Investigación e Innovación en Nutrición Acuícola

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Comparative Analysis of Soybean Meal Effects in Commercial Strains of Rainbow Trout *Oncorhynchus mykiss*

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Summary

A strain of rainbow trout (CX strain) at the Hagerman Fish Culture Experiment Station has been selected for growth on a plant-based diet for ten generations. We Compared fish from the CX strain that were age (CXA) and size (CXS) matched to three commonly available commercial strains selected for growth (RBT1, RBT2, RBT3). We compared differences in overall growth, oxidative stress and intestinal inflammation between fish fed a fishmeal (FM) or soybean-meal (SBM) diet for 12 weeks. Fish of each strain were randomly assigned to FM or SBM diet groups and fed daily to satiation. Tissues from each experimental group were sampled every four weeks to assess gene expression and growth parameters. Both CX strains had higher growth rates compared with similar feed consumption among all groups. Expression of intestinal and hepatic biomarkers for oxidative stress varied between liver and intestine. Expression varied between CXA and CXS fish despite being from the same strain indicating size at age affects gene expression and care should be taken when comparing different strains with different growth rates. Expression of calcium binding protein S100I2 in the intestine was elevated initially at 4 weeks, diminished at 8 weeks, then elevated again by 12 weeks. This pattern was also observed in intestinal SOD expression and GPx expression in the liver. Overall, these results provide further information on current commercial strains of rainbow trout to help improve the utilization on plant protein sources in their diets.

Keywords: *Enteritis, Rainbow trout, Salmonids, soybean meal, S100 gene*

1. Introduction

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

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

while trying to avoid impairing growth performance and survival in rainbow trout (Boucher *et al*., 2012; Callet *et al.*, 2017; Cheng *et al*., 2003).

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

In this study, we compared three current commercial strains of rainbow trout to the CX strain of rainbow trout in a 12-week feeding trial. We wanted to assess how different strains of rainbow trout might show differences in resistance to SMIE and differences in utilization of plant proteins when compared to a fishmeal diet. To understand the effect of this dietary alteration on oxidative stress, expression levels of three genes (SOD, superoxide dismutase; glutathione peroxidase, GPx; and catalase) were compared in the liver and distal intestine in each RBT strain during each time point. We also examined calcium-binding protein genes expressed in the intestine (S100I2) and in the liver (S100V2) as inflammatory markers.

2. Material and Methods

2.1. Diets

Two experimental diets were formulated to provide 40% digestible protein and 20% crude lipid containing either fishmeal (FM diet) or soybean meal (PM diet) as the primary protein source (Table 1). Both diets were balanced for digestible protein and supplemented with essential amino acids to reach or exceed known nutrient requirements (NRC *et al*., 2011).

Table 1. Formulations and proximate composition of control and experimental diets used in a 12 week, comparative feeding trial of rainbow trout strains.

Ingredient	FM	PM
Menhaden Fish Meal	30.00	0.00
Soybean meal	0.00	40.00
Chicken meal	11.50	11.50
Corn protein concentrate	11.50	11.50
Menhaden fish oil	14.72	16.35
Wheat gluten meal	0.16	1.30
Wheat flour	23.29	5.54
Lecithin	1.00	1.00
Stay-C 35	0.15	0.15
Vitamin premix ARS 702	1.00	1.00
TM ARS 640	0.10	0.10
NaCl	0.28	0.28
Magnesium Oxide	0.06	0.06
Potassium chloride	0.56	0.56
Monocalcium phosphate	0.60	4.80
Choline chloride 50%	1.00	1.00
DL-Methionine	0.33	0.63
Lysine HCl	2.17	2.56
Threonine	0.40	0.49
Taurine	1.00	1.00
Yttrium oxide	0.10	0.10
Astaxanthin	0.08	0.08
TOTAL	100.00	100.00
Proximate analysis (analyzed)		
Protein (%DM)	46.7	48.0
Lipid (%DM)	18.7	17.6

2.2. Fish culture and feeding

Three, same age, commercial strains of rainbow trout (RBT1, RBT2, RBT3) and size-matched Hagerman selected strain rainbow trout (CXS; approximately 2 weeks younger) collectively averaging 6.8 ± 0.49 g were stocked separately into 140 L poly tanks along with age-matched Hagerman strain rainbow trout (CXA) averaging 7.87 ± 0.14 g. Fish from each strain were

randomly assigned to 4 replicate tanks (2 tanks/diet, 30 fish/tank) in a flow-through system with spring water inflow. The water temperature was maintained at 15 °C with a 14:10 light: dark cycle during the experiment. Tanks were randomly assigned the FM or PM diet and fed twice daily to apparent satiation, 6 days per week, for 12 weeks. All fish were counted and bulk weighed every 4 weeks.

