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Selection on a plant-based diet reveals changes in oral tolerance, microbiota and growth in rainbow trout (*Oncorhynchus mykiss*) when fed a high soy diet

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ABSTRACT

A strain of rainbow trout at the Hagerman Fish Culture Experiment Station has undergone a long-term selection program for growth on a plant-based diet (HGM strain). Comparing fish from the F_8 generation to a commonly available commercial strain selected for growth (CSS), we sought to investigate potential differences in oral tolerance, intestinal microbiota richness and overall growth when fed a 40% SBM diet. Triplicate tanks (initial mean fish weight 77.9 ± 1.1 g) of each strain were fed diets containing either 0% or 40% SBM over 12 weeks in a factorial design. Fish were weighed every four weeks and subsampled to assess gene expression in the distal intestine. Fish were also sampled at the beginning and end of the study to assess gut histology and characterize intestinal microbiota. At twelve weeks, the HGM strain showed superior growth on both diets ($p < .001$). Gene expression related to gut health and inflammation show reduced inflammatory response in the HGM fish compared to the CSS strain. Significant effects of strain and diet were also observed on metrics of gut microbiota diversity. Together, these results suggest selection for growth on a plant-based diet also drives selection for increased oral tolerance for dietary soybean meal inclusion.

1. Introduction

Increasing cost and limited availability of fishmeal (FM) and fish oil (FO) has driven continuing efforts to reduce the aquaculture industry's dependence on these ingredients in favor of cheaper and more sustainable alternatives. While partial or complete replacement of FM and FO has been a goal, issues with health and diminished growth performance using increasing levels of plant-based sources has meant continued reliance on FM and FO in feeds for many carnivorous fishes. In salmonids, inclusion of soybean meal (SBM) produces notable distal intestine enteritis when dietary levels rise much above 20% and results in characteristic inflammation, blunting of intestinal folds and the intrusion of leukocytes into the submucosa and thickened lamina propria (Burrells et al., 1999; Krogdahl et al., 2003; Silva et al., 2015). Despite the favorable amino-acid profile of SBM, inflammatory responses arising from its use in aquafeeds becomes a limiting factor to increased utilization of this alternative plant-based ingredient in salmonids (Baeverfjord and Krogdahl, 1996; Refstie et al., 2000; Hart et al., 2007; Collins et al., 2013; Krogdahl et al., 2015; Silva et al., 2015).

Causes for the inflammatory effects related to soy-induced distal intestine enteritis have been attributed to various anti-nutritional factors commonly present in plant-based ingredients (Francis et al., 2001; Krogdahl et al., 2010). In an effort to address poor performance using plant-based diets, a strain of rainbow trout (*Oncorhynchus mykiss*) has been selected for growth on a 100% plant-meal based feed containing 19.6% SBM and 25.6% soy protein concentrate, and is now in its 9th generation (Overturf et al., 2013; Abernathy et al., 2017). In addition to improved growth performance, this selected strain has shown concomitant resistance to the development of distal intestine enteritis (Venold et al., 2012).

Previous studies have shown *IL*-17 expression is a component of the acute mucosal inflammatory response in the distal intestine of salmonids, as well as in other fishes in response to dietary soy (Marjara et al., 2012; Krogdahl et al., 2015; Miao et al., 2018). Marjara et al. (2012) observed a 200-fold increase in the two paralogs for interleukin-17A/F2 (*IL*-17A/F2) expression in Atlantic salmon fed a diet containing 20% SBM. Members of the IL-17 family of cytokines are associated with T helper 17 cells (Th17). In salmonids, genes of the IL-17 family are

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characterized into the subgroups of A/F, N, C, and D (Kumari et al., 2009; Wang et al., 2010a; Monte et al., 2013; Wang et al., 2015).

The recognized signals for Th17 cell differentiation in higher vertebrates are interleukin 6 (IL-6) and transforming growth factor beta (TGF-β) (Miossec et al., 2009). TGF-β was initially recognized as an anti-inflammatory cytokine associated with regulatory T helper cells (Tregs) but was discovered to also induce the development of Th17 cells (Mangan et al., 2006). In the presence of IL-6, TGF-β induces the differentiation of naïve T cells into Th17 cells (Kimura and Kishimoto, 2010). In addition, IL-6 actively suppresses the ability of TGF-β to induce forkhead box P3 (*Fox*p3) expression, the master transcription factor required for the induction of T regulatory cells (Tregs). As the anti-inflammatory cytokines produced by Tregs are necessary for the development and maintenance of oral tolerance to innocuous antigens and the resident microbiome, their dysfunction is implicated in many autoimmune and inflammatory bowel disorders (Faria and Weiner, 2005; Pabst and Mowat, 2012). Observed variation in oral tolerance may involve differences in resident leukocyte populations within the intestinal epithelium before the development of enteritis.

Immunohistochemistry methods utilizing cell T cell surface markers have helped characterize the location of T cells before and during intestinal inflammation, but there is a lack of cell surface markers available for differentiation of subsets of T helper cells (Bakke-McKellep et al., 2007; Lilleeng et al., 2009). However, the master regulator genes of Th17 (*ROR*γ) and Tregs (*Fox*P3) have been characterized in rainbow trout (Wang et al., 2010b; Monte et al., 2012) along with the cytokines associated with each (Harun et al., 2011; Wang and Secombes, 2013; Benedicenti et al., 2015). Despite these advances, Tregs and anti-inflammatory cytokine involvement in oral tolerance to innocuous antigens remains less understood in fish. Resistance to distal intestinal enteritis observed in the Hagerman selected-strain rainbow trout provide an excellent contrast and model for further characterization of immunogenic markers, including the apparent induction of Th17 cells and the repression of Tregs. In order to test the response of Tregs and anti-inflammatory cytokines to dietary SBM in rainbow trout, differences in gene expression between selected and non-selected strains were evaluated when fed a diet with no SBM versus a diet with 40% SBM. The results enhance our understanding of the salmonid mucosal immune system that lead to development of chronic enteritis and transitory enteritis observed in other fishes (Urán et al., 2008; Wu et al., 2018).

2. Materials and methods

2.1. Diets

Two diets were formulated on a digestible nutrient basis to provide 40% digestible protein and 20% crude fat, while containing 0% or 40% SBM (Table 1). Both diets were balanced for digestible protein and supplemented with methionine, lysine, and threonine to meet or exceeded all known nutrient requirements (National Research Council, 2011).

Diets were mixed and co-ground without fish oil and lecithin using an air-swept pulverize (model 18-H, Jacobson, Minneapolis, USA) to a particle size of less than 250 μm. Diets were manufactured using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with six barrel sections and 18-s transit time through the barrel. The material was not steam conditioned prior to extrusion and water was added to barrel Section 2. The 3.5- mm floating pellets were dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) at 102 °C until moisture levels were below 7%, and cooled prior to vacuum oil infusion (A.J. Mixing, Ontario, Canada). Diets were stored in a cool dry environment until utilized.

Moisture content was measured as loss in drying for diets, feces and whole-body samples and was performed according to standard methods (AOAC, 1995). Crude protein (N x 6.25) was determined in ingredients, diets, and whole-body samples by the Dumas method (AOAC 1995) on a Leco TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, Michigan, USA). Total energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, Illinois, USA). Lipid was determined by petroleum ether extraction using an Ankom XT10 (Ankom Technologies, Macedon, New York, USA).Yttrium oxide was included as an indigestible inert marker to quantify nutrient digestibility, and apparent digestibility coefficients (ADCs) were calculated following the methodology of Cho et al. (1982).

Table 1
Formulation (g/100 g dry weight) of experimental diets fed to a commercial strain and a selected strain of rainbow trout for 12 weeks in a 2 × 2 factorial design.

Ingredient	Soy 0	Soy 40
Menhaden fish meal[a]	30.00	0.00
Soybean meal[b]	0.00	40.00
Chicken meal[c]	11.50	11.50
Corn protein concentrate[d]	11.50	11.50
Menhaden fish oil[e]	14.72	16.35
Wheat gluten meal	0.16	1.30
Wheat flour	23.29	5.54
Lecithin	1.00	1.00
Stay-C 35[f]	0.15	0.15
Vitamin premix ARS 702[g]	1.00	1.00
TM ARS 640[h]	0.10	0.10
NaCl	0.28	0.28
Magnesium Oxide	0.06	0.06
Potassium chloride	0.56	0.56
Monocalcium phosphate	0.60	4.80
Choline chloride 50%	1.00	1.00
DL-Methionine	0.33	0.63
Lysine HCl	2.17	2.56
Threonine	0.40	0.49
Taurine	1.00	1.00
Yttrium oxide	0.10	0.10
Astaxanthin	0.08	0.08
Total	*100*	*100*
Proximate analysis (analyzed)		
Protein (%DM)	46.7	48.0
Lipid (%DM)	18.7	17.6
Energy (cal/g DM)	5268.1	5280.9

[a] Menhaden Special Select, Omega Proteins, Reedville, VA, USA.
[b] Archer Daniels Midland Co., St. Louis, MO, USA.
[c] American Dehydrated Foods, Springfield, MO, USA,
[d] Cargill, Inc., Empyreal 75, Blair, NE, USA.
[e] Omega Proteins, Inc., Virginia Prime, Reedville, VA, USA.
[f] DSM Nutritional Products, Basel, Switzerland.
[g] Provides per kg diet before processing: vitamin A, 9650 IU; vitamin D, 6600 IU; vitamin E, 132 IU; vitamin K3, 1.1 mg; thiamin mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate DL-calcium, 46.5 mg; cyanocobalamin, 0.03 mg; nicotinic acid, 21.8 mg; biotin 0.34 mg; folic acid, 2.5 mg; inositol, 600 mg.
[h] Contributed in mg kg^{-1} of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4.

