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A nutrigenomic analysis of intestinal response to partial soybean meal replacement in diets for juvenile Atlantic halibut, *Hippoglossus hippoglossus*, L.

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ABSTRACT

Aquaculture feeds for carnivorous finfish species have been dependent upon the use of fish meal as the major source of dietary protein; however, the increasing demands upon the finite quantity of this high-quality protein source requires that feeds become increasingly comprised of alternative plant and/or animal protein. Soybean meal has been has been used to partially replace fish meal in the diets of several fish but it is known to cause enteritis in Atlantic salmon, *Salmo salar*. We have compared two groups of juvenile (207.2 ± 6.6 g) Atlantic halibut, *Hippoglossus hippoglossus*, L., fed diets containing fish meal (FM; control) or 30% soybean meal (SBM; experimental) as a protein source for 3 weeks. No detectable difference in feed intake or palatability was evident with the SBM diet relative to the FM diet. Histological examination of the distal intestine was performed to examine leukocyte infiltration of the lamina propria and other changes in morphology commonly observed with soybean-induced enteritis of salmonids. No significant difference was found between fish fed the FM and SBM diets. Global gene expression profiling performed using a high-density oligonucleotide microarray containing 9260 unique features, printed in quadruplicate, from Atlantic halibut revealed subtle underlying changes in the expression of several immune genes and genes involved in muscle formation, lipid transport, xenobiotic detoxification, digestion and intermediary metabolism. These results indicate that SBM can be used successfully as a replacement for animal protein in diet for juvenile Atlantic halibut, although long-term effects on the immune system may ensue.

1. Introduction

Atlantic halibut (*Hippoglossus hippoglossus* L.) is a highly valued food fish and it shows good potential for coldwater aquaculture (Berg, 1997; Mangor-Jenson et al., 1998). Although some progress has been made on diet development for halibut, information on the nutritional requirements of most coldwater marine fish including halibut is limited. Proteins and their constituent amino acids are essential components of marine fish diets. The dietary protein requirements of coldwater marine and salmonid finfish for maximum growth generally range from 40–55% assuming a sufficient and appropriate supply of available energy. Flatfish, such as plaice (Cowey et al., 1972) and turbot (Danielssen and Hjertnes, 1993) require 50% dietary protein and several studies have determined the optimal dietary

protein levels for halibut to be similar, although somewhat higher amounts (~60%) are required by younger fish (Aksnes et al., 1996; Grisdale-Helland and Helland, 1998; Helland and Grisdale-Helland, 1998; Hamre et al., 2003). In general, smaller fish require more dietary protein and are more sensitive to dietary carbohydrates (Hamre et al., 2003; Hatlen et al., 2005).

Fish meal (FM) is a major source of protein in fish feeds. However, the increasing demands of the world's aquaculture production upon the finite quantity of this high-quality protein source necessitates that fish feeds become increasingly comprised of alternative economical and highly digestible protein sources of plant and/or animal origin that support similar fish performance and concurrently have little or no adverse effects upon the environment. Numerous studies have investigated the potential of alternate plant proteins, particularly soybean meal (SBM) and canola meal or their concentrates in rainbow trout and other salmonid fish diets (see Higgs et al., 1995; Storebakken et al., 2000 for reviews). Research conducted in the past two decades has

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established the maximum amounts of some plant protein sources that carnivorous fish can tolerate and the negative effects of their antinutritional factors or toxicants (depending upon the source some or most of the following may be present i.e., protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, alkaloids, tannins, cyanogens, glucosinolates, etc.) that adversely affect digestion, absorption and physiological utilization of protein and amino acids. Some differences in the anatomy of the digestive tract of flatfish, cod and salmonids exist; however, the effects of dietary protein sources of plant and animal origin and physiological differences among these fish on nutrient absorption mechanisms from the digestive tract are not known. The site of lipid and protein absorption in the digestive tract of turbot appears to be the hindgut and rectum where lipolytic activity is relatively high (Koven et al., 1997). However, in halibut fat absorption occurred to a greater extent in the anterior part of the intestine and may be linked to a few pyloric caeca located in the anterior part of the digestive tract (Martins et al., 2009).

Soybean protein has been used successfully for some species such as Atlantic cod, *Gadus gadus* (Hansen et al., 2006; Refstie et al., 2006b), Indian carp, *Cirrhinus mrigala* (Jose et al., 2006), three genera of catfishes (Usmani et al., 2003; Evans et al., 2005), yellowtail, *Seriola quinqueradiata* (Shimeno et al., 1997), Japanese flounder, *Paralichthys olivaceus* (Kikuchi, 1999), and Egyptian sole, *Solea aegyptiaca* (Bonaldo et al., 2006). In some species, morphological changes have been noted in fish fed high levels of soy protein. These include the liver of Asian sea bass, *Lates calcarifer* (Boonyaratpalin et al., 1998) and mangrove red snapper, *Lutjanus argentimaculatus* (Catacutan and Pagador, 2004) and distal intestine of sea bass, *Dicentrarchus labrax* (Penn et al., 2007) and carp (Uran et al., 2008).

In salmonids, inflammation of the distal intestine (van den Ingh et al., 1991; Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996) and ulcer-like lesions in the stomach (Refstie et al., 2006a) caused by antinutritional factors in the plant protein has presented problems such as reduced intestinal absorptive ability and increased disease susceptibility (Krogdahl et al., 2000; Bakke-McKellep et al., 2007a). Proliferation of distal intestine enterocytes of SBM-fed Atlantic salmon, *Salmo salar* was observed using antibodies against PCNA (Sanden et al., 2005) and changes in trypsin activity and gene expression have also been observed in both Atlantic salmon (Krogdahl et al., 2003; Lilleeng et al., 2007b) and rainbow trout, *Oncorhynchus mykiss* (Romarheim et al., 2006). In addition to morphological studies, analyses of immunological (Bakke-McKellep et al., 2000, 2007a), metabolic and hormonal factors (Bakke-McKellep et al., 2007b) have been performed to investigate the salmonid response to soy protein. Proteomics has also been used to analyse global changes in protein expression in response to the introduction of soy protein diet (Martin et al., 2003) or other plant protein diet (Vilhelmsson et al., 2004).

Studies with Atlantic halibut have shown that up to 36% full-fat SBM can be added to the diet without adversely affecting growth, feed efficiency or intestinal histology (Grisdale-Helland et al., 2002). Inclusion of 28% soy protein concentrate also did not affect growth or protein digestibility but feed utilization was lower (Berge et al., 1999). Although the morphology of the Atlantic halibut digestive system has been well-characterized (Murray et al., 1993, 1994, 1996) as has the ontogeny of digestive enzyme production (Gawlicka et al., 2000; Murray et al., 2006), the effect of feeding non-fish based diets on gut histology has only received limited attention (Grisdale-Helland et al., 2002) and there are no reports of changes in intestinal gene expression in this species in response to the inclusion of plant protein in the diet. In this study we use a combination of morphological observations and nutrigenomics with a custom-made Atlantic halibut oligonucleotide microarray to assess changes in intestinal gene expression in juvenile Atlantic halibut over the first 3 weeks after introduction of a diet containing SBM.

2. Materials and methods

2.1. Fish rearing

Atlantic halibut juveniles (average weight 207.2 ± 6.6 g) were cultured at Scotian Halibut Ltd. (Clarks Harbour, Nova Scotia, Canada) on October 18, 2006. Each of six tanks was stocked with 42 fish and the fish raised under constant incandescent light (approximately 1000 lx at the surface) in 0.26 m^3 tanks with flow-through oxygenated salt water (30 ppt) maintained at 11 ± 0.2 °C using a heat exchanger. The halibut were hand fed to satiation on a commercial FM diet (North East Nutrition Inc., Truro, NS, CAN) three times daily from the time of metamorphosis until the initiation of the trial. On October 24, 2006, the SBM diet was introduced to fish in three randomly assigned tanks whereas FM diet was introduced to fish in the three remaining tanks. Sampling of fish tissues occurred after 1, 10 and 21 days. The mean weight of the fish in each tank was calculated at the beginning and the end of the trial and medians for each tank used to determine weight gain. Feed consumption weights (amount of food administered), oxygen saturation and temperature were measured daily. All animals were maintained and sampled according to the guidelines set by the Canadian Council of Animal Care (Olfert et al., 1993).

