture, with 739.5 thousand tonnes produced in 2020 (FAO, 2022). Specifically, 37.42 million trout were sold in United States, with 11.0 tonnes of produced in Idaho in 2022 (USDA and NASS, 2023a, 2023b). As a carnivorous species, rainbow trout, and in broader terms, salmonids, are reliant in fishmeal (FM) as protein source to achieve optimal growth, nonetheless, their farming is still more ecologically efficient than catching wild salmonids (Shepherd and Jackson, 2013). Out of the total FM destined to aquafeed production, the salmonid sector consumes approximately 13.7% (Fig. 1.2).

For years, aquafeeds have been reliant in FM as the primary source of protein, the so-called "gold standard" in aquafeed formulation to achieve optimal growth and fillet quality (Hardy, 2010; Turchini et al., 2019). The estimated commercial feed usage in 2020 was 56.99 thousand tonnes, with salmon as one of the major fed aquaculture species, experiencing a 5.4% growth between 2000 and 2018 (Tacon et al., 2022). Nowadays, increased aquaculture operations are resulting in 69% of FM coming from capture fisheries being destined to aquaculture feeds (Naylor et al., 2021). However, the aforementioned stagnation of capture fisheries limits the reliance on FM, resulting in prohibitive prices for this ingredient, that are expected to continue increasing (Jannathulla et al., 2019; Naylor et al., 2021). Moreover, the sustainability goals throughout the supply chain further challenge the use of this resource in formulations, as it is increasingly criticized by scientists and environmental groups (Naylor et al., 2000; Turchini et al., 2019). Therefore, although extensive effort has been made to minimize the use of FM in aquafeeds, it is imperative to find novel ways that allow reducing FM while ensuring optimal production of high-quality food fish.

Alternative protein sources: soybean meal

Two of the biggest issues in today's aquaculture industry are aquafeed costs (Hardy 2010) and ensuring sustainable formulations (Colombo et al., 2022). Efforts to reduce FM in aquafeeds have been in place for decades (Turchini et al., 2019). The strategic use of FM inclusion in feeds according to the development stage of the fish has been an initial approach in the quest to reduce FM inclusion and maintain competitive prices (Hardy, 2010; Shepherd and Jackson, 2013). Substantial attention has been put in plant protein sources as a FM alternative due to their balanced amino acid profile, worldwide availability and affordability, placing them as good candidates to produce high quality fish in a sustainable manner (Gatlin et al., 2007). Several sources of plant protein have been considered for aquafeed formulation for their high content in protein such as canola, cottonseed, corn, sunflower, rapeseed and soybean. Soybean (*Glycine max* L. Merr.) is a

legume commercially produced across thirty-five countries with Brazil, United States, Argentina, China, and India as the five leading producers (Karr-Lilienthal et al., 2004). The world production was estimated 335 million tonnes in 2018 (Galvan et al., 2016; Voora et al., 2020); and the United states Department of Agriculture estimated a production of 112 million tonnes in 2020 in that country. Hence, soybeans are an available resource, projected to continue this growing in the coming years (Voora et al., 2020). Soybean meal (SBM) is the by-product obtained from soybean seed through solvent extraction processing, and has been identified as one of the most promising options due to their high crude protein content (~ 500 g Kg^{-1}) and essential amino acid balance (Table 1.1) (Gatlin et al., 2007); as well as its adequate fatty acid profile showing 61% C_{18} n- 3 LC-PUFA, highly required by fish (Trushenski et al., 2013). Importantly, the deficiency in the essential amino acids methionine and lysine needs to be accounted for when using SBM in practical feed formulations in order to meet the fish amino acid requirements (Galvan et al., 2016). With over 6 million tons (i.e., 5.4 million tonnes), SBM is the plant protein source most used in aquafeeds (Fig. 1.3) (Troell et al., 2014). However, the SBM content of certain substances that interfere with nutrient digestion and absorption have been the biggest limitation for its use in aquafeeds (Ghosh and Ray, 2017). These substances, referred to as antinutritional factors (ANFs), have been extensively reviewed and were defined by Francis et al. (2001) as "substances which by themselves or through their metabolic products arising in living systems, interfere with food utilization and affect the health and production of animals".

These ANFs may cause reduced feed intake, depress growth performance, and negatively impact health in fish. For instance, more than 50% FM replacement by soy products can result in feed conversion rates increasing beyond 5% in salmonids (Cottrell et al., 2020). Classic examples of ANFs include phytates, oligosaccharides, protease inhibitors, isoflavones, saponins and lectins among others (Table 1.2). Phytates reduce the availability of divalent and trivalent positively charged minerals ions like Mg^{2+}, Cu^{2+}, Zn^{2+}, Cu^{3+} by chelating with them due to the negatively charged phosphate and similarly chelating positively charged proteins insolubilizing them, reducing the availability of these nutrients to the fish (Francis et al. 2001; Krogdahl and Bakke 2015). Oligosaccharides increase the viscosity of the chyme, interfering with nutrient absorption while lectins are agglutinins that bind glycoproteins in membrane surface, similarly interfering with nutrient absorption (Gatlin et al., 2007). Among protease inhibitors, trypsin inhibitors have been broadly characterized in plant ingredients (Francis et al., 2001); by inhibiting this protease, protein digestion in the lumen is markedly decreased, saturating enterocyte pinocytosis of proteins and intracellular digestion. Inhibition of trypsin may also affect other digestive enzymes that require trypsin activation such as lipases or chymotrypsin, overall reducing nutrient absorption (Halver and Hardy, 2002). Saponins have received

increasing attention in recent years and there is growing evidence that these may be the causal agents of intestinal inflammation and reduced digestibility associated to SBM-based diets. These polycyclic triterpene glycosides can bind cholesterol, including cholesterol forming cell membranes, eventually forming holes, and potentially increasing cellular permeability. Saponins can also interfere with membrane enzymatic activity, impacting mineral absorption and lipid digestibility (Francis et al., 2001; Krogdahl and Bakke 2015). Detrimental effects of saponins have been described in commercial fish species including Atlantic salmon (*Salmo salar*) (Gu et al., 2014; Knudsen et al., 2007; Krogdahl et al., 2015) and turbot (*Scophthalmus maximus*) (Gu et al., 2018, 2018). Saponins can also form complexes with bile acids and cholesterol, interfering with their synthesis regulation as seen in rainbow trout and Atlantic salmon (Kortner et al., 2013; Murashita et al., 2018; Romarheim et al., 2008). Isoflavones, in turn, may have an impact in lipid synthesis by binding sterol regulatory elements as these effects were reported in mammalian hepatocyte cell culture (Mullen et al., 2004).

The gastrointestinal tract of fish

Morphology and function

Morphologically, the GIT of rainbow trout resembles the general description of this organ in carnivorous teleost (Bjørgen et al., 2020). It is comprised of a short esophagus connecting with the cardiac portion of the stomach which shows blind diverticula called pyloric caeca that connect with the proximal intestine. The proximal intestine connects with the distal intestine, which can be distinguished by a gradual increase in diameter and a darker color (Burnstock, 1959; Verdile et al., 2020). Orchestrated action of both proximal and distal segments happens via hormonal regulation which regulates the digestive process as well as osmoregulation, immunity and appetite (Buddington and Krogdahl, 2004). The two primary roles of the GIT are ensuring nutrient absorption and providing a defensive function to prevent pathogens, antigens, proinflammatory factors and harmful substances from reaching the inner environment of the gut (Ray and Ringo, 2014). The lumen of the GIT comprises a huge area of interaction with the environment, with great antigen affluence, so ensuring inner homeostasis becomes crucial. The different cellular types and extracellular components that form the GIT ensure maintenance of homeostasis. Enterocytes are cuboidal shaped epithelial cells that form the epithelial lining. They are polarized cells, distinct on their apical surface than on their basal surface. The apical membrane faces the intestinal lumen and shows the characteristic folding of intestinal cells, called microvilli. The lumen harbors a specific microbial population or microbiota formed by commensal bacteria, and is covered by mucus, synthesized by goblet cells (Bjørgen et al., 2020). The basolateral membrane leads to the subepithelial region and the

lamina propria, which is connected to vasculature which contains absorbed nutrients, and harbors innate immune cells such as macrophages (Bjørgen et al., 2020; Salvo-Romero et al., 2015).

Enterocytes (Fig. 1.4) serve the important function of nutrient absorption and barrier function, adhering together to ensure epithelial integrity while maintaining selective permeability (Cain and Swan 2010; Gómez and Balcázar 2008; Salvo-Romero et al. 2015). Barrier function was defined by Marchiando et al. (2010) as: the ability of epithelial and endothelial-lined surfaces to restrict free passage of water, ions and larger molecules. This ability relies on regulation of intercellular junction proteins located between enterocytes, among which, three types can be distinguished:

(i) Tight junctions (TJ) are located on the most apical intercellular space (Fig. 1.4) and are a rate limiting step in antigen passage to the basolateral membrane region. Examples of these include the claudin family of TJ proteins, occludin and zonula occludens (ZO-1). In mammals the claudin family has been reported to include twenty-seven members in a wide variety of organs, and they function to regulate epithelial barrier integrity either by providing sealing functions or increasing permeability (Markov et al., 2015). In fish, Loh et al. (2004) identified fifty-six claudin genes in the teleost *Fugu rubripes* somewhat expressed in a tissue specific manner. Importantly, claudins in the fish intestine are additionally involved in maintaining osmotic homeostasis (Loh et al., 2004). Occludin has been identified as a transmembrane TJ and its biochemical composition (e.g. level of phosphorylation), determines its subcellular localization and thus its sealing or permeability enhancing function (Wong, 1997). Zonula-occludens 1 (Zo1) is known as a cytoplasmic adaptor protein (scaffolding protein) (Cummins, 2012) that facilitates protein interactions in the junction and claudin recruitment (Marchiando et al., 2010). The Zo1 links intercellular occludin to actin cytoskeleton, involved in cellular contraction processes as well as cell-cell partnering (Cummins, 2012).

(ii) Adherens junctions: provide anchoring between adjacent enterocytes by connecting both cytoskeletons; together with tight junctions, they are part of the apical junctional complex and are necessary for TJ assembly and maintenance (Salvo-Romero et al. 2015). Connexins, associated with adherens junctions as well as TJ play a role in maintenance of these junctions and intervene in epithelial cell differentiation.

(iii) Desmosomes: located in the basolateral membrane, desmosomes are transient and dynamic structures that provide strong cell adhesion (Salvo-Romero et al. 2015).

Lastly, communicating junctions or GAP junctions provide communication between two enterocytes through channel-like structures between membranes (i.e., substance or nutrient, particle

exchange), but they are also considered to have a role in barrier function maintenance through their interaction with TJ.

Nutrient absorption in enterocytes requires fine regulation through selective permeability based on particle size and charge. Hence, nutrients diffuse through the intestinal barrier by two means of transport (Jiang et al. 2015; Salvo-Romero et al. 2015):

(i) Transcellular transport occurs on the apical and basolateral membranes of the cell; this can happen by diffusion or by active transport through transmembrane transporters like the glucose/Na^+ cotransport system, or other amino acid transporters like glutamine transporter; and, through pumps and channels.

(ii) Paracellular transport involves passive transport between two adjacent epithelial cells, and only small molecules and ions, solutes and fluids can reach the blood this way. Permeability in this case is regulated by different TJ synergies that work to provide a tightening or a pore forming function. Cellular contraction is an important process to consider during paracellular transport. The actin cytoskeleton contraction is key in regulation of permeability, and it relies on myosin light chain II (MLC) phosphorylation by myosin light chain kinase (Mylk). This enzyme undergoes several ways of activation under physiological conditions such as the formation of a the Ca^{2+}/calmodulin complex that binds and activates Mylk (Kamm and Stull, 1985a) or the activation of the apical glucose/Na^+ cotransport system which induces Mylk during nutrient absorption (Cunningham and Turner, 2013). Phosphorylation of MLC is also regulated via Rho kinase which inhibits MLC phosphatase, increasing MLC phosphorylation (Capaldo and Nusrat, 2009). Lastly, protein kinase C (PKC) may also play a role in regulating transepithelial resistance by inhibiting Mylk by modulating calmodulin availability (González-Mariscal et al., 2008). Activated MLC induces cell contraction, reduces barrier function and facilitates nutrient passage when transcellular transporters are saturated (Clayburgh et al., 2004). This leads to increased paracellular permeability through reorganization of perijunctional actin, Zo1 and occludin, with an observed increase in TJ length, in accordance with an increase in paracellular permeability in a size-selective way, allowing passage of small molecules (Clayburgh et al. 2004; He et al. 2012; Cunningham and Turner 2013; Liu et al. 2018; Wang et al. 2015).

Immune function

The GIT of fish immune function distinguishes both primary or innate response, and secondary or adaptive response (Cain and Swan, 2010). The primary or innate response comprises the combined action of effector cells and molecules. Effector cells include phagocytes (i.e., macrophages), granulocytes (i.e., neutrophils), non-specific cytotoxic cells, dendritic cells, antibodies,

goblet cells, B cells and epithelial cells. Effector molecules comprise enzymes (i.e., proteases, phosphatases, oxidases), cytokines and chemokines (Salinas and Parra, 2015).

Epithelial cells have a key role in innate immunity via pattern recognition receptors (PRRs) like toll like receptors (TLR) present in their apical and basolateral membrane. These can recognize pathogen-associated molecular patterns (PAMPs) and microbial-associated molecular patterns (MAMPs) such as lipopolysaccharide (LPS) found in bacterial cell walls and synthesize cytokines (Salvo-Romero et al., 2015). In addition to nutrient absorption, gut epithelial cells perform signal-transduction functions through apical receptors that receive stimuli from luminal contents and can modulate immune cells in the lamina propria (Khor et al., 2011). Goblet cells synthesize mucus, formed of glycoproteins like mucin, and immunoglobulin M and T (IgM, IgT), cytokines, lysozyme, proteases, ant its functions as chemical barrier against infectious agents (Salinas and Parra, 2015). Among cytokines, both pro-inflammatory cytokines such as interleukin 8 (Il8), tumor necrosis factor alpha (Tnfa) or interleukin 1 beta (Il1b); and anti-inflammatory cytokines like interleukin 10 (Il10) can be distinguished. Both types of cytokines interact to maintain the GIT immune balance and their coordinated action prevents the perpetuation of uncontrolled inflammatory reactions (Marchiando et al., 2010; Salinas and Parra, 2015).

Although the immune system in fish relies on the innate immune response through creating a chemical and physical barrier, the GIT of teleost can also elicit an adaptive immune response, although this is less developed than it is in mammals (Cain and Swan, 2010). The adaptive immune response is activated once the initial innate response has taken place, and activated macrophages and neutrophils act as antigen presenting cells in fish. In mammals, specialized structures called Peyer's patches act as antigen retrieval centers (Bjørgen et al., 2020; Cain and Swan, 2010). In fish, the site for antigenic presentation has been shown to occur at diffuse gut associated lymphoid tissue (GALT) present in the distal intestine (Cain and Swan, 2010). Macrophage and neutrophils activated during the innate response present antigenic components to B and T lymphocytes effector cells in adaptive immune response. This causes B lymphocytes to proliferate and synthesize antibodies: IgM and IgT which can infiltrate the epithelium during an immune response and block pathogenic infections (Salinas and Parra, 2015).

The GIT also harbors commensal bacteria forming the gut microbiota which play a role both in immunity and digestive processes (Romero et al., 2014). Gut bacteria provide additional digestive enzymes that may aid in absorption of nutrients (Talwar et al., 2018) and other processes such as bile acid deconjugation, 7ihydroxylation and desulfation (Franzosa et al., 2019). Mucosal microbes provide immune protection by competitive exclusion and inhibition of pathogens (Cain and Swan, 2010; Romero et al., 2014; Salinas, 2015),send signals to adaptive immune effector cells

(Salinas, 2015; Salinas and Parra, 2015) and affect gene expression in the GIT (Balcázar et al., 2006). Bacteria forming the microbiota are also susceptible of being recognized by apical PRRs, stimulating the secretion of antimicrobial peptides and maintenance of tolerance to inflammation (Salvo-Romero et al. 2015). Importantly, the presence of the microbiota is central to the full development and maturation of intestinal tissues, including cell proliferation rates, formation of glycocalyx and function of digestive brush border enzymes (Tuma and Hubbard, 2003).

Overall, the immune function of the GIT relies on the integrity of the intestinal barrier to protect against foreign substances; a specific microbiota that serve a role in both digestive processes and intestinal immune activity; and the composition of the mucus and activation of the innate and adaptive immune responses. Under normal conditions this is finely regulated to discern between commensal bacteria and harmless antigens that can be ingested in the diet and potentially harmful proinflammatory factors.

Soybean meal-induced enteritis (SBMIE)

Intestinal inflammatory mechanisms

Inflammatory bowel diseases (IBD) comprise a series of intestinal disorders that encompass both a perpetual inflammatory response and the loss of epithelial barrier function (Maloy and Powrie, 2011), and are thought to be a result of an autoimmune response to the host commensal microbes (Khor et al., 2011). In higher vertebrates, IBDs have been extensively studied, and the bases of the underlying molecular mechanisms are beginning to be understood. Substantial evidence indicates that enhanced expression of the pro-inflammatory cytokine Tnfa is responsible for higher transcription rates of Mylk via the nuclear factor kappa B (Nfkb) pathway (He et al. 2012; Cunningham and Turner 2013). Increased expression of Mylk leads to cellular hypercontractility and loss of barrier function.

The transcription factor Nfkb has a central role in immune homeostasis of the intestine and it directs transcription of inflammatory cytokines upon exposure to toxins, pathogens or other stress signals (Wang et al. 2015; de Oliveira et al. 2016). Nfkb is formed by two subunits: p50 and p65, located in the cytosol bound to inhibitor kappa B (Ikb) which keeps the dimer inactive (Neumann and Naumann, 2007; Xiao et al., 2006). Under normal conditions, activation of Nfkb is followed by an autoregulatory feedback loop that activates Ikb, ensuring transient nuclear expression of Nfkb and avoiding deleterious conditions such as acute inflammatory scenarios (Xiao et al., 2006). During IBD, Tnfa expression is abnormally elevated (Ye et al., 2005), and, in these conditions, binding to its receptor on the apical membrane of enterocytes elicits a signal transduction mechanism to continuously activate Nfkb (Fig. 1.5) (Cunningham and Turner, 2012). This initiates a molecular

signaling cascade that phosphorylates Ikb leading to its proteasomal degradation (Lesueur et al. 2012; Lodish et al. 2012; Cunningham and Turner 2013; Xiong et al. 2017). Released Nfkb p50/p65 heterodimer exposes the nuclear localization signals and translocate to the nucleus to control gene expression (Lodish et al. 2012; Wang et al. 2015; de Oliveira et al. 2016). Nfkb has a binding motif in the promoter region of Mylk, leading to its increased transcription, thus increased cell contractility and permeability and, eventually, barrier function loss (He et al. 2012; Cunningham and Turner 2013; Xiong et al. 2017; Su et al. 2019). Hence, it is suggested that Mylk increased transcription occurs by Nfkb activation in response to Tnfa stimulation (Ma et al., 2005; Ye et al., 2005). However, there are other factors involved. As stated before, the coordinated balance of both pro-inflammatory and anti-inflammatory cytokines determines the immune status and gut health (Salinas and Parra, 2015). The anti-inflammatory cytokine Il10 has received research attention, and decreased expression levels have been documented in IBD studies (Bijjiga and Martino, 2011). While a normal expression of Il10 may ensure the correct regulation of an immune response, defects to its expression may lead the development of a chronic inflammatory status, with increase in Tnfa expression and inflammation amplification (Marchiando et al., 2010).

The study of TJ proteins during IBD is also crucial to understand the disease and barrier dysfunction. It has already been covered how Mylk expression is increased in association with Tnfa, however, cytokines may also intervene in TJ remodeling or alter their composition (Capaldo and Nusrat, 2009). In mammals, IBD has been associated to increased claudin-2 levels and Mylk activity, as well as decreased claudin-1, claudin-8, claudin-4, claudin-5, Zo1 and occluding (Cunningham and Turner 2013; Salvo-Romero et al. 2015; Xiong et al. 2017). Abnormal expression of Mylk has a negative impact on TJ protein by altering their expression patterns or cellular localizations. Studies in IBD models in mammals have described an upregulation of TJ protein claudin-2 as well as occludin endocytosis (Cunningham and Turner 2013; Marchiando et al. 2010; Xiong et al. 2017). A study by He et al. (2012) using the human intestinal epithelial cell line Caco-2 revealed the impact of Tnfa treatment in loss of barrier integrity and its involvement in decreased expression of Zo1 and occludin, and increased Mylk expression, as well as reorganization of actin cytoskeleton. However, these effects were not observed in mutant cells expressing I-κB resistant to degradation, evidencing the role of Tnfa-activated Nfkb in loss of barrier integrity (He et al., 2012).

In summary, intestinal loss of barrier function clearly correlates Tnfa activation of Mylk with TJ remodeling and increased permeability, which in contrast to Na^+ glucose cotransport, allows paracellular infiltration of small but also large molecules (Cunningham and Turner 2013; Marchiando et al. 2010). Nonetheless, it is not clear whether the pro-inflammatory signals appear first, leading to increased Mylk activity and permeability thus filtrating more luminal material into the lamina propria

and thus activating the inflammatory response; or if there is barrier dysfunction is caused first due to changes in microbiota or genetic predisposition function which eventually leads to activation of an inflammatory cycle. In both instances, failure to balance the inflammatory response or altered microbiota could contribute development of disease (Marchiando et al. 2010).

SBMIE

An inflammatory reaction is developed in the distal intestine of fish fed 20 – 40% SBM diets (depending on whether these are carnivores or omnivores) which is referred to as soybean meal-induced enteritis or SBMIE (Gu et al., 2016a; Heikkinen et al., 2006; Romarheim et al., 2008; Uran et al., 2008; Urán et al., 2008; Zhang et al., 2021). Baeverfjord and Krogdahl (1996) defined this phenomenon as non-infectious inflammation of the distal intestine. Hence, SBMIE is studied and described herein as the inflammatory process observed in fish fed SBM based diets. The ANFs described previously, alone or in combination, are believed to act as causative agents of SBMIE (Baeverfjord and Krogdahl 1996; Bakke-McKellep et al. 2000; Bjørgen et al., 2020; Fuentes-Quesada et al. 2018; Gajardo 2016; Gu et al. 2016; Knudsen et al. 2007; Krogdahl et al. 2003; Krogdahl et al. 2020).

It has been stated that SBMIE in fish share similarities with IBDs (Abernathy et al., 2019; De Santis et al., 2015; Grammes et al., 2013), although in contrast to IBD, SBMIE is not caused as a continuing immune response against commensal microbiota but, instead, triggered by the combination of several ANFs (Zhou et al., 2018). In fish, signs of enteritis have been reported in a large variety of both marine and freshwater species including omnivores and carnivores (Zhou et al., 2018), and it has been studied in susceptible commercial species including turbot and salmonids (Baeverfjord and Krogdahl 1996; Gu et al. 2016, 2018; Heikkinen et al. 2006; Å Krogdahl, Bakke-McKellep, and Baeverfjord 2003; Åshild Krogdahl et al. 2020; Merrifield et al. 2009); and to a lesser extent in hybrid sturgeons, European seabass (*Dicentrarchus labrax*), Asian seabass (*Lates calcarifer*), Jian carp (*Cyprinus carpio* var. Jian), and largemouth bass (*Micropterus salmoides*) among others. A range of several effects caused by different dietary soy sources in a range of commercial species is shown in Table 1.3.

Decrease of feed intake typically in connection with depressed growth rates and feed efficiencies are well described signs of SBMIE in commercial species (Zhou et al., 2018). Additionally, histopathological analysis in different fish species have helped study and define the signs of SBMIE, which encompass inflammatory cell infiltration, lamina propria thickening, altered villi morphology, and increased numbers of goblet cells and large vacuoles (Bjørgen et al., 2020). Such symptoms have been largely evidenced in several species, including rainbow trout (Barnes et

al., 2015; Bruce et al., 2018; Heikkinen et al., 2006; Merrifield et al., 2009; Mosberian-Tanha et al., 2018; Rumsey et al., 1994; Venold et al., 2012), Atlantic salmon (Bakke-Mckellep et al., 2000; Gajardo, 2016; Jacobsen et al., 2018; Krogdahl et al., 2003; Venold et al., 2013), even after short term exposure (Navarrete et al., 2013; Uran et al., 2008); turbot (*Scophthalmus maximus*) (Gu et al., 2016, 2017a, 2018; Liu et al., 2018), European seabass (*Dicentrarchus labrax*) (Rimoldi et al., 2016), the omnivore Jian carp (Yan and Qiu-zhou, 2006), largemouth bass (*Micropterus salmoides*) (Romano et al., 2021), and totoaba (*Totoaba macdonaldi*) (Fuentes-Quesada et al., 2018).

Gene expression analyses aiming to identify both inflammatory markers and barrier integrity have also been performed in commercial species, but data remain inconclusive. In common carp (*Cyprinus carpio* L.), higher levels of Tnfa and Il1b were found after 1, 3 and 5 weeks of exposure to 20% SBM diets (Urán et al., 2008); and Tnfa was similarly upregulated in the distal intestine of Jian carp in response to the ANF glycinin (Jiang et al., 2015b) after 42 days. Sahlmann et al. (2013), using microarray technology, recorded an upregulation of Nfkb/p52 precursor, p100, whose processing is required for the so-called alternative pathway of Nfkb activation (Neumann and Naumann, 2007). Higher expression of of Nfkb, Tnfa, and Il8 have been found in turbot fed ~40% SBM diets for 8-12 weeks (Bai et al., 2017; Gu et al., 2017; Liu et al., 2018). Instead, expression of the pro-inflammatory cytokines Tnfa and Il8 was unaffected in rainbow trout fed a 21% level SBM diet (Kumar et al., 2021). In totoaba, increased levels of Il8 have been reported (Fuentes-Quesada et al., 2020, 2018).

Studies on TJ and cell contractility markers are scarce in fish. In turbot, Liu et al. (2018) found consistently lower expression of TJ proteins claudin-4, occludin and Zo1 after 2, 4 and 8 weeks feeding a high SBM diet. This same study also reported increased expression of Mylk in the SBM group, but only 4 weeks of dietary exposure. Instead, by week 8 Mylk appeared upregulated in the FM (control) diet. In Jian carp, upregulation of claudin-7 was found in the distal intestine in response to glycinin, and this was related to the pore-forming function of said claudin. However, same study also showed a consistent upregulation of claudin-11, thought to display sealing properties, in distal intestine as well as proximal of Jian carp fed the SBM (glycinin) diet (Jiang et al., 2015a).

Other well characterized SBMIE signs are changes in important brush border digestive enzyme activities. Alkaline phosphatase shows reduced reactivity in both apical membrane and intracellularly in salmonids fed SBM, which imply reduced capacity for nutrient absorption and are a sign of malnourishment in salmonids fed SBM diets or low FM (Bakke-McKellep et al., 2000). Reduced alkaline phosphatase activities have been seen also in rainbow trout fed both SBM or fermented SBM (Choi et al., 2020; Yamamoto et al., 2007); gilthead seabream (*Spaurs aurata)* fed

high levels of plant protein (Estensoro et al., 2016a); Asian seabass fed 30% SBM (Ma et al., 2019); and totoaba fed high level SBM diets (Fuentes-Quesada et al., 2020, 2018).

Under normal conditions, intestinal cell renewal occurs at the base of the intestinal folds, in the intervillous pockets or crypts, until reaching full cell maturity (Bakke-McKellep et al., 2000; Bjørgen et al., 2020), in a process that entails TJ reorganization and apoptosis (Williams et al., 2015). During disease, such as IBD, cell shedding may occur at faster rates, with increased apoptosis, and stimuli caused by TNF-α is thought to play a critical role (Marchiando et al., 2011a; Williams et al., 2015). In Atlantic salmon, SBMIE studies revealed increased proliferative cell nuclear antigen (PCNA)-positive cells and increased number of immature cells that migrate to the apical fold, as a mechanism to counteract cell loss due to increased apoptosis, and causing hyperplasia of immature cells (Bakke-McKellep et al. 2007; Gajardo 2016; Grammes et al. 2013); and this has similarly been reported in gilthead seabream fed high level plant based diets (Estensoro et al., 2016).

The GIT microbiota has also been shown to suffer changes during SBMIE, such as reduction of microbial diversity and differences in relative abundance of different bacteria phyla observed in a range of species fed soybean products (Bjørgen et al., 2020; Zhou et al., 2018). Changes in microbiota during SBMIE are similar to those observed in IBD, with an increase of gram positive bacteria and decrease in antimicrobial secretion as observed in Atlantic salmon fed SBM as compared to those fed FM diets (Grammes et al., 2013). In rainbow trout, bacterial numbers and microbiota diversity were decreased after feeding a SBM diet (Heikkinen et al., 2006), while Atlantic salmon displayed a more diverse microbial population in the distal intestine (Bakke-McKellep et al. 2007). However, another comparing Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*) only pointed out differences in the intestinal microbiome between species, but no difference between fish fed a SBM or a FM diet (Booman et al., 2018). A recent study in totoaba demonstrated microbiome changes in both in the distal intestine and feces (Fuentes-Quesada et al., 2020).

Lastly, apart from SBMIE, and other described symptoms in the distal intestine, feeding high inclusion levels of SBM can also cause deleterious effects in the liver. Higher hepatic vacuolization has been described in Atlantic salmon (Gu et al., 2014), turbot (Gu et al., 2017), and totoaba (Fuentes-Quesada et al., 2018); as well as atrophied hepatocytes in rainbow trout (Murashita et al., 2013; Iwashita et al., 2008; Yamamoto et al., 2007). Moreover, changes in cholesterol and bile physiology have also been reported. ANFs like isoflavones may interfere with nuclear regulatory elements of bile and sterol metabolism in the liver (Ricketts et al., 2005). Cholesterol reserves are depleted in Atlantic salmon fed SMB, and this has been attributed to the effect of saponins, which can bind and form complexes with bile and cholesterol in the intestine, limiting their absorption in the intestine (Francis et al., 2002; Kortner et al., 2013). Impacted lipase activity has been reported in

tilapia fed SBM or combined sources of plant protein including SBM (Jiang et al., 2018; Wangkahart et al., 2022); yellowtail (*Seriola quinqueradiata*) (Nguyen et al., 2013); pompano (*Trachinotus blochii*) (Nguyen and Van Do, 2021). In rainbow trout, changes in reduced plasma cholesterol and changes in bile acid levels in intestine and gall bladder have been reported (Murashita et al., 2018, 2013; Romarheim et al., 2008). Finally, abnormal expression of fatty acid binding protein two (Fabp2) has been described in Atlantic salmon, rainbow trout, and gilthead seabream which can also interfere with lipid absorption (Estensoro et al., 2016; Krogdahl et al., 2015; Venold et al., 2013, 2012).

Feed additives and functional feeds

Introduction

To increase the nutritive value of SBM, reduction of ANFs through mechanical or biochemical treatment of SBM such as heating or fermentation during the extrusion process yield more digestible and improved protein profiles of diets (Ghosh and Ray, 2017). For example, enzymatically processed SBM such as EnzoMeal renders a product with reduced content of certain ANFs while maintaining protein content and amino acid levels, which has been reported to beneficially affect growth and digestive capacity in species like rainbow trout and Asian seabass (Kumar et al., 2020; Ma et al., 2019; Murashita et al., 2013; Romano et al., 2021). Other soy derived products include soy protein concentrate (SPC) in which the carbohydrate fraction is removed; and fermented soybean meal (FSBM) which is treated with microorganisms rendering higher crude protein and amino acid contents (Hooft and Bureau, 2021; Zhou et al., 2018). More recently, the use of feed additives has received increasing attention as it can be an effective way to boost immunity, enhance nutrient digestion and absorption, overall improve the welfare of the animal, and enhance nutritional quality of the final product (Dawood et al., 2018; Encarnação, 2016; Tacon et al., 2020). The use of additives has led to the development of functional feeds, which are described as foods with dietary ingredients that provide growth, health, environmental and economic benefits beyond traditional feeds (Soto et al., 2015). With the intensification of aquaculture, and feed representing 20-40% of production costs, feed additives seem a great solution to maintain high productivity while ensuring animal welfare and maintaining affordability of feeds.

There are various types of additives that have been considered in aquafeeds which include amino acids, emulsifiers, vitamins, probiotics and prebiotics, yeast products, organic acids, nucleotides, and phytogenics among others (Dawood et al., 2018; Encarnação, 2016). Thus, the use of feed supplements in high level SBM diets for carnivorous species can be a valuable strategy to combat against SBMIE, and a wide range of additives have been studied with the intent to increase

tolerance to high SBM diets (Bai et al., 2017; Carballo et al., 2019; Chen et al., 2019; Cheng et al., 2012; Fuentes-Quesada et al., 2020; Gu et al., 2017, 2021; Kumar et al., 2021; Rimoldi et al., 2016; Romano et al., 2021; Yoshida et al., 2016).

The work presented herein focuses on three types of additives:

(i) Amino acids: functional amino acids are commonly supplied in crystalline form to fulfil a requirement and improve quality of the product (Dawood et al., 2018). For instance, in SBM-based diets, the essential amino acids methionine and lysine are supplemented following NRC recommendations to meet nutritional requirements. Taurine has been shown to be critical in the formulated diets of marine species (Salze and Davis, 2015). Arginine, leucine, and glutamine have received attention due to their growth and immune stimulatory properties. Arginine has been associated with increased growth and protein deposition (Cheng et al., 2011; Pohlenz et al., 2013) as well as shown to stimulate channel catfish (*Ictalurus punctatus*) macrophage phagocytic activity in vitro (Pohlenz et al., 2012) and, combined with glutamine, protect against SBMIE in turbot (Gu et al., 2017). Arginine is also substrate of nitric oxide synthesis, which has a role in intestinal health (Rhoads and Wu, 2009). Leucine is also involved in intestinal health and has been tested in grass carp (*Ctenopharyngodon idella*) during SBMIE (Jiang et al., 2015a) and has been proposed to exert beneficial signaling in the intestine in combination with arginine and glutamine. Glutamine has been widely studied both in mammals and in fish for its protective role in the intestine and will be explained in detail in this section (Achamrah et al., 2017; Bortoluzzi et al., 2018; Ewaschuk et al., 2011; Newsholme, 1996; Wang et al., 2011).

(ii) Bile acids: bile acids and emulsifiers have gained attention as they represent a good option to enhance lipid absorption and reduce the need for *de novo* synthesis of cholesterol (Romano et al., 2022; J. S. Zhou et al., 2018). In fish, different types of bile or emulsifiers have been tested including bovine bile salts, soy lecithin, cholyltaurine or gall powder (Gu et al., 2017; Iwashita et al., 2008; Lin et al., 2022; Murashita et al., 2018; Yamamoto et al., 2007). The supplementation with taurine has also been reported to enhance lipid utilization (Nguyen et al., 2013; Nguyen and Van Do, 2021). However, the involvement of bile acids in intestinal health has been studied less in fish, thus, this aspect will be covered in the work presented herein.

(iii) Vitamins: vitamins are a dietary requirement for fish and one of the most expensive ingredients (Dawood et al., 2018). The antioxidant roles of vitamins C and E are well known and studied (Dawood et al., 2016; El-Sayed and Izquierdo, 2022). A role in

remediating waterborne iron toxicity has also been shown for vitamin C (Yadav et al., 2020). Vitamins K and D have been tested for their roles in bone health, especially at the larval developmental stages (Dominguez et al., 2022; Sivagurunathan et al., 2022). Vitamin D has more recently been suggested to exert beneficial effects in the intestine of turbot and boost immune status after bacterial challenge (Shao et al., 2022). This work aimed to further characterize vitamin D as a suitable additive candidate for SBMIE attenuation.

Glutamine (Gln)

Glutamine (Gln) is a non-essential neutral amino acid that has been identified as the main source of energy in enterocytes and almost all dietary Gln is used by enterocytes (de Oliveira et al., 2016). Gln serves an important role in maintaining the tricarboxylic acid cycle (TCA) via its conversion to glutamate (Glu) in proliferative cells and provides source of nitrogen intermediates important for purine and pyrimidine synthesis nucleic acids synthesis in dividing cells. Importantly, given the involvement of Glu in satiety signaling and feed intake regulation, Gln may play an important role by supporting the generation of free Glu in the brain (where Glu is produced *de novo*) via Gln-Glu cycle (Delgado, 2013; Torii et al., 2013). In addition, the involvement of Glu in digestive enzyme activation also emphasizes the importance of both dietary Gln and Glu in digestive function (Torii et al., 2013). Further, Gln can also be metabolized into glutathione, which acts as a co-factor of glutathione peroxidase enzyme. Glutathione peroxidase intervenes in antioxidant metabolism, preventing uncontrolled apoptosis and protecting the cell from reactive oxygen species (ROS) (Kern and Kehrer, 2005; Wang et al., 2015).

Importantly, Gln interacts with many of the IBD markers detailed above, making it a good candidate to mitigate inflammatory and barrier integrity symptoms (Kim and Kim, 2017). As reviewed by Wang et al. (2015) and Rhoads and Wu (2009), Gln intervenes in a wide range of molecular signaling cascades that induce cell survival, cell division, reduce apoptosis, boost immunity and positively impact TJ regulation. Activation of mitogen-activated protein kinases (MAPK/ERK) in the kinase cascade pathway is one of the mechanisms of action of Gln, since MAPK phosphorylates nuclear transcription factors that take part in cell proliferation. In addition, Gln-induced activation of the phosphatidylinositol – 3 kinase/protein kinase B (PKB)/Akt (PI3K/Akt) pathway which is known modulate cell survival by reducing apoptosis during cellular stress, as well as controlling glucose homeostasis. Larson et al. 2007 found positive effects of Gln on cell survival as a result of MAPK/ERK-induced anti-apoptotic effects. However, that study dismissed the activation of

PI3K/Akt Gln, and activation of this pathway is attributed to cellular stress coping mechanisms in the absence of Gln.

Indeed, there are other studies supporting the activation of PI3K/Akt pathway in the context of Gln deprivation. Li and Neu (2009) investigated the effects of PI3K inhibitors on Caco-2 cells in the presence or absence of Gln and under no stress conditions. Their results show that the absence of Gln increased protein expression of p85 component of PI3K and its downstream products (Akt), while the presence of Gln mimicked the effects of PI3K inhibitors. Claudin-1 expression appeared to be reduced in the absence of Gln, but this effect was reversed both by the presence of Gln and PI3K inhibitors, suggesting Gln may maintain claudin-1 expression through inhibition of the PI3K/Akt pathway in normal conditions. Another study in rats where intestinal hyperpermeability and TJ alterations were induced, demonstrated increased expression of claudin-1, occludin and in the presence of Gln, and this was related to activation of MAPK/ERK and downstream modulation of phosphorylation of Ikb (Beutheu et al., 2014). This mechanism of activation leads to the modulation of the activation of Nfkb transcription factor, critical in the control of inflammation (Brasse-Lagnel et al., 2007; Lesueur et al., 2012).

Brasse-Lagnel et al. 2007 confirmed the Gln-induced nuclear decrease of Nfkb p65 in human Caco-2 cells in normal conditions within 4 hrs; furthermore, these authors suggested that these effects were caused by Gln via its conversion to Glu, as this metabolite appeared increased in treated cells, and was able to cause the same effects as Gln. A subsequent study by the same group (Lesueur et al. 2012) studied the mechanisms behind Gln-induced inhibition of Nfkb in cultured Caco-2 cells in normal conditions and found that p65 degradation happened in the nucleus via de nuclear ubiquitin proteasomal pathway. This process starts in the cytoplasm where Glu activates Ikb phosphorylation via inhibitor kappa B kinase (Ikk) activation. This releases Nfkb which is translocated to the nucleus; but Ikk also causes phosphorylation of p65 at a specific serine (Ser 536) which leads to nuclear ubiquitination and degradation. Other studies have linked Gln modulation of Nfkb with reduced levels of pro-inflammatory cytokines like Tnfa among others. Ewaschuk et al. 2011 studied the effects of Gln in piglets exposed to enterotoxigenic *Escherichia coli* (LPS-induced) for a period of 2 weeks. While Gln had a positive effect in reducing mRNA expression of pro-inflammatory cytokines like Il6 and Il8, no impact on occludin and claudin-2 protein expression was detected, in contrast to other studies. Other studies have related the modulation of Nfkb by Gln with the expression of heat shock proteins (Xue et al., 2011).

Intriguingly, while most studies on intestinal diseases investigate the expression of Mylk (Clayburgh et al., 2004; Feighery et al., 2008; He et al., 2012a; Xiong et al., 2017), studies focusing on the beneficial effects of Gln on intestinal diseases do not examine Mylk but mostly focus on Nfkb,

and TJ among other parameters (Beutheu et al. 2014; Brasse-Lagnel et al. 2007; Ewaschuk et al. 2011; Haynes et al. 2009; Jiang et al. 2015; Larson et al. 2007; Lesueur et al. 2012; Molinari 2020; Li and Neu 2009; Yan and Qiu-zhou 2006). Knowing the effect of Gln on Nfkb, its control over inflammatory cytokine expression and the association of Tnfa and Mylk during IBD, it would be interesting to further investigate expression of both Tnfa and Mylk under Gln treatment would provide evidence to draw more conclusions.

A plethora of studies have been carried out in commercial fish species addressing the role of dietary Gln in improving growth performance and, especially in SBMIE mitigation (Table 1.4). The study by Liu et al. (2018) showed that 2.0% Gln supplementation in a SBM-based diet fed to trout had positive effects on turbot growth performance. Moreover, these authors described reduced expression of Tnfa and Nfkb after 12 weeks. Furthermore, dietary Gln was also able to maintain TJ claudin-4 expression levels similar to those of the FM (control) group throughout the trial. These authors also recorded lower cytokine expression as well as Nfkb in Gln supplemented diets. Furthermore, these authors examined Mylk expression, which was decreased in fish fed a SBM diet supplemented with Gln compared to fish fed a non-supplemented SBM diet, although this was not maintained at all time points, in accordance with lower Nfkb. The protective effect of Gln in that study was also supported by histopathology analysis of the distal intestine showing higher degree of resemblance between fish fed the FM diet and the Gln supplemented SM diet. In rainbow trout, Yoshida et al. (2016) studied the effects of 1.0-2.0% dietary Glu and revealed positive effects on growth performance and distal intestine histology.

Jiang, et al. (2015) studied the specific effects of the ANF glycinin in Jian carp (*Cyprinus carpio* var Jian) intestinal health and the protective effects of Gln. After 42 days they found that 1.2% dietary Gln was able to counteract the negative impact caused in antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase as well as reduced reactive oxygen species, compared to those observed in the control group. These authors observed some changes in TJ expression patterns like increased claudin-11 in distal intestine in the presence of glycinin, which was reversed by Gln, but they fail to explain the role of Gln and of each TJ in intestinal health and disease. Wang et al. (2011) investigated the effects of different levels of dietary Gln in hybrid sturgeon (*Acipenser schrenkii* x *A. baerii*) although this study was not conducted in the context of SBMIE, they observed improved growth performance parameters with increasing Gln levels, as well as antioxidant enzymes, with a 30% increase SOD activity from 0.0% to 1 % Gln supplementation. Molinari et al. (2020) observed similar effects of Gln on fish fed SBM diets were observed in terms of decreased inflammatory markers in zebrafish (*Danio rerio*) as well as increased expression of Fabp2 and improved histology of the proximal intestine. Improved growth and

histology were also described in carp but the authors did not specify the portion of the GIT that was studied (Yan and Qiu-zhou, 2006); none of these studies investigated effects on Mylk.

Other studies have examined the combined effects of Gln and arginine. Beutheu et al. (2014) concluded that the combination of these two amino acids did not show the same beneficial effects of Gln alone. However, a positive outcome was seen in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) and red drum (*Sciaenops ocellatus*) fed SBM-based diets in terms of growth performance, distal intestine histopathology, and antioxidant metabolism, but with exceptions (Cheng et al., 2012, 2011). For instance, for hybrid striped bass, Gln seemed to be beneficial in combination with arginine or alone at 1.0% but not alone at 2.0%. (Cheng et al., 2012). In contrast, dietary Gln at 2.0% alone, as well as its combination with arginine at 1.0% of the diet improved feed efficiency in red drum (Cheng et al., 2011).

Bile acids (BAs)

Bile acids are amphipathic molecules synthesized from cholesterol in the liver and stored in the gall bladder until secreted into the duodenum upon cholecystokinin (CCK) stimulation. Their primary function is to aid in digestion and absorption of lipids and fat soluble vitamins like vitamin A, D, E and K; as well as maintaining cholesterol homeostasis (Hofmann, 1999). BAs are subjected to a finely regulated physiological recycling process known as enterohepatic circulation. Bile acids synthesis from their substrate cholesterol follows either of two pathways: the classic or the alternative pathway (Chiang, 2013). Cholesterol 7 – α hydroxylase (Cyp71a) is the rate limiting enzyme and its transcriptional activation and deactivation are subjected to two nuclear receptors: liver X receptor (Lxr) and farnesoid X receptor (Fxr) respectively. The Lxr become activated by oxysterols (cholesterol metabolites) which, as observed in mammals, recruits hepatocyte nuclear factor 4 alpha (Hnf4a) and activates Cyp71a transcription in the hepatocyte (of note, glucose can also activate Cyp7a1 by histone acetylation) (Chiang, 2013, 2009). Synthesized primary BAs include cholic acid (CA) and chenodeoxycholic acid (CDCA). These BA are conjugated with taurine or glycine (in fish it is mainly taurine) to form bile salts. Bile salts are pumped out through bile salt export pump (Bsep), a transporter present in the canalicular membrane, and stored in the gall bladder (Chiang 2013). Upon CCK stimulation, bile salts are released into the proximal intestine where they intervene in fat emulsification by helping in lipase activation and micelle formation. Intestinal microbiota deconjugates bile salts and secondary BAs are formed such as deoxycholic acid (DCA) and lithocholic acid (LCA). Of these, 95% are resorbed in the distal intestine and transported back to the liver via portal vein circulation. Resorption of bile salts occurs via apical sodium-dependent bile salt transporter (Slc10a2, previously known as Asbt) present in the apical membrane of distal enterocytes;

however, unconjugated BAs like secondary bile acids can passively diffuse. The baso-lateral organic solute transporter alpha and beta dimer (OSTα/β) which is activated by enterocyte Fxr, previously activated by BAs. BA-dependent activation of Fxr in the enterocyte is also responsible for fibroblast growth factor 19 (Fgf19) synthesis, which also reaches the liver via portal vein circulation. Back in the liver, resorbed BAs are transported into the hepatocyte via Na^+ dependent taurocholate transport peptide or Na^+ independent organic anion transporter protein (Oatp). In the hepatocyte, returning BAs activate Fxr which acts as negative feedback regulator of *cyp7a1* transcription. Fgf19, identified in zebrafish, is also involved in repressing Cyp7a1 upon reaching the hepatocyte membrane and binding its receptor fibroblast growth factor four receptor (Fgfr4) by activating the MAPK pathway, but further downstream inhibiting mechanisms remain unknown (Chiang 2009; Thomas et al. 2008).

Among all types and forms of BAs, it is critical to consider their chemical properties, for example whether they are in conjugated or unconjugated form as this can affect their level hydrophobicity, critical to determine absorption in the intestine and also their potential cytotoxic at high concentrations (Hofmann, 1999).

More recently, BAs have been identified as signaling molecules with a key role in inflammatory processes via the nuclear receptor Fxr (Matsubara et al. 2013; Nijmeijer et al. 2011; Vavassori et al. 2009) and the G protein-coupled receptor (GPCR), Takeda G protein-coupled membrane receptor (Tgr5, also known as GPBAR1) (Perino and Schoonjans, 2015; Pols et al., 2011). Tgr5, is the first GPCR identified to be activated by BAs, and it is also referred to as G protein bile acid receptor 1 (GPBAR-1) or as M-BAR (Cipriani et al. 2011; Chiang 2013). Tgr5 is expressed in many tissues including adipocytes, myocytes, nervous system, immune cells like macrophages; gallbladder and intestine (Perino and Schoonjans, 2015). When stimulated by BAs, Tgr5 activates membrane bound enzyme adenylyl cyclase and this increases the synthesis of cAMP as second messenger which activates protein kinase A (PKA) which induces further downstream signaling (Pols et al., 2011).

Through Tgr5/cAMP activation in intestine may have an anti-inflammatory effect by inhibiting cytokine production by macrophages through the Nfkb pathway explained above, hence protecting intestinal barrier integrity in mice and humans (Chiang 2013; Perino and Schoonjans 2015). Cipriani et al. (2011) showed, in a robust experiment in mice, that Tgr5 expressed in colonic tissue was involved in intestinal homeostasis and inflammation and described a higher expression of this receptor in colonic tissue of patients with Crohn's disease. Lack of Tgr5 in KO mice led to TJ dysregulation and permeability increase. Further, the authors showed that a synthetic ligand of TGR5 could decrease expression of proinflammatory cytokines Tnfa, Il6 and Il1β when colitis was induced, although Nfkb was not examined.

Regulation of intestinal inflammation via Fxr has been suggested to occur in mammals by impacting proinflammatory cytokines in intestinal macrophages and colonic enterocytes. A thorough study in mice and human observed decreased Fxr expression in the intestine of diseased subjects. Colonic activation of Fxr by a semi-synthetic ligand led to reduced mRNA levels of Tnfa and Il8 among others, attenuating inflammation associated to Crohn's disease. Fxr was detected in both colonic enterocytes as well as intestinal macrophages, where Fxr repressed expression of genes regulated by TLR induction and transcriptionally controlled by Nfkb (Vavassori et al., 2009). Similarly, Gadaleta et al. (2011) observed a reduction of proinflammatory cytokines in intestinal cells of mice exhibiting colitis when Fxr was activated together with reduced Tnfa and restored permeability levels. A more recent study corroborated the anti-inflammatory effects of taurocholic acid and glycoholic acid in a LPS-induced zebrafish model (Ge et al., 2023). Interestingly, activation of Fxr by CDCA has also been related to elimination of Mylk overexpression in addition to enhanced Occln and Cldn expression (Song et al., 2019).

These signaling cascades may potentially attenuate chronic inflammation as has been suggested in mammals with IBD (Cipriani et al., 2011; Fiorucci et al., 2018). The role of BAs in this context might become even more critical considering the changes to the microbiota during the disease and the role it plays in BA processing through deconjugation and dihydroxylation (Biagioli et al., 2021). Changes in the microbiota may cause an imbalance in intestinal bile composition, aiding in continuing the disease (Biagioli et al., 2021) Given the similarities between IBD and SBMIE, this opens a door to further explore the potential of BA in SBMIE mitigation in fish.

A summary of studies testing the role of BAs in fish fed plant-based diets is presented in Table 1.5. Based on the lipid emulsifier function of BAs and the negative effects that SBM-based diets have on lipid absorption, most studies have focused on the potential that BAs could have as a dietary supplement in high inclusion SBM diets. Murashita et al. (2018) did not observe any significant impact of 1.0% dietary BA (taurocholic acid) supplementation in high inclusion level SBM diet in terms of growth and BA reabsorption and synthesis in rainbow trout. A study in largemouth bass took the approach of using 1.0% supplemental BA to increase the carbohydrate levels of the diet to be used as main source of energy and spare protein and lipid and maximize lipid absorption. The results showed that dietary BA improved growth performance in low lipid/high starch diets (Romano et al., 2022). A similar study in that same species fed high starch diets showed that 350 mg Kg^{-1} (0.03%) dietary BA led to improved growth performance and a significant increase in antioxidant enzyme activity (Guo et al., 2020). Overall, the supplementation of lecithin or different forms of BA appears to enhance lipid digestibility in several commercial species (Jiang et al., 2018; Lin et al., 2022; Nguyen et al., 2013; Nguyen and Van Do, 2021; Yao et al., 2021).

Although to a lesser extent, investigations of the role of dietary BAs on intestinal inflammation exist in fish. In rainbow trout, dietary bovine bile salts (1.5% - 2.0%), cholyltaurine (1.0%), and soybean lecithin (2.0%), were able to mitigate detrimental effects of SBM-based diets on intestinal and liver histology and promoted phospholipid absorption and digestion in addition to growth (Iwashita et al., 2008; Yamamoto et al., 2007). However, contradictory results were found in Atlantic salmon, as no signs of improved growth nor diminished intestinal inflammation were observed after different supplemental combinations of bovine bile salts, lecithin and taurocholate, suggesting possible cytotoxic effects of BAs in the intestinal mucosa, since higher levels of hydrophobic BAs are found in bovine bile salt (Kortner et al., 2016).

Vitamin D

Vitamin D is a fat-soluble secosteroid hormone, hence an organic substance that is essential to normal metabolism and which deficiency may lead to associated diseases (Dawood et al., 2016). It has been extensively studied due to its crucial role in skeletal tissue homeostasis through regulation of calcium and phosphorus absorption in the intestine and resorption in the kidney (Haussler, 1986). The term vitamin D is broad, and some clarification is needed to distinguish its roles and functions. Firstly, one can separate ergocalciferol (vitamin D_2, VD_2) and cholecalciferol (vitamin D_3, VD_3) (Jones and Pike, 2019). The former is barely detected in circulation whereas the latter has been shown to be an inactive structural precursor for the active metabolites that are involved in physiological regulation (Vieth, 2020). In short, activation of vitamin D (cholecalciferol, VD_3 hereafter) occurs through hydroxylation in the liver to 25-hydroxyvitamin D_3 ($25(OH)_2D_3$, $25D_3$), also referred to as calcidiol; and a second hydroxylation in the kidney, carried out by the enzyme 1-alpha hydroxylase, to render 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$; $1,25D_3$), also known as calcitriol (Lock et al., 2010). $1,25D_3$ is the specific ligand of the vitamin D receptor (Vdr), a nuclear receptor that acts as a transcription factor, regulating gene expression upon recognition of VD_3 responsive elements in the genome. A key difference in fish is that both hydroxylation steps take place in the liver, making $25(OH)_2D_3$ plasma levels considerably lower as compared to mammals, although kidney hydroxylation is also possible (Fraser, 2018; Takeuchi et al., 1991). Another metabolite of VD_3 that is found in fish is 24,25 – dihydroxyvitamin D_3 ($24,25(OH)_2D_3$), and carbon 24 hydroxylation is considered the initial step in the inactivation of the secosteroid hormone, induced by the hormone itself (Fraser, 2018; Lock et al., 2010). The final metabolite is calcitroic acid which is excreted in the bile and shown to be subjected to enterohepatic circulation (Yu and Arnold, 2016).