2.2. Fish culture and feeding

Thirty rainbow trout (77.9 ± 1.1 g) per tank of either a commercially-selected strain (CSS; Troutlodge Inc., Sumner, WA) or the 8th generation Hagerman strain (HGM; USDA-ARS/University of Idaho, Hagerman, ID) were stocked into a recirculating system consisting of twelve 320-L poly tanks connected to a common bio-filter with spring water inflow of approximately 20% makeup. Water temperatures were maintained at 15 °C with a 14:10 light:dark cycle for the duration of the experiment. A sample of 10 fish taken from the initial population were euthanized with an overdose of MS-222 (250 ppm, pH 7.4, Western Chemical Co., Ferndale, WA) and stored frozen at −20 °C for chemical analysis to determine protein and energy retention efficiencies at the

experiment's completion. Fish were fed twice daily, 6 days per week, for 12 weeks to apparent satiation. Consumed feed was recorded weekly. All fish in each tank were counted and weighed every 4 weeks. All fish were cultured and sampled under the approved protocol #2017–19 from the University of Idaho, Institutional Animal Care and Use Committee.

2.3. Sample collection

At weeks 4, 8, and 12, five fish from each tank were euthanized and ~5 mm samples of distal intestine removed for gene expression analysis; distal being defined as the point from the where intestinal diameter and smooth muscle visually increase in size (approximately half the distance from the last pyloric cecum to the vent). Intestinal samples were flushed with ice-cold sterile saline, snap frozen in liquid nitrogen and stored frozen at −80 °C until processed for gene expression. At the termination of the trial (week 12), three fish per tank were sampled for whole-body proximate composition and three were dissected to quantify relative liver size (hepatosomatic index, HSI), and viscerosomatic index (VSI). Subsamples of muscle were removed and stored frozen (−20 °C) for proximate analysis. Samples of distal intestine were excised, transferred to 10% formalin for histological analysis.

Feces from remaining fish were collected 16-h post feeding to measure nutrient digestibility by sedating the fish in 50 ppm MS-222 until equilibrium was lost, removing the fish from the water and drying the fish to prevent water contamination of the feces. Gentle pressure was then applied to the abdomen approximately one third of the distance from the anus to the gill plate. Fecal matter was excreted into a weigh boat and transferred into micro-centrifuge tubes, snap frozen in liquid nitrogen, and stored at −80C for analysis. The remaining feces were lyophilized, and nutrient and yttrium content was measured to calculate apparent nutrient absorption and digestibility (Gaylord et al., 2009).

Growth performance and condition indices were calculated using the following formulas:

$$\text{Percent weigh gain} = \frac{\textit{Mean fish mass gain (g)}}{\textit{Initial mean fish mass (g)}} \text{ x } 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{g dry feed fed}}{\text{g wet weight gain.}}$$

$$\text{Feed Intake (\%bw/day)} = \frac{\text{g dry feed fed}}{\text{mean fish mass (g)}} \div 84\ \textit{days}$$

$$\text{Hepatosomatic index (HSI)} = \frac{\text{liver mass (g)} \times 100}{\text{fish mass (g)}}$$

$$\text{Viscerosomatic index (HSI)} = \frac{\text{viscera mass (g)} \times 100}{\text{fish mass (g)}}$$

$$\text{Fillet Yield (\%)} = \frac{\text{fillet mass (g)X 2} \times 100}{\text{fish mass (g)}}$$

2.4. Histological analysis of intestine

Sections of dissected distal intestine were dehydrated in ethanol, flushed with xylene, and paraffin embedded. Tissue from each section was sliced (3 μm) crosswise on the longitudinal axis and stained with hematoxylin and eosin (H&E). Slides were imaged using light microscopy (Zeiss Axioscope A1, Carl Zeiss Ltd., Cambridge, UK) and measurements of the thickness of the lamina propria and submucosa were made using ImageJ version 1.52 from two slides per fish and three fish per tank (Brown et al., 2016). Briefly, measurements of the thickness of the submucosa were made from the region spanning the stratum compactum to the circular muscles. Measurements of the lamina propria were taken halfway between the tip of the mucosal fold and sub-epithelial mucosa. A semi-continuous scoring system (ranging from 0 to 5) adapted from Urán et al. (2008) and Venold et al. (2012) was used to evaluate goblet cell number, supranuclear vacuolization, eosinophilic granulocyte infiltration, and sub-epithelial mucosa appearance in the intestine. A score of 0–2 represented normal variation in morphology and scores > 2 indicating morphological changes consistent with increasing severity of intestinal inflammation.

2.5. Quantitative PCR analyses

Total RNA was extracted from each tissue with TRIzol® reagent (ThermoFisher Scientific, Waltham, USA). Briefly, intestinal tissue samples were placed in 2-mL round-bottom centrifuge tubes containing 1 mL of TRIzol® and homogenized in a bead mill (Mixer Mill 200, Retsch GmbH, Haan, Germany). The homogenate was centrifuged at 12,000 *g* at 4 °C for ten minutes, with the supernatant being transferred to fresh 1.5 mL tubes. Two hundred uL of chloroform was added to each tube, which were then vigorously shaken for 15 s before being incubated at room temperature for ten minutes. Tubes were then centrifuged at 12,000*g* at 4 °C for fifteen minutes, with the supernatant being transferred to fresh 1.5 mL tubes. Five hundred uL of isopropanol was added to each tube, which were then mixed gently before incubating overnight at −20 °C. The precipitated RNA was pelleted by centrifuging at 12,000 *g* at 4 °C for fifteen minutes, washed with cold 75% ethanol, and resuspended in nuclease-free water. RNA quantity and purity were determined spectrophotometrically (Nanodrop 2000, ThermoFisher Scientific, Waltham, USA) and pooled by tank. Samples were treated with DNase (DNase I, Invitrogen, Carlsbad, USA) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, ThermoFisher Scientific, Waltham, USA) according to manufacturer's instructions. The resulting cDNA was aliquoted and stored at −80 °C for later analysis. Primers (Table 2) were designed to be intron

Table 2
Primer sequences of rainbow trout genes used for quantitative PCR and the sequence for the V3-V4 locus used for microbiome analysis in a 12-week study comparing a 0% SBM diet to a 40% SBM diet between two rainbow trout strains.

Gene	Accession	Primers
IL-17A/F1a	KJ921977.1	*f. TTG GTG ATG ATG ATG ATG ATG G* *r. GTG TTT GGA GCT GAA GGC*
IL-17A/F1b	KJ921978.1	*f. CAG AGT CAG AGT CTT CAG GTC* *r. GGG GAG ATT GAA TGG TTG TAA A*
IL-17A/F2a	AJ580842.1	*f. ACC CTG GAC CTG GAA AAG CAC* *r. GCT GAA GTG TAG AGT ACC ACG ACC TG*
IL-17A/F2b	KJ921979.1	*f. CCA AAG GGG AGG TAG GGG A* *r. GGT TCT TCA CTG TAA TGG GTT TCC A*
IL-17A/F3	KJ921980.1	*f. CTG GTG CTG GGT CTG ATC ATG T* *r. GGT CTC ATC GTA TGT GTC GCT GTA TG*
IL-17 N	KJ921981.1	*f. CCG CCT GGA ACT ACG TGG AAA* *r. ACT TTG TCT GGA AGT GGC GCA*
*S*100I2	XM_021598338.1	*f. GCT TGG AGA GAT CAT GGG GAA AA* *r. GCC ATC TGA GTT AGC GTC CA*
*Fox*P3–1	NM_001246333.1	*f. ATC TGG ACA CAA ATC AAC TGT C* *r. GAC ACT CCA CCT CGG TTC*
*Fox*P3–2	NM_001246332.1	*f. CCT GTC TGA ACA CAA ATC AAC T* *r. GCC GGG GAT GAG TAC TG*
*ROR*γ	NM_001199827.1	*f. ACA GAC CTT CAA AGC TCT TGG TTG TG* *r. GGG AAG CTT GGA CAC CAT CTT TG*
TGF-β1a	KF870471.1	*f. GTG GGA AAC CAA TGG AGG AA* *r. TGT ATC TCC CCT CAG CTT GTT CA*
IL-10a	NM_001245099.1	*f. AAG GGA TTC TAC ACC ACT TGA A* *r. GTT GTT GTT CTG TGT TCT GTT G*
IL-10b	NM_001246350.1	*f. AAG GGG ATT CTA GAC CAC AT* *r. GTT CCA TTG TTG TTG TTC ATG G*
*RPS*15	NM_001165174.2	*f. ACA GAG GTG TGG ACC TGG AC* *r. AGG CCA CGG TTA AGT CTC CT*
V3-V4 Locus		*f. tag-CCT ACG GGN GGC WGC AG* *r. tag-GAC TAC HVG GGT ATC TAA TCC*