2.2. Diet composition

We have used two experimental diets and, since information on the amino acid requirements of halibut is unavailable, formulation of these diets was based on the amino acid requirements of salmonids (NRC, 1993). The FM and SBM diets were isonitrogenous and isocaloric, containing 50% total protein and 22% total lipid (Table 1). In addition, the fibre and carbohydrate compositions were designed to be similar. The two diets differed in the partial replacement of FM with SBM at an inclusion level of 30%. Solvent-extracted dehulled SBM was used. Dry ingredients of the diets were finely ground (<800 μm) using a Perten Laboratory Mill (Model 3100, Perten Instruments, Huddinge, Sweden) before being combined with the liquid ingredients

Table 1
Formulation of the experimental diets (as-fed basis).

	Fish meal diet (g/kg)	Soybean meal diet (g/kg)
Ingredient		
Fish meal[a]	640.0	472.0
Soybean meal (dehulled)[b]	0.0	300.0
Wheat middlings[c]	150.0	0.0
Whey[d]	30.0	30.0
Fish oil[e]	152.0	170.0
Vitamin mixture[f]	18.0	18.0
Mineral mixture[g]	10.0	10.0
Calculated analysis		
Crude protein	503.5	499.9
Lipid	220.8	220.5
Fibre	16	13
Carbohydrates	183	199
Gross energy (MJ/kg)	22.0	22.1

[a] Scotia Garden Seafood Incorporated, Yarmouth, NS.
[b] Bunge Canada, Oakville, ON.
[c] Walker's Livestock Feeds, Dartmouth, NS.
[d] Farmer's Co-operative Dairy Ltd., Truro, NS.
[e] Stabilized with 0.06% ethoxyquin. Commeau Seafood, Saulnierville, NS.
[f] Vitamin added to supply the following (per kg diet): vitamin A, 8000 IU; vitamin D3, 4500 IU; vitamin E, 300 IU; vitamin K$_3$, 40 mg; thiamine HCl, 50 mg; riboflavin, 70 mg; d-Ca pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin B$_{12}$, 0.15 mg; niacin, 300 mg; pyridoxine HCl, 20 mg; ascorbic acid, 300 mg; inositol, 400 mg; choline chloride, 3000 mg; butylated hydroxy toluene, 15 mg; butylated hydroxy anisole, 15 mg.
[g] Mineral added to supply the following (per kg diet): calcium phosphate (mono), 6000 mg; manganous sulphate (32.5% Mn), 40 mg; ferrous sulphate (20.1% Fe), 30 mg; copper sulphate (25.4% Cu), 5 mg; zinc sulphate (22.7% Zn), 75 mg; sodium selenite (45.6% Se), 1 mg; cobalt chloride (24.8% Co), 2.5 mg; sodium fluoride (42.5% F), 4 mg.

(choline chloride and herring oil). Micronutrients (vitamins and minerals) were pre-mixed with ground wheat as a base, using a twin-shell blender (Paterson-Kelly, East Stroudsburg, PA, USA) prior to being added to the main ingredient mixture. All ingredients were mixed in a Hobart mixer (Model H600T, Rapids Machinery Co., Troy, OH, USA) and steam-pelleted into 5.0 mm pellets (California Pellet Mill Co., San Francisco, CA, USA). The pellets were dried in a forced-air drier at 80 °C for 90 min to form dry, sinking pellets and stored in air-tight containers at −20 °C until use. Diets were screened to remove fines prior to feeding.

2.3. Sampling

Five individual fish from each of the six tanks (three experimental, three control) at each of the three sampling times were euthanized with an overdose of TMS-Aqua MS-222 (Syndel, Vancouver, BC, CAN). Fork length and total weight were measured prior to dissection. Subsequently, the body cavity was opened and the gastrointestinal tract was partially removed and photographed to determine any gross morphological changes and to assess the presence of food in the gut.

The distal intestine was dissected away from the anal pore for ease of sampling and divided longitudinally into two pieces: half of the tissue from a given fish was kept for gene expression analysis and half for histological examination. Similarly, an area was chosen approximately half way between the pyloric sphincter and the rectal/intestinal junction for sampling of mid intestine and divided longitudinally into two pieces (Fig. S1 in the Appendix). All tissue samples for gene expression studies were preserved in RNALater (Ambion, Austin, TX, USA) and stored at −80 °C until use. Samples for histology were fixed overnight at 4 °C by immersion in 4% (v/v) paraformaldehyde in Tris-HCl (pH 7.8), and processed for paraffin embedding (Murray et al., 2006). For this study, intestine samples were used from three separate fish from each sampling point.

2.4. Microscopy

Three separate fish from each of the six tanks sampled at each of the three time points were analysed by microscopy. Paraffin-embedded distal and mid intestine were serially sectioned at 7 µm, mounted on uncoated glass slides, dried briefly and then incubated overnight at 60 °C to enhance adherence to the slides. For general histological examination, sections were deparaffinized, rehydrated and then stained with hematoxylin and eosin using standard procedures. Eight slides were prepared from each fish, each containing approximately 8 contiguous serial sections. Each slide was examined for preservation of structure, thickness of lamina propria, vacuolation of enterocytes and extent of leukocyte infiltration into the mucosa of the intestinal folds.

The extent of leukocyte infiltration was noted for each section per slide. Those sections that contained less than 5 leukocytes per 0.35 mm^2 (field of view for 20× objective) were classified as low and given a numerical score of 1, those with 5–15 were classified as medium and given a numerical score of 3, and those more than 15 were classified as high and given a numerical score of 5. Scores from each set of slides were averaged and used in statistical analysis. Images were taken of representative sections.

2.5. RNA extraction

One fish from each of the six tanks sampled at each time point was used for RNA extraction. Tissue samples (approximately 0.1 g each) were homogenized in 1.5 ml of Trizol (Invitrogen, Burlington, ON, CAN) using a Polytron on setting 5 until the sample was turbid and no large material was observed. Extraction of total RNA was performed as recommended by the manufacturer, with a final resuspension of the RNA pellet in 75 µl of RNAse-free water. A 2 µl aliquot was quantitated

using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and 100 µg was processed using the RNeasy kit (Qiagen, Mississauga, ON, CAN), finally eluting in 75 µl of RNAse-free water. The integrity of all RNA samples was determined using a BioAnalyser (Agilent Technologies, Mississauga, ON, CAN) and samples were stored at −80 °C until use.

2.6. Microarray experiments

Eighteen slides in total, representing three individual control fish and three individual experimental fish from each of the three time points, were analysed (Fig. 1). cDNA was prepared from 1 µg of total RNA from each individual using the Array 900 kit (Genisphere, Hatfield, PA, USA) and labeled with Alexa Fluor™ 647 dye. This was hybridized with cDNA prepared from 1 µg of a pooled universal RNA consisting of equal amounts of RNA from five developmental stages from hatching until post-metamorphosis and labeled as above with the Alexa Fluor™ 546 dye. Pooled universal RNA is recommended for fish microarray studies (Cossins et al., 2006) to account for their high genetic variability. The formamide-based hybridization buffer was used and hybridization to an Atlantic halibut oligonucleotide array (Douglas et al., 2008) was performed overnight at 43 °C. Capture reagents #1 and #2 (2.5 µl of each) were added in 60 µl of hybridization buffer and the microarray incubated at 52 °C for 4 h.