In fish, $24,25D_3$ which seemed to have a role during transfer of rainbow trout to salt water, facilitating adaptation to an environment rich in calcium, decreasing binding of $1,25D_3$ to its

intestinal receptor (Lock et al., 2010). In Atlantic salmon, VD_3 requirements were determined to be higher in seawater rather than freshwater (Prabhu et al., 2019). However, this effect is not limited to anadromous fish species, as it was also detected in Atlantic cod (*Gadus morhua*) and has been shown to exert a counteractive effect to $25D_3$ and $1,25D_3$, reducing calcium uptake in the intestine (Fraser, 2018).

VD_3 uptake also differs between fish and mammals: in mammals VD_3 can be synthesized in the skin by exposure to ultraviolet (UV) radiation and through diet. In fish VD_3 can also be synthesized through sunlight exposure, as the precursor 7 – dehydrocholesterol has been detected in the skin of several species including tilapia and rainbow trout (Fraser, 2018). However, VD_3 obtention through the photochemical pathway is considered unsignificant due to limited exposure to sunlight in natural habitats. Hence uptake must happen through the diet which is done from phytoplankton (VD_2) and zooplankton (VD_3) in the wild; or via dietary supplementation of aquaculture feeds (Fraser, 2018; Rao and Raghuramulu, 1996). Absorption of VD_3 occurs in the intestine, associated with dietary fat, and transported to the liver in chylomicron lipid (Fraser, 2018). Storage of VD_3 occurs in the liver and adipose tissue, which is also in contrast to mammals, and it is often readily metabolizable (Lock et al., 2010).

A VD_3 endocrine system as described in mammals has been shown in fish, thus regulating calcium and phosphate absorption and resorption towards bone turnover and homeostasis, all mediated by hormones (Fraser, 2018). The active metabolite $1,25D_3$ enhances calcium uptake in the intestine and increases expression of Na^+ dependent phosphorus co transporters in fish (Verri and Werner, 2019). $1,25D_3$ is not the sole regulator of calcium and phosphorus homeostasis but has a synergistic action together with parathyroid hormone (Pth), which is synthesized in the brain. Both PTH and 1,25D3 are stimulated when serum calcium levels are low, and consequently promote calcium release from bone, absorption in the intestine and resorption in kidney, with the key difference that $1,25D_3$ also promotes phosphorus resorption in the kidney while Pth enhances phosphorus renal excretion (Haussler et al., 2013). Other important factors involved in this regulation are fibroblast growth factor 23 (Fgf23), which is also present in fish, produced in the corpuscles of Stannius (CD), and released in the presence of Pth and during periods of high phosphatemia and to repress synthesis of $1,25D_3$. Further hormones are in control of calcium homeostasis in fish. Stanniocalcin is synthesized in the CS in response to hypercalcemia, to reduce calcium uptake in the intestine and gills and increase phosphorus resorption as well as enhancing calcium release. Calcitonin has hypocalcemic effects also, and its role it not well described and considered less relevant or secondary (Verri and Werner, 2019).

Transportation of VD_3 occurs via vitamin D binding protein (Dbp) whereas its absorption and endocrine effects occur through binding to the Vdr. In fish, Dbp shows a higher affinity for D_3 as compared to D_2, and most of the VD_3 in circulation is bound to DBP in the form of $1,25D_3$, as that is the predominant metabolite of VD_3 found in fish plasma (Lock et al., 2010).

The Vdr is a receptor belonging to the large family of nuclear receptors, although it can be expressed in the cellular basolateral membrane too (Lock et al., 2010). In fish, nuclear Vdr is widely expressed and can be found in intestine, liver, kidney, pituitary gland, gills and corpuscles of Stannius; later research showed its expression in the nervous system, osteoblasts and chondrocytes in gill filaments and bile duct epithelia (Craig et al., 2008; Haussler, 1986; Lock et al., 2010). As a nuclear receptor, Vdr is found in the cytoplasm complexed with heat shock proteins and remains inactive (Lodish et al., 2012). Upon binding to its ligand, the Vdr is transported to the cell nucleus where, via the formation of a heterodimer with the retinoid X receptor (Rxr), modulates gene expression through recognition of VD_3 responsive elements in the DNA (Lodish et al., 2012). Interestingly, studies in mammalian intestinal cells found that localization of the Vdr in the nucleus as well as Vdr abundance was not always affected by ligand treatment (Peleg and Nguyen, 2010).

It is important to consider that the Vdr is a phosphoprotein and contains sites for potential phosphorylation PKC. When phosphorylated at a specific serine residue, this could lead to its inactivation (Hsieh et al., 1991). PKC is activated by increasing levels of intracellular calcium, which could mean the Vdr is subjected to a desensitization mechanism by its own effect on increased calcium uptake (Haussler, 1986; Hsieh et al., 1991; Krishnan and Feldman, 1991). In addition, catabolism of $1,25D_3$ to calcitroic acid is controlled by Vdr as widely demonstrated in mammals (Jones and Pike, 2019) as well as fish (Berntssen et al., 2015; Lock et al., 2010), consequently desensitizing the target cell to excessive $1,25D_3$ stimulation. In turn, the Vdr conformation can suffer changes depending on the VD_3 analogue binding to it, and consequently impact Vdr signalling (Jones and Pike, 2019).

The role of VD_3 in immune response activation and regulation has gained importance over the last years, suggesting direct applications in the development of functional diets. Findings in mammals indicating the presence of the Vdr as well as the enzyme 1-α hydroxylase in dendritic cells and macrophages, and hence the ability to produce and respond to $1,25D_3$, suggest an autocrine or paracrine function of vitamin D during immune response and infection (Limketkai et al. 2017, Cantorna et al. 2019). The involvement of $1,25D_3$ and Vdr in the immune system led researchers to explore its function in autoimmune diseases as well as colon cancer (Sun, 2010; Y. Zhang et al., 2013).

Several mechanisms have been suggested in mammals by which VD_3 may exert immunomodulation. Synthesis of $1,25D_3$ and activation of Vdr expression is triggered differently in the immune system, as compared to its normal activation and synthesis in kidney (in mammals). During infection, studies in mice show that synthesis and activation of $1,25D_3$/Vdr in immune cells happens through cytokine ligands and T cell receptors stimulation in macrophage and innate immune cells at 3 days post-infection (Cantorna, et al. 2019). In this review, Cantorna et al., (2019) model the role of vitamin D in immunity as a factor that controls immune response after infection, protecting from immunopathology and tissue injuries. Mechanistically, the model established in that review points out an effect of $1,25D_3$ in cytokine release, promoting anti-inflammatory over pro-inflammatory cytokines. During early stages of infection, it stimulates macrophage production of the anti-inflammatory cytokine IL-10. In addition, as the infection is cleared at later stages, T cells start producing $1,25D_3$ locally, which will decrease the production of Il17, Tnfa and interferon gamma (Ifng), preventing chronic inflammation. The Nfkb, is also known to be a target of the Vdr and it was observed in salmonella infected mice that Vdr could bind the p65 subunit of Nfkb, preventing its translocation to the nucleus; but this did not happen in Vdr knockout mice (Wu et al., 2010). These same authors found increased expression of Vdr in intestinal epithelium, showing its active role mediating immune response. Moreover, they observed that Vdr expression in epithelial cells was increased following bacterial infection alone, in the absence of its traditional ligand $1,25D_3$. Other mechanisms involve interactions with Pth to increase synthesis of the antimicrobial peptide cathelicidin (Aranow, 2017; Haussler et al., 2013; Y. Zhang et al., 2013).

Interestingly, there is extensive research done in mammals in the context of intestinal immune disorders, with a special focus on IBD, and the influence of VD_3. Studies have described a correlation between intestinal immunopathology and deficiency of VD_3, although it is not clear whether VD_3 deficiency is a cause or a consequence of intestinal damage (Limketkai et al., 2017). In addition, *vdr* expression is decreased in patients with IBD (Wu et al., 2010). Ligands of the Vdr have been suggested to regulate inflammatory signaling as well as tight junction proteins that are key to maintaining intestinal barrier function and are also severely affected during IBD (Cantorna et al., 2019a, 2019b; Y. Zhang et al., 2013). In addition to the effects mentioned above, VD_3 and Vdr agonists have also been related to decreased Tnfa (Marchiando *et al.* 2011, Cantorna *et al.* 2019). Nonetheless, these observations should be interpreted with caution, as the Vdr could be inactivated by PKC, a protein that also seems to be expressed during IBD, supporting the observed reduced expression of VDR in patients with IBD as mentioned above.

Beneficial effects of VD_3 on IBD may also happen through improving altered TJ expression, by increasing the expression levels of claudin-2 and 12 (Y. Zhang et al., 2013). Studies in

mice testing the effects on VD_3 administered by colorectal injection showed an amelioration of the disease with no associated hypercalcemia (Sun, 2010). These observations in IBD suggest a potential application of dietary VD_3 supplementation in SBM-based diets to tackle SBMIE. The involvement of VD_3 in regulating immune response has also been documented in fish. A study in Atlantic salmon showed that VD_3 improved the anti-bacterial activity of macrophages against *Aeromonas salmonicida* challenge *in vitro*, reduced percentage of pathogen attachment to macrophages, enhanced expression of genes involved in neutrophil recruitment, and decreased growth of the pathogen. However, high doses of VD_3 (10,000 ng/mL) compromised macrophage viability, suggesting its potential toxic effects (Soto-Dávila et al., 2020). Other challenge studies were performed in yellow catfish (*Pelteobagrus fulvidraco*) (Cheng et al., 2020) and turbot (Liu et al., 2021). In turbot, scientists observed an upregulation of genes controlling T cell expression, while IL-17 was downregulated which is in accordance with Cantorna et al. (2019) model. In yellow catfish, survival was higher at higher dietary levels of VD_3, and this was accompanied of lower expressions of the pro-inflammatory cytokines Il6 and the Tnfa as well as upregulation of Il10 in the head kidney. A different study was carried out in European seabass in which immunomodulatory effects of VD_3 were observed in terms of increased survival rate and serum peroxidase activity after four weeks in a dose and time dependent manner, together with upregulation of immune related genes in the gut, but there was no pathogen challenge (Dioguardi et al., 2017). Lastly, in black carp (*Mylopharyngodon piceus*), a dietary dose of 13.3 μg Kg^{-1} had a positive impact on growth performance as well as antioxidant enzyme activities and increased gene expression of lysozyme (Wu et al., 2020). Initial signs of a positive effect of dietary VD_3 in modulating intestinal immunity have been recently shown in turbot subjected to a pathogen challenge source of stress (Shao et al., 2022). However, the effects of dietary VD_3 on SBMIE modulation have not been investigated in fish.

The supplementation of VD_3 in fish feeds is controlled and kept within stipulated ranges to ensure safe fish products for consumers (Prabhu et al., 2019). It is known that excess may lead to deformities, reduced growth and mineralization of the skeleton (Craig et al., 2008), but bone resorption has also been seen in cichlids (Wendelaar Bonga et al., 1983). For rainbow trout and Atlantic salmon the requirement is currently 0.04 and 0.06 mg Kg^{-1} diet, respectively, and the maximum limit in the feed is at 75 μg Kg^{-1} diet (Jakobsen et al., 2019; Prabhu et al., 2019). Previous experiments in Atlantic salmon fry have shown tolerance to dietary VD_3 doses to be as high as 57 mg Kg^{-1}, and found no signs of detrimental effects in terms of skeletal deformities (Graff et al., 2002). In rainbow trout, Mattila et al. (1999) showed that feeding higher VD_3 levels translated in higher contents in the liver, but no significant increase was found in the fillets. In agreement, Horvli et al. (1998) indicated that liver, intestine and plasma had the highest VD_3 concentrating abilities in

Atlantic salmon. These observations suggest a reduced risk of hypervitaminosis D in these species. Nonetheless, high doses of dietary VD_3 should be carefully examined in different species, as initial signs of deformities have been detected in gilthead seabream (Dominguez et al., 2021).

In support of increasing dietary VD_3 for its use as an additive, studies revising VD_3 requirements in the diet of salmonids have pointed out that higher levels would be required when feeding plant-based diets to fulfill the requirement (Prabhu et al., 2019). As these authors indicated, the use of alternative sources of protein other than FM made it necessary to reassess micronutrient requirements such as vitamin D in the diets of farmed salmon. They concluded that, for salmon, VD_3 requirement was 1.5 -2-fold higher than what had been reported earlier, reaching a saturation point in tissue at 0.08 mg Kg^{-1} diet. This is in accordance to guidelines in European Union, where 0.075 mg Kg^{-1} is the maximum allowed in fish feeds (Jakobsen et al., 2019). All this information suggests that VD_3 supplementation could be a good area of study for feeding rainbow trout SBM-based diets, given the involvement of VD_3 in intestinal health, the apparent tolerance of this species to this micronutrient, together with low rates of deposition in the muscle, and considering the increased requirement when feeding a high-level plant-based diet.

Additive interactions

Given the broad range and complexity of signaling pathways in which some of the described additives are involved in, it is advisable to consider possible crosstalk, interactions, and synergies.

Bile acids, vitamin D and vitamin A

Vitamin D as well as vitamin A, another fat-soluble protein, require BAs for their absorption; however, it has been questioned whether both can affect bile synthesis regulation. As explained above, synthesis of BAs is regulated in a feedback-loop mechanism upon binding the nuclear receptor FXR, which forms a heterodimer with Rxr (Chiang, 2009). The Fxr-Rxr heterodimer is activated by BAs both in the enterocyte and the hepatocyte and acts as a transcription factor to activate expression of Fgf19 which inhibits expression of the BA biosynthetic enzyme in the liver (Chiang, 2013; Romano et al., 2020). On the other hand, vitamin A activates its nuclear receptor retinoic acid receptor (Rar) in the liver which also partners with RXR (Schmidt et al., 2010). Indeed, vitamin A, when sufficiently acquired from the diet, may control (suppress) BA synthesis in the liver by activating Rxr-containing heterodimers or homodimers, which has been described to affect expression of BA synthesis enzymes as well as BA transporters in both liver an intestine (Saeed et al., 2017). Conversely, a vitamin A deficiency scenario would lead to stimulation of BA synthesis and

export (Saeed et al., 2017). Intuitively, because Rxr is an obligate partner of Fxr, Vdr, and Rar - among others- one can think of possible crosstalk in regulatory pathways. It is known, for instance, that Vdr is a non-permissive receptor, which, upon binding to Rxr will not allow binding to Rar (Haussler et al., 2013).

The mechanisms by which both vitamins regulate BA synthesis is through Fgf19 transcriptional activation in the intestine by distinct mechanisms but both inducing repression of BA synthesis. Schmidt *et al.* (2010) found that $1,25D_3$ treated mice showed higher expression of *fgf15* (ortholog of human *fgf19*) in ileum enterocytes and consequent decreased expression of *cyp7a1* in the liver. Moreover, Vdr seemed to be required for normal *fgf15* expression and BA levels. In the same study, vitamin A was shown to exert negative feedback on BA synthesis through Fgf15 via the Fxr-Rxr heterodimer.

More recent studies have revealed additional mechanisms of regulation by vitamin A in human, which include synergistic action with BAs through the Fxr-Rxr complex, but also a direct way of vitamin A-mediated Fgf19 upregulation through Rar-Rxr, independently of Fxr (Jahn et al., 2016). Of note, Fgf19 as well as Fxr signaling have been described in zebrafish, with conserved bile regulatory mechanisms (Wen et al., 2021), which could open an area of study in fish to further understand additional functions of vitamin D and other lipid soluble vitamins. An important fact to take into consideration, however, is that, as observed by Saeed et al. (2019) both depletion or activation of Fxr in the liver by a pharmacological Fxr agonist led to impaired liver vitamin A storage, hence, the therapeutic role of BAs should be carefully considered regarding its possible associated adverse effects on vitamin A metabolism.

Lastly, LCA, a secondary BA is known to bind the Vdr, and that Vdr can act as a sensor to promote LCA detoxification and maintain bile homeostasis by initiating transcription of enzymes for their catabolism (Makishima et al., 2002; Teske et al., 2016). Studies *in vitro* have confirmed anti-inflammatory and barrier enhancing effects of LCA to be mediated via Vdr, rendering reduced expression levels of *tnfa*, *nfkb* as well as increasing TJ expression (Yao et al., 2019). In addition, the final metabolite of VD_3, calcitroic acid, has also been shown to interact with Vdr, initiating detoxification of excess BAs in the intestine, as well as anti-inflammatory responses (Teske et al., 2016; Yu et al., 2021; Yu and Arnold, 2016). The use of LCA should be considered with caution, as its high hydrophobicity confers a higher degree of cytotoxicity (Hofmann, 1999).

Conclusion and Objectives

Efforts made over the years towards more sustainable aquafeeds have helped in the transition from SBM being an alternative ingredient to rendering it an essential ingredient in practical feed formulations (Kari et al., 2023). However, limitations still exist, especially when it comes to carnivorous species like rainbow trout, which remain rather sensitive to high inclusion levels of SBM. Therefore, it is imperative to explore novel approaches that allow improving performance and ensuring optimal welfare. Feed additives have gained attention as they represent an effective way to enhance feed formulations while maintaining affordability (Dawood et al., 2018). Thus, the overarching goal of this dissertation was to evaluate the development of SBMIE during long-term time periods by identifying key biomarkers and exploring the impact of different dietary additives in SBMIE mitigation.

The specific objectives and hypotheses were:

1. The use of the amino acid Gln as a dietary supplement in a 30% inclusion level SBM diet could ameliorate detrimental effects of SBM in the intestine and promote growth and compare performance of two commercial rainbow trout strains. The additional hypothesis of whether Gln effects are maintained in the long term were evaluated.
2. The use of BAs as dietary supplements in 30% and 40% inclusion level SBM diets to mitigate detrimental effects of SBM in intestine and liver and evaluate possible cytotoxic effects of bile in the short and long term.
3. Supplementation of high doses of VD_3 in the diet could attenuate SBMIE and promote growth without causing signs of hypervitaminosis D or hypercalcemia. As an additional hypothesis, the possible combined action of VD_3 and Gln and whether that combination could further improve SBMIE symptoms and related deleterious effects was also examined.

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Table 1.1. Crude protein composition and essential amino acid composition on dry matter (g Kg^{-1}) of soybean meal and other plant protein ingredients. Soybean meal and canola meal are solvent extracted; all other ingredients are heat extracted (Kumar 2011).

Proximate composition (g Kg^{-1})		Essential amino acids (g Kg^{-1})								
Ingredients	Crude protein	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine
Soybean meal*	518	42	14	23	44	28	9	27	24	24
Canola meal*	431	32	26	3	25	41	30	27	16	78
Sunflower meal	422	36	11	17	26	12	7	20	13	23
Corn gluten	602	19	13	25	102	10	14	38	21	28
Cottonseed meal	414	45	12	13	25	17	7	22	14	18
Pea meal	252	19	11	14	41	27	6	19	17	14
Rapeseed meal	385	21	11	14	24	20	8	16	15	17
Wheat meal	145	6	3	4	9	3	7	2	4	5

Table 1.2. Antinutritional factors (ANFs) found in soybean meal (SBM), their respective amounts and physiological effect.

ANF	Amount (g/Kg)	Physiological effects	Source
Phytates	15 - 18 *	Chelate minerals, reduced nutrient availability	Gatlin et al. 2007
Oligosaccharides	50 *	Increase chyme viscosity, impaired nutrient uptake	Gatlin et al. 2007
Trypsin inhibitors	0.01 **	Reduced protein digestibility	Gatlin et al. 2007

Table 1.2. Cont.

Isoflavones	0.67 - 1 ***	Bile acid and cholesterol synthesis imbalance	Krogdahl and Bakke 2015
Saponins	5 *	Intestinal inflammation	Knudsen et al. 2007
Lectins	< 0.0001	Bind glycoproteins in intestinal epithelia	Gatlin et al. 2007

* Data taken from the review of Zhou et al. (2018).
** Data taken from the chapter of Galvan et al. (2016).
*** Data taken form the study of Sudar et al. (2012).

Table 1.3. Summary of studies that investigated different effects of dietary soybean products and related soybean meal-induced enteritis (SBMIE) symptoms.

Enteritis effect	Species	Soybean product	Reference
Reduced growth performance	Atlantic salmon (*Salmo salar*)	~30% SBM	Baeverfjord and Krogdahl 1996 Krogdahl et al. 2003
	Rainbow trout (*Oncorhynchus mykiss*)	20% SBM, 20% FSBM	Choi et al. 2019; Venold et al. 2012; Yamamoto et al. 2007; Rumsey et al. 1994
	Turbot (*Scophthalmus maximus*)	~35-40% SBM	Liu et al., 2018; Gu et al., 2017; Bai et al. 2017
	Jian carp (*Cyprinus carpio* var. Jian)	Glycinin/β-conglycinin (0.8%)	Jiang et al. 2015; Zhang et al. 2013
	Gropuer, hybrid (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*)	SBM/FSBM (7.5-37%/ 12-24%)	Zhang et al. 2019, 2021; He et al. 2020
	Asian seabass (*Lates calcarifer*)	SBM, FSBM, full-fat soy ≥35%	Tola et al. 2022; Hong et al. 2020; Ma et al. 2019; Ilham et al. 2016; Boonyaratpalin et al. 1998
	Sturgeon, hybrid	SBM >35%	Yue et al. 2022; Wang et al. 2011; Qiyou et al. 2011
	Snakehead (various)	SBM/FSBM (7-28%/ 17-70%)	Duan et al. 2022 Miao et al. 2018; Hien et al. 2015

Table 1.3 cont.

	Largemouth bass (*Micropterus salmoides*)	~ 40-45% SBM/BSBM	Romano et al. 2021
	Totoaba (*Totoaba macdonaldi*)	16-50% SBM	Fuentes-Quesada et al. 2018, 2020
	Red drum (*Scianeps ocellatus*)	~ 30% SBM	Cheng et al. 2011
Histopathology of distal intestine	Atlantic salmon	20 – 30% SBM	Bakke-McKellep et al. 2000; Krogdahl et al. 2003; Uran et al. 2008 Venold et al. 2013; Baeverfjord and Krogdahl 1996 Sahlmann et al. 2013
	Rainbow trout	20-40% SBM, 27% FSBM	Barnes et al., 2015; Romarheim et al. 2008 Yamamoto et al. 2007; Bruce et al., 2018; Heikkinen et al., 2006; Merrifield et al., 2009; Mosberian-Tanha et al., 2018; Rumsey et al., 1994; Venold et al., 2012; Choi et al. 2019
	Turbot	26-54% SBM, saponins (0.25-1.5%)	Gu et al., 2018, 2017, 2016; Liu et al., 2018; Bai et al. 2017
	Common carp (*Cyprinus carpio* L.)	20% SBM	Uran et al. 2008
	Grouper, hybrid	SBM >25% approx.	Zhang et al. 2021; He et al. 2020

Table 1.3 cont.

	Asian seabass	21% SBM, ~28% full-fat soy	Boonyaratpalin et al. 1998
	Sturgeon, hybrid	20% SBM	Qiyou et al. 2011
	Beluga sturgeon (*Huso huso*)	20-40% SBM	Sohrabnezhad et al. 2017
	Totoaba	SPC/SBM >20%	Fuentes-Quesada et al. 2018, 2020
	Snakehead	SBM/FSBM (7-28%/ 22-70%)	Duan et al. 2022; Miao et al. 2018
	Red drum	~ 30% SBM	Cheng et al. 2012
	Largemouth bass (*Micropterus salmoides*)	~ 40-45% SBM/BSBM	Romano et al. 2021
	European seabass (*Dicentrarchus labrax*)	Full-fat soy/SPC (12.8/13.6%)	Rimoldi et al., 2016
Inflammatory cytokine upregulation in distal intestine	Atlantic salmon	20% SBM	Sahlman et al. 2013; Tsai et al., 2023
	Rainbow trout	21% SBM	Kumar et al. 2021; Blaufuss et al., 2019; Tsai et al., 2022
	Turbot	26-54% SBM, saponins (0.25-1.5%)	Liu et al., 2018; Gu et al., 2017; Bai et al. 2017
	Common carp	20% SBM	Uran et al. 2008
	Jian carp	Glycinin/β-conglycinin (0.8%)	Jiang et al. 2015; Zhang et al. 2013
	Grouper, hybrid	SBM/FSBM (7.5-37%/ 12-24%)	Zhang et al. 2021; He et al. 2020
	Totoaba	16-50% SBM	Fuentes-Quesada et al. 2018

Table 1.3 cont.

	Snakehead	22-70% SBM	Miao et al. 2018
Other observations			
Mineral status	African catfish (*Clarias gariepinus*)	30-60% SBM	Toko et al. 2008
Digestive enzymes	Atlantic salmon	30% SBM	Bakke-McKellep et al. 2000
	Rainbow trout	20% SBM	Choi et al. 2019; Yamamoto et al. 2007
	Grouper, hybrid	12-24% FSBM	Zhang et al. 2021
	Asian seabass	30-50% SBM	Ma et al. 2019
	Totoaba (*Totoaba macdonaldi*)	16-50% SBM	Fuentes-Quesada et al. 2018, 2020
	Snakehead	7 – 28% FSBM	Duan et al. 2022
Antioxidant metabolism	Jian carp	0.8% β-conglycinin	Zhang et al. 2013
	Sturgeon, hybrid	20% SBM	Qiyou et al. 2011
	Snakehead	7 – 28% FSBM	Duan et al. 2022
	Red drum	~ 30% SBM	Cheng et al. 2011

Table 1.4. Summary of studies that have investigated the effects of different dietary levels glutamine (Gln) for different time periods in commercial fish species.

Species	Dietary glutamine (%)	Duration (weeks)	Physiological impact	Reference
Carp (*Cyprinus carpio* var. Jian)	0.0, 0.4, 0.8, 1.2, 1.6, 2.0	11	Healthier intestine morphology	Yan and Qiu-Zhou 2006
Carp (*Cyprinus carpio* var. Jian)	1.2	6	Enhanced expression of CAT and GPx; Healthier DI morphology	Jiang et al. 2015
Hybrid sturgeon (*Acipenser shrenkii* × *A. baerii*)	0.0, 0.25, 0.50, 0.75, 1.0	7	Enhanced expression of SOD	Wang et al. 2011
Hybrid sturgeon (*Acipenser schrenkii* × *Huso dauricus)*	0.3 - 1.5	8	Increased antioxidant metabolism; Digestive enzymes activity; Healthier DI morphology	Qiyou et al. 2011
Hybrid sturgeon (*Acipenser baerii* × *A. shrenkii*)	1.0	8	Improved weight gain; Improved liver health	Yue et al. 2022
Turbot (*Scophthalmus maximus*)	2.0	12	Lower pro-inflammatory cytokines; Higher MLCK expression; Higher TJ expression; Helathier DI morphology	Liu et al. 2018

Table 1.4 cont.

Turbot (*Scophthalmus maximus*)	1.5 (glutamine and arginine)	8	Enhanced weight gain and feed efficiency; Non-specific immune response; Healthier DI morphology	Gu et al. 2017
Gilthead seabream (*Sparus aurata*)	4.0	7	Protein retention	Caballero-Solares 2015
Red drum (*Sciaenops ocellatus*)	1.0, 2.0; glutamine+arginine	7	Improved weight gain; Non-specific immune response; Healthier DI morphology	Cheng et al. 2012
Hybrid striped bass (*Morone chrysops* × *Morone saxatilis)*	1.0, 2.0 glutamine/arginine or glutamine + arginine	8	Improved wight gain and feed efficiency; Non-specific immune response	Cheng et al. 2012
Zebrafish (*Danio rerio*)	1.0	4	Lower pro-inflammatory cytokines; Increased FABP2; Helathier PI morphology	Molinari et al. 2020

Table 1.5. Summary of studies that have investigated the effects of dietary emulsifiers and taurine in different commercial species fed SBM-based diets.

Species	SBM	Bile supplementation	Duration	Effect of bile on SBM related effects	Reference
Rainbow trout (*Oncorhynchus mykiss*)	SBM	1.5%, bovine bile salt	10 weeks	Improved growth performance; Increased bile content; Improved distal intestine morphology; Restored hepatocyte size	Yamamoto et al. 2007
	SBM (total FM replacement)	1.0% taurocholate	10 weeks	Improved growth performance	Murashita et al. 2018
	SBM	1.0% cholytaurine / 2.0% soy lecithin / 1.50% bovine salts	10 weeks	Improved growth performance; Healthier hepatic and intestinal morphology	Iwashita et al. 2008
Atlantic salmon (*Salmo salar*)	SBM	1.8% sodium taurocholate/1.8% bovine bile salt/1.5% lecithin	9 weeks	No signs of improved growth nor diminished intestinal inflammation	Kortner et al. 2016
Japanese yellowtail kingfish (*Seriola quinqueradiata*)	SBM, FSBM	1.5% taurine	8 weeks	Improved lipid digestibility; Higher liver and muscle lipid content and higher bile in content in gallbladder and intestine	Nguyen et al. 2013
Grouper (*Epinephelus lanceolatus*)	SBM	1.0% soy lecithin	6 weeks	Higher hepatic lipid content and improved liver health	Lin et al. 2021
Nile tilapia (*Oreochromis niloticus*)	SBM (combined with FM and CGM)	0.03% nutritional emulsifier	8 weeks	Improved growth performance; Reduced oxidative stress; Increased lipase activity	Wangkahart et al. 2022

Table 1.5 cont.

Nile tilapia, genetically improved (Oreochromis niloticus)	SBM 22%	0.05 - 1.35 g/Kg taurocholate	9 weeks	Improved growth performance; Normal liver morphology; Increased activity of liver and intestine lipases	Jiang et al. 2015
Pompano (*Trachinotus blochii*)	SBM, DSP	1.5% taurine	8 weeks	Improved growth performance; Increased lipid digestibility and lipase activity in DSP + taurine	Nguyen and Do 2021
Common carp (*Cyprinus carp*)	Plant proteins	0.6 % / 6.0% bile acid	11 weeks	Decreased FCR; Healthier distal intestine morphology; Upregulation of anit-inflammatory markers in distal intestine;	Yao et al. 2021

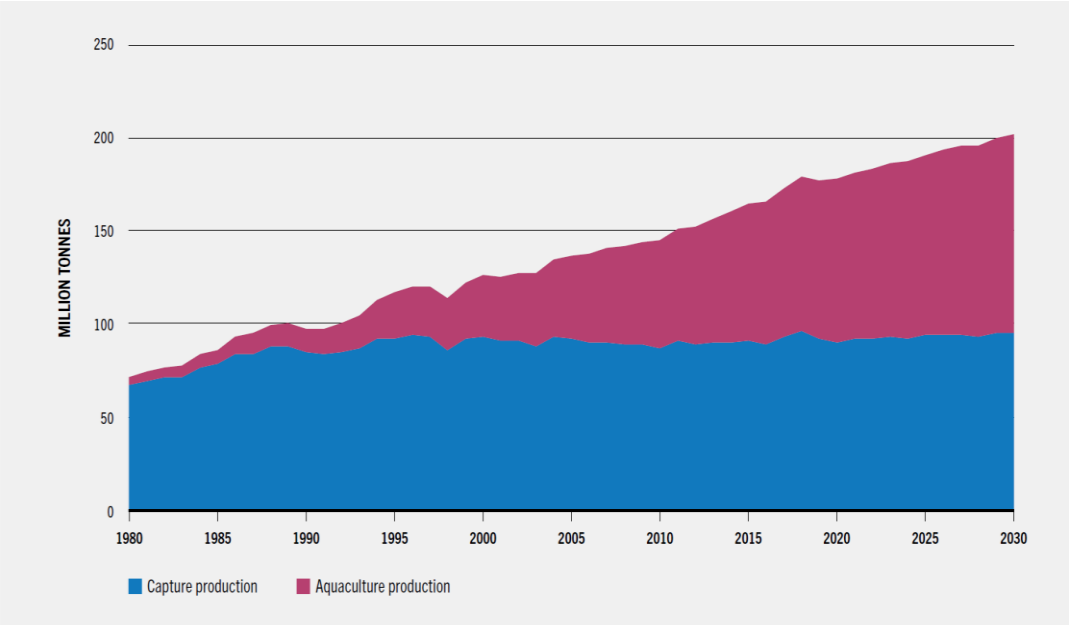

Figure 1.1. World capture fisheries and aquaculture (FAO, 2022).

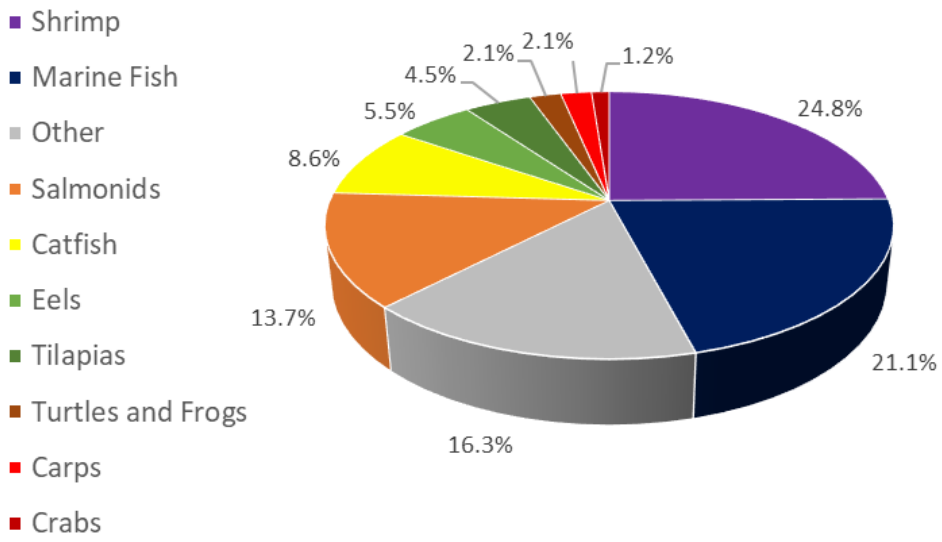

Figure 1.2. Global fishmeal consumption by aquaculture sector in 2020.
Source: IFFO 2020

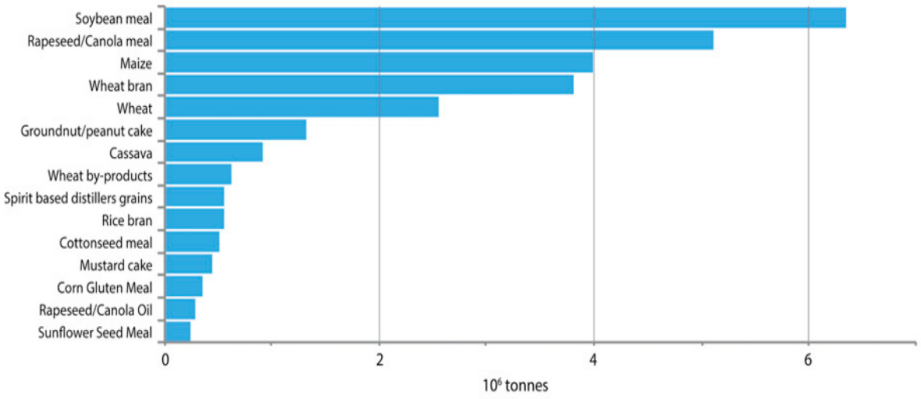

Figure 1.3. Crop feed ingredients used in aquaculture feeds (Troell et al., 2014).

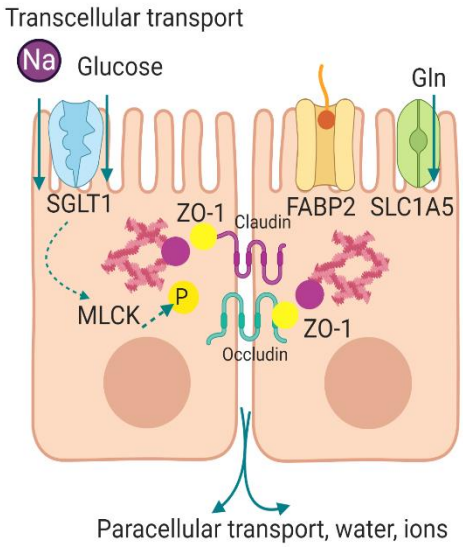

Figure 1.4. Schematic representation of enterocyte structure and shape including apical membrane transporters, tight junction proteins and actin cytoskeleton.

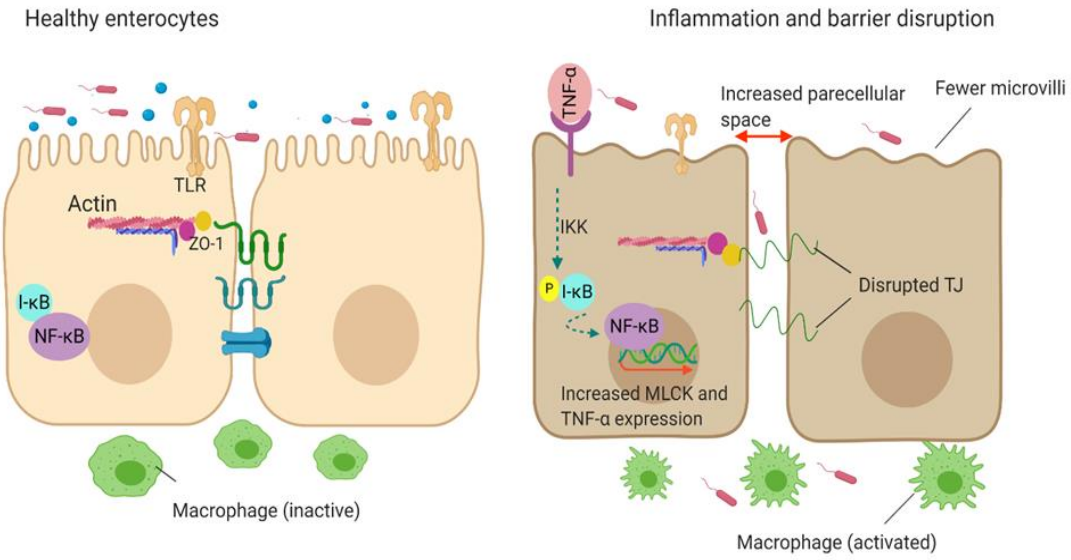

Figure 1.5. Comparison of endothelial barrier function in healthy enterocytes and loss of integrity in enterocytes in an inflammation context.

Chapter 2:

Evaluating long term effects of soybean meal-induced enteritis on growth and histology of two rainbow trout (*Oncorhynchus mykiss*) strains and the potential mitigating effects of the non-essential amino acid glutamine

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The following chapter is being prepared as an article for submission to Aquaculture Nutrition

Abstract

A long-term feeding trial was conducted to monitor the progression of soybean meal-induced enteritis (SBMIE) in two commercial strains of rainbow trout (strain R and strain T; average weight = 24.3 ± 1.0 g) and evaluate the potential protective role of the non-essential amino acid glutamine (Gln) on the severity of SBMIE. Three experimental diets were formulated to include a fishmeal diet (FM), a soybean meal diet (SBM, 30% inclusion level) and a SBM diet supplemented with 2.0% Gln (SBM-Gln). Diets were fed to both strains in triplicate for 30 weeks. Feed intake (FI) was recorded daily, and growth performance, feed conversion ratio (FCR) and distal intestine histology were recorded at six, twelve, eighteen, twenty-four and thirty weeks of the trial (W6, 12, 18, 24 and 30). FI was affected by diet type until W18, and an interaction between diet and strain was present at all time points after W6, where strain T fed the SBM diet had the lowest FI while the equivalent group in strain R had the highest. Weight gain of strain R fed the SBM diet was higher compared to the FM group after eighteen weeks, and significantly higher than both FM and SBM-Gln by week thirty. An interaction between diet and strain was observed at this point, where the FM and the SBM group of each strain followed opposite growth trends. FCR was affected by the interaction between diet and strain at W18 and W30, with significantly higher FCR in strain R fed the SBM diet by W30. Histopathology results show that both strains developed SBMIE, as evidenced by significantly shorter and wider villi, as well as goblet cell and inflammatory cell abundance as the most notable signs. Both strains similarly developed SBMIE, regardless of the interaction observed in weight gain and FI at various time points. Supplementation with Gln improved distal intestine morphology by ameliorating all observed parameters but did not reach control diet appearance. It can be concluded that a 30% inclusion level SBM does not compromise growth of commercial strains of rainbow trout, however SBMIE was evident in both strains. Dietary supplementation of 2.0% Gln in SBM based feed mitigated, to some extent, SBMIE in rainbow trout.

Introduction

The growing demand for protein sources from the aquaculture industry is posing an increasing need to shift from fish meal (FM) as the major source of protein in aquafeeds to more available and cost-effective alternatives (Hua et al., 2019; Little et al., 2016; Tacon et al., 2022). Soybean meal's (SBM) adequate protein content and amino acid composition, as well as reduced cost have made this an integral ingredient in fish feed formulations (Gatlin et al., 2007; Zhou et al., 2018). However, research is still needed when it comes to increasing inclusion levels above 25% in salmonid feeds (Barrows et al., 2008a; Krogdahl et al., 2003). As is the case with other plant-derived ingredients, SBM is known to contain a wide range of antinutritional factors (ANFs) such as trypsin inhibitors, phytates, non-starch polysaccharides and glycinin to name a few (Francis et al., 2001; Krogdahl and Bakke, 2015). Of these, heat-stable, alcohol soluble factors like saponins and isoflavones, which are not eliminated during ingredient processing and feed manufacture, may be of concern to some species. For instance, saponins have been shown to interfere with health and growth in both Atlantic salmon (*Salmo salar*) (Gu et al., 2015; Knudsen et al., 2007; Krogdahl et al., 2015) and turbot (*Scophthalmus maximus*) (Gu et al., 2018).

Rainbow trout (*Oncorhynchus mykiss*) is farmed for human consumption worldwide (FAO, 2020), and constitutes a good example of a carnivore exhibiting reduced tolerance to SBM-based diets, which leads to lower growth rates and protein utilization as well as morphological changes in the distal intestine epithelium (Baeverfjord and Krogdahl, 1996; Rumsey et al., 1994; Rungruangsak-Torrissen et al., 2006). This set of symptoms have been termed soybean meal-induced enteritis (SBMIE) (Krogdahl et al., 2020). Histopathological analysis in different fish species have helped study the signs of SBMIE, which encompass inflammatory cell infiltration, lamina propria thickening, altered villi morphology, and increased numbers of goblet cells and absorptive vacuoles (Bjørgen et al., 2020). Such symptoms have been largely evidenced in several species, including rainbow trout (Barnes et al., 2015; Bruce et al., 2018; Heikkinen et al., 2006; Merrifield et al., 2009; Mosberian-Tanha et al., 2018; Rumsey et al., 1994; Venold et al., 2012), Atlantic salmon (Bakke-Mckellep et al., 2000; Gajardo, 2016; Jacobsen et al., 2018; Krogdahl et al., 2003; Venold et al., 2013), even after short term exposure (Navarrete et al., 2013; Uran et al., 2008); turbot (*Scophthalmus maximus*) (Gu et al., 2018, 2017, 2016; Liu et al., 2018), European seabass (*Dicentrarchus labrax*) (Rimoldi et al., 2016), the omnivore Jian carp (*Cyprinus carpio* var. Jian) (Yan and Qiu-zhou, 2006), largemouth bass (*Micropterus salmoides*) (Romano et al., 2021a), and totoaba (*Totoaba macdonaldi*) (Fuentes-Quesada et al., 2018).

To improve production rates and enhance disease prevention, the use of functional feeds in aquaculture has become increasingly popular (Hua et al., 2019; Lulijwa et al., 2022). Functional

feeds are typically formulated to include a diverse range of additives such as organic acids, probiotics, amino acids, trace minerals and vitamins among others (Dawood et al., 2018; Li et al., 2020). Amino acids are known for being an energy source in metabolic pathways for protein synthesis in fish, with a consequent impact on growth and protein utilization in different species (Hou et al., 2015; Li et al., 2020). The neutral, non-essential amino acid glutamine (Gln) has been studied in higher vertebrates for its benefits in epithelial health, with direct applications on intestinal health (Kim and Kim, 2017; Li et al., 2020; Wang et al., 2015). Dietary Gln is also important considering its conversion to glutamate (Glu) and the role free Glu plays in digestive function and satiety signaling in the brain (Torii et al., 2013). This aspect may be critical for feed intake and increased capacity of protein retention (Caballero-Solares et al., 2012). Some examples of Gln benefits in higher vertebrates include pigs (Ewaschuk et al., 2011; Haynes et al., 2009) and chicken (Bortoluzzi et al., 2020; Soltan, 2008), where Gln proved beneficial either in terms of immune performance, growth and blood biochemistry, and intestinal health both during weaning periods or enteric infections. Studies with fish cells have shown anti-inflammatory effects of Gln *in vitro* in channel catfish macrophages (Pohlenz et al., 2012) and in rainbow trout leukocytes (Li et al., 2019). Several studies have pointed out the protective effects of Gln when supplemented in SBM based diets, in terms of growth performance and remission of SBMIE in the distal intestine. Some examples include red drum (*Scianepos ocellatus*) (Cheng et al., 2011), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Cheng et al., 2012), Jian carp (Jiang et al., 2015; Yan and Qiu-zhou, 2006), turbot juveniles (Gu et al., 2017; Liu et al., 2018), seabream (*Sparus aurata*) juveniles (Caballero-Solares et al., 2015), hybrid sturgeon (*Acipenser schrenckii* × *A. baerii*) larvae (Wang et al., 2011), hybrid sturgeon juveniles (Yue et al., 2022) and hybrid sturgeon (*Acipenser schrenckii* × *Huso dauricus*) (Qiyou et al., 2011). However, all these studies were carried out for a maximum period of twelve weeks, raising the question of whether these benefits can be maintained in the long term, for instance during a typical grow-out period as would take place in aquaculture farm operations. Furthermore, while the susceptibility of salmonids to SBMIE has been well described as discussed above, more research is necessary in species like rainbow trout to examine the suitability of Gln supplementation towards SBMIE amelioration. Therefore, the goal of this study was to examine the development of SBMIE in a long-term experimental period of thirty weeks and assess the potential benefits of Gln supplementation. Two different commercial strains of rainbow trout were selected in this trial to identify possible differences among strains and/or diet-strain interactions.

Materials and Methods

Experimental diets

The dietary formulation and proximate composition are presented in Table 1, and amino acid profile of the experimental feeds is shown in Table 2. Three isonitrogenous and isolipidic diets were formulated to contain 45% crude protein and 16% crude lipid. Experimental diets included a fish meal (FM) or control diet; a 30% inclusion level soybean meal diet (SBM) and the same SBM diet supplemented with 2.0% Gln (SBM-Gln). Glutamine (Ajinomoto Co., Inc.) was added to the diet as a glutamine-alanine dipeptide to account for thermolability of glutamine during the extrusion process.

All diets were produced with commercial manufacturing methods using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) at the Bozeman Fish Technology Center, Bozeman, MT. Diet mash was exposed to an average of 114°C for 18-s in five-barrel sections, and the last section was water cooled to an average temperature of 83°C. Pressure at the die head was approximately 450 psi. The pellets were then dried in a pulse bed drier (Buhler AG) for 25 min at 102°C and cooled at ambient air temperatures to reach final moisture levels of < 10%. Feed pellet size was 2.5 mm for the first 12 weeks, and 3.5 mm from week 13 to week 30 of the feeding trial. Fish oil was top-dressed using vacuum coating (A.J Flauer Mixing, Ontario Canada) after the pellets were cooled. Diets were stored in plastic lined paper bags at room temperature until used. Appropriate feed size was used throughout the feeding trial. All diets were fed within 4 months of manufacture.

Long-term feeding trial and sampling

For this trial, two commercial strains of rainbow trout, namely R and T, were transferred to the Cold-Water Lab at the Aquaculture Research Institute, University of Idaho (Moscow, ID). A total of 2,250 juvenile rainbow trout were randomly distributed into each of eighteen 350 L tanks (125 fish per tank), incorporating three replicate tanks per diet and strain. Fish were acclimated for one week, after which initial weights were 24.32 ± 1.0 g on average. Fish were maintained in a recirculating aquaculture system at 14.0 ± 1.0 °C and a 12 h dark/ 12 h light photoperiod. Water was supplied at a constant flow rate of 180 mL sec^{-1}. Oxygen was supplied by normal aeration and maintained at 5.73 ± 0.73 mg L^{-1}; pH was kept at 7.61 ± 0.16 and ammonia (NH3) and nitrite (NO2) were kept at < 0.1 mg L^{-1}. All parameters were monitored using commercial kits (LaMotte, Chestertown, MD, USA). During the long-term feeding trial, fish were fed to apparent satiation six days a week for a total of thirty weeks.

Fish were sampled every six weeks 12 h after the last meal, for a total of five times throughout the trial. Samples were taken at six (W6), twelve (W12), eighteen (W18), twenty-four (W24) and thirty (W30) weeks of the feeding trial. The initial stocking density per tank was 8.70 ± 0.4 Kg m^3.. To maintain optimal densities in the long-term, at a maximum limit of 80.20 Kg m^3 per tank, excess fish were pulled out from all tanks at week sixteen and week twenty-one. During sampling, fish were euthanized with a lethal overdose of tricaine methanesulfonate MS-222 (Syndel Inc., Ferndale, WA) at 250 mg L^{-1} buffered to pH 7.0-7.5. At each sampling point, fish weight and length, liver weight, and distal intestine samples were taken from three fish per tank. Only intestines containing digesta were sampled for this study to ensure dietary exposure.

Prior to starting the feeding trial, all experimental protocols, including fish handling and sampling were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2020-34).

Performance indices

The following performance indices were calculated at each sampling point:

- Weight gain (WG, g) = average final weight (g) – average initial weight (g)
- Specific Growth Rate (SGR, % day^{-1}) = $\frac{(\ln(final\ weight)^{i} - \ln(initial\ weight))\ x\ 100}{days^{ii}}$
- Feed intake (FI/fish day^{-1}) = $\frac{feed\ intake\ (g) x\ average\ fish\ weight\ (g)}{days^{ii}}$
- Feed Conversion Ratio (FCR) = total feed intake (g)/weight gain (g)
- Condition factor (*K*) = $(10^4\ x\ fish\ weight\ (g))/[(fork\ length\ (cm)\ x\ 10)^3]$
- Hepatosomatic index (HSI) = (liver weight (g)/fish weight (g)

[i]Final weight as recorded at each sampling point

[ii]Cumulative number of days passed until a given sampling point

Proximate composition of experimental diets and fish whole-body and amino acid composition of the diets

Crude protein, crude lipid, ash and moisture of the feeds and fish fillet were analyzed following standard methods (AOAC, 2000). Briefly, dry matter and moisture content were determined by drying feed or fillet samples overnight (12 h) in a convection oven (105 °C) to a constant weight. Ash was assessed after ignition at 500°C for 12h, according to AOAC (1995). Afterwards, crude protein content in feed samples was analyzed using the Kjeldahl method (N x 6.25) (Kjeltec 8100, FOSS, Denmark) and crude protein in fillet was analyzed using a combustion method with a nitrogen determinator (rapidN exceed, Langenselbold, Germany). Crude lipid in feed samples

was calculated gravimetrically using SoxtecTM (FOSS, 2050 automated analyzer, Denmark) and petroleum ether extraction following acid hydrolysis on SoxCapTM (FOSS, Denmark). Crude lipid in fillet samples was analyzed similarly by hydrolysis using an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY) and petroleum ether extraction was done using an ANKOM XT15 extractor. Total energy content was determined using a Parr bomb calorimeter (Parr Instrument Co., Moline, IL, USA).

The amino acid composition of the feed was determined after acid hydrolysis and derivatization by AccQ-Tag™ Ultra, according to the amino acid analysis application solution (Waters Corporation, Milford, MA, USA). DL-Norvaline (Sigma) 2.5 mM was used as an internal standard and ultra-performance liquid chromatography (UPLC) was performed on an Acquity system (Waters Corporation) equipped with PDA detector. The detection wavelength was set at $\lambda = 260$ nm and the column used was a Waters' BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm). The flow rate was set at 0.7 ml/min and the column temperature were kept at 55 ºC. Lastly, peak identification and quantitation were performed by the software Empower v.2.0 (Waters) using an amino acid standard (Waters) as an external standard. All analyses were performed in duplicate. Tryptophan was not quantified due to its susceptibility to acid hydrolysis, forming a disulfide bridge to produce cystine because of cysteine-cysteine interactions. Similarly, during acid hydrolysis, asparagine is converted to aspartate (ionic form of aspartic acid) and glutamine to glutamate (ionic form of glutamic acid), thus, the reported values for these amino acids (Asx and Glx) represent the sum of both amino acids.

Histopathology of distal intestine

Distal intestine samples were collected from three fish per tank (a total of nine fish per dietary treatment and strain) at each of the five sampling points. Dissected sections of distal intestines, delimited proximally by the first complex fold and distally by the anus opening, were fixed in Bouin's solution for less than 24 h, and subsequently transferred to 70% ethanol for preservation. Distal intestine samples were then placed in cassettes and dehydrated at increasing concentrations of ethanol followed by removing ethanol in xylene and then embedded in paraffin wax. Sections were cut (5μm) using a rotary microtome (HM 340E, Thermo Scientific) and then stained with hematoxylin and eosin and the slides were mounted with DPX solution. Sections were taken with a microscope mounted with camera at x 200 magnification. Both the villi length and width (VL and VW) were measured using a Leica Application Suite software after the pictures were taken and prior calibration. Histological scoring of the distal intestine was done by blind evaluation of slides as described in Palma et al. (2021). The variables assessed were mucosal fold fusion, goblet cells, inflammatory cells, vacuoles prevalence, and infiltration of the lamina propria. Five measurements

were taken randomly from each individual sample (n = 15) and the mean of each replicate tank was used for statistical analysis.