spanning using Primer3 version 2.6 and were checked for specificity by performing BLAST searches against the most recently published rainbow trout genome assembly (Untergasser et al., 2012). Pooled samples in plates were amplified in duplicate (ViiA 7 Real-Time PCR System, Life Technologies, Carlsbad, USA) according to manufacturer's instructions (Fast SYBR™ Green Master Mix, Life Technologies, Carlsbad, USA) with a melt curve to check for product specificity. Expression efficiencies were 97–103%. Genes of interest were normalized against the ribosomal reference gene *RPS*15 (Bustin and Nolan, 2006) and quantified using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001). For each gene, the expression of the HGM strain fed the 0% SBM diet was set to a baseline of 1, with the expression of all other treatments being presented relative to it at each time point.

2.6. Microbiome analysis

DNA from fecal samples of nine fish per treatment (3 fish/tank) was extracted from 0.1 g subsamples using a commercial kit (QIAmp® PowerFecal®, Qiagen, Germantown, USA). Similarly, DNA from samples of each diet (N = 3/diet) and 50 mL of inflow water (N = 3) were also extracted. Extracted DNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific Inc.). First round PCR used degenerate primers, targeting the 16S ribosomal V3 and V4 region (Table 2). Attached to these primers were custom tag sequences designed by the University of Idaho Genomics Resources Core (UIGRC) to use with the custom barcoding sequencing system developed in partnership with Fluidigm Corporation (San Francisco, CA). First round PCR products were checked for suitability on 1% agarose gels and amplicons were diluted 1:15 before undergoing a second round of PCR, where sample specific barcodes and Illumina indexes were attached as per the Illumina 16S Metagenomic Sequencing Library Preparation Manual (part# 15044223 Revision B, Illumina, San Diego, CA). Barcoding primers were obtained from the UIGRC. Second round amplification products were again verified on 1% agarose gels and quantified using the Quant-iT™ PicoGreen® dsDNA assay (Thermo Fisher Scientific Inc., Waltham, MA). All PCR was conducted using the KAPA HiFi HotStart Kit (KAPA Biosystems, Wilmington, USA). Barcoded amplicons were pooled and purified using the Agencourt AMPure XP system (Beckman Coulter Inc., Atlanta, GA) and quantified. Resulting amplicons were sequenced on an Illumina MiSeq next generation sequencer platform using a MiSeq Reagent Kit v3 (Illumina, Inc., San Diego, USA).

Raw reads were demultiplexed and trimmed of primers using dbcAmplicons v0.9.0 (github.com/msettles/dbcAmplicons) and custom python scripts. DADA2 v1.14 (Callahan et al., 2016) was then used to trim forward (280 bp) and reverse reads (205 bp), quality filter to 2 expected errors, error-correct, and chimera filter the resulting reads into denoised amplicon sequence variants (ASVs). Taxonomy was applied to ASVs using a naïve Bayesian classifier trained on the Silva v.132 database (Quast et al., 2013), and a phylogenetic tree was assembled using Decipher v2.12 (Wright, 2016) and phangorn v2.5.5 (Schliep, 2011). Singleton ASVs and those assigned to the phylum Cyanobacteria or the family Mitochondria were removed as contaminating reads derived from non-functional plant and animal organelle present within the diets or derived from the host. Rarefaction analysis was conducted to ensure sequencing depth was sufficient to adequately characterize the microbial communities. All samples reached an appropriate asymptote in microbial richness within the current sampling depth. Alpha diversity was assessed using the observed richness (Observed ASVs), Shannon Index, and Simpson Index. Beta diversity was assessed using the phylogenetically informed distance metrics of weighted and unweighted UniFrac calculated on a proportionally transformed abundance table (relative abundance). All microbiota data filtering and diversity analyses were conducted using phyloseq v1.28 (McMurdie and Holmes, 2013) and vegan v2.5.6 (Oksanen et al., 2019).

2.7. Statistical assessment of data

Growth, histological measures, and gene expression were analyzed by two-way ANOVA using the PROC-GENMOD procedure of SAS® version 9.4 (SAS Institute, Cary, USA), with Tukey-Kramer *post-hoc* tests when interactions were significant. Non-parametric Mann-Whitney Rank Sum Tests with a Yates continuity correction (Sigmaplot 14.0, Systat Software, Inc. San Jose, CA) were used to evaluate differences in semi-continuous histological scores (goblet cell number, supranuclear vacuolization, eosinophilic granulocyte infiltration and sub-epithelial mucosa appearance) with significance assigned at $\alpha < 0.05$. A paired *t*-test was used to test for differences in microbiota alpha diversity among the two experimental diets, between the initial pre-trial gut communities of the two fish strains, as well as to compare the initial and end of trial gut samples. Alpha diversity among the end of trial fish samples was assessed by fitting a two-way linear model (Diet * Fish Strain + Diet:Strain). Beta diversity UniFrac distance matrices of the end point gut microbiota samples were tested for multivariate dispersion by individual factor (Strain and Diet) and a distance based permutational MANOVA (PERMANOVA) was conducted (Diet * Fish Strain + Diet:Strain), with permutations stratified by tank to adjust for nestedness in the design. ANCOM2 (Kaul et al., 2017) was used to separately evaluate differential abundance of ASVs by Treatment (CSS0, CSS40, HGM0, HGM40), Strain (CSS vs. HGM) and Diet (0% Soy vs. 40% Soy) using a 60% prevalence cut-off and multiple comparisons correction. Significance was determined at $\alpha \leq 0.05$ for all statistical tests.

3. Results

3.1. Growth performance

Regardless of diet, HGM fish had significantly higher final weights, percent weight gain, SGR, and condition factors (Table 3). Significant differences attributable to both strain and diet were observed with feed intake and feed conversion ratio. There was a significant decrease in survival in HGM fish fed the 0% SBM diet, primarily attributed to starve-outs, as the weight of the majority of mortalities were significantly below the average individual fish weight. Apparent digestibility coefficients of dry matter, lipid, and energy were higher in strains fed the 0% SBM diet, while that of protein was higher in strains fed the 40% SBM diet.

There were no differences in whole-body moisture between treatments (Table 4). Whole-body fat and energy were significantly lower in the HGM strain fed the 40% SBM diet. Significant differences in HSI attributable to both diet and strain were observed, being lower in the CSS strain and in fish fed the 40% SBM diet while there were no differences in VSI between treatments. Fecal protein was higher in strains fed the 0% SBM diet, and fecal energy was lower in strains fed the 0% SBM diet, with an almost significant interaction. There were no differences in fecal lipid among the treatment groups.

3.2. Histology

We observed a significant interaction in parametric measurements of the thickness of the lamina propria and submucosa. Neither were significantly different between strains fed the 0% SBM but were higher in both strains fed the 40% SBM diet (Table 3). Between strains fed the 40% SBM diet, thickness of the lamina propria and submucosa were significantly higher in the CSS strain. Combined semiquantitative scores for goblet cell number, supranuclear vacuolization, eosinophilic granulocyte infiltration and sub-epithelial mucosa appearance did not differ between strains fed the 0% SBM diet (HGM = 1.1 ± 0.2; CSS = 1.0 ± 0.2) or the HGM strain fed the 40% SBM diet (1.5 ± 0.4). However, a significant increase was observed in the CSS strain fed the 40% SBM diet (2.9 ± 0.3) indicating mild inflammation

Table 3
Growth and feeding performance after consuming a 0% SBM or 40% SBM diet for 12 weeks compared between a commercial strain of rainbow trout (CSS) and a strain previously selected for growth on an all plant-based diet (HGM). Differences were considered statistically significant with $\alpha \leq 0.05$. Different letters mark significant differences between treatment groups when a significant interaction is present.

