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Early response of gene expression in the distal intestine of Atlantic salmon (*Salmo salar* L.) during the development of soybean meal induced enteritis

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ABSTRACT

Plant products in general and soybeans in particular can challenge the function and health of the intestinal tract. Salmonids develop an intestinal inflammation when fed diets containing soybean meal (SBM) and certain other legume ingredients. In the present study a 44K oligonucleotide salmonid microarray, qPCR and histology were used to investigate early response mechanisms in the distal intestine of Atlantic salmon (*Salmo salar* L.) during the first week of oral exposure to a diet containing 20% extracted SBM. The distal intestine transcriptome was profiled on days 1, 2, 3, 5 and 7 and compared to a control group fed fishmeal as the sole protein source. Histological evaluation of the distal intestine revealed the first signs of inflammation on day 5. The most prominent gene expression changes were seen on days 3 and 5. Up-regulation in immune-related genes was observed during the first 5 days, including GTPase IMAP family members, NF-kB-related genes and regulators of T cell and B cell function. Many functional genes involved in lipid metabolism, proteolysis, transport, metabolism and detoxification were initially up-regulated on days 1–3, possibly as an attempt by the tissue to compensate for the initiating immune response. Cell repair and extracellular matrix remodeling genes were up-regulated (*heparanase*, *collagenase*) on days 3 and 5. Down regulation of genes related to endocytosis, exocytosis, detoxification, transporters and metabolic processes from day 5 indicated initiation of dysfunction of digestive and metabolic functions that may occur as a result of inflammation or as a response to the introduction of soybean meal in the diet. This is the first study conducting transcriptomic profiling to characterize early responses during the development of SBMIE. Switching Atlantic salmon from a fishmeal to a 20% SBM diet resulted in rapid changes to the intestinal transcriptome, indicating an immune reaction with subsequent impaired epithelial barrier function and other vital intestinal functions.

1. Introduction

Atlantic salmon (*Salmo salar* L.) is carnivorous in nature and the inclusion of plant materials in their diet exposes the digestive system to a large number of substances that are not typically part of their natural diet [1–3]. In most experiments, Atlantic salmon fed diets containing more than 5–10% fullfat or defatted (extracted) soybean meal (SBM) develop inflammation in the distal part of the intestine [4,5]. Other teleost species, such as rainbow trout (*Oncorhynchus mykiss* W.) and common carp (*Cyprinus carpio* L.), also react to SBM in a similar way [6–8]. Histopathological characteristics of SBM-induced enteritis (SBMIE) include decreased amounts of supranuclear vacuoles in enterocytes, widened lamina propria and submucosa with increased leukocyte infiltration and decreased height of mucosal folds [5]. The first histological signs of inflammation are apparent after 2–5 days of SBM feeding and the severity escalates with extended exposure time, reaching a maximum after about three weeks [4,5].

The specific components in SBM causing intestinal inflammation have not been conclusively identified. Plant feed ingredients, in general, contain antinutritional factors (ANFs) and many can be found in SBM, including lectins, protease inhibitors, phytosterols, saponins and more [1,3]. Potential effects on fish metabolism from these ANFs include increased gut permeability, interference with

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lipid and protein digestion, and alteration of cholesterol and bile salt absorption and metabolism (reviewed in Krogdahl et al. [3]). Other proposed causes include unidentified antigens in SBM that could induce the immune response [9], or alterations of intestinal microbial communities caused by SBM as observed in Atlantic salmon, rainbow trout and Atlantic cod (*Gadus morhua* L.) [10–12]. A shift in the microbial community could allow for an increase in harmful bacteria, triggering the inflammation [10]. However, when diets for Atlantic salmon were simultaneously supplemented with SBM and the broad-spectrum antibiotic, oxytetracycline, the inflammatory response was not mitigated [13]. But cultivatable bacteria were still present in the distal intestine even with oxytetracycline treatment. Thus, a combination of tissue responses to ANFs/antigens and altered microbial community as a cause of the inflammation cannot be excluded at present.

Only two previous studies have characterized the progression of SBMIE, studying the histomorphology [5] and transcriptional changes (qPCR) of some selected immune factors [14]. On the other hand, numerous studies reported histochemical, biochemical and molecular responses during chronic stages of SBMIE [9,13,15–18].

To further elucidate mechanisms leading to the development of SBMIE, the present study examined the transcriptomic responses in the distal intestine of Atlantic salmon during the first 7 days of feeding with a SBM-containing diet using a 44K salmonid oligonucleotide microarray. Given that histological signs of the inflammation are apparent after only a few days of SBM feeding [5], an early screening of transcriptional responses may give new indications of how the inflammation is triggered, and thus aid in finding means of mitigating the condition in the future.

2. Material and methods

2.1. Feeding trial

The feeding trial was conducted in accordance with laws and regulations that control experiments and procedures in live animals in Norway, as overseen by the Norwegian Animal Research Authority. A detailed description of the experiment can be found in other recent publications [19,20]. In brief, Atlantic salmon (*S. salar* L.) of the Sunndalsøra breed were held in 1 m^3 fiberglass tanks with running seawater (25–30 fish per tank) at the research facilities of Nofima Marin, Sunndalsøra, Norway. The fish had an initial body weight of 500–600 g. Two diets were formulated, the reference (FM) diet contained 563 g kg^{-1} fishmeal as the sole protein source while the test diet contained 200 g kg^{-1} hexane-extracted (defatted) SBM, partially replacing the fishmeal in the FM diet. Both diets were approximately iso-nitrogenous and iso-energetic on a crude protein and gross energy basis, containing 28% lipid and 43% crude protein. All fish were adapted to the FM diet for 7 days. Day 0 in this experiment marks the sampling of FM fed fish. Duplicate tanks of the remaining fish received the SBM test diet from day 0 and were sampled after 1, 2, 3, 5 and 7 days of exposure. Fish were euthanized by a combination of deep anesthesia with a high dose of tricaine methanesulphonate (MS 222; Argent Chemical Laboratories, Redmond, WA, USA) and a sharp blow to the head.

For RNA extraction, sections of distal intestinal tissue (distalmost region of the post-gastric intestinal tract defined by appearance of annular mucosal folds and widening of intestinal diameter) were dissected, carefully rinsed in phosphate-buffered saline and immediately placed in *RNAlater* at 4 °C for 24 h and then stored at −80 °C. For histology, sections were fixed in 4% phosphate-buffered formaldehyde solution for 24 h and subsequently transferred to 70% alcohol and stored at 4 °C until further processing. To ensure exposure to SBM, only fish that had content throughout the intestinal tract were sampled.