Statistical analysis

All statistical analyses were performed using the statistical Program R (v. 3.6.2). All data were analyzed for homogeneity of variance using the Levene's test, and for normality using the Shapiro-Wilk's test. Growth performance (WG, SGR, FCR, K and HSI), FI, and muscle proximate composition were analyzed by two-way analysis of variance (ANOVA) with diet (FM, SBM and SBM-Gln) and strain (R, T) set as independent factors; and Tukey's Honest Difference was used as post-hoc test. If significant differences or interaction effect were found using two-way ANOVA, a one-way ANOVA was also performed for each strain separately, followed by the same post-hoc test. Histological data were analyzed separately for each strain using Kruskal non-parametric test followed by a Dunn's multiple comparison test. Possible diet and strain interactions were evaluated using interaction plots in R statistical software with VL, VW or cumulative scores as dependent variables and diet and strain as independent variables. A one-way ANOVA test was used to look for differences in time within individual histology parameters, followed by Tukey's post hoc. Differences among groups were considered significant at $p < 0.05$.

Results

Growth performance

All analyzed growth parameters are summarized in Table 2.3. FI was significantly impacted by diet at W6, 12 and 18, and by both diet and strain at W12. There was a significant interaction of diet and strain from W12 onwards (all $p < 0.05$). These interactions can be explained by the fact that both FM and SBM treatments result in opposite FI levels in each strain. WG, SGR and FCR did not show any differences between diets and strains at W6, W12 and W24 ($p > 0.05$). However, a significant interaction between diet and strain was seen for WG at W18 ($p = 0.059$,) and W30 (all $p = 0.00$); and at W30 for SGR and FCR ($p = 0.001$, $p = 0.022$, respectively). At W30 there was also a significant effect of diet ($p = 0.006$) and strain ($p = 0.028$) on FCR.

Despite differences between diets at the first three sampling points of the trial, one-way ANOVA revealed no differences between dietary treatments in strain T throughout the entire trial (all $p > 0.05$). In strain R, a shift from FI being higher in the FM group to it being higher in the SBM group was noted from W6 to W12, and this was maintained until W30.

At W18, fish from strain R fed the SBM diet had significantly higher WG and SGR (p = 0.028, p = 0.037, respectively) than fish fed the FM diet. These fish also displayed higher WG and SGR than their counterparts from strain T, although this was not significant. At W30, one-way ANOVA revealed that strain R fed the SBM diet had significantly higher WG and SGR (all p = 0.005) than fish fed both the FM and SBM-Gln diets. At W30, strain R fed the SBM and SBM-Gln treatments had higher FCR than the FM group. This is explained by significantly lower FI in the FM group compared to those fish in the SBM and SBM-Gln diets (p = 0.004) as per one-way ANOVA analysis. No significant effects of diet on neither WG, SGR and FI were observed on strain T by one-way ANOVA analysis.

One-way ANOVA revealed a significant effect of diet on *K* at W12 (p = 0.022) and W30 (p = 0.015) in strain R. Two-way ANOVA showed a significant effect of strain on condition factor (*K*), which was maintained at all time points except for W18. *K* was consistently higher in strain R than strain T, and significantly higher in the SBM group of strain R than all dietary groups in strain T; at W24, *K* was also higher in the SBM-Gln in strain R compared to the same group in strain T. Interaction between diet and strain was only significant at W12 of the trial.

One-way ANOVA showed that HSI in strain T was significantly higher in the FM diet than both SBM diets at W6 (p = 0.002) and W18 (p = 0.011) and only higher than the SBM diet at W24. In strain R this same pattern was maintained only at W6 where HSI was higher in the FM group compared to SBM. Two-way ANOVA revealed that HSI was significantly impacted by diet at all sampling points except for W12, while strain only had a significant effect at W6. No interaction of strain and diet were observed throughout the trial.

Proximate composition of fillets

Muscle lipid and protein after thirty-week dietary exposure varied between diets and showed different trends between strains, which could indicate an interaction effect (Table 2.4). Fish from both strains fed the SBM had higher muscle lipid than their counterparts fed the FM or the SBM-Gln diets (p < 0.001); and in case of strain R, the SBM-Gln group also had significantly higher lipid than the FM group. This can be explained by the slightly higher lipid level of the SBM diet (Table 2.1). In turn, protein content was highest in the fillets of fish fed the FM diet in strain R, however, in strain T, highest protein content was observed in fish fed either SBM or SBM-Gln diets, driving the interaction observed between strain and dietary treatment (p < 0.001). For both strains, protein content was the same in SBM and SBM-Gln groups.

No differences in ash content were observed, but moisture content was significantly higher in fish from both strains fed the FM and SBM-Gln diets as compared to the SBM. There was no interaction effect in this case.

Histopathology of distal intestine

Histopathological analyses of the distal intestine showed that SBM inclusion affected all parameters assessed, namely length and width of intestinal villi (VL and VW) and scoring variables mucosal fold fusion, Goblet cells, inflammatory cells, vacuoles prevalence, and infiltration of the lamina propria. However, no interaction between strain and diet was observed at any time point as per interaction plots. Based on microscopic observations (Fig. 2.1-2.5), feeding a 30% SBM diet led to morphological differences among all three dietary treatments. Throughout the trial, a similar trend was maintained in which, regardless of strain, the SBM dietary groups were different from the FM group and demonstrated signs of SBMIE including shorter and thicker villi (all $p < 0.05$) and higher histology scores accounting for mucosal fold fusion, lamina propria infiltration, prevalence of goblet cells and vacuoles and inflammatory cells (all $p < 0.05$). Cumulative scores were reduced when Gln was supplemented, although these were not restored to the control diet levels (Fig. 2.6). However, the degree of mucosal fold fusion was reduced significantly at W12 ($p = 0.014$) in both SBM-Gln diets, matching the control diet in both strains (Fig. 2.2c, 2.2f; Fig. 2.6) as well as the decrease of vacuoles at W24 in both strains ($p = 0.015$) (Fig. 2.4c, 2.4f; Fig. 2.6), and goblet cells in strain T ($p = 0.003$) by W24 (Fig. 2.4f; 2.6b). The height and width of villi are shown in Fig. 2.7. In strain T, VW was significantly improved to resemble the control diet at W6 ($p = 0.009$) (Fig. 2.7d), as well as VL in strain R at W24 ($p = 0.008$) (Fig. 2.7a). However, at W18, the SBM-Gln treatment group in strain T shows reduced VL and is slightly different from the FM treatment ($p =0.048$) (Fig. 3f; Fig. 2.7b). Only VW appears unchanged between all three treatments and both strains by W24 ($p > 0.05$) (Fig. 2.7b, 2.7d).

Rainbow trout strains responded differently to SBM and SBM-Gln treatments at W6 for cumulative scores, VL and VW (all $p < 0.05$). At W12 and W18 the cumulative score was significantly higher in strain R than T when fed SBM ($p = 0.008$, $p = 0.046$ respectively.). Lastly, length of villus was higher in strain T as compared to strain R under the SBM-Gln treatment ($p = 0.016$).

One-way ANOVA was used to examine changes of each parameter in each diet through the five time points. Individual analysis of histological parameters was done for all except VL and VW which will show the obvious significant increase in time due to fish normal growth. Gln supplementation led to a significant improvement ($p = 0.015$) in T as per goblet cells in W24

compared to W6 and in strain R ($p = 0.013$) at W18 compared to W6. In strain R inflammatory cells were increased at W24, but Gln protective effect is noteworthy at W12 and W30 ($p = 0.004$). Interaction plots revealed no apparent interaction except for VW. The R strain appears to perform worse for this parameter compared to strain T at W6. An interaction might be present for VL at W12, when strain R shifts from a slightly better performance at SBM compared to T, to a worse performance at SBM-Gln, where it is worse than T. In contrast, strain T seems to show a more pronounced improvement compared to R. After W18, however, the interaction reverted, being strain R the one showing slightly worse performance in the SBM group and a more evident improvement in the SBM-Gln treatment, compared to strain T. This is maintained until W30.

Discussion

The use of SBM in commercial aquafeed formulations has become routine in order to minimize the use of FM, a limited and more expensive resource; and achieve more affordable and sustainable formulations (Albrektsen et al., 2022; Tacon et al., 2022; Turchini et al., 2019). This is, however, not exempt from constraints and challenges. Reduced productivity, and higher risk of disease are among the biggest concerns when shifting from FM to plant protein sources like SBM (Bjørgen et al., 2020). Efforts to eliminate, reduce, prevent and/or minimize these limitations have followed several strategies, from different forms of feed processing to the use of feed additives as well as genetic selection of fish. The work presented here addressed the intestinal inflammation caused by high dietary SBM inclusions and evaluated the potential protective role of the amino acid Gln for a period of thirty weeks. The long-term nature of this research allowed to expand insights on the suitability of Gln as an effective feed additive as well as potential adaptations to dietary SBM after longer times of exposure.

Growth performance and feed utilization

The present study showed that growth performance was not negatively affected by SBM inclusion at a 30% level regardless of the commercial strain examined or at any time point over thirty weeks. Furthermore, strain R demonstrated improved growth in this dietary treatment after W18, and this became more obvious by W30, both relevant time points from a commercial production perspective (market size >300 g) (Fornshell, 2002; Goeritz et al., 2013; Leeds et al., 2016). Differences in FCR were only detected at W30 of the trial, with strain R fed SBM and SBM-Gln diets showing higher values compared to the FM group. This can be explained by the lower overall feed intake recorded in the in the FM group from W12 onwards, suggesting better acceptance of SBM by strain R. Unaffected growth performance by SBM is opposite to what would be expected, but it has

been reported by Gu et al. (2015) and Refstie et al. (1997). Rainbow trout fry reached higher final weight at the end of a fourteen-week trial compared to a FM control group, but this was at a 16.7% inclusion level (Gu et al., 2015). Good growth performance on high SBM diets has also been described for rainbow trout juveniles after adaptation periods of twenty-eight days (Refstie et al., 1997). Although not significant, there could be a degree of adaptation in strain R in the current study, showing numerically higher WG and SGR values after W6. There are studies that report minimal negative impacts on growth performance of different commercial species when fed high levels of SBM (Magalhães et al., 2016; Navarrete et al., 2013; Sealey et al., 2015; Yigit et al., 2018). However, in the majority of cases it is common to observe growth retardation as a result of high SBM feedstuffs unless diets are enhanced with additives, including enzyme mixes and attractants (Ilham et al., 2016; Yeşilayer and Kaymak, 2020) or by the inclusion of processed sources of soy such as soy protein concentrate (SPC) or bioprocessed soybean (Barnes et al., 2015; Bruce et al., 2017; Kumar et al., 2020; Voorhees et al., 2019; Yamamoto et al., 2012). Both strategies would considerably increase feed cost as in the case of SPC (Hardy, 2010) or substantially change ingredient composition that reduces predictability during feed formulation and ensuring nutritional requirements are met (Hua et al., 2019; Jannathulla et al., 2019). Furthermore, for higher levels of inclusion, neither the use of additives nor bioprocessed SBM improved growth performance to a level comparable to FM based diets, as is the case of largemouth bass (Romano et al., 2021). The enhanced growth observed in the strain R in this trial is likely to be a consequence of genetic adaptations after growth and feed utilization selection processes as seen previously in rainbow trout (Barnes et al., 2015; Venold et al., 2012).

In the present study, Gln supplementation did not affect the weight gain of either of the two commercial rainbow trout strains in a period of thirty weeks, which is in contrast with what has been reported for several other species. Some examples include red drum (*Scianepos ocellatus*) (Cheng et al., 2011), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Cheng et al., 2012), Jian carp (Jiang et al., 2015; Yan and Qiu-zhou, 2006), turbot juveniles (Gu et al., 2017; Liu et al., 2018), seabream (*Sparus aurata*) juveniles (Caballero-Solares et al., 2015), hybrid sturgeon (*Acipenser schrenckii* × *A. baerii*) larvae (Wang et al., 2011), hybrid sturgeon juveniles (Yue et al., 2022) and hybrid sturgeon (*Acipenser schrenckii* × *Huso dauricus*) (Qiyou et al., 2011). All those studies were able to demonstrate a positive effect of supplementary Gln on growth. Nonetheless, the extrapolation and interpretation of data remains challenging considering the varying conditions regarding levels of FM and SBM inclusion as well as the duration of the study.

A more holistic approach to the analysis of growth performance can potentially be done by considering other growth metrics like visceral and organosomatic indexes, condition factor,

carcass, or fillet weight (Du and Turchini, 2022). *K* and HSI indices (among others) are routinely used as an indicator of the healthy and nutritional condition of animals (Bolger and Connolly, 1989). In this study, *K* and HSI were taken at each of five sampling points. Fish had a significant impact on *K*, with overall higher *K* values in strain R than strain T, suggesting a more healthy and balanced growth of the former. In strain T, *K* was maintained rather homogeneous in all three dietary groups in accordance with the similarities in WG. HSI observations point out larger liver size in the FM fed group in both strains, suggesting improved nutritional status, as liver size could be associated with enhanced nutrient accumulation and growth (Jobling, 1988; Tolussi et al., 2010) while reduced HSI can be a sign of stress in salmonids (Basrur et al., 2010). Rainbow trout also showed lower HSI after being fed plant-based diets including a mixture of wheat gluten, corn gluten, peas and soy, as compared to a FM control diet, both short and long-term (Brinker and Reiter, 2011; De Francesco et al., 2004). Reductions of HSI in relation to vegetable protein sources could be due to lower lipid/glycogen accumulation in the liver in the SBM groups, possibly as a result of impaired bile acid synthesis and intestinal resorption as similarly observed in Atlantic salmon (Kortner et al., 2013). However, lower HSI is not always a consequence of feeding SBM or plant-based diets. The study by Barrows et al. (2008b) which tested different sources of SBM did not find any impact on HSI. In another study with two rainbow trout strains fed high SBM diets, no differences were observed in HSI, however there was a tendency towards enlarged livers in fish fed increasing levels of fermented SBM (Barnes et al., 2015). These discrepancies make it challenging to draw conclusions on the significance of HSI variations.

Supplementation with dietary Gln did not seem to have a clear and consistent effect on either *K* or HSI. Gln appeared only slightly beneficial around W6 and W24 for strain R and W24 for T. Marked differences between strains were not present for HSI, in contrast to what was seen with *K*, and values tended to be lower in the SBM diet groups, which is in contrast to other growth parameters displayed by strain R. Largemouth bass HSI and *K* was significantly influenced by the type of SBM (i.e., commercial SBM or bioprocessed SBM) at 45% inclusion level after eight weeks compared to those fed a FM-based diet (Romano et al., 2021). Wang et al. (2011) aimed to identify an optimal supplementation level of Gln in starter diets for hybrid sturgeon and did not observe any effect on *K* by up to 1.0% Gln after fifty-six days.

Nutritional composition of the fillets

Proximate composition can help determine the quality of the product for human consumers. In this study, fish fed 30% SBM diets had significantly higher muscle lipid and lower protein content compared to the other two dietary treatments (FM and SBM-Gln), likely due to the

slightly higher lipid content in the SBM diet. When it came to protein, this was significantly lower in fish from strain R fed the SBM diet and fish from strain T fed the FM diet, which coincided with the groups of each strain that had shown the highest growth performance. De Francesco et al. (2004) similarly observed higher lipid content in rainbow trout fed plant protein. There is evidence that replacement of FM by plant protein sources such as corn gluten meal and soya protein concentrates increases hepatic lipogenic enzyme activities in seabass (Kaushik et al., 2004), which led to higher whole body lipid. In salmonids, increases found in whole body fat content with the use of dietary plant proteins were explained by imbalances in amino acid concentrations (Bjerkeng et al., 1997; Kaushik et al., 2004). Furthermore, it is suggested that an unbalanced amino acid composition influences energy metabolism (Vilhelmsson et al., 2004). Vilhelmsson et al. (2004) reported an up-regulation of several proteins involved in energy metabolism in rainbow trout liver when fed a mixture of plant (maize gluten meal, wheat gluten, extruded whole heat, extruded peas, and rapeseed meal) proteins and concluded that these plant proteins increased the energy demands of fish. Thus, a possible reason could be that the higher supply of some dispensable amino acids, such as glutamic acid, in excess by the plant protein–based diets could have led to higher lipid retention (Barrows et al. 2008). It may be noted that in our study, glutamic acid concentration in SBM- based diets was higher. In the present study, the supplementation of Gln helped increase protein deposition, especially in strain T as compared to SBM, which was accompanied of a decrease in fat content as noted above. Moisture content was affected in a similar fashion, also exhibiting an inverse relationship with crude lipid. Although, it appears that the supplementation with Gln led to a higher resemblance of whole-body composition to fish fed the FM diet, this may be attributed to these two diets having the same level of lipid while the SBM formulation had higher lipid levels.

Histopathology of distal intestine

Histological analyses of the distal intestine of both strains show the development of SBMIE in both strains as evidenced by increased leukocyte infiltration, swelling, and shortening of the villi, and increased prevalence of vacuoles and goblet cells that likely compromised barrier function. SBMIE was partially attenuated by the dietary supplementation of Gln throughout the trial. This observation is supported by improved VL and width and overall scores at all sampling points. This is, except for villi shortening in strain T by W18, where no mitigating effect is noted. The significant reduction presumably caused by Gln in fold fusion by W12 and in goblet cells (strain T), VL (strain R) and vacuoles abundance (both T and R) by W24 could indicate Gln action targets. In contrast to growth data, no interaction between strain and diet was noted in the histopathological analysis, indicating that SBMIE may affect SBM-fed rainbow trout strains in similar ways. Navarrete

et al. (2013) also attempted to monitor changes in the intestine of Atlantic salmon through five sampling points but over a short-term trial of thirty-five days. They reported that feeding a 50% SBM diet supplemented with lactic acid bacteria led to a slight improvement of intestinal morphology but did not elucidate which histological parameter(s) were modulated. Rimoldi et al. (2016) studied the effects of dietary taurine and butyrate in European sea bass and described an effect of both additives on increasing villi length. The histology results from the present study make it challenging to speculate on a specific mechanism of action that restores morphology to a better state. Because significant histological parameters affected in this trial are more involved in barrier function (i.e., VL and VW) and absorptive capacity (i.e., goblet cells and vacuoles), one could argue that Gln specifically aids in improving intestinal integrity thus potentially minimizing the barrier leakiness. Other studies where dietary Gln was supplemented also show a general mitigation to the adverse effects of high SBM, driven by reduced goblet cells in turbot (Liu et al., 2018), villi length in largemouth bass, hybrid striped bass red drum (Romano et al., 2021; Cheng et al., 2012, 2011), and Jian carp intestinal folds (Yan and Qiu-zhou, 2006).

Intriguingly, despite the significant morphological alterations accompanied by inflammatory cells, increased vacuoles, and goblet cells, not only there is no negative impact on growth performance, but strain R still thrived on the SBM diet. There was also no interaction between diet and strain in our histology analysis, indicating that SBMIE affects the distal intestine in an equal manner, and that the optimal growth performance observed in strain R could be a result of other physiological adaptations. The opposite was observed when studying two strains of rainbow trout in which fermented SBM negatively impacted growth but this was not accompanied by SBMIE in one of the strains (Barnes et al., 2015). The present study was performed on two commercial strains of rainbow trout which likely accounts for the level of SBM tolerance observed. Previous studies with selected strains of rainbow trout bred for high SBM tolerance, have shown that improved growth would be accompanied by normal histological scores when fed high levels of plant protein (Abernathy et al., 2019; Venold et al., 2012). This was, however, not the case in our study, as clear histopathological signs of SBMIE developed despite optimal growth performance.

Commercial strains and SBMIE adaptation

Selective breeding has been used in aquaculture for decades to improve certain traits such as disease resistance and enhanced growth (Houston et al., 2020). With the growing demand for alternative protein sources for carnivorous species, selective breeding of rainbow trout towards being more tolerant to plant based diets has been in focus (Abernathy et al., 2019; Gaylord et al., 2010). This has led to the development of increased tolerance to higher inclusion levels of SBM in the diets,

when years ago, growth performance of salmonids was significantly reduced at inclusion levels of 20% or higher (Krogdahl et al., 2003; Rumsey et al., 1994; Venold et al., 2012). Although selective breeding efforts have demonstrated better growth performance and histology outcome (Overturf et al., 2013; Venold et al., 2012), these observations belong to relatively early sampling times (8-12 weeks). In this study, the commercial strain R demonstrated similar weight gain trends to that of FM or even higher after eighteen weeks, or, as observed in strain T, not negatively impacted throughout the study. The idea that the extrusion process could improve the digestibility of soy proteins by denaturation could help explain the lack of a detrimental effect on growth (Morken et al., 2012, 2011). Another strategy is acclimating juveniles through early nutrition stimuli during starter feeding periods, a developmental stage that appears to confer a higher tolerance when fed diets with a low inclusion level of SBM (Gu et al., 2015; Sealey et al., 2009). However, no long-term benefits have been reported when feeding SBM to rainbow trout later on during grow-out phases (Sealey et al., 2009). When working with different strains (and improved strains), one could consider whether that strain possesses adaptations that lead to a lower requirement of specific nutrients (i.e., amino acids) that may be deficient in the nutritional composition of the diet to which they have been adapted. That could explain, hypothetically, the lack of effect of Gln in growth performance of strains already adapted to grow well when fed high SBM. In turn, Gln does have a mitigating effect of SBMIE in the distal intestine, supporting the hypothesis that, enhanced growth can occur despite adverse effects in the intestine through other physiological adaptations such as differential gene regulation in muscle and liver (Abernathy et al., 2019). It could be speculated that the only role of Gln was to be used by enterocytes to cope with the stress induced by dietary SBM. If this is the case, it is likely that Gln was not directed as extra source of energy for cells or endocrine functions but rather exerted a paracrine or autocrine effect in the gut. Consequently, Gln beneficial effects are only reflected on the partially improved distal intestine histopathology of SBMIE but not on improved growth.

Conclusion

In conclusion, this study demonstrates that two commercial strains of rainbow trout are not negatively impacted by dietary SBM inclusion up to a 30% level, and that good growth rates are maintained long-term. Despite this, the development of SBMIE remains evident with no signs of attenuation due to adaptation to the diet. Nonetheless, dietary supplementation of 2.0% with the non-essential amino acid Gln has shown a clear role in mitigating SBMIE, maintaining a similar degree of effect throughout thirty weeks.

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Table 2.1. Dietary formulation and proximate composition of the three experimental diets fed to two commercial strains of rainbow trout for thirty weeks.
FM: fish meal; **SBM**: soybean meal; **SBM-Gln**: soybean meal + 2.0% glutamine.

Ingredients	FM	SBM	SBM-Gln
Fish meal[1]	25.0	10.0	10.0
Soybean meal[1]	0.0	30.0	30.0
Canola meal[1]	12.2	2.2	2.7
Wheat gluten meal[1]	5.7	6.2	5.6
Corn protein concentrate[2]	6.2	6.2	5.6
Blood meal[1]	5.6	10.0	8.7
Wheat flour[1]	21.3	12.0	12.5
Poultry meal[1]	5.4	2.7	2.3
Fish oil[1]	15.7	17.4	17.4
Dicalcium phosphate[1]	1.2	1.2	1.2
Choline chloride (60%)[1]	0.6	0.6	0.6
Vitamin premix[3]	0.8	0.8	0.8
Trace mineral mixture[4]	0.1	0.1	0.1
Vitamin C, Stay C-35)[5]	0.2	0.2	0.2
Lysine[6]	0.0	0.2	0.2
Methionine[6]	0.0	0.1	0.1
Ala-Gln[7]	0.0	0.0	2.0
Calculated composition (%)			
Protein	44.7 ± 0.2	45.5 ± 0.1	45.1 ± 0.0
Lipid	15.1 ± 0.0	16.7 ± 0.0	15.2 ± 0.0
Fiber + N-Free extract	32.0	29.1	30.7
Ash	4.3 ± 0.0	4.0 ± 0	4.2 ± 0.0
Moisture	3.7 ± 0.0	4.5 ± 0.0	4.6 ± 0.0
Energy (MJ kg^{-1})	22.0	22.4	22.0

1 Rangen Inc., Buhl, ID, USA
2 Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
3 Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B12, 50 µg; and inositol as meso-inositol, 400 mg.
4 US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as ZnSO4.7H2O), 75; Mn (as MnSO4), 20; Cu (as CuSO4.5H2O), 1.54; I (as KIO3), 10
5 Skretting USA, Tooele, UT, USA.
6 Sigma Aldrich, St. Louis MQ, USA
7 Ajinomoto Health & Nutrition North America, Inc. Raleigh, NC, USA.

Table 2.2. Essential and non- essential amino acid (AA) profile of the three experimental diets.
FM: fish meal; **SBM**: soybean meal; **SBM-Gln**: soybean meal + 2.0% glutamine.

Essential AA (g/100g feed)	FM	SBM	SBM-Gln
Lysine	2.84 ± 0.08	2.96 ± 0.11	2.76 ± 0.15
Methionine	0.96 ± 0.03	0.78 ± 0.02	0.77 ± 0.03
Histidine	1.27 ± 0.09	1.62 ± 0.11	1.47 ± 0.05
Isoleucine	1.62 ± 0.00	1.58 ± 0.02	1.52 ± 0.02
Leucine	4.24 ± 0.23	4.47 ± 0.18	4.28 ± 0.28
Phenylanine	2.29 ± 0.06	2.45 ± 0.16	2.35 ± 0.19
Threonine	1.84 ± 0.07	1.77 ± 0.04	1.75 ± 0.05
Valine	2.26 ± 0.02	2.39 ± 0.05	2.23 ± 0.07
Arginine	2.13 ± 0.03	2.21 ± 0.07	2.15 ± 0.08
Non-Essential AA (g/100g feed)	FM	SBM	SBM-Gln
Taurine	0.07 ± 0.01	0.07 ± 0.03	0.03 ± 0.00
Tyrosine	1.27 ± 0.07	1.33 ± 0.09	1.29 ± 0.10
Cysteine	0.28 ± 0.00	0.27 ± 0.02	0.28 ± 0.00
Hydroxiproline	0.13 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
Serine	2.23 ± 0.08	2.27 ± 0.06	2.20 ± 0.08
Alanine	**2.54 ± 0.13**	**2.53 ± 0.08**	**3.00 ± 0.75**
Proline	2.47 ± 0.07	2.54 ± 0.04	2.40 ± 0.04
Glutamic Acid	**7.70 ± 0.48**	**7.95 ± 0.30**	**8.65 ± 0.57**
Aspartic Acid	3.72 ± 0.00	4.11 ± 0.03	3.95 ± 0.12
Glycine	1.93 ± 0.03	1.92 ± 0.03	1.82 ± 0.08

Table 2.3. Growth performance and growth metrics.

Strain	Diet	IW (g)	WG (g)				
	Week	0	6	12	18	24	30
R	FM	25.09 ± 0.04	72.94 ± 1.73	156.12 ± 7.90	254.91 ± 12.60 [a]	487.70 ± 27.20	653.02 ± 29.8 [a]
	SBM	25.12 ± 0.27	69.58 ± 6.09	172.74 ± 25.60	356.05 ± 26.83 [b]	583.62 ± 38.75	874.79 ± 36.9 [b]
	SBM-Gln	24.37 ±1.28	65.46 ± 1.99	172.62 ± 12.14	328.01 ± 18.40 [ab]	526.59 ± 27.54	742.96 ± 21.0 [a]
T	FM	23.25 ±0.87	72.44 ± 3.2	165.75 ± 1.65	307.76 ± 13.93	531.08 ± 42.58	786.52 ± 30.9
	SBM	24.16 ± 0.77	65.61 ± 1.03	154.59 ± 13.04	292.44 ± 22.74	508.38 ± 32.18	700.65 ± 2.62
	SBM-Gln	23.94 ± 1.4	65.99 ±2.64	177.42 ± 16.02	345.36 ± 32.16	511.43 ± 35.00	731.22 ± 36.3
			SGR (%/day)				
	R	FM	3.24 ± 0.04	2.38 ± 0.05	1.93 ± 0.04 [a]	1.81 ± 0.03	1.58 ± 0.02 [a]
		SBM	3.15 ± 0.14	2.47 ± 0.16	2.17 ± 0.06 [b]	1.91 ± 0.04	1.71 ± 0.02 [b]
		SBM-Gln	3.11 ± 0.09	2.51 ± 0.09	2.14 ± 0.06 [ab]	1.87 ± 0.02	1.65 ± 0.00 [b]
	T	FM	3.37 ± 0.04	2.52 ± 0.02	2.12 ± 0.05	1.90 ± 0.06	1.70 ± 0.03
		SBM	3.13 ± 0.06	2.41 ± 0.10	2.05 ± 0.07	1.85 ± 0.04	1.63 ± 0.01
		SBM-Gln	3.15 ± 0.12	2.56 ± 0.13	2.18 ± 0.09	1.86 ± 0.03	1.65 ± 0.04
			FI (g)/fish/day				
	R	FM	1.17 ± 0.02 [a]	1.42 ± 0.02 [a]	2.34 ± 0.05 [a]	4.44 ± 0.08 [a]	6.06 ± 0.16 [a]
		SBM	1.11 ± 0.01 [ab]	1.62 ± 0.04 [b]	2.78 ± 0.03 [b]	5.36 ± 0.04 [b]	7.12 ± 0.13 [b]
		SBM-Gln	1.08 ± 0.02 [b]	1.57 ± 0.02 [b]	2.69 ± 0.03 [b]	5.06 ± 0.10 [b]	6.95 ± 0.14 [b]
	T	FM	1.06 ± 0.03	1.44 ± 0.04	2.55 ± 0.10	4.93 ± 0.28	6.87 ± 0.37
		SBM	1.00 ± 0.1	1.42 ± 0.03	2.38 ± 0.07	4.42 ± 0.10	6.15 ± 0.17
		SBM-Gln	1.03 ± 0.02	1.52 ± 0.05	2.62 ± 0.09	4.79 ± 0.09	6.93 ± 0.19
			FCR				
	R	FM	0.67 ± 0.03	0.70 ± 0.03	0.81 ± 0.02	0.77 ± 0.02	0.77 ± 0.01 [a]
		SBM	0.68 ± 0.06	0.75 ± 0.08	0.76 ± 0.04	0.82 ± 0.02	0.82 ± 0.01 [b]
		SBM-Gln	0.69 ± 0.03	0.71 ± 0.04	0.77 ± 0.03	0.82 ± 0.02	0.83 ± 0.01 [b]
	T	FM	0.61 ± 0.04	0.67 ± 0.02	0.76 ± 0.02	0.79 ± 0.01	0.78 ± 0.01

Table 2.3 cont.

	SBM	0.64 ± 0.02	0.71 ± 0.04	0.76 ± 0.03	0.77 ± 0.03	0.78 ± 0.01
	SBM-Gln	0.66 ± 0.03	0.67 ± 0.04	0.71 ± 0.04	0.78 ± 0.02	0.80 ± 0.01
		K				
R	FM	1.60 ± 0.03	1.48 ± 0.03 [a]	1.43 ± 0.02	1.52 ± 0.00	1.54 ± 0.00 [a]
	SBM	1.66 ± 0.13	1.70 ± 0.04 [b]	1.52 ± 0.01	1.63 ± 0.03	1.72 ± 0.06 [b]
	SBM-Gln	1.60 ± 0.02	1.56 ± 0.01 [a]	1.50 ± 0.03	1.56 ± 0.05	1.57 ± 0.02 [ab]
T	FM	1.43 ± 0.02	1.35 ± 0.01	1.32 ± 0.02	1.40 ± 0.03	1.42 ± 0.05
	SBM	1.44 ± 0.02	1.33 ± 0.02	1.33 ± 0.02	1.43 ± 0.00	1.51 ± 0.04
	SBM-Gln	1.44 ± 0.01	1.39 ± 0.02	1.47 ± 0.15	1.37 ± 0.04	1.45 ± 0.02
		HSI				
R	FM	1.63 ± 0.03 [a]	1.28 ± 0.13	1.37 ± 0.02	1.30 ± 0.06	1.32 ± 0.03
	SBM	1.35 ± 0.03 [b]	1.08 ± 0.03	1.17 ± 0.05	1.12 ± 0.04	1.23 ± 0.18
	SBM-Gln	1.54 ± 0.06 [ab]	1.16 ± 0.06	1.15 ± 0.05	1.26 ± 0.06	1.17 ± 0.02
T	FM	1.63 ± 0.05 [a]	1.47 ± 0.10	1.58 ± 0.10 [a]	1.44 ± 0.06 [a]	1.53 ± 0.16
	SBM	1.29 ± 0.04 [b]	1.22 ± 0.09	1.14 ± 0.09 [b]	1.06 ± 0.03 [b]	1.09 ± 0.03
	SBM-Gln	1.35 ± 0.00 [b]	1.17 ± 0.13	1.05 ± 0.05 [b]	1.21 ± 0.03 [ab]	1.12 ± 0.04
Main effects		WG (g)				
	Diet	ns	ns	ns	ns	ns
	Strain	ns	ns	ns	ns	ns
	Diet x Strain	ns	ns	0.059	ns	0
		SGR (%/day)				
	Diet	ns	ns	0.046	ns	ns
	Strain	ns	ns	ns	ns	ns
	Diet x Strain	ns	ns	0.041	ns	0
		FI (g)/fish/day				
	Diet	0.023	0.022	0.025	ns	ns
	Strain	0	0.023	ns	ns	ns
	Diet x Strain	ns	0.023	0.002	0	0.004

Table 2.3 cont.

	FCR				
Diet	ns	ns	ns	ns	0.006
Strain	ns	ns	ns	ns	0.028
Diet x Strain	ns	ns	ns	ns	0.022
	K				
Diet	ns	0.022	ns	ns	0.015
Strain	0.001	0	ns	0	0
Diet x Strain	ns	0.003	ns	ns	ns
	HSI				
Diet	0	ns	0	0	0.034
Strain	0.044	ns	ns	ns	ns
Diet x Strain	ns	ns	ns	ns	ns

Values are presented as means of three replicate tanks (n =9). Values within columns not sharing common superscript letters are statistically different at $p \leq 0.05$ as per one-way ANOVA on the Tukey multiple comparison test.
IW = initial weight; **WG** = weight gain; **FCR** = feed conversion ratio; ***K*** = condition factor; **HSI** = hepatosomatic index.
FM: fish meal; **SBM**: soybean meal; **SBM-Gln**: soybean meal + 2.0% glutamine.
W6 n = 18; W12, W18, W24 n = 6; W30 n = 9

Table 2.4. Muscle proximate composition of two commercial strains of rainbow trout after being fed three experimental diets for thirty weeks.

Analyses (% wet weight)	Lipid	Protein	Ash	Moisture
Diet	Strain R			
FM	10.1 ± 0.5 ^{a}	20.4 ± 0.2 ^{b}	1.74 ± 0.03	68.4 ± 0.4 ^{b}
SBM	12.8 ± 0.1 ^{c}	19.8 ± 0.1 ^{a}	1.70 ± 0.04	66.1 ± 0.1 ^{a}
SBM-Gln	11.6 ± 0.1 ^{b}	19.6 ± 0.1 ^{a}	1.66 ± 0.04	67.4 ± 0.1 ^{b}
	Strain T			
FM	11.3 ± 0.3 ^{y}	19.5 ± 0.1 ^{a}	1.74 ± 0.04	67.7 ± 0.3 ^{z}
SBM	12.6 ± 0.1 ^{z}	20.2 ± 0.2 ^{b}	1.66 ± 0.04	65.9 ± 0.2 ^{y}
SBM-Gln	10.7 ± 0.3 ^{y}	20.2 ± 0.2 ^{b}	1.66 ± 0.03	67.8 ± 0.3 ^{z}
Main effects				
Diet	$p < 0.001$	ns	ns	$p < 0.001$
Strain	ns	ns	ns	ns
Diet x Strain	0.001	$p < 0.001$	ns	ns

Means of three replicate tanks. Values within columns with different superscript letters represent significant differences between dietary treatments for each strain as per one-way ANOVA at $p \leq 0.05$ based on the Tukey's comparison test.
FM: fish meal; **SBM**: soybean meal; **SBM-Gln**: soybean meal + 2.0% glutamine.

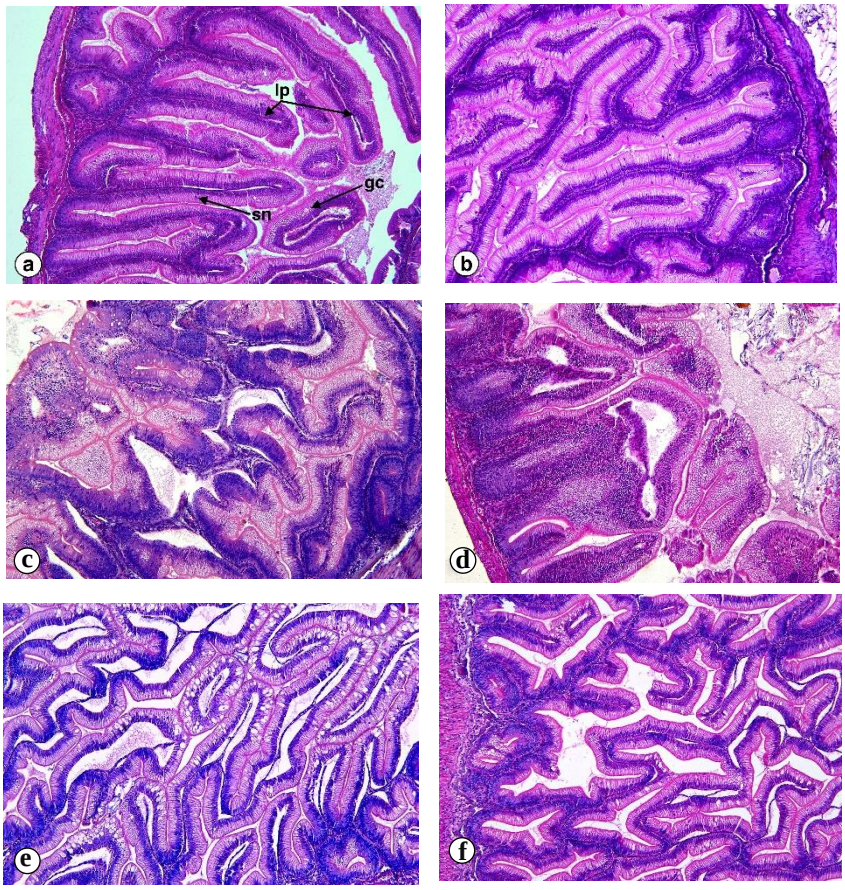

Figure 2.1. Distal intestine histopathology of rainbow trout after six weeks of feeding trial (W6).
Images are representative of five sections analyzed from each of three fish/replicate (*n* = 15). **A, C, E**: FM, SBM and SBM-Gln strain R. **B, D, F**: FM, SBM and SBM-Gln strain T. Staining was performed using hematoxylin and eosin. Magnification x 200.

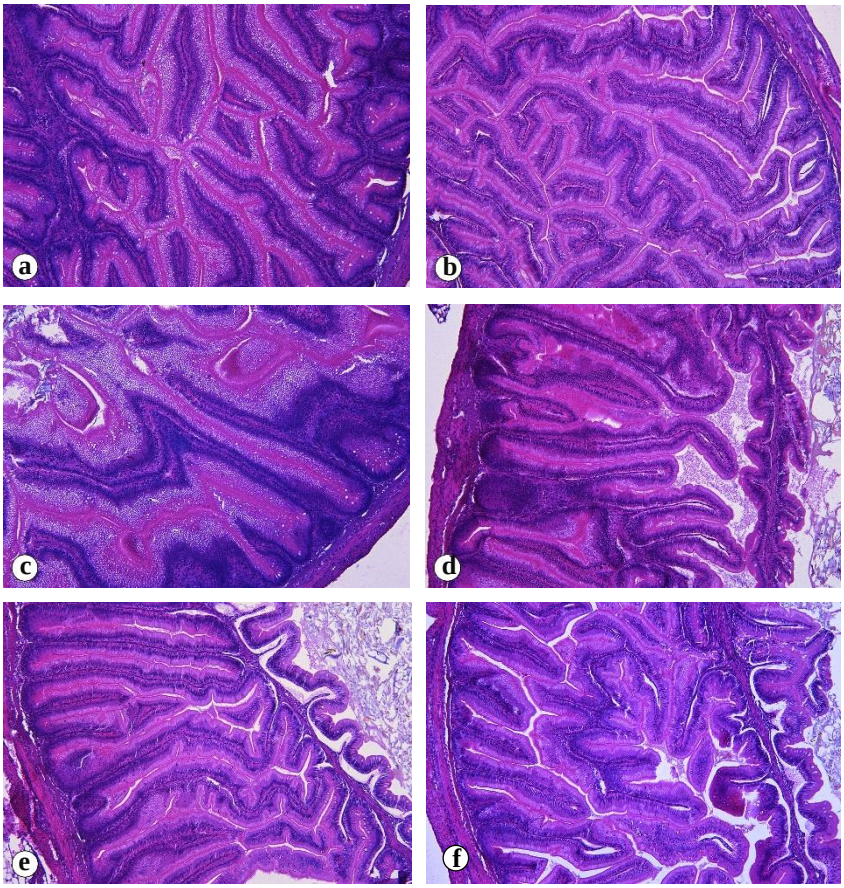

Figure 2.2. Distal intestine histopathology of rainbow trout after twelve weeks of feeding trial (W12).
Images are representative of five sections analyzed from each of three fish/replicate (*n* = 15). **A, C, E**: FM, SBM and SBM-Gln strain R. **B, D, F**: FM, SBM and SBM-Gln strain T. Staining was performed using hematoxylin and eosin. Magnification x 200.

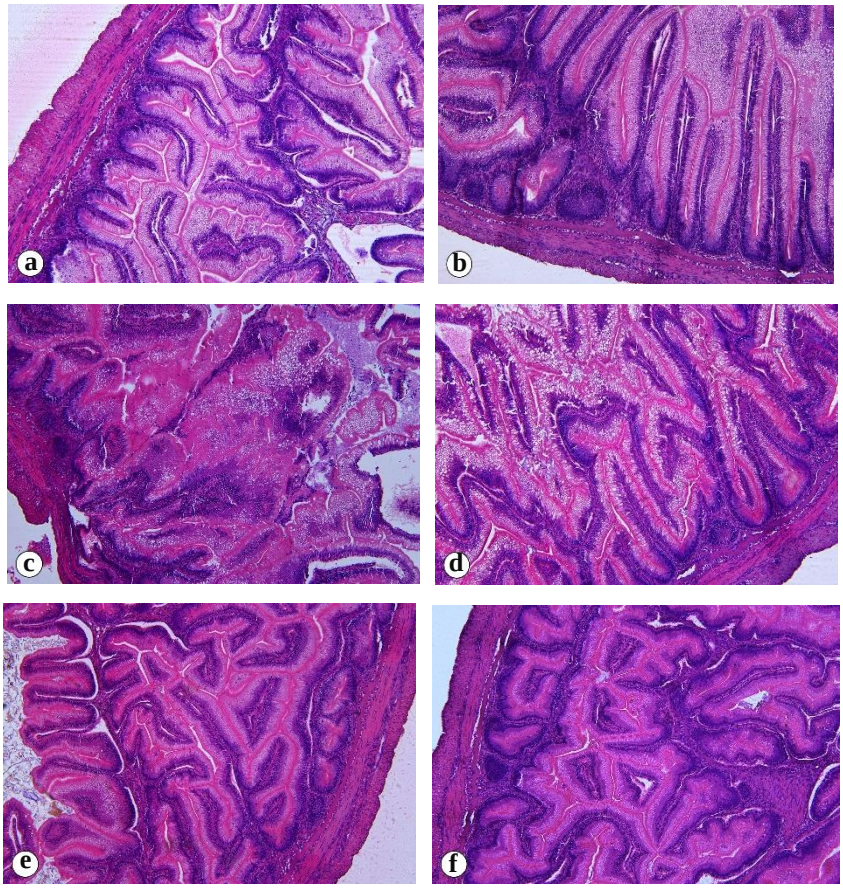

Figure 2.3. Distal intestine histopathology of rainbow trout after eighteen weeks of feeding trial (W18).
Images are representative of five sections analyzed from each of three fish/replicate (n = 15). **A, C, E**: FM, SBM and SBM-Gln strain R. **B, D, F**: FM, SBM and SBM-Gln strain T. Staining was performed using hematoxylin and eosin. Magnification x 200.

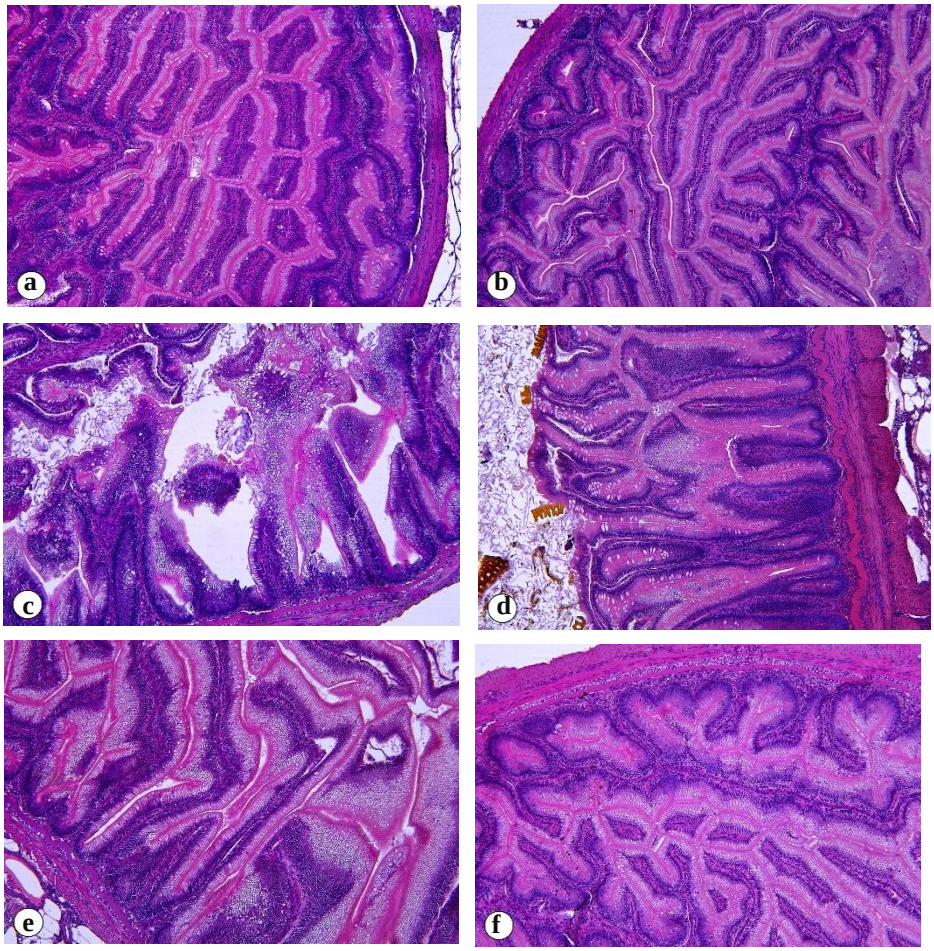

Figure 2.4. Distal intestine histopathology of rainbow trout after twenty-four weeks of feeding trial (W24).
Images are representative of five sections analyzed from each of three fish/replicate (*n* = 15). **A, C, E**: FM, SBM and SBM-Gln strain R. **B, D, F**: FM, SBM and SBM-Gln strain T. Staining was performed using hematoxylin and eosin. Magnification x 200.

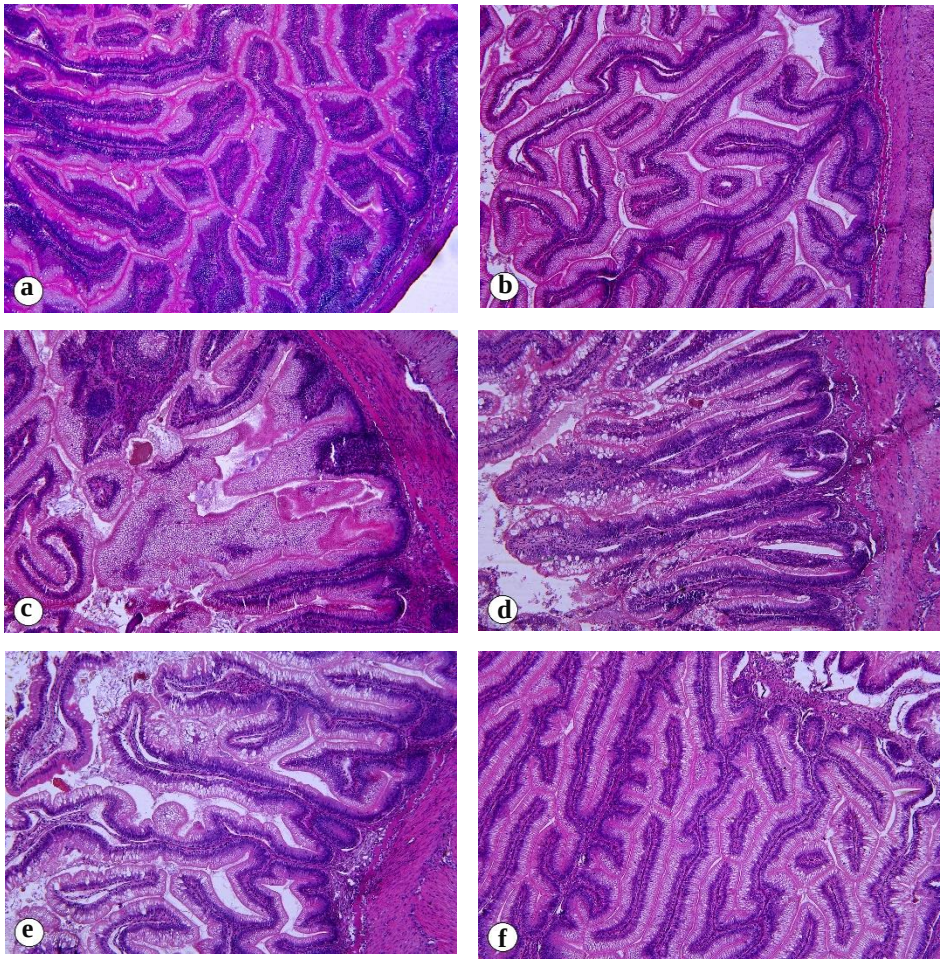

Figure 2.5. Distal intestine histopathology of rainbow trout after thirty weeks of feeding trial (W30).
Images are representative of five sections analyzed from each of three fish/replicate (n = 15). **A, C, E**: FM, SBM and SBM-Gln strain R. **B, D, F**: FM, SBM and SBM-Gln strain T. Staining was performed using hematoxylin and eosin. Magnification x 200.

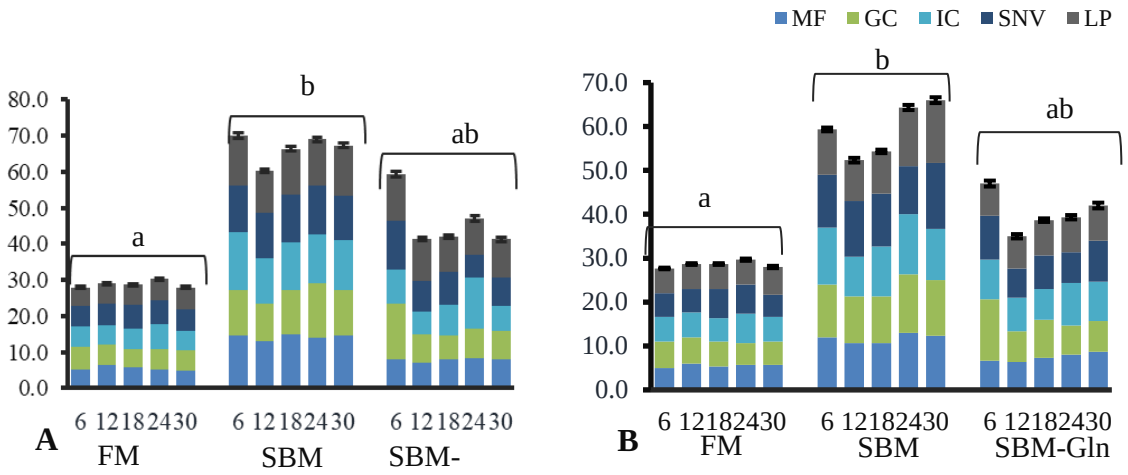

Figure 2.6. Cumulative histology scores at each 6, 12, 18, 24 and 30 weeks of experimental trial.

A strain R; **B** strain T. Values represent the cumulative score calculated as the sum of individual observations in each parameter. Statistical analysis was done on the sum of each parameter obtained for each replicate. Higher scoring indicates higher increase in severity. Graph bars not sharing common letters are significantly different as per Dunn's test ($p < 0.05$), n = 15.
MF = mucosal fold fusion; GC = goblet cells; IC = inflammatory cells; SNV = supranuclear vacuoles; LP = lamina propria infiltration.