	Soy 0		Soy 40		p		
	HGM	CSS	HGM	CSS	Diet	Strain	Diet x strain
Growth							
Initial mass (g)	77.9 ± 0.50	77.7 ± 0.60	77.3 ± 0.40	78.7 ± 1.00	*0.7324*	*0.2876*	*0.1924*
Final mass (g)	487.6 ± 7.50	411.2 ± 7.20	471.1 ± 5.10	405.6 ± 6.20	*0.0573*	< ***0.0001***	*0.3225*
Weight gain (%)	526.2 ± 6.14	429.0 ± 5.84	509.4 ± 4.15	415.6 ± 5.10	*0.0743*	< ***0.0001***	*0.8266*
SGR[1] (%)	2.18 ± 0.03	1.98 ± 0.02	2.15 ± 0.02	1.95 ± 0.01	*0.0666*	< ***0.0001***	*0.9541*
Condition factor	1.56 ± 0.06	1.36 ± 0.07	1.56 ± 0.08	1.39 ± 0.05	*0.6653*	***0.0002***	*0.6603*
Survival (%)	0.88 ± 0.05	1.00 ± 0.00	0.97 ± 0.02	1.00 ± 0.00	*0.0547*	***0.0028***	*0.0547*
Feeding							
FI[2] (%BW/day)	2.04 ± 0.02	1.99 ± 0.02	2.16 ± 0.01	2.09 ± 0.02	< ***0.0001***	***0.0007***	*0.4239*
FCR[3]	0.99 ± 0.01	1.03 ± 0.01	1.05 ± 0.01	1.09 ± 0.01	< ***0.0001***	***0.0003***	*0.6259*
Digestibility							
ADC DM (%)	67.8 ± 0.69	68.6 ± 0.33	61.0 ± 1.25	60.8 ± 0.76	< ***0.0001***	*0.4701*	*0.6581*
ADC Protein (%)	81.8 ± 0.35	82.0 ± 0.37	84.5 ± 0.60	84.3 ± 0.22	< ***0.0001***	*0.5692*	*0.9835*
ADC lipid (%)	91.5 ± 0.99	91.2 ± 1.16	86.9 ± 1.25	89.7 ± 0.09	***0.0022***	*0.0835*	*0.1311*
ADC energy (%)	77.8 ± 0.84	78.3 ± 0.43	72.6 ± 0.67	72.4 ± 0.13	< ***0.0001***	*0.5887*	*0.8033*
Histology							
Lamina Propria (px[4])	13.11 ± 0.72[a]	13.35 ± 0.78[a]	18.61 ± 0.54[b]	24.16 ± 0.66[c]	< ***0.0001***	***0.0076***	***0.0126***
Submucosa (px)	26.15 ± 0.35[a]	25.70 ± 0.29[a]	31.64 ± 1.40[b]	36.86 ± 1.45[c]	< ***0.0001***	*0.2723*	***0.0338***

[1] Specific Growth Rate, = 100 × ($\ln W_f - \ln W_i$)/t; where W_f represents initial fish body weight, W_i represents the initial fish body weight, and t represents the total number of days of feeding.
[2] Feed Intake, = g dry feed consumed/average fish biomass (g) /culture days * 100.
[3] Feed Conversion Ratio, = g dry feed consumed / g wet weight gained.
[4] Pixel.

consistent with the onset of distal intestinal enteritis ($p < 0.001$, $U = 799.3$).

3.3. Gene expression

The main effect of strain was significant at week 8 for *IL*-17A/F1a, with the expression of the HGM strain being higher than the CSS strain (Fig. 1). A significant interaction was present with the expression of *IL*-17A/F1b at week 4, with lower expression in the CSS strain fed the 0% SBM diet. Both main effects were significant at week 8, with higher expression in the HGM strain and in fish fed the 40% SBM diet. The main effect of diet was significant at all three time points for both *IL*-17A/F2a and b, with higher expression in fish fed the 40% SBM diet when no significant interaction was present (Fig. 1). A significant interaction was observed with the expression of *IL*-17A/F2b at week 4, with the expression of the CSS strain fed the 40% SBM diet being higher than the other treatments. The main effect of strain was significant at week 12 for both paralogs, with higher expression in the CSS strain. Both main effects were significant for *IL*-17A/F3 at weeks 8 and 12, with significant interaction only observed at week 8, where expression was highest in the HGM strain fed the 40% SBM diet and lowest in the CSS strain fed the 0% SBM diet (Fig. 1). At week 12, expression was higher in the CSS strain and in both strains fed the 40% SBM diet. We did not observe expression of *IL*-17 N in either strain at any time point.

Both main effects were significant for *ROR*γ at week 8, with higher expression in the HGM strain and in fish fed the 40% SBM diet (Fig. 1). Only diet was significant at week 12, with higher expression in fish fed the 0% SBM diet. Both main effects were significant at week 4 for *IL*-10a, with higher expression in the HGM strain and in fish fed the 40% SBM diet (Fig. 2). At week 8, diet was significant in both *IL*-10a and b, with higher expression in fish fed the 40% SBM diet. The effect of diet was significant in *Fox*P3–1 at week 8, with higher expression in fish fed

Table 4
Whole body and fecal proximate composition after consuming a 0% SBM or 40% SBM diet for 12 weeks compared between a commercial strain of rainbow trout (CSS) and a strain previously selected for growth on an all plant-based diet (HGM). Differences were considered statistically significant with $\alpha \leq 0.05$. Different letters mark significant differences between treatment groups when a significant interaction is present.

	Soy 0		Soy 40		**p**		
	HGM	CSS	HGM	CSS	Diet	Strain	Diet x strain
Whole-body							
Moisture (%)	66.6 ± 0.56	66.1 ± 0.44	67.3 ± 0.36	66.0 ± 0.78	*0.6656*	*0.1125*	*0.4393*
Fat (%)	14.3 ± 0.37[a]	13.8 ± 0.30[a]	12.2 ± 0.24[b]	13.7 ± 0.60[a]	***0.0141***	*0.2630*	***0.0274***
Protein (%)	17.5 ± 0.48	17.9 ± 0.21	18.5 ± 0.54	18.8 ± 0.42	***0.0328***	*0.4530*	*0.8621*
Energy (cal/g)	2292 ± 18.0[a]	2328 ± 20.5[a]	2142 ± 33.2[b]	2320 ± 25.6[a]	***0.0072***	***0.0008***	***0.0128***
HSI[1]	1.49 ± 0.06	1.29 ± 0.09	1.12 ± 0.06	1.03 ± 0.03	< ***0.0001***	***0.0020***	*0.1585*
VSI[2]	10.63 ± 0.90	10.65 ± 0.31	10.65 ± 1.30	9.81 ± 0.24	*0.3963*	*0.3976*	*0.3806*
Fillet yield (%)	54.9 ± 1.12	57.5 ± 0.59	55.8 ± 0.74	58.5 ± 0.83	*0.0612*	***0.0001***	*0.9800*
Fecal							
Protein (%)	23.8 ± 0.44	25.0 ± 0.19	17.9 ± 0.29	17.6 ± 0.30	< ***0.0001***	*0.2342*	*0.0949*
Fat (%)	4.47 ± 0.46	4.87 ± 0.55	4.31 ± 0.09	5.52 ± 0.55	*0.6730*	*0.1879*	*0.4886*
Energy (cal/g)	3268 ± 20.9	3406 ± 20.4	3462 ± 48.6	3443 ± 24.4	***0.0157***	*0.1514*	*0.0716*

[1] Hepatosomatic Index, = liver mass x 100 / fish mass.
[2] Vicerosomatic Index, = viscera mass x 100 / fish mass.

