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Transcriptional regulation of IL-17A and other inflammatory markers during the development of soybean meal-induced enteropathy in the distal intestine of Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Progression of soybean meal (SBM)-induced enteropathy in Atlantic salmon (*Salmo salar* L.) distal intestine (DI) was studied to investigate pathophysiological mechanisms and immune responses. Seawater-adapted salmon were fed an extracted SBM-containing diet (200 g kg^{-1}) from day 1–21 and compared with fish fed a fishmeal-based diet (day 0). Histological evaluation of the DI revealed signs of inflammation from day 5, which progressively increased in severity and affected more fish with increasing SBM exposure time. The expression profiles of 16 genes were analyzed by quantitative PCR. The pro-inflammatory cytokines interleukin 17A (IL-17A), IL-1β, interferon α (IFNα) and IFNγ, as well as IL-17A receptor, T-cell receptor γ (TCRγ), cluster of differentiation 4α (CD4α), CD8β, transforming growth factor β (TGFβ), trypsin, protease-activated receptor 2 (PAR2) and myeloid differentiation primary response gene 88 (MyD88) were significantly up-regulated during early and/or late inflammation stages, whereas interferon-γ-inducible lysosomal thiol reductase (GILT) was downregulated. Up-regulation of TCRγ from day seven suggests proliferation of intraepithelial γδ T cells. IL-17A, up-regulated by 218-fold during early inflammation, indicates involvement of T helper 17 cells in the pathogenesis of the SBM-induced inflammatory response.

1. Introduction

To secure a sustainable aquaculture industry, the inclusion of alternative feed ingredients, especially plant crops, in formulated feeds is increasing. There are a number of benefits to using plant ingredients, including relatively low prices and favorable market availability. However, there are also a number of concerns – they may contain anti-nutritional factors and antigens that can lead to reduced feed intake and otherwise inhibit feed utilization, as well as compromise fish health and welfare [1–5]. Full-fat and extracted soybean meal (SBM), for example, induce inflammation (enteropathy) in the distal intestine of salmonids [6–10] and common carp, *Cyprinus carpio* L. [11]. The causatory component(s) are present in the ethanol soluble fraction of SBM [12–14]. Higher inclusion levels of pea products cause a similar condition in Atlantic salmon [15].

The role of saponins – ethanol-soluble, amphipathic molecules present in both soybeans and peas – have been investigated and they appear to be involved in the pathogenesis of the condition [16,17], although not alone but in combination with other, yet to be identified factors. The saponins also appear to increase the permeability of the intestinal tissues [16] and thus may increase tissue exposure to other antinutritional factors, immunostimulatory compounds and/or microbiota present in the intestine. Similarities have been suggested between the legume-induced enteropathies in fish and enteropathies in mammals/humans, such as celiac disease, a hypersensitivity to gluten, and inflammatory bowel disorders (IBDs), an apparent loss of tolerance to the microbiota of the gut.

The pathohistological changes that occur in the distal intestine of SBM-fed Atlantic salmon include loss of the supranuclear vacuolization of the epithelial cells (enterocytes), thickening of both the lamina propria and submucosa due to an infiltration of inflammatory cells such as T-cells, eosinophilic granular cells (mast cells), macrophages, neutrophilic granulocytes and diffuse immunoglobulin M, and shortening and fusing of the mucosal folds [6,9,10,14,18–22]. The onset of observable inflammation occurs around day 2–3 following initial dietary exposure to SBM, and at day 7 all the signs of an inflammation are visible [9,22]. In Atlantic

Abbreviations: DI, distal intestine; SBM, soybean meal; FM, fishmeal; CD, cluster of differentiation; Hprt, hypoxanthine phosphoribosyltransferase; IFN, interferon; GILT, interferon-γ inducible lysosomal thiol reductase; IL, interleukin; MyD88, myeloid differentiation factor 88; PAR2, proteinase activated receptor 2; TLR, toll-like receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

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salmon the inflammation is reversible as long as SBM is eliminated from the diet [9]. In common carp, the inflammation is apparently transient, resolving some weeks after the SBM feeding has been started without the need of eliminating SBM from the diet [11].

To date, the mechanisms behind the development of the SBM-induced enteropathy in salmon are not well understood, partially due to the lack of tools to specifically identify immune cells, cytokines, interleukins, interferons and other factors involved in immunological responses in fish. The current hypothesis is that $CD4^+$ and $CD8^+$ effector T cells are involved, indicating that it may be a T cell-mediated hypersensitivity reaction [19,22]. It appears to be initiated by serine protease activation of proteinase-activated receptor 2 (PAR2) in the apical membrane of epithelial cells or antigen-presenting cells [23], as has been demonstrated in intestinal inflammatory conditions in mammals [24]. Among the relatively few immune-relevant genes linked to inflammation in the distal intestine of Atlantic salmon that have been investigated by real time PCR to date are interleukin 1β (IL-1β), transforming growth factor β (TGFβ), interferon-γ inducible lysosomal thiol reductase (GILT), and clusters of differentiation (CD) CD3, CD4α, and CD8α and β of T cells. These have altered transcriptional profiles in SBM-fed compared to fishmeal-fed salmon [19,22].