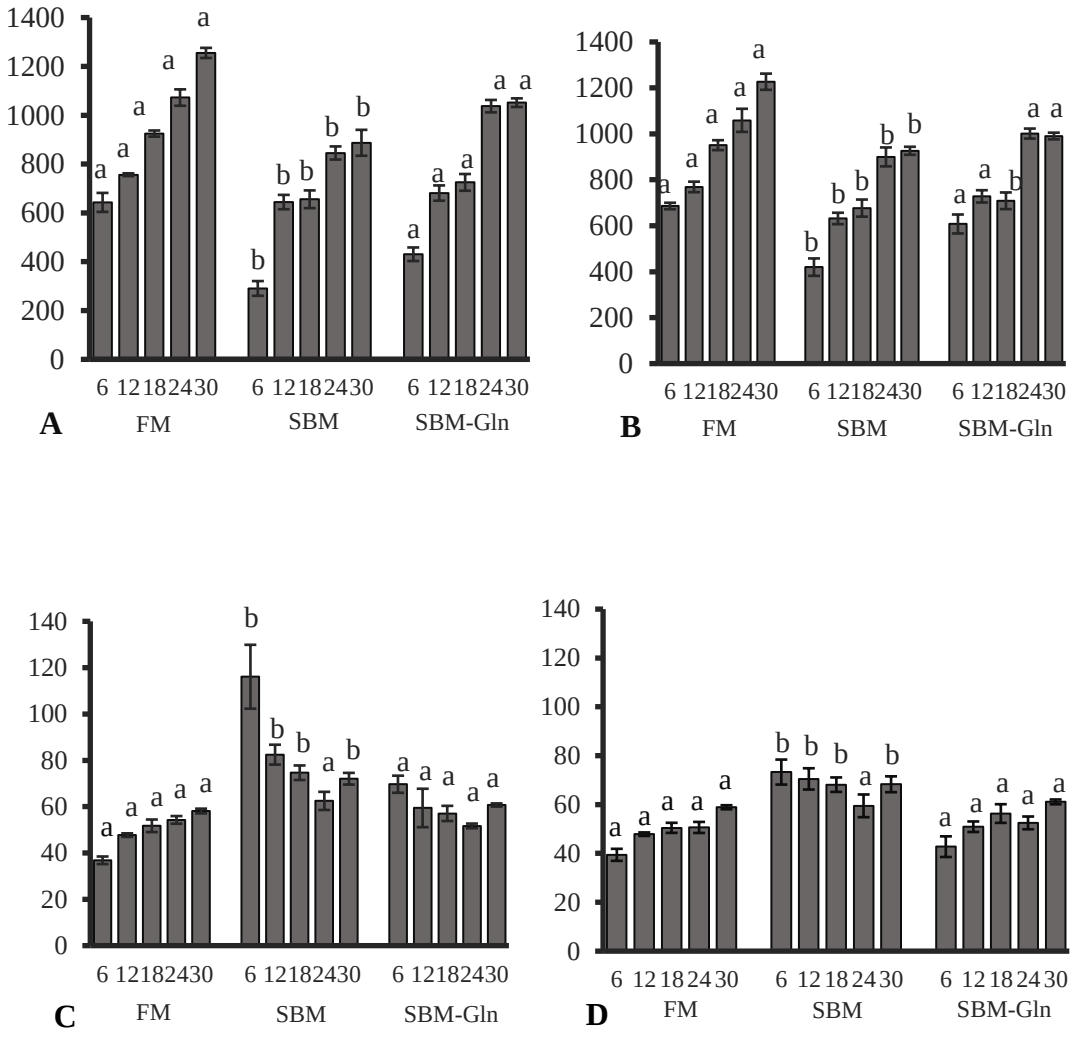

Figure 2.7. Villi length and width (µm) at each of 6, 12, 18, 24 and 30 weeks of experimental trial.

A and C: VL and VW, respectively, strain R; **B and D**: VL and VW, respectively, strain T. Graph bars of same time point and different diet not sharing common letters are significantly different as per Dunn's test ($p < 0.05$), n = 15.

VL = villi length; VW = villi width

Chapter 3:

Glutamine supplementation of soybean meal diets: effects on gene expression, antioxidant metabolism and amino acid composition in two strains of rainbow trout (Oncorhynchus mykiss) over a long-term feeding trial

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Abstract

The use of soybean meal (SBM) as a replacement for fishmeal (FM) in the diet of commercial carnivorous fish species remains troublesome. The development of soybean meal-induced enteritis (SBMIE) can compromise fish growth and health status. The non-essential amino acid glutamine (Gln) has been intensively studied for its protective role in the intestine by both mitigating inflammation and restoring epithelial barrier function. This study aimed investigated the role of SBM and the potential effects of Gln in a thirty-week feeding experiment. Two rainbow trout strains (namely R and T) initially weighing 24.32 ± 1.0 g were fed three experimental diets, including a fish meal (FM) diet (25%, control); a 30% inclusion level SBM diet, and the same SBM diet supplemented with 2.0% Gln (SBM-Gln). Fish were sampled every six weeks for a total of five times at week six (W6), twelve (W12), eighteen (W18), twenty-four (W24) and thirty (W30). Samples were taken from the distal intestine for glutamate quantification, glutathione peroxidase (GPx) activity measurement and gene expression of inflammatory markers (Nfkb, Tnfa, Il8 and Il10); barrier integrity markers (Cldn12 and Ocln); cellular contractility (Mylk); and neutral amino acid transporters (Slc1a5). Additionally, plasma samples were also taken for amino acid profile analysis. The results showed that Gln supplementation contributed to a sustained increase in GPx activity within time in strain T with significantly higher activity in the long term (W24) compared to FM and SBM groups. The pro-inflammatory cytokine *il8* and the anti-inflammatory cytokine *il10* were more highly expressed in strain T fed the SBM diet than in R by W24. Gene expression of *tnfa* was also significantly higher in strain T fed the SBM diet as compared to strain R by W30. Similarly, higher expression of *slc1a5* was seen for strain T fed the SBM diet at W24 compared to strain R. In conclusion, dietary Gln can exert long-term effects in ameliorating SBMIE in rainbow trout, however more research is needed to clarify or confirm its modulation of TJ and Mylk, as well as the involvement of Mylk in SBMIE in this species.

Introduction

Soybean meal (SBM) inclusion in aquafeeds has been widely studied for its optimal composition to serve as an alternative source of protein to substitute fish meal (FM), and has indeed become an essential ingredient in fish feed formulations (Colombo et al., 2022; Eroldoğan et al., 2022). Nowadays, the main challenge concerning SBM revolves around increasing inclusion levels in aquafeeds, especially considering carnivorous species. Historically, diets containing high SBM levels have been linked to reduced feed intake and growth rates, and only bioprocessed SBM sources or soy protein concentrates would yield good fish performance due to their reduced levels of antinutritional factors (ANFs) compared to commercial SBM (Gaylord et al., 2010; Morken et al., 2012; Overturf et al., 2013; Sealey et al., 2009). Compromised growth performance has been linked to the development of soybean meal enteritis (SBMIE), a pathology well reported in carnivores. SBMIE entails inflammation of the distal intestine and loss of epithelial barrier function (Baeverfjord and Krogdahl, 1996), and both events have been widely described in a range of species (Fuentes-Quesada et al., 2018; Gu et al., 2016a; Krogdahl et al., 2015; Urán et al., 2008). In fish, specific molecular mechanisms remain elusive, and gaining a better understanding of what key players are involved would help in the development of solutions against SBMIE.

In higher vertebrates, numerous studies have been conducted to examine the underlying mechanisms of intestinal bowel diseases (IBD), which affect human, and similarly course with intestinal inflammation, morphological alterations, and increased cell contractility, leading to hyperpermeability and in turn, loss of barrier function. This raises the question of whether shared physiological mechanisms exist between IBD and SBMIE. Research in this topic have identified overexpression of inflammatory cytokines such as tumor necrosis factor alpha (Tnfa), interleukins one-beta, eight, six, two (Il1b, Il8, Il6, Il2); altered expression of barrier integrity markers such us tight junction (TJ) proteins including claudins (Cldn) and occludin (Ocln); and changes in paracellular transport and enterocyte contraction (Marchiando et al., 2010). Cellular contractility is tightly regulated, and its occurrence and frequency are related to the action of myosin light chain kinase (Mylk), involved in myosin light chain phosphorylation leading to cytoskeletal contraction. Studies in vitro have shown an association between Tnfa and Mylk, via nuclear factor kappa B (Nfkb) activation, and this can ultimately interfere with TJ (Ma et al., 2005; Ye et al., 2005). In fish fed SBM based diets, evidence shows correlation with IBD pathology, with regards to higher expression of Tnfa, Il8, Il1b, all of which are under transcriptional regulation of Nfkb. Some examples include turbot (*Scophthalmus maximus*, L.) (Liu et al., 2018); Atlantic salmon (*Salmo salar*) (Sahlmann et al., 2013); hybrid sturgeon (*Acipenser baerii* × *A. shrenckii*) (Yue et al., 2022); and gilthead seabream (*Sparus aurata* L.) (Pérez-Sánchez et al., 2015). Other SBMIE signs identified lower expression of the fatty acid binding protein

two (Fabp2) in the distal intestine of Atlantic salmon (Gajardo, 2016; Venold et al., 2012), or the reduced activity of antioxidant enzymes such as glutathione peroxidase (GPx), impacted by the ANF β-conglycinin (Zhang et al., 2013).

Gene expression insights in fish regarding barrier integrity markers is scarce, but some studies have analyzed *mylk* expression in turbot (Liu et al., 2018); expression of *cldn* and *ocln* in Jian carp (*Cyprinus carpio* var. Jian) (Jiang et al., 2017, 2015a, 2015b) and gilthead seabream (Pérez-Sánchez et al., 2015); and myosin light chain in Atlantic salmon (Sahlmann et al., 2013).

There is accumulating evidence that the neutral non-essential amino acid glutamine (Gln) plays a protective role in intestinal tissue of vertebrates. Being a major energy substrate for the enterocyte, Gln may be beneficial for maintenance of barrier function, mitigation of dysregulated inflammation and control of cellular stress (Kim and Kim, 2017; Li et al., 2020; Wang et al., 2015). Moreover, Gln deprivation may also lead to compromised gut health (Li et al., 2004; Li and Neu, 2009). Some of the proposed mechanisms of action of Gln include signal transduction pathways that lead to inhibition of the NF-κB, reducing cytokine abundance (Lesueur et al., 2012); and TJ regulation, although much of these processes remain unclear (González-Mariscal et al., 2008). Hence, the use of Gln in the development of functional fish feeds has been a focus of study to improve intestinal health (Caballero-Solares et al., 2015; Jiang et al., 2015b; Li et al., 2019; Liu et al., 2018; Qiyou et al., 2011; Yan and Qiu-zhou, 2006; Yue et al., 2022) (Cheng et al., 2012, 2011; Yoshida et al., 2016). While studies have shown encouraging results when it comes to inflammation attenuation, less data is available on the specific molecular markers driving SBMIE in fish with regards to transcript abundance of inflammatory cytokines and, especially TJ proteins and Mylk.

The aim of this study was to monitor the impact of high inclusion levels of SBM on the expression of key inflammatory and barrier genes through a long-term feeding trial. Additionally, the benefits of using Gln as a dietary supplement were studied from a molecular perspective to assess its capacity to modulate gene expression of SBMIE markers and enhance antioxidant metabolism. Thus, the suitability of Gln to mitigate SBMIE was assessed both in the short and long term. This trial was conducted in two commercial strains of rainbow trout, for which growth performance and distal intestine histopathology results have already been described in Chapter 2.

Materials and Methods

Experimental diets

The dietary formulation and proximate composition of the experimental feeds is shown in Table 2.1; and the amino acid profile is detailed in Table 2.2. Three isonitrogenous and isolipidic diets were formulated to include 45% crude protein and 16% crude lipid. A fishmeal (FM) diet (25%

inclusion) was used as a control diet. Two more diets included a 30% inclusion level of SBM of which, one was 2.0% glutamine-alanine supplemented (SBM and SBM-Gln, respectively) (Gln-Ala, Ajinomoto Co., Inc.). Gln was supplemented as a dipeptide to avoid thermal degradation of Gln during extrusion process.

Fish rearing conditions and sampling

Two commercial rainbow trout strains, namely R and T were transferred to the Cold-Water Lab at the Aquaculture Research Institute, University of Idaho (Moscow, ID). A total of 2,250 juvenile rainbow trout were randomly distributed into each of eighteen 350 L tanks (125 fish per tank) incorporating three replicate tanks per diet and strain. Fish were acclimated for one week, after which initial weights were 24.32 ± 1.0 g on average. Animals were maintained in a recirculating aquaculture system at 14.0 ± 1.0 °C and a 12 h dark/ 12 h light photoperiod. Water was supplied at a constant flow rate of 180 mL s^{-1}. Oxygen was supplied by normal aeration and maintained at 5.73 ± 0.73 mg L^{-1}; pH was kept at 7.61 ± 0.16 and ammonia (NH3) and nitrite (NO2) were kept at < 0.1 mg L^{-1}. All parameters were monitored using commercial kits (LaMotte, Chestertown, MD, USA). During the long-term feeding trial, fish were fed to apparent satiation six days a week for a total of thirty weeks. During the long-term feeding trial, fish were fed to apparent satiation six days a week for a total of thirty weeks.

Fish were sampled every six weeks 12 h after the last meal, for a total of five times throughout the trial. Samples were taken at six (W6), twelve (W12), eighteen (W18), twenty-four (W24) and thirty (W30) weeks of the feeding trial. The initial stocking density per tank was 8.70 ± 0.4 Kg m^3. To maintain optimal densities in the long-term, at a maximum limit of 80.20 Kg m^3 per tank, excess fish were pulled out from all tanks at week sixteen and week twenty-one. During sampling, fish were euthanized with a lethal overdose of tricaine methanesulfonate MS-222 (Syndel Inc., Ferndale, WA) at 250 mg L^{-1} buffered to pH 7.0-7.5. At each sampling point, fish weight and length, liver weight, and distal intestine samples were taken from three fish per tank. Only intestines containing digesta were sampled for this study to ensure dietary exposure.

Prior to starting the feeding trial, all experimental protocols, including fish handling and sampling were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2020-34).

Glutamate quantification

Intracellular glutamate (Glu) concentrations in the distal intestine were determined by using the Glutamate Assay Kit (ab83389, Abcam, Cambridge, UK or MAK004, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Distal intestine samples were collected from three fish per tank (n = 9) 12 to 18 h after the last feeding. Tissue samples taken at W12, W18 and W30 were rinsed with phosphate buffered saline (1 X, pH 7.2, Cytiva Hyclone™) snap-frozen at the time of sampling using liquid nitrogen. Tissue samples (10 mg) were lysed using a dounce homogenizer in assay buffer provided with the kit and homogenates were then pooled per tank. The assay included two technical replicates per pooled sample. The amount of Glu was quantified by measuring the absorbance at 450 nm using a microplate reader (SpectraMax® i3X, Molecular Devices LLC., San Jose, CA, USA). Glu concentrations were calculated according to the manufacturer's calculation guide and stated in µM. For every experiment a standard curve and background measurements of control groups were calculated and accounted for calculations.

$$Sa = \left(\frac{Corrected\ absorbance - (y - intercept)}{Slope}\right)$$

$$[Glu] = \left(\frac{Sa}{Sv}\right) * D$$

Where:

Sa = amount of sample (nmol) from the standard curve

Sv = volume of sample (µL) added to the well

D = sample dilution factor

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined using the Glutathione Peroxidase Assay kit from Cayman Chemicals (#703102). Briefly, distal intestine samples were obtained from three fish per tank (n = 9) at W6, W18 and W24, rinsed with phosphate buffered saline (1 X, pH 7.2, Cytiva Hyclone™) and snap-frozen in liquid nitrogen. Tissue samples (10 mg) were placed in cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT), centrifuged at 10,000 x g for 15 minutes at 4 °C, and the supernatant kept for analysis. Homogenized samples (20 µL) were placed in assay buffer, and NADPH, glutathione and glutathione reductase were added to a final volume of 190 µL. The reaction was initiated by the addition of cumene hydroperoxide, and absorbance at 340 nm was recorded every minute for five time points using a microplate reader (SpectraMax® i3X, Molecular Devices LLC., San Jose, CA, USA). Changed in absorbance were determined and used to calculate GPx activity using the following formula provided by the kit manufacturer:

$$GPx\ activity = \frac{\Delta A_{340}/min}{0.00373\ \mu M^{-1i}}\ x\ \frac{0.19\ mL}{0.02\ mL} = \ nmol/min/mL$$

[i] Adjusted extinction coefficient for NADPH at 340 nm as provided by the kit manufacturer.

RNA extraction, cDNA synthesis and real-time qPCR

Distal intestine samples were taken from three fish per tank (n = 9) at each of five sampling points, and snap-frozen in liquid nitrogen until analysis. Samples were taken only when digesta was present in the distal intestine, to ensure exposure to the diet. Tissue samples were homogenized using a motor-pestle homogenizer in Trizol® and RNA was extracted following manufacturer instructions. RNA purity and quantity was determined using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE), and quality (integrity) was assessed by agarose gel electrophoresis. Samples were treated with DNase (Invitrogen, Waltham, MA, USA) when appropriate prior to reverse transcription. Extracted RNA (0.5 µg) was reverse-transcribed to cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). RT-qPCR reactions were carried out using PowerUp ™ SYBR Green Master Mix green detection method (Applied Biosystems, Waltham, MA, USA) on a StepOnePlus real-time thermocycler (Applied Biosystems, Foster City, CA, USA). Duplicate reactions were performed for each cDNA sample analyzed (i.e., six technical replicates per tank). The reactions were set on a 96-well optical plate, and each contained: 1.5 µL of diluted (1/5) cDNA, 5 µL of 2X concentrated PowerUp ™ SYBR Green Master Mix, 0.5 µM forward primer and 0.5 µM reverse primer. Gene expression analyses were performed for inflammation marker genes: *nfkb*, *tnfa, il8*, *il10*; barrier integrity makers: *ocln* and *cldn12*; cellular contractility gene *mylk*; and neutral amino acid brush border membrane transporter solute carrier family 1 member 5 (*slc1a5*). Primer sequences were obtained from the literature or designed using Primer3Plus (https://bioinformatics.nl/). Primers for the target genes *cldn12*, *ocln*, *nfkb*, and *mylk* were based on rainbow trout sequences or on conserved regions of known teleost sequences paralogues in the GenBank database, using NCBI BLAST software (http://blast.ncbi.nlm.nih.gov) or Clustal Omega 1.2.4 multiple alignment tool (https://ebi.ac.uk/Tools/msa/clustalo/).

Cyclophilin A (*cypa*, pepdidyl-prolyl-cis-trans isomerase A) was selected as housekeeping gene as its expression is maintained during disease and always showed a variation coefficient < 5.0%. The qPCR programme consisted of initial denaturation program (2 min at 50 °C followed by 2 min at 95 °C); an amplification and quantification program (repeated 40 times, 15 s at 95 °C, 20 s at 57-60 °C, and 30 s at 72 °C); and a melting curve 60 – 95 °C, 0.15 °C s^{-1}). Gene expression was determined using the ΔC_t method described by Pfaffl (Pfaffl, 2001), where the genes of interest were normalized

to the C_t value of the housekeeping gene *cypa*. . Both the standard curves and primer efficiencies were obtained by serial dilutions of a random cDNA mixture of control samples. All primer sets were validated on a 1% agarose gel (125 V for 35 min) to validate successful amplification product formation. Table 3 shows primer sequences and efficiencies.

Statistical analysis

Data were analyzed using R studio statistical software (v. 3.6.2) and presented as means ± standard error of the mean (SEM). Normality of distribution was analyzed using a Shapiro-Wilk test, and a Levene's test was performed to describe homogeneity of the variance. Gene expression data were log-transformed to meet the assumption of homogeneity of variance. A two-way analysis of variance (ANOVA) was done to identify statistical differences among the means, with diet (FM, SBM and SBM-Gln) and strain (R, T) set as independent factors. Tukey's post-hoc test was used to assess statistical differences. If significant differences were found using the two-way ANOVA on glutamate quantification and GPx activity, a one-way ANOVA test was performed to test differences between dietary treatments for each fish strain. A one-way ANOVA was also used to examine differences across time points within each dietary treatment. Level of significance was set at $\alpha = 0.05$.

Results

Glutamate quantification in distal intestine

Distal intestine Glu concentrations per strain and diet at W12, W18 and W30 are shown in Fig. 3.1. The Glu levels in distal intestine enterocytes differed between fish strains at W12 (p = 0.0294), and this was marked by differences in the FM diet which were 411.16 µM (highest) in strain T and 264.36 µM (lowest) in strain R. Moreover, the latter was significantly lower compared to measurements taken at W18 and W30 in fish from strain R fed the FM diet (p = 0.013). By W30, the SBM-Gln group of strain T showed the highest Glu concentration, however this was not statistically significant.

Glutathione peroxidase enzymatic activity in distal intestine

The GPx activity measurements showed little impact from either diet or fish strain at the early stages of the trial, but significant effects of both strain and diet were seen at W18 (Fig. 3.2). At this point, GPx activity in strain R fed the SBM-Gln diet (96.85 nmol min^{-1} mL^{-1}) was significantly higher than the FM and SBM (52.21 and 64.97 nmol min^{-1} mL^{-1}, respectively) (p = 0.001). Overall, recorded enzyme activities were lower in strain R than strain T at this point. More obvious

divergencies appeared at W24, where all diets followed opposite trends in each strain, leading to a significant interaction of these two effects. For instance, the SBM-Gln-fed strain T fish reached 124.53 nmol min^{-1} mL^{-1} at W24, being significantly higher ($p < 0.001$) compared with the FM and SBM groups (61.84 and 92.00 nmol min^{-1} mL^{-1}, respectively). The opposite was observed in strain R, as the SBM-Gln showed an inflection point, decreasing GPx activity after W18, to similar levels as FM and SBM.

Recorded enzymatic activity showed an increasing trend in strain T, with significantly higher GPx activity in the SBM and SBM-Gln groups at W18 and W24 compared to W6 ($p = 0.003$ and $p = 0.081$, respectively). This contrasted with what was observed in strain R, where GPx activity in the FM and SBM groups remained rather static between W6 and W18, and only the SBM-Gln group demonstrated a more noticeable increase between these two time points, although not significant ($p > 0.05$) (Fig. 3.2). In turn, the FM and SBM groups, which activity had remained stable, suffered a more pronounced increase in the time comprised between W18 and W24 but this was also not significant ($p > 0.05$).

Gene expression of SBMIE markers and slc1a5

Gene expression results of barrier integrity genes of both strains is shown in Fig. 3. There were no significant differences in mRNA transcript level of *ocln* (Fig. 3.3A, 3.3B) and *cldn12* (Fig. 3.3C, 3.3D) across diets and strains at any of the five time points (all $p > 0.05$). Levels of *mylk* expression seemed to be significantly lower at W6 in strain R compared to strain T ($p = 0.0496$), but there were no differences among dietary treatments (Fig. 3.3E, 3.3F). Gene expression of immune response genes is displayed in Fig. 3.4 The transcription factor *nfkb* showed slightly higher mRNA transcript levels in in SBM diets, and significantly higher expression in the SBM-Gln group from strain T at W6 ($p = 0.001$); and in the SBM group from the same strain at W24 ($p = 0.005$) (Fig. 3.4A, 3.4B). By W30, the pro-inflammatory cytokine *tnfa* had significantly higher expression levels in the SBM group in strain T compared to strain R, with a significant effect of strain ($p = 0.0312$) (Fig. 3.4C, 3.4D). Similar results were obtained for *il8 and il10* at W24 with significant differences between strain R and T fed the SBM diet ($p = 0.048$, 0.0213 respectively), and this pattern was maintained towards W30, although differences were not statistically significant at this point ($p =$ 0.0649, 0.059 respectively) (Fig. 3.4E, 3.4F; Fig. 3.4G, 3.4H, respectively). Lastly, *slc1a5* (Fig.3.5) also showed higher expression in strain T fed the SBM diet at W24 compared to R ($p = 0.0308$) but there were no differences between dietary treatments (Fig. 3.5A, 3.5B).

Discussion

The results presented herein are a continuation of the growth and distal intestine histology results obtained as part of the same 30-week long-term feeding trial. Hence, it has been reported in Chapter 2 that growth performance was not positively affected by Gln throughout 30 weeks. Furthermore, both weight gain (WG) and specific growth rate (SGR) were significantly higher in strain R fed the SBM diet as compared to FM and SBM-Gln, and higher than all three dietary groups in strain T. Despite no major detrimental effects to the growth performance of fish fed the SBM diet, that study reported substantial morphological changes in the distal intestine which were also accompanied by inflammation (i.e., SBMIE). Furthermore, feeding the SBM-Gln diet achieved partial SBMIE mitigation in both rainbow trout strains, which was maintained long-term for 30 weeks. The data presented here is focused on further exploring the effects of supplemental Gln at the molecular level in terms of gene expression of inflammation and barrier markers; and Glu quantification and GPx activity. The aim was therefore to gain more in-depth knowledge on the key players modulated by Gln towards SBMIE amelioration in rainbow trout.

Glutamate quantification and glutathione peroxidase activity

In a robust study that aimed to clarify the role of Gln in *nfkb* downregulation, Lesueur et al. (2012) proposed a mechanism of action of Gln in which its intracellular conversion to glutamate (Glu) would interact with the signaling cascade that leads to the activation of the Nfkb transcription factor by promoting its eventual degradation in the cell nucleus. Downregulation of *nfkb* would revert the inflammation during SBMIE as it would, presumably hamper the *tnfa* and *mylk* overexpression.

The quantification of Glu in the distal intestine was done as a first step in understanding the fate of dietary Gln. Results showed significant differences between strains at W12, where the FM group in strain T had the highest Glu concentration and the SBM group had the lowest and vice versa in strain R. These differences at W12 suggest that even the control group is modulated differently for each strain. At W18, the very similar concentrations of Glu between dietary treatments in strain T, showing a tendency towards lowering levels, is in accordance with the apparent decrease in transcript abundance of IL-8 and IL-10 at this time point. All three dietary treatments seemed to follow the same trend in strain T, and concentration was highest in the Gln supplemented group by W18, although this was not significant. In contrast, in strain R, the decreasing trend in Glu concentration from W12 to W18 in the SBM group is opposed to the increasing trend of SBM-Gln and FM between these two times. While the FM group steadily increased at each time point, both SBM diets follow divergent trends again from W18 to W30, with a tendency to increase Glu concentration in the SBM group as opposed to SBM-Gln. Nonetheless, none of these variances were significant, complicating

interpretation. It should be kept in mind that our analysis was done in samples taken 12 h after feeding. Apart from ins conversion to Glu, Gln has also been proposed to target NF-κB inactivation by increased expression of heat shock proteins (Xue et al., 2011), and this pathway deserves more attention in future fish feeding trials.

Apart from the involvement of Glu in the hypothesized mechanism of NF-κB nuclear degradation, intracellularly generated Glu can be used for GSH synthesis (Wu et al., 2004). GSH could be crucial in maintenance of gut health during episodes of inflammatory stress during which reactive oxygen species accumulate. Although this does not target the pro-inflammatory cycle directly, increased GPx activity would be key to increase cell survival. In the present study we found that, for strain T, only the SBM-Gln fed fish showed a continued increase in enzymatic activity across the three time points, with significantly higher GPx activity by W24. Observations in strain T GPx activity also seem to correlate with Glu concentrations. This is especially true at W18, where the slightly lower concentrations of Glu in fish fed all diets seemed to correlate with the GPx activity increase trend taking place at that time. These observations support the role of supplemental Gln in antioxidant enhancement. However, this conclusion becomes questionable when comparing it with strain R. Indeed, both strains showed differences at W18, with overall higher GPx activity in strain T although both strains showed the highest activity in the SBM-Gln group. The significant interaction between diet and strain at W24 with opposing increase/decrease tendencies among all three dietary groups points out the high variability that exists among strains of the same species, let alone across species. Strain R demonstrated higher growth performance in the SBM group after W18 of the trial, and this adaptation to SBM could be driving differences observed at the metabolic level (Brezas et al., 2021), and this could also be the case for the observations presented in Chapter 2. The highly similar GPx activity levels between dietary treatments and strains at W6 also deserve attention. This could be indicating that either physiological responses to cope with oxidative stress derived from exposure to SBM develop only after longer exposure times; or that the 30% inclusion level does not lead to a scenario of physiological stress until later stages. Jiang et al. (2015) found an improved antioxidant response in Jian carp fed Gln supplemented diets, with a marked increase in GPx activity as compared to a glycinin-based diet, and this was after 42 d exposure only. Another study in Jian carp demonstrated a 97% increase in GPx activity by 0.8% Glu dietary supplementation after 9 weeks in a lipopolysaccharide-driven oxidative stress scenario (Jiang et al., 2017).

Cytokine gene expression

The expression of *nfkb* tended to be higher in the SBM and SBM-Gln groups at W6 and W24, especially in strain T. Because samples were taken from distal intestine that contained digesta, this could be stimulating expression of *nfkb* 12 – 18 h after feeding. The higher expression levels observed in the SBM-Gln group could be due to the transient increase in *nfkb* expression before its downregulation in the cell nucleus triggered by Gln as Lesueur et al. (2012) postulated. Conversely, these results could also suggest no effect of the dietary Gln in decreasing Nfkb. Protein expression analysis would help in getting more informative results, as *nfkb* expression is dynamic and subject to multiple activation pathways (Xiao et al., 2006). Because the hypothesized effect of Gln on reducing Nfkb activity entails a mechanism in which p65 is transiently increased in the cell nucleus before its degradation, the detection of any differential expression would be highly time-sensitive. A study was carried out in rainbow trout to evaluate acute effects of oral Glu administration and found significant upregulation of nucleic acid-synthesis genes only 30 min after Glu exposure (Yoshida et al., 2016).

The Tnfa has been identified as a key player during intestinal inflammation in mammals, contributing to the perpetuation of inflammation (Cunningham and Turner, 2013; Marchiando et al., 2010); and it has been studied in SBMIE in salmonids and other species (Gu et al., 2016; Kumar et al., 2021). In the present study, expression of *tnfa* was higher in strain T fed the SBM diet as compared to their counterparts in strain R by W24 and W30, although differences were only significant at W30. This pattern of expression was similarly recorded for the pro-inflammatory cytokine *il8* and the anti-inflammatory cytokine *il10* both of which were significantly higher in strain T at W24 and higher at W30 as well, although statistical significance was not detected. Nonetheless, considering observations of *tnfa* expression and the near 0.05 *p* values obtained, these differences at W30 should also receive attention. All three cytokines also showed highest transcript levels at W12 for both strains, but this was never significant. After this point, strain R maintains low expression levels of *tnfa*, *il8*, and *il10*, which contrasts with strain T after W18. These differences between strains might be showing the degree of adaptability to the SBM diet of each strain. In strain T, the SBM-Gln group demonstrated higher, although non-significant, *il8* and *il10* levels until W12, after which it remains low and similar to FM group expression levels, supporting a possible beneficial role of Gln after W12, which is the time when strain R shows more stable expression patterns for all three cytokines in the three dietary treatments. Il8 is a neutrophil recruiting cytokine which is produced by various cell types during episodes of acute inflammation (Grimm et al., 1996), and its increased expression at later stages in strain T could be indicative of more severe inflammation in strain T, although histology analysis showed similar changes in distal intestine morphology for both strains, as described in Chapter 2.

Sahlmann et al. (2013), Sahlmann et al. (2013), using microarray technology, recorded an upregulation of *nfkb*/p52 precursor, p100, whose processing is required for the so-called alternative pathway of Nfkb activation (Neumann and Naumann, 2007). However, this pathway only responds to few activation stimuli, and detection of p100 does not confirm activation of the transcription factor (Xiao et al., 2006). Liu et at. (2018) found higher expression of *nfkb* and *tnfa* in distal intestine of turbot after 12 weeks. Gene expression of the pro-inflammatory cytokines *tnfa* and *il8* was unaffected in rainbow trout fed a 21% level SBM diet, however the time of sampling was not stated by the authors (Kumar et al., 2021). These authors also used beta actin (*bactin*) as reference gene, whose expression may be modulated by enteritis itself (Kortner et al., 2011). Gu et al. (2016) were able to find higher expression of both *tnfa* and *il8* in turbot fed SBM diets containing >30% inclusion levels. In contrast, Jiang et al. (2015) reported the highest *tnfa* expression in turbot fed a Gln supplemented diet and a slight but significant downregulation of IL-10 in fish fed a glycinin (SBM antinutritional factor) -based diet; this study also used beta-actin and only 42 days exposure to high glycinin levels in Jian carp. In common carp (*Cyprinus carpio* L.), higher levels of *tnfa* and *il1b* were found after 1, 3 and 5 weeks of exposure to 20% SBM diets (Urán et al., 2008). However, none of these authors specify how much time had passed since the last feeding before samples were taken. In juvenile salmons, differences in expression levels of pro-inflammatory cytokines have been described at 6 and 24 h after a bacterial challenge (Fast et al., 2007).

TJ, mylk and slc1a5 gene expression

It has been proposed that Gln regulates TJ directly by influencing phosphorylation patterns and cellular localization, hence maintaining controlled permeability (Beutheu et al., 2014; Seth et al., 2004). The TJ proteins Cldn12 and Ocln studied herein did not show any significant differences. The expression level of *cldn12* seemed higher in SBM fed fish of strain R until W12 and in both SBM and SBM-Gln in strain T, with maintained higher levels in the latter strain through W24, although these slight differences were not significant. The role of Cldn12 is not fully understood and it has been classified as an ambiguous function type of claudin which function in each tissue may depend on the presence of other claudins (Amasheh et al., 2011). As such, its physiological role in either barrier forming, or mediating permeability could highly depend on formation of claudin clusters by interaction with other claudins, and subjected to specific tissue localization (Markov et al., 2015). In intestinal tissues, Cldn12 has been identified to associate with Cldn2, a well-defined pore-forming TJ (Amasheh et al., 2011; Capaldo and Nusrat, 2015) to form paracellular pores (Fujita et al., 2008). Furthermore, Fujita et al. (2008) hypothesized the role of Cldn12 in enhanced expression of *cldn2*. The combined action of Cldn2 and 12 is observed during paracellular calcium absorption in

mice enterocytes. These observations could support a possible role of Cldn12 in hyperpermeability observed during SBMIE.

The physiological function of Ocln, on the other hand, remains enigmatic (González-Mariscal et al., 2008). Its phosphorylated status dictates its localization in the junction or the cytoplasm (Wong, 1997). Its implication in IBD has been more related to displacement from the junction and its internalization in the cell (Marchiando et al., 2010), although a correlation between higher ocln expression levels and improved permeability has also been shown in human cells *in vitro* (Beutheu et al., 2014). Paradoxically, Ocln is equally expressed in kidney cells with different transepithelial resistance, so Ocln may be present in tissues with different transepithelial resistance. Translating this notion to a SBMIE case, it could be the case that changes in transepithelial resistance does not affect Ocln abundance, in which case it would not be a good target for gene expression analysis. Instead, examining the basic biochemistry of Ocln (i.e., phosphorylated status or cellular localization) would help elucidate its role in enteritis in fish.

Pérez-Sánchez et al. (2015) found an upregulation of *cldn12* and *cldn15* in the distal intestine of gilthead seabream fed a functional feed, together with an upregulation of *il6*, all in all supporting barrier function and anti-inflammatory effect of the investigated additives. In Jian carp, supplementation with Glu was able to modulate the expression of *cldn11*, compensating for the effects caused by soy glycinin (Jiang et al., 2015b) and the amino acid leucine could impact expression levels of *ocln* (Jiang et al., 2015a). However, despite the ample research that has been done in SBMIE in salmonids, insights into salmonid TJ regulation in this context remain scarce.

The Mylk also constitutes a signaling pathway in TJ regulation, by regulating cell contractility by MLC phosphorylation within the acto-myosin ring (González-Mariscal et al., 2008; Kim and Kim, 2017; Marchiando et al., 2010). In this study, differences between fish strains were recorded at W6, with lower expression levels in strain R as compared to those of strain T. No further differences were seen throughout the study, and this could be due to several reasons. Firstly, as discussed for other markers examined herein, the timing of sampling chosen for this study could have been inadequate to detect mRNA. Secondly, the isoform investigated could not be primarily involved in SBMIE. In mammals, robust studies including protein sequencing, protein expression and localization as well as stimuli for activation have identified a specific isoform involved in IBD and hypercontractility, facilitating its investigation or therapeutic targeting (Graham et al., 2019). On the other hand, studies in fish addressing Mylk characterization are scarce, but an upregulation of *mylk* was reported by Liu et al. (2018), in correlation with higher *nfkb* and *tnfa*. Sahlman et al. (2013) found upregulation of the cytoskeleton protein myosin, a Mylk target, involved in cell contractility. Activation of Mylk is dependent on calcium and calmodulin (Kamm and Stull, 1985). Calcium can be obtained from

intracellular reserves or through extracellular calcium entry via calcium channels. Hence, the *mylk* overexpression that occurs during IBD can lead to depletion of calcium reserves (Kamm and Stull, 1985; Stull et al., 1993). Concomitantly, the extracellular sequestration of calcium and other divalent cations, part of the innate immune response (Blaufuss et al., 2019; Hood and Skaar, 2012; Kehl-Fie et al., 2011), can aggravate the calcium shortage under an inflammation scenario. It is known that low extracellular calcium concentrations lead to TJ disassembly in the intestine and increased paracellular permeability (González-Mariscal et al., 2008). As noted above, Cldn12 and 2 could be involved in enhancing paracellular calcium transport in these conditions.

Apart from TJ functional interaction driving barrier and permeability functions and Mylk, cytokines can also modulate TJ expression (Capaldo and Nusrat, 2015). In this study, expression level of *il8* and *il10* was modulated by dietary SBM at W6 and W24 and although this did not reflect on changes in TJ expression at the same time points, the distal intestine histopathology results.

Changes in amino acid transporter systems have been identified as a mechanism of adaptation to maximize nutrient absorption when fed alternative protein-based feeds (Song et al., 2017). The Slc1a5 (also known as ASCT2) functions as a neutral amino acid transporter (i.e., for Gln transport), and it is of further relevance in the absorption of methionine, a limiting amino acid in SBM diets (To et al., 2019). Gene expression of *slc1a5* has been shown increase by the use of meat and bone meal as alternative protein source to FM, but not by dietary supplementation with essential amino acids (Song et al., 2017). In that 4-week study, upregulation was reported at 2-8 h after feeding. Brezas et al. (2021) also showed *slc1a5* postprandial modulation in a selected and non-selected strain of rainbow trout, with higher expressions recorded 18 h after feeding in the non-selected strain. Results obtained from the current study also showed higher expression in strain T fed the SBM-Gln diet compared to the R strain at 12-18 h post-feeding at W6, but this was not significant. Instead, there was a significantly higher expression of this transporter at W24 in the SBM group of strain T which differed from strain R. This can be interpreted as a response reduced nutrient absorption during SBMIE and an attempt to enhance Gln absorption. Considering our previously reported results showing superior growth performance of strain R in the SBM diet, these observations agree with those reported by Brezas et al. (2021), where expression levels of *slc1a5* were affected by rainbow trout strain. Although no further differences were seen after W6, nor differences between dietary treatments, strain T did show slightly higher *slc1a5* expression in the SBM group throughout the trial, whereas the SBM-Gln group seemed to stabilize expression to control levels.

All gene expression analysis reported herein were performed using *cypa* as a reference gene. A potential limitation of using *cypa* could be the presence of multiple cyclophilin homologous sequences representing related genes (Schmid et al., 2003), which in fish could be even more critical given the

whole genome duplication events (Panserat et al., 2019). This is of further relevance considering that Sybr green dye, which binds to DNA non-specifically was used for this analysis and that can affect the accuracy of quantification.

Conclusion

Dietary supplementation with Gln has been intensively studied both in higher vertebrates and teleost for its protective role in the intestine by modulating both inflammatory and barrier markers. The present study aimed to expand the knowledge on the role of Gln during mitigation of SBMIE in two rainbow strains trout in a 30-week long-term trial. Gene expression of the pro-inflammatory cytokine IL-8 was significantly increased by SBM in strain T compared to strain R at W24 and seemingly downregulated by Gln at this time point. Strain T also showed consistently increasing GPx activities in the SBM-Gln diet, reaching significantly higher levels by W24, which supports the line of action of Gln through antioxidant pathways. Unconclusive data was obtained with regards to Tnfa as well as TJ proteins and Mylk. Since previous studies showed significant distal intestine morphological abnormalities (Chapter 2), further research will be needed to elucidate which key players are involved at the molecular level.

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Table 3.1. The primer list and accession numbers of target and reference genes and efficiencies of the PCR reactions.

Gene	Primer sequence (5' --> 3')	Accession no.	Reference	Efficiency (%)
tnfa	F: CAAGAGTTTGAACCTCATTCAG R: GCTGCTGCCGCACATAAAG	AJ279069	Bridle et al. (2006)	110.0
il8	F: CACAGACAGAGAAGGAAGGAAAG R: TGCTCATCTTGGGGTTACAGA			103.1
il10	F: CGACTTTAAATCTCCCATCGAC R: GCATTGGACGATCTCTTTCTTC		Pérez-Sánchez et al. (2011)	95.3
nfkb	F: GGGAAAGACTGCAAACATGG R: AAACTCCTCCTCCCACACCT	AY163839.1		108.2
mylk	F: GCACTACATGCAGCAGATCG R: AGCCACAAACTCTGGTGTCC	XM_021557724.2		93.8
cldn12	F: AAAACGCCAAGAACATCAGC R: GACATGCCTGCCATACACAG	BK007967.1		105.8
ocln	F: CGGAATCCAATGGCTACG R: AAGATCCCCACACAGAGCAC	GQ476574.1		108.6
slc1a5	F: AGCCTGCACTAATTCTCACTG R: CTGCGACTCTTAGCCTAGTGAA		To et al. (2019)	105.8
cypa	F: GCAAGTCCATCTACGGCAAT R: TGCTAGCGATGATGTTGAGG	MN722644.1		110.0

cypa: cyclophilin A, peptidyl-prolyl cis-trans isomerase A; ***tnfa***: tumor necrosis factor alpha; ***il8***: interleukin 8; ***il-10***: interleukin 10; ***nfkb***: nuclear factor kappa B; ***mylk***: myosin light chain kinase; ***cldn12***: claudin-12; ***ocln***: occludin; ***slc1a5***: solute carrier transporter family 1 member 5.

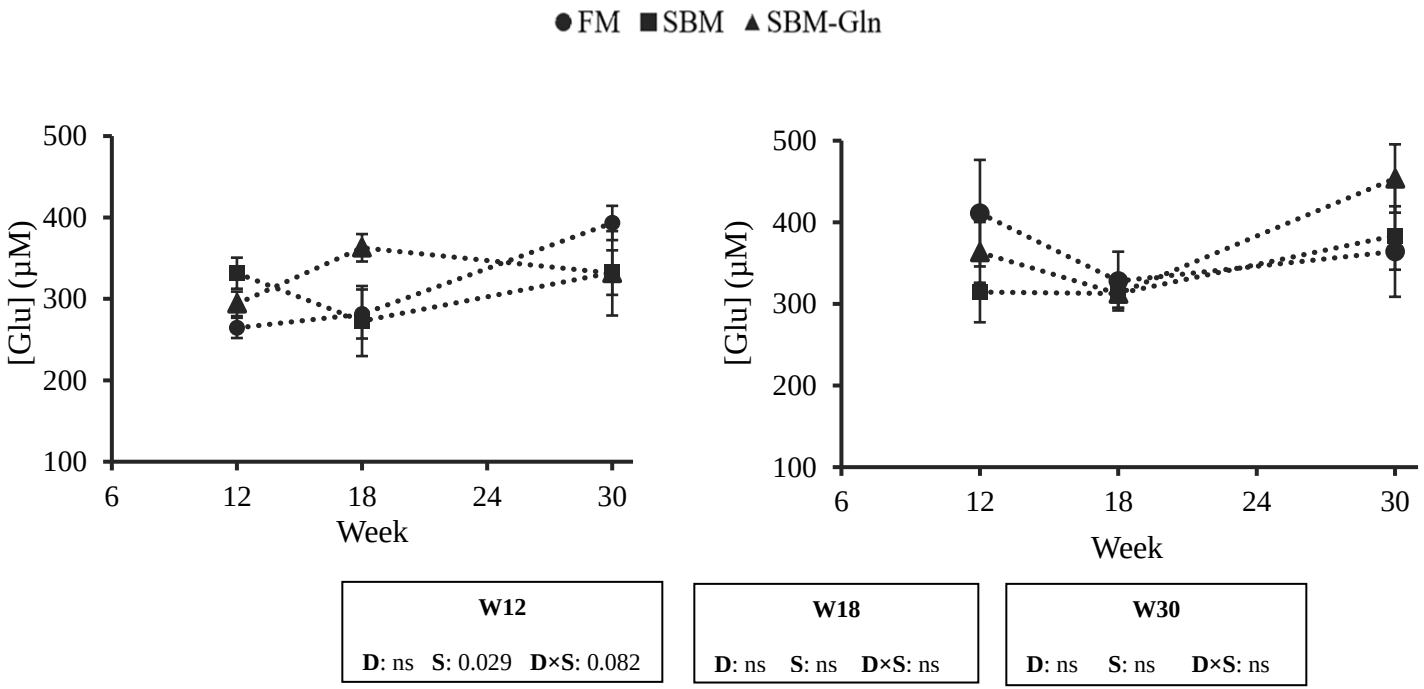


Figure 3.1. Glutamate concentration levels (µM) found in distal intestine enterocytes at W12, W18 and W30 of the feeding trial.

Data from fish of strain R (A) and T (B), fed three experimental diets: FM, SBM meal – glutamine SBM-Gln are shown as means ± SD, $n = 9$. Displayed under the graph are p – values of factors diet (D), strain (S), and interaction (D × S).
FM: fishmeal; **SBM**: soybean meal; **SBM-Gln**: soybean meal-glutamine; W = week number of the feeding trial.

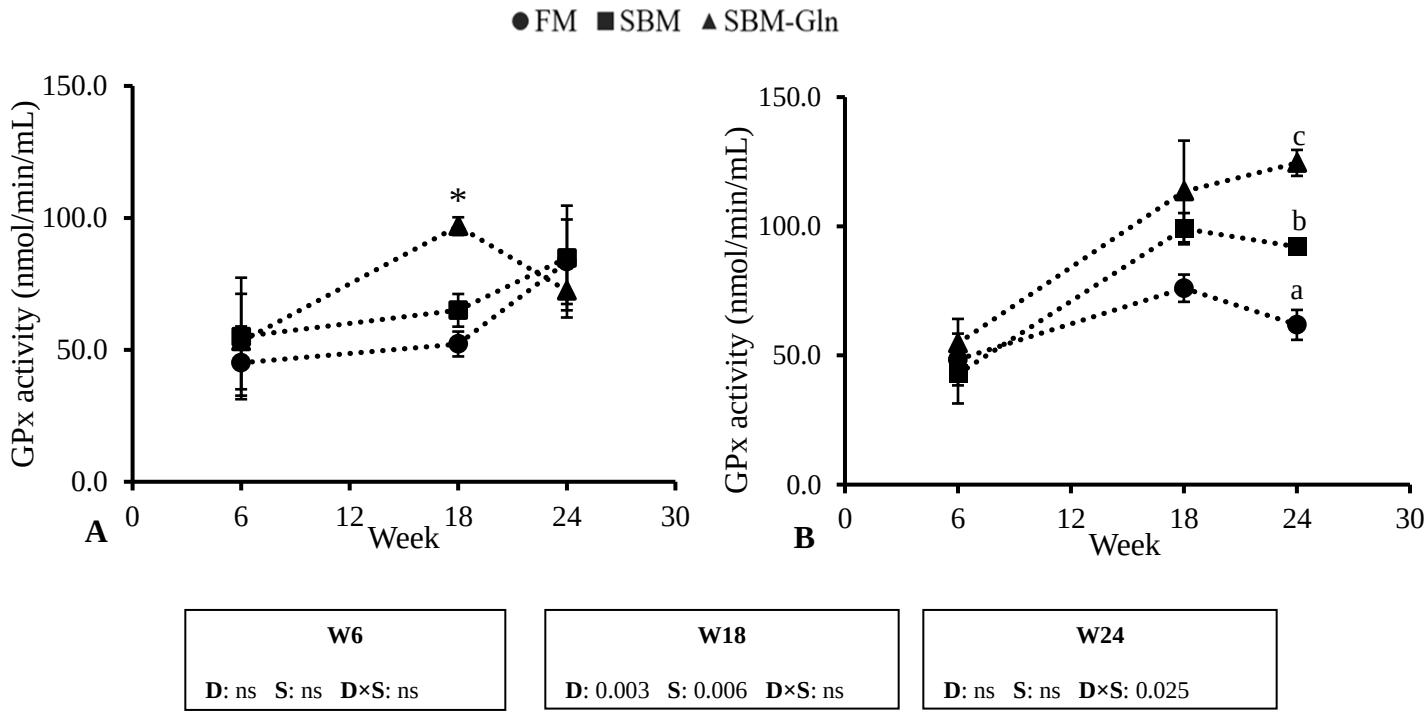
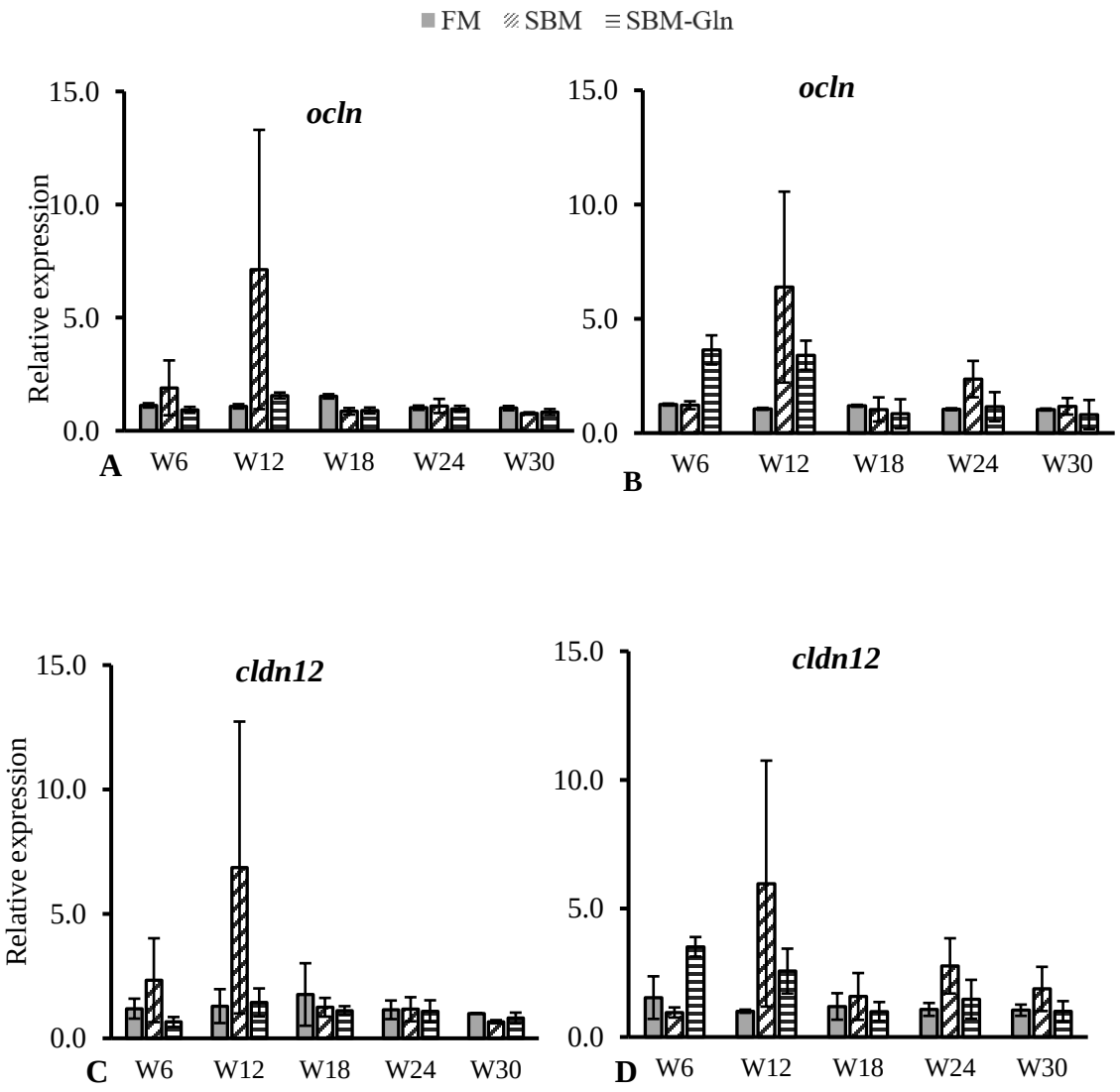


Figure 3.2. Measurement of glutathione peroxidase (GPx) activity (nmol/min/mL) found in distal intestine enterocytes at W6, W18 and W24 of the feeding trial.

Data from fish of strain R (A) and T (B), fed three experimental diets: FM, SBM meal – glutamine SBM-Gln are shown as means ± SD, $n = 9$. Displayed under the graph are p – values of factors diet (D), strain (S), and interaction (D × S). Different letters or asterisk represent significant differences between dietary treatment at a given time point for a specific strain as described by one-way ANOVA.

FM: fishmeal; **SBM**: soybean meal; **SBM-Gln**: soybean meal-glutamine; **W**: week number of the feeding trial.