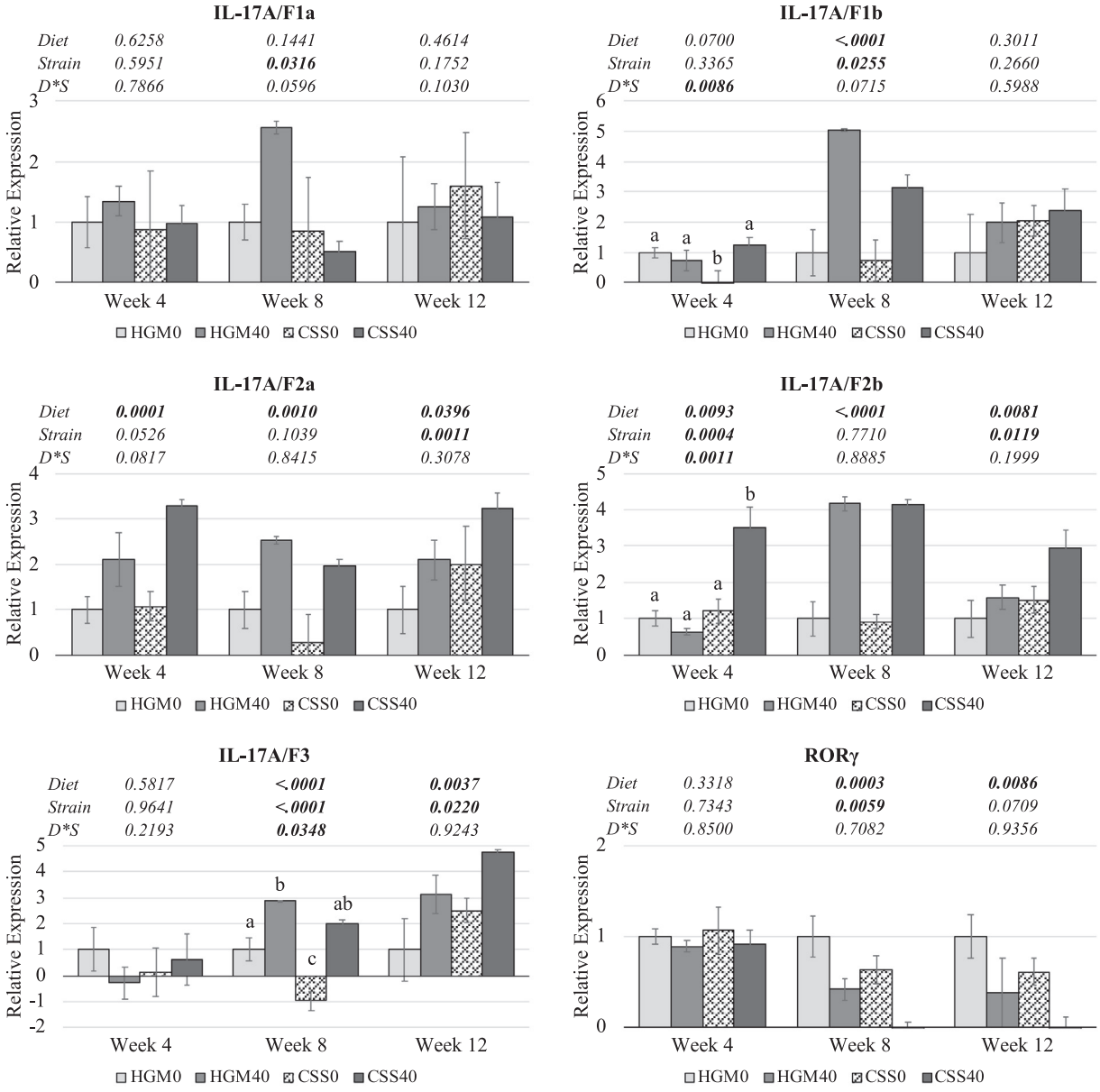

Fig. 1. Relative expression of genes in distal intestine in a common commercial strain of rainbow trout (CSS) and a strain selected on an all plant diet for eight generations (HGM) after being fed a diet containing 0% or 40% soybean meal for 12 weeks. Differences were considered statistically significant with $\alpha \leq 0.05$. Different letters mark significant differences between treatment groups when a significant interaction is present. Error bars represent sample SEM.

the 40% SBM diet (Fig. 2). It was also significant in *Fox*P3–2 at week 4, where expression was higher in fish fed the 0% SBM diet. There was significant interaction in *Fox*P3–2 at week 12, with lower expression in the HGM strain fed the 0% SBM diet.

Both main effects were significant for the expression of *TGF*-β1a at week 4, with higher expression in the HGM strain and in fish fed the 0% SBM diet (Fig. 2). At week 12, only diet was significant, with lower expression in fish fed the 40% SBM diet. Both main effects were significant for *S*100I2 at all three time points, with a significant interaction at week 12 (Fig. 2). At weeks 4 and 8, expression was higher in the CSS strain and in fish fed the 40% SBM diet. At week 12, expression was higher in the CSS strain fed the 40% SBM diet.

3.4. Microbiome analysis

A total of 1,339,188 raw sequence reads were generated for all microbiota samples (environmental samples, initial pre-trial samples, and end point treatments). After data processing a total of 987,331 reads remained, with an average read depth of 18,283 ± 7789 (mean ± SD) per sample. Removal of singleton ASVs and those assigned to Mitochondria or Chloroplast reduced the read depth to 12,183 ± 8387 (mean ± SD), although, rarefaction analysis indicated that the microbial richness reached an asymptote under the current sequencing depth in all samples (Supplementary Fig. S1). A total of 2465 unique ASVs were detected in the full dataset, with 1434 ASVs detected among the end of trial fish gut samples.

In terms of alpha diversity, no differences were detected among the two treatment diets in microbial richness according to a Student's paired *t*-test, with an average of 106 and 108 ASVs detected among the 0% soy and 40% soy treatment diets (Fig. 3). The inflow water samples showed the greatest alpha diversity among all samples with an average observed richness of 233 ASVs (Fig. 3). Initial gut microbiota samples taken from the two fish strains prior to initiation of the 12-week feeding trial showed no differences in alpha diversity (Fig. 3), though the pre-

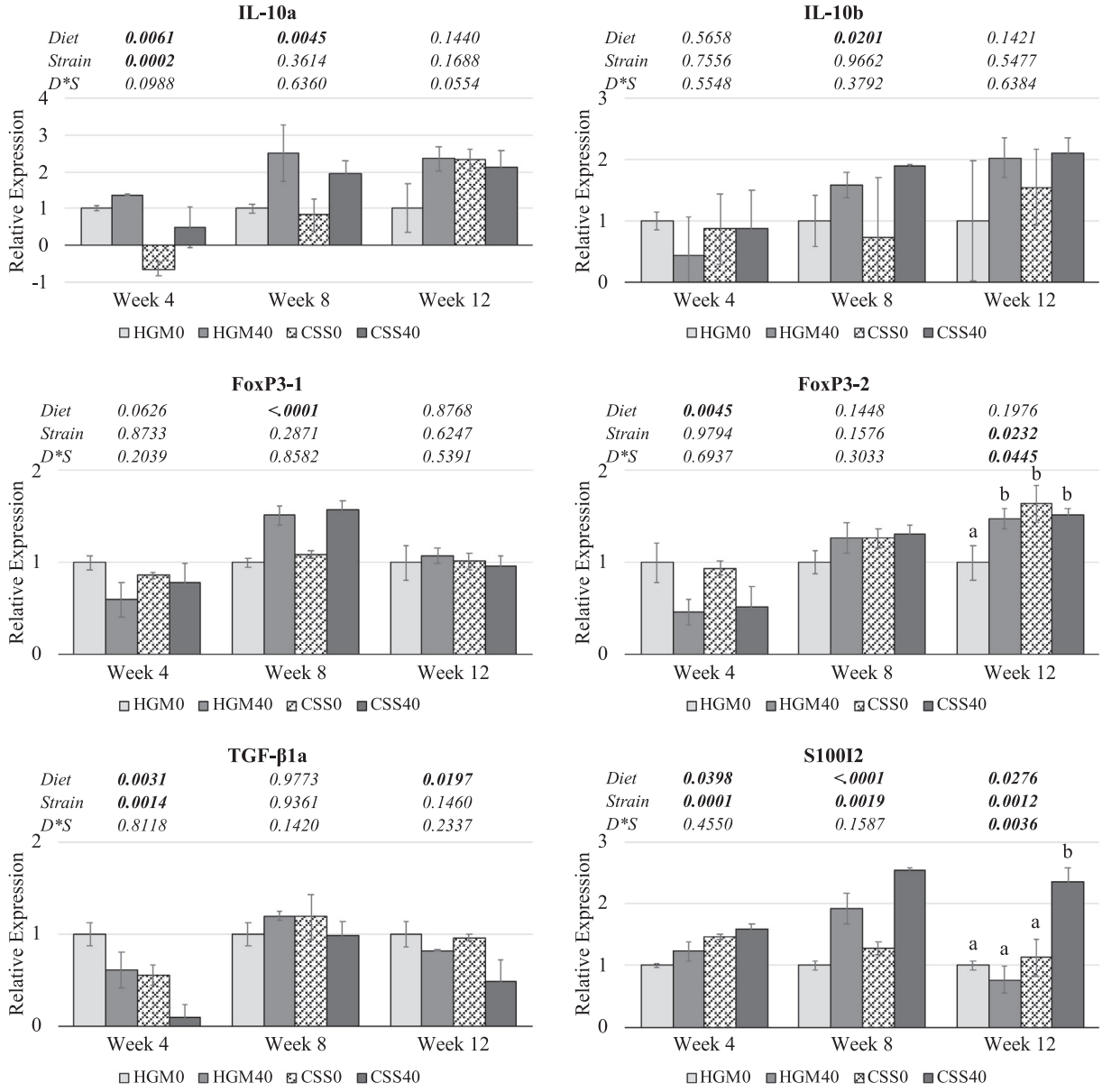

Fig. 2. Relative expression of genes in distal intestine in a common commercial strain of rainbow trout (CSS) and a strain selected on an all plant diet for eight generations (HGM) after being fed a diet containing 0% or 40% soybean meal for 12 weeks. Differences were considered statistically significant with $\alpha \leq 0.05$. Different letters mark significant differences between treatment groups when a significant interaction is present. Error bars represent sample SEM.

trial gut microbiota samples did show significantly greater microbial richness then that of the end of trial samples ($p \leq .01$). When comparing the gut microbiota at the conclusion of the 12-week feeding trial using a two-way ANOVA, fish strain was found to have an influence on the number of observed ASVs ($p = .050$), while diet ($p = .915$) and the interaction of the two terms (Diet:Strain, $p = .129$) were found to have less of an influence on gut microbiota alpha diversity (Fig. 3).

