Etiology of soybean-induced enteritis in fish

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Etiology of soybean-induced enteritis in fish

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Abstract

The inclusion of soybean meal (SBM), especially in the diet of Atlantic salmon, induces an inflammatory response of the distal intestinal mucosa, known as SBM-induced enteritis. A semi-quantitative scoring system was developed to assess the extent of the morphological changes observed in this study. The influence of SBM feeding has been investigated taking into account several dietary and non-dietary factors possibly involved in the induction of the disorder. It has been found that the severity of enteritis and its kinetics are dose-dependent. Electron microscopy studies indicated a block of the endocytosis process and a strong decrease of the microvilli length. Comparative studies were carried out in an omnivorous species and for the first time ever reported, the results suggested that the symptoms of enteritis also occur in common carp. Contrary to the observations in studies with Atlantic salmon, the common carp started to recover from week four onwards. Several cytokines were presumed to influence this process and they were correlated to the modulation of the inflammatory process triggered by the SBM-containing diet. The influence of different factors was measured according to the degree of enteritis developed. Low temperature (8 °C vs. 12 °C) seem to delay the onset of the symptoms. On the other hand, it was suggested that SBM-induced enteritis was not strongly influenced by either salinity or age. The extent of enteritis in Atlantic salmon depends on the origin and/or the processing of the soybeans. The morphological changes observed were induced when soyasaponins were fed to Atlantic salmon alone or in combination with other soybean components suggestion their possible role on the induction of enteritis. The actual causative components and its mechanisms of action need further research. It is concluded that the etiology and further development of SBM-induced enteritis is related to dietary factors rather than non-dietary factors. SBM inclusion levels and the commercial source used for the diet formulation have a great impact on the severity of the disorder, mainly affecting the endocytosis process. This thesis evidenced that the endocytosis block is directly related to the disappearance of the supranuclear vacuoles, which can be considered as the most striking feature in the onset of enteritis.

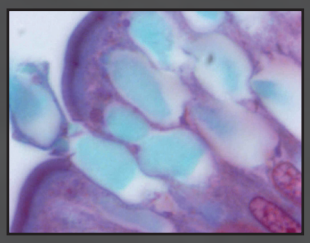
Abbreviations

AMP	antimicrobial peptides
ANFs	anti-nutritional factors
BG	basophilic granulocytes
Ct	cycle threshold
E	efficiency
EG	eosinophilic granulocytes
EM	electron microscopy
FM	fishmeal
FO	fish oil
GAR-HRP	goat-anti-rabbit-HRP
GC	goblet cells
GOI	gene of interest
HKG	house-keeping gene
HSP	heat shock proteins
IBD	inflammatory bowel diseases
IEL	intra-epithelial lymphocytes
LM	light microscopy
LP	lamina propria
MF	mucosal folds
MFAA	methanol, formalin and acetic acid
Mv	microvilli
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PBS-t	phosphate buffered saline-tween
RQ-PCR	real time quantitative-polymerase chain reaction
R	relative expression ratio
SB	soybean
SBM	soybean meal
SM	sub-epithelial mucosa
SNV	supranuclear vacuoles

Contents

GENERAL INTRODUCTION

Chapter 1



Fishmeal and fishmeal replacement

Fishmeal (FM) and fish oil (FO) are important commodities for the production of animal feeds. FM is an important source of protein for fish and is often the only source that complies with most of their nutritional requirements. In addition, FO is a rich source of polyunsaturated fatty acids which constitutes the main source of lipids, particularly in fish. FM and FO are derived from a natural resource composed of wild-caught pelagic fish from the sea. Since 1985, world production of FM has stabilized at six to seven million tonnes and for FO at one million tonnes. Aquaculture presently accounts for 35 percent of the world's FM consumption. In recent decades aquaculture has been growing at a higher rate than all other animal food-producing sectors, with an average annual growth rate of 8.8 percent per year (FAO 2006). At this strong growth, pressures on the fish stocks supporting the production of FM and FO will continue or even increase while they are already being utilized at their maximum level of exploitation. Therefore, a strong competition on the market can be expected for FM and FO resources, possibly leading to high prices and low availability. Knowing that feed often comprises more than 50% of the total production costs (El-Sayed 1999; Fagbenro 1999), for both economic and sustainability reasons, a cheap and reliable source of protein is needed to ensure a cost-effective and sustainable aquaculture. Therefore the replacement of FM for fish diets is a high priority. Among the alternatives, plant-based formulations are the cheapest, and many have a suitable protein profile and will be available in the long term (Carter & Hauler 2000; Francis *et al*. 2001; Glencross *et al*. 2004; Gatlin *et al*. 2007). These alternative protein sources have to ensure an excellent growth performance and health status of the cultured species but also have to meet requirements for taste, odour, and the consumer's acceptability.