FM
SBM
SBM-Gln
ocln
Relative expression
15.0
10.0
5.0
0.0
A
W6
W12
W18
W24
W30
ocln
B
cldn12
C
cldn12
D

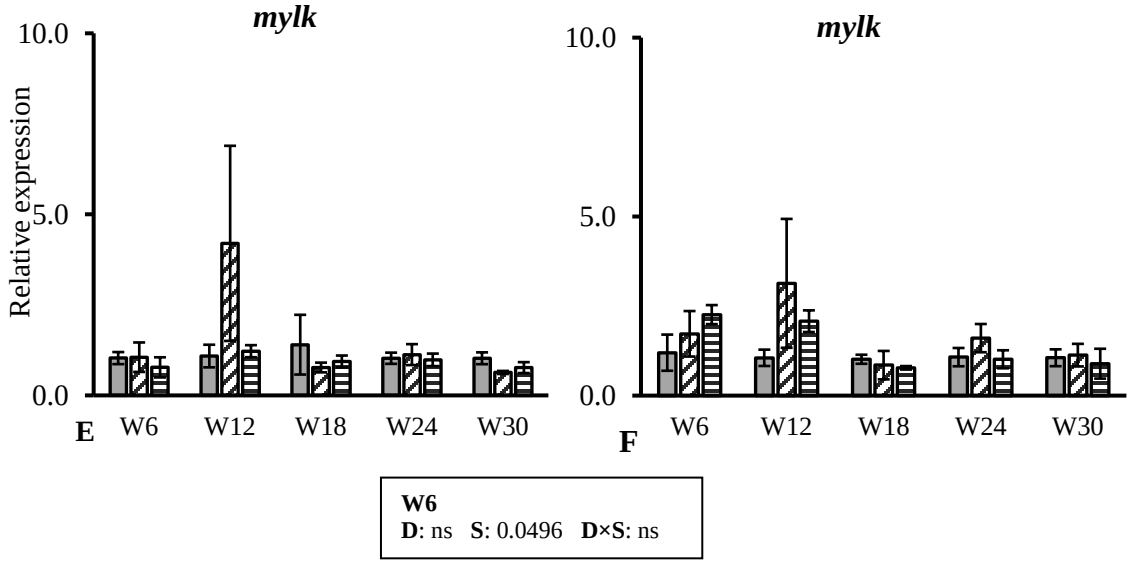
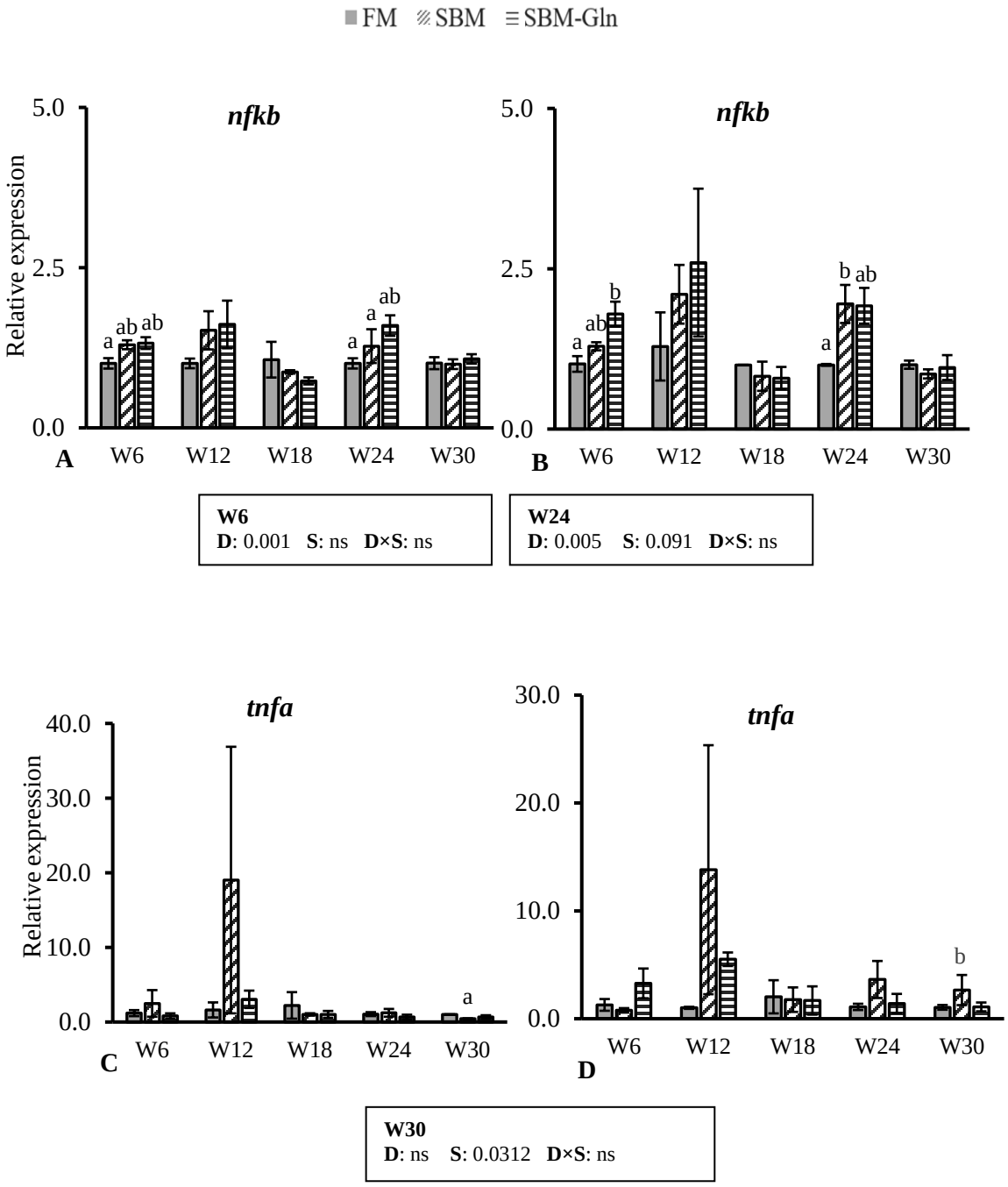


Figure 3.3. Relative gene expression ratio of barrier integrity makers in distal intestine of rainbow trout strain R (A, C, E) and strain T (B, D, F) fed three experimental diets.
Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, W18, W24 and W30) of the feeding trial. Values are shown as means ± SEM, $n = 9$. Displayed under the graph are p – values of factors diet (D), strain (S), and interaction (D×S).
FM: fishmeal; **SBM**: soybean meal; **SBM-Gln**: soybean meal-glutamine; **W**: week number of the feeding trial; ***cypa***: cyclophilin A.


FM SBM SBM-Gln
nfkb
Relative expression
5.0
2.5
0.0
a ab ab
a a ab
A
W6 W12 W18 W24 W30
nfkb
5.0
2.5
0.0
a ab b
a b ab
B
W6 W12 W18 W24 W30
W6
D: 0.001 S: ns D×S: ns
W24
D: 0.005 S: 0.091 D×S: ns
tnfa
Relative expression
40.0
30.0
20.0
10.0
0.0
a
C
W6 W12 W18 W24 W30
tnfa
30.0
20.0
10.0
0.0
b
D
W6 W12 W18 W24 W30
W30
D: ns S: 0.0312 D×S: ns

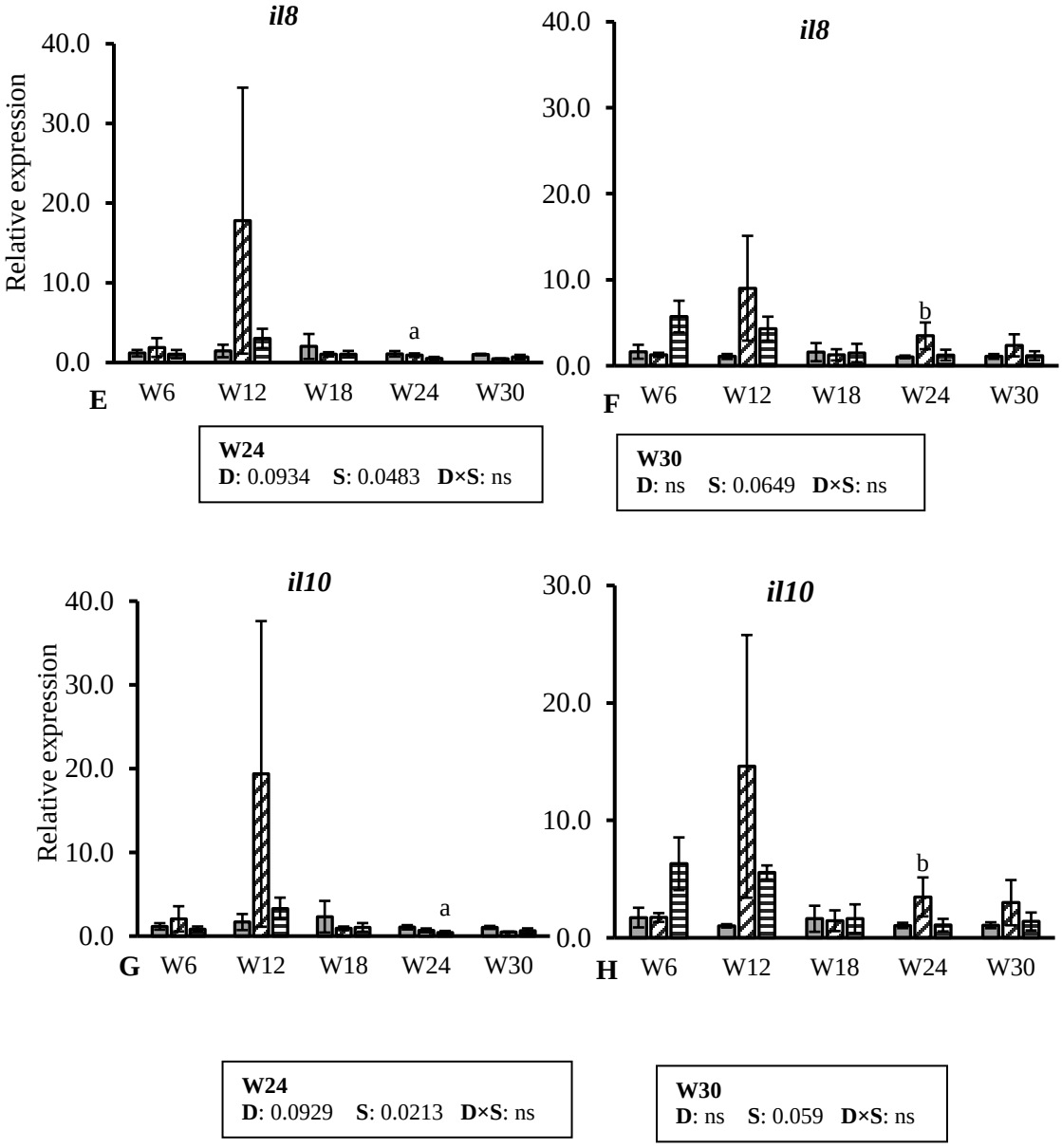


Figure 3.4. Relative gene expression ratio of immune makers in distal intestine of rainbow trout strain R (A, C, E, G) and strain T (B, D, F, H) fed three experimental diets.

Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, W18, W24 and W30) of the feeding trial. Values are shown as means ± SEM, $n = 9$. Displayed under the graph are p – values of factors diet (D), strain (S), and interaction (D × S). Significant differences are marked with an asterisk (*).

FM: fishmeal; **SBM**: soybean meal; **SBM-Gln**: soybean meal-glutamine; **W**: week number of the feeding trial; ***cypa***: cyclophilin A.

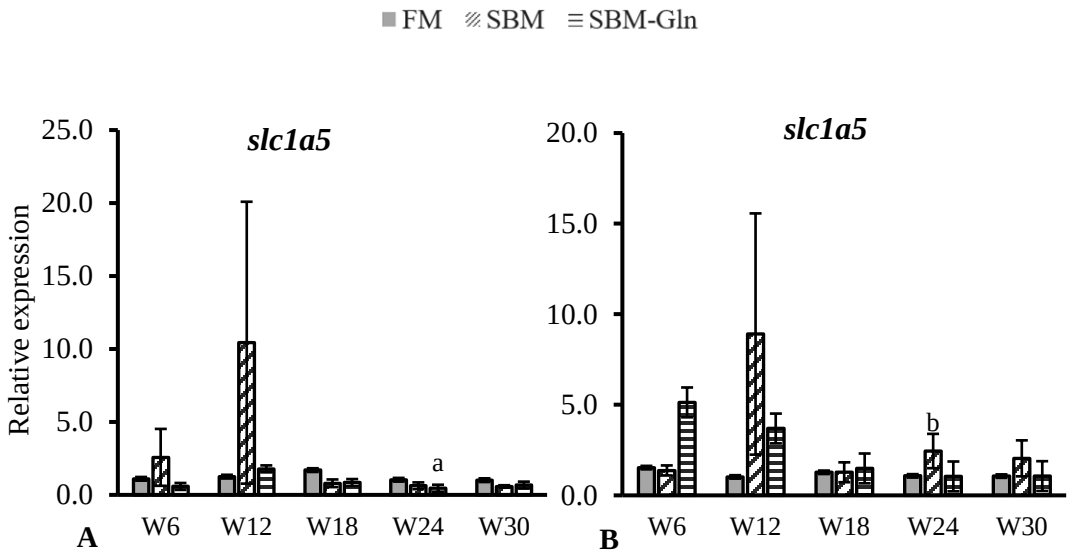


Figure 3.5. Relative gene expression of neutral amino acids transporter in distal intestine of rainbow trout strain R (A, C, E) and strain T (B, D, F) fed three experimental diets.
Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, W18, W24 and W30) of the feeding trial. Values are shown as means ± SEM, n = 9. Displayed under the graph are p – values of factors diet (D), strain (S), and interaction (D × S). Significant differences are marked with an asterisk (*).
FM: fishmeal; **SBM**: soybean meal; **SBM-Gln**: soybean meal-glutamine; **W**: week number of the feeding trial; ***cypa***: cyclophilin A.

Chapter 4:

The potential role of bile acids in soybean meal-induced enteritis mitigation in rainbow trout (*Oncorhynchus mykiss*) fed two levels SBM diets over an 18 - week feeding trial

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Abstract

An 18-week feeding trial was designed to examine the effects of two levels of soybean meal (SBM) and dietary supplementation with bile acids (BAs) on growth performance, feed efficiency, distal intestine soybean meal-induced enteritis (SBMIE), and histological condition of the liver in rainbow trout (*Oncorhynchus mykiss*). Five isonitrogenous and isolipidic diets were formulated including a control diet (FM) diet containing 25% FM protein; a 30% and a 40% inclusion level SBM diets (i.e., SBM30 and SBM40); and the same SBM30 and SBM40 each supplemented with 1.5% BAs (i.e., SBM30-BA and SBM40-BA). Growth performance was recorded at week 6 (W6), week 12 (W12), and week 18 (W18) of the trial; and liver and distal intestine samples were taken for histopathological analysis at the same points. There were no significant differences in weight gain, specific growth rate, hepatosomatic index, and condition factor among the dietary groups. There were no differences in the fillet proximate composition nor the protein and lipid retention. Feed conversion ratio (FCR) was significantly higher in the SBM40 diet at W18 compared to the FM, SBM30, and SBM30-BA treatments, indicating lower efficiency of this diet, as there were no differences in feed intake at any time point. Histopathological examination of the distal intestine revealed the occurrence of SBMIE in both SBM30 and SBM40 diets, with the severity proportionally increased with the level of SBM. Dietary supplementation with BAs contributed to partially mitigating the inflammation throughout the trial. Hepatic histopathology indicated that, despite initial signs of inflammation at W6, fish livers adapted to the SBM30 diet after this time point, showing similar liver morphology compared to the FM group. However, this was not the case in the SBM40 group, with livers showing signs of haemorrhaging and necrosis. The BA supplement in SBM30-BA diet improved fish liver health at the initial stages (W6) and throughout the entire trial in the SBM40-BA group, although this did not reach the same condition compared to fish fed the FM diet. These results indicate that SBM inclusion at levels above 30% increase FCR and cause intestinal and liver alterations; and that 1.5% BA supplementation to SBM-based diets exerts a protective effect in both intestinal and hepatic tissues in rainbow trout.

Introduction

Alternative plant protein ingredients like soybean meal (SBM) are vital in supporting the continued growth of the aquaculture industry. In the last twenty years, SBM has become an essential source of protein to replace the more expensive, traditionally used fishmeal (FM). The inclusion of SBM in commercial fish feed formulations, nonetheless, brings intricate challenges. It is well documented that high inclusion levels of SBM lead to gastrointestinal disorders, which are often associated with growth retardation, and to which salmonids are particularly sensitive (Gu et al., 2014; Murashita et al., 2013; Romarheim et al., 2008; Yamamoto et al., 2012). This condition has been termed 'soybean meal induced enteritis' (SBMIE), which occurs with an inflamed distal intestine and compromised barrier integrity (Baeverfjord and Krogdahl, 1996). Susceptibility to SBMIE is principally observed in carnivorous fish species, which may exhibit shorter and thicker intestinal villi, leukocyte infiltration of the lamina propria, increased number of goblet cells, and reduced absorptive capacity. In addition to SBMIE, digestibility of lipids can also be affected by dietary SBM, potentially compromising growth or fillet quality (Bruni et al., 2021; Kaushik et al., 1995). Most of these effects are attributed to a combination of anti-nutritional factors (ANFs) present in SBM, such as saponins and isoflavones (Francis et al., 2001; Krogdahl and Bakke, 2015). In particular, dietary saponins have been linked to hypocholesterolaemia in various vertebrate species as previously reviewed (Francis et al., 2002; Milgate and Roberts, 1995), probably by inhibiting cholesterol absorption via insoluble complex formation and promoting faecal bile loss (Coulson and Evans, 1960). In addition, dietary fibre can interact and form complexes with bile (Gunness and Gidley, 2010), which would contribute to a lowering of cholesterol. In fish, SBM diets up to 20% or higher, have been shown to cause hypocholesterolaemia, decreased bile levels in intestinal digesta, and altered liver or distal intestine morphology. Some or all of these symptoms have been described in a wide range of species including hybrid snakehead (*Channa argus* × *Channa maculata*) (Duan et al., 2022), rainbow trout (Iwashita et al., 2008; Murashita et al., 2013; Yamamoto et al., 2007), Atlantic salmon (*Salmo salar*) (Gu et al., 2014; Kortner et al., 2016, 2013), turbot (*Scophthalmus maximus*) (Gu et al., 2017), grouper (*Epinephelus lanceolatus*) (Lin et al., 2022), Japanese yellowtail (*Seriola quinqueradiata*) (Nguyen et al., 2013), pompano (*Trachinotis blochii*) (Nguyen and Van Do, 2021), Nile tilapia (*Oreochromis niloticus*) (Jiang et al., 2018; Wangkahart et al., 2022), and hybrid sturgeon (*Acipenser baerii* × *Acipenser schrenckii*) (Yue et al., 2022).

Bile acids (BAs) are biological detergents synthesized in the liver from cholesterol, conjugated with glycine or taurine and stored in the gall bladder until secreted into the intestine during digestion. Up to 95% of BAs released in the intestine, are reabsorbed in the distal intestine,

and returned to the liver via portal vein circulation. This is known as the enterohepatic circulation of BAs, which is critical to replenish the BA pool in the liver and regulate their synthesis by a feedback loop mechanism (Romano et al., 2020). Maintaining enterohepatic circulation homeostasis is therefore key to ensuring that hepatic cholesterol reservoirs are maintained at normal levels.

As emulsifiers, BAs have gained attention for their potential to enhance the utilization of lipids, while relying on less expensive ingredients (e.g. carbohydrates) as an energy source (Guo et al., 2020; Romano et al., 2022; Staessen et al., 2020; Yu et al., 2019) or when using alternative sources of dietary oil (Wangkahart et al., 2022). Other studies, have used supplemental BAs as a tool to restore bile and cholesterol physiology (Jiang et al., 2018; Murashita et al., 2018). Apart from their emulsifying properties, BAs may also act as signalling molecules that are involved in a wide range of cellular and physiological mechanisms. Importantly, BAs have been described in higher vertebrates to modulate intestinal inflammation in intestinal bowel disease models (Cipriani et al., 2011; Fiorucci et al., 2018; Gadaleta et al., 2011; Pols et al., 2011; Thomas et al., 2022). In fish, the first evidence came from a study carried out in rainbow trout in which bovine bile salts were supplemented to SBM diets. These authors were able to demonstrate improved growth performance and restored liver and distal intestine morphology (Yamamoto et al., 2007). This finding was further evidenced in a later study carried out on the same species, (Iwashita et al., 2008), and more recently in a study on common carp (*Cyprinus carpio*) where similar beneficial effects on the liver and intestine were observed (Yao et al., 2021).

Based on the above, BAs could make a robust functional feed additive in SBM based diets by mitigating SBMIE while compensating for faecal bile loss and improving hepatic function. However, none of those studies aimed to investigate whether these outcomes can be maintained over a longer period, and thus, fail to determine if BA supplementation would be effective during full grow-out periods at farm operations and to dismiss potential cytotoxic effects. To further examine these effects in the long term, an eighteen-week feeding trial was performed to investigate the effects of SBM-based diets on growth performance, feeding efficiency as well as on liver and intestinal histopathology of rainbow trout. Two levels of SBM inclusion, 30% and 40% were selected, and a mixture of BAs was added to each at 1.5%. Growth and histopathology results were recorded every six weeks and are therefore presented for three study time points.

Materials and Methods

Experimental diets

Five experimental diets were formulated to have a 43% crude protein level and 16% lipid content. A 25% fish meal-based diet (FM) was selected as a control diet. Soybean meal (SBM) was used as the main source of protein for the remaining dietary treatments at the expense of FM, poultry meal, and canola meal. Two diets were formulated to contain SBM at an inclusion level of 30% (SBM30) or 40% (SBM40); and two more diets included both SBM30 and a SBM40 supplemented with 1.5% bile acids (BAs), composed of 3.0% cholic acid (CA), 21.0% deoxycholic acid (DCA) and 6.0% chenodeoxycholic acid (CDCA) (Redox Inc., Los Angeles, CA, USA). The dietary formulation and proximate composition of the experimental diets are detailed in Table 4.1, and the amino acid profile is shown in Table 4.2.

All diets were produced following commercial manufacturing methods by using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) at the Bozeman Fish Technology Center, Bozeman, MT. Diet mash was exposed to an average of 114°C for 18-s in five-barrel sections, and the last section was water cooled to an average temperature of 83°C. Pressure at the die head was approximately 450 psi. The pellets were then dried in a pulse bed drier (Buhler AG) for 25 min at 102°C and cooled at ambient air temperatures to reach final moisture levels of < 10%. Feed pellet size was 2.5 mm for the first 12 weeks, and 3.5 mm from week 13 to week 30 of the feeding trial. Fish oil was top-dressed using a vacuum coating (A.J Flauer Mixing, Ontario Canada) after the pellets were cooled. Diets were stored in plastic lined paper bags at room temperature until use. Appropriate feed sizes were used throughout the feeding trial. All diets were fed within 4 months of manufacture.

Experimental design and sampling

Rainbow trout were obtained from Troutlodge (Sumner, WA) and kept at the Cold-Water Lab at the Aquaculture Research Institute at the University of Idaho (Moscow, ID). A total of two thousand fish were randomly distributed into twenty 350 L tanks (100 fish per tank) in a recirculating aquaculture system, incorporating four replicate tanks per dietary treatment. After one week acclimation, fish initially weighing 40.4 ± 1.3 g were fed each of five experimental diets to apparent satiation six days per week for a period of eighteen weeks. Feed intake was calculated daily. Rearing conditions were maintained at a constant photoperiod 12 hrs. dark/12 hrs. light at an average temperature of 14.33 ± 1.07 °C. Water was supplied at a constant rate of 160 mL sec^{-1} and oxygenation was accomplished by continuous aeration to each tank, and dissolved oxygen was kept at

5.55 ± 0.59 mg L^{-1}. Ammonia nitrogen, nitrite and pH were maintained at 0.03 ± 0.02 mg L^{-1}, 0.19 ± 0.18 mg L^{-1} and 7.58 ± 0.16, respectively. All water quality parameters were monitored weekly using commercial kits (LaMotte, Chestertown, MD, USA).

Throughout the feeding trial, fish were sampled every six weeks after 12 h after receiving the last meal, for a total of three times. Samplings took place at week 6 (W6), week 12 (W12) and week 18 (W18), during which fish were euthanized using an overdose of tricaine methanesulfonate (Syndel Inc., Ferndale, WA) at 250 mg L^{-1} buffered to pH 7.0-7.5. A total of nine fish per tank were euthanized at W6 and W18, and a total of six fish per tank at W12. During each sampling, fish weight and length were recorded individually, and livers were dissected and weighed for hepatosomatic index (HIS) calculation. Liver and distal intestine samples were taken from three fish per tank (n = 12) for histopathology analysis. A stocking density of 35 Kg m^{-3} was maintained throughout the trial. To ensure optimal stocking densities in the long term, an additional number of fish were pulled randomly out of each tank at week 7.

All experimental procedures involving live fish were carried out with prior approval from the Institutional Animal Care and Use Committee, University of Idaho (IACUC-2021-49).

Growth, condition and feeding efficiency

Growth performance was evaluated following the below indices calculation at each sampling point (W6, 12 and 18), except for protein and lipid retention which were calculated only at W18:

- Weight gain (WG, g) = average final weight (g) – average initial weight (g)
- Specific Growth Rate (SGR, %/day) = $\frac{\ln(final\ weight)^{i} - \ln(initial\ weight)}{100 * days^{ii}}$
- Feed intake (FI/fish/day) = $\frac{feed\ intake\ (g) x\ average\ fish\ weight\ (g)^{i}}{days^{ii}}$
- Feed Conversion Ratio (FCR) = total feed intake (g)/weight gain (g)
- Condition factor (K) = $(10^4\ x\ fish\ weight\ (g))/[(fork\ length\ (cm)\ x\ 10)^3]$
- Hepatosomatic index (HSI) = (liver weight (g)/fish weight (g)
- Protein retention (PR, %) = $\frac{fillet\ protein\ gain\ (g)}{protein\ intake\ (g)}$
- Lipid retention (LR, %) = $\frac{fillet\ lipid\ gain\ (g)}{lipid\ intake\ (g)}$

[i]Final weight as recorded at each sampling point
[ii]Cumulative number of days passed until a given sampling point

Proximate composition of feed and fish fillet samples

The proximate composition of the experimental feeds as well as initial and final fillet samples was carried out following the methods by AOAC (1995). In feed samples, protein content was determined using the Kjeldahl method (N x 6.25) (Kjeltec 8100, FOSS, Denmark) and crude fat was calculated by petroleum ether extraction following acid hydrolysis on SoxCapTM (FOSS, 2050, Denmark). In fillet samples, crude protein was analyzed using a combustion method with a nitrogen determinator (rapid N exceed, Langenselbold, Germany), and crude lipid was determined by hydrolysis using an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY, USA) and petroleum ether extraction sample extraction was done using an ANKOM XT15 extractor. Moisture content was measured after drying the feed and fillet samples at 105°C for 24 h and ash was assessed after ignition at 500°C for 12h. Total energy content was determined using a Parr bomb calorimeter (Parr Instrument Co., Moline, IL, USA).

The amino acid profile of the feeds was determined after acid hydrolysis (6N HCl, 110^{o}C, 24h) and derivatization by AccQ-Tag™ Ultra, according to the amino acid analysis application solution (Waters Corporation, Milford, MA, USA). An internal standard (DL-Norvaline, Sigma) of 2.5 mM was used and ultra-performance liquid chromatography (UPLC) was performed on an Acquity system (Waters Corporation) equipped with a PDA detector. A Waters' BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm) was used at a temperature of 55 °C, the detection wavelength was set at $\lambda = 260$ nm and the flow rate at 0.7 mL min^{-1}. The software Empower v.2.0 (Waters) was used for peak identification and quantitation, using an amino acid external standard (Waters). All analyses were performed in duplicate.

Liver and distal intestine histopathology

Liver and distal intestine segments from three fish per tank were placed in tubes containing Bouin's solution and tissue samples were fixed for less than 24 h, after which samples were transferred and immersed in 70% ethanol for preservation. Distal intestine samples were then placed in cassettes and dehydrated at increasing concentrations of ethanol followed by removing ethanol in xylene and then embedded in paraffin wax. Sections were cut (5μm) using a rotary microtome (HM 340E, Thermo Scientific) and then stained with hematoxylin and eosin and the slides were mounted with DPX solution. Sections were taken with a microscope mounted with camera at x 200 magnification. Both the villi length and width were measured using a Leica Application Suite software after the pictures were taken and prior calibration. Histological scoring of the distal intestine was done by blind evaluation of slides as described in Palma et al. (2021). In distal intestine, the

variables assessed were mucosal fold fusion, goblet cells, inflammatory cells, vacuoles prevalence, and infiltration of the lamina propria. In liver, tissue samples were assessed for vacuoles, inflammatory cells, necrosis, haemorrhaging, and congested vessels. Slides were visually scored (1-best, 5-worst). Twelve observations were taken randomly from each diet replicate for each variable, and the sum of twelve observations was used in the calculation of the cumulative score of all variables per diet group used for statistical analysis. For villi length and width, twelve measurements were taken randomly from each diet replicate and the mean of each was used for statistical analysis.

Statistical analysis

All growth performance parameters including WG, SGR, HIS, *K*, PR and LR; fillet proximate composition and FI data were subjected to one-way analysis of variance (ANOVA) and the differences between treatment means were compared by Tukey's honest significance test. Histology data were analysed using non-parametric tests to determine significant differences between dietary treatments and significant differences within a specific diet over time. Hence, Kruskal-Wallis followed by Dunn's post-hoc test where applied. Normality and homogeneity of variance of all data presented were tested by Shapiro-Wilk and Levene's test, respectively. Differences among groups were considered significant at $p < 0.05$. All statistical analyses were performed using R statistical software (v. 3.6.2).

Results

Growth performance

All growth performance parameters are summarized in Table 4.3. There were no significant differences in WG, SGR, FI, HIS and *K* between dietary treatments throughout the experimental trial ($p > 0.05$). Although not significant, WG was numerically the lowest in the SBM40 group at all three sampling points, and this pattern was similarly observed for SGR at W6 and W12. SBM40-BA WG and SGR figures, however, were higher and closer to the other dietary treatments, but this divergence between SBM40 and SBM40-BA was also not significant. FCR was significantly impacted by dietary treatment at W18 ($p < 0.001$), with highest FCR recorded for the SBM40 diet, followed by SBM40-BA, SBM30 and SBM30-BA. Both SBM30 and SBM30-BA appeared to mirror the FM group FCR values, which were the lowest ($p > 0.05$). Throughout the trial, no differences were seen in FI among dietary groups ($p > 0.05$).

Proximate composition

Proximate composition of fillet samples obtained at W18 as well as PR and LR indices are shown in Table 4.4. The FM diet showed the highest PR and LR and although there was no significant difference observed among dietary treatments, the lowest lipid content and LR happened in the SBM40 group, which also had higher protein and moisture content in this dietary treatment.

Histopathology of distal intestine

Overall, intestinal morphology of rainbow trout was more severely impacted by the SBM40 diet than by the SBM30 diet. Intestinal villi were significantly shorter and thicker in fish fed the SBM40 diet than those in the FM control, and significantly thicker than those in the SBM30-BA treatment at all sampling points (all $p \leq 0.05$) (Fig. 4.1). Similar types of differences between SBM40 and SBM30 treatments were seen throughout the trial, but only the length of villi differed significantly at W6. Fish fed the SBM30 diet appeared to have a more negative impact on villi width, which were significantly thicker than those of fish fed the FM at W6 and W12 (all $p \leq 0.05$), but this seemed to be less pronounced by W18 ($p \geq 0.05$). Instead, the length of villi resembled the control group at W6 and W12 ($p \geq 0.05$), but significantly shortened at W18 ($p < 0.001$).

The supplementation with BA led to a significant improvement in intestinal morphology in the SBM30-BA group, which mirrored ($p \geq 0.05$) the FM diet in terms of villi length and width (Fig. 4.1). The SBM40-BA also resembled the FM diet at W6 and W12 for villi width and length respectively ($p \geq 0.05$). However, at W18, the villi length (Fig. 4.1a) of fish fed SBM40-BA diet was the same as the SBM30 and SBM40 treatments, and the same as the SBM30 treatment in case of villi width (Fig. 4.1b). Instead, at this same time point, the SBM30-BA group shared similarities with both the FM and SBM30 treatments ($p \geq 0.05$) (Fig. 4.1).

Cumulative histological scores in the distal intestine are in line with morphological observations (Fig. 4.2a). Highest scores were recorded at the SBM40 dietary treatment, followed by the SBM30, and both were significantly different that the FM control at all sampling points (all $p \leq 0.05$). The supplementation with BA significantly contributed to lower the scores in the SBM30-BA, which mirrored those of the FM group ($p > 0.05$), showing superior performance compared to both SBM30 and SBM40 (all $p \leq 0.05$). The SBM40-BA also showed signs of improvement although, apart from W6, in which it shared commonalities with the FM group ($p > 0.05$), it represented an intermediate point resembling the SBM30-BA at W6 and W12 ($p > 0.05$) and was the same as SBM30 by W18 (Fig. 4.2a).

The degree of mucosal fold fusion did not show significant differences across diets at W6 and W12 (all $p > 0.05$), however, it was significantly increased in both SBM40 and SBM40-BA diets at W18, both of which differ from the FM diet (all $p \leq 0.05$). Supra nuclear vacuole abundance seemed to be consistently higher in non-supplemented SBM diets, with a proportional improvement in both BA diets at W6 and W12 (Fig. 4.2a, 4.3 and 4.4). However, this effect was lost at the end of the trial, where neither of the BA supplemented diets resembled the FM group ($p \leq 0.05$), but rather, were the same as the SBM-based diets (all $p > 0.05$) (Fig. 4.5). The abundance of goblet cells appeared to be modulated by BA in the SBM30 and SBM40 groups, reaching similar levels as those of the FM diet at all three sampling points ($p > 0.05$) (Fig. 4.2a; Fig. 4.3 – 4.5). This contrasts with SBM30 and SBM40, which always remained significantly higher than the FM group (all $p \leq 0.05$).

To compare individual diet performance in time, each parameter included in the cumulative histology scoring was assessed individually for a given dietary treatment at each of the three sampling points. In the FM diet, supranuclear vacuoles were higher at W6 compared to W12 and W18 ($p = 0.010$). The SBM30 group showed a significant improvement in lamina propria infiltration at W18 compared to W6 and 12 ($p = 0.002$). The SBM40 diet tended to become worse at W18 as per the level of mucosa fold fusion ($p < 0.001$). In BA diets, inflammatory cells were less abundant in the SBM30-BA diet at W6, but they were significantly increased at W12 and 18 ($p = 0.015$), and supranuclear vacuoles were significantly higher at W12 and 18 compared to W6 ($p = 0.003$). Cumulative histological score was also increased by W18 compared to W6. Lastly, the degree of fold fusion in the mucosa was significantly higher in the SBM40-BA diet at W18 compared to the two earlier time points ($p = 0.004$), in a similar way that observed for SBM40. All this together indicates that SBMIE is being worsened proportionally to the time of exposure.

Histopathology of liver

Cumulative histological scores were significantly higher in the SBM40 group than in the FM control group during the entire feeding trial (all $p \leq 0.05$) (Fig. 4.2b). In turn, feeding a SBM30 diet led to a significant negative impact on hepatic tissue at W6 and W12 compared to FM ($p \leq 0.05$), however, by W18 this detrimental effect faded away and tissue resembled the FM group outcome at this time point ($p > 0.05$). Indeed, there was an abating trend of the score at W12, which was different from SBM40 at this point, as well as at W18 (all $p \leq 0.05$). Supplemental BA to SBM30 feed did have a protective effect on the liver, as scores reflect the same pattern as FM at W6 and W18 ($p > 0.05$), while W12 shares similarities with both FM and SBM30 scores. In the SBM40-BA diet, the protective effect, although present, did not lead to comparable liver health as that of the FM diet at

any sampling point (all $p \leq 0.05$). However, scores were improved to some extent after W12 and through W18 sharing similarities with both SBM30 and SBM40, respectively.

Analysis of individual scores per dietary treatment and time points showed there was higher liver vacuolization in the FM group at W6 compared to W18 ($p = 0.013$). Furthermore, the cumulative score is significantly smaller at W18 compared to W6 ($p = 0.002$). Both in the SBM30 and SBM30-BA group, the cumulative score was significantly reduced at W12 and W18 (all $p = 0.000$), as well as reduced inflammation, necrosis, and haemorrhaging (all $p \leq 0.05$) in the former; and a significant decrease in necrosis and haemorrhaging in the latter (all $p \leq 0.05$).

Presence of necrotic tissue in the SBM40 diet was significantly accentuated at W18 compared to W6 (Fig. 4.2b, 4.6d, 4.8d) ($p = 0.022$). Hepatic haemorrhaging was improved at W12 (Fig. 4.2b, 4.7d), but this was not maintained when measure at W18, where significantly higher haemorrhaging was recorded compared to W12 ($p = 0.009$). The SBM40-BA dietary group showed decreased necrosis at W12 compared to W6 ($p = 0.006$), but this was not maintained towards W18. Haemorrhaging was improved at first, showing significantly lower scored from W6 to W12 ($p < 0.001$), but this was not maintained towards W18 ($p \geq 0.05$).

Discussion

The susceptibility of commercial carnivorous species like rainbow trout to SBMIE is a cause for concern when shifting from FM to more sustainable plant-based protein sources, like SBM (G. Baeverfjord and Krogdahl, 1996; van den Ingh et al., 1991). This has fuelled extensive research in the functional feed sector, in order to identify novel additives that act as immunostimulants (Dawood et al., 2018). In the present study, the potential role of BAs in promoting anti-inflammatory effects was tested in dietary treatments of up to 30 and 40% SBM inclusion levels. Moreover, the selected eighteen-week experimental period allowed the investigation into how persistent any beneficial effect of BAs could last, as well as the emergence of any physiological signs of adaptation to a high SBM diet over time.

Growth performance

In many instances, the first potential sign of SBMIE is reduced feed acceptance followed by inferior growth performance. In the present study, only FCR was impacted by the SBM40 diet after W12, and this was presumably caused by suboptimal feed utilization, as FI consistently remained similar among dietary groups. Results from this study concerning the SBM30 group are in line with our previous study (presented in Chapter 2), where no differences in WG were observed

after thirty weeks in a similar commercial strain. While this could indicate a certain level of tolerance to SBM, a more notable negative effect on growth was expected after increasing the SBM inclusion from 30 to 40%. Instead, despite growth being lower in the SBM40 group, these differences were not significant; furthermore, the supplemental BAs did not show any effect on growth. The lack of significant differences on WG can be explained by the increased variation within diet groups. Nevertheless, BA inclusion led to a slightly better FCR in the SBM40-BA diet compared to SBM40 diet, suggesting BA improved feed utilization. A positive effect of bile on FCR was similarly observed in carp, when bile doses as low as 60 and 600 mg/Kg were included in the diets (Yao et al., 2021). Although not significant, FI was slightly lower in the SBM30-BA diet, however, this group showed about the same or slightly higher WG and SGR values. Other studies in rainbow trout have previously been able to improve WG and SGR in addition to feed efficiency after a ten week feeding trial using different sources of bile as a dietary supplement (Iwashita et al., 2008; Yamamoto et al., 2007). In Atlantic salmon, neither supplemental bile compounds nor cholesterol were able to restore lower SGR from high SBM diets that was comparable to those fed a control diet (Kortner et al., 2016).

The lack of difference between dietary treatments in proximate composition of the fillets at the end of the trial, together with the lack of differences for PR and LR suggests that neither high dietary SBM nor BA would potentially impact fillet quality. These results are similar to those found by Yamamoto et al. (2007) in rainbow trout but are, nonetheless, in contrast to what would be expected, as several studies have demonstrated otherwise. For instance, our previous study with rainbow trout pointed to a higher lipid accumulation in SBM-fed fish, associated with slightly lower protein deposition (Chapter 2) as opposed to the lower lipid content in the fillet found in trout by Brinker and Reiter (2011) and Hang et al. (2022) in rainbow trout. Seibel et al. (2022) found both protein and lipid to be decreased in the whole body of rainbow trout fed increasing inclusion levels of SBM, although differences in lipid content were not significant. In Japanese yellowtail and pompano, feeding different forms of SBM led to lower lipid deposition in both liver and muscle, compared to a FM control diet (Nguyen et al., 2013; Nguyen and Van Do, 2021).

Hang et al. (2022) also explored the effects of supplemental bile and showed that lipid content did not vary between an enzymatically hydrolysed SBM diet and the supplemented diet, both of which were significantly lower than the FM group; however, the 0.02% bile supplementation level selected in that study may have been too low to exert an effect. In turbot, whole body lipid was also reduced in the plant protein group; furthermore, this was reverted by 0.5% taurocholate supplementation (Gu et al., 2017a). In tilapia, different levels of bile supplementation all lead to lower

lipid content in muscle, liver and whole body but this was compared to a plant-based control diet (Jiang et al., 2018). Variations in the source of dietary lipids used in each study as well as the type of dietary bile may complicate reaching consistent conclusions.

Other growth metrics such as HSI and *K* were not influenced by dietary SBM levels, which is in contrast with previous observations made in a similar setting, as smaller liver sizes were described by after feeding SBM diets for thirty weeks (Chapter 2). In general, smaller liver sizes in rainbow trout are often associated with SBM-based diets, presumably due to lower fat content in the liver (Brinker and Reiter, 2011; Bruni et al., 2021; Kaushik et al., 1995; Yamamoto et al., 2007); as was also determined in similar studies using giant grouper (Lin et al., 2022). However, a lack of effect of high SBM on HSI in rainbow trout has also been the case in some instances (Bruni et al., 2021; Seibel et al., 2022). In the present study, no effect of bile on HSI was reported, and variations exist in the literature regarding the role of bile in this case. For instance, Yamamoto et al. (2007) found lower HIS in rainbow trout fed SBM diets after only ten weeks, and bile salt supplementation did not revert this effect. In grouper, supplementation with soy lecithin did not help restore HSI to FM values (Lin et al., 2022). Only Iwashita et al. (2008) reported an increase in rainbow trout HSI to match a FM control diet when ~ 1.0% cholyltaurine, was supplemented to FM-free diets with high SBM inclusion. Similarly, Gu et al. (2017) found that 0.5% taurocholate increased the HIS in turbot fed plant-based diets.

The lack of variation in HSI was unexpected in the present study since hepatic histopathology analysis revealed alterations, as discussed under the histopathology of liver section.

Histopathology of distal intestine

Histology of the distal intestine in the FM dietary treatment was expected to remain stable throughout the entire trial. However, supra nuclear vacuoles were higher during the first six weeks of the trial, which could be an adaptive strategy to a new diet and should be kept in mind when evaluating other dietary treatments. The results shown here demonstrate a clear SBM-dependent change to the distal intestine morphology in terms of length and width of villi. These seemed to be accentuated in a dose-dependent manner to the level of dietary inclusion of SBM, with more acute SBMIE symptoms in the SBM40 group. Keeping this in mind, the mitigating power of supplemental BA seems proportional to the damage caused by the primary dietary protein source. The results obtained here made it challenging to point at a single factor that might be particularly affected by the inclusion of soy or be a target for a BA mitigating effect. A primary beneficial effect of BAs seems to be on goblet cell abundance, as these were maintained at FM levels in both BA supplemented diets

throughout the entire trial. The amount of vacuolization appears modulated by both BA diets until W12 but reduces progressively until no differences are seen at W18. Interestingly, despite clear changes on the length and width of the mucosal villi, fusion of mucosal folds did not appear affected by the diet until W18, where inclusion of SBM40 starts to have an impact, regardless of the dietary BA. This is opposite to histopathological analysis done after twelve weeks of feeding rainbow trout a SBM30 (Chapter 2). In that study a 30% inclusion of SBM showed an increase in mucosal fold fusion while the use of 2.0% glutamine helped reduce this effect. These observations were, however, limited to only one time point (W12) in a study that looked at five time points for thirty weeks. This happened similarly with vacuolization and goblet cells, showing changes at one specific time point, and, in the case of goblet cells, only limited to one strain.

One of the key objectives of this study was to determine if supplemental dietary BAs would have a mitigating effect, at which point in time this effect becomes strong enough to make a significant difference, and for how long it could be maintained. The change over time for histology markers in fish fed the SBM30-BA and SBM40-BA diets showed that the effect of BA supplementation may start diminishing over time, possibly at around W12. The shared trend in mucosal fold fusion observed in both SBM40 and SBM40-BA diets, in which it worsened at W18 could indicate a lack of a BA mitigating effect on this parameter, as indeed, there were no differences between the two diets for this parameter throughout the trial. Similarly, there seems to be a reduction in BA effectiveness on maintaining villi length and width in the SBM40-BA diet by the end of the trial, but this was less evident in fish fed SBM30-BA diets. The study by Iwashita et al. (2008) supports this protective effect of bile in the distal intestine of rainbow trout, and mucosal fold and number of absorptive vacuoles were identified as bile-improved targets (Iwashita et al., 2008). Similar beneficial effects of bile have been shown in carp (Yao et al., 2021), but not in turbot fed a 40% SBM diet (Gu et al., 2017a). Opposite results are nonetheless found in Atlantic salmon fed a high SBM diet, in which bovine bile salt supplementation did not show any signs of enteritis mitigation after 77 days (Kortner et al., 2016).

Histopathology of liver

In the liver, the FM control group displayed somewhat elevated vacuolization at W6 which then diminished; apart from that, the appearance remained normal throughout the trial. While hepatic tissue appears compromised at W6 in the SBM30 diet, these effects began diminishing at W12 and mirror the FM appearance by W18. The question, however, of whether these adaptations are due to how long the fish have been exposed to the SBM30 diet, or whether it is solely dependent on

fish size, remains to be answered. Supplemental BAs made a significant improvement at W6 and W12, but seemed unnecessary after W12 as a more FM-like appearance was recovered in the SBM30 group by W18. It is important to point out that BAs did not cause cytotoxic effects in the liver nor led to excessive vacuolization, evidenced by the similarities shared between the FM, SBM30 and SBM30-BA treatments. However, similar to the possible adaptation to SBM discussed above, it is unknown if the impact of BAs on the liver is dependent on time of exposure to SBM or the size of the fish (i.e., bigger fish being more resistant), making it challenging to establish a feed management protocol for the use of supplemental BAs. The evolution of hepatic health in the SBM40 group can help shed light on this. Although a reduction in haemorrhaging in SBM40 at W12 led to a lower cumulative score, liver abnormalities worsened again after W12, suggesting size did not necessarily help in tissue adaptation. In this case, the addition of BAs moderately improved liver histopathology, especially by ameliorating haemorrhaging, but this still differed from the control group.

The level of haemorrhaging and necrosis observed in the present study has not been previously reported in salmonids. Studies carried out in Atlantic salmon have shown different effects on liver. For instance, a 21 day short term exposure to SBM led to early decrease of glycogen/lipid deposition plus increased number of bile ducts (Kortner et al., 2013) while a longer term exposure of 10 weeks led to significant hepatic steatosis (Gu et al., 2014). In turbot fed 40% level SBM, Gu et al. (2017) found atrophied hepatocytes with reduced numbers of vacuoles, but taurocholate supplementation partially reverted liver histology to a FM appearance.

As mentioned above, this contrasts with the lack of differences in HSI, as a reduction in liver weight would be expected when significant necrosis is taking place. Discrepancies exist among studies because the use of SBM or plant protein sources has been associated both with enlarged and smaller livers. Hence, some studies deem beneficial that a particular additive reduce HSI based on the premise that plant protein sources diets cause liver hypertrophy (Jiang et al., 2018; Wangkahart et al., 2022; Yao et al., 2021) while other studies would take the opposite positively, as a sign of higher lipid and glycogen stores as discussed above (Gu et al., 2017a; Iwashita et al., 2008). Hence, as a crude indicator of liver health, HIS would not be a reliable indicator to draw conclusions on either the effect of high SBM or supplemental BAs.

There are fundamental differences in experimental design in the studies carried out in tilapia and carp versus the study presented here, as well as the cited examples with rainbow trout, Japanese yellowtail, turbot and pompano. While Jiang et al. (2018) report that a 1.35 g Kg^{-1} dietary BA can lead to excess hepatocyte vacuolization, Iwashita et al. (2008) showed that a supplemental dose of 1.5% helped reduce liver histological abnormalities such as atrophied nuclei, which supports

the results presented here. This makes it challenging to compare results, let alone draw conclusions on optimal BA doses or risk of cytotoxicity. Given the broad range of bile acid chemical forms, more attention should be also given to which type of bile or which combinations could render optimal results. Insights provided by Iwashita et al. (2008) and Gu et al. (2017) point out cholyltaurine, which is a primary bile acid most abundant in fish (Iwashita et al., 2008; Murashita et al., 2013) as a successful bile additive both in terms of growth and liver health in fish. This raises the question of which specific bile acid, bile combination or even source, impacts liver health and how. It has been demonstrated that SBM-based diets alter the biliary bile composition in rainbow trout by shifting ratios of cholyltaurine and chenodeoxycholyltaurine (Yamamoto et al., 2012). Furthermore, it is known that changes in the intestinal microbiota happen during SBMIE (Merrifield et al., 2009). These differences may impact the capacity to synthesize secondary BAs in the distal intestine, leading to an imbalance between primary and secondary BAs (Biagioli et al., 2021). In the current study, the BA mixture used contained predominantly DCA, which is a secondary BA. In mammals, certain BA such as DCA or taurolithocholic acid have been shown to elicit different responses in different target cells (Thomas et al., 2022). Therefore, future investigations should address the effects of specific types of bile as well as the specific BA composition in the intestine of fish both in normal conditions and during SBMIE, in order to make informed decisions as to which BA or BA combination may serve most effectively.

Conclusion

The results presented herein serve as further evidence that despite marginal differences in growth performance (WG, SGR) and FI, dietary SBM negatively affected the liver and distal intestine of rainbow trout. Eventually, FCR was higher when a 40% SBM formulated diet was fed, while SBMIE was notable starting at a 30% SBM inclusion, with severity proportionally increased with increasing SBM. SBMIE was apparent from start to end of the trial, but the dietary addition of BA to both SBM30 and SBM40 feed helped mitigate inflammation throughout the trial. Detrimental effects of SBM in liver health were obvious in the SBM40 group throughout the trial, however these were less notable in the SBM30 at W6 and were almost fully gone by the end of the trial, indicating that hepatic tissues might have coped with SBM to a certain extent. In the SBM40 group, BAs had a beneficial role but, this rendered moderate liver histology, with a partial mitigation of deleterious effects, contrasting with the SBM30-based treatments. Nonetheless, the mitigating effect of the dietary BAs on liver histology seemed to slightly diminish towards the end of the trial, questioning the ability of BAs to maintain long term protective effects. However, this study showed that BA did

not exert any negative effects on either the distal intestine or liver over a long-term period of 18 weeks, which aids dismissing the potential cytotoxic effect of bile with extended exposure times. Since a slight loss of BAs effect could be happening in the long term, it could be interesting to explore higher supplementation levels in the 40% SBM diets. Overall, these results indicate that BA supplementation at a 1.5% level could be a good strategy for functional feed development in SBM-based diets with no apparent risk of cytotoxicity. Further research should focus on identifying whether specific combinations of BAs serve a more durable outcome or enhanced attenuation of inflammation in intestine and liver.

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Table 4.1. Ingredients and proximate composition of five experimental diets expressed as percentages (%).
FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

Ingredient	FM	SBM30	SBM40	SBM30-BA	SBM40-BA
Fish meal[1]	25.0	10.0	10.0	10.0	10.0
Soybean meal[1]	0.0	30.0	40.0	30.0	40.0
Canola meal[1]	13.5	5.0	2.0	5.0	2.0
Wheat gluten meal[1]	3.5	3.5	3.3	3.5	3.3
Corn protein concentrate[2]	4.3	5.5	4.7	5.5	4.7
Blood meal[1]	8.5	7.6	7.4	7.6	7.4
Wheat flour[1]	19.8	11.6	8.35	11.57	8.35
Poultry meal[1]	7.0	5.2	2.5	5.2	2.5
α-Cellulose[3]	0.0	1.5	1.5	0.0	0.0
Fish oil[1]	15.5	16.52	16.65	16.52	16.65
Dicalcium phosphate[1]	1.2	1.2	1.2	1.2	1.2
Choline chloride (60%)[1]	0.6	0.6	0.6	0.6	0.6
Vitamin premix[4]	0.8	0.8	0.8	0.8	0.8
Trace Mineral mixture[5]	0.1	0.1	0.1	0.1	0.1
Vitamin C, Stay C-35)[6]	0.2	0.2	0.2	0.2	0.2
Lysine[3]	0.0	0.49	0.45	0.49	0.45
Methionine[3]	0.0	0.26	0.27	0.26	0.27
Bile acid[7]	0.0	0.0	0.0	1.5	1.5
Analyzed composition (± SD)					
Crude protein (%)	43.0 ± 0.1	43.7 ± 0.1	43.1 ± 0.3	43.3 ± 0.1	42.6 ± 0.07
Lipid (%)	16.7 ± 0.3	16.6 ± 0.1	15.3 ± 0.0	16.3 ± 0.00	15.6 ± 0.00
Crude Fiber + N free extract (%)*	27.7	29.0	32.0	29.6	29.7
Ash (%)	8.8 ± 0.0	6.7 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.4 ± 0.0
Moisture	3.6 ± 0.0	3.8 ± 0.0	3.8 ± 0.0	3.6 ± 0.0	4.7 ± 0.0
Gross energy (MJ Kg^{-1})**	21.6	21.9	21.2	21.8	21.3

1 Rangen Inc., Buhl, ID, USA
2 Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
3 Sigma Aldrich, St. Louis MQ, USA
4 Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as

nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B12, 50 µg; and inositol as meso-inositol, 400 mg.

[5] US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as ZnSO4.7H2O), 75; Mn (as MnSO4), 20; Cu (as CuSO4.5H2O), 1.54; I (as KIO3), 10

[6] Skretting USA, Tooele, UT, USA.

[7] RUNEON Feed Bile Acids® was obtained from Redox Inc. (Los Angeles, CA, USA).

[*]Calculated by difference: 100 - (%protein + %fat + %ash + %moisture) (i.e., N-free extractives + crude fibre).

[**]Gross energy was calculated using combustion values for protein 23.6 MJ kg−1, lipid 39.5 MJ kg−1 and carbohydrate 17.2 MJ kg^{-1}, respectively.

Table 4.2. Proximate amino acid composition of each experimental diet.