2.2. Histology

For histological evaluation, distal intestinal sections (n = 4–6 per tank and time point) were prepared using standard histological methods. In brief, the samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. Longitudinal cuts (i.e. perpendicular to the macroscopically visible circular folds) of approximately 5 μm were stained with hematoxylin and eosin and examined under a light microscope. The sections were analyzed by experienced personnel in two independent blinded evaluations. A detailed histopathological interpretation of these samples has been reported previously [20].

2.3. RNA extraction

Samples were randomized for all RNA procedures. From ten fish per time point (five from each tank duplicate) approximately 20 mg of sample tissue was immersed in 1 ml TRIzol® (Invitrogen), homogenized with a mixermill (Retsch MM301) and total RNA was extracted as per manufacturer's instructions. Total RNA was purified using RNeasy columns as per manufacturer's instructions (QIAGEN), and included the on-column DNase I digestion step to remove any remaining genomic DNA. Total RNA was quantified by spectrophotometry (NanoDrop1000™, Thermo Fisher Scientific) and quality checked by agarose gel electrophoresis. Absence of genomic DNA was confirmed by PCR with and without reverse transcriptase. Purified total RNA was stored at −80 °C until further use.

2.4. Microarray

Reverse transcription and dye labeling was performed according to Agilent's two-color Low Input Quick Amp labeling protocol (Version 6.5, May 2010). In brief, 200 ng total RNA was reverse transcribed into cDNA, then Cy3- (reference) and Cy5- (samples) labeled cRNA was amplified from the cDNA by T7 RNA Polymerase (Agilent Technologies). The labeled cRNA was then purified using RNeasy spin columns (QIAGEN) and quantified by spectrophotometry (NanoDrop1000™, Thermo Fisher Scientific). All samples had specific activity >6 pmol dye per μg cRNA. Labeled cRNA was stored at −80 °C in the dark until hybridization. A common reference design was constructed by pooling equimolar amounts of Cy3-cRNA synthesized from three fish from each condition (total in reference = 18 fish). An equal amount of this reference was hybridized to each array with the experimental samples (n = 10 per each of the 6 time points, total number of arrays: 60).

Each experimental sample and reference pool (825 ng each) was fragmented and hybridized to a cGRASP 44K salmonid oligonucleotide microarray [21] as per manufacturer's instructions (Agilent Technologies). The arrays were hybridized for 17 h in a hybridization oven (10 rpm at 65 °C, Agilent Technologies). Slides were washed immediately after hybridization as per manufacturer's instructions with the additional use of Stabilization and Drying Solution (Agilent Technologies) to prevent ozone-related problems. Slides were then kept in the dark at low ozone (<9 ppb) and scanned as soon as possible.

Slides were scanned using a ScanArray® Express scanner (PerkinElmer®; 5 μm resolution; PMTs: Cy5: 60, Cy3: 65) in a low-ozone environment (<9 ppb). Scanned images were processed in the ImaGene® software (v8.0; Biodiscovery) using the cGRASP 44K salmonid GAL file (11/09) (http://web.uvic.ca/grasp/microarray/array.html). The average of the median signal values for negative control spots per array was subtracted from each probe median intensity value, control spots were removed, and the experimental values were imported into GeneSpring GX11.5 (Agilent

Technologies). Annotation files and details for the microarray can be found on the cGRASP microarray page (http://web.uvic.ca/grasp/microarray/array.html). In order to remove negative expression values, background corrected probe median values were thresholded to 1, and each array was normalized with an intensity-dependent LOWESS normalization. Each probe was then normalized by the median value of that probe across all conditions (per-gene normalization: baseline to median; Agilent). Probes with poor quality flags designated by ImaGene® (v8.0; Biodiscovery) in any biological replicate were filtered out for all samples. Probes were also filtered to retain only those for which at least 80% of the biological replicates had raw values ≥500 in any one of six time points. After quality filtering, there were 10,671 probes remaining for analysis. Probes were tested for differential expression from each time point against the control (Mann–Whitney $p \leq 0.05$; fold change ≥1.5). Principal component analysis (PCA) was performed to identify potential clustering of expression data by tank and other technical aspects (e.g. hybridization day). Complete data files were deposited in NCBI's Gene Expression Omnibus under the accession number GSE37457.

2.5. Quantitative real-time PCR (qPCR)

Total RNA (1.0 μg) was reverse-transcribed to cDNA using SuperScript® III Reverse Transcriptase (Invitrogen) in 20 μl reactions and primed with Oligo$(dT)_{20}$ as per manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or reverse transcriptase. Synthesized cDNA samples were diluted 1:10 and stored at −20 °C. Primers for qPCR were designed with Primer3 (http://frodo.wi.mit.edu/primer3) (Supplementary data 1) [22]. PCR reaction efficiency (E) for each gene was determined using 2-fold serial dilutions of pooled cDNA originating from randomly selected samples used in the experiment (starting dilution 1:5).

qPCR was run on a LightCycler® 480 system (Roche Diagnostics) with the following conditions: pre-incubation 95 °C (5 min); amplification (40 cycles) 95 °C (10 s), 60 °C (10 s), 72 °C (15 s); melt curve 95 °C (5 s), 65 °C (1 min), ramp to 97 °C (0.11°/s, 5 acquisitions/degree). Each 10 μl DNA amplification reaction contained 2 μl PCR-grade water, 5 μl Lightcycler 480 SYBR Green I Master (Roche Diagnostics), 2 μl 1:10 diluted cDNA template and 0.5 μl (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control and an inter-run plate calibrator. Quantification cycle (Cq) values were calculated using the second derivative method (Roche Diagnostics). Melt curve analysis and agarose gel inspection of products confirmed amplicons were only a single product. Target gene expression was normalized to the geometric mean of *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), *RNA polymerase II* (*rnapolII*) and *hypoxanthine-guanine phosphoribosyltransferase 1* (*hprt1*) expression as evaluated elsewhere [19]. Quantification of the target was performed using the method of Pfaffl [23]. Finally, data were expressed as fold change to fishmeal control group (day 0). The samples were tested with the non-parametric Steel-with-control test as part of the statistical software JMP 9.0 (SAS) with a significant level of $p < 0.05$ [24].

3. Results

3.1. Histology

No morphological changes in the distal intestine were observed from day 0 to day 3 of SBM exposure (Fig. 1). First signs of SBM-induced inflammation, characterized by shortening of the mucosal folds, decreased enterocyte vacuolization, increased number of intraepithelial lymphocytes (IELs), and/or cellular infiltration into the lamina propria and submucosa, were visible at day 5 in 3 of 8 fish and at day 7 in about half the fish. The feeding trial was continued until day 21 and all sampled fish at this time point had developed inflammation in the distal intestine. Readers are referred to Marjara et al. [20] for a detailed description of the histomorphological changes.