2.3. Sample collection

Initially and at weeks 4, 8, and 12, five fish from each tank were euthanized with MS-222 (250 ppm, buffered to pH 7.4; Western Chemical Co., Ferndale, WA). Samples of distal intestine and liver (~100 mg) were removed from each fish and placed in 1 ml TRIzol® (ThermoFisher Scientific, Waltham, USA) and stored frozen at -80 °C until RNA was extracted for gene expression.

2.4. RNA extraction and cDNA synthesis

For gene expression analysis, total RNA from sample tissues were extracted in the TRIzol® reagent. The liver or distal intestine tissues were put in 2-mL round-bottom centrifuge tubes and homogenized in a bead mill (MixerMill 200, Retsch GmbH, Hann, Germany). After homogenization, tubes were centrifuged at 12,000 g at 4 °C for ten minutes and the supernatant transferred to new 1.5 mL tubes. Chloroform (200 ul) was added to each tube and vigorously shaken for 15 seconds before incubation at room temperature for ten minutes. Tubes were then centrifuged at 12,000 g at 4 °C for 15 minutes, and the supernatant transferred to a new 1.5 mL tube. Isopropanol (500 ul) was added and mixed well before incubation at -20 °C overnight. RNA was pelleted by centrifuging at 12,000 g at 4 °C for 10 minutes, then washed with cold 75% ethanol, and resuspended in nuclease-free water. RNA quantity and purity were examined spectrophotometrically using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA). RNA sample concentrations were adjusted with nuclease-free water to 100 nM, treated with DNase (DNase I, Invitrogen, Carlsbad, USA), and reverse transcribed into cDNA (High-Capacity cDNA Reverse Transcription kit, ThermoFisher Scientific, Waltham, USA) using the manufacturer's instructions. Resulting cDNAs were stored at -80 °C for further analysis.

2.5. Quantitative PCR and data analysis

All samples were run in duplicate using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Fast SYBR™ Green Master Mix (Life Technologies, Carlsbad, USA) was used according to the manufacturer's instructions along with 40nM of each primer and 10 ng of template cDNA. After amplification, a melt curve was used to check the specificity of the

qPCR product. A standard curve with a 7-point, fivefold dilution series was used with pooled total RNA from all samples. Expression efficiencies ranged from 97-103%. Primers for reference and target genes are shown in Table 2. Target genes were normalized against the reference gene RPS15 and quantified using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Growth parameters and gene expression were analyzed with one-way and two-way ANOVA using the "AOV" procedure in R Studio® version 1.2.1335 (R Studio, Inc, Boston, MA), with "Tukey HSD" *post-hoc* tests when significant differences were observed. Significance was defined at $\alpha < 0.05$ for all statistical tests.

Table 2. Primer sequences of rainbow trout genes used for qPCR in a 12 week, comparative feeding trial of rainbow trout strains.

Gene	Accession	Primers
SOD[1]	NM_001124329	*f. GGC ACG AGG GCA AGT AGG A*
		r. GCC TTT GAG CAC GCA AAC A
GPx[2]	AF281338	*f. CGC CCA CCC ACT GTT TGT*
		r. GCT CGT CGC TTG GGA ATG
S100I2	XM_021598338.1	*f. GCT TGG AGA GAT CAT GGG GAA AA*
		r. GCC ATC TGA GTT AGC GTC CA
S100V2	XM_021572132.1	*f. TTA CGA CTG GAG CGT CAG A*
		r. CCT CCA GAA GTG ATT GAA GGT G
Catalase	XM_021557350.2	*f. GGC TTT GCA GTT AAG TTC TAC*
		r. AGC ATT GCG TCC CTG ATA AA
RPS-15	NM_001165174.2	*f. ACA GAG GTG TGG ACC TGG AC*
		r. AGG CCA CGG TTA AGT CTC CT