Comparison of the overall microbial composition among all samples according to weighted and unweighted UniFrac showed a great deal of compositional overlap among all gut and water samples, with the treatment diets clustering separately, with the exception of two gut samples which showed high similarity to the dietary microbiota (Supplementary Fig. S3B). When comparing the end of trial gut microbiota samples, a great deal of overlap among treatments was still apparent (Fig. 4), though differences among groups were detected in both multivariate dispersion and centroid location according to a PERMANOVA (Table 5). Abundance weighted beta diversity showed a significant impact of diet ($p = .002$) on the overall gut microbiota composition, while beta diversity based on presence and absences of ASVs (unweighted UniFrac) showed significant effects of both strain ($p = .011$) and diet ($p = .015$), although data also suggested multivariate dispersion was not equivalent among the two dietary treatments ($p = .028$) (Table 5).

The most abundant bacterial phyla detected among all samples was Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes (Supplemental Fig. S4). At the genus level, *Cetobacterium, Clostridium, Mycoplasma, Shewanella*, *Turicibacter,* and *Weissella* were among the most abundant microbes found in the end of trial gut microbiota samples (Supplemental Fig. S5). Differential abundance analysis conducted on the end point gut microbiota samples suggested *Shewanella* to be differentially abundant by treatment, diet, and fish strain, while *Streptococcus* was different among treatments and fish strains and *Lactobacillus, Geobaccilllus,* and *Leuconostoc* were different among treatments and diet (Fig. 5). Differential abundance analysis showed that overall, dietary treatment yielded the greatest number of differential microbes (Fig. 5).

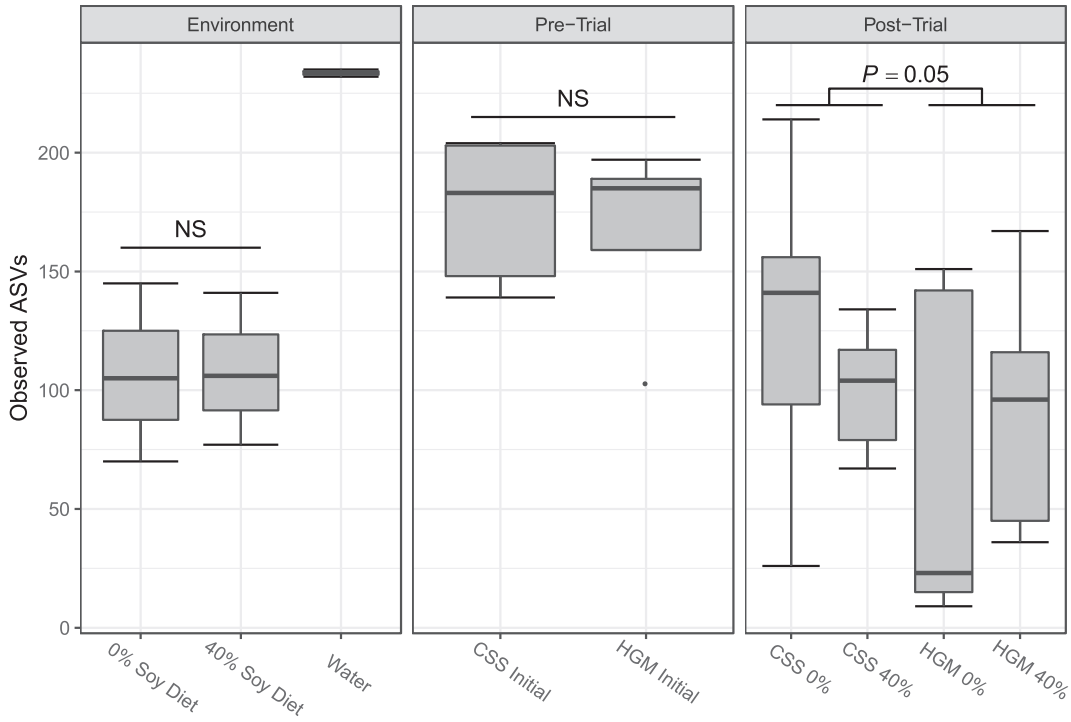

Fig. 3. Microbial richness (observed ASVs) detected among environmental (diet and water) and fish gut samples. Pre-trial samples were collected to evaluate any differences in gut microbiota between the fish strains prior to the 12-week feeding trial. Differences in alpha diversity between the diet samples and the initial pre-trial gut samples were tested by a paired *t*-test. Post-trial gut microbiota samples collected after 12-weeks were compared by fitting a two-way linear model (Diet * Strain + Diet:Strain).

4. Discussion

Regardless of diet, the HGM strain displayed superior growth compared to the CSS strain after twelve weeks (Table 3). These findings indicate the improved performance observed in previous trials with HGM strain is maintained when fish meal is replaced with SBM up to a total of 40% (Overturf et al., 2013). In addition, our histological findings also provide further evidence of improved tolerance to the inclusion of SBM for HGM strain trout in relation to the development and severity of enteritis in the distal intestine. This improvement was first reported by Venold et al. (2012), who measured expression levels of intestinal fatty-acid binding protein and enterocyte proliferation rate to determine the degree of difference in response between 4th generation HGM fish.

Table 5
Statistical analysis of beta diversity among two strains of rainbow trout, a common commercial strain of rainbow trout (CSS) and a strain selected on an all plant diet for eight generations (HGM), after being fed a diet containing either 0% or 40% soybean meal for 12 weeks.

Distance Metric	Factor	Multivariate dispersion	PERMANOVA
Weighted UniFrac	Fish strain	0.112	0.116
	Diet	0.264	***0.002***
	Strain:diet	–	0.687
Unweighted UniFrac	Fish strain	0.096	***0.011***
	Diet	***0.028***	***0.015***
	Strain:diet	–	***0.072***

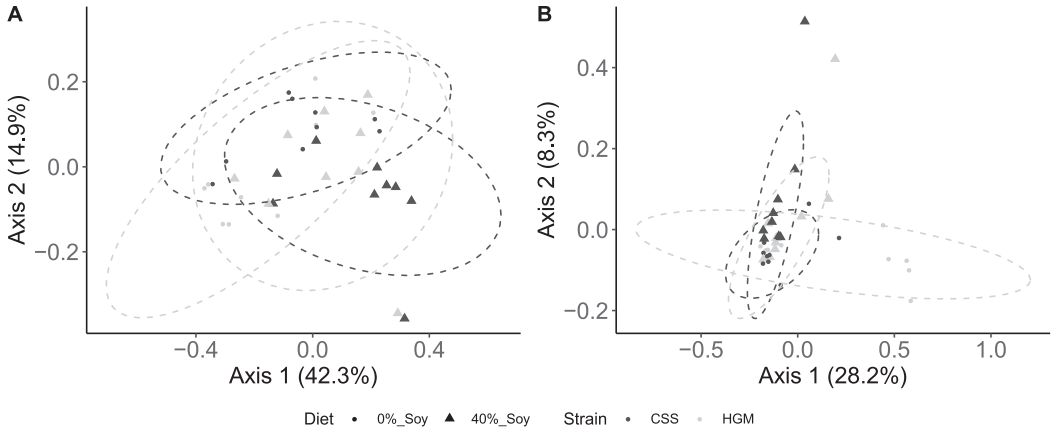

Fig. 4. Principle coordinates analysis (PCoA) plots of gut microbiota beta diversity in a common commercial strain of rainbow trout (CSS) and a strain selected on an all plant diet for eight generations (HGM) after being fed a diet containing 0% or 40% soybean meal for 12 weeks. Data are displayed according to (A) weighted and (B) unweighted UniFrac with 95% confidence ellipses.