3.2. Microarray

There was a large amount of variation in expression levels, even though the biological replication was relatively high ($n = 10$ per time point). Only a few genes responded at multiple time points over the course of the experiment. The highest number of differentially expressed genes (DEGs) was observed on day 3 and day 5 (Table 1). PCA analysis revealed no clustering of samples by tank number or other technical aspects (data not shown).

Although few genes were responding during the first two days, several immunity and transport-related genes were differentially expressed (Table 2). Of particular interest were two genes encoding GTPase IMAP proteins (family members 4 and 7), *Ig kappa chain V-IV region B17 precursor* and *proto-oncogene tyrosine-protein kinase*

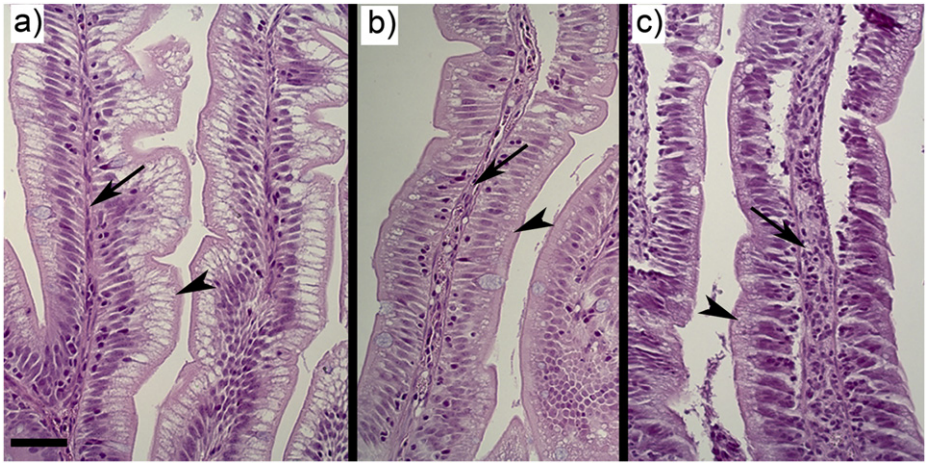

Fig. 1. Light microscopic images depicting morphological changes, a) healthy condition: normal lamina propria (arrow), high degree of enterocyte vacuolization (arrowhead), b) day 5, first signs of inflammation are becoming visible: widening of the lamina propria (arrow) and reduction in enterocyte vacuolization (arrowhead), c) day 7, signs of inflammation are increasing in severity: widening of the lamina propria (arrow) and reduction in vacuolization (arrowhead). Bar = 50 μm.

Table 1
Differentially expressed genes (DEGs) at each time point of SBM feeding. Numbers represent uniquely annotated genes (excluding unknowns).

Day	Number of DEGs	Up-regulated	Down-regulated
1	7	4	3
2	7	5	2
3	44	38	6
5	48	26	22
7	5	2	3

fyn, which all are considered important for B and/or T cell function [25,26].

On day 3, a larger number of DEGs were observed (Table 3). Genes involved in several important general functions were identified, including binding, cell integrity and extracellular matrix (ECM), immune response, lipid metabolism, proteolysis and transport. The genes with the largest fold change were involved in lipid metabolism: *fatty acid synthase* (FC = 5.2 up) and *acyl-coa binding protein* (FC = 6.4 down). Most of the up-regulated genes ($n = 8$) were immune-related, including *gtpase imap family member 7* [26,27], *chemokine-like receptor 1* [28] and *ig mu chain c region membrane-bound form*. *Ig kappa chain V-IV region B17 precursor*, found up-regulated on day 1, was also up-regulated on day 5. Of note was also the up-regulation of *switch-associated protein 70*, which is specifically expressed in mature B-cells [29,30]. Increased expression was found for several transporters that can take part in intestinal absorption of various ions and nutrients, including taurine and choline. Other up-regulated genes belonged to functional categories involving proteolysis, apoptosis (*caspase-14 precursor*, *apoptosis facilitator bcl-2-like protein 14*), cell repair/extracellular matrix-related (*mucin-2 precursor*, *myoferlin*), cytoskeleton (*myosin-VII*), transporters and xenobiotic/antioxidant processes. Reduced expression levels were observed for *cytochrome P450 26A1* (*cyp26a1*), which regulates cellular concentrations of retinoid acid via oxidative metabolism.

The highest number of DEGs was observed on day 5 (Table 4). Functions included ECM disassembly, cell adhesion, proteolysis, transport, antioxidation and immune response. Up-regulated genes were related to ECM disassembly (*collagenase 3 precursor*) and cell adhesion (*heparanase precursor*). However, *protocadherin-20 precursor*, also related to cell adhesion, was down-regulated (FC = 3.9). Genes related to proteolysis (*calpain-2 catalytic subunit*, *serine protease 23 precursor*, *calpain small subunit 1*) were up-regulated. *Solute carrier family 22 member 5*, which is a carnitine transporter associated with systemic primary carnitine deficiency in humans [31], was down-regulated, while *epithelial chloride channel* was up-regulated. Most genes ($n = 4$) that were down-regulated were related to xenobiotic metabolism and anti-oxidation activity (*glutathione s-transferase alpha 4*, *bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthetase 2*, *glutathione s-transferase p1*). As in day 3 responses, several immune-related genes were up-regulated. Among them were *cd99 antigen*, *leukocyte elastase inhibitor* and *traf-interacting protein*.

Interestingly, on day 7 (Table 5) the number of DEGs decreased markedly. Apoptosis-related gene *bcl2/adenovirus e1b 19kda protein-interacting protein 3* and a transcription-related ATP-dependent RNA helicase were up-regulated. Immune response-related genes *radical s-adenosyl methionine domain-containing protein 2*, *protein bat5* and *interferon-induced protein 44* were down-regulated. The complete list of DEGs for each day can be found in Supplementary data 2.

3.3. qPCR

To validate microarray results using qPCR, 12 genes related to key functional groups showing differential expression on one or more of the sampling days were selected (Supplementary data 1). Two genes were quantified as genes of interest without showing significant differential expression on the microarray; these genes were *b-cell lymphoma 3-encoded protein* and *collectrin*. In general, qPCR analysis confirmed the expression profiles of the microarray results.