[1] SOD, superoxide dismutase; [2] GPx, glutathione peroxidase

3. Results

3.1. Growth performance

On the FM diet, CXA fish averaged the highest final weight (Table 3). On PM diet, both CXA and CXS fish showed higher final weight compared to the commercial strains, and RBT3 showed significantly lower final mass despite a greater feed intake. Although not significant, the CXA fish fed both the FM and PM diets had a lower FCR compared to the other strains. The survival rate was between 0.76-0.93 ± 0.02 % with no significant difference among strain and diet.

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

Diet	Strain	Initial Mass (g)	Final Mass (g)	Weight Gain (%)	SGR[1]	Feed Intake[2]	FCR[3]
Fishmeal	CXS	7.47	128.83^{b}	1624^{b}	3.37^{b}	1.71	0.96
	CXA	7.87	194.95^{a}	2377^{a}	3.80^{a}	1.46	0.76
	RBT1	4.88	131.50^{b}	2592^{a}	3.91^{a}	1.70	0.80
	RBT2	7.25	137.66^{b}	1798^{b}	3.50^{b}	1.61	0.86
	RBT3	7.27	137.54^{b}	1790^{b}	3.49^{b}	1.74	0.85
Plant meal	CXS	7.47	152.38^{x}	1939^{xy}	3.58^{x}	1.73^{xy}	0.91
	CXA	7.87	155.09^{x}	1870^{yz}	3.54^{xy}	1.52^{xy}	0.76
	RBT1	4.88	120.28^{y}	2362^{x}	3.80^{x}	1.64^{xy}	0.88
	RBT2	7.25	116.28^{y}	1503^{yz}	3.28^{y}	1.48^{y}	1.05
	RBT3	7.27	112.74^{y}	1449^{z}	3.24^{y}	1.92^{x}	0.99
Pooled SEM		0.53	7.66	123.5	0.07	0.04	0.03
P	Strain		<0.001	<0.001	<0.001	0.006	0.27
	Diet		0.005	0.007	0.008	0.78	0.25
	S × D		0.004	0.014	0.007	0.57	0.72