Essential AA (g/100g feed)	FM	SBM30	SBM40	SBM30-BA	SBM40-BA
Lysine	2.60 ± 0.06	3.17 ± 0.06	2.76 ± 0.00	2.74 ± 0.10	3.07 ± 0.01
Methionine	0.85 ± 0.04	0.72 ± 0.01	0.74 ± 0.01	0.81 ± 0.02	0.67 ± 0.00
Histidine	1.43 ± 0.01	1.25 ± 0.04	1.39 ± 0.03	1.36 ± 0.01	1.15 ± 0.01
Isoleucine	1.35 ± 0.01	1.45 ± 0.01	1.52 ± 0.04	1.43 ± 0.01	1.40 ± 0.00
Leucine	3.74 ± 0.01	3.83 ± 0.03	3.79 ± 0.00	3.84 ± 0.03	3.65 ± 0.00
Phenylalanine	2.16 ± 0.08	1.92 ± 0.07	2.26 ± 0.04	2.28 ± 0.11	1.89 ± 0.01
Threonine	1.75 ± 0.01	1.64 ± 0.04	1.66 ± 0.02	1.71 ± 0.01	1.57 ± 0.01
Valine	2.09 ± 0.01	2.06 ± 0.02	2.11 ± 0.04	2.01 ± 0.03	1.93 ± 0.02
Arginine	2.11 ± 0.04	2.01 ± 0.11	2.20 ± 0.03	2.22 ± 0.08	1.98 ± 0.00
Non Essential AA (g/100g feed)	FM	SBM30	SBM40	SBM30-BA	SBM40-BA
Taurine	0.13 ± 0.02	0.04 ± 0.01	0.04 ± 0.00	0.28 ± 0.01	0.25 ± 0.00
Tyrosine	1.27 ± 0.05	1.13 ± 0.07	1.23 ± 0.01	1.33 ± 0.08	1.07 ± 0.00
Cysteine	0.28 ± 0.00	0.24 ± 0.02	0.27 ± 0.01	0.30 ± 0.01	0.25 ± 0.01
Hydroxyproline	0.39 ± 0.03	0.16 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.12 ± 0.01
Serine	2.05 ± 0.09	2.13 ± 0.17	2.11 ± 0.01	2.17 ± 0.04	2.04 ± 0.01
Alanine	2.38 ± 0.01	2.36 ± 0.04	2.14 ± 0.02	2.19 ± 0.03	2.23 ± 0.00
Proline	2.44 ± 0.04	2.37 ± 0.01	2.35 ± 0.04	2.41 ± 0.03	2.29 ± 0.01
Glutamic Acid	6.59 ± 0.06	7.61 ± 0.00	7.05 ± 0.16	7.04 ± 0.18	7.50 ± 0.01
Aspartic Acid	3.31 ± 0.03	3.94 ± 0.04	3.69 ± 0.06	3.59 ± 0.13	4.05 ± 0.01
Glycine	2.43 ± 0.08	1.89 ± 0.10	1.99 ± 0.04	2.03 ± 0.08	1.78 ± 0.01

FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

Table 4.3. Growth performance of rainbow trout fed five experimental diets over an eighteen-week feeding trial.

Diet	IW (g)	WG (g)		
Week	0	6	12	18
FM	39.88 ± 1.02	132.22 ± 4.44	311.47 ± 23.36	608.79 ± 6.60
SBM30	40.50 ± 0.71	133.69 ± 5.33	324.90 ± 19.62	585.61 ± 40.15
SBM40	41.04 ± 0.88	124.33 ± 6.85	276.21 ± 17.62	536.47 ± 27.04
SBM30-BA	40.10 ± 0.83	129.08 ± 2.74	312.23 ± 26.31	539.75 ± 34.92
SBM40-BA	40.48 ± 0.70	131.64 ± 7.66	321.05 ± 24.84	566.87 ± 42.68
		SGR (%/day)		
	FM	3.48 ± 0.06	2.76 ± 0.04	2.23 ± 0.02
	SBM30	3.47 ± 0.07	2.65 ± 0.08	2.20 ± 0.05
	SBM40	3.31 ± 0.06	2.46 ± 0.08	2.14 ± 0.04
	SBM30-BA	3.41 ± 0.04	2.61 ± 0.08	2.19 ± 0.05
	SBM40-BA	3.44 ± 0.12	2.63 ± 0.09	2.21 ± 0.05
		FI (g)/fish/day		
	FM	2.10 ± 0.02	6.66 ± 0.04	7.68 ± 0.21
	SBM30	2.16 ± 0.05	6.68 ± 0.23	7.74 ± 0.56
	SBM40	2.16 ± 0.08	6.69 ± 0.25	7.53 ± 0.42
	SBM30-BA	2.02 ± 0.06	6.26 ± 0.19	6.73 ± 0.45
	SBM40-BA	2.17 ± 0.10	6.69 ± 0.36	7.53 ± 0.62
		FCR		
	FM	0.67 ± 0.01	0.74 ± 0.01	0.74 ± 0.00 a
	SBM30	0.68 ± 0.02	0.73 ± 0.01	0.76 ± 0.00 ab
	SBM40	0.73 ± 0.02	0.80 ± 0.02	0.80 ± 0.00 c
	SBM30-BA	0.66 ± 0.00	0.73 ± 0.02	0.75 ± 0.01 ab
	SBM40-BA	0.69 ± 0.02	0.75 ± 0.01	0.79 ± 0.00 bc
		Hepatosomatic index (HSI)		
	FM	1.07 ± 0.02	1.18 ± 0.40	1.08 ± 0.02
	SBM30	0.99 ± 0.04	1.03 ± 0.04	1.02 ± 0.03
	SBM40	1.01 ± 0.03	1.09 ± 0.04	0.99 ± 0.05
	SBM30-BA	1.01 ± 0.03	1.11 ± 0.05	0.99 ± 0.04
	SBM40-BA	1.05 ± 0.03	1.05 ± 0.04	1.04 ± 0.04
		Condition factor (*K*)		
	FM	1.46 ± 0.01	1.55 ± 0.10	1.73 ± 0.02
	SBM30	1.56 ± 0.05	1.58 ± 0.04	1.71 ± 0.03
	SBM40	1.50 ± 0.02	1.54 ± 0.05	1.72 ± 0.05
	SBM30-BA	1.51 ± 0.02	1.64 ± 0.02	1.70 ± 0.02
	SBM40-BA	1.52 ± 0.04	1.56 ± 0.02	1.75 ± 0.04

Values are presented as means of four replicate tanks ± SD. Values within columns sharing different superscript letters are significantly different following Tukey's multiple comparison test at $p \leq 0.05$. Week 6 (W6) $n = 9$; Week 12 (W12) $n = 6$; Week 18 (W18) $n = 9$.
FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

Table 4.4. Proximate composition of initial (W0) and final (W18) fillet samples (% wet weight), and nutrient retention (PR: protein retention, %; LR: lipid retention, %).

	Initial	FM	SBM30	SBM40	SBM30-BA	SBM40-BA
Lipid	4.07 ± 0.14	9.65 ± 0.56	9.58 ± 0.60	8.55 ± 0.62	9.85 ± 0.62	9.26 ± 0.42
Protein	21.82 ± 0.19	20.44 ± 0.22	20.37 ± 0.08	20.94 ± 0.86	20.48 ± 0.42	20.05 ± 0.35
Ash	1.62 ± 0.35	1.73 ± 0.03	1.75 ± 0.03	1.70 ± 0.06	1.53 ± 0.03	1.64 ± 0.04
Moisture	71.92 ± 0.47	68.67 ± 0.60	68.73 ± 0.67	69.30 ± 0.83	68.62 ± 0.70	68.86 ± 0.65
	PR (%)	29.93 ± 1.24	27.76 ± 1.23	27.72 ± 1.63	28.08 ± 0.97	27.31 ± 1.14
	LR (%)	37.77 ± 2.35	36.03 ± 2.91	33.29 ± 2.89	37.38 ±1.74	36.18 ± 2.42

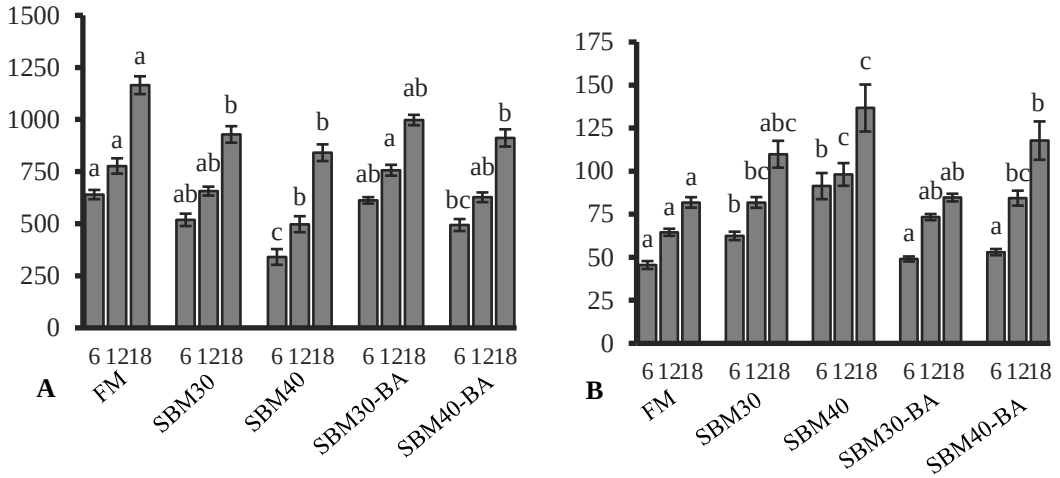

Figure 4.1. Villi length (µm) (A) and width (µm) (B) of rainbow trout fed each of five experimental diets.

Values represent means of tank replicates (n = 12). Different letters between diets at a given time point of the trial (6, 12 or 18 weeks) indicate significant differences. Statistical significance was identified by the Dunn's test at $p \leq 0.05$.

FM: fish meal; **SBM**: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

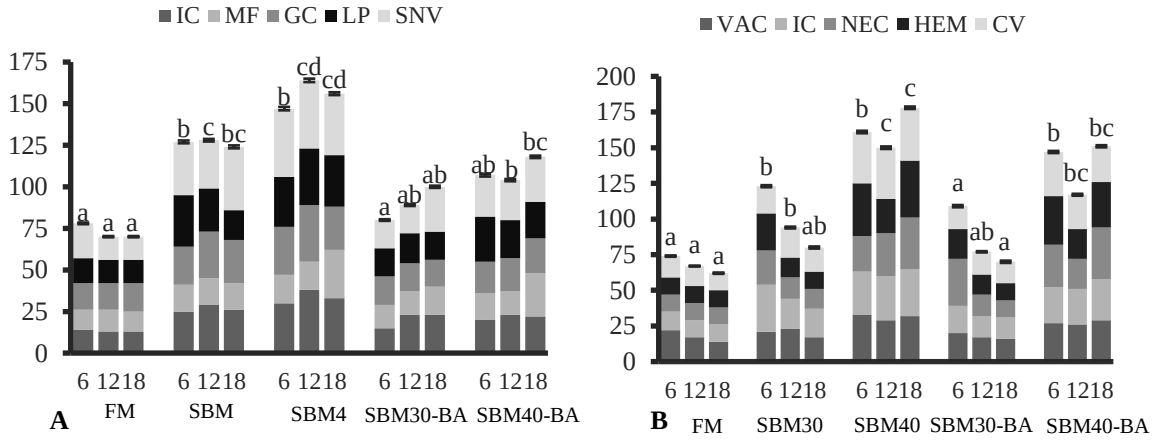

Figure 4.2. Cumulative histological scoring in distal intestine (A) and liver (B) of rainbow trout fed each of five experimental diets.

Values represent the total sum each parameter score per treatment (n = 12). Different letters between diets indicate statistical significance at a given time point (6, 12 or 18 weeks) of the trial. Significant differences were identified by the Dunn's test at $p \leq 0.05$.

FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

A: Distal intestine. IC = inflammatory cells; MF = mucosal fold fusion; GC = Goblet cells; LP = lamina propria infiltration; SNV = supranuclear vacuoles.

B: Liver. VAC = vacuolization; IC = inflammatory cells; NEC = necrosis; HEM = haemorrhaging; CV = congested vessels.

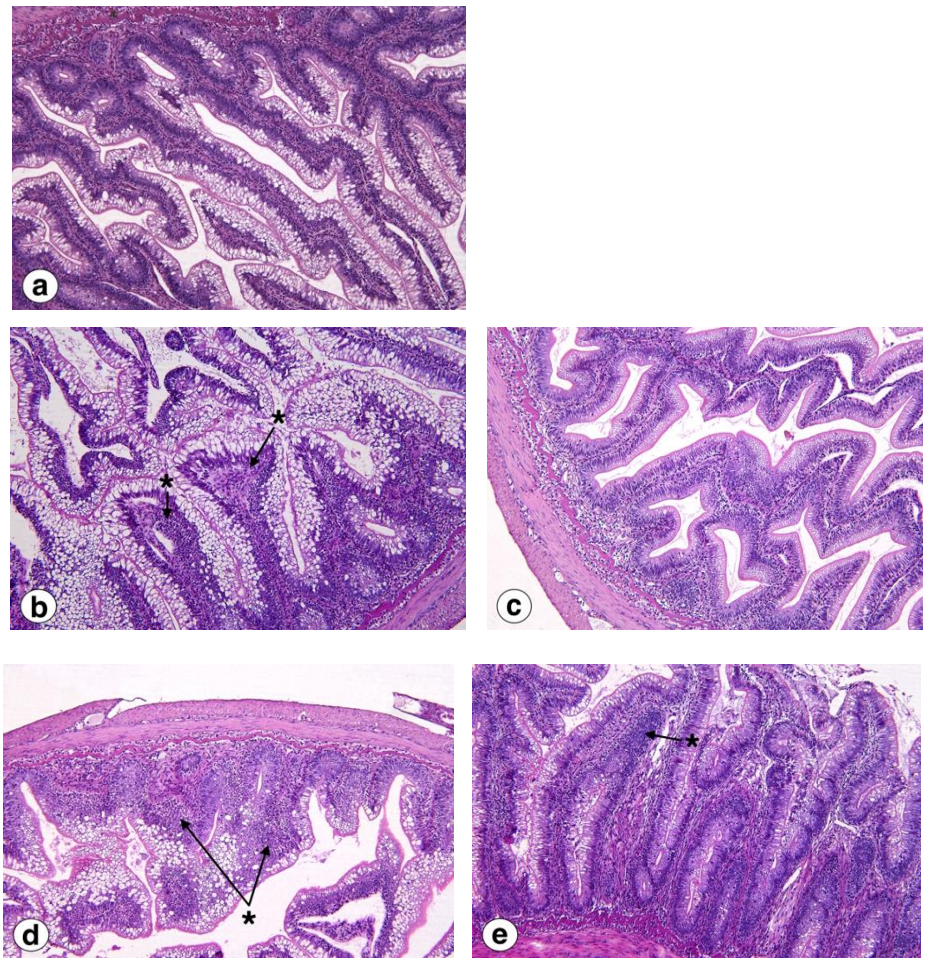

Figure 4.3. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after six weeks dietary exposure (W6).

A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets respectively. Note the villi widening and shortening in D, and lymphocytic infiltration in B, D and, to some extent in E. Staining was performed using haematoxylin and eosin. Magnification x 200.

FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

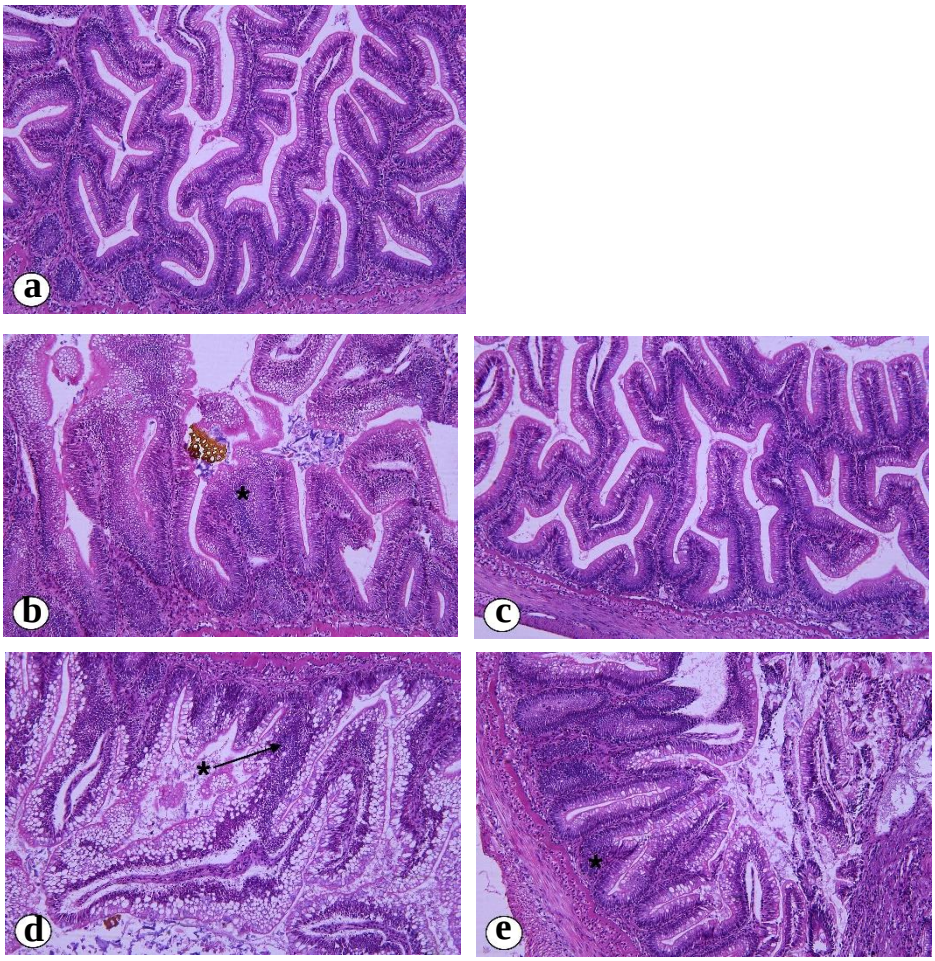

Figure 4.4. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after twelve weeks dietary exposure (W12).

A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets, respectively. Note the lymphocytic infiltration in B, D and, to some extent in E. Staining was performed using haematoxylin and eosin. Magnification x 200.

FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

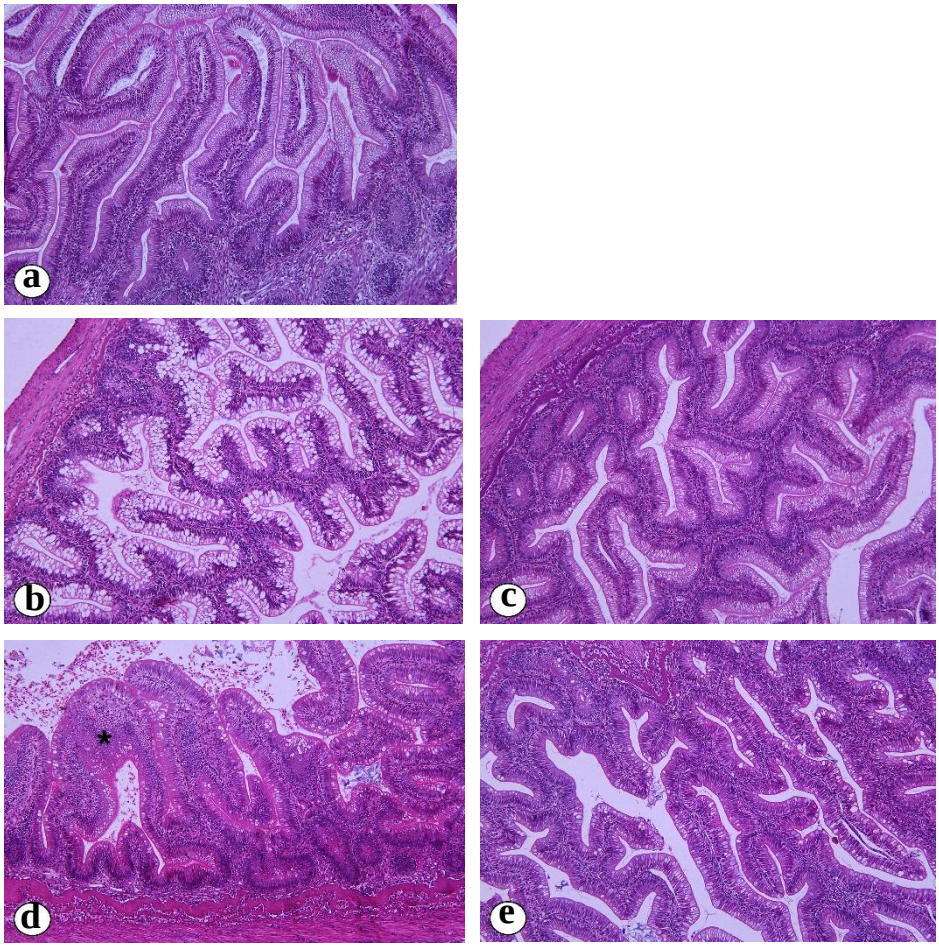

Figure 4.5. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on distal intestine histological traits of rainbow trout after eighteen weeks dietary exposure (W18).
A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets, respectively. Note the Goblet cell abundance in B, and lymphocytic infiltration in D. Staining was performed using haematoxylin and eosin. Magnification x 200.
FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

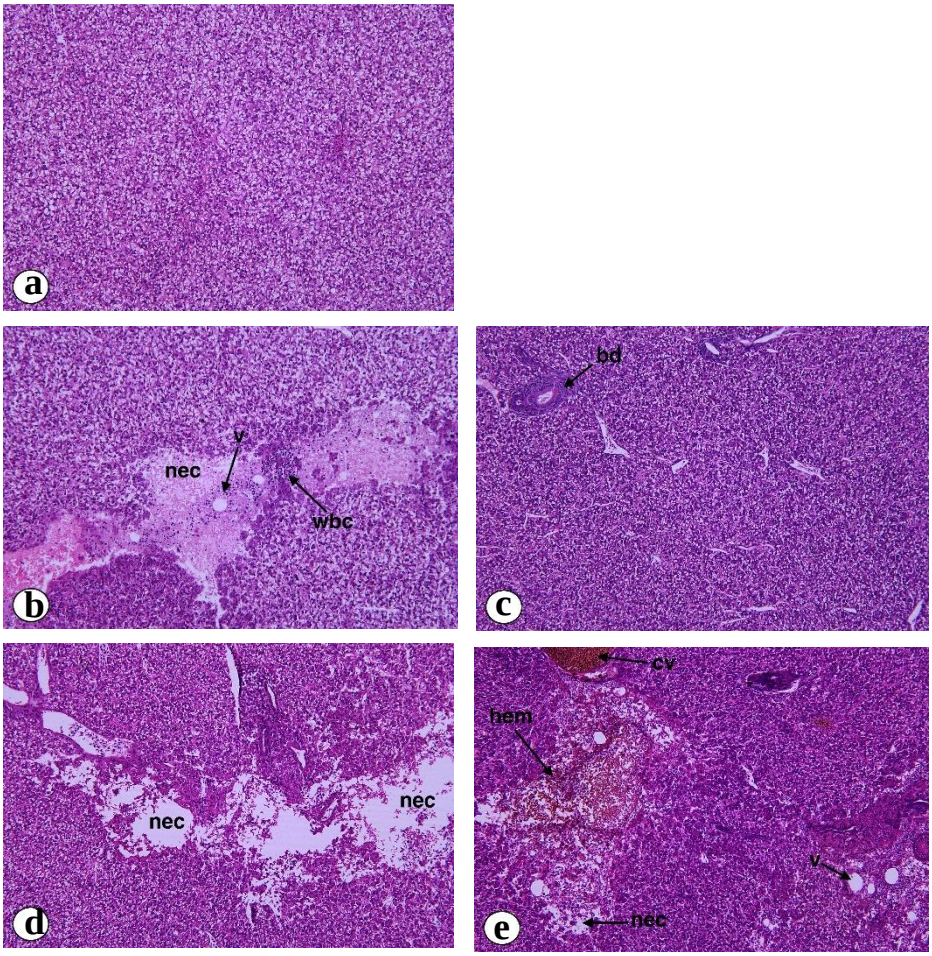

Figure 4.6. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after six weeks dietary exposure (W6).

A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets, respectively. Note the necrosis in B, D and necrosis combined with haemorrhaging in E. Staining was performed using haematoxylin and eosin. Magnification x 200. **FM**: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

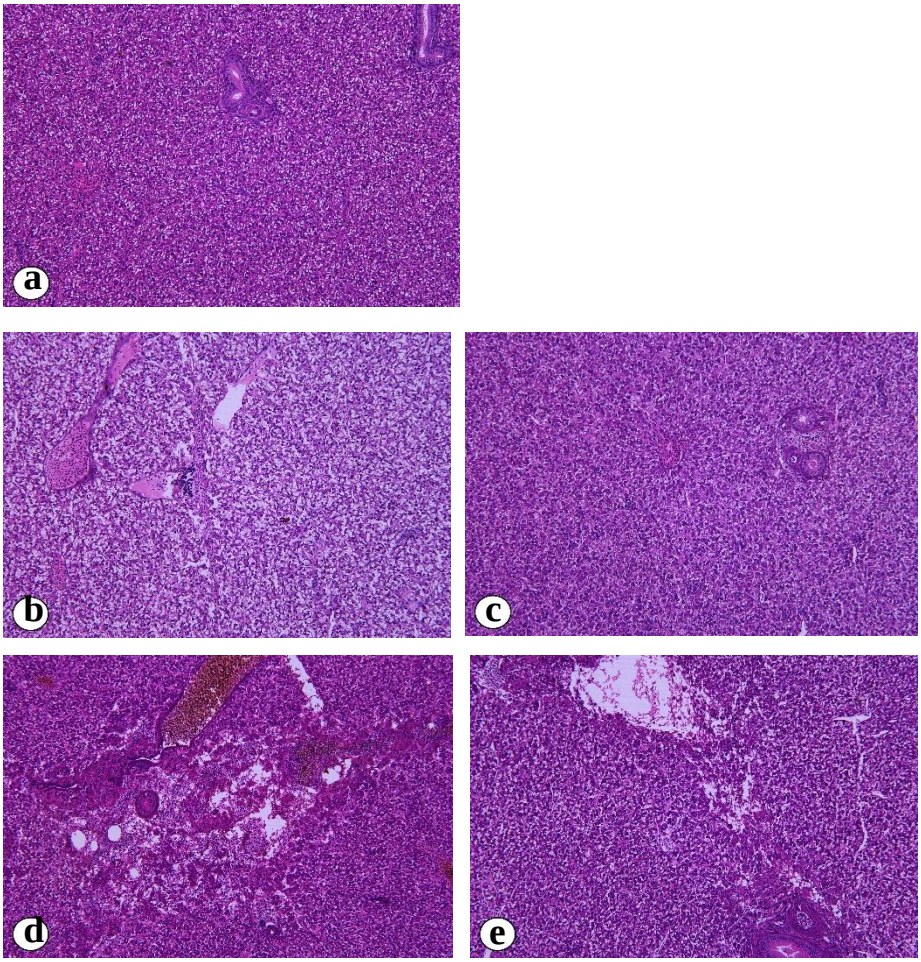

Figure 4.7. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after twelve weeks dietary exposure (W12).
A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets, respectively. Note the necrosis in B and E, and combined necrosis, vacuolization and haemorrhaging in D. Staining was performed using haematoxylin and eosin. Magnification x 200.
FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

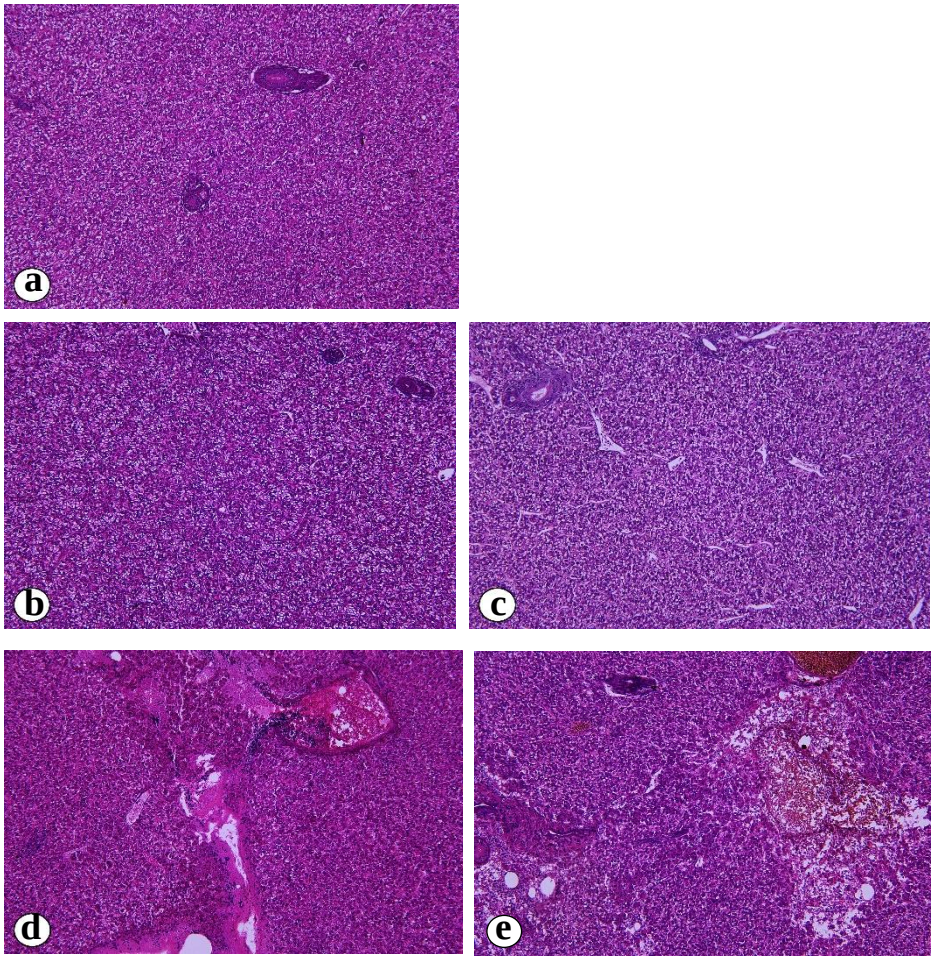

Figure 4.8. Effect of 30 and 40% dietary SBM inclusion, and the effect of BA supplementation on liver histological traits of rainbow trout after eighteen weeks dietary exposure (W18).
A: representative image of fish fed the FM diet; **B, C:** representative images of fish fed the SBM30 and SBM30-BA diets, respectively; **D, E:** representative images of fish fed the SBM40 and SBM40-BA diets, respectively. Notice the similarities between B and C. Staining was performed using haematoxylin and eosin. Magnification x 200.
FM: fish meal; SBM: soybean meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 + 1.5% bile acid; **SBM40-BA**: SBM40 + 1.5% bile acid.

Chapter 5:

Long-term effects of two levels soybean meal dietary inclusion, with or without bile acid supplementation, on plasma bile and cholesterol, digesta bile and gene expression in liver and intestine of rainbow trout (*Oncorhynchus mykiss*)

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The following chapter is being prepared as an article for submission to the journal Aquaculture.

Abstract

Current practices in aquaculture nutrition tend to shift from the use of fishmeal (FM) in feed formulations and increase the content of plant derived ingredients such as soybean meal (SBM). However, this may alter cholesterol and bile acid levels and plasma, reduce bile acid levels in intestinal digesta, and impair hepatic capacity for bile and cholesterol synthesis regulation. Furthermore, higher expression of pro-inflammatory markers in the distal intestine as well as changes to barrier function indicators have also been described in fish.

Bile acids (BA) are biological detergents that are involved in a wider range of molecular signalling pathways. Thus, BAs are becoming promising therapeutic agents that could aid in both maintaining cholesterol and bile physiology homeostasis in the liver and reducing inflammation in the intestine. Five isonitrogenous and isolipidic diets were formulated including a control or fishmeal (FM) diet containing 25% FM protein; a 30% and a 40% inclusion level SBM diets (SBM30 and SBM40); and the same SBM30 and SBM40 each supplemented with 1.5% BA were fed to rainbow trout juveniles over an 18-week feeding trial. Plasma, liver, and distal intestine samples were taken sequentially throughout the trial at 6, 12 and 18 weeks (W6, W12 and W18). The distal intestine gene expression profile of the apical sodium-dependent bile acid transporter (*slc10a2*) showed initial modulation by BAs in the SBM40/SBM40-BA treatments. Plasma cholesterol levels showed a ~30% increase in both BA supplemented diets compared to the SBM40, with the SBM30-BA treatment showing a significant increase. Plasma bile concentrations were significantly decreased in the SBM40 group at W6, which correlated with lower *slc10a2* expressions. Intestinal digesta BA concentration was measured in the proximal and distal regions at W18, but only differences between segments were found, no differences between dietary treatments. Overall, these data indicate that dietary BA played a role in restoring plasma bile and cholesterol levels at the initial stages of the trial, and that BA intestinal reabsorption and hepatic synthesis might be compromised after 6 weeks dietary exposure to a 40% SBM diet.

Introduction

Nowadays, the use of soybean meal (SBM) as the main protein source in fish feeds to replace the more expensive, less available fishmeal (FM) does not come without challenge. The presence of antinutritional factors (ANFs) in SBM is well known to negatively impact feed efficiency and overall health of fish (Francis et al., 2001; Krogdahl and Bakke, 2015). Specifically, saponins, which are higher in SBM than other plant feedstuffs, are known to bind cholesterol and bile, forming insoluble complexes that can interfere with lipid metabolism (Francis et al., 2002). Bile acids (BAs) are amphipathic molecules synthesized in the liver from cholesterol, conjugated with glycine and taurine, and stored in the gall bladder as bile salts until released into the intestine upon cholecystokinin hormonal stimulation. BAs are well known for their role in fat emulsification, by aiding micelle formation and activating the co-lipase enzyme, facilitating its access to the micelle. During their passage through the gastrointestinal tract, bile salts undergo deconjugation to become primary bile acids, as well as further bacterial enzymatic alterations to become secondary bile acids. Importantly, 95% of the BAs in the intestine are reabsorbed in enterocytes via the apical bile acid sodium dependent transporter (Slc10a2) and exported basolaterally via the organic solute transporter dimer (OSTα/β) to be returned to the liver via portal vein circulation. In the liver, BAs regulate their own synthesis via a negative feedback mechanism involving activation of their farnesoid X nuclear receptor (FXR, also known as NR1H4), and subsequently inhibiting cholesterol 7-α hydroxylase (Cyp7a1), the rate-limiting enzyme in BA biosynthesis (Chiang, 2009; Hofmann, 1999; Romano et al., 2020).

In the presence of saponins, BAs are sequestered and made unavailable for micelle formation but also for reabsorption. Reduced reabsorption, in turn, interferes with enterohepatic circulation, which would cause an increase in BA synthesis in the liver. Under these conditions, cholesterol absorption would also be compromised, hepatic cholesterol reserves would be depleted, causing hypocholesterolaemia as a result. Apart from interfering with BA and cholesterol physiology, saponins are related to inflammation in the distal intestine, and as causative agents of increased permeability, allowing for other allergen or pathogen infiltration (Krogdahl and Bakke, 2015).

In fish, there is substantial evidence pointing out the detrimental effects of SBM (and soya-saponins) to the liver and gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*) (Iwashita et al., 2008; Yamamoto et al., 2007), Atlantic salmon (*Salmo salar*) (Gu et al., 2014; Krogdahl et al., 2015); and turbot (*Scophthalmus maximus*) (Gu et al., 2017), among others. Common effects found in carnivores include both reduced glycogen/lipid deposition (Kortner et al., 2013) and increased lipid droplet accumulation in the liver (Gu et al., 2014); decreased vacuolization and

atrophied hepatocytes (Gu et al., 2017), and overall abnormal morphology of livers; increased hepatic cholesterol synthesis capacity and depressed bile acid synthesis (Murashita et al., 2013, 2018; Romarheim et al., 2008). In the distal intestine, SBM diets have caused changes in morphology, accompanied by inflammation, uncontrolled cell proliferation, changes in fatty acid absorption capacity and BA concentrations, as well as increased expression of pro-inflammatory cytokines (Baeverfjord and Krogdahl, 1996; Gu et al., 2018, 2014; Knudsen et al., 2007; Krogdahl et al., 2015; Romarheim et al., 2008).

Presently, it is well known that BAs are ligands to several membrane and nuclear receptors and are therefore involved in a variety of cellular signalling cascades (Biagioli et al., 2021). Studies in mammals have provided substantial evidence regarding the implication of BAs in promoting anti-inflammatory responses and restoring permeability in mammalian intestinal bowel disease (IBD) studies. The involvement of the Fxr (Gadaleta et al., 2011) and Takeda G protein-coupled membrane receptor (TGR5, also known as GPBAR1) (Cipriani et al., 2011) as BAs targets has attracted attention for investigation. Indeed, a key event resulting upon activation of these receptors is the downregulation of the nuclear factor kappa B (Nfkb). Given the critical role that Nfkb plays in the transcriptional regulation of pro-inflammatory cytokines such as tumour necrosis factor alpha (Tnfa) and interleukin 8 (Il8), its downregulation has become a therapeutic target in IBDs. In addition, the expression of myosin light chain kinase (Mylk), a key regulator of cellular contractility, which appears over-expressed during IBD, also falls under Nfkb regulation in a Tnfa driven inflammatory scenario (Ma et al., 2005; Ye et al., 2005). Lastly, tight junction proteins such as claudins (Cldn) and occludin (Ocln), key players in intestinal permeability and epithelial integrity, have also been identified as BA targets promoting barrier function (Cipriani et al., 2011). Some of the aforementioned markers, such as Tnfa, interleukins, and to a lesser extent, Mylk and tight junction proteins have shown signs of similar dysregulation in teleost during SBM induced enteritis (De Santis et al., 2015; Grammes et al., 2013; Gu et al., 2016; Jiang et al., 2015; Liu et al., 2018; Rimoldi et al., 2016; Seibel et al., 2022; Skugor et al., 2011; Zhang et al., 2021).

It has been found that in fish, BAs act as regulators of cholesterol and bile physiology, enhancers of crude fat digestibility, and as protective agents in liver and intestine in a SBM context (Gu et al., 2017; Yamamoto et al., 2007). However, the role of BAs as modulators of inflammation and barrier function has received little attention in fish at the gene expression level. Specifically, expression of pro-inflammatory cytokines tight junctions and their modulation by both SBM and dietary BA deserved more attention in fish.

The aim of the present study was to evaluate the integrated effects of dietary BAs in restoring bile and cholesterol physiology, as well as their anti-inflammatory and barrier enhancing properties in the distal intestine of rainbow trout in a SBM enteropathy scenario. Further, the ability of supplemental BA to maintain beneficial effects in the long term was assessed in an 18-week feeding trial.

Materials and Methods

Experimental diets

Five experimental diets were formulated to a 43% crude protein level and 16% lipid content. A 25% fish meal-based diet (FM) was selected as control diet. Soybean meal (SBM) was used as the main source of protein for the remaining dietary treatments. Two diets were formulated to contain SBM at an inclusion level of 30% (SBM30) or 40% (SBM40); and two more diets included both a SBM30 and a SBM40 supplemented with 1.5% bile acids (BAs) (Redox Inc., Los Angeles, CA, USA). The dietary formulation and proximate composition of the experimental diets are detailed in Table 4.1, and the amino acid profile are shown in Table 4.2.

Fish rearing and sampling conditions

Rainbow trout were obtained from Troutlodge (Sumner, WA) and kept at the Cold-Water Lab at the Aquaculture Research Institute at the University of Idaho (Moscow, ID). A total of two thousand fish were distributed into twenty (100 fish/tank) 350 L tanks in a recirculating aquaculture system, incorporating four replicate tanks per dietary treatment. After one week acclimation, fish initially weighing 40.4 ± 1.3 g were fed each of five experimental diets to apparent satiation six days per week for a period of eighteen weeks. Rearing conditions were maintained a constant photoperiod 12 h dark/12 h light at an average temperature of 14.33 ± 1.07 °C. Water was supplied at a constant rate of 160 mL sec^{-1} and oxygenation was accomplished by continuous aeration to each tank, and dissolved oxygen was kept at 5.55 ± 0.59 mg L^{-1}. Ammonia nitrogen, nitrite and pH were maintained at 0.03 ± 0.02 mg L^{-1}, 0.19 ± 0.18 mg L^{-1} and 7.58 ± 0.16, respectively. All water quality parameters were monitored weekly using commercial kits (LaMotte, Chestertown, MD, USA).

Throughout the feeding trial, fish were sampled every six weeks after 12 h after receiving the last meal, for a total of three times. Samplings took place at week 6 (W6), week 12 (W12) and week 18 (W18), during which fish were euthanized using an overdose of tricaine methanesulfonate (Syndel Inc., Ferndale, WA) at 250 mg/L buffered to pH 7.0-7.5. Liver and distal intestine samples

were taken from three fish per tank (n = 12) for gene expression analysis. Plasma and digesta samples were also taken from the same three fish. The initial stocking density was 11.54 Kg m^{-3}. To ensure a maximum stocking density of ~ 50 Kg m^{-3} in the long term, excess fish were pulled out of each tank at week 7. To maintain optimal densities in the long-term, at a maximum limit of 80.20 Kg m^3 per tank, excess fish were pulled out from all tanks at week sixteen and week twenty-one.

All experimental procedures involving live fish were carried out with prior approval from the Institutional Animal Care and Use Committee, University of Idaho (IACUC-2021-49).

RNA extraction, cDNA synthesis and RT-qPCR

Distal intestine and liver samples were taken from three fish per tank (n = 12) at each of three sampling points, and snap-frozen in liquid nitrogen until analysis. Distal intestine samples were taken only when digesta was present in the distal intestine, to ensure exposure to the diet. Tissue samples were homogenized using a motor-pestle homogenizer in Trizol® and RNA was extracted following manufacturer instructions. RNA purity and quantity was determined using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE), and quality (integrity) was assessed by agarose gel electrophoresis. Samples were treated with DNase (Invitrogen, Waltham, MA, USA) when appropriate prior to reverse transcription. Extracted RNA (0.5 µg) was reverse-transcribed to cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). RT-qPCR reactions were carried out using SYBR green detection method on a StepOnePlus real-time thermocycler (Applied Biosystems, Foster City, CA). Duplicate reactions were performed for each cDNA sample analyzed (i.e., six technical replicates per tank). The reactions were set on a 96-well optical plate, and each contained: 1.5 µL of diluted (1/5) cDNA, 5 µL of 2X concentrated PowerUp ™ SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA), 0.5 µM forward primer and 0.5 µM reverse primer. Distal intestine gene expression analyses were performed on inflammation marker genes: *tnfa*, *il8*, *il10*; barrier integrity makers: *ocln* and *cldn12*; cell contractility gene *mylk*; the bile acid transporter *slc10a2*. In liver, qPCR was performed on bile acid synthesis rate limiting enzyme *cyp7a1* and cholesterol synthesis rate limiting enzyme β-hydroxy- β-methyl-glutaryl-CoA (*hmgc*). Primer sequences were obtained from the literature or designed using Primer3Plus (https://bioinformatics.nl/). Primers for the target genes *cldn12*, *ocln*, *mylk* and *hmgc* were based on rainbow trout sequences or on conserved regions of known teleost sequences paralogues in the GenBank database, using NCBI BLAST software (http://blast.ncbi.nlm.nih.gov) or Clustal Omega 1.2.4 multiple alignment tool (https://ebi.ac.uk/Tools/msa/clustalo/).

Cyclophilin A (*cypa*, pepdidyl-prolyl-cis-trans isomerase A) and elongation factor 1-alpha (*ef1a*) were selected as housekeeping genes for distal intestine and liver, respectively. The qPCR programme consisted of initial denaturation programme (2 min at 50 °C followed by 2 min at 95 °C); an amplification and quantification program (repeated 40 times, 15 s at 95 °C, 20 s at 57-60 °C, and 30 s at 72 °C); and a melting curve 60 – 95 °C, 0.15 °C s^{-1}). Gene expression was determined using the ΔC_t method described by Pfaffl (Pfaffl, 2001), where the genes of interest were normalized to the C_t value of the housekeeping genes *cypa* (distal intestine samples) and *ef1a* (liver samples). Both the standard curves and primer efficiencies were obtained by serial dilutions of a random cDNA mixture of control samples. Both the standard curves and primer efficiencies were obtained by serial dilutions of a random cDNA mixture of control samples. All primer sets were validated on a 1% agarose gel to check for amplification product (125 V for 35 min) and validate successful product formation. Table 5.1 shows primer sequences and efficiencies.

Plasma bile and cholesterol levels

Three fish per tank (n = 12) were anesthetised in MS-222 and blood was drawn via caudal venepuncture using a heparinized syringe (Sigma Aldrich®, 1,500 IU/mL), placed into tubes and subsequently centrifuged (2 min., 10,000 *g*). Plasma separated in the supernatant fraction was collected into a tube, snap frozen in liquid N_2 and stored at -80 °C until further analysis. Cholesterol was determined using the Total Cholesterol Assay (catalogue #STA-384, Cell Biolabs). Bile acid was determined fluorometrically using the Total Bile Acid Assay (catalogue #STA-631, Cell Biolabs). Samples were assayed in a 96-well plate using a microplate reader (SpectraMax® i3X, Molecular Devices LLC., San Jose, CA, USA).

Proximal and distal intestine digesta bile quantification

Digesta samples were collected from three fish per tank (n = 12) from proximal and distal intestine by carefully squeezing the digesta from the intestine into 1.5-mL tubes and immediately frozen in liquid N_2. Distal intestine was delimited proximally by the first complex fold and distally by the annulo-spiral septum. The proximal intestine was delimited proximally by the pyloric caeca and distally by the first complex fold of the distal intestine.

The percentage of reduction of BA from proximal to distal intestine regions was calculated as follows:

BA reduction rate (%) = $\frac{[proximal\ BA\ (mM) - distal\ BA\ (mM)]}{proximal\ BA\ (mM)}\ x\ 100$

Statistical analysis

All data are presented as mean ± standard deviation (SD). Intestinal digesta was analysed using a two-way ANOVA, with diet and intestinal segment (proximal or distal) as independent variables. If significant differences were found, a one-way ANOVA was conducted to analyse proximal and distal intestine results separately. All other data were analysed using one way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test. Gene expression data were subjected to log transformation to meet the assumption of homogeneity of variance (Levene's test). Normal distribution of the data was confirmed using the Shapiro-Wilk normality test. Statistical significance was set at $p < 0.05$ and all analyses were performed using R (v. 3.6.2) statistical package.

Results

Plasma bile and cholesterol levels

Total plasma cholesterol was not impacted at W6 of the trial but differences between dietary treatments were observed at W12 and W18 (Fig. 5.1a). At W12, cholesterol concentrations were significantly lower ($p = 0.005$) in the SBM40 group (6.27 mM) compared to both the FM (9.29 mM) and the SBM30-BA (8.41 mM) groups which had the highest levels. Mean cholesterol levels recorded for the SBM40-BA diet were similar to the SBM30-BA (8.29 mM), and higher than the SBM40 levels, but not significantly ($p > 0.05$), and this was similarly observed in the SBM30 diet (7.71 mM). At W18, plasma cholesterol was overall lower than observed at W12. The SBM40 group remained significantly lower than the FM group (5.32 and 7.38 mM, respectively) ($p = 0.004$), while the SBM30-BA dramatically decreased to significantly lower levels compared to the FM diet (5.75 mM) ($p = 0.027$). Again, no difference was seen between the SBM30 and SBM40-BA (6.20 and 6.38 mM, respectively) and the rest of the dietary treatments.

Plasma BA levels were significantly higher in the SBM40-BA at W6 compared to the SBM30 and SBM40 groups ($p = 0.005$) but not in the SBM30-BA group (Fig. 5.1b). No further differences among dietary treatments were seen from W12 onwards (all $p > 0.05$). Although not significant, higher levels of BA in the plasma of fish fed SBM30-BA and SBM40-BA are observed.

Within time, plasma cholesterol levels changed in the FM group at all time points ($p = 0.0002$), with the highest levels at W12, after which, cholesterol concentrations decreased again. This pattern was similarly observed in both the SBM30-BA and SBM40-BA groups (all $p < 0.01$). For the SBM30 and SBM40, cholesterol levels also significantly increased from W6 to W12 ($p = 0.005$ and p

= 0.048, respectively), however, cholesterol concentrations remain within the same range after this time point until W18 (all $p > 0.05$).

In case of bile plasma levels, all dietary treatments showed the same variation within time, with significant lower levels after W6 (all $p < 0.05$).

Proximal and distal intestine bile quantification

In intestinal digesta, BA concentrations were analysed in samples taken at W18 from both proximal and distal segments (Table 5.2). Results showed significantly higher BA concentrations in the proximal region in all dietary treatments, as expected ($p < 0.001$). One-way ANOVA showed no differences between dietary treatments for either proximal or distal portions (all $p > 0.05$). For each diet, the concentration observed in the distal intestine was subtracted from that observed in the proximal segment to calculate the percentage of reabsorption. No differences were observed in reabsorption rates, but fish fed the SBM30 and SBM40 treatments displayed the highest percentages of reabsorption.

Gene expression analysis in distal intestine and liver

No differences were seen in the gene expression of either the pro-inflammatory cytokines *tnfa*, *il8*, and the anti-inflammatory cytokine *il10* (all $p \geq 0.05$) (Fig. 5.2). However, at W12, the expression of the three cytokines appeared to be higher in the SBM30-BA and SBM40-BA.

Transcription levels of *cldn12* and *ocln* were not differentially expressed between the dietary treatments and throughout the trial (all $p \geq 0.05$) (Fig. 5.3). The SBM40-BA group showed the highest *cldn12* expression levels at W12 and 18.

Relative gene expression of the *mylk* (Fig. 5.3), involved in enterocyte contraction, appeared to be slightly lower in the SBM40, SBM30-BA and SBM40-BA groups compared to the FM and SBM30 at W6, but this was not significant at this point ($p \geq 0.05$). No differences were found at W12, but a different pattern from that of W6 was observed at W18, with significantly lower *mylk* expression in the SBM30-BA group compared to the SBM40-BA ($p = 0.0308$).

The distal intestine bile acid transporter *slc10a2*, showed significant differences in expression at W6 (Fig. 5.3), with a significant downregulation in the SBM40 group group ($p = 0.0158$) compared to the FM group. No other differences in relative expression were seen at W12 and W18 (all $p \geq 0.05$).

In the liver, genetic expression of the cholesterol synthesis enzyme *hmgc* did not show any differences (all $p \geq 0.05$), but its transcriptional expression appeared slightly higher in the

SBM40-BA group at W6 (Fig. 5.4). At W12, the highest expression was seen in the SBM40-BA group although this was not significant and all groups seemed downregulated by W18, also not significantly. The *cyp7a* bile synthesis enzyme also did not show significant differences based on treatment (all $p \geq 0.05$); however, the SBM40 group showed the lowest expression levels throughout the entire trial while the SBM30 and SBM40-BA shared the most resemblance with the FM group at all time points (Fig. 5.4).

Discussion

Bile acids appear to be pivotal in the study of the SBM-related pathologies that carnivorous fish species experience when fed high levels of this ingredient. Indeed, changes in their availability, enterohepatic circulation and regulation can have implications for both hepatic and intestinal tissues. Moreover, BAs can also act as modulators of inflammation and, furthermore, tight junction protein and *mylk* expression, both of which are central in the study of enteropathies derived from high level SBM feeds. The results presented herein are a continuation of the set of observations made on the same 18-week feeding trial. As previously reported, feeding the SBM40 diet to rainbow trout led to an eventual significant increase in feed conversion ratio (FCR) by W18, while the BA supplement prevented this. Liver and distal intestine histopathology showed significant improvement at different stages due to BA protective action, however, in some instances, this effect was not maintained in the longer term as described in Chapter 4.

Bile acids quantification in plasma and digesta; and cholesterol quantification in plasma

As noted above, both cholesterol and bile physiology are modulated by SBM diets in salmonids, with reduced plasma levels of both observed in turbot and Atlantic salmon fed SBM diets ranging 20% - 40% inclusion levels (Gu et al., 2017; Kortner et al., 2013, respectively) or a plant based diet containing lupin, soya saponin and wheat gluten (Gu et al., 2014); and rainbow trout fed different types of SBM (Murashita et al., 2013; Romarheim et al., 2008). According to Kortner et al. (2013), this impact on both cholesterol and bile can be seen in Atlantic salmon plasma as soon as 15 days after initiating feeding with SBM while in rainbow trout hypocholesterolaemia became significant after 40 days (Romarheim et al., 2008). In grouper (*Epinephelus lanceolatus*) fed SBM for 6 weeks no differences in plasma cholesterol were seen as well as no beneficial effect of lecithin, and this was in samples taken 24 h postprandially (Lin et al., 2022). In the present study, a significant decrease in plasma cholesterol was not observed until W12 of the trial, with approximately ~ 30% reduction in the SBM40 compared to the control diet, which were then maintained in the long term

(until W18). Instead, plasma bile was reduced in the SBM30 and SBM40 at the earlier stages but showed no differences at W12 and W18; nonetheless, lower bile concentration in those two diets could indicate poor reabsorption capacity in the distal intestine. The supplementation with BAs rendered higher cholesterol levels in both supplemented groups at W12, but this effect seemed to diminish at W18. Similarly, the slightly higher (non-significant) levels of plasma bile in both BA supplemented diets maintained after W6 could be indicative of a beneficial role in compensating for reduced reabsorption. The study carried out in turbot supports the ability of supplemental taurocholate in increasing plasma levels of total cholesterol and, especially, bile to similar levels as observed in the FM-control group (Gu et al., 2017). The loss of effect of BA in rescuing total cholesterol levels in the long term and lack of differences in BA levels in plasma after W6 obtained in the present study suggest that dietary levels of BA could be proportionally increased and adjusted in the diet according to fish growth, especially considering the histology results obtained from this same trial reported in Chapter 4.

The lack of differences in the BA concentration in the digesta of fish fed different dietary treatments was unexpected. This was true for measurements taken both in the proximal and distal segments of the gastrointestinal tract. While studies have found that SBM reduces BA concentration in the proximal region of the intestine rather than the distal, as seen in pompano (*Trachinotus blochii*) (Nguyen and Van Do, 2021), yellowtail (*Seriola quinqueradiata*) (Nguyen et al., 2013), and rainbow trout (Murashita et al., 2013; Romarheim et al., 2008); Iwashita et al. (2008) found SBM-related reduction both in the proximal and distal segments in the latter species. When it comes to the impact of BA as dietary supplements more discrepancies arise. For instance, in rainbow trout, total BA content in the gall bladder was reduced in fish fed a plant based diet (SBM and corn gluten meal), but not impacted by bovine bile salts (Yamamoto et al., 2007); however, both bovine gall powder and taurocholate did increase proximal and distal intestine digesta bile quantities of in this species fed SBM diets after 10 weeks (Iwashita et al., 2008). Similarly in turbot, Gu et al. (2017) reported taurocholate to rescue BA concentration in proximal intestine digesta in a plant-based diet as early as 2 h postprandially.