The objective of the current study was to further investigate the development of the SBM-induced enteropathy in Atlantic salmon over time, taking samples of the distal intestine at ten different time points following 0–21 days of dietary SBM exposure. Pathohistological changes and gene expression profiles of 22 immune-relevant genes by quantitative real time PCR (qPCR) were studied in an attempt to increase understanding of the mechanisms, immune cells, and signaling factors involved in the induction and further development of the inflammatory response.

2. Material and methods

2.1. Diets, fish and sampling

Two experimental diets were formulated as shown in Table 1; a reference diet containing fishmeal as the sole protein source (FM) and a test diet containing 200 g kg^{-1} hexane-extracted (defatted) soybean meal (SBM). Diets were formulated to be approximately iso-nitrogenous and iso-energetic on a crude protein and gross energy basis, and to contain 28% lipid and 43% crude protein.

Table 1
Formulation and calculated chemical composition of the fishmeal (FM) and soybean meal (SBM) diets on an as fed basis.

Diet	FM	SBM
Ingredient (g kg^{-1})		
Fish meal (108/07)	563	435
Extracted SBM (252/06)	0	200
NorSeaOil	234	243
Wheat (174/07)	179	98
Vitamin mix	20	20
Mineral mix	4	4
Yttrium oxide	0.1	0.1
Carophyll pink 10%	0.4	0.4
Proximate compostion (g kg^{-1})		
Crude protein	427	426
Lipid	280	279
Carbohydrate	142	148
Ash	88	81
Water	67	70
Gross energy (MJ kg^{-1})[a]	23.7	23.8

[a] Gross energy was calculated according to Tacon, 1987 using the energy content of 39.5 kJ g^{-1} for lipid, 23.6 for protein, and 17.2 for starch.

Seawater-adapted Atlantic salmon (*Salmo salar* L.) with initial body weight of 500–600 g were held at Nofima's research station in Sunndalsøra, Norway. The fish were allocated to 14 tanks (1 m^3), 25–30 fish per tank, containing running seawater at 8–10 °C. The fish were fed the reference FM diet for a 7-day adaptation period. The control fish received the FM diet only and are referred to as time point 0. Fish in randomly selected tanks then received the SBM diet for 1, 2, 3, 5, 7, 10, 14, 17 and 21 days, duplicate tanks per time point.

All efforts were made to avoid unnecessary stress to the fish and thus ensure adequate feed intake. A shortage of available tanks, however, made it necessary to use some tanks of fish for multiple time point samplings. The control fish fed the FM diet only (0 days) were sampled first. The remaining fish in those two tanks were subsequently fed the SBM diet for 1 and 2 days, followed by respective samplings. The remaining fish following sampling after the 3-day exposure to SBM continued on the same diet and were subsequently sampled following a total of 5 days of SBM exposure. To ensure intestinal exposure to SBM, only fish with digesta throughout the intestinal tracts were sampled.

Prior to sampling, fish were anaesthetized with tricaine methanesulphonate (MS 222; Argent Chemical Laboratories, Redmond, WA, USA), weighed, measured and killed with a sharp blow to the head. Distal intestine (DI) samples were taken from 8 to 12 fish at each of the 10 different time points, 4–6 fish per tank. All adipose and mesenteric tissue was removed from the serosal surface. The intestine was then opened longitudinally and the intestinal content carefully removed prior to collection of tissue samples.

For histology, DI samples of approximately 5 × 10 mm size were fixed in 4% phosphate-buffered formalin for approximately 24 h and subsequently stored in 70% ethanol until further processing.

For total RNA extraction, approximately 300 mg DI samples were carefully rinsed in PBS (phosphate-buffered saline), placed in RNAlater, and subsequently stored at −20 °C.

2.2. Histology

Fixed DI tissue was dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin using standard histological techniques. Longitudinal sections (i.e. perpendicular to the macroscopically visible circular folds) approximately 5 μm thick were prepared. After staining the sections with haematoxylin and eosin, light microscopy examination was performed to study the development and progression of SBM associated enteropathy, using the criteria described previously [9]. Two independent, blinded evaluations were performed as a quality control.

A semi-quantitative evaluation was employed that was based on changes in mucosal fold height, enterocyte vacuolization, leukocyte infiltration of the submucosa and lamina propria, and number of intraepithelial lymphocytes in each fish sampled for each time point. An overall score representing the severity of the SBM-induced enteritis was computed for each time point based on the mean scores.

2.3. RNA extraction

Trizol was used to extract RNA from 100 mg of DI samples, 8 fish per time point, according to the protocol from the manufacturer (Invitrogen). The samples were then DNAse treated using the Turbo DNA–free kit from Ambion® (Life Technologies Corporation, Carlsbad, CA, USA). Quality control of the RNA samples was performed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the amount of RNA.