After washing, microarrays were scanned at 543 and 633 nm using a ScanArray® 5000XL Microarray Acquisition scanner (Packard Bioscience, Billerica, MA, USA) at a resolution of 10 µm. Laser power was set at levels between 80 and 100% and photomultiplier tube settings were set at values ranging from 70 to 95% to adjust Alexa555 and Alexa647 channels on individual slides. Spot intensities were measured using SpotReader version1.3 (Niles Scientific, Portola Valley, CA, USA) software, and the CSV files loaded into the ArrayPipe (Hokamp et al., 2004) server at the Institute for Marine Biosciences.

Markers (blanks and those containing Arabidopsis controls) were flagged and the remaining spots were corrected for background using the "limma normexp BG correction" option. Background-corrected spots were normalized using the "limma loess (subgrid)" option and data from quadruplicate spots were merged. Data from the triplicate arrays from each diet and each time point were separately merged and the "limma eBayes mod *t*-test (within group)" was applied to test
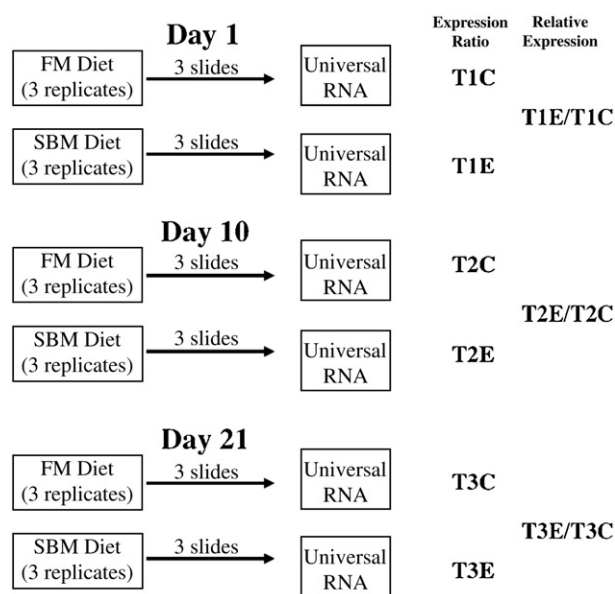

Fig. 1. Summary of microarray experiments conducted using RNA from juvenile fed FM diet compared to SBM diet and sampled 1, 10 and 21 days after initiation of feeding SBM diet to experimental animals.

for significant spots. Spots with *P*-values <0.05 in either the control FM diet group or the experimental SBM diet group, and fold changes <−2 or >+2 in both groups were retained for further analysis.

Significance Analysis of Microarrays (SAM; Tusher et al., 2001) was also performed for each set of triplicate arrays in order to select differentially expressed genes that were statistically significant. The false discovery rate was set at <1%.

Average fold change values for each spot for fish fed the control FM diet relative to the universal reference RNA (T1C, T2C, T3C) and for fish fed the experimental SBM diet relative to the universal reference RNA (T1E, T2E, T3E) were compared with one another to obtain a relative expression value for the experimental SBM diet versus the control FM diet (see Fig. 1; T1E/T1C, T2E/T2C, T3E/T3C). This comparison was performed for each of the three time points, thereby yielding a list of genes that were differentially expressed in response to the experimental SBM diet at each time point.

The relative expression values were entered into the Multiple Expression Viewer (Saeed et al., 2003) in order to cluster genes according to their expression values over the three time points. The KMC (K-means/K-medians Clustering) module was used with the following parameters: medians calculated, 50 maximum iterations, and Euclidean distance. The number of clusters was varied between 5 and 10 and expression graphs assessed for each. Figure of Merit calculations were performed to obtain a measure of fit for the KMC clusters. The maximum number of clusters was set to 20 and the maximum number of iterations was set to 50. The KMS (K-means/K-medians Support) module was also run with a threshold of 80% to assess the significance of the consensus clusters.

2.7. Quantitative RealTime-PCR (qRT-PCR)

Validation of microarray data was performed using qRT-PCR on one representative gene from each of the seven clusters identified above. Primers (approximately 20mers) were designed based on the Atlantic halibut EST corresponding to the microarray spot, using PrimerQuest[SM] software (www.idtdna.com/Scitools/Applications/Primerquest/) and synthesized by Integrated DNA Technologies (Toronto, ON, CAN). Amplicon sizes were set to 140–160 bp and the optimum Tm was set to 58 °C (Table 2). Two housekeeping genes that were found to be stable in expression levels over a variety of tissues in Atlantic halibut (Infante et al., 2008), elongation factor 1 alpha (EF1A1) and 40S ribosomal protein S4 (Rps4), were used as reference genes for all normalizations of expression levels. First-strand cDNA was generated from 1 μg of the

same RNA samples as used in the microarray experiments using Super-script III First-strand Synthesis Super mix (Invitrogen). All qRT-PCR reactions were performed in a 20 μl total reaction volume (18 μl master mix and 2 μl PCR product/cDNA template). The master mix contained 6.4 μl H_2O, 0.8 μl of each primer (0.4 μM final concentration), and 10.0 μl of the SYBR Green mix (Roche Applied Science, Laval, PQ, CAN). The same cycling parameters were used for all tested genes: (i) denaturation, 5 min at 95 °C; (ii) amplification repeated 40 times, 10 s at 95 °C, 10 s at 55 °C, 15 s at 72 °C with ramp rate of 4.4, 2.2 and 4.4 °C/s, respectively; (iii) melting curve analysis, 1 min at 95 °C, 1 min at 55 °C with ramp rate of 4.4 and 2.2 °C/s, respectively, then up to 95 °C at a rate of 0.1 °C/s; (iv) cooling, 10 s at 40 °C, with ramp rate of 2.2 °C/s. Each sample was analysed in duplicate and reactions were performed in a Light Cycler (Roche). Crossing-point (Cp) values were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool (REST) 384 v. 2 (Pfaffl et al., 2002).

2.8. Statistical analysis

The data were analysed statistically to determine if there were any differences in descriptive parameters, *i.e.*, initial weight, weight gain and feed consumption over the experimental period. Because of limited sample sizes, we performed a robust two-sided Wilcoxon's signed rank test of the hypothesis that the differences in initial weights as well as weight gains come from a distribution whose median is zero. Feed consumption per tank was compared using a Bonferroni corrected *t*-test for multiple comparisons where the average consumption in each tank was compared to the average consumption in every other tank. The null hypothesis tested was that there was no difference in the consumption of feed by fish in any of the six tanks regardless of the feed type. 95% confidence intervals were calculated with 23 degrees of freedom for this test statistic, such that, $CI = \bar{x}_i - \bar{x}_j \pm t_{\alpha/(k(k-1)/2)} \times \frac{s}{\sqrt{n}}$, for all $i = 1, 2, \ldots k$ and $j = 1, 2, \ldots k$ tanks. $\bar{x}$ represents the mean and s is the pooled estimate of the standard deviation between n measurements in any two tanks. The significance level, α, was set at 0.05 while k, the number of tanks was 6. Statistical analyses of the scores for leukocyte infiltration were performed using the Mann–Whitney non-parametric test. Single factor ANOVA of leukocyte infiltration scores was used to assess tank effects between the three replicate tanks for control and experimental diets.

3. Results

3.1. Fish performance

As seen from Table 1, both of the diets were isonitrogenous (50%), isolipidic (22%) and isoenergetic (22 MJ kg^{-1}). A Wilcoxon signed rank test showed that the median weight gain of the fish (Table 3) from the SBM-fed group (39 g) was not significantly different from that of the FM-fed group (40 g). The median daily consumption was 26 g per tank per day for both groups of fish. From the results of the Bonferroni corrected *t*-test, the 95% confidence interval for all tests straddled zero implying that there was no significant difference in

Table 2
Primer sequences for genes selected for qRT-PCR analysis.