Soybean as Fishmeal replacement

Oilseeds, in particular, soybean (*Glycine max* L.), and grain products have great potential as protein and/or oil sources for fish feeds (Alexis & Nengas 2001). Nonetheless, SB contains anti-nutritional factors (ANF's) which may inhibit nutrient utilization and digestibility. Oligosaccharides, non-starch polysaccharides, saponins, protease inhibitors, antigenic compounds, lectins, phytic acid, tannins, phytoestrogens alkaloids, gossypols are well known ANF's (Alexis & Nengas 2001; Francis *et al*. 2001). Diverse feed processing

techniques like dry or wet heating, aqueous-extraction and the addition of supplements can reduce the final content of ANF's (Rumsey *et al*. 1994; Buttle *et al*. 2001; Refstie *et al*. 2005) and reduce their negative impacts (Francis *et al*. 2001).

The negative effects of SB products inclusion also depend on the source and type of this product and the level of replacement. According to literature, commercial diets for salmonids may contain about 34% to 47% protein and 28% to 40% lipid (Refstie *et al*. 2001). Different SB products could fulfil this high protein demand, however, the problem remains that the more refined the formulations are, the more expensive the feed becomes. Many studies have searched for the optimum inclusion level with the lowest amount of noxious factors. Different treatments of SB and inclusion levels have been tested. Some of the results indicate that for salmonids, diets containing SB protein concentrate shows a growth performance as good as the high quality FM (Olli *et al*. 1995; Storebakken *et al*. 1998). This is followed in performance by full-fat, dehulled solvent-extracted and solvent-extracted SB meal. The latter seems to reduce growth with increasing levels of inclusion (Krogdahl *et al*. 2003). In African catfish (*Clarias gariepinus* Burchell) diets, replacement of FM by dehulled solvent-extracted SB meal was possible up to the level of 50% (even 75% when methionine supplementation was used) without compromising growth and feed utilization efficiency (Fagbenro & Davies 2001). For Atlantic halibut (*Hippoglossus hippoglossus*) 36% full-fat SB meal may be added to the diets without negative effects on growth, feed efficiency or intestinal morphology (Grisdale-Helland *et al*. 2002). Egyptian sole (*Solea aegyptiaca*) can be fed with a diet containing up to 30% SB meal without any reduction in the growth rate or induction of histopathology of the gut (Bonaldo *et al*. 2007).

SB-induced enteritis in Atlantic salmon

In Atlantic salmon (*Salmo salar* L.), replacing 20% fish meal protein by dehulled solvent-extracted SB meal does not impair growth (Olli *et al*. 1994, 1995). This seems to corroborate with the results of several other authors, who all demonstrated that replacing small amounts of FM (e.g. 20% or less) by SB products does not lead to significant growth depression (Bjerkeng *et al*. 1997; Refstie *et al*. 2001; Opstvedt *et al*. 2003). However, Krogdahl *et al*. (2003) showed that at low inclusion levels, dietary SB meal could induce intestinal disorders, which on their turn could lead to growth depression later on. These

disorders were first described by van den Ingh *et al*. (1991, 1996) and named by Baeverfjord & Krogdahl (1996) as "non- infectious sub-acute enteritis". The typical signs of this intestinal disorder are: a shortening of the mucosal folds, loss of the normal supranuclear vacuolisation; a thickening of both lamina propria and sub-epithelial mucosa with a severe infiltration of inflammatory cells (particularly macrophages and eosinophilic granulocytes) and increased numbers of goblet cells in the epithelium (van den Ingh *et al*. 1991, 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001).