Fewer studies have examined the reduction rates and differences associated with dietary treatments. In the present study, there were no differences in the rate of reduction across the five experimental diets. By supplementing BA in the diet, an initial premise would be to observe higher BA in the proximal digesta of fish fed the supplemented diets. Another basis would be to find higher BA in the distal intestine digesta of fish fed the SBM30 and SBM40, as reabsorption is less effective due to the presence of ANFs. In pompano, Nguyen et al., (2021), found lower reduction rates after 8

weeks in fish fed a SBM diet compared to a FM diet. This is contrary to what we determined here, with higher (although non-significant) reduction rates in SBM30 and SBM40 diets. Nonetheless, our analysis was done in digesta taken at W18. Considering that there were also no BA differences in plasma samples at this point, it could be the case that the late stage at which our samples were taken is related to the lack of impact. This could indicate a capacity to balance bile physiology within time. In all other studies, digesta bile was measured after shorter overall SBM exposure times (~ 10 weeks). In the study conducted by Romarheim et al., (2008) in rainbow trout, dietary SBM seemed to cause BA concentrations to be higher at very early times after initial exposure (i.e., 5 days), in the pyloric region and proximal intestine, but did lead to lower BA quantities after this point. After day 10, concentrations were significantly reduced in all intestine segments. Of note, it has also been observed that different processed forms of SBM can affect this differently. The study in pompano showed that a digested source of soy protein did not cause this reduction. Murashita et al. (2013) also found that proximal intestine digesta was differentially impacted according to the SBM source and processing method.

Distal intestine gene expression

The lack of differences in relative gene expression levels of all inflammatory markers in distal intestine was contrary to expected. Essentially, the low expression levels of both the pro-inflammatory cytokines *tnfa* and *il8* as well as the anti-inflammatory *il10* at W12 and W18 is in disagreement with the idea of an inflammation scenario during SBMIE firstly (as no sign of inflammation is seen based solely on gene expression results), and with the hypothetical anti-inflammatory effect of BA secondly. However, the mRNA levels of the three cytokines seemed slightly higher in the SBM40 group at W6, and although this was not significant, it could indicate more severe inflammation at this point. Furthermore, at W12 higher expression levels of *tnfa*, *il8*, and *il10* were seen in the SBM30-BA and SBM40-BA groups, suggesting a possible negative reaction to BA. However, this was not significant and was not maintained towards W18. Little is known about how the anti-inflammatory cytokine *il10* may be impacted by BAs. In human, the activation of TGR5 by BAs leads to a shift in the activated macrophages from a pro-inflammatory phenotype to an anti-inflammatory one, demonstrated by the higher levels of *il10*, which would drive the resolution of the immune response (Haselow et al., 2013). On this basis, higher transcriptional expression of *il10*, as observed at W12, could signify a beneficial effect of dietary BA at initial stages of insult which is when acclimation would be taking place. However, this was not significant, and was accompanied by a similar expression pattern of the pro-inflammatory cytokines *tnfa* and *il8*. The results presented in

Chapter 3 showed that a SBM 30 % diet led to an upregulation trend of *il8* and *il10* after 12 weeks and later, after 24 weeks, in a commercial rainbow trout strain but also no differences between dietary treatments. In turn, *tnfa* showed an upregulation pattern both short and long term but did not differ between dietary treatments. These results are partially in agreement with those obtained in this study, to the extent that no differences between diets were seen, but the different trends observed make drawing conclusions a challenging task. Furthermore, comparison with histology results obtained in Chapter 4 questions this interpretation. Indeed, although histology results attained from the same experimental fish indicated that 1.5% dietary BAs were capable of significantly mitigating tissue inflammation (both in distal intestine and liver), the gene expression results presented herein did not match these observations at a molecular level.

The disagreement with results reported in Chapter 2 can be attributed to slight difference in rearing conditions or feed effects. However, all feeds were manufactured under the same conditions and subjected to extrusion. The divergence between histological observations from Chapter 4 and gene expression results reported here is nonetheless harder to explain since experimental conditions were the same. In the present study, transcriptional expression analysis of the pro-inflammatory cytokine *tnfa* could benefit from sampling on a shorter time scale after feeding, as similarly concluded in our previous study using glutamine as an additive (Chapter 2).

Although there is accumulating evidence of this role of BAs in higher vertebrates, advances in piscine species are scarce. The studies carried out by Iwashita et al. (2008) and Yamamoto et al. (2007), in which rainbow trout liver and distal intestine histology was improved by BA dietary supplementation to SBM diets constitute a promising basis on this premise. However, there is no evidence of the modulating effect of dietary bile on cytokine expression. A study in zebrafish (*Danio rerio*) showing the implication of taurocholic acid and glycoholic acid in an anti-inflammatory response (both *in vitro* and *in vivo*) mediated by Fxr activation, constitutes the only example of the effect of BA in teleost (Ge et al., 2023). In that study, these two BAs are demonstrated to activate both Fxr and TGR5 in macrophages, leading to the subsequent decrease in transcriptional expression of *tnfa* and *il6*. However, BAs administration was done parenterally. Another study performed in largemouth bass (*Micropterus salmoides*) pointed out the ability of dietary BA supplementation as low as 0.035% to stimulate innate response, but this was seen as increased plasma lysozyme activity and immunoglobulin titers in an 8 week high starch diet context (Guo et al., 2020).

The *mylk* expression was opposite to expected, as its expression is thought to be excessively increased during SBM related enteropathies, leading to hypercontractility. In this study, the SBM40 is however showing the lowest transcriptional expression at W6, but non-significantly.

Instead, the SBM40-BA group recorded the highest *mylk* expression at W18, and this was significantly higher than the SBM30-BA group which had the lowest level of expression. This difference between both BA supplemented diets makes it challenging to elucidate the role of BAs in this and suggests a possible interaction between level of inclusion of SBM and BA dietary supplementation. The study presented in Chapter 3, which was carried out on similar rainbow trout commercial strains also did not support this hypothesis of *mylk* overexpression. All together, these observations could suggest that this type of gene expression analysis might not be the most adequate or that there are other enzymes mediating myosin light chain phosphorylation in fish during intestinal pathologies. The relative expressions of TJ did not support the idea of a downregulation event happening during enteritis nor with the potential role of dietary BA in increasing expression levels. The expression of *ocln* appeared to increase within time in the SBM40-BA, which evolved from having the lowest expression at W6 (non-significant) to higher levels, comparable to the FM group by W18. The shift from lower expression levels of *cldn12* in the BA supplemented diets at W6 to increasing expression levels at W12 is somewhat similar to what was recorded for *ocln*, and both TJ show a similar pattern. The lack of similarities with previous study makes in cytokine and TJ gene expression patterns makes it challenging to come to conclusions, as there might be additional governing factors influencing regulation of expression. However, both the present study and the study presented in Chapter 3, reported similar expression patterns for both cytokines and TJ, suggesting that, indeed, there may be external factors derived from experimental conditions affecting these observations, thus complicating comparisons.

The distal intestine bile acid transporter *slc10a2* showed differences in expression at W6, with significantly lower mRNA levels in the SBM40 diet as would have been expected under the premise of reduced rates of bile reabsorption during SBMIE and presence of saponins (Gu et al., 2014, Kortner et al., 2013). In addition, studies in mammals have shown that the *slc10a2* is subjected to negative feedback regulation mediated by BAs (Neimark et al., 2004). Studies in rainbow trout have shown a short upregulation of *slc10a2* after 1 h exposure to taurocholate, as well as 4 days after fasting; however, evidence from the same studies pointed out a possible role of BAs also on the negative feedback regulation of *slc10a2* (Murashita et al., 2014). Studies investigating the acute and chronic effects of a high SBM diet in rainbow trout revealed a short term upregulation of the *slc10a2* 6 h after feeding and no difference with a FM control group whereas a longer term exposure led to a significantly lower *slc10a2* expression in the SBM group compared to the FM after the same amount of time had passed since feeding (Murashita et al., 2018). Furthermore, the supplementation with 1.0% taurocholate did not make any difference with respect to the SBM group in that study. These

data are, for the most part, in accordance with what was observed in our study, since a significant downregulation was observed at W6 in the SBM40 group, with samples taken between 12 and 18 hours after feeding. However, this difference was not maintained through W12 and W18. The presence of digesta in the distal intestine at the time of sampling also indicates the possible role of BAs in the observed downregulation, and for said downregulation to be more pronounced in the SBM40 group due to, presumably, the increased bile concentrations resulting from poor reabsorption rates in this group, although this is not supported by the BA measurements taken at W18 in this study. In addition, as discussed above, studies in rainbow trout and other species have predominantly shown changes in BA concentration in the proximal segment of the intestine and at earlier time points; however, the *slc10a2* expression was analysed in the distal intestine. As already mentioned, both expression of *slc10a2* and changes in BA concentrations could be more subjected to changes at the early stages of SBM exposure. Interestingly, the SBM40-BA showed some level of resemblance to the FM, SBM30 and SBM30-BA groups, indicating that the 1.5% BA supplementation level could be more effective compared to a lower level of 1.0% tested by Murashita et al. (2018).

Hepatic gene expression

Synthesis of cholesterol and BA in the liver did not seem to be impacted by either SBM inclusion nor BA supplementation as no differences were observed in the relative expression of *hmgc* and *cyp7a1*. The lower expression of *cyp7a1* in the SBM40 group at W6, although not significant, correlates with the significantly lower BA concentration in plasma and the significantly lower *slc10a2* expression at this point. Other studies have pointed out the upregulating effect that SBM diets have on cholesterol biosynthesis based on enhanced gene expression of *hmgc*, which is what would be expected as a response to lower plasma cholesterol. Hence, reduced plasma cholesterol is accompanied by an increase in cholesterol hepatic synthesis capacity. A slight tendency towards higher *hmgc* relative expression after W6 seemed present, especially in the SBM40 group, but this observation, does not correspond to reduced plasma cholesterol at W12 and W18, since significantly reduced plasma cholesterol was not observed until W12.

In rainbow trout, the capacity for BA synthesis, based on *cyp7a1* expression, seems to be mildly upregulated during the fasted state, upregulated 6 h after feeding compared to a 48 hour fasted state; and downregulated after 24 h when fed a FM diet (Murashita et al., 2018). In turbot and Atlantic salmon, results coincide in their observations of an upregulation of *hmgc* and downregulation of *cyp7a1* after feeding SBM; however, these studies fail to specify if these analysis were done on fasted fish or after a specific time since the last feeding (Gu et al., 2017, 2014, respectively). Studies

in mammals show increased *cyp7a* activity during the fasting states (De Fabiani et al., 2003) which is in line with what Murashita et al. (2018) observed in rainbow trout. Given the dependency of BA synthesis on fed/fasted status, investigations where these factors are taken into consideration would greatly aid reaching conclusions. It would be expected that BA synthesis is downregulated postprandially via Fxr activation by reabsorbed bile both in enterocytes and hepatocytes. Hypothetically, the reduced bile reabsorption rates resulting from the SBM30 and SBM40 diets in the present study would enhance BA synthesis postprandially, since less bile would be entering enterohepatic circulation. This would eventually cause cholesterol depletion, and increased *hmgc* activity would be seen. The SBM30, but not the SBM40, could be supporting this hypothesis at W6. Although not significant, expression was highest in the SBM40-BA group, followed by the SBM30 and FM groups at W6. Overall, our results indicate lower levels of expression after 12 – 18 h feeding from W12 until the end of the trial. Since there were no significant differences nor a specific pattern of expression, it is not possible to tell the effect of the supplemental bile.

Other attempts to study the effect of BA on *cyp7a1* in a SBM dietary background have shown a partial recovery of relative expression as in turbot (Gu et al., 2017); no difference between supplemented diets and SBM as in rainbow trout (Murashita et al., 2018) nor in largemouth bass fed conventional or bioprocessed sources of SBM (Romano et al., 2021). In a study investigating high/low fat and starch levels in the diet and bile acid supplementation in largemouth bass, higher expression of *cyp7a1* was reported in all BA supplemented diets compared to non-suppleme1nted diets. However, this was during fasted conditions after an 8 week trial (Romano et al., 2022). This could indicate that the BA dietary supplement replenishes the bile reserves in the liver/gall bladder, reducing energy expenditure during BA synthesis.

Importantly, BAs are not the only regulators of *cyp7a1*, and inflammatory cytokines can also interfere with its transcription. Indeed, the inflammation observed in the liver of these experimental fish could be playing a role in this expression patterns (Chapter 4). Moreover, BA may reduce the stability of *cyp7a1* mRNA (Chiang, 2009). Nonetheless, it should be kept in mind that BA synthesis via *cyp7a1* is not the only one pathway of bile acid synthesis.

Conclusion

Future research should focus on the characterization and profiling of bile acids found in the intestine of fish and how different diets can modulate the specific composition. It is known that bile composition varies in the gut during intestinal bowel diseases, well reviewed by Biagioli et al. (2021). This can be a consequence of dysbiosis occurring in such conditions, with changes in

microbial flora resulting in reduced capacity to enzymatically synthesize certain BAs. In these conditions of BA unbalance, knowing which specific BA is depleted or which synthetic pathway is interrupted could shed light into the investigation of which specific bile to supplement. Moreover, if BA profiling done in plasma shows a level of correlation with changes observed in the distal intestine, this could serve as a non-lethal indicator of gut health status and inform decisions as to which type of bile additive or mixture to supplement. Furthermore, the already known dysbiosis in gut microbiota taking place during SBM related enteropathies, suggests that microbiome research would be pivotal to select which specific bacteria strains could be beneficial as probiotics/prebiotics, allowing the corresponding enzymatic transformations of bile to occur.

In conclusion, our data show that BAs can successfully restore total plasma cholesterol and bile in the initial stages of feeding rainbow trout a 40% level SBM diet, and these effects can be maintained at least for 12 weeks. For longer term SBM exposure, higher dietary BA doses could counteract the loss of effect occurring in the present study after W12, but this will require further investigation. In turn, the significantly lower expression of *slc10a2* and BA content in plasma, together with the downregulation of *cyp7a1* (although non-significant) at W6 suggest early impacts of a 40% SBM diet, although these seem to disappear in the longer term. Nonetheless, these observations could not be supported by the BA concentrations quantified in digesta which were not measured at W6 but W18, and no differences were seen in *slc10a2* expression and plasma bile either at this point. The downregulation and lack of differential expression of all pro-inflammatory markers and the absence of statistical differences in BA and cholesterol synthesis enzymes make it an arduous task to fulfil the aim of this study to provide more insight into the role of dietary BA on modulating SBM-induced enteritis markers and bile and cholesterol physiology.

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Table 5.1. Primer pair sequences, efficiency for the genes used for qPCR.

Gene	Primer sequence (5' --> 3')	Accesion no.	Reference	Efficiency (%)
cypa	F: GCAAGTCCATCTACGGCAAT R: TGCTAGCGATGATGTTGAGG	MN722644.1		110
eef1a	F: TGCCCCTGGACACAGAGATT R: CCCACACCACCAGCAACAA		Murashita et al. (2018)	
tnfa	F: CAAGAGTTTGAACCTCATTCAG R: GCTGCTGCCGCACATAAAG		Bridle et al. (2006)	110.0
il8	F: CACAGACAGAGAAGGAAGGAAAG R: TGCTCATCTTGGGGTTACAGA	AJ279069		103.1
il-10	F: CGACTTTAAATCTCCCATCGAC R: GCATTGGACGATCTCTTTCTTC		Pérez-Sánchez et al. (2011)	95.3
slc10a2	F: TGGCTGGATGGAGACATGGACCTCAGT R: TGGATGGTGTCAGCAGAGGTCCAGACAG		Murashita et al. (2018)	108.2
mylk	F: GCACTACATGCAGCAGATCG R: AGCCACAAACTCTGGTGTCC	XM_021557724.2		93.8
cldn12	F: AAAACGCCAAGAACATCAGC R: GACATGCCTGCCATACACAG	BK007967.1		105.8
ocln	F: CGGAATCCAATGGCTACG R: AAGATCCCCACACAGAGCAC	GQ476574.1		108.6
hmgc	F: TCAGAACCGGACAGTCGTTG R: CTCAGCCGGCTCAAAGAAGA	XM_036977084.1		114.3
cyp7a1	F: AGGCCAACACGCTCCCGACTG R: CCGGGAGAGAGTGAGTTGTGGTTTGCT		Murashita et al. (2018)	97.5

cypa**:* cyclophilin A, peptidyl-prolyl cis-trans isomerase A; ***eef1a: elongation factor 1-alpha; ***tnfa***: tumor necrosis factor alpha; ***il8***: interleukin 8; ***il-10***: interleukin 10; ***slc10a2***: apical Na-dependent bile acid transporter; ***mylk***: myosin light chain kinase; *cldn12*: claudin-12; ***ocln***: occludin; ***hmgc***: β-hydroxy-β-methyl-glutaryl-CoA; ***cyp7a1***: cholesterol 7-alpha-hydroxylase, cytochrome P450 7A1.

.

Table 5.2. Total BA levels in intestinal digesta collected from proximal and distal segments and reduction rates (%). Values presented are as means ± standard deviation (*n* = 12).

Dietary groups	Proximal intestine digesta (mM)	Distal intestine digesta (mM)	Intestinal BA reduction rate (%)
FM	2.14 ± 0.13	1.54 ± 0.21	28.92 ± 5.92
SBM30	1.93 ± 0.10	1.24 ± 0.20	35.15 ± 11.56
SBM40	2.01 ± 0.03	1.37 ± 0.16	31.88 ± 7.43
SBM30-BA	1.98 ± 0.15	1.40 ± 0.14	27.36 ± 9.37
SBM40-BA	1.99 ± 0.17	1.54 ± 0.08	21.55 ± 4.56

FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

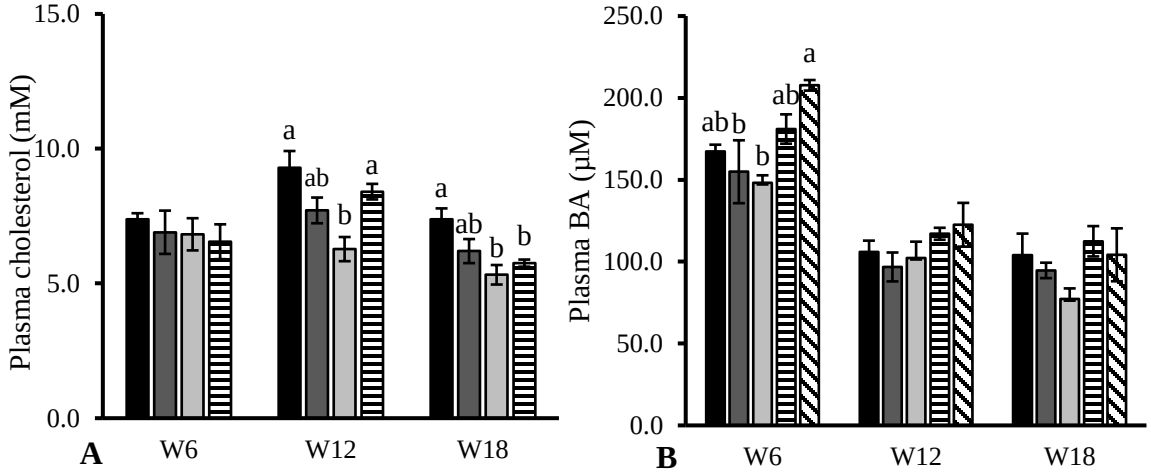

Figure 5.1. Plasma cholesterol (A) and BA (B) concentrations at each of three sampling points (W6, W12 and W18).

Values presented are as means ± standard deviation (n = 12). Different letters indicate significant differences between dietary treatments at that time point ($p \leq 0.05$).

FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

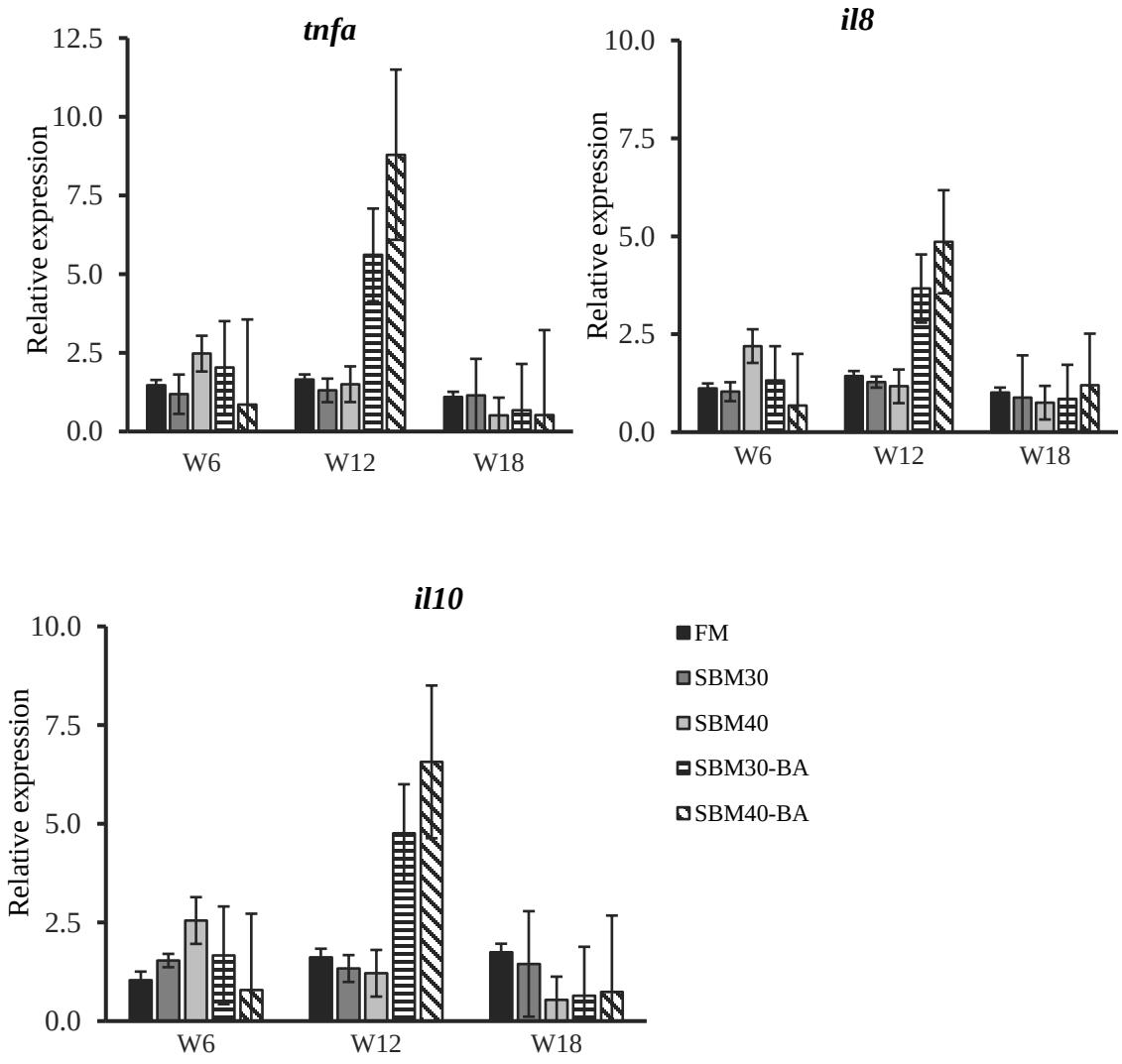

Figure 5.2. Distal intestine gene expression of the cytokines Tnfa, Il8, and Il10. Values are presented as means ± standard deviation (*n* = 12).
Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, and W18) of the feeding trial.
tnfa*:** tumour necrosis factor alpha; ***il8: interleukin eight; ***il10***: interleukin ten.
FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

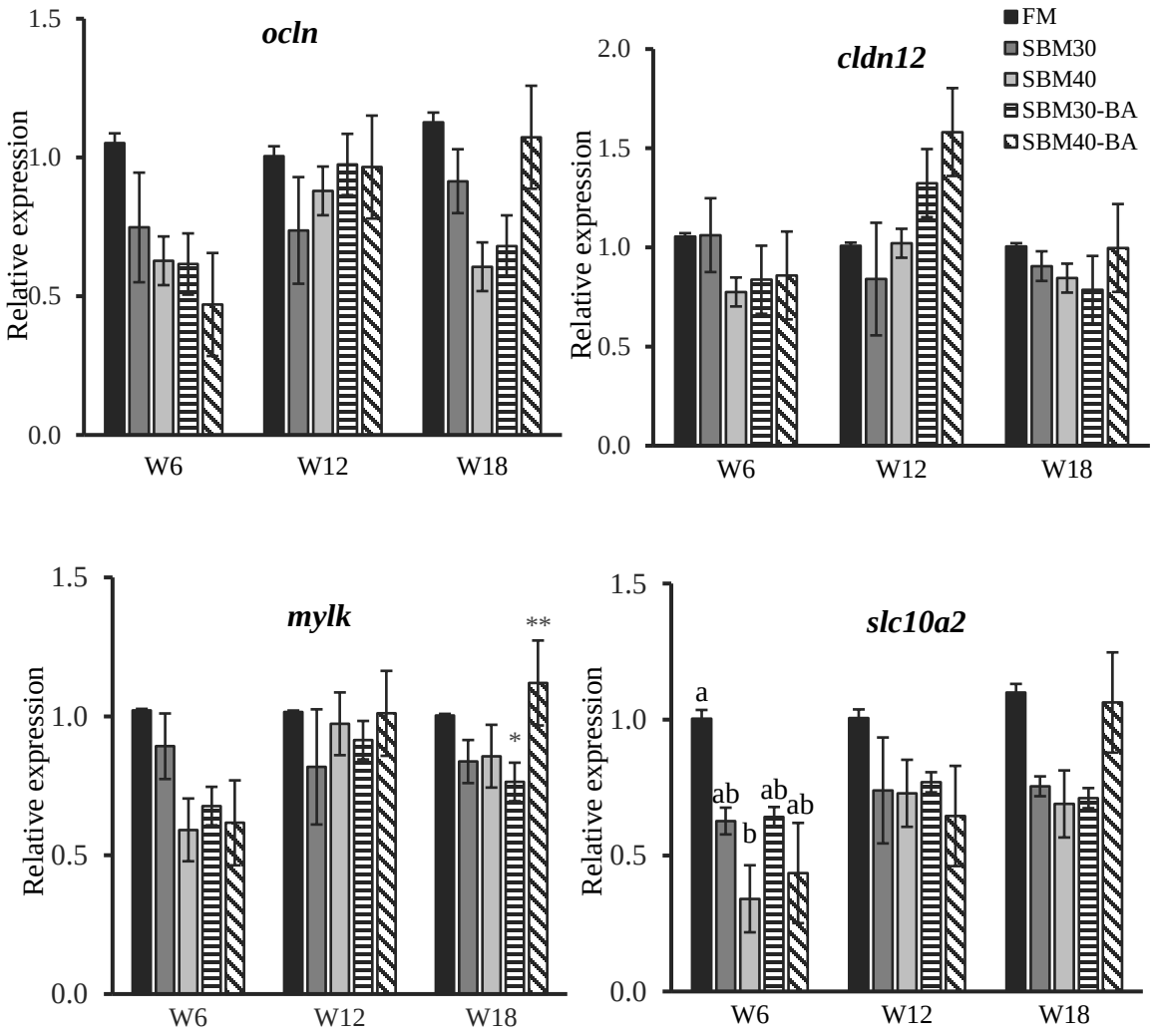

Figure 5.3. Distal intestine gene expression of the tight junction proteins Cldn12 and Ocln; the enzyme Mylk; and the bile acid transporter Slc10a2. Values are presented as means ± standard deviation (*n* = 12).

Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, and W18) of the feeding trial.

cldn12: claudin-12; ***ocln***: occludin*;* ***mylk***: myosin light chain kinase; ***slc10a2***: apical sodium dependent bile acid transporter.

FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

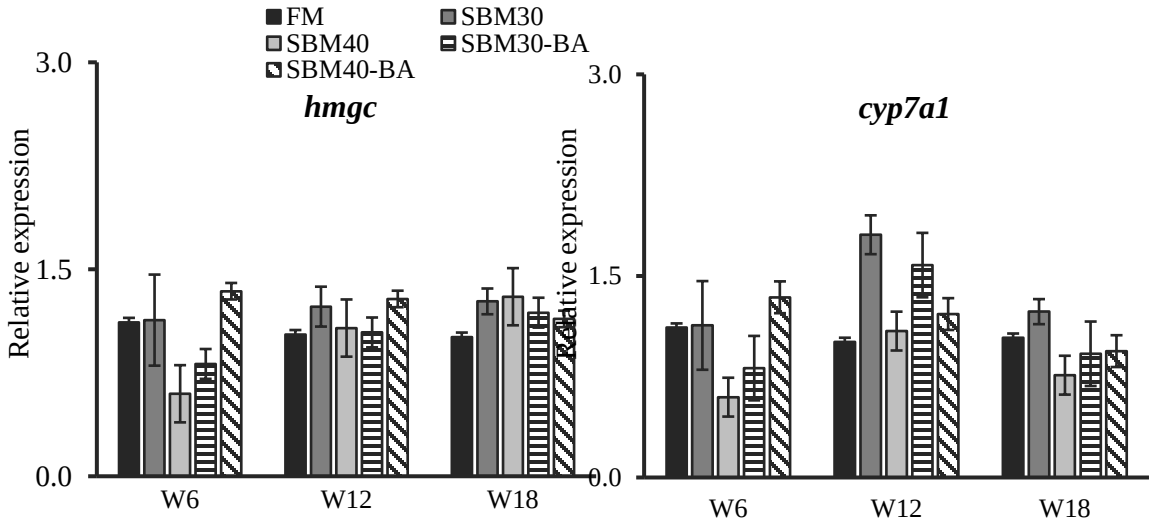

Figure 5.4. Hepatic gene expression of cholesterol and bile acid synthetic enzymes Hmgc and Cyp7a1.

Values are presented as means ± standard deviation (n = 12).

Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *ef1a* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W6, W12, and W18) of the feeding trial.

hmgc: hydroxy-methylglutaryl coenzyme A; ***cyp7a1***: cholesterol 7-alpha hydroxylase.

FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

hmgc: hydroxy-methylglutaryl coenzyme A; ***cyp7a1***: cholesterol 7-alpha hydroxylase.

FM: fishmeal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-BA**: SBM30 supplemented with bile acid; **SBM40-BA**: SBM40 supplemented with bile acid.

Chapter 6:

Mitigation of soybean meal-induced enteritis in rainbow trout (*Oncorhynchus mykiss*) using vitamin D and glutamine as dietary additives

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Abstract

Soybean meal (SBM) has become an essential ingredient in fish feed formulations, reducing the aquafeed industry reliance on fishmeal (FM). However, challenges remain when using SBM as the major source of protein, especially for carnivorous species. Reduced growth, and the occurrence of intestinal inflammation and loss of barrier function, referred to as soybean meal-induced enteritis (SBMIE), are signs of limited tolerance to SBM.

Vitamin D (VD) or cholecalciferol is well known for its role in mineral balance, maintaining calcium (Ca) and phosphorus homeostasis. More recently, evidence in mammalian studies has highlighted a role in intestinal immunity and barrier integrity. We therefore hypothesized its potential use as feed additive in SBM-based diets towards SBMIE attenuation. In addition, possible synergistic effects of VD and the non-essential amino acid glutamine (Gln), known for its protective action during intestinal inflammation were also examined in the present study.

A 10-week feeding trial was conducted in a commercial strain of rainbow trout. Seven experimental diets (42% isonitrogenous and 20% isolipidic) including a FM diet (control), a SBM30 and SBM40 recorded, g Kg^{-1} and 40 g Kg^{-1} inclusion level, respectively); two SBM30 and two SBM40, each supplemented with either VD or VD-Gln (50 mg Kg^{-1} VD or 50 mg Kg^{-1} VD + 2 g Kg^{-1} Gln) were formulated; growth was recorded and tissue samples were taken after 5 (W5) and 10 (W10) weeks of the feeding trial.

Fish fed the SBM30-VD-Gln diet demonstrated superior growth performance by W10 with higher weight gain and specific growth rate and reduced feed conversion ratio, but there were no differences in whole body proximate composition nor protein and lipid retention. No differences were seen among dietary treatments in the Ca levels measured in bone, kidney, muscle, and serum. Histopathology analysis in distal intestine showed a slight improvement as per lamina propria thickness and large vacuoles abundance in the SBM30-VD-Gln group compared to SBM40, SBM40-VD, and SBM30 dietary groups. Gene expression analysis in distal intestine demonstrated higher expression of *il1b* in fish fed a SBM40 diet compared to FM by W10 of the trial. Expression of the VD receptor (*vdr*) was higher in the FM group compared group compared to both SBM30-VD and SBM40-VD at W5, and higher than the SBM40 and SBM30-VD-Gln groups at W10.

This study provides evidence of the benefits of supplemental Gln at a 2.0% level in SBM30 diets. More research is needed to further elucidate the effects of high dietary VD doses in rainbow trout.

Introduction

The current trend in aquaculture nutrition to substitute fishmeal (FM) -based ingredients by more affordable plant derived feedstuffs such as soybean meal (SBM), translates in reduced growth performance in carnivorous fish species such as rainbow trout (*Oncorhynchus mykiss*). Moreover, high inclusion levels of SBM in fish feeds causes inflammation of the distal intestine, as widely demonstrated in histopathological examinations in Atlantic salmon (*Salmo salar*) (G. Baeverfjord and Krogdahl, 1996; Booman et al., 2018b; Krogdahl et al., 2015; Uran et al., 2008) and rainbow trout (*Oncorhynchus mykiss*) (Heikkinen et al., 2006; Kumar et al., 2021; Romarheim et al., 2008; Seibel et al., 2022). In addition to inflammation, changes in epithelial barrier integrity have also been demonstrated in terms of altered tight junction (TJ) protein expression in other carnivores such as turbot (*Scophthalmus maximus*) (Liu et al., 2018; Zhao et al., 2019); or even in the omnivores common carp (*Cyprinus carpio* L.) (Urán et al., 2008), and Jian carp (*Cyprinus carpio* var Jian) (Jiang et al., 2017, 2015b). This set of abnormalities related to high SBM diets are known as SBM-induced enteritis (SBMIE).

Vitamin D, a fat-soluble secosteroid hormone is a nutritional requirement in fish as the ultraviolet light exposure synthetic pathway, although present, is thought to be of minimal contribution in fish (Lock et al., 2010). In natural conditions, fish can obtain vitamin D in the form of vitamin D_2 also known as ergocalciferol; or as vitamin D_3 or cholecalciferol (hereafter referred to as VD). The former is barely detected in circulation whereas the latter constitutes the most abundant storage form found in fish and is an inactive structural precursor for the active VD metabolites (Vieth, 2020). (Mattila et al., 1999; Prabhu et al., 2019; Vielma et al., 1998). In fish, VD is metabolised in the liver through subsequent hydroxylation reactions to produce 25-hydroxyvitamin D_3 ($25(OH)_2D_3$, $25D_3$), also referred to as calcidiol; and 1α,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, $1,25D_3$), also known as calcitriol, the active hormone (Takeuchi et al., 1991). Of note, in mammals, the second hydroxylation takes place in the kidney, which is also possible in fish, although this happens to a much lesser extent (Fraser, 2018). Calcitriol is an important regulator of calcium and phosphorus intestinal uptake and renal excretion, as well as bone remodelling (Verri and Werner, 2019). These functions are transcriptionally regulated upon binding of $1,25D_3$ to the vitamin D receptor (VDR), a nuclear receptor. The VDR is widely expressed in teleost and is found in intestine, liver, pituitary glands, gills, corpuscles of Stannius, and bile duct epithelia (Craig et al., 2008; Lock et al., 2010).

Studies in higher vertebrates have shown a link between VD levels and development of IBDs, which share similarities with SBMIE observed in fish. Indeed, a deficiency in VD is present during IBD, suggesting that it could be a predisposing factor contributing to the development of the

disease (Cantorna et al., 2014; Limketkai et al., 2017). The pro-inflammatory cytokine tumour necrosis factor alpha (Tnfa), which has been central in the study of IBDs (Cunningham and Turner, 2012; Marchiando et al., 2010) was demonstrated to supress *vdr* expression in a human colon cancer cell line, further driving mucosal inflammation (Chen et al., 2014). On the other hand, treatment with $1,25D_3$ leads to nuclear factor kappa B (Nfkb) suppression, which is a central point in the transcriptional control of Tnfa and other inflammatory cytokines such as interleukin one beta (Il1b) (Liu et al., 2013; Wu et al., 2010). Additionally, stimulation of Vdr has also been linked to increased expression of TJ proteins such as occluding (Ocln), and claudins (Cldn) (Assa et al., 2014; Chen et al., 2014). The involvement of Vdr in immune responses is further supported by its expression in immune cells such as macrophages. Several lines of evidence suggest that the activation of Vdr in immune cells plays a role in reducing tissue damage and having an ability to at least partly attenuate detrimental effects, and anti-inflammatory cytokines like interleukin 10 and -22 (Il10, Il22) appear to be targets of this regulation (Froicu et al., 2006). Hence, $1,25D_3$/VDR could play a role in ameliorating the severity of the pathology and avoiding a perpetuation of the immune responses as those that characterise IBDs (Cantorna et al., 2019a).

In Atlantic salmon, *in vitro* treatment of isolated macrophages with VD decreased pathogen attachment and increased neutrophil attachment (Soto-Dávila et al., 2020). The involvement of dietary VD in innate immunity modulation in fish has been seen in yellow catfish (*Pelteobagrus fulvidraco*) (Cheng et al., 2020), European seabass (*Dicentrarchus labrax* L.) (Dioguardi et al., 2017), and black carp (*Mylopharyngodon piceus*) (Wu et al., 2020) in terms of improved phagocytic activity or antioxidant capacity. Initial signs of a positive effect of dietary VD in modulating intestinal immunity have been recently shown in turbot subjected to a pathogen challenge source of stress (Shao et al., 2022). However, the effects of dietary VD on SBMIE modulation have not been investigated in fish.

The supplementation of VD in fish feeds is controlled and kept within stipulated ranges to ensure safe fish products for consumers. For rainbow trout and Atlantic salmon the requirement is currently 0.04 and 0.06 mg Kg^{-1} diet, respectively, and the maximum limit in the feed is at 75 µg Kg^{-1} diet (Jakobsen et al., 2019; Prabhu et al., 2019). Previous experiments in Atlantic salmon fry have shown tolerance to dietary VD doses to be as high as 57 mg Kg^{-1}, and found no signs of detrimental effects in terms of skeletal deformities (Graff et al., 2002a). In rainbow trout, Mattila et al. (1999) showed that feeding higher VD levels translated in higher contents in the liver, but no significant increase was found in the fillets. In agreement, Horvli et al. (1998) indicated that liver, intestine and plasma had the highest VD concentrating abilities in Atlantic salmon. These observations suggest a reduced risk of hypervitaminosis D in these species.

Materials and Methods

Experimental diets

A total of seven experimental diets were formulated to contain ~43% crude protein and 20% crude lipid as detailed in Table 6.1. A 25% fishmeal (FM) diet was selected as a control. Soybean meal diets (SBM) were formulated at two inclusion levels, 30% (SBM30) and 40% (SBM40). Each SBM30 and SBM40 were supplemented with 50 mg Kg^{-1} vitamin D_3 (Bulk Supplements, Hard Eight Nutrition LLC., Henderson, NV, USA) (SBM30-VD and SBM40-VD) or supplemented with 50 mg Kg^{-1} VD plus 20 g Kg^{-1} Gln (Ajinomoto Co., Inc.) (SBM30-VD-Gln and SBM40-VD-Gln). Ingredients were mixed for 20 min prior to the addition of fish oil, and cold-pelleted at the University of Idaho's Hagerman Fish Culture Experiment Station (HFCES) using a laboratory-scale California pellet mill fitted with a 2.0-mm die. Twenty percent water was added to the diets prior to extrusion. Pelleted particles were then placed in a forced-air dryer at 35°C until moisture levels were less than 10%, and sorted in plastic bags at room temperature until fed.

Experimental design and sampling

Rainbow trout were obtained from Troutlodge (Sumner, WA) and transported to the Aquaculture Research Institute Cold- Water Laboratory at the University of Idaho, Moscow, USA. A total of three hundred and seventy-eight fish (initial body weight: 9.47 ± 0.47 g) were randomly distributed into twenty-one, 60-L tanks in a recirculating aquaculture system, incorporating three replicates per diet. Each tank was supplied with well water at a flowrate of 6.0 – 7.5 L min^{-1} and constant temperature (15.0 °C). Rearing conditions were maintained at a constant photoperiod 12 h light/12 h dark, and oxygen was supplied by normal aeration and maintained at 7.35 ± 0.65 mg L^{-1}. Water quality parameters including pH, NO_2 and NH_3 were measured weekly using commercial kits (LaMotte Chestertown, MD, USA) and maintained at 7.5 ± 0.17, 0.19 ± 0.05 mg L^{-1} and < 0.05 mg L^{-1}, respectively.

Rainbow trout were fed twice daily six days per week to apparent satiation for a total of ten weeks, and feed intake was recorded weekly. During the feeding trial, samples were taken from 6 fish per tank after five weeks (W5) and from 9 fish per tank after ten weeks (W10), 12 h after the last feeding. Fish were euthanized using an overdose of tricaine methanesulfonate (Syndel Inc., Ferndale, WA) at 250 mg L^{-1} buffered to pH 7.0-7.5. A total of six fish were sampled at W5 and nine fish at W10. Fish weight and length were recorded at each sampling point, and livers were dissected and weighed for hepatosomatic index (HIS) calculation. Samples were taken from distal intestine for gene

expression and histopathology analysis. At W10, extra samples from muscle, vertebra, kidney and serum were taken for calcium analyses.

All experimental procedures involving live fish were carried out with prior approval from the Institutional Animal Care and Use Committee, University of Idaho (IACUC-2021-57).

Growth performance parameters

Growth performance was evaluated following the below indices calculation at each sampling point (W5 and 10), except for protein and lipid retention which were calculated only at W10:

- Weight gain (WG, g) = average final weight (g) – average initial weight (g)
- Specific Growth Rate (SGR, %/day) = $\frac{\ln(final\ weight)^{i} - \ln(initial\ weight)}{100 * days^{ii}}$
- Feed intake (FI/fish/day) = $\frac{feed\ intake\ (g) x\ average\ fish\ weight\ (g)^{i}}{days^{ii}}$
- Feed Conversion Ratio (FCR) = total feed intake (g)/weight gain (g)
- Condition factor (*K*) = $(10^4\ x\ fish\ weight\ (g))/[(fork\ length\ (cm)\ x\ 10)^3]$
- Hepatosomatic index (HIS) = (liver weight (g)/fish weight (g)
- Protein retention (PR, %) = $\frac{fillet\ protein\ gain\ (g)}{protein\ intake\ (g)}$
- Lipid retention (LR, %) = $\frac{fillet\ lipid\ gain\ (g)}{lipid\ intake\ (g)}$

[i]Final weight as recorded at each sampling point
[ii]Cumulative number of days passed until a given sampling point

Proximate composition of whole-body and diets; and amino acid composition of diets

Crude protein, crude lipid, ash and moisture of the feeds and fish fillet were analyzed following standard methods (AOAC, 2000). Briefly, dry matter and moisture content were determined by drying feed or fillet samples overnight (12 h) in a convection oven (105 °C) to a constant weight. Ash was assessed after ignition at 500°C for 12h, according to AOAC (1995). Afterwards, crude protein content in feed samples was analyzed using the Kjeldahl method (N x 6.25) (Kjeltec 8100, FOSS, Denmark) and crude protein in fillet was analyzed using a combustion method with a nitrogen determinator (rapidN exceed, Langenselbold, Germany). Crude lipid in feed samples was calculated gravimetrically using SoxtecTM (FOSS, 2050 automated analyzer, Denmark) and

petroleum ether extraction following acid hydrolysis on SoxCapTM (FOSS, Denmark). Crude lipid in fillet samples was analyzed similarly by hydrolysis using an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY) and petroleum ether extraction sample extraction was done using an ANKOM XT15 extractor. Total energy content was determined using a Parr bomb calorimeter (Parr Instrument Co., Moline, IL, USA).

The amino acid composition of the feed (Table 6.2) was determined after acid hydrolysis and derivatization by AccQ-Tag™ Ultra, according to the amino acid analysis application solution (Waters Corporation, Milford, MA, USA). DL-Norvaline (Sigma) 2.5 mM was used as an internal standard and ultra-performance liquid chromatography (UPLC) was performed on an Acquity system (Waters Corporation) equipped with PDA detector. The detection wavelength was set at $\lambda = 260$ nm and the column used was a Waters' BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm). The flow rate was set at 0.7 ml/min and the column temperature was kept at 55 ºC. Lastly, peak identification and quantitation were performed by the software Empower v.2.0 (Waters) using an amino acid standard (Waters) as an external standard. All analyses were performed in duplicate. Tryptophan was not quantified due to its susceptibility to acid hydrolysis, forming a disulfide bridge to produce cystine because of cysteine-cysteine interactions. Similarly, during acid hydrolysis, asparagine is converted to aspartate (ionic form of aspartic acid) and glutamine to glutamate (ionic form of glutamic acid), thus, the reported values for these amino acids (Asx and Glx) represent the sum of both amino acids.

Calcium analysis

Muscle, kidney, vertebra, and serum samples were collected from three fish per tank (n = 9). Firstly, fish were anaesthetised in MS-222 and blood was drawn from the caudal vein using a 1 cc syringe with 26 ½G needle (AirTite Products Co., Inc., Virginia Beach, VA, USA) and placed into 1.5 mL microcentrifuge tubes and allowed to clot overnight at 4.0 °C. Serum was collected by centrifugation at 15,000 x *g* for 5 min., and stored at -20 °C until further analysis. Muscle, kidney, and vertebra samples were oven dried at 70 °C for 24 h, digested at 115 °C for 30 min in 4 mL of trace metal-grad nitric acid (HNO_3) (69%). Hydrogen peroxide (H_2O_2, 30%) was added (0.1 mL) with 40 mL Milli-Q water to all samples. This solution was used for calcium (Ca) determination on a flame atomic absorption spectrophotometer (AAS) (iCE 3000 series, ThermoScientific, USA) with deuterium lamp background correction. Calibrations were made with single element standards (CPI International, Santa Rosa, CA, USA).

Histopathology analysis of distal intestine

Histopathological analyses were done on distal intestine (delimited proximally by the first complex fold and distally by the anus opening) samples taken from three fish per tank (n = 9) at W10 of the trial. Samples were kept in Bouin's fixative for less than 24 h and dehydrated in 70% ethanol. Samples were processed at Washington Animal Disease Diagnostic Laboratory (WADDL; Washington State University, Pullman, WA). Haematoxylin – eosin (H&E) staining was used and samples were evaluated following the histological scoring system established by (Barnes et al., 2014). Briefly, morphology of the DI was evaluated for lamina propria thickness (LP); connective tissue thickness (CT) and prevalence of large vacuoles (VAC). The ranking criteria goes from 1 (healthiest morphology) to 5 (most damaged morphology). A total of six observations were taken per tank (n = 18). Individual scores for each variable were summed. Statistical analysis was done on the mean calculated for each dietary treatment.

RNA extraction, cDNA synthesis and RT-qPCR

Distal intestine samples were collected from three fish per tank (n = 9) at W5 and W10, snap frozen in liquid N_2 and stored at -80 °C until analysis. Trizol (Waltham, MA, USA) was used for RNA extraction following the manufacturer's instructions. Quantity of RNA was measured using Nanodrop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) and purity was determined based on the OD_{260}/OD_{280} absorption ratio (>1.95). The quality of the RNA was analysed using agarose gel electrophoresis and samples were DNase (Invitrogen, Waltham, MA, USA) treated when appropriate.

Next, 0.5 µg RNA were reverse transcribed to cDNA using a a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). RT-qPCR reactions were carried out using SYBR green detection method on a StepOnePlus real-time thermocycler (Applied Biosystems, Waltham, MA, USA). Duplicate reactions were performed for each cDNA sample analyzed (i.e., six technical replicates per tank). The reactions were set on a 96-well optical plate, and each contained: 1.5 µL of diluted (1/5) cDNA, 5 µL of 2X concentrated PowerUp ™ SYBR Green Master Mix, 0.5 µM forward primer and 0.5 µM reverse primer. The reaction protocol consisted of an initial denaturation step (2 min at 50 °C and 2 min at 95 °C); an amplification and quantification program (repeated 40 times, 15 s at 95 °C, 20 s at 57 - 60 °C, and 30 s at 72 °C); and a melting curve analysis to verify presence of single products that had specific melting temperatures. Negative controls were run for each gene to ensure absence of non-specific products or primer-dimer formation.

Cyclophilin A (*cypa*, pepdidyl-prolyl-cis-trans isomerase A) was selected as reference gene as it had the lowest coefficient of variation across treatments (< 3.5%). Gene expression was determined using the ΔC_t method described by Pfaffl (Pfaffl, 2001), where the genes of interest were normalized to the C_t value of the housekeeping genes *cypa*. Both the standard curves and primer efficiencies were obtained by serial dilutions of a random cDNA mixture of control samples. Gene expression analysis were performed on the inflammation markers tumour necrosis factor alpha (*tnfa*), interleukin one beta (*il1b*), interleukin 10 (*il10*); barrier integrity markers: occludin (*ocln*), and claudin-12 (*cldn12*); the cellular contractility enzyme gene myosin light chain kinase (*mylk*); and the nuclear vitamin D receptor (*vdr*). All primer sets were validated on a 1% agarose gel to check for amplification product (125 V for 35 min) and validate successful product formation. Table 3 shows primer sequences and efficiencies.

Statistical analysis

All data were analysed using R statistical software (v. 3.6.2). Mean ± standard deviation (SD) was calculated for growth performance, mineral and gene expression data. The Shapiro-Wilk and Levene's test were used to test the normality of data distribution and homogeneity of the variance, respectively. When data did not follow a normal distribution, a logarithmic transformation was carried out. A one-way analysis of variance (ANOVA) was used to determine the effect of different dietary treatments. Post-hoc analysis was done using Tukey's honest significant test. Differences were considered significant at $p \leq 0.05$.

Results

Growth performance and whole-body proximate composition

All analysed growth performance indicators are presented in Table 6.4. No differences were seen for any of the studied parameters at W5 (all $p \geq 0.05$). However, significant differences in WG between dietary treatments were seen at W10 ($p < 0.001$), with fish fed the SBM30-VD-Gln diet showing the highest growth rates, followed by the FM, SBM40-VD-Gln and SBM30 diets. This pattern was similarly obtained for SGR, although differences were not significant ($p \geq 0.05$). No differences were recorded in FI, however, FCR was significantly lower for both SBM30 and SBM30-VD-Gln, followed by the SBM40-VD-Gln and FM diets whereas the SBM40, SBM30-VD and SBM40-VD had the highest FCR ($p = 0.008$). No significant differences were seen at any time point in *K* and HSI the highest indices were seen in the FM group followed by the SBM30-VD-Gln.

Whole body proximate composition of moisture content, ash, protein, and lipid were not affected by either the inclusion level of SBM, VD or the VD and Gln combination (all $p \geq 0.05$) (Table 6.5). There was also no impact of dietary treatment on PR and LR after the 10 weeks feeding trial (all $p \geq 0.05$).

Calcium analysis

Kidney, vertebra, muscle, and serum samples collected at W10 showed no difference in Ca level among dietary treatments (all $p \geq 0.05$) (Table 6.6).

Histology of distal intestine

Distal intestine histopathology results from samples taken at W10 are summarised in Table 6.7. There were no differences in connective tissue thickness between the base of folds and stratum compactum among fish fed any of the seven dietary treatments ($p \geq 0.05$). Robustness of the lamina propria of fish fed all dietary treatments resembled the FM group. However, fish fed the SBM30-VD-Gln diet scored the lowest, followed by the SBM30-VD diets, the former being significantly lower than the SBM30, SBM40 and SBM40-VD groups ($p = 0.001$); while the latter was lower than both SBM40 and SBM40-VD groups ($p = 0.041$). Large vacuoles were least abundant in the distal intestine of fish fed the FM diet, and this was statistically the same as the SBM40, SBM30-VD, SBM30-VD-Gln, and SBM40-VD-Gln. Significantly higher vacuole abundance was observed in the SBM30, and SBM40-VD dietary groups compared to the control (FM) ($p = 0.026$).

Gene expression analysis

The relative gene expression of the *tnfa* and *il10* in distal intestine did not show any changes throughout the trial (all $p \geq 0.05$) (Fig. 6.1). The expression of the pro-inflammatory cytokine *il1b* was not changed at W5 ($p \geq 0.05$) but showed a significantly higher level at in the SBM40, SBM30-VD-Gln and SBM40-VD-Gln W10 compared to the FM fed fish ($p = 0.0084$) (Fig. 6.1). Gene expression of the TJ proteins *cldn12* and *ocln* showed no significant differences at any time point (all $p \geq 0.05$) (Fig. 6.2). The transcriptional levels of *mylk* were markedly reduced in all dietary treatments at W5 except for the FM group ($p = 0.00317$), although the SBM30-VD-Gln shared statistical resemblance to the control (Fig. 6.3). The mRNA transcript level remained significantly lower in the SBM40 group at W10 compared to the FM group ($p = 0.034$). Expression of the *vdr* showed changes both at W5 and W10, with the FM fed fish showing the highest levels at both time points ($p = 0.0336$ and $p = 0.013$) (Fig. 6.3). At W5, the SBM40-VD-Gln group showed significantly

lower *vdr* expression levels than the FM group, whereas at W10 the lowest transcriptional levels were recorded for the SBM40 and SBM40-VD-Gln groups.