Cluster	Gene	Primer sequence (5′>3′)	Amplicon size (bp)
A	Creatine kinase muscle isoform 1	F: AGCAGACGGAGTAAGAATCGCA R: AGCACCTTGGACATGTGGTT	124
B	Similar to Aquaporin 8	F: ATCAGTGGCTCCCACTTCAA R: ATCTGCAGGGCACATCATCT	140
C	β-2 microglobulin	F: AGCTTGGTGAGTGGACTTCTTC R: TGTTGTTTGGTTCTGGACAGGC	150
D	Adenylate kinase	F: TCTAGAGCCACACGACAAGAGAGA R: TTGCCACTATCTTCTCACACTGGG	147
E	Fast skeletal muscle troponin T	F: GTTCTGCCTCATCAGTCTCTCCAT R: GCAAGCAACTCAACATCGACCATC	128
F	Cytochrome P450 3A40	F: ATGGAAGAGGATTCGCAGTGT R: TTCATCCGCAGCGACTTTCTT	130
G	α-2-macroglobulin	F: TAAAGACGTACGCCAACAGAGC R: TGCTTACATCACTGCCGGTTTC	140
HKG	Elongation factor 1A1	F: AAGAGGACCATCGAGAAGTT R: GTCTCAAACTTCCACAGAGC	141
HKG	Ribosomal protein S4	F: GCCAAGTACAAGCTGTGCAA R: AGGTCGATCTTGACGGTGTC	138

HKG, housekeeping gene.

Table 3
The performance of Atlantic halibut fed the experimental diets.[a]

	Fish meal diet	Soybean meal diet
Median initial weight (g)	206	210
Median weight gain (g)	40	39
Median feed cons. (g)	26	26
FCR[b]	0.65	0.67

[a] No significant differences between treatments.
[b] FCR, Feed conversion ratio = feed consumption/wet weight gain.

feed consumption between any of the tanks. Feed conversion ratios were also not significantly affected by the dietary treatments and both the diets were efficiently utilized during this short-term study.

3.2. Microscopy

Sections through the distal intestine of fish fed SBM and FM diets are shown in Figs. 2 and 3. The lamina propria appeared to be marginally narrower in the fish fed FM diet but there did not appear to be any difference in vacuolation of the enterocytes. Leukocytes appeared as small ovoid-shaped cells with dark-stained nuclei. In both cases, there are areas with low, medium and high numbers of
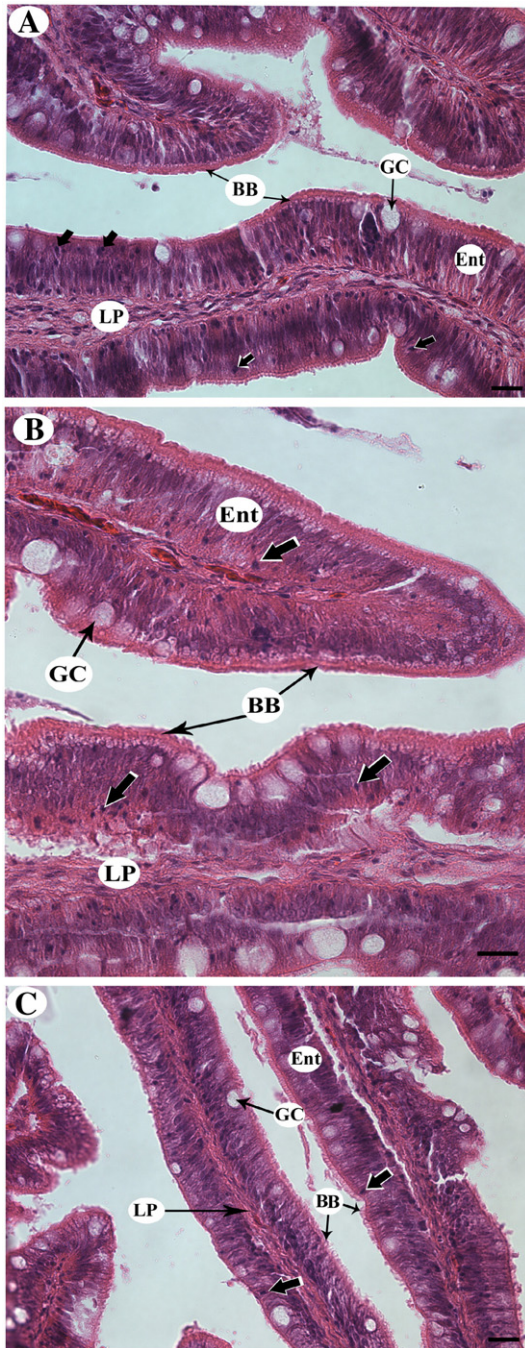

Fig. 2. Sagittal sections through the distal intestinal villi from an Atlantic halibut fed SBM diet showing relative abundance of leukocytes (thick arrowheads) within the enterocyte layer. (A) High relative abundance; (B) medium relative abundance; (C) low relative abundance. BB, brush border; GC, goblet cell; LP, lamina propria; Ent, enterocyte layer.

Fig. 3. Sagittal sections through the distal intestinal villi from an Atlantic halibut fed FM diet showing relative abundance of leukocytes (thick arrowheads) within the enterocyte layer. (A) High relative abundance; (B) medium relative abundance; (C) low relative abundance. BB, brush border; GC, goblet cell; LP, lamina propria; Ent, enterocyte layer.
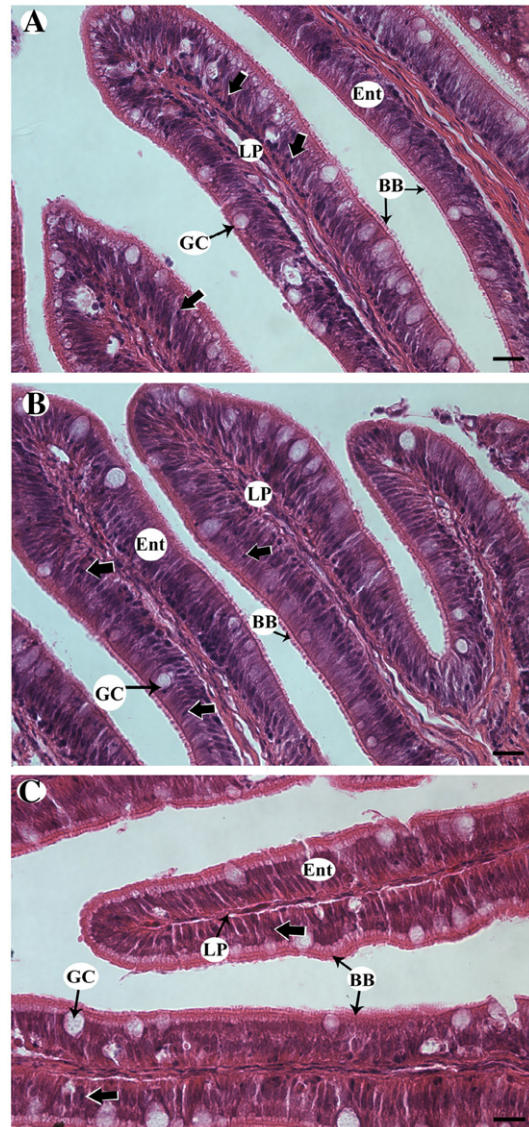
leukocytes in the enterocyte layer, but statistical analysis of the scores for leukocyte infiltration showed no significant difference between the FM-fed and SBM-fed fish at all three time points ($P<0.3$, $\alpha=0.05$) and no significant tank effects ($P<0.5$, $\alpha=0.05$) in all three control and experimental tanks (Fig. 4). Sections through the mid intestine showed very low numbers of leukocytes in all fish in the first two time points; only one control and two experimental fish exhibited low to medium leukocyte infiltration at the last time point (data not shown).