Up to now, the degree of enteritis and its impact on the epithelial mucosa was mainly described as either slight, moderate or severe, usually based on qualitative analyses only (Refstie *et al*. 2000, 2001; Sanden *et al*. 2005; Bakke-Mckellep *et al*. 2007). The majority of studies (Krogdahl *et al*. 2000, 2003; Refstie *et al*. 2005; Lilleeng *et al*. 2007) used an end point approach, with a response analysis not earlier than 20 days after SB meal feeding. Such an approach does not provide information on the development process of the disorder, while this is crucial information for the comparison of species and for different husbandry and environmental parameters.

It is still unclear how SB products really cause enteritis in Atlantic salmon. The development of enteritis may be related to the risk for secondary diseases, which could be facilitated by the SB-induced morphological changes in the intestine. Krogdahl *et al*. (2000) studied the disease resistance and local immune response in Atlantic salmon fed different SB products during a cohabitation challenge using *Aeromonas salmonicida* ssp. s*almonicida*. In animals with clinical signs of enteritis, gut permeability increased. This could have facilitated the colonization of the epithelium by pathogens, which in combination with other factors such as diarrhoea, reduced nutrient digestibility and reduced growth, make fish more vulnerable to disease outbreaks.

The main structure affected seems to be the proximal part of the distal intestine, also called the second gut segment. This gut segment is considered as more sensitive to food-borne enteropathies because it is the major site of endocytosis of intact proteins (Stroband *et al*. 1979, Stroband & van der Veen 1981; Rombout *et al*. 1985; Sire & Vernier 1992; Bakke-McKellep *et al*. 2000). Endocytosis could well play an essential role in the development of the intestinal disorder, but until now, data are not available to support this hypothesis. This could also be related to the fact that most of the observations on SB-induced enteritis are

restricted to light rather than to electron microscopy. It is clear that, if the etiology of the SB-induced enteritis process needs to be understood, more information on the function of the second gut segment, particularly on its role in absorption and immunity, has to be gathered. More knowledge on the early development of enteritis will contribute to the elucidation of the mechanisms behind this disorder. The identification of the early symptoms of enteritis can be used not only as indicators of the severity and/or speed at which the disorder develops but also as a tool to assess the development of the inflammatory process. Since most research on the impact of SB products, and in particular, of SB meal, focused on salmonids, it is of high importance to investigate whether enteritis also occurs in other species. In this regard, omnivorous fish species may be highly interesting since they are usually exposed to SB products. Common carp (*Cyprinus carpio* L.) is an example of an omnivorous fish. In addition, it is a representative of species lacking the stomach (Fig. 1). Therefore, it may not only provide information of why and how some species respond stronger than others to SB products, but also common carp can be an interesting model to compare with stomach containing species like Atlantic salmon.

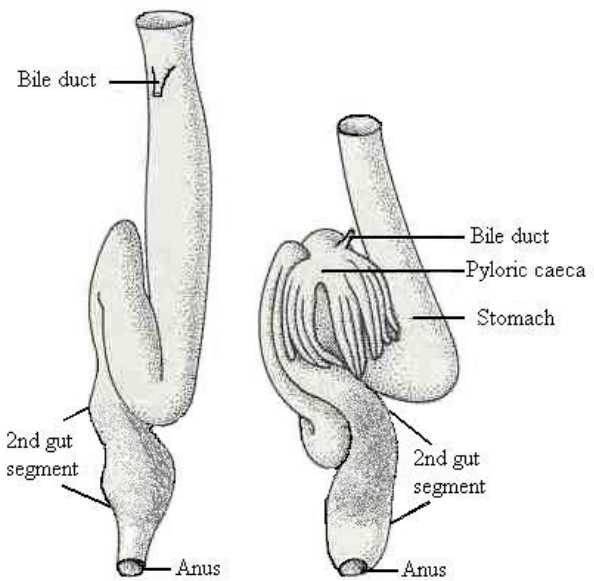


Figure 1 Scheme of different intestinal tract a stomachless fish (left) and a more complex stomach containing fish (right). (Adapted from Stroband *et al*. 1980).

Up to now only qualitative methods were used to assess enteritis. These methods are insufficient to study the etiology of the disorder and to compare it between species, environmental factors and different SB products. The mentioned ambitions can only be realized if a quantitative assessment method is developed and used as an instrument to compare the effects of SB-induced enteritis.