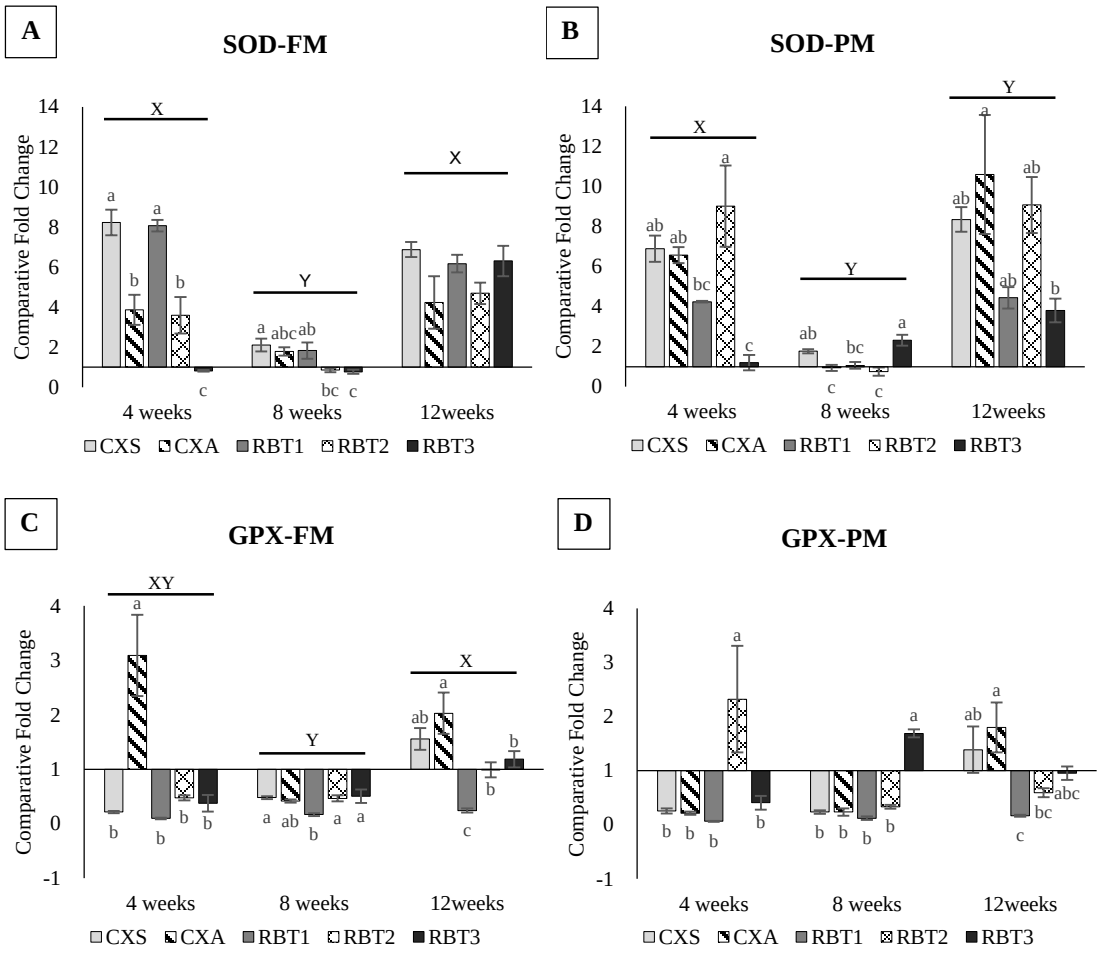
[1] Specific Growth Rate, = $100 \times (\ln W_f - \ln W_i) / t$; W_f: final fish body weight, W_i: initial fish body weight, and t: total number of days of feeding

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

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

3.2. Gene expression

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



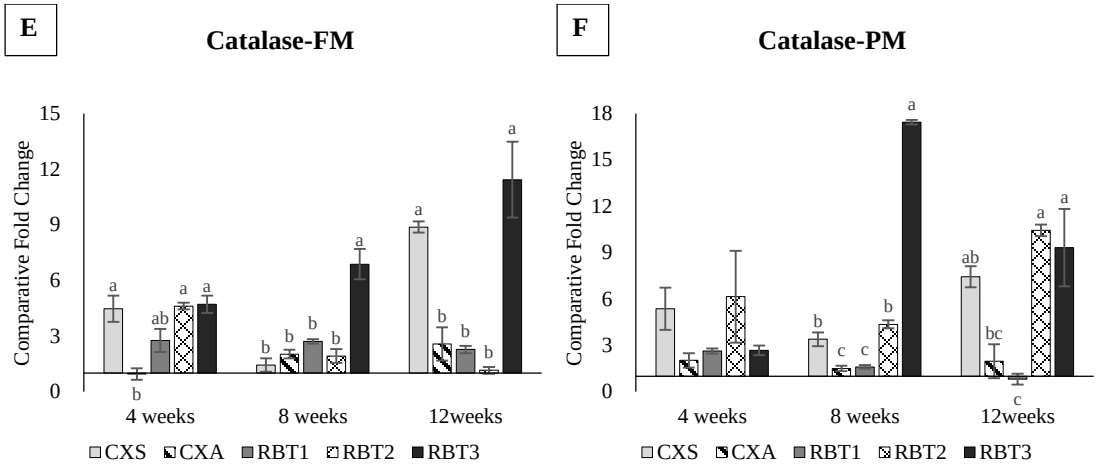


Figure 1. Comparative fold change of gene expression in distal intestine of rainbow trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different letters indicate significant differences among strains within diets. Bars indicate significant differences between time points. Error bars indicate SEM.

In the liver, SOD expression was downregulated among strains on both diets except for RBT3 which showed increased expression by week 12 on the FM diet (Fig. 2A) and week 8 on the PM diet (Fig. 2B). The RBT2 strain also showed increased expression at week 12 on the PM diet (Fig. 2B). The expression of GPx was significantly different over time with all strains on both the FM and PM diets with lower expression again observed at week 8 (Fig. 2C and D). A significant time effect was present with the expression of catalase in both FM and PM diets, with higher expression at week 4 and expression diminishing to initial levels (time 0) by week 8 in both diets (Fig. 2E and F).