Three immune response related genes were quantified (Fig. 2): *cd99* was significantly up-regulated on day 3 while *b-cell lymphoma 3-encoded protein* (*bcl3*) and *chemokine-like receptor 1* (*cmklr1*) were significantly up-regulated on days 3 and 5. *Fatty acid synthase* (*fasn*), involved in lipid metabolism, was significantly up-regulated on days 3 and 5 (Fig. 3). Three genes with ECM and cytoskeletal related functions were quantified (Fig. 3). The expression of *collagenase 3 precursor* (*mmp13*) was not found to be significantly

Table 2
Differentially expressed genes ($p < 0.05$) on days 1 and 2 vs. control.

Category/gene name	Day	Fold change	Direction	*p*-value	ProbeID
Amino acid biosynthesis					
Branched-chain-amino-acid aminotransferase, cytosolic	1	1.52	Down	0.019	C153R142
Cell communication					
Proto-oncogene tyrosine-protein kinase Fyn	2	1.64	Up	0.013	C060R072
Immune response					
GTPase IMAP family member 7	1	2.35	Up	0.041	C024R155
Ig kappa chain V-IV region B17 precursor	1	1.58	Up	0.023	C173R127
GTPase IMAP family member 4	1	5.33	Down	0.019	C130R168
Protein BAT5	2	1.51	Down	0.023	C200R093
mRNA processing					
Cleavage and polyadenylation specificity factor subunit 5	2	1.55	Down	0.016	C219R142
Protein folding					
DnaJ homolog subfamily A member 4	1	1.51	Down	0.049	C120R137
Retinol metabolic process					
Beta,beta-carotene 15,15′-monooxygenase	1	1.55	Up	0.049	C121R069
Transcription					
Transcription factor HES-1	1	1.58	Up	0.034	C249R035
Transport					
Cyclic nucleotide-gated cation channel alpha-3	2	1.73	Up	0.004	C114R137
Potassium channel subfamily K member 6	2	1.59	Up	0.028	C210R045
Unknown					
Zinc finger CCCH domain-containing protein 15	2	2.29	Up	0.049	C103R050
Zinc finger protein 69	2	1.59	Up	0.004	C075R061

Table 3
Differentially expressed genes ($p < 0.05$) on day 3 vs. control.

Category/gene name	Fold change	Direction	*p*-value	Probe ID
Apoptotic process				
Apoptosis facilitator Bcl-2-like protein 14	1.51	Up	0.010	C093R166
Binding				
Seminal vesicle major clotting proteins precursor	1.75	Up	0.005	C199R135
Switch-associated protein 70	1.70	Up	0.049	C180R060
Tctex1 domain-containing protein 2	1.66	Up	0.034	C217R086
Lactose-binding lectin l-2 precursor	1.66	Up	0.016	C026R144
TPR repeat-containing protein C1orf34 homolog	1.52	Up	0.019	C260R027
Cell repair/extracellular matrix related				
Mucin-2 precursor	2.22	Up	0.002	C068R032
Myoferlin	1.64	Up	0.007	C146R047
Cell differentiation				
Calcium/calmodulin-dependent protein kinase type 1	1.56	Up	0.019	C105R165
Negative regulation of cell growth				
Cyclin-dependent kinase inhibitor 1B	1.55	Down	0.041	C136R072
Cytoskeleton				
Myosin-VIIb	1.77	Up	0.008	C163R074
Myosin-VIIa	1.53	Up	0.007	C026R057
Immune response				
GTPase IMAP family member 7	2.77	Up	0.010	C024R155
Chemokine-like receptor 1	1.89	Up	0.049	C113R072
Ig mu chain C region membrane-bound form	1.88	Up	0.028	C012R159
Nuclear factor NF-kappa-B p100 subunit	1.75	Up	0.023	C254R141
NF-kappa-B inhibitor zeta	1.70	Up	0.023	C240R073
Ig kappa chain V-IV region B17 precursor	1.66	Up	0.034	C247R066
Ig kappa chain V-IV region STH	1.65	Up	0.023	C039R106
Tyrosine-protein kinase Lyn	1.58	Up	0.028	C119R166
Lipid metabolism				
Fatty acid synthase	5.22	Up	0.049	C173R048
Lipolysis-stimulated lipoprotein receptor precursor	1.78	Up	0.002	C056R131
Acyl-CoA-binding protein	6.44	Down	0.010	C121R122
Acyl-CoA desaturase	2.14	Down	0.010	C239R110
Mitochondrion				
Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial precursor	2.40	Up	0.041	C140R113
DNA polymerase subunit gamma-2, mitochondrial precursor	1.65	Down	0.002	C227R140
Proteolysis				
Caspase-14 precursor	1.73	Up	0.049	C119R149
Aminopeptidase N	1.57	Up	0.034	C132R154
Dipeptidyl-peptidase 1 precursor	1.53	Up	0.034	C214R116
High choriolytic enzyme 1 precursor	1.94	Down	0.041	C106R090
Retinoic acid metabolic process				
Cytochrome P450 26A1	1.53	Down	0.023	C221R104
Signal transduction				
Probable G-protein coupled receptor 101	1.62	Up	0.041	C195R030
Transcription				
Fos-related antigen 2	1.74	Up	0.023	C246R117
Host cell factor 2	1.63	Up	0.028	C254R152
Translational elongation				
60S acidic ribosomal protein P2	1.61	Up	0.034	C070R123
Transport				
Epithelial chloride channel protein	2.58	Up	0.013	C081R050
Potassium channel subfamily K member 6	1.80	Up	0.013	C210R045
Sodium- and chloride-dependent taurine transporter	1.77	Up	0.010	C159R025
Choline transporter-like protein 4	1.57	Up	0.001	C014R126
Ubiquitination				
Ubiquitin	1.61	Up	0.016	C255R144
Xenobiotic/antioxidant				
ATP-binding cassette sub-family D member 4	1.54	Up	0.004	C056R028
Egl nine homolog 1	1.53	Up	0.019	C212R089
Unknown				
Leucine-rich repeat-containing protein 8E	1.63	Up	0.013	C044R118
Uncharacterized protein KIAA0174	1.68	Up	0.049	C207R047

altered but showed similar trends as observed in the microarray; *keratin, type I cytoskeletal 18* (*krt18*) was significantly up-regulated on day 5 and *mucin 2* (*muc2*) was significantly up-regulated on day 3. Two transport related genes were tested (Fig. 4). The expression of *sodium and chloride dependent taurine transporter* (*slc6a6*) was not found to be significantly altered but showed similar trends as observed in the microarray while *epithelial chloride channel* (*ecc*) was significantly up-regulated on days 3 and 5. *Collectrin* (*tmm27*), involved in cell replication, was also significantly up-regulated on days 3 and 5; the expression of *epidermal retinal dehydrogenase 2* (*rdhe2*), associated with vitamin metabolism, was not significantly altered, while *glutathione s-transferase alpha-4* (*gsta4*), related to xenobiotic metabolism, was significantly down-regulated on day 5 (Fig. 4).