Aim and scope of this Thesis

The overall objective of the present study was to elucidate the mechanism behind the inflammatory process induced by SB products. Based on previous observations, it is hypothesised that the altered endocytosis process is the driving mechanism behind the SB meal-induced enteritis. Since the development of enteritis has been mainly studied in relation to the type of SB diet, more attention will be paid to non-diet related factors like i.e. husbandry conditions and animal-intrinsic factors like the endocytosis process. It is reasonable to consider that the hampered endocytosis can also occur in other fish species. Therefore, the etiology of the disorder will be investigated both in Atlantic salmon and in common carp.

In this study the following objectives/aspects are addressed:

- Development of a scoring system which can be used as a tool to further study and compare the kinetics of the disorder under different conditions.
- The etiology of the SB meal-induced enteritis, focusing on the early development of the disorder.
- Evaluation of the effects of non-diet related factors on the development of the disorder such as water temperature.
- Evaluation of the effects of diet related factors on the development of the disorder such as SB inclusion levels, commercial sources, soyasaponins content.
- Investigation of possible enteritis in an omnivorous fish species (common carp) and a better identification of the mechanism responsible for the possible induction of the disorder.

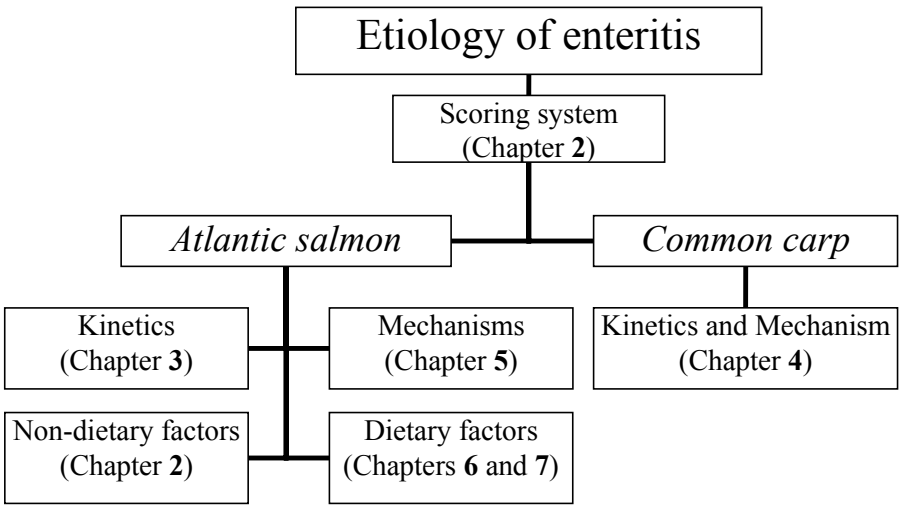


Figure 2. Summary of the thesis setup.

In **Chapter 2,** the impact of water temperature on the development of enteritis is assessed by means of a semi-quantitative scoring system. In **Chapter 3,** the onset of the enteritis development is described and the kinetics of the enteritis process is analysed at two different SB meal inclusion levels. This chapter gives also information on the changes at the ultrastructural level in the epithelium of the distal intestine. In **Chapter 4**, the effect of dietary SB meal is compared in omnivorous common carp and carnivorous Atlantic salmon. In this study common carp was continuously fed on animal protein before being transferred to the SB meal diets (20% inclusion level). At this inclusion level, Atlantic salmon usually develops severe clinical signs of enteritis. The kinetics of several cytokines are included to illustrate the regulation of the inflammatory process. In **Chapter 5**, an attempt is made to link the morphological changes observed at the light microscopical level and the changes observed at ultrastructural level (endocytosis). Young salmon were used to establish whether age and freshwater conditions can have any influence on the severity of SB meal-induced enteritis described previously for older fish kept in seawater. **Chapter 6** aims to clarify if different commercial sources of SB meal can result in dissimilar severity degrees of the enteritis

process in Atlantic salmon using the mentioned semi-quantitative scoring system. In **Chapter 7,** attention is paid to possible causative components of the SB-induced enteritis, by means of phase separation of the SB molasses and the biochemical composition of the saponin-containing sub-fractions. Finally in **Chapter 8**, the overall results obtained from this study will be summarized and discussed, together with possible explanations and indications for the underlying mechanisms involved in the development of the SB-induced inflammatory response.