3.3. Microarray analysis of gene expression

Since there was no evidence of leukocyte infiltration in the mid intestine, microarray analysis was performed only on distal intestine. Three replicate microarrays were performed for each sampling time and diet, as in past studies using this microarray (Douglas et al., 2008; Murray et al., in press). Approximately 100 μg of total RNA was obtained from each sample and RIN values were generally over 8.5. Three samples had lower values (7.4–7.8).
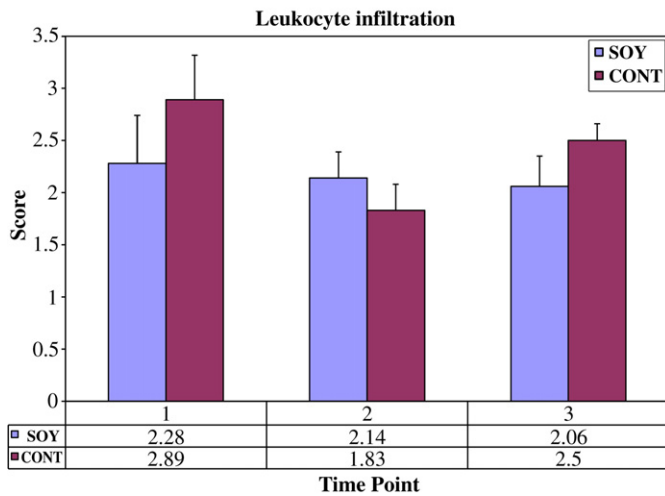
Fig. 4. Leukocyte infiltration in distal intestine of Atlantic halibut at three different timepoints after introduction of SBM diet. 1, day 1; 2, day 10; 3, day 21.

Of the 39,936 spots on the Atlantic halibut microarray, approximately 2500–10,000 passed quality control for the 18 microarrays used in the analysis (Table 4). After merging data from quadruplicate spots and data within triplicate arrays, approximately 3000–5500 were retained for the control FM and experimental SBM samples at each of the three time points. Of these, approximately 600 were significantly differentially expressed in the three replicate arrays using the limma eBayes mod *t*-test (*P* value of <0.05) in either the control FM or experimental SBM samples at each time point, whereas approximately 400 had a *P* value of <0.05 in both the control FM and experimental SBM samples. In general, there were more genes that were >2 fold up-regulated than down-regulated in SMB compared to FM samples.

Of the approximately 600 spots that had a *P* value of <0.05 in either the control FM or experimental SBM samples at each time point, 243 were common to all three time points. Using the K-medians module of MeV, seven main clusters were resolved and retained for qPCR validation (Fig. 5). These consisted of two clusters that were up-regulated over time to greater (Cluster G; 10 spots) and lesser

Table 4
Numbers of spots passing quality control for each microarray at different stages of data analysis.

Array	Background Corr/Norm	Merged	Common to TM1, TM2 or TM3	P<0.05	>2 Up	>2 Down	Common to TM1, TM2, and TM3
TM1-C TM1-C TM1-C	6698 10,767 5692	5544					
TM1-Ex TM1-Ex TM1-Ex	5376 4952 5000	3412	TM1	626	162	102	
TM2-C TM2-C TM2-C	3776 6185 4401	3548					
TM2-Ex TM2-Ex TM2-Ex	4683 5631 9496	4586	TM2	676	189	96	243
TM3-C TM3-C TM3-C	5086 2497 4388	2989					
TM3-Ex TM3-Ex TM3-Ex	6143 4865 7426	4511	TM3	622	157	140	

Abbreviations: C, control FM diet; Ex, experimental SBM diet; TM, time point after introduction of experimental diet; P, probability eBayes value.

(Cluster C; 39 spots) extents and two clusters that were down-regulated over time to greater (Cluster E; 28 spots) and lesser (Cluster A; 63 spots) extents. In addition, there were two clusters that were up-regulated at 10 days relative to the other two time points to greater (Cluster F, 23 genes) and lesser (Cluster D; 35 genes) extents, and one cluster that remained largely unchanged over time (Cluster B; 45 genes). The results of KMS showed that clusters E, F and G were present in 80% of the runs. Approximately half of the microarray spots in each cluster corresponded to known annotated genes (Table 5A, 5B, 5C, 5D).

3.4. qRT-PCR validation of candidate differentially expressed genes

Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis. Both EF1A and Rps4 housekeeping genes showed similar levels of expression in both diet conditions at all three times (average 20.2 and 20.18 Cp, respectively) relative to the universal control (18.23 and 19.88 Cp, respectively). This justified the use of both EF1A and Rps4 as reference genes for the REST analysis.

For all seven candidates, both qPCR and microarray data were consistent in terms of up- or down-regulation of genes in fish fed the two diets compared to the universal control (Table 6). Aquaporin, β-2 microglobulin and cytochrome P450 3A450 showed high levels of expression in fish fed either of the two diets relative to the universal control, whereas the other genes showed very low levels of expression relative to the universal control.

4. Discussion

Following feeding of the 30% SBM-containing experimental diet for 3 weeks, there was no statistically significant difference in food consumption or weight gain compared to fish fed the FM diet. This result is in agreement with previous studies with Atlantic salmon (Hemre et al., 1995; Heikkinen et al., 2006; Bakke-McKellep et al., 2007b), cod (Forde-Skjaervik et al., 2006), sea bass (Tibaldi et al., 2006), Egyptian sole (Bonaldo et al., 2006), channel catfish, *Ictalurus punctatus* (Evans et al., 2005), Japanese flounder (Kikuchi, 1999; Choi et al., 2004), Senegalese sole, *Solea senegalensis* (Aragão et al., 2003), and turbot, *Scophthalmus maximus* (Day and Gonzalez, 2000) where no significant changes in growth were detected over the trial periods.