Table 4
Differentially expressed genes ($p < 0.05$) on day 5 vs. control.

Category/gene name	Fold change	Direction	*p*-value	ProbeID
Apoptosis				
Receptor-interacting serine/threonine-protein kinase 3	1.64	Up	0.023	C124R132
CTP biosynthetic process				
CTP synthase 1	1.67	Up	0.049	C256R099
Cell cycle				
G1/S-specific cyclin-D1	1.59	Up	0.016	C021R077
Nucleolin	1.54	Up	0.028	C140R148
Caprin-2	2.36	Down	0.049	C123R133
Ubiquitin carboxyl-terminal hydrolase 2	1.67	Down	0.049	C143R139
Cell adhesion				
Heparanase precursor	2.87	Up	0.028	C127R104
Myelin-associated glycoprotein precursor	1.57	Up	0.041	C108R085
Protocadherin-20 precursor	3.90	Down	0.004	C238R016
Cytoskeleton				
Annexin A2-B	1.94	Up	0.033	C026R110
Keratin, type I cytoskeletal 18	1.52	Up	0.049	C035R143
Endocytosis				
EH domain-containing protein 1	1.63	Down	0.019	C256R017
Exocytosis				
Guanine nucleotide exchange factor for Rab3A	2.01	Down	0.005	C053R106
Vesicle-associated membrane protein 8	1.52	Down	0.041	C090R013
Extracellular matrix disassembly				
Collagenase 3 precursor	3.16	Up	0.049	C133R147
Immune response				
CD99 antigen	1.83	Up	0.028	C167R016
Leukocyte elastase inhibitor	1.80	Up	0.011	C166R163
TRAF-interacting protein	1.78	Up	0.049	C021R124
CD276 antigen precursor	1.53	Up	0.034	C194R109
Iron metabolism				
Ferritin, middle subunit	1.75	Down	0.041	C133R102
Methyltransferase				
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	2.28	Down	0.023	C162R161
Methyltransferase type 11 domain-containing protein	1.52	Down	0.010	C130R069
Mitochondrion				
5-aminolevulinate synthase, nonspecific, mitochondrial precursor	1.99	Up	0.031	C066R041
Cytochrome c oxidase subunit VIIa-related protein, mitochondrial precursor	1.59	Down	0.019	C223R087
Proteolysis				
Calpain-2 catalytic subunit precursor	2.03	Up	0.041	C114R108
Serine protease 23 precursor	1.93	Up	0.009	C127R147
Calpain small subunit 1	1.55	Up	0.023	C196R049
Retinol metabolic process				
Epidermal retinal dehydrogenase 2	2.47	Down	0.041	C065R057
Thyroid hormone regulation				
Mu-crystallin homolog	3.40	Down	0.028	C179R097
Transcription				
ETS domain-containing transcription factor PEA3	1.66	Up	0.023	C083R028
General transcription factor II-I repeat domain-containing protein 2B	1.77	Down	0.049	C072R126
C-Myc-binding protein	1.62	Down	0.028	C085R112
Tripartite motif-containing protein 16	1.56	Down	0.023	C175R087
Transport				
Epithelial chloride channel protein	1.71	Up	0.049	C081R050
Solute carrier family 22 member 5	1.90	Down	0.019	C219R044
Ubiquitination				
E3 ubiquitin-protein ligase RNF138	1.84	Up	0.043	C134R112
Xenobiotic/antioxidant				
Glutathione S-transferase alpha-4	1.70	Down	0.049	C253R096
Bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthetase 2	1.63	Down	0.049	C207R105
Glutathione S-transferase P 1	1.61	Down	0.041	C138R089
Probable thiopurine S-methyltransferase	1.50	Down	0.049	C264R107
Unknown				
Zinc finger CCCH domain-containing protein 15	2.23	Up	0.041	C103R050
Hematopoietic SH2 domain-containing protein homolog	1.74	Up	0.028	C006R057
Tsukushin precursor	1.75	Down	0.028	C151R086

4. Discussion

In the present study, histology of the distal intestine revealed progression of typical characteristics of SBMIE over the seven day period, which was also reflected in the transcriptomic changes that can be linked to inflammatory changes and subsequent dysfunction. In general, transcriptional changes observed in the distal intestine of SBM-fed Atlantic salmon showed an early response of genes with immune and epithelial barrier related functions. On day 7, fewer DEGs were observed, which could imply that the response to the stimulus (i.e. the new diet) was leveling off. Results of the histological observation, however, also showed that only about half of the investigated fish showed signs of inflammation. No direct correlation analysis between histology and mRNA expression was

Table 5
Differentially expressed genes ($p < 0.05$) on day 7 vs. control.

Category/gene name	Fold change (FC)	Direction of FC	*p*-value	ProbeID
Apoptosis				
BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	1.72	Up	0.023	C116R035
Immune response				
Radical S-adenosyl methionine domain-containing protein 2	1.75	Down	0.041	C139R032
Protein BAT5	1.51	Down	0.049	C200R093
Interferon-induced protein 44	1.50	Down	0.034	C260R153
Transcription				
Probable ATP-dependent RNA helicase DDX5	1.53	Up	0.041	C125R117

performed as different fish were sampled for the respective analyses. Furthermore, the fish were not force-fed in order to avoid stress. It was therefore not possible to follow the feed intake of individuals that were sampled, i.e. not all fish may have started feeding on the new diet from the first day of feeding. This variable exposure time to SBM may have contributed to the variation in the data and offer an explanation for the relatively few and variable DEGs observed during the seven day period. However, from the same experiment Marjara et al. [20] found increased mRNA expression of immune-related genes in the distal intestine after 10 days of SBM exposure. This could mean that changes in gene expression in the distal intestine may occur during the first days of SBM exposure and subsequently level off for a short period. The same may be said for the signs of dysfunction.

4.1. Immune response

The gastrointestinal epithelium acts as a first line of defense in the body. It is exposed to a mixture of substances taken up during feeding and a diverse microbial community residing in the gut as well as in the environment of the fish. The immune system of the intestinal epithelium has to successfully identify and react to potentially harmful substances and pathogens. Switching Atlantic salmon from a fishmeal to a 20% SBM diet resulted in a rapid response of immune-related genes.