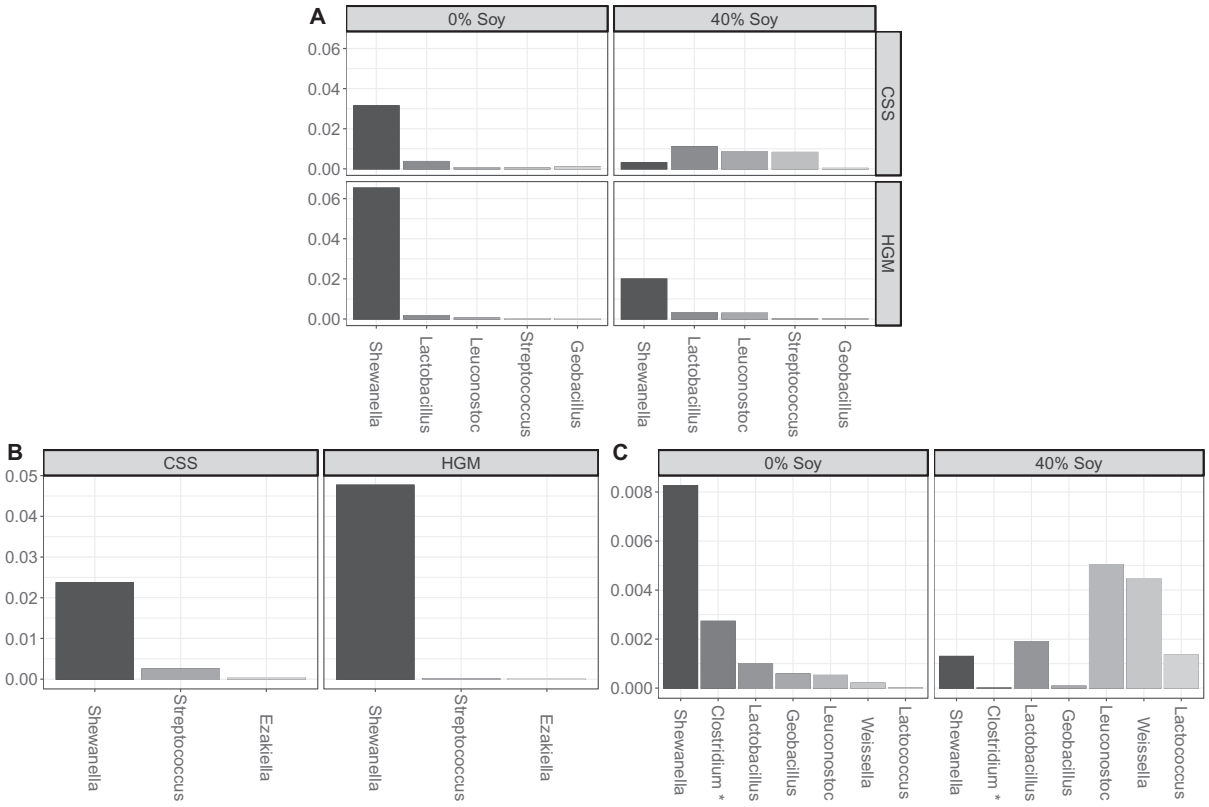

Fig. 5. Microbiota detected as differentially abundant among experimental groups. A common commercial strain of rainbow trout (CSS) and a strain selected on an all plant diet for eight generations (HGM) were fed a diet containing 0% or 40% soybean meal for 12 weeks. Statistical tests were conducted using ANCOM2 to test for differential abundance among (A) treatments (CSS0, CSS40, HGM0, HGM40), (B) fish strain (CSS vs. HGM), or (C) diet (0% Soy vs. 40% Soy). Data are displayed in units of relative abundance (%). Clostridium * - Clostridium_sensu_stricto_1.

We also sought to determine if we could detect differences in other genes related to inflammation, specifically those of the *IL*-17A/F family. Previous work has shown that *IL*-17 expression is a component of the acute mucosal inflammatory response in the distal intestine of salmonids, as well as in other fishes (Marjara et al., 2012; Krogdahl et al., 2015; Miao et al., 2018). Marjara et al. (2012) observed a 200-fold increase in the two paralogs for interleukin-17A/F2 (*IL*-17A/F2) expression in Atlantic salmon fed a diet containing 20% SBM. Additional evidence for the importance of IL-17A/F2 was reported by Krogdahl et al. (2015), reporting a $>$ 30-fold increase in *IL*-17A/F2 expression in Atlantic salmon fed a FM diet containing 10 g/kg purified soy saponin. In northern snakehead, *Channa argus*, Miao et al. (2018) observed increased expression of *IL*-10 and *IL*-17F after 63 days of feeding diets containing 47 and 70% SBM.

Rainbow trout *IL*-17A/F2 was characterized by Monte et al. (2013), where highest constitutive expression was observed in the intestine. IL-17A is recognized in higher vertebrates for its importance in inflammatory responses in mucosal tissue (Miossec et al., 2009). As the key cytokine expressed by type 17 helper T cells (Th17), the Th17 immune response is characterized by high numbers of neutrophils. The intrusion of eosinophilic/neutrophilic granulocytes is also a significant characteristic of soybean meal-induced enteritis (Baeverfjord and Krogdahl, 1996).

We observed significant differences in expression of members of the *IL*-17A/F family both between strains and levels of dietary SBM (Fig. 1). Our expression data show that the ohnologues of *IL*-17A/F2 were the first to respond to the dietary treatment, with the CSS strain showing a greater response at week 4 than the HGM strain. At week 8, both strains showed significant increases in the expression of the ohnologues of *IL*-17A/F2 in response to the 40% SBM diet. The only significant response at week 4 with either ohnologue of *IL*-17A/F1 was a minor decrease in the expression of *IL*-17A/F1b in the CSS strain fed the 0% SBM diet. At week 8, a significant increase in expression was observed with both ohnologues, with the main effect of strain being significant for *IL*-17A/F1a and both main effects significant for *IL*-17A/F1b. The pattern of expression for *IL*-17A/F3 shows similarities to the other paralogs of *IL*-17A/F, being more similar to those of *IL*-17A/F1 at week 4 and those of *IL*-17A/F2 at weeks 8 and 12.

The expression of *IL*-17A/F genes have been observed by others to increase in response to a variety of stimuli. Ribeiro et al. (2010) observed an increase in *IL*-17A/F2 gene expression in the head kidney of common carp, *Cyprinus carpio*, 3 weeks post-infection with the protozoan parasite *Trypanoplasma carassi*. Zhang et al. (2014) examined expression of *IL*-17A/F genes in zebrafish, *Danio rerio*, and turbot, *Scophthalmus maximus*, mucosal tissues in response to vaccination with live attenuated *Vibrio anguillarum*. Expression of *IL*-17A/F genes in the gut rose significantly in zebrafish and turbot administered the vaccine by bath. Expression of *IL*-17A/F2 in zebrafish peaked at over a 100-fold increase over control on day 21, but quickly decreased to approximately a 3-fold increase by day 28. *IL*-17A/F1 and 3 were also significantly increased in zebrafish, but much more modestly, peaking at approximately 3.5- and 2.5-fold, respectively. Expression of all three increased in response to a bath challenge with live *V. anguillarum*. In turbot, expression of *IL*-17A/F in the gut of fish vaccinated by bath peaked on day 14 at 20-fold over control but by day 21 expression was no different than control.

Further investigations into differences in gene expression and protein abundance between selected and non-selected strains will assist in understanding characteristics of the salmonid mucosal immune system that lead to the development of chronic distal intestinal enteritis observed in salmonids and the transitory enteritis observed in common carp and grass carp, *Ctenopharyngodon idella* (Urán et al., 2008; Wu

et al., 2018). The investigation into induction and recovery by Wu et al. (2018) found that recovery from exposure to a 40% SBM occurred by week 7 but, continued worsening in fish fed diets containing 70% SBM. They also examined expression of *IL*-17 protein in the gut and found that it was increased in the lamina propria (LP) but not in the intraepithelial lymphocytes (IEL) at weeks 3 and 5 in fish fed a 40% SBM diet but returned to pre-exposure levels by week 7. In contrast, the level of *IL*-17 protein remained elevated at week 7 in fish fed a 70% SBM diet. The expression of *IL*-10 protein was elevated in both the LP and IEL of fish fed the 40% SBM diet at weeks 3 and 5. By week 7, *IL*-10 remained elevated in the LP and returned to baseline levels in the IEL. Urán et al. (2008) reported increased expression of *IL*-10 in common carp after 1 week on a 20% SBM diet but returning to the baseline level by week 3. We also observed increased expression of *IL*-10 genes at weeks 4 and 8 in response to the 40% SBM diet, but not at week 12 (Fig. 2). This pattern is similar to observations in cyprinids and other species (Miao et al., 2018).

Considerable research has focused on the antagonism between Th17 cells and another type of T helper cell, regulatory T-cells (Tregs), which are anti-inflammatory through the action of the IL-10 (Mucida et al., 2007; Eisenstein and Williams, 2009). We sought to measure the potential activity of these two cell types through the expression of the master transcription factors associated with each (*ROR*γ and *Fox*P3), along with *IL*-10. At week 8, we observed that the expression of *ROR*γ was lower, and the expression of *Fox*P3–1, *IL*-10a/b, and several paralogs of *IL*-17A/F were higher in fish fed the 40% SBM diet (Figs. 1-2). There are several interesting observations to be made from these results. The first is that while the expression of *IL*-17 increased in fish fed the 40% SBM diet, the expression of *ROR*γ was lower. This contrasts with our observation that when there were significant differences in the expression of both *IL*-10 and *Fox*P3, that the differences were in the same direction. That is to say, when *Fox*P3 was elevated by dietary SBM, *IL*-10 tended to be elevated as well. This may be evidence that Tregs is modulating the activity of Th17 cells through the action of IL-10 (Faria and Weiner, 2005; Guo, 2016). This research adds to what is already known about the cytokine networks in teleosts, but caveats with our research and that of others requires further research in order to come to any definitive conclusions (Castro et al., 2011; Kono et al., 2011; Laing and Hansen, 2011; Secombes et al., 2011; Zou and Secombes, 2016).