In contrast to salmonids, which show marked shortening of villous folds, abnormal vacuolation of enterocytes, and thickening of the lamina propria and submucosa when fed full-fat or solvent-extracted plant SBM (Heikkinen et al., 2006; Bakke-McKellep et al., 2007b), the distal intestine of halibut fed the SBM diet did not show significant histological changes. This is consistent with results obtained by (Grisdale-Helland et al., 2002), also with Atlantic halibut. Similarly, the massive infiltration of leukocytes in salmon intestine evident after ingestion of SBM-containing diets (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003), was not observed with halibut. A sub-acute inflammatory response in the distal intestine of Atlantic salmon and rainbow trout is often associated with reduced growth performance and nutrient utilization, as well as diarrhea in a dose-dependent manner (Baeverfjord and Krogdahl, 1996). These effects were not found in the present study; however the duration of the study was relatively short to study the effects of dietary changes on fish performance. In salmonids, the inflammation is histologically detectable in a matter of days following exposure and also recedes quickly following removal of SBM from the diet (Baeverfjord and Krogdahl, 1996). Recent studies have demonstrated the involvement of a mixed population of T lymphocytes and increased numbers of epithelial cells undergoing apoptosis, proliferation and stress responses in the affected distal intestine (Bakke-McKellep et al., 2007a,b). However, the aetiological agent or agents in soybeans causing the enteropathy is unknown. It is obvious that species differences exist between halibut and salmonid fishes in response to SBM.
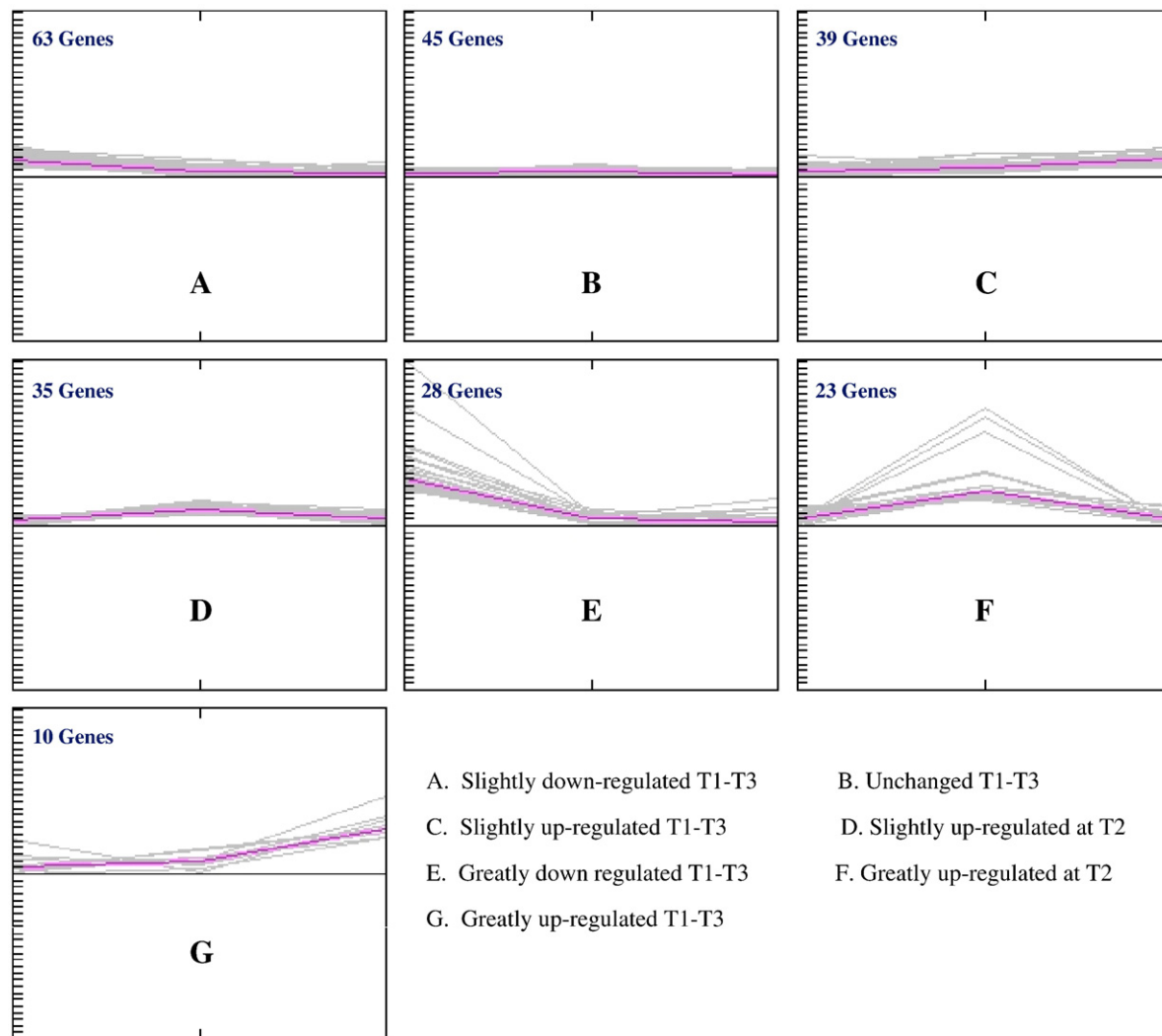
Fig. 5. Seven clusters resolved by MeV that were used for subsequent qPCR validation. Grey lines represent relative intensity values for experimental SBM diet versus control FM diet at three different time points. The pink line represents the centroid line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Although there were no obvious histological effects in the distal intestine, differential gene expression indicates that physiological responses do occur when SBM is added to the feed. Two clusters of genes were down-regulated over time and were involved in muscle structure and physiology (myosin, tropomyosin, troponin, parvalbumin, muscle creatine kinase, atp2A, titin N2-B, muscle-specific protein, lamin B2, catenin alpha E, sarcoplasmic/endoplasmic reticulum calcium ATPase 1A). qPCR analysis confirmed the very low transcript levels of two representative genes, creatine kinase and troponin 2. A decrease in muscle protein and myosin heavy chain mRNA in cod fed SBM (von der Decken and Lied, 1993) before there were any measurable changes in growth rate has also been noted. It is quite likely that the muscle proteins detected in our study were involved in intestinal motility rather than skeletal muscle growth. Our microarray analysis also showed that genes for a number of proteins involved in protein turnover and folding and cell growth were down-regulated. These included aurora kinase A-interacting protein, HIRA-interacting protein 5, protein disulfide isomerase A2, translation elongation factor 1 delta, cathepsin L, carboxypeptidase A2, elastase A, UMOD zymogen granule membrane protein, proteinase inhibitor, apoptosis inhibitor, plasminogen, FK506 binding protein 1b, evectin, Rab5 GDP/GTP exchange factor, 26S protease regulatory subunit 7, MADP-1, MAD2A and MAD2B. Two spots for warm-temperature-

acclimation-related-65 kDa-protein, which is involved in iron homeostasis, and one for transferrin, which transfers iron from the intestine to proliferating cells were also down-regulated. This generalized down-regulation of protein synthesis and cell growth, particularly in muscle, is in agreement with the finding in soybean-fed trout where liver structural proteins such as beta tubulin and keratin were down-regulated (Martin et al., 2003).

The expression of many of the genes involved in lipid transport and metabolism also decreased over time (phosphodiesterase 3A, 3-ketoacyl-coenzyme A thiolase, betaine homocysteine methyltransferase, N-acylsphingosine amidohydrolase, benzodiazepine receptor, apolipoprotein AI, 14 kDa apolipoprotein, fatty acid binding proteins) or remained constant (phospholipase A2, apolipoproteins B, CII and EI). Plant proteins including soybean have been shown to induce hypocholesteremia in mammals (De Schrijver, 1990). Little is known about the hypocholesteremia-inducing effects of proteins in fish although soybean protein has been shown to lower cholesterol in sea bream, *Sparus aurata* (Venou et al., 2006), sea bass (Kaushik et al., 2004), and rainbow trout (Kaushik et al., 1995) and non-starch polysaccharides reduce the digestibility of fats and energy in diets based on SBM (Olli and Krogdahl, 1995; Reftsie et al., 1999). The reduced expression of genes for proteins involved in lipid transport found in our study may reflect induction of hypocholesteremia by the

Table 5A
Genes whose expression is continuously down-regulated after introduction of experimental SBM diet.

	Cluster A	Cluster E
Unknown	19	8
EST/hyp	5	3
Similar	Phosphodiesterase 3A[c]	HIRA-interacting protein 5[b]
	Aurora kinase A-interacting protein[b]	Protein disulfide isomerase A2[b]
	CGRP-receptor component protein	
	Cytokine induced apoptosis inhibitor 1[b]	
	Deltex 4 homolog	
	Translation elongation factor 1 delta[b]	
	Mitotic spindle assembly checkpoint protein MAD2A[b]	
	MADP-1 protein[b]	
	Intestinal mucin 2	
	Muscle-specific protein[a]	
	Titin isoform N2-B[a]	
Known	Adenosine monophosphate deaminase	Apolipoprotein AI[c]
	Betaine homocysteine methyltransferase[c]	14 kDa apolipoprotein[c]
	Fatty acid binding protein 6[c]	Fatty acid binding protein[c]
	Myosin heavy chain 3, myosin heavy chain[a]	Myosin heavy chain[a]
	Myosin light chain 2[a]	Myosin light chain[a]
	Creatine kinase muscle isoform 1[a,*]	Tropomyosin 2[a]
	Troponin 1-like protein[a]	Troponin-I isoform 2[a]
	Troponin C[a]	Troponin T (2)[a,*]
	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1A[a]	Atp2a1[a]
	Parvalbumin[a]	N-acylsphingosine amidohydrolase[c]
	Catenin alpha E[a]	26S protease regulatory subunit 7[b]
	Lamin B2[a]	MAD2B[b]
	Warm-temperature-acclimation-related-65 kDa-protein	Warm-temperature-acclimation-related-65 kDa-protein
	Transferrin	Plasminogen[b]
	Cathepsin L1 precursor[b]	
	Elastase A precursor[b]	
	Carboxypeptidase A2[b]	
	Pancreatic serine proteinase inhibitor homolog[b]	
	FK506 binding protein 1b[b]	
	Evectin (pleckstrin homology domain-containing protein)[b]	
	UMOD zymogen granule membrane glycoprotein 2[b]	
	Rab5 GTP/GDP exchange factor[b]	
	Dehydrogenase/3-ketoacyl-coenzyme A thiolase[c]	
	Benzodiazepin receptor (peripheral) Bzrp[c]	
	Leukocyte immune-type receptor 3	
	Neurogenin 1	
	Nuclease diphosphate kinase B	
	Pyruvate kinase	

Asterisks indicate representative genes selected for qPCR assay. Genes involved in muscle physiology are indicated by superscript a, protein synthesis/cell growth by superscript b, and lipid transport/metabolism by superscript c.

addition of SBM or the removal of FM, as suggested by (Kaushik et al., 1995).