During the first three days of SBM exposure, several DEGs related to T and B cell regulation were observed (*GTPase IMAP*, *transcription factor hes-1*, *cd99, cd276*, *proto-oncogene tyrosine-protein kinase fyn*, *tyrosine-protein kinase lyn*). The family of *GTPase IMAP* genes consists of members with various functions. *GTPase IMAP family members 4* (down-regulated) and *7* (up-regulated) are considered important genes for pro- and anti-apoptotic T cell pathways and T helper (T_H) cell differentiation [27,32]. A down-regulation of *gtpase imap family member 4* (*gimap4*) has previously been associated with differentiation of T_H2 cells [33], whereas *gimap7* (up-regulated in the current study) is expressed in both CD4+ and CD8+ T cells, more highly in the former, and in cells isolated from rat thymus (T cells only), spleen and mesenteric

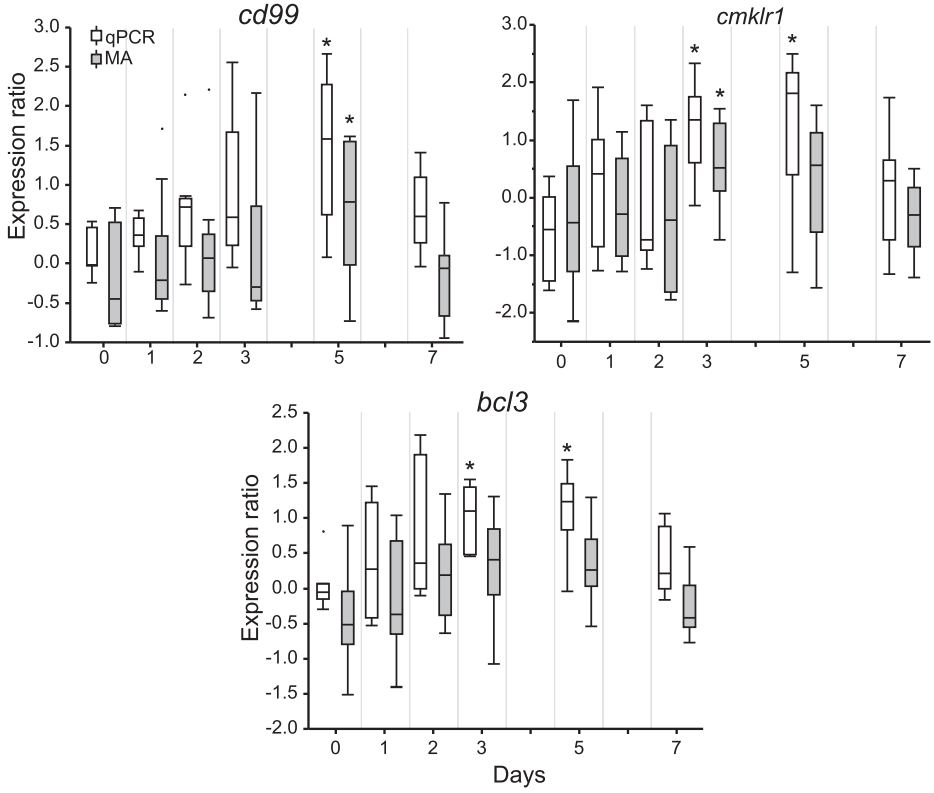

Fig. 2. Box-and-whisker plots showing $\log_2$ microarray and qPCR relative mRNA expression ratios of immune related genes. qPCR expression ratios are normalized to the geometric mean of expression ratios of *gapdh*, *hprt1* and *rnapolII*. Boxes mark the interquartile range, the line dividing the boxes denote the median, whiskers extend to 1.5× interquartile range, points outside the whisker range are displayed as outliers, asterisks mark significant differences from day 0 ($p < 0.05$). Abbreviations: *cluster of differentiation 99* (*cd99*); *chemokine-like receptor 1* (*cmklr1*); *b-cell lymphoma 3-encoded protein* (*bcl3*).

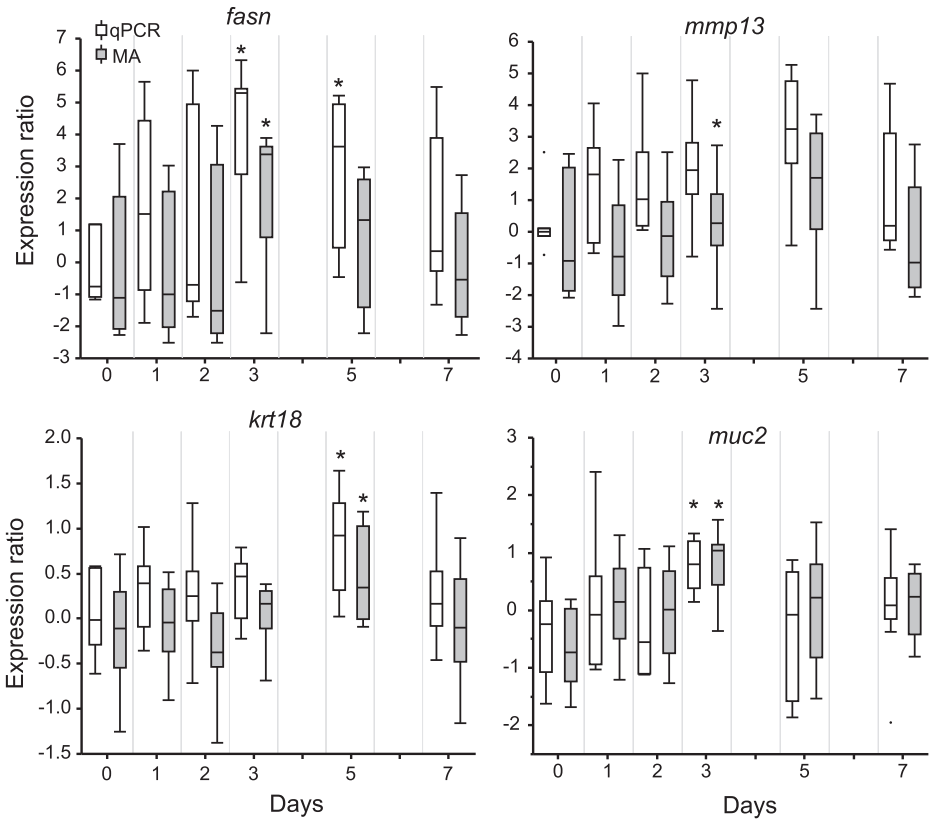

Fig. 3. Box-and-whisker plots showing $\log_2$ microarray and qPCR relative mRNA expression ratios of lipid metabolism, cytoskeleton and extracellular matrix related genes. qPCR expression ratios are normalized to the geometric mean of expression ratios of *gapdh*, *hprt1* and *rnapolII*. Boxes mark the interquartile range, the line dividing the boxes denotes the median, whiskers extend to 1.5× interquartile range, points outside whisker range are displayed as outliers, asterisks mark significant differences from day 0 ($p < 0.05$). Abreviations: *fatty acid synthase* (*fasn*), *collagenase 3 precursor* (*mmp13*), *keratin, type I cytoskeletal 18* (*krt18*), *mucin 2* (*muc2*).