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Soybean meal-induced enteritis in Atlantic salmon *(Salmo salar L.)* at different temperatures

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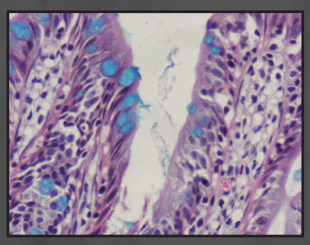
Chapter 2



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Abstract

This study evaluates the effect of temperature on the development of intestinal disorders when Atlantic salmon are fed soybean meal (SBM). In this study 20% of the dietary fishmeal (FM) was replaced by solvent-extracted Hipro SBM. Atlantic salmon reared at two different water temperatures (8 ºC and 12 ºC), were fed a control diet and an experimental diet for 20 days. Samples were taken at days 7 and 20. The extent of the morphological changes was assessed using a semi-quantitative scoring system developed for this purpose. The study demonstrates that enteritis is affected by temperature. The intestinal disorders were more severe in fish reared at 12 °C compared to those reared at 8 °C. It can be concluded from this study that temperature changes the speed but not the type of SBM-induced enteritis expressed as a delay on the response when Atlantic salmon are kept at lower temperatures.

Introduction

Soybean meal (SBM) has been suggested as one of the best alternatives to replace fishmeal (FM) for salmonid diets. However, the inclusion of SBM induces enteritis in Atlantic salmon (*Salmo salar* L.). Baeverfjord & Krogdahl (1996) described this condition as "a non- infectious sub-acute inflammation of the distal intestine". These pathological changes seem to be particularly present on the distal intestinal segment rather than on the proximal as reported in several studies on salmonids (van den Ingh *et al*. 1991; Burrells *et al*. 1999; Nordrum *et al*. 2000; Buttle *et al*. 2001).

The symptoms that define the condition are: a shortening of the mucosal folds; a loss of the normal supranuclear vacuolisation of the absorptive cells in the intestinal epithelium; a widening of the central stroma within the mucosal folding, with increased amounts of connective tissue; a profound infiltration of inflammatory cells in the lamina propria (van den Ingh *et al*. 1991; van den Ingh *et al*. 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001); an increased presence of IgM (Bakke-McKellep *et al*. 2000), an increased amount of goblet cells in the epithelium, as well as a decreased height of the microvilli together with increased microvillar vesicle formation (van den Ingh *et al*. 1991).

The inclusion levels, varieties, origins and processing techniques of the different soybean (SB) products along with husbandry conditions influence the occurrence of these symptoms. Previous studies have primarily focused on the impact of diet formulation on fish performance. An exception is the study of Nordrum *et al*. (2000) who investigated the effect of salinity on the development of enteritis in salmonids. Several studies have demonstrated that the absorptive capacity in salmonids is negatively influenced by high inclusion levels of SBM in the diet. Rumsey *et al*. (1994) suggested that antigenic soya protein affects non-specific defence mechanisms, growth performance and protein utilization in rainbow trout. Nordrum *et al*. (2000) found that the effects of SBM on the intestinal morphology of rainbow trout were of less magnitude than for salmon. Atlantic salmon seem to develop a more severe enteritis condition than other fish species. What more, despite the importance of this species, little information is available on the impact of different management-related factors on the occurrence of enteritis. In the present study the effects of temperature were evaluated.

Preliminary studies (unpubl. observ.) suggested that enteritis becomes less severe at higher temperatures. The current study will investigate whether temperature can indeed affect the enteritis process either by influencing the metabolic rate or by having a direct impact on the normal nutrient absorption process. For this purpose a semi-quantitative scoring system is introduced. This scoring system has great potential as a diagnostic tool for the histological evaluation of an inflamed intestine. The possible effects of temperature on the aggravation of the mentioned symptoms will be investigated.

Material and Methods

Fish rearing conditions

The experiment was carried out at the Skretting fish trials station, Lerang, Jørpeland, Norway. For this experiment, 40 Atlantic salmon (AquaGen strain) were sampled for gut histology measurements. The Atlantic salmon used originated from a stock of fish present at the research station. The experiment consisted on a 14-day adaptation period and a 20-day experimental period. At the start of the adaptation period the fish weighed approximately 300 g. Four indoor tanks with a diameter of 1 m each were used. The water volume in the tanks was 400 L. The stocking density was 50 fish per tank. Each tank was kept at flow rate of 12 to 15 L min^{-1}. Seawater pumped from 90 m depth in the fjord, with a salinity of 34 ‰ and an oxygen concentration above 9 ppm, was used as the inlet water. The temperature of the inlet water was 8 °C or 12 °C depending on the experimental treatment (2 tanks per water temperature). Prior to the adaptation period fish were kept at 8 °C. The applied photoperiod was 18L : 6D.