One major caveat of our research is that we only sampled at 4 week intervals, when it is known that differences in expression of these cytokines can occur much sooner (Urán et al., 2008; Marjara et al., 2012; Miao et al., 2018). We observed significant effects of diet on the expression of several *IL*-17A/F genes at week 4, but we do not know how soon after introduction of the high-SBM diet that significant differences in expression of these genes first became detectable. Marjara et al. (2012) sampled fish a total of nine times over a three- week period, but in order to accomplish this they had to increase the total number of tanks, stagger their samplings, and utilize only two tanks per sample. For their gene expression analyses, they pooled their samples into composite timepoints of pre- (days 1 and 2), early (days 3, 5, and 7), and late (10, 14, 17, and 21) exposure. With this methodology, Marjara et al. (2012) were able to greatly increase their sample sizes, albeit with them being unbalanced. As we used one feeding trial to obtain both performance data and as a means to obtain tissue samples for gene expression analysis, we wanted to minimize the impact of handling stress and its subsequent effect of reducing feed intake on our performance data. That Marjara et al. (2012) were able to detect such significant changes in the expression of *IL*-17 due to dietary SBM in Atlantic salmon within days may be expressive of how much more severe the response to SBM is in this species.

Considering the stark differences in response between rainbow trout and Atlantic salmon, it would be enlightening to see if what we has been observed in these trials would be observed in Atlantic salmon, considering the severe enteritis produced in this species. Furthermore, how would different inclusion levels of SBM affect the timing and significance of *IL*-17 expression in Atlantic salmon, along with that of *ROR*γ, *Fox*P3, and *IL*-10? Such an experiment would do much to further our understanding, as 40% is about the maximal inclusion level of SBM possible in rainbow trout diets that allows for meeting their nutritional requirements and target levels of protein and fat.

Expression of *TGF*-β1a was significantly lower in the CSS strain and fish fed the 40% SBM diet at week 4, with no differences at week 8, and reduced in fish fed the 40% SBM diet at week 12 (Fig. 2). In Atlantic salmon, the decrease in *TGF*-β1a occurred within the first three days upon introduction of a diet containing 46% SBM and remained low at day 7 (Lilleeng et al., 2009). Interestingly, Marjara et al. (2012) observed the expression of *TGF*-β1a increasing in Atlantic salmon experiencing enteritis over a three-week period on a 20% SBM diet. It is important to note that several *TGF*-β isoforms are present in fishes and other vertebrates. While we looked at the expression of one of them, we cannot speculate as to the expression of other isoforms (Maehr et al., 2013).

Following previous work in our laboratory that showed an effect of high SBM diets on the expression of *S*100 genes in the distal intestine, we measured expression of *S*100I2 (Blaufuss et al., 2019). Remarkably, it followed a similar pattern to *IL*-17 genes at week 8, with both strains fed the 40% SBM diet showing increased expression. However, by week 12, expression of *S*100I2 was no longer elevated in the HGM strain fed the 40% SBM diet, while it remained elevated in the CSS strain fed the 40% SBM diet. S100I is an abundant protein in the skin and mucus of Atlantic salmon and channel catfish (Karsi et al., 2002; Easy and Ross, 2009). Data reported by Easy and Ross (2009) was the first evidence of presence of an extracellular S100 protein in a teleost. The protein they identified as ictacalcin is S100I2, and it was found to be present in mucus of both infected and noninfected fish. In channel catfish, *S*100I was the most abundant mRNA transcript detected by Karsi et al. (2002), accounting for almost 5% of all transcripts. In addition to the skin, *S*100I expression is elevated in the gills of Atlantic salmon infected with sea lice (Easy and Ross, 2009; Marcos-López et al., 2017). It is unknown how the physiological differences related to adaptations to a marine environment would affect their expression in the gut of Atlantic salmon. As the gut is a major site of calcium uptake and homeostasis in marine fish, expression patterns of the *S*100 genes may be quite different (Wilson and Grosell, 2003; Gregório and Fuentes, 2018). Therefore, differences in the severity of enteritis in response to dietary SBM between Atlantic salmon and rainbow trout provide an avenue for furthering our understanding of *S*100 gene expression in the gut.

As a result of the salmonid specific whole-genome duplication event, salmonids possess multiple copies of many of these genes (Benedicenti et al., 2015). Depending on how primers are designed, they may amplify multiple paralogs, some of which may be uncharacterized. For instance, the primers used by Marjara et al. (2012) amplified both paralogs of *IL*-17A/F2. This can complicate interpretation of current and previous results, and it's important to take this into consideration. In addition, it may be difficult or impossible to design primers that are robust and capable of amplifying individual paralogs, such as with *ROR*γ (Monte et al., 2012). Characterizing the effect of SBM inclusion on the expression of all paralogs of *IL*-17, in addition to the paralogs of other important immunogenic genes, will be required to fully understand their influence on the intestinal inflammatory response. Another open question relates to how important it is to examine each paralog. In this work, we examined the members of the *IL*-17A/F family, although there are three additional *IL*-17 members that are not as well studied (C and D). The paralogs of salmonid *IL*-17C were characterized by Wang et al. (2010a) and *IL*-17D by Kumari et al. (2009). Further investigations into differences in gene expression and protein abundance between selected and non-selected strains will assist in understanding characteristics of the salmonid mucosal immune system that lead to the development of chronic enteritis observed in salmonids and the transitory enteritis observed in cyprinids (Urán et al.,

2008; Wu et al., 2018).

Much work has been done examining microbiomes of rainbow trout (Desai et al., 2012; Ingerslev et al., 2014; Lyons et al., 2017a; Michl et al., 2017; Betiku et al., 2018). In this study, dietary treatment showed the greatest influence upon intestinal microbial differences. Strain did have an effect on microbial richness by the end of the trial. Similar to our findings, Desai et al. (2012) observed an increase in the ratio of Firmicutes to Proteobacteria in fish fed a plant-based diet. Ingerslev et al. (2014) also noted an increase after first feeding in *Leuconostoc* and *Weisella* in fish fed plant-based diets. These microbial genera were also differentially abundant in this study when diets were compared (Fig. 5C). Even so, the abundance of *Shewanella* was significantly different between strains and between dietary treatments in our results and accounted for a greater proportion of bacteria found in fecal samples. Bacterial species within the *Shewanella* genus have been previously isolated in fresh water fish, including salmonids, with the ability to produce omega-3 fatty acids (Dailey et al., 2016). Alternatively, at least one species in the *Shewanella* genus, *Shewanella putrefaciens*, has been shown to be pathogenic and often noted in farm-raised rainbow trout (Pękala et al., 2014). We did not observe any overt disease symptoms during the study that would lead to a potential association with this microbe and its abundance. Likewise, we did not measure omega-3 fatty acid content in fillets but overall, percent fat was lower in fish fed the 40% SBM diet (with a significant interaction between diet x strain) and Lipid ADCs were also lower.

Gajardo et al. (2017) observed an increase in the abundance of *Weissella* in the digesta of Atlantic salmon fed a 30% SBM diet. In a study where the microbiomes of farmed rainbow trout were found to be largely similar regardless of where fish were cultured, Lyons et al. (2017b) found *Weissella* abundance to be discriminatory between farmed and tank-raised rainbow trout. Although the relative abundance of *Weissella* (≈1%) was much less than we observed, Lyons et al. (2017b) suggests more "sporatic taxa" unique to a particular environment (such as a tank or raceway) could colonize the gut and play an important role in digestive physiology. Similar to the diverse effects from species within the *Shewanella* genus, at least one species of *Weissella*, *Weissella ceti,* has been determined to be the causative agent in outbreaks of hemorrhagic septicemia in commercial rainbow trout farms in Asia and the Americas (Liu et al., 2009; Figueiredo et al., 2012; Ladner et al., 2013; Castrejon-Najera et al., 2018). Conversely, Jesus et al. (2017) found that supplementing *Weissella cibaria* in diets for the hybrid catfish (*Pseudoplatystoma reticulatum x P. corruscans*) appeared to improve intestinal health and immune function. In this study, differential abundance of *Leuconostoc* and *Lactobacillus* in fish fed the 40% SBM diet warrants further investigation since species within these two microbial genera have been shown to enhance immune function in rainbow trout when used as probiotics (Vendrell et al., 2008).

5. Conclusions

Overall, our results highlight the importance of sampling time on the expression of *IL*-17 genes and complicates the interpretation of data related to them. Our initial hypothesis surmised that that observed improvement in enteritis observed in the HGM strain may be due to improved oral tolerance through increased activity of T-regulatory cells. Our current findings provide further evidence for the involvement IL-17 associated with distal intestine enteritis, but many questions remain regarding the differences in response between different species and between different strains within a species.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735287.

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