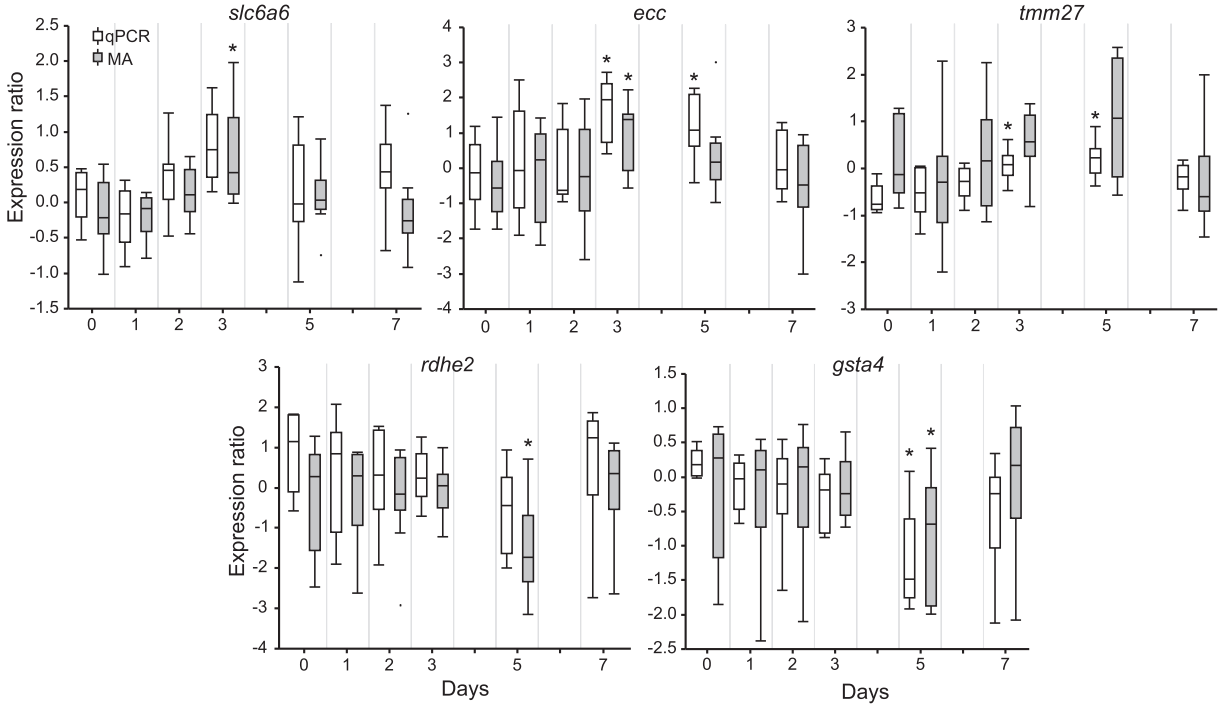

Fig. 4. Box-and-whisker plots showing $\log_2$ microarray and qPCR relative mRNA expression ratios of transport and xenobiotic metabolism related genes. qPCR expression ratios are normalized to the geometric mean of expression ratios of *gapdh*, *hprt1* and *rnapolII*. Boxes mark the interquartile range, the line dividing the boxes denotes the median, whiskers extend to 1.5× interquartile range, points outside whisker range are displayed as outliers, asterisks mark significant differences from day 0 ($p < 0.05$). Abbreviations: *sodium and chloride dependent taurine transporter* (*slc6a6*), *epithelial chloride channel* (*ecc*), *collectrin* (*tmm27*), *epidermal retinal dehydrogenase 2* (*rdhe2*), *glutathione s-transferase alpha-4* (*gsta4*).

lymph nodes [26]. Development of IL-17-producing γδ T cells was indicated by up-the regulation of *Transcription factor hes-1*. γδ T cells constitute a major population among intestinal intraepithelial lymphocytes [34,35]. Increased expression of T cell markers CD4α and CD8β, as well as T cell receptor γ (TCRγ) and IL-17A, previously reported from the same feeding trial [20], support the findings in the current microarray study and suggest that IL-17 producing γδ T cells may be triggered early after the introduction of the SBM diet and may play an important part during the development of the SBMIE. Furthermore, up-regulation of *cd99* and *cd276* may indicate increased T-cell adhesion, migration and activation [36,37] important for T cell recruitment to the tissue as a response to the injurious stimulus. Modulation of T and/or B cell signaling pathways was indicated by the up-regulation of *proto-oncogene tyrosine-protein kinase fyn* and *tyrosine-protein kinase lyn* [25,38]. These observations of DEGs related to T-cell regulation along with the increasing numbers of IELs observed in the distal intestinal tissue [20] support the hypothesis of the involvement of T lymphocytes during the development of SBMIE, as has been stated previously [9,14,20].

The up-regulation of the p100 subunit of NF-kB (*nf-kb2*), *nf-kappa-b inhibitor zeta* (*iκb-ζ*), *b-cell lymphoma 3-encoded protein* (*bcl3*) and *chemokine-like receptor 1* (*cmklr1*) indicate an alteration of the NF-kB signaling pathway [39,40]. This alteration is considered a general cellular response to a variety of stimuli including various stressors, infection and antigens [41]. NF-kB also plays an important role in the development and maintenance of an inflammatory response within cells [42,43].

An early B-cell response was indicated by the up-regulation of *ig kappa chain V-IV region b17 precursor* on days 1 and 3, as well as up-regulation of *switch-associated protein 70*, which is specifically expressed in mature B-cells. The up-regulation of other immunoglobulin related genes and *tyrosine-protein kinase lyn*, an important regulatory gene for B-cell activation and neutrophil stimulation [44], with increasing SBM exposure time indicates B-cell activation on day 3. Increased expression of Ig mu chain indicates an increase of IgM production in distal intestinal tissue. Earlier findings have shown increased IgM protein levels in the distal intestinal mucosa of salmon with SBMIE [15,45], but no changes in the number of IgM+ B-cells were observed after 3 weeks of feeding SBM compared to a control fishmeal-based diet [45]. Tests for specific antibody responses in plasma against soy proteins were previously found to be negative in rainbow trout fed SBM or immunized with soy proteins [6]. However, systemic antibody responses in fishes appear to be variable, even in successfully vaccinated fish (see review Magnadottir [46]) and local/mucosal immunity may be of greater importance. IgM plays a critical part in the defense against pathogenic bacteria [47]. Activated T-cells as well as other antigens may also trigger B-cell activation and Ig production. Further research is needed to identify the involvement of B-cells and different classes of Ig in the early stages of SBMIE.