Diets and feeding

The diets were produced at Skretting Feed Technology Plant (Stavanger, Norway). Two diets were formulated: a control diet (0SBM) and an experimental diet (20SBM) (Table 1). The major ingredients in the control diet, 0SBM, were: FM (protein content above 70%), fish oil (FO) and wheat. This control diet did not contain SBM. The experimental diet, 20SBM, contained 20% solvent-extracted Hipro SBM (Cargill, The Netherlands). In the

20SBM diet, FM, FO as well as wheat were exchanged for 20% SBM compared to the control diet. Diets were formulated to be iso-nitrogenous and iso-energetic on a crude protein and a crude lipid basis. Diets were supplemented with a standard vitamin and mineral premix. Feed was produced as extruded 4 mm sinking pellets.

Prior to the experiment the fish were fed a commercial salmon diet (Skretting, Stavanger, Norway), without any SB products. During the adaptation period all fish were fed with the control diet (0SBM). At the start of the experimental period (day 1), fish of one of the tanks at each water temperature were fed the experimental diet (20SBM) while fish in the two others continued to be fed with 0SBM. Fish were fed 20% in excess. Feed was divided into two meals per day and it was provided by automatic feeders.

Table 1 Ingredients and chemical composition of the experimental diets.

	Diets[1]	
	0SBM	**20SBM**
Ingredients (g kg^{-1})		
Fishmeal[2]	564.3	475.3
Extracted soybean meal[3]	0	200
Wheat	210.6	70
Fish Oil[4]	222.6	252.1
Vitamin premix	1.3	1.3
Mineral premix	1.3	1.3
Pigment premix		
Yttrium oxide	0.1	0.1
Carophyll Pink	0.6	0.6
Chemical composition (by analysis)		
Crude Protein (g kg^{-1})	429.8	450.6
Crude Lipid (g kg^{-1})	277.1	301.4
Target dry matter (%)	95	95
Fat NMR (%)	30.5	32.8
Protein (%)	43.1	45.2
Moisture (%)	5.1	4.7
Ash (%)	7.2	7.3

[1] Amount of fish meal replaced by Soybean meal (SBM) in percent.

[2] LT North Atlantic, from Egersund, Norway.

[3] Cargill, The Netherlands.

[4] Northern hemisphere.

Chemical analysis of diets

The nutrient composition of the experimental diets was determined using standard techniques for proximate analyses. Crude protein content was determined by the Kjeldahl nitrogen measurement according to the Nordic Committee on Food Analysis, Method No. 6, 4th edition 2003. Crude fat content was measured by low field nuclear magnetic resonance. Moisture content in samples was measured by drying to constant weight at 102-105 °C for 16-18 h. Ash content was measured by combustion at 540 °C for 16-18 h, after which the remaining residues were weighed, both according to the Nordic Committee on Food Analysis Method No. 23, 3rd edition 1991. The preceding analyses were carried out at the Skretting Aquaculture Research centre, Stavanger, Norway. (See Table 1 for chemical composition).

Sampling for intestinal morphology

During the experimental period, fish gut was sampled for a histological assessment of enteritis developed at 7 and 20 days after SBM feeding. At each sampling moment, five fish per treatment group were sampled (per water temperature two experimental diets). The samples were taken from alternate tanks to avoid drops in feed intake due to sampling stress. Directly after the morning meal, the fish were anaesthetized using 0,05 g L^{-1} metacaine (Argent chemical laboratories, USA) and thereafter killed by a sharp blow to the head. The distal intestine was dissected from the point were the intestinal diameter increases, the mucosa becomes darker and annular rings are clearly noticeable. A two-centimeter section of dissected distal intestine of each fish was taken and gently rinsed with cold (4ºC) saline water. Samples were fixed in a 4% phosphate-buffered formalin with a pH of 7.2 and stored at room temperature. After dehydration by standard procedures, samples were embedded in paraffin. Transverse sections of 5 µm thickness were cut using a Microm HM 350 rotary microtome (Heidelberg, Germany) and thereafter mounted on glass slides. Each slide contained from 3 to 4 sections of a complete cut of an annular ring of distal intestine where all layers were visible and well represented. After de-paraffination, sections were stained using a mix of Haematoxylin/Eosin and Alcian blue pH 2.5. Alcian blue staining enhances the contrast between goblet cells and the supranuclear vacuoles. Slides were blindly evaluated after randomization.