Discussion

The occurrence of SBMIE in commercial carnivorous species is a topic of concern in modern aquaculture as it can lead to significant losses. The formulation of functional feeds using additives is gaining attention to tackle this issue. Amino acids, minerals, and vitamins are good examples of popular compounds used to supplement diets due to their beneficial properties. The amino acid Gln has been extensively studied in the context of SBMIE. However, despite mounting evidence on the role of VD in mucosal health in higher vertebrates, little to no research has been done to further examine its effect on piscine species. We hereby examined how a high dose of VD supplementation alone or in combination with Gln could modulate intestinal health in a high SBM dietary level scenario.

Growth performance and proximate composition

In the present study, rainbow trout fed the SBM30-VD-Gln showed the highest WG by W10 of the trial, and although it did not statistically differ from the FM diet, it showed higher WG compared to the control (i.e., 119.74 vs. 106.47 g, respectively), and this was similarly observed for FCR (0.78 vs. 0.83, respectively). Comparing the SBM30-VD-Gln with a non-supplemented SBM30 diet, the beneficial role of the additives was evident, maintaining similar FI and FCR but reaching significantly higher growth in the VD-Gln supplemented diet. Nonetheless, interpretation of these results must be done cautiously. Since there was no significant difference between both SBM30, SBM40 and these two diets supplemented solely with VD, it could be that either VD had no impact on growth performance or that VD was not adequately mixed with the diet at manufacture. In either case, the superior growth results obtained for the SBM30-VD-Gln diet would be a result of Gln supplementation and not the combined action with VD. Interestingly, both SBM30 and SBM40-VD still showed a level of resemblance to the FM diet while the SBM30-VD and SBM40 had the lowest WG. It is expected for fish fed a SBM40 diet to display inferior growth performance; however, the addition of VD in the SBM30 not only did not seem to help but also slightly negatively impact growth. As noted in other discussions (Rubio-Benito et al., Paper I and III), once a level of adaptation is reached, changes in metabolism to cope with the new ingredient may interfere with the hypothetical beneficial signalling of the additive being tested.

In general, there is substantial evidence of the positive impact of Gln on growth performance enhancement in a variety of fish species including rainbow trout (Yoshida et al., 2016),

turbot (Liu et al., 2018), red drum (*Sciaenops ocellatus*) (Cheng et al., 2011) hybrid sturgeon (*Acipenser schrenckii* × *Huso dauricus*) (Qiyou et al., 2011), and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Cheng et al., 2012). However, less data is available with regards to the effects of VD on growth. In rainbow trout, Hilton and Ferguson (1982) reported no effect of excess VD in rainbow trout, which is in contrast with the decreased WG observed in the same species by Vielma et al. (1998). In Atlantic salmon fry, a mega dose of VD (57 mg Kg^{-1}) did not impact SGR in a period of up to 104 days (Graff et al., 2002a), but yellow catfish fed a 0.4 mg Kg^{-1} VD supplemented led to the highest weight gain percentage compared to lower VD supplemental levels after 12 weeks (Cheng et al., 2020a). Further positive impacts of a supplemental VD dose ranging from 0.09 to 0.93 mg Kg^{-1} were shown in European seabass after 4 weeks, with significantly lower FCR compared to a commercial diet (Dioguardi et al., 2017). In turbot, a significant increase in both WG and SGR was seen after feeding diets containing 0.4 and 0.8 mg Kg^{-1} dietary VD for 8 weeks, but not at higher levels (Shao et al., 2022). Similarly, gilthead seabream showed a proportional increase in these parameters with increasing dietary VD doses (ranging from 0.14 to 0.65 mg Kg^{-1}) after 70 days, although this was not significant (Dominguez et al., 2021). Importantly, that study also revealed signs of deformities in gilthead seabream, which contrasts with what was observed by Graff et al. (2002) in Atlantic salmon fry fed extra high VD doses, although both studies confirmed higher VD levels in the liver of both species. Overall, there seems to be a positive effect of dietary VD in growth performance of different species. This may be especially true when considering plant-based diets. As reported by Prabhu et al. (2019), an increased requirement for VD may exist, at least for salmonids, fed low level fishmeal practical diets.

No further differences were seen in proximate composition of whole body by the end of the present study, together with no impact on PR and LR. Based on our previous results, it would have been expected that at least the 2.0% Gln supplementation increased the protein content, counteracting the slightly lower protein deposition that results from dietary SBM. However, none of these premises were met. Instead, these results are in line with what was obtained from our previous study which also included a similar SBM30 and SBM40 diets and where no differences in either lipid content, protein content, PR and LR were determined. In Siberian sturgeon (*Acipenser baeri*), higher dietary VD (0.04 – 0.08 mg Kg^{-1}) led to increased lipid and ash content, but these numbers decreased again with the highest dose (0.25 mg Kg^{-1}) (Wang et al., 2017).

Calcium analysis

Given the role of VD in promoting mineral homeostasis and bone development, it was deemed necessary to monitor the Ca levels in vertebra, muscle, kidney, and serum in the present study.

Analysis of Ca in vertebra could give an idea of the risk of hypercalcinosis associated to the elevated dose in our dietary treatments which could lead to deformations. Although it has been shown that this risk is reduced in salmonids and that excess VD is stored in the liver rather than in the fillet (Graff et al., 2002a; Jakobsen et al., 2019; Mattila et al., 1999), an excess VD would typically lead to bone mineralization and, thus, higher Ca levels, as well as increased kidney Ca levels also known as nephrocalcinosis; and hypercalcaemia. The results obtained herein did not show any difference in Ca levels in any tissue studied among dietary treatments. Discrepancies exist on the effect of excess VD in bone among different fish species, as both increased bone mineralization and bone resorption have been observed (Lock et al., 2010). However, many of these studies were carried out using intraperitoneal injection as way of exposure to VD. Dominguez et al. (2021) showed that surpassing the VD requirement level in gilthead seabream fed plant feedstuffs could lead to skeletal anomalies as well as myocarditis when dietary VD was 500 µg Kg^{-1} (Dominguez et al., 2021). On the other hand, no signs of deformities were seen in Atlantic salmon fed extra high VD doses (57 mg Kg^{-1}) (Graff et al., 2002a) as well as no change in bone Ca content (Graff et al., 2002b). Furthermore, neither of these studies found changes on kidney Ca. All these findings are in agreement with the results of the present study.

This study reports no changes in serum Ca levels, which is consistent with what was reported in turbot, although with lower VD doses, ranging from 0.03 – 1.5 mg Kg^{-1} (Shao et al., 2022). However, studies in different fishes including tilapia, catfish (*Clarias batrachus*) and Atlantic cod (*Gadus morhua*) have typically shown a rise in plasma or serum Ca levels, but these observations were made on fish that had received either VD or $1,25D_3$ injections (Fraser, 2018). In American eel (*Anguilla rostrata*) it was shown that increased Ca in plasma happened in fish that had previously been fed, attributing the rise in Ca to enhanced intestinal uptake rather than bone resorption (Fenwick et al., 1984). However, increased serum Ca levels can also be as a result of bone demineralization due to prolonged administration of VD as observed in cichlids (Wendelaar Bonga et al., 1983), a notion that is also widely accepted in mammals under supraphysiological VD doses (Carlsson, 1952; Suda et al., 2003).

Apart from the possible impact that a high dietary dose of VD could cause, the present study also showed no effect of the high SBM inclusion levels. Little is known about mineral, and particularly Ca status in fish fed plantstuffs. Prabhu et al. (2019) showed an increased requirement in

micro-minerals such as zinc and manganese as well as VD in salmonids fed low level FM diets. Blaufuss et al. (2019) identified the expression of Ca binding proteins in the distal intestine of rainbow trout fed 40% SBM. This could potentially result in lower Ca levels in serum as a consequence of decreased uptake. However, the lack of differences in serum free Ca obtained in the present study questions the impact of Ca sequestration in the intestine. However, serum Ca levels could be maintained by bone resorption, as suggested in studies in mice in an IBD context involving gut VDR insufficiency where Ca serum levels did not differ (He et al., 2018). Still, our results did not seem to point out an impact on Ca homeostasis of neither high SBM nor VD. As a last consideration, these results could also support the surmise that no or little VD was present in the experimental diets.

Histopathology of distal intestine

Histopathological analysis could have been a determining factor in elucidating the role of VD in SBMIE mitigation or discerning if VD was successfully supplemented in the diets on the present study. However, our results did not aid in clarification. While slight improvements were seen in lamina propria infiltration and large vacuole abundance switching from SBM30 to SBM30-VD, the SBM40-VD showed increased abundance of large vacuoles compared to the SBM40 diet, while the connective tissue thickness and lamina propria infiltration remained the same between fish fed either treatment. Remarkably, fish fed the SBM30-VD-Gln displayed optimal performance in terms of lamina propria, even compared to the FM group, and shared the highest similarity with the FM fed fish for connective tissue and large vacuoles abundance, followed by the SBM40-VD-Gln. These observations indicate that any slight improvement detected in the present study could be attributable to dietary Gln rather than VD. There is abundant evidence of the beneficial effects of dietary Gln in gut histopathology in a SBMIE context (Beutheu et al., 2014; Gu et al., 2017d; Qiyou et al., 2011; Yan and Qiu-zhou, 2006). Our previous study in rainbow trout showed that the same Gln supplementation level 2.0% led to partial mitigation of the SBMIE.

In turbot, no changes in villus height were seen for any VD level, dismissing the idea of possible VD detrimental effects in the intestine.

Gene expression

Distal intestine gene expression of *tnfa* and *il10* did not show any differences at any time point, however, at W5 *tnfa* showed slightly higher expression levels in the SBM30 and SBM40 groups than other dietary treatments, while *il10* had slightly lower levels in those same dietary treatments at W5. The pro-inflammatory cytokine *tnfa* is thought to play a critical role in IBD and, similarly so in SBMIE in fish (Gu et al., 2021, 2017, 2016; Liu et al., 2018b; Ma et al., 2005;

Marchiando et al., 2010; Urán et al., 2008; Zhang et al., 2013, 2021), therefore, high transcriptional levels would have been expected in the SBM30 and SBM40 groups. In turn, Il1b, which is involved in inflammatory process regulation expression in fish, and whose local effect also increases Tnfa (Zou and Secombes, 2016) showed significant upregulation at W10 in the SBM40, SBM30-VD-Gln and SBM40-VD-Gln groups. This was in line with what would be expected after feeding rainbow trout SBM for this amount of time, however, it also points out higher inflammation in both double supplemented diets, suggesting a lack of anti-inflammatory effect of the supplements. A lack of difference in the expression of *tnfa* and *il8* was encountered in the same commercial rainbow trout strain fed similar SBM30 and SBM40 diets throughout a longer period of 18 weeks, as shown in Chapter 5. Increased expression levels of *il10*, or other anti-inflammatory cytokines, would indicate that the immune response has the ability to counteract an otherwise uncontrolled and perpetual inflammatory response. In the current study, *il10* showed the highest expression levels in the SBM30-VD-Gln group by W10, and although this was not significant, it could be indicative of increased anti-inflammatory capacity in fish under that dietary treatment. In common carp, a species that is routinely fed plant-based diets, SBMIE was observed to happen from the first to the third week of dietary exposure, and to clear out towards the fifth week; and an upregulation of *il10* in the distal intestine was recorded during the first week (Urán et al., 2008). This could indicate that the anti-inflammatory action at the initial stages is a "requirement" for enteritis recovery in that species. In the present study, the similar *tnfa*, and *il10* expression levels between supplemented treatments also showed no modulation resulting from dietary VD or Gln. Further, the significantly higher expression of il1b in both double supplemented diets together with the SBM40 also questions the effectiveness of the additives used. However, studies in fish have previously documented the role of Gln in the modulation of these cytokines (Gu et al., 2017; Jiang et al., 2015; Liu et al., 2018). Dissimilarities between juvenile salmons have been proved in liver gene expression patterns of inflammatory cytokines depending on the time after exposure to an exogenous stimulus (Fast et al., 2007). In the present study, samples were taken 12 – 18 h after feeding, which is similar to what was done in our previous trial on the same fish strain, and the same *tnfa* and *il10* downregulation resulted from SBM30 and SBM40 exposure (Chapter 5). Therefore, in the present study, it could be possible that significant upregulation of *tnfa* occurs at an earlier or later time after exposure. Liu et al. (2018) also identified fluctuations in the expression trends of, for instance, *tnfa* as modulated by SBM diets at different time points (i.e., 2, 4 and 8 weeks) throughout a 12-week period.

Relative expression of *ocln* and *cldn12* was not significantly affected by SBM inclusion, VD or VD and Gln. In the current study there were no signs of VD modulation on *cldn12* expression For *cldn12*, the highest transcriptional levels were observed for the SBM30-VD-Gln and SBM40-VD-

Gln group at W5, and especially the latter by W10. In turn, at W10, fish fed the SBM30-VD-Gln diet showed the closest resemblance to the control diet, which could be a positive sign, considering the excellent growth performance of fish fed that diet, but this is merely speculative. The difference between SBM30-VD-Gln and SBM40-VD-Gln at W10, although unsignificant, could be explained by insufficient Gln supplementation in the SBM40 diet. It could be argued that a higher level of Gln supplementation would be required in 40% SBM diets to obtain similar *cldn12* expression levels as observed in the SBM30-VD-Gln. However, since expression levels are slightly higher in the SBM40-VD-Gln at W10 than all other dietary treatments, it could also be speculated that this was due to Gln, in which case it would be hard to explain the divergence between both double supplemented dietary groups. The lack of differences between both SBM30 and SBM40 and all other dietary treatments, challenges the possible association with a hypothetical SBM impact on TJ protein expression. Little is known in teleost about the role of VD in TJ protein modulation in fish. In turbot, *ocln* and zonula occuldens gene expression levels were not changed in response to dietary VD, although that study did not examine TJ expression in a plant-based diet context (Shao et al., 2022). Based on observations in higher vertebrates, VD could potentially modulate TJ expression in the intestine (Assa et al., 2014; Cantorna et al., 2019b; Chen et al., 2014; Liu et al., 2013; Wu et al., 2010). Interestingly, a relationship between VD and Cldn12 has been observed *in vitro* in which 1,25VD_3 induces Cldn12 and that their combined action is crucial for VD-induced Ca absorption (Fujita et al., 2008). Changes in Ca levels during SBMIE have been little studied, but evidence in mammalian studies point out the role played by calgranulins or calcium binding proteins during auto-immune diseases such as IBDs (Manolakis et al., 2011, 2010). In fish, the increased expression of some of these was shown in the intestine of rainbow trout fed 40% SBM diets (Blaufuss et al., 2019).

The inter-relation between the loss of barrier function, the decreased availability of extracellular Ca due to calgranulins, the involvement of Cldn12 in Ca absorption in the intestine and the barrier-promoting role of VD is a conundrum that warrants further attention. It can be speculated that either the SBM30-VD-Gln is performing better with regards to maintaining barrier function and hence, *cldn12* remains lower, similar to FM, because there is also no Ca imbalance going on in the intestine of fish fed these diets. Perhaps 2.0% Gln is not enough in the SBM40-VD-Gln diet and *cldn12* expression is promoted (although not via VD but maybe Gln) to restore Ca levels AND/OR support Mylk activity during the associated hypercontractility that accompanies loss of barrier function, since cellular contraction requires Ca. Indeed, Mylk constitutes another marker in this interplay. While an enhanced Mylk activity is what would be expected based on mammalian studies (Ma et al., 2005; Marchiando et al., 2010; Ye et al., 2005) and as described for turbot (Liu et al., 2018), the opposite was obtained in the present study at W5, with significantly higher expression

levels in the FM group compared to all other dietary treatments with the exception of SBM30-VD-Gln. Similarly, at W10, *mylk* expression was significantly lower in the SBM40 group compared to FM, again contrary to expected, but maintaining the same pattern observed at W5. These results are, however, in accordance with our previous studies on SBMIE in rainbow trout, as none of them showed signs of *mylk* increased expression in SBM diets. In the present study, the fact that the FM and SBM30-VD-Gln show the most similar expression at W5 could be interpreted as a good sign, considering the optimal growth performance of fish fed the SBM30-VD-Gln diet. As discussed in Papers II and IV, a rise in Mylk activity would require increased Ca uptake by the cell. Our results could be showing a downregulation resulting from inability to keep up with the enteritis-induced hypercontractility. If Ca levels are so limiting that cellular contraction cannot keep up, synthesis of Mylk could decrease as a means of saving energy. The *mylk* transcriptional level in the SBM40 group is significantly lower than in the FM group at both W5 and W10. Since the SBM40 treatment represents the highest level of stress, it could be indicating that at these stages (i.e., W5 and W10 of SBM40 exposure and 12 h postprandially), Mylk activity has diminished, and that overexpression can be captured at earlier time points either after shorter exposure to SBM or less time after feeding. The study carried out by Liu et al. (2018) also reported higher expression levels of *mylk* in fish fed a FM diet compared to those fed a SBM and a Gln supplemented SBM diet after 8 weeks, which is in line with what was observed in the current study. However, at 4 and 12 weeks, these authors reported higher *mylk* expression in the SBM group compared to both FM and their SBM-Gln supplemented diet (Liu et al., 2018).

The *vdr* showed the highest expression in the control group at W5, being significantly higher than the SBM40-VD-Gln, although all other treatments also showed lower levels compared to the FM group (non-significant). This was except for the SBM30-VD-Gln which appeared to have a slightly higher expression level, being the closest to the FM group. At W10, significantly lower expressions were detected in the SBM40, SBM30-VD-Gln, and SBM40-VD-Gln groups, and overall expression was lower in all SBM dietary groups compared to the FM and expression levels in the SBM30-VD-Gln decreased from W5 to W10. When evaluating these results, it is crucial to consider possible desensitization mechanisms involved in Vdr regulation. Indeed, catabolism of $1,25D_3$ to calcitroic acid is controlled by Vdr as widely demonstrated in mammals (Jones and Pike, 2019) as well as fish (Berntssen et al., 2015; Lock et al., 2010), consequently desensitizing the target cell to excessive $1,25D_3$ stimulation. In turn, the Vdr conformation can suffer changes depending on the VD analogue binding to it, and consequently impact Vdr signalling (Jones and Pike, 2019). Since the VD dietary levels used in the present study were considerably higher than what is normal for salmonids, a desensitization phenomenon as explained above could have resulted, however our gene expression

results did not point in that direction. Another factor to take into account in *vdr* gene expression analysis is the Vdr long half-life, as it is present in the cell cytosol in its inactive form (Lodish et al., 2012). This implies that, for instance, higher expression levels would not always necessarily correlate with increased Vdr activity. Furthermore, as observed in intestinal mammalian cells, neither Vdr abundance or its nuclear/cytosolic localization was exclusively affected by ligand treatment (Peleg and Nguyen, 2010). It is interesting to mention, however, mitogenic signalling could also be involved in Vdr downregulation, in a process related to protein kinase C (PKC) activity (Krishnan and Feldman, 1991). Proliferating cell nuclear antigen (Pcna) has been identified as a SBMIE marker, showing significant upregulation in Atlantic salmon fed SBM diets (Krogdahl et al., 2015), while PKC plays a role both in Mylk activation and as well as inflammation during colitis (González-Mariscal et al., 2008; Nagahama et al., 2008). The interplay between Vdr, PKC and Pcna in the context of SBMIE could be a target of study in future investigations. All in all, the data gathered in the present study is insufficient to make conclusions on *vdr* expression patterns and/or desensitization during SBMIE or high dietary VD.

Conclusion

The results presented herein demonstrate that a supplementation level of 2.0% Gln helps improve growth performance and distal intestine histopathology in rainbow trout fed SBM30 diets. However, it appears that, although helpful, this supplementation level might not be sufficient for SBM40 diets. The data are inconclusive with regards to the possible effects of high dietary VD on intestinal health and potential Ca toxicity, however, the growing body of literature supporting the role of VD in intestinal health encourages the performance of more studies to examine the effects of dietary VD in SBMIE. This is especially true in salmonids as they appear to be VD tolerant. Nonetheless, variation among species, especially with regards to possible toxic effects should be carefully studied and considered (as gilthead seabream study showed hypervitaminosis D signs and the Atlantic salmon fry revealed higher VD content in whole body analysis.

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Table 6.1. Dietary formulation of the experimental diets expressed as percentages (%).

Ingredients	FM	SBM30	SBM40	SBM30-VD	SBM40-VD	SBM30-VD-Gln	SBM40-VD-Gln
Fish meal	25	10	10	10	10	10	10
Soybean meal	0	30	40	30	40	30	40
Canola meal	13.5	2.25	0.15	2.25	0.15	2.25	0.15
Wheat gluten meal	3.5	2.8	1	2.8	1	2.8	1
Corn protein concentrate	4.3	7.2	6	7.2	6	7.2	6
Blood meal	8.5	7.9	9	7.9	9	7.9	9
Wheat flour	19.8	11.5	10.5	11.5	10.5	11.5	10.5
Poultry meal	7	6	1	6	1	6	1
α-cellulose		2.01	2.01	2	2		
Fish oil	15.5	16.7	16.65	16.7	16.65	16.7	16.65
Dicalcium phosphate	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Choline chloride (60%)	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Vitamin premix	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Trace mineral mixture	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin C, Stay C-35)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Lysine	0	0.55	0.5	0.55	0.5	0.55	0.5
Methionine	0	0.26	0.3	0.26	0.3	0.26	0.3
Glutamine						2	2
Vitamin D (mg Kg^{-1})	0			50	50	50	50
Analysed composition (± SD)							
Crude protein (%)	41.49 ± 0.03	41.17 ± 0.17	40.16 ± 0.69	40.28 ± 0.20	40.36 ± 0.04	42.68 ± 0.59	43.22 ± 0.46

Table 6.1 cont.	19.21 ± 0.03	18.77 ± 0.0	17.87 ± 0.00	19.00 ± 0.19	17.84 ± 0.00	18.28 ± 0.00	18.05 ± 0.00
Lipid (%)							
Crude Fiber + N free extract (%)	25.28	27.26	29.53	27.70	28.35	25.75	26.60
Ash (%)	8.30 ± 0.15	6.62 ± 0.09	6.26 ± 0.18	6.65 ± 0.20	6.31 ± 0.15	6.69 ± 0.11	6.18 ± 0.12
Moisture	5.71 ± 0.09	6.18 ± 0.06	6.17 ± 0.01	6.22 ± 0.02	7.15 ± 0.08	6.61 ± 0.00	5.95 ± 0.12
Gross energy (MJ Kg^{-1})	21.73	21.82	21.62	21.83	21.45	21.72	21.91

FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
*Calculated by difference: 100 - (%protein + %fat + %ash + %moisture) (i.e., N-free extractives + crude fibre).
**Gross energy was calculated using combustion values for protein 23.6 MJ kg−1, lipid 39.5 MJ Kg-1 and carbohydrate 17.2 MJ Kg-1, respectively.

Table 6.2. Proximate amino acid composition of each experimental diet (g/100 g feed).

Essential AA	FM	SBM30	SBM40	SBM30-VD	SBM40-VD	SBM30-VD-Gln	SBM40-VD-Gln
Lysine	2.69 ± 0.06	2.92 ± 0.04	2.77 ± 0.00	2.80 ± 0.13	2.70 ± 0.04	2.90 ± 0.14	2.78 ± 0.08
Methionine	0.76 ± 0.04	0.73 ± 0.04	0.77 ± 0.01	0.78 ± 0.06	0.82 ± 0.03	0.81 ± 0.06	0.76 ± 0.04
Histidine	1.28 ± 0.11	1.39 ± 0.16	1.47 ± 0.01	1.46 ± 0.00	1.81 ± 0.01	1.49 ± 0.23	1.46 ± 0.05
Isoleucine	1.62 ± 0.05	1.62 ± 0.04	1.65 ± 0.02	1.60 ± 0.01	1.67 ± 0.04	1.59 ± 0.03	1.64 ± 0.00
Leucine	3.39 ± 0.04	3.48 ± 0.11	3.49 ± 0.06	3.48 ± 0.10	3.55 ± 0.11	3.47 ± 0.06	3.43 ± 0.03
Phenylanine	1.82 ± 0.13	2.01 ± 0.15	2.23 ± 0.03	2.12 ± 0.16	2.49 ± 0.11	2.09 ± 0.31	2.20 ± 0.02
Threonine	1.71 ± 0.08	1.66 ± 0.08	1.73 ± 0.06	1.70 ± 0.04	1.85 ± 0.06	1.70 ± 0.11	1.70 ± 0.03
Valine	1.99 ± 0.06	1.91 ± 0.09	1.94 ± 0.03	1.88 ± 0.02	1.97 ± 0.06	1.87 ± 0.03	1.91 ± 0.00
Arginine	2.00 ± 0.13	2.11 ± 0.13	2.25 ± 0.04	2.17 ± 0.15	2.45 ± 0.11	2.15 ± 0.19	2.26 ± 0.02
Non Essential AA							
Taurine	0.11 ± 0.01	0.07 ± 0	0.06 ± 0.01	0.09 ± 0	0.08 ± 0	0.07 ± 0.01	0.06 ± 0
Tyrosine	1.14 ± 0.06	1.22 ± 0.09	1.34 ± 0.04	1.30 ± 0.06	1.54 ± 0.08	1.33 ± 0.20	1.35 ± 0.01
Cysteine	0.24 ± 0.01	0.26 ± 0.01	0.27 ± 0.00	0.28 ± 0.01	0.32 ± 0	0.28 ± 0.04	0.26 ± 0.01
Hydroxiproline	0.31 ± 0.01	0.2 ± 0.01	0.12 ± 0	0.25 ± 0.05	0.15 ± 0.01	0.2 ± 0.01	0.12 ± 0.01
Serine	1.82 ± 0.06	2.03 ± 0.2	2.00 ± 0.04	2.02 ± 0.02	2.14 ± 0.07	2.03 ± 0.13	2.04 ± 0.04
Alanine	2.33 ± 0.03	2.17 ± 0.09	2.06 ± 0.03	2.15 ± 0.11	2.09 ± 0.07	2.87 ± 0.01	2.75 ± 0.05
Proline	2.38 ± 0.00	2.32 ± 0.08	2.10 ± 0.01	2.35 ± 0.06	2.13 ± 0.01	2.34 ± 0.04	2.09 ± 0.02
Glutamic Acid	6.87 ± 0.18	6.86 ± 0.01	6.40 ± 0.05	6.77 ± 0.1	6.40 ± 0.17	8.19 ± 0.07	7.64 ± 0.11
Aspartic Acid	3.22 ± 0.06	3.41 ± 0.03	3.41 ± 0.04	3.32 ± 0.08	3.47 ± 0.12	3.40 ± 0.05	3.47 ± 0.08
Glycine	2.10 ± 0.09	1.92 ± 0.13	1.83 ± 0.03	1.98 ± 0.09	1.97 ± 0.01	1.94 ± 0.11	1.83 ± 0.01

FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.

Table 6.3. Primer sequences used for each gene of interest in qPCR analyses.

Gene	Primer sequence (5' --> 3')	Accession no.	Reference	Efficiency (%)
cypa	F: GCAAGTCCATCTACGGCAAT R: TGCTAGCGATGATGTTGAGG	MN722644.1		110
tnfa	F: CAAGAGTTTGAACCTCATTCAG R: GCTGCTGCCGCACATAAAG		Bridle et al. (2006)	110.0
il1b	F: ACCGAGTTCAAGGACAAGGA R: CATTCATCAGGACCCAGCAC	AJ223954		95.0
il10	F: CGACTTTAAATCTCCCATCGAC R: GCATTGGACGATCTCTTTCTTC		Pérez-Sánchez et al. (2011)	95.3
vdr	F: AGATGAGGAGGTGCAGAGGA R: GCGTGAGAAGTCGGAGTAGG	AY526906.1		98.0
mylk	F: GCACTACATGCAGCAGATCG R: AGCCACAAACTCTGGTGTCC	XM_021557724.2		93.8
cldn12	F: AAAACGCCAAGAACATCAGC R: GACATGCCTGCCATACACAG	BK007967.1		105.8
ocln	F: CGGAATCCAATGGCTACG R: AAGATCCCCACACAGAGCAC	GQ476574.1		108.6

cypa: cyclophilin A, peptidyl-prolyl cis-trans isomerase A; ***tnfa***: tumor necrosis factor alpha; ***il1b***: interleukin 1- beta; ***il10***: interleukin 10; ***vdr***: vitamin D receptor; ***mylk***: myosin light chain kinase; ***cldn12***: claudin-12; ***ocln***: occludin.

Table 6.4. Growth performance parameters calculated at W5 and W10 of the feeding trial.

	FM	SBM30	SBM40	SBM30-VD	SBM40-VD	SBM30-VD-Gln	SBM40-VD-Gln	
				W5				*p value*
IW (g)	9.61 ± 0.24	9.76 ± 0.10	9.63 ± 0.18	9.07 ± 0.23	8.91 ± 0.09	9.64 ± 0.00	9.64 ± 0.28	> 0.05
WG (g)	32.85 ± 1.74	33.60 ± 1.13	29.25 ± 2.47	34.03 ± 2.21	32.20 ± 0.09	38.03 ± 2.84	30.88 ± 0.53	> 0.05
SGR (% day $^{-1}$)	4.24 ± 0.11	4.26 ± 0.09	3.99 ± 0.17	4.45 ± 0.18	4.37 ± 0.02	4.56 ± 0.18	4.10 ± 0.09	> 0.05
FI (g fish day^{-1})	1.05 ± 0.05	1.01 ± 0.03	1.02 ± 0.03	1.01 ± 0.01	1.07 ± 0.03	1.14 ± 0.02	1.07 ± 0.04	> 0.05
FCR	1.12 ± 0.01	1.06 ± 0.06	1.23 ± 0.06	1.05 ± 0.07	1.16 ± 0.03	1.06 ± 0.07	1.21 ± 0.06	> 0.05
HSI	1.72 ± 0.04	1.42 ± 0.05	1.58 ± 0.08	1.56 ± 0.16	1.47 ± 0.13	1.61 ± 0.06	1.53 ± 0.47	> 0.05
K	1.43 ± 0.01	1.41 ± 0.01	1.37± 0.00	1.41 ± 0.04	1.39 ± 0.01	1.45 ± 0.01	1.39 ± 0.04	> 0.05
				W10				
WG (g)	106.47 ± 0.98^{ab}	102.43 ± 5.26^{bc}	87.87 ± 5.71^{c}	86.96 ± 2.45^{c}	97.71 ± 2.87^{bc}	119.73 ± 1.04^{a}	104.76 ± 1.48^{ab}	< 0.001
SGR (% day $^{-1}$)	3.65 ± 0.02^{ab}	3.47 ± 0.06^{abc}	3.48 ± 0.06^{c}	3.44 ± 0.11^{c}	3.55 ± 0.04^{bc}	3.68 ± 0.10^{a}	3.62 ± 0.05^{ab}	< 0.001
FI (g fish day $^{-1}$)	2.27 ± 0.07	1.98 ± 0.07	2.20 ± 0.10	2.07 ± 0.02	2.16 ± 0.09	2.37 ± 0.07	2.17 ± 0.11	0.07
FCR	0.83 ± 0.02^{ab}	0.78 ± 0.01^{a}	0.88 ± 0.03^{b}	0.84 ± 0.01^{ab}	0.89 ± 0.03^{b}	0.78 ± 0.01^{a}	0.82 ± 0.01^{ab}	< 0.01
HSI	1.53 ± 0.03	1.36 ± 0.01	1.35 ± 0.02	1.44 ± 0.11	1.34 ± 0.01	1.40 ± 0.09	1.38 ± 0.04	> 0.05
K	1.59 ± 0.01	1.48 ± 0.02	1.46 ± 0.04	1.48 ± 0.04	1.44 ± 0.02	1.50 ± 0.00	1.46 ± 0.03	> 0.05

Values are calculated as means ± SD. W5: $n = 6$; W10: $n = 9$. Different superscript letters indicate statistical significance as determined by Tukey's pairwise comparison test at $p \leq 0.05$.
FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
W5: week 5; **W10**: week 10.

Table 6.5. Whole body proximate composition (% wet weight) of rainbow trout fed seven experimental diets at W10.

	Initial	FM	SBM30	SBM40	SBM30-VD	SBM40-VD	SBM30-VD-Gln	SBM40-VD-Gln
Moisture	70.40 ± 0.23	64.55 ± 0.52	64.04 ± 0.86	64.94 ± 1.07	62.95 ± 0.52	64.54 ± 0.62	63.38 ± 0.23	64.41 ± 0.20
Ash	3.26 ± 0.04	2.62 ± 0.05	2.41 ± 0.09	2.39 ± 0.08	2.52 ± 0.03	2.63 ± 0.09	2.49 ± 0.01	2.51 ± 0.02
Protein	18.30 ± 0.15	18.92 ± 0.04	18.36 ± 0.27	18.23 ± 0.18	18.88 ± 0.06	18.89 ± 0.13	18.20 ± 0.23	18.58 ± 0.11
Lipid	6.75 ± 0.11	12.54 ± 0.60	13.52 ± 0.77	12.80 ± 0.90	13.88 ± 0.50	12.11 ± 0.86	13.92 ± 0.08	13.00 ± 0.11
	PR (%)	30.68 ± 0.42	30.82 ± 0.74	27.32 ± 1.93	27.33 ± 1.03	29.37 ± 1.15	31.10 ± 1.66	29.80 ± 0.95
	LR (%)	43.76 ± 2.24	52.40 ± 4.00	44.00 ± 4.28	45.10 ± 1.87	41.38 ± 4.57	52.07 ± 2.94	49.87 ± 1.34

Values are calculated as means ± SD.
FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
W10: week 10.

Table 6.6. Calcium (Ca2+) concentrations measured in kidney, vertebra, and muscle (mg g-1 dry weight); and serum (nmol mL-1) from pooled samples from fish sampled at W10 of the feeding trial.

Dietary group	Kidney	Vertebra	Muscle	Serum
FM	1.73 ± 0.52	15. 89 ± 0.91	1.17 ± 0.23	3.20 ± 0.06
SBM30	0.59 ± 0.13	16.13 ± 0.32	1.37 ± 0.18	3.10 ± 0.12
SBM40	0.80 ± 0.23	16.41 ± 0.63	1.24 ± 0.38	3.40 ± 0.00
SBM30-VD	0.43 ± 0.08	15.87 ± 0.42	1.21 ± 0.42	3.20 ± 0.10
SBM40-VD	0.43 ± 0.02	16.17 ± 0.45	0.99 ± 0.12	3.20 ± 0.17
SBM30-VD-Gln	0.50 ± 0.13	15.28 ± 0.40	1.28 ± 0.31	3.33 ± 0.27
SBM40-VD-Gln	0.67 ± 0.12	15.33 ± 0.57	1.20 ± 0.28	3.30 ± 0.36

Values are calculated as means ± SD (n = 9). Different superscript letters within columns indicate statistical significance as determined by Tukey's pairwise comparison test at $p \leq 0.05$.
FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
W10: week 10.

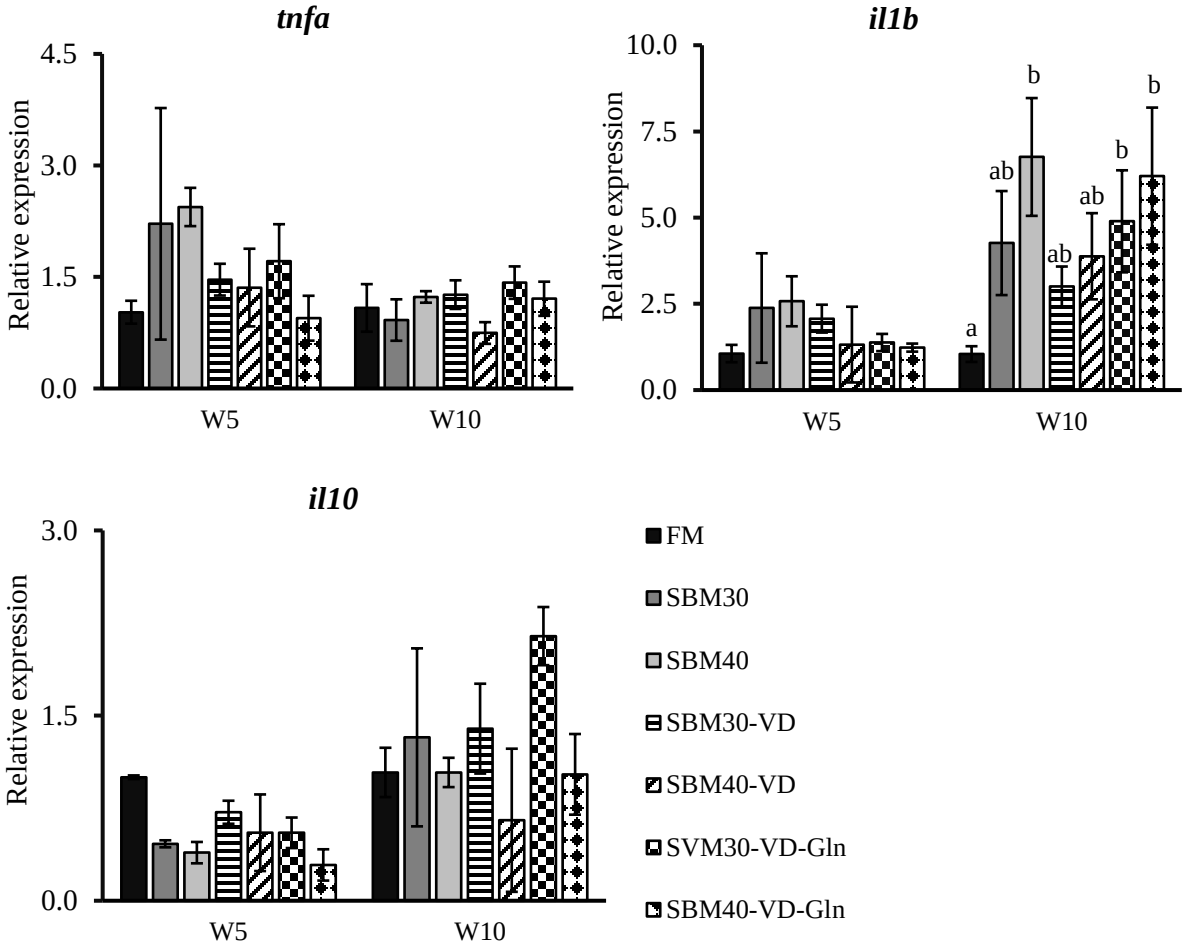

Figure 6.1. Distal intestine relative gene expression of the cytokines Tnfa, Il1b, and Il10 of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9).
Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W5 and W10) of the feeding trial. Different letters represent significant differences between dietary treatments at a given time point as determined by Tukey's pairwise comparison test at $p \leq 0.05$.
tnfa*:** tumour necrosis factor alpha; ***il1b: interleukin one-beta; ***il10***: interleukin ten.
FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
W5: week 5; **W10**: week 10.

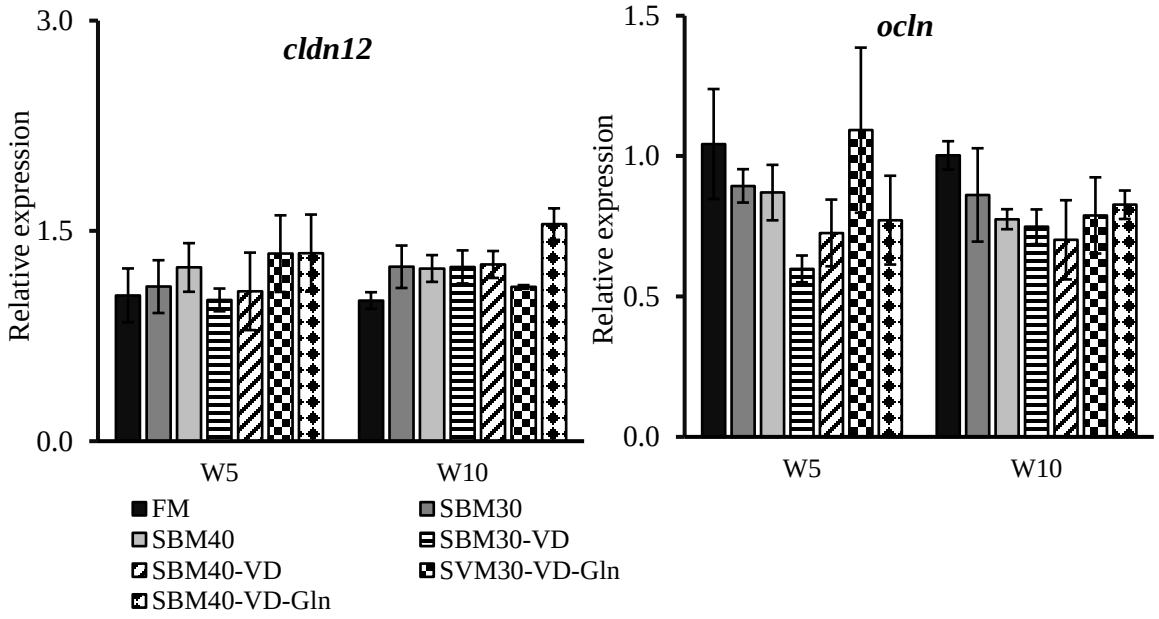

Figure 6.2. Distal intestine relative gene expression of the tight junction proteins Cldn12 and Ocln of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9). Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W5 and W10) of the feeding trial.
Cldn-12: claudin twelve; **Occl**: occludin.
FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.
W5: week 5; **W10**: week 10.

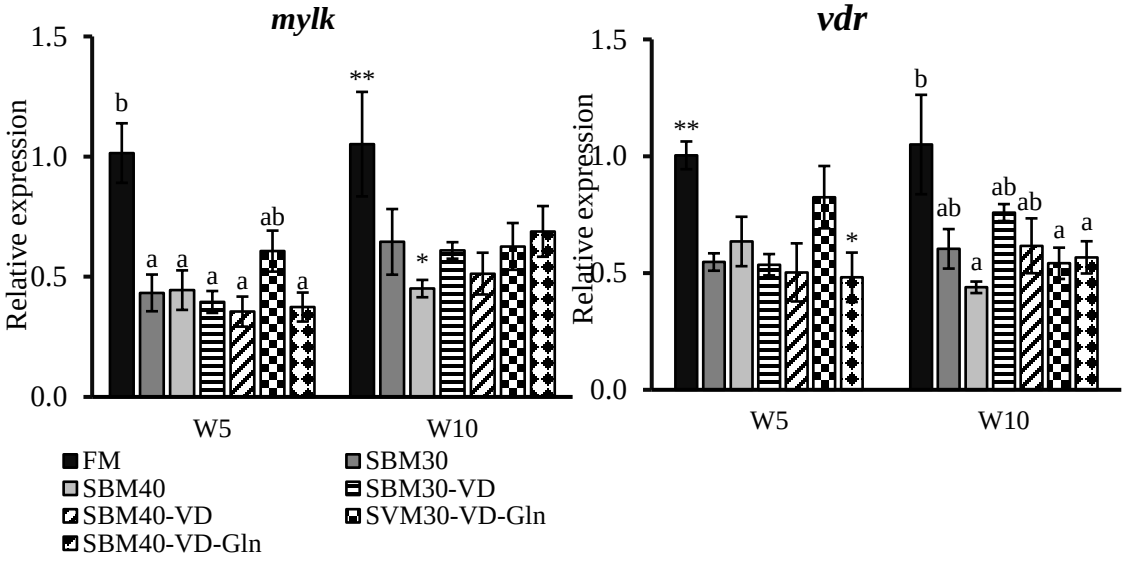

Figure 6.3. Distal intestine relative gene expression of Mylk and Vdr of rainbow trout fed the experimental diets. Values are presented as means ± SD (*n* = 9).

Data is expressed as ΔC_t relative to the FM group (control) and normalized to the level of *cypa* reference gene (which was not affected by diet). Gene expressions were recorded at each sampling point (W5 and W10) of the feeding trial. Different letters/asterisks represent significant differences between dietary treatments at a given time point as determined by Tukey's pairwise comparison test at $p \leq 0.05$.

mylk*:** myosin light chain kinase; ***vdr: vitamin D receptor.

FM: fish meal; **SBM30**: soybean meal 30% inclusion; **SBM40**: soybean meal 40% inclusion; **SBM30-VD**: SBM30 + 50 mg Kg^{-1} vitamin D; **SBM40-VD**: SBM40 + 50 mg Kg^{-1} vitamin D; **SBM30-VD-Gln**: SBM30-VD + 2.0% glutamine; **SBM40-VD-Gln**: SBM40-VD +2.0% glutamine.

W5: week 5; **W10**: week 10.

Chapter 7: Conclusion

Synthesis of Research Findings

This research aimed to identify effective feed additives that could mitigate detrimental effects derived from high inclusion level SBM diets fed to carnivorous fish such as rainbow trout, and to evaluate whether those benefits could be maintained in the long-term. Based on the data collected, it can be concluded that both Gln and BAs could serve as protective agents against SBM related detrimental effects at different physiological levels throughout the grow-out period. In case of high VD dose as dietary additive, further research would be required to draw a solid conclusion on its SBMIE mitigating capacity. In addition, the research presented in this dissertation had the objective to further elucidate key markers involved in the pathogenesis of SBMIE. Although the data gathered in this work was inconclusive for some of the presumed critical players such as the inflammatory cytokine TNF-α or the enzyme MLCK, observations remained rather uniform among the three feeding trials that were carried out. This uniformity can set the basis for future experiment design and stating hypotheses. Results obtained from this investigation on other potential markers could serve to estimate the impact of high levels of SBM in the diets in this species. The principal remarks from each trial will be summarized for each of the nutritional additives that have been evaluated.

Glutamine experiment

The role of 2.0% dietary Gln on SBMIE mitigation was analyzed in two commercial strains of rainbow trout for a period of thirty weeks. Firstly, growth data collected in this experiment confirmed the existence of adaptations of certain strains to diets containing a 30% SBM inclusion level, although differences between strains were only significant for FCR and not for WG or SGR. It became apparent that strain R thrived when fed the SBM diet, while strain T did so when fed the FM diet and both strains performed similarly when fed a SBM-Gln diet. This probably drove the interactions observed. Importantly, Gln supplementation did not lead to any growth improvement at any time point. Because growth data was collected on individual fish for the first four sampling points (W6, 12, 18 and 24), one could argue that if bulk weights had been taken, more pronounced differences could have been found. However, WG and SGR data were always slightly lower than the best performing group in each strain (i.e., the SBM group in strain R and the FM group in strain T).

Strikingly, even though strain R performed significantly better when fed the SBM diet, the histopathology of the distal intestine showed a similar level of SBM-related deleterious effects for both strains. These results came as a surprise, since a better intestinal condition would have been expected in strain R, in relation to enhanced growth performance. Indeed, it has been described

previously for rainbow trout after twelve weeks fed high levels of SBM, that a selected strain would show improved growth accompanied by significantly lower cumulative histological scores (Abernathy et al., 2019). However, comparisons between these cases must be made carefully. Firstly, strain R used in this research had not been previously identified as a selected strain. Secondly, the plant-based diet used in that study had a higher inclusion level of soy protein concentrate (SPC), reducing the amount of ANFs present in the diet (Francis et al., 2001; Zhou et al., 2018), while only SBM was used in the work presented here.

Hence, this research raises the question of which type of adaptations lead to superior performance in SBM diets and which life history conditions could determine that. Importantly, however, even if strain R shows optimal growth in a SBM-based formulation, the compromised intestinal morphology which shows significant signs of inflammation, could come in association with complications down the road when translated to intensive farm conditions, increasing susceptibility to disease outbreak.

Bile acids

The supplementation with 1.5% BAs to 30% and 40% inclusion level SBM diets appeared to overall exert beneficial effects in a SBMIE context and be exempt of cytotoxicity for a period of eighteen weeks. Although BAs did not influence growth significantly throughout the trial, slightly higher growth was seen, especially comparing the SBM40 and SBM40-BA diets, and this also translated into (unsignificant) higher lipid content in final fillet at W18, as well as percentage LR, both similar to the FM group. Instead, growth performance was barely affected by BAs in 30% SBM diets, which was in line with the absence of effect of Gln on growth observed in the previous trial. This could reveal a degree of adaptation of these commercial strains to this level of SBM.

In the BAs experiment, it became clear that raising the SBM inclusion level from 30% to 40% had more significant effects in bile and cholesterol physiology, with a 32.5% and 27.9% reduction in plasma cholesterol in the SBM40 group compared to FM by W12 and W18 respectively; as well as a significant reduction of 11.4% in plasma BA at the initial stages (W6). Furthermore, the results obtained in this experiment confirmed a beneficial effect of the dietary BAs in reverting these concentrations to normal levels.

One of the objectives of this experiment was to determine whether the protective action of BAs could be maintained long term without cytotoxic effects. Although signs of cytotoxicity were dismissed, the results provide insights into the fact that there could be a tendency to diminish mitigating capacity after W12 of continued exposure. Said loss of effectiveness of BAs was reflected on equal decrease in villi length in fish fed either SBM40 and SBM40-BA by W18; slight increase in

cumulative histological scores both in liver and distal intestine from W12 to W18 in the SBM40-BA diet, marked by an increase in the mucosal fold fusion in the intestinal villi and the degree of necrosis and hemorrhage observed in the liver. Additionally, the apparent loss of effect of dietary BA in SBM40-BA diets suggests that increasing the supplementation level could be necessary in >30% inclusion level SBM-based diets. These observations could help make informed feed management decisions in an aquaculture farm context, such as determining which points during grow-out periods it would be more advisable to offer a BA-supplemented diet.

The use of dietary BAs in SBM shows promise for their role both in SBMIE mitigation as well as maintaining liver health and plasma cholesterol levels. Moreover, there are further areas of study to optimize their use in diets. Knowing the role of intestinal microbiota of BA transformation to form unconjugated and secondary BAs opens the door to determining BA profiles in the intestine and identifying unbalanced ratios between primary and secondary bile as well as deficiencies. That information could ultimately be used to tailor BA supplements for aquaculture or to determine which bacterial strains would be beneficial as probiotics for their involvement in the synthesis of specific types of BAs.

Vitamin D

This experiment aimed to investigate the potential effects of high level (50 mg Kg^{-1}) dietary VD on SBMIE mitigation as well as its combination with Gln in a medium-term experiment of ten weeks, using 30% and 40% SBM diets. The incorrect mixing of VD with the other ingredients prevented this study from answering the initial research questions. However, positive observations were made with regards to the superior growth performance that displayed by fish fed a 30% level SBM diet supplemented with 2.0% Gln (and VD). These fish showed significantly higher WG in comparison with the FM and SBM30 counterparts; and even lower FCR as compared to the FM group. This, however, contrasts with the little effect that 2.0% Gln had on growth performance in the thirty-week feeding trial. This observation could be pointing out that the water quality parameters, which were optimal in the Vitamin D trial and just on the range limit on the other two trials, could lead to the lack of effects of the dietary supplements on growth performance.

Considering the histology examinations in the distal intestine it appears that the lowest scores were obtained for the diets that had the VD + Gln supplement, aside from the FM group, further suggesting that the diets were probably void of VD, as not much improvement was seen in the SBM diets supplemented solely with VD. Nonetheless, these results should not discourage further attention to VD as a suitable candidate for SBMIE mitigation in rainbow trout.

Gene expression of SBMIE markers

Gene expression of key inflammatory markers was, in many cases, opposite to what would have been expected, with lacking a significant increase in the expression of pro-inflammatory cytokines such as Tnfa, Il8 or the anti-inflammatory Il10 in the BA and VD experiments. This was not the case, however, in the Gln experiment, where higher mRNA levels were detected for *tnfa* and *il8* of rainbow trout from strain T fed a SBM diet after thirty and twenty-four weeks, respectively. This could be indicative of inferior long-term response to a 30% SBM diet as compared to strain R which seemed to show less inflammatory signaling. In the BA experiment, high variability and lack of significant differences between dietary treatments made it difficult to pinpoint a clear marker for SBMIE. The absence of a significant upregulation of gene expression levels of pro-inflammatory markers could be a consequence of depletion of cytokine producing cells, in an environment of prolonged stress (Klenerman et al., 2002). The pro-inflammatory cytokine Il1b, which was only examined in the VD experiment, did show significantly higher mRNA levels in fish fed a 40% inclusion level SBM diet after ten weeks. Because the time of sampling was between 12-18 h postprandially for all three experiments, it could be that this specific cytokine maintains expression for longer time periods after dietary exposure. Other gene expression analysis done in a SBMIE background have attributed the low expression to the degree of damage in the distal intestine (Bakke-McKellep et al., 2007; Fuentes-Quesada et al., 2018; Lilleeng et al., 2009).

An important limitation of the methodology used for SBMIE marker identification in these experiments is the reliance on transcript level measurements. Indeed, gene expression analyses only provide an initial sign of the possible implication of a given marker being studied. However, mRNA abundance does not have a strong correlation with protein cellular concentration, and post-transcriptional reactions as well as translation of mRNA is also subject to regulatory mechanisms that may or may not lead to the synthesis of a final, functional product (Vogel and Marcotte, 2012). Instead, protein expression analysis could yield a more accurate representation, although protein abundance regulation and half-life of target protein would be important factors to consider (Vogel and Marcotte, 2012). Another consideration would be related to the use of Sybr green dyes for gene expression analysis. Since this is a type of dye that binds DNA non-specifically, there is an associated risk of inaccuracy during mRNA quantification for a given product. This is further complicated in teleost fish, which have undergone several rounds of whole-genome duplication events, increasing genome complexity; and especially salmonids, which suffered a lineage-specific genome duplication. Consequently, a gene of interest may have several paralogues which may have been subjected to, for instance, neo-functionalization, further increasing the likelihood of reaching inaccurate results when

using non-specific dyes (Panserat et al., 2019). Using fluorescent oligonucleotide probes that bind specifically to the gene of interest would render a much higher specificity of gene expression assays.

In the future, research aiming to elucidate specific mechanisms underlying SBMIE could consider sampling points that cover the earlier postprandial scenario to capture what is occurring at the starting point, as well as in vitro studies that allow for even more controlled experimental conditions. Once specific markers are well identified, future research would be required to determine whether SBMIE markers can be detected using non-lethal ways. This will allow in-farm monitoring of fish health and SBMIE status and adjust feeding schemes ensuring fish welfare.

Finally, from an aquaculture perspective, where farmers strive to obtain maximum benefits while ensuring sustainable practices and food security, the use of additives such as the ones examined in the research developed here will play a pivotal role.

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