A characteristic of SBMIE is increased trypsin activity in the distal intestine [17,48]. Up-regulation of *serine protease 23 precursor* on day 5 supports the extra-pancreatic production of serine proteases in inflamed tissue during SBMIE in salmon [17]. Serine proteases play an important part in immune responses in the intestine of mammals as well as fish and increased expression levels of serine protease 23 precursor have been related to various types of cancer [49–52]. In mammals it has been shown that the serine proteinase trypsin activates proteinase-activated receptor 2 (PAR-2), which acts as a pro-inflammatory mediator [51]. A similar correlation has been proposed for Atlantic salmon, in which two isoforms of PAR-2 have been identified [52]. Up-regulation of PAR-2 during the development of SBMIE has been reported in an earlier gene expression analysis from this feeding trial [20].

4.2. Epithelial barrier

An intact mucosal barrier is key to maintaining tissue homeostasis and a successful immune defense against pathogens and feed antigens. Mucus is secreted from epithelial goblet cells and provides the first layer of protection critical for the maintenance of intestinal homeostasis. In this study, increased mucus production after 3 days of SBM feeding was indicated by increased expression levels of *mucin-2*, which has been found to aid in the defense against several intestinal pathogens [53]. Up-regulation was also seen for other genes that are involved in remodeling and repairing cellular membranes, such as *myosin* and *myoferlin*. Functional integrity of the gastrointestinal mucosal barrier is also dependent on cellular proliferation to replace lost cells. Several genes involved in cellular growth and proliferation were up-regulated, and this is in line with previously made observations that SBM-induced enteritis increases proliferation of epithelial cells in salmon [13]. Of particular note was the notch signaling *transcription factor hes-1* (up-regulated at day 1). Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells, and in the intestinal epithelium Hes 1 is predominantly expressed in proliferating crypt cells [54]. Furthermore, Hes-1 has recently been shown to mediate induction of matrix metalloproteinase 13 (*mmp13*) which is involved in ECM degradation in disease processes [55]. On day 5, *mmp13* was up-regulated indicating that the epithelial barrier could be affected at this stage. Using qPCR for confirmation, *mmp13* up-regulation was found to be marginally significant ($p = 0.0501$). Up-regulation of MMPs has been observed during immune responses to ectoparasites in pink salmon (*Oncorhynchus gorbuscha* W.) and Atlantic salmon [56,57], as well as during a soyasaponin and pea protein concentrate mediated inflammation in the distal intestine of Atlantic salmon [58]. Degradation and remodeling of the ECM was further indicated by the up-regulation of *heparanase* (up-regulated day 5), which is an endoglycosidase that breaks down heparan sulfate, an oligosacharide that is part of the ECM. *Heparanase* has been detected in leukocytes and can aid leukocyte infiltration into the tissues by degrading the endothelium of blood vessels [59,60]. Another up-regulated group of genes related to the ECM was the *calpain* group. Calpains are involved in remodeling of cell membranes and regulation of apoptosis (reviewed by Goll et al. [61]). This supports reports of increased numbers of enterocytes positive for an apoptosis marker (*caspase-3*) during SBMIE in salmon [9]. *Calpain 2* was also found to be up-regulated in a previous report on SBM induced gene expression changes in the distal intestine of Atlantic salmon [18].

4.3. Functional and metabolic processes

Many DEGs involved in cell growth/repair/differentiation, digestion, transport, metabolism and detoxification processes were initially up-regulated on days 1–3, possibly as an attempt by the tissue to compensate for the initiating immune response. From day 5, indications of tissue malfunction became more apparent, most likely as a result of the onset of inflammation. Decreased transcription levels were identified in a number of genes involved in endocytosis, exocytosis, transport, iron, retinol and other metabolic processes as well as xenobiotic detoxification. Two members of the glutathione S-transferase (*gst*) family, *gstp1* and *gsta4*, were among the latter group of genes. The down-regulation of *gst* genes could leave the epithelial cells vulnerable to oxidative stress as these genes are important during detoxification processes in the cell [62]. Similar observations have been observed in salmon and rainbow trout hepatic transcriptome after restricted feeding, SBM feeding and infection [18,58,63,64]. Expression of genes for transport proteins, ion channels and ion pumps, important for

nutrient absorption and maintaining cell homeostasis were altered. This is in accordance with the decreased nutrient transport and increased permeability of the distal intestinal epithelium that has been reported earlier [65,66]. In previous studies, legume based diets have been associated with decreased lipid digestibility, reduced bile salt levels and hypocholesterolemia in fish [16,67–70], which may be a result of both dysfunction of the inflamed tissue and other disturbances in the digestion and absorption of nutrients due to ANFs. In the present study, the early up-regulation of taurine and choline transporters could be interpreted as a response to altered transcription levels for several genes involved in lipid metabolism. This may have had consequences for the biosynthesis of conjugated bile salt levels as well as lipid absorption and turnover. Another interesting finding was the reduced levels of solute carrier family 22 member 5 (*slc22a5*, carnitine transporter). Carnitine plays a key role in fatty acid metabolism, and seems to be important for intestinal homeostasis [71]. Furthermore, mutations in carnitine transporter genes including *slc22a5* increase the risk of developing Crohn's disease [71], an inflammatory bowel disease that shows similarities to SBMIE [20]. Decreased expression of genes involved in endocytosis apparently supports earlier findings that link the loss of supranuclear vacuoles during SBMIE with decreased macromolecular uptake of ferritin by endocytosis [72].

4.4. Conclusions

In this study, replacement of FM with 20% SBM in Atlantic salmon diets resulted in a rapid transcriptomic response in the distal intestine. Further work on the etiology and early pathogenesis should specifically focus on the involvement of CD4+ T_H2 cells, γδ T cells (IELs) and dendritic cell activation. The apparent up-regulation of genes involved in Ig production also merits further investigation, as significant involvement of IgM-producing B cells during SBMIE seems unlikely [45] but the involvement of other Ig-types cannot be ruled out. The microarray data indicates that dysregulation of barrier function and other vital intestinal functions on day 5 may have occurred as a result of the inflammatory response initiated during the first three days.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2012.11.031.

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