Scoring system

For this study a semi-quantitative scoring system was used. In this scoring system six separate parameters of soybean-induced enteritis were quantified independently, according to: 1) the appearance and length of the mucosal folds (MF); 2) the presence and size of supranuclear vacuoles (SNV); 3) the abundance of goblet cells (GC); 4) the degree of infiltration abundance and of eosinophilic granulocytes into the lamina propria and into the sub-epithelial mucosa (EG); 5) the degree of widening of the lamina propria (LP); and 6) the degree of thickening of the sub-epithelial mucosa (SM). Each of these parameters was scored on a scale from 1 to 5, including half values between categories. An increasing scoring value represents a more severe enteritis condition. Sections were photographed with an Olympus DP 50 digital camera connected to a Nikon Microphot-FXA light microscope (Badhoevedorp, The Netherlands). The pictures were processed and analysed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany). A detailed description of the morphological/histological appearance per characteristic for the different scoring values from 1 to 5 is given in Table 2. Different degrees of enteritis are shown in Figure 1. An overall value of the degree of enteritis was calculated by averaging the scores of the six separate parameters (MF, SNV, GC, EG, LP and SM). (For illustrations of the different scores see annex or check at http://www.afi.wur.nl/UK/Publications/).

Statistics

Preliminary analysis of the slides showed that SBM-induced enteritis was not present in those fish fed the control diet (0SBM). Therefore, the effect of water temperature and sampling moment (i.e., days after changing to the SBM diet) on scorings of the separate enteritis parameters (MF, SNV, GC, EG, LP and SM) as well as the overall mean enteritis scoring were analysed by a 2-way ANOVA of the fish at the experimental diet (20SBM). Furthermore, it was assessed as to how development of enteritis was related to the combined effect of days and water temperature, after exposing the fish to the 20SBM diet by using degree-days. This was done by a linear regression of degree-day on the mean enteritis score. These analyses were done using the general linear model procedure of SAS (1999). Error term analysis using the univariate procedure of SAS (1999) showed that scoring values of all

separate parameters and the overall mean score were normally distributed. The level of significance was established at $P < 0.05$.

Table 2 Description of the semi-quantitative scoring system using different parameters to assess the degree of enteritis developed by Atlantic salmon fed a soybean meal-containing diet

Score	Parameter	Score	Parameter
	Mucosal folds (MF)		**Supranuclear vacuoles (SNV)**
1	Basal length	1	Basal SNV size
2	Some shrinkage and bloating	2	Some size reduction
3	Diffused shrinkage and onset of tissue disruption	3	Diffused size reduction
4	Diffused tissue disruption	4	Onset of extinction
5	Total tissue disruption	5	No SNV
	Goblet cells (GC)		**Eosinophilic granulocytes (EG)**
1	Scattered cells	1	Few in SM basal small quantity
2	Increased number and sparsely distributed	2	Increased number in SM and some migration into LP
3	Diffused number widely spread	3	Increased migration into LP
4	Densely grouped cells	4	Diffused number in LP and SM
5	Highly abundant and tightly-packed cells	5	Dense EG in LP and SM
	Lamina propria (LP)		**Sub-epithelial mucosa (SM)[1]**
1	Normal size LP	1	Normal SM
2	Increased size of LP	2	Increased size SM
3	Medium size LP	3	Medium size SM
4	Large LP	4	Large SM
5	Largest LP	5	Largest SM

[1]Other common names used by different authors to describe the intestinal sub-epithelial mucosa in fish:

Submucosa: Rumsey *et al*. 1994; Baeverfjord & Krogdahl 1996; Burrells *et al.* 1999; Olsen *et al*. 2000; Sitjà-Bobadilla *et al*. 2005.

Connective tissue: van den Ingh *et al.* 1991, 1996; Reite 1997.

