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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