Genes involved in the immune system (α-2 macroglobulin, β-2 microglobulin, MHC IIb, galectin, LR8-like protein, placenta-specific 8 protein, C1q-like factor, IgM heavy chain) were up-regulated over time, indicating that although there were no gross morphological changes indicative of inflammation, the immune system may have been stimulated by the SBM diet or possibly by the presence of increased numbers of pathogenic bacteria in a sub-optimally healthy gut. β-2 microglobulin and MHC IIb are both involved in antigen presentation required for production of antibodies (IgM), and galectin-2, through its ability to recognize and bind β-galactoside on pathogens, is a master regulator of inflammation and host–pathogen interactions. It has been localized to the brush border of enterocytes in

Table 5B
Genes whose expression is continuously up-regulated after introduction of experimental SBM diet.

	Cluster C	Cluster G
Unknown	14	2
EST/hyp	3	1
Similar	LR8-like protein[a]	0
	Membrane-spanning 4-domain, subfamily A, member 4a	
	Transmembrane 4 superfamily member L6	
	Myosin regulatory light chain 2, smooth muscle isoform	
	Transcription factor E2F3	
Known	Beta-2-microglobulin (2)[a,*]	Alpha-2-macroglobulin[a,*]
	Galectin 2[a]	IgM heavy chain[a]
	MHC class IIb[a]	Ovary-specific C1q-like[a]
	Glutathione peroxidase	Placenta-specific 8 protein (onzin)[a]
	Retinol binding protein 2a	Cathepsin Z[b]
	Proline dipeptidase[b]	Aminopeptidase P[b]
	Pepsinogen A1[b]	Transcobalamin II[b]
	Ependymin (2)	
	Pancreatic protein with 2 somatomedin B domains[b]	
	Lactate dehydrogenase A	
	5-aminolevulinate synthase	
	Folate receptor 1	
	Adaptor-related protein complex 1 mu 1 subunit	
	Reticulon 2-A	
	Sorting nexin10 protein	

Asterisks indicate representative genes selected for qPCR assay. Genes involved in the immune system are indicated by superscript a and digestion by superscript b.

pigs and confers protection to the intestine by ensuring optimal cross-linking of membrane glycolipids and glycoproteins (KamppThomsen et al., 2009). Placenta-specific 8 protein, also known as onzin, is

Table 5C
Genes whose expression peaks at 10 days after introduction of experimental SBM diet.

	Cluster D	Cluster F
Unknown	9	10
EST/hyp	3	2
Similar	Claudin-12	Macrophage inflammatory protein 3 beta (MIP3b; CCL19)[a]
	Collagen, type X, alpha 1 precursor isoform 2	Cytochrome P450 3A13[c]
	Cortactin isoform 1	
	Cellular disintegrin-related protein ADAM9	
	ES1 protein	
	Esophagus specific gene 1 protein	
	Membrane-spanning 4-domains, subfamily A, member 4 isoform a	
Known	Cytochrome P450 2K5[c]	Cytochrome P450 3A40[c,*]
	PDZ domain-containing 1-like	Cytochrome P450 3A27[c]
	Microtubule-based motor protein	Calponin 3
	Myosin heavy	Myosin heavy
	Transgelin	Tropomyosin
	Cathepsin B[b]	Trypsinogen[b]
	Carboxyl ester lipase[b]	High choriolytic enzyme I
	MHC II invariant chain[a]	MHC Ia[a]
	Aldolase A fructose-bisphosphate	Transmembrane serine protease 2
	Retinol binding protein-like	
	Solute carrier family 35, member B4	
	Adenylate kinase*	
	Interferon gamma inducible lysosomal thiol reductase[a]	
	CD63[a]	
	Myosin light polypeptide 6, smooth muscle	
	Stomatin	

Asterisks indicate representative genes selected for qPCR assay. Genes involved in immunity are indicated by superscript a, digestion by superscript b, and detoxification by superscript c.

Table 5D
Genes whose expression remains constant after introduction of experimental SBM diet.

	Cluster B
Unknown	6
EST/hyp	4
Similar	Apolipoprotein B
	Aquaporin 8*
	Mitochondrial elongation factor G2 isoform 1
	Nebulin
	Phosphofructokinase, muscle
	Retinol-binding protein II, cellular
	Ribosomal protein L19
	Tetraspan 1
	Tetraspanin-13
	Transforming protein bmi1
Known	Alpha amylase
	Apolipoprotein C-II
	Apolipoprotein E1
	Phospholipase A2
	Cytosolic nonspecific dipeptidase
	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
	Alpha-2-HS-glycoprotein
	Carbonyl reductase-like 20beta-hydroxysteroid dehydrogenase
	Putative tyrosine phosphatase
	Creatine kinase brain isoform
	Myo-inositol monophosphatase
	Sodium/potassium ATPase alpha subunit isoform 1
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
	Fast skeletal muscle troponin T
	Myosin, heavy polypeptide 2, fast muscle specific
	Skeletal/cardiac alpha-actin
	Gamma fibrinogen
	CD81
	Glutathione S-transferase
	Itm2b protein
	Legumain
	Periostin
	Secreted phosphoprotein 24
	Selenoprotein W2a
	Urod protein

Asterisk indicates representative gene selected for qPCR assay. Shaded boxes indicate genes involved in lipid transport and metabolism.

expressed at high levels in mammalian intestinal epithelial cells and during differentiation of myeloid cells and participates in intracellular killing of pathogens (Ledford et al., 2007). LR8-like protein is a marker of a subpopulation of lung fibroblasts present in the dermis (Lurton et al., 1999) but its function is unknown. C1q enhances chemotaxis of immature dendritic cells from the blood to peripheral inflammatory tissues as well as of mature dendritic cells to secondary lymphoid tissues via MIP3 beta (Liu et al., 2008). MIP3 beta, also known as chemokine CCL19, was maximally up-regulated at 10 days. In addition, four genes involved in antigen presentation showed peak expression at 10 days and further indicate that the immune system has been stimulated — MHCIa, MHC II invariant chain, CD63 (a lysosome-associated membrane glycoprotein that forms com-

plexes with MHCII; Hammond et al., 1998), and interferon gamma inducible lysosomal thiol reductase (which participates in MHCII antigen presentation). The up-regulation of glutathione peroxidase, which protects cell membranes from oxidative stress is also consistent with early stages of inflammation. In addition to inflammation-induced changes of immune gene expression, it has been shown that phosphorus deficiency modulates the immune response of European white fish *Coregonus lavaretus* (Jokinen et al., 2003) and channel catfish (Eya and Lovell, 1998); possibly the SBM diet in this study contained nutrient deficiencies that contributed indirectly to immune stimulation.

Genes for four isoforms of cytochrome P450 (2K5, 3A13, 3A27 and 3A40), which are involved in detoxification, also showed peak expression at 10 days after introduction of SBM diet. qPCR analysis confirmed that cytochrome P450 3A40 was highly expressed at all time points and was up-regulated in response to SBM diet at T1 and T3. Cytochrome P450 3A40 was also found to be significantly up-regulated in distal intestine of cod fed 24% SBM (Lilleeng et al., 2007a). It is known that soy contains phytoestrogens, isoflavones and phorbol esters (Storebakken et al., 2000); generally, most of the heat labile antinutritional factors associated with complex carbohydrates are denatured in dehulled solvent extracted SBM. However, cytochrome P450 may be responsible for detoxifying any remaining antinutritional factors.