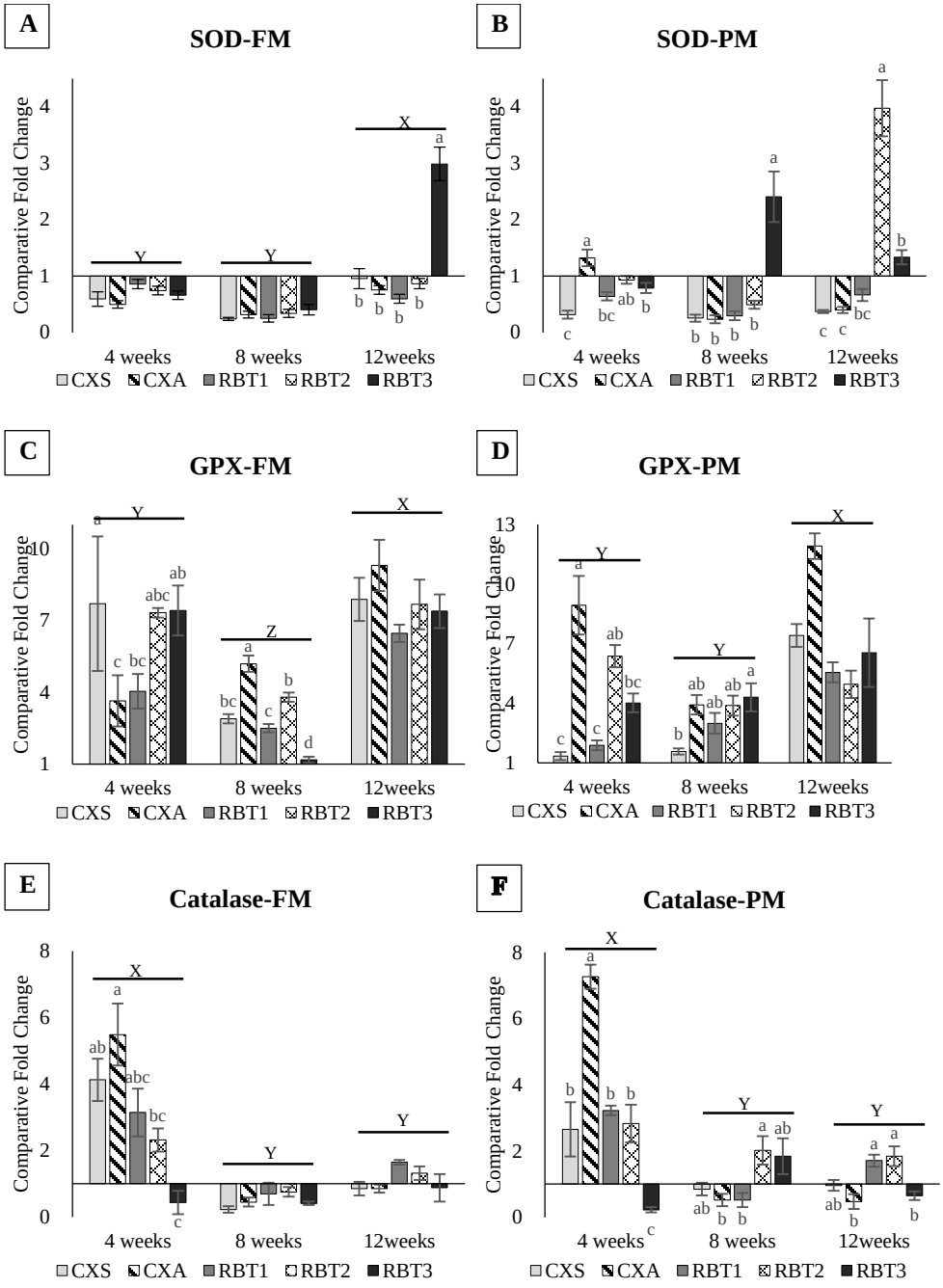


Figure 2. Comparative fold change of gene expression in the liver of rainbow trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different letters indicate significant differences among strains within diets.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