Other genes that were up-regulated over time include ependymin, which is involved in cell adhesion, and several genes that control cell division and differentiation. Retinol binding protein 2a is specifically involved in enterocyte differentiation, folate receptor 1 is involved in erythroid differentiation, and transcription factor E2F3, transmembrane 4 superfamily L6, and myosin regulatory light chain 2 regulate overall cell division. Sequences similar to a putative transmembrane 4 superfamily member protein and a cell cycle ras-related GTPase were also represented in a suppression subtractive library constructed from cod fed 24% SBM (Lilleeng et al., 2007a).

Several genes potentially involved with digestion were differentially regulated; these included enzymes involved in proteolysis, vitamin transport, carbohydrate breakdown and sugar transport, and lipases and lipid transport molecules. Trypsinogen, aminopeptidase P, proline dipeptidase, cathepsins B and Z, carboxyl ester lipase, pepsinogen A1 and the pancreatic protein with two somatomedin B domains increased after introduction of the SBM diet. Aminopeptidase M/aminopeptidase N also showed an increase in transcription in Atlantic cod fed 24% SBM (Lilleeng et al., 2007a). Interestingly, introduction of SBM-containing diet to Atlantic salmon did not cause any change in pancreatic transcription of two trypsin isoforms despite higher levels of trypsin enzymatic activity in the distal intestine (Lilleeng et al., 2007b) and in faeces (Krogdahl et al., 2003; Romarheim et al., 2006). However, in rainbow trout there was an increase in pancreatic protease activity that was presumed to be in response to trypsin inhibitors present in SBM (Dabrowski et al., 1989). The decreased expression of an endogenous proteinase inhibitor in our study may also be in response to the exogenous trypsin inhibitors

Table 6
Comparison of expression ratios of FM diet and SBM diet samples compared to the universal control derived using qPCR (first number) and microarray (second number) methods.

Cluster	Gene	T1 FM	T1 SBM	T2 FM	T2 SBM	T3 FM	T3 SBM
A	Creatine kinase	<0.01/0.01	<0.01/0.02	<0.01/<0.01	<0.01/<0.01	<0.01/0.03	<0.01/0.02
B	Aquaporin 8	33/8	54/6	54/15	33/8	49/15	37/7
C	β-2 microglobin	297/13	596/10	473/18	235/30	354/5	334/11
D	Adenylate kinase	<0.01/0.03	0.02/0.03	0.01/0.03	0.01/0.04	0.01/0.11	0.02/0.08
E	Troponin 2	0.01/<0.01	<0.01/0.08	<0.01/0.01	0.01/0.02	<0.01/0.05	<0.01/0.02
F	Cyt P450 3A40	57/35	73/10	71/4	35/72	38/38	46/20
G	α-2 macroglobulin	0.01/0.16	0.01/0.15	0.01/0.13	0.01/0.46	0.01/0.04	0.01/0.29

Both EF1A and Rps4 were used as reference genes for the qPCR analysis.

in the SBM diet. Transcobalamin II, which is responsible for transporting cobalamin/vitamin B12 in plasma was up-regulated both in this study and in cod (Lilleeng et al., 2007a).

The expression of alpha amylase remained constant. Because of the low levels of carbohydrate in the diet, gluconeogenesis is the main process by which carnivorous fish meet their glucose requirements (Cowey et al., 1977). The carbohydrates in SBM diet are more complex than carbohydrates such as starch and cellulose supplied by the wheat middlings in the control FM diets. SBM contains 30% carbohydrates but the main constituents are the oligosaccharides sucrose (5%), raffinose (1%) and stachyose (4%) and non-starch polysaccharides (~20%) (Bach Knudsen, 1997). It is therefore unlikely that amylase plays a major role in digestion of this diet. Fish fed soybean protein have an increased energy demand, with coincident changes in expression of proteins, such as aldolase B, that participate in intermediary metabolism (Vilhelmsson et al., 2004). Similar to rainbow trout fed soybean protein, halibut exhibited a decrease in pyruvate kinase expression and an increase in aldolase B, indicating an increased emphasis on catabolism versus anabolism (Martin et al., 2003).

The number of microarray spots that passed threshold after background correction and normalization (14.3%) is similar to our previous studies on larval development, where an average of 11% of spots passed threshold at five different developmental stages (Douglas et al., 2008), and on the effect of early introduction of microdiet where 15.1% passed threshold at four different time points (Murray et al., in press). The Atlantic halibut microarray contains spots representing eight different tissues (including intestine) and five different developmental stages, and many would not be expected to hybridise with RNA derived only from a single tissue, in this case the distal intestine. In a recent microarray study on the effect of phosphorus deficiency on the expression of liver genes from rainbow trout, 50% of spots were above threshold but an additional 30% were eliminated as false positives based on a self–self hybridization, yielding 21 genes that were differentially expressed (Kirchner et al., 2007). Similarly, in a microarray study of gene expression in trout liver in response to inflammation, 36 of 1400 spots on the array passed threshold and were more than two-fold up- or down-regulated (Gerwick et al., 2007).

The approximately 600 spots representing genes that showed statistically significant differential expression ($P < 0.05$) at each of the three time points is in keeping with other microarray experiments using wild fish for nutritional studies. It is known that there is high genetic variability among fish of a given species (Gerwick et al., 2007). Furthermore, unequal feeding rates in fish culture due to fish hierarchies means that gene expression changes can vary considerably among individuals (Jobling and Koskela, 1996). The use of universal RNA for the reference in all of the microarray experiments serves to minimize some of these effects (Kirchner et al., 2007). In our experiments, we used a universal RNA that contained equal amounts of RNA from five developmental stages of Atlantic halibut from hatching until post-metamorphosis.

Data from gene expression analyses determined by microarray and qPCR showed general agreement in terms of differential expression levels (up- or down-regulated). However, the magnitude of the differential expression often varied between qPCR and microarray, with values determined by pPCR being consistently higher (for up-regulated genes) or lower (for down-regulated genes). Similarly, gene expression differences in rainbow trout fed low phosphate diet compared to high phosphate diet were generally more than two-fold higher when determined by PCR compared to microarray (Kirchner et al., 2007). The lower signal–noise ratio of gene expression from a population of genetically diverse fish results in a lower sensitivity of the microarray method. This sometimes results in the ratios of genes from SBM-fed fish vs FM-fed fish being different from those derived by qPCR. Discordance between qPCR and microarray data can be particularly evident in experiments involving unrelated wild animals

due to the large amount of genetic variation and inter-individual differences in expression (Gerwick et al., 2007). In the study by Kirchner et al. (2007), 45% of microarray results were not confirmed by qPCR.

5. Conclusions

Although several studies have reported changes in growth, performance and gut morphology in fish in response to the inclusion of SBM in the diet, few have investigated gene expression changes and underlying mechanisms. Nutrigenomics, by providing global gene expression patterns under different dietary conditions, can provide an improved understanding of how overall nutrition or the inclusion of specific dietary ingredients influences metabolic pathways and, potentially, animal performance. This approach has been successfully used to investigate changes in gene expression in Atlantic salmon liver resulting from partial replacement of fish oil with vegetable oil (Jordal et al., 2005; Leaver et al., 2008) and growth hormone transgenesis (Rise et al., 2006), in rainbow trout liver after varying dietary fish oil levels (Panserat et al., 2008) or total replacement of fish protein and oil with vegetable products (Panserat et al., 2009), and in intestine of rainbow trout deficient in phosphorus (Kirchner et al., 2007). We now have identified genes that are differentially regulated in Atlantic halibut in response to replacement of FM with SBM. Although morphological examination of the distal intestine did not reveal enteritis or an influx of leukocytes, the expression of several immune markers was increased, indicating a subtle effect on immunity, especially initiation of the adaptive immune system. Genes involved in detoxification were also highly expressed, perhaps in order to counteract antinutritional factors present in the SBM. Down-regulation of genes involved in lipid transport are consistent with previous reports of hypocholesterolemia in sea bass, sea bream and rainbow trout in response to replacement of FM with SBM, and down-regulation of numerous genes involved in muscle function indicates that intestinal muscle metabolism and motility may be affected.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquaculture.2009.11.001.

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