Strain and time effect were significant for expression of S100I2 in the intestine of fish fed both FM and PM diets. Overall expression was lower by week 8 with significant upregulation of S100I2

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

4. Discussion

Rainbow trout selected for increased utilization of plant protein diets (CXS, CXA) showed superior growth compared to the other commercial strains (RBT1, RBT2, and RBT3) on both FM and PM diets after twelve weeks (Table 3). Similar results have been observed in previous feeding trials using this selected strain when fishmeal was replaced with up to 40% SBM (Blaufuss *et al*., 2019; 2020; Overturf *et al*., 2013). Advantages of these selected rainbow trout was first reported by Venold *et al*., 2012, where they examined the level of fatty acid binding protein and enterocyte proliferation rate to define the different responses with 4th generation CX select line fish and non-selected rainbow trout. Callet *et al*., 2021 recently showed transcriptome profiles differ along with growth performance within three isogenic lines of RBT with varying sensitivity to SBM suggesting sufficient genetic variation is present in RBT lines to perhaps select for further improvement in oral tolerance of SBM.

Maintaining homeostasis with variable generation of reactive oxygen species (ROS) is important for preventing oxidative injury and is maintained by superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase as the first line of antioxidant defense reducing the oxidative stress in an organism (Hoseinifar *et al*., 2021; Livingstone, 2003). SOD, GPx and catalase are easily induced by oxidative stress, therefore their expression and enzyme activities have been used as a biomarkers to define oxidative stress in cells (van der Oost et al., 2003). Anti-nutritional factors found in SBM have been linked to damage of the antioxidant system in fish (Zhang *et al*., 2013).

In the distal intestine, we observed significant up-regulation in expression of the SOD gene in fish fed with the FM or PM diet (Fig. 1A and B). At week 8, both diet and strains showed lower SOD expression than at other time points. This up and down regulation over time has been previously observed (Blaufuss et al 2019) and may indicate an adaptive response by the fish to the experimental and control diets which are different than the diet fish were fed prior to the study. The CXA and RBT1 strain showed greater expression of SOD than other strains in week 4 while fed FM diet but this pattern was unresolved by week 12. The expression of GPx in the distal

intestine was highly variable and showed overall down-regulation regardless of the diet or strain (Fig. 1C and D). At week 12, CXS and CXA fish on both FM and PM diets showed up-regulation in GPx in the distal intestine. The expression of catalase data in the distal intestine was observed a significant effect between strain and up-regulation among all-time points in FM or PM diets. Catalase expression generally increased over time but interestingly, expression differences were significant between CXS and CXA strains on both diets at all time points (Fig. 1E and F). This suggests that despite these fish being from the same cohort and same strain, significant differences remain in genetic variability between families and expression of catalase can be very time and age dependent. Comparison to similar work shows intestinal antioxidant activity was significantly higher in largemouth bass after 8 weeks fed a 28% SBM diet. Specifically, bass fed on a high-SBM diet showed about two-fold greater activity of SOD and GPx in the intestine than bass fed an FM diet (Chen *et al.*, 2021). In addition, Blaufuss et al., (2019) observed up-regulation in other genes related to inflammation in distal intestine of RBT while fed a 40% SBM diet. In that study, upregulation of IL-17 was related to the commonly observed mucosal inflammatory response in salmonids. RBT fed on a 40% SBM diet showed greater expression on IL-17A/F2a, F2b, and F3 than the RBT fed an FM diet at weeks 4, 8, and 12 (Blaufuss *et al*., 2019).

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

Catalase expression in the liver was variable with both time and strain and there was a general tendency for upregulation of catalase by week 12 regardless of diet. Catalase expression between the CXA and CXS was significantly different by week 12 again suggesting as observations of GPx expression did, that age and size differences between these cohorts of the same strain convey significantly different responses to the same diet. In the liver of Atlantic salmon, increases in expression and enzyme activity of catalase was positively correlated with a plant-based diet after day 17 (Olsvik *et al*., 2011). Catalase processes H_2O_2 to water, but GPx can also reduce lipid peroxides (Ighodaro & Akinloye, 2018). The patterns of SOD, GPx and catalase expression observed between diets and among strains may indicate variation in changes to metabolism that strains encounter over time as they detoxify H_2O_2 or reduce fatty acid peroxides.

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

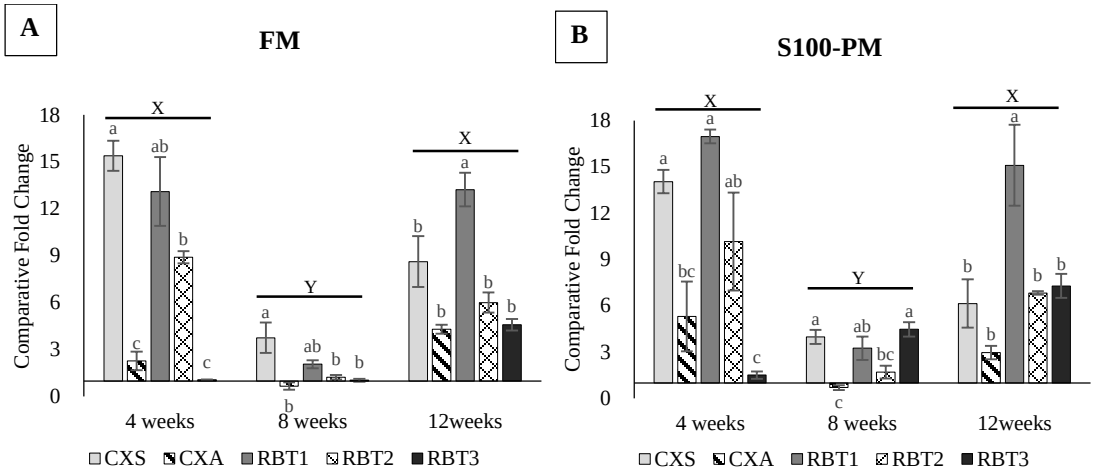


Figure 3. Comparative fold change of S100I2 gene expression in the intestine of rainbow trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different letters indicate significant differences among strains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

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

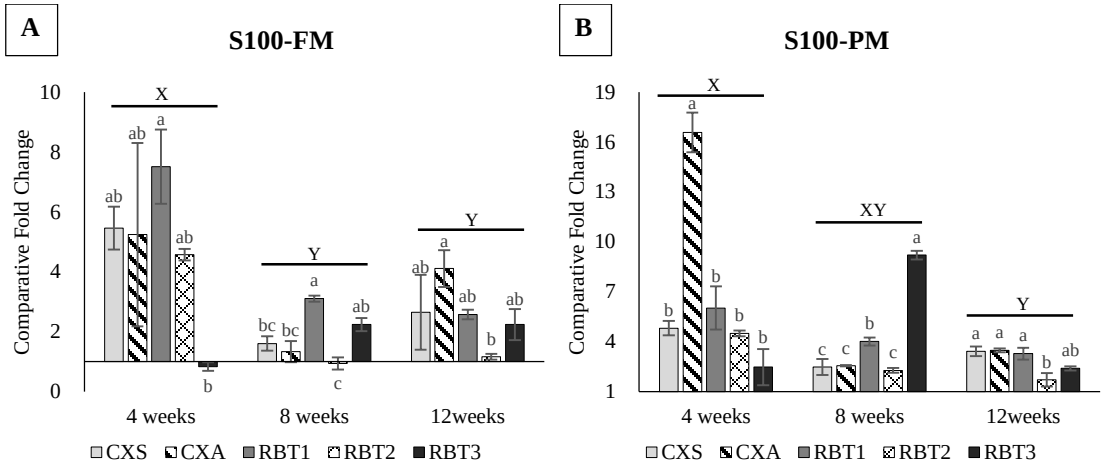


Figure 4. Comparative fold change of S100V2 gene expression in the liver of rainbow trout strains after being fed a fishmeal (FM) or plant meal (PM) diet for 12 weeks. Differences were considered significant with $\alpha \leq 0.05$. Different letters indicate significant differences among strains within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

Conclusions

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

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