Underlying connective tissue: Reite & Evensen 2006.

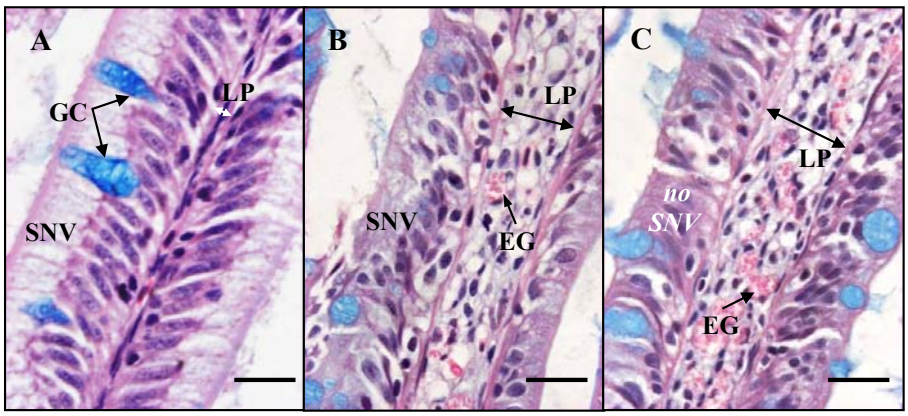

Figure 1 Distal intestine of Atlantic salmon during the enteritis process (for more details see annex or check at http://www.afi.wur.nl/UK/Publications/). Supranuclear vacuoles **SNV**, goblet cells **GC**, lamina propria **LP**, eosinophilic granulocytes **EG,** mucosal folds **MF** and sub-epithelial mucosa SM (not shown). **A**) normal epithelium with tall finger-like MF; SNV are normally aligned. Some scattered GC in normal amount; LP is a thin and delicate core of cells. Scores are considered as basal values. **B**) SNV are present as small vesicles, GC and EG population is increased. **C**) completely disturbed epithelium, showing infiltration of inflammatory cells especially EG into the LP; SNV are not longer present, GC are highly abundant; mucosal folds MF have a stubby appearance. (H & E, Alcian blue staining). Bar is 20 µm.

Results

Qualitative description of morphological changes

Figure 2 shows the intestinal morphology of salmon fed the SB diet (20SBM) at day 7 and day 20 for both water temperatures (8 °C and 12 °C) in comparison to salmon fed the control diet at 12 °C at day 7 and day 20 of the experiment. The control diet (0SBM), formulated to contain 100% FM as sole protein source did not induce any sign of enteritis. Water temperature did not affect the morphology of the distal intestine of those fish fed the 0SBM diet.

All fish fed the SBM-based diet developed enteritis. It was observed after 7 days of feeding that even a 20% inclusion level was enough to induce enteritis at both temperatures.

The degree of enteritis increased over time at both temperatures, expressing a progressive condition after 20 days of feeding but the reaction was stronger in those fish reared at 12 °C. The observed changes were related to the loss of the regular alignment of the SNV, the increased infiltration of inflammatory cells in the SM and LP, and the increased number of GC among the enterocytes. After 20 days of SBM feeding, a more progressive response at both temperatures was observed. The width of the SM had increased steadily and the vacuolization had been completely disturbed. At the lower temperature, these parameters had been less affected, indicated by the presence of less shortened MF, less infiltrated SM and LP and less increased EG and GC. Indeed, supranuclear vacuolization is somehow less disrupted compared to that found in fish reared at 12 °C.

During the trial, no mortality was observed for any of the treatments.

Semi-quantitative scoring results

The scoring of MF, SNV, GC, EG, LP, SM, and the mean score were all significantly different between sampling moments ($P < 0.01$) and for all parameters except for LP they were all significantly different between temperatures, ($P < 0.05$) (Table 3).

Furthermore, the scoring of enteritis parameters like SNV and GC were the most affected by the water temperature ($P < 0.01$). For all parameters a higher value was scored at a water temperature of 12 °C compared to 8 °C. The interaction effect between sampling moment and water temperature was not present for any of the parameters scored. Plotting the mean score value against degree-day shows that the SBM-induced enteritis is delayed when fish are kept at lower temperatures. The mean score was linearly related to the degree-day (R^2= 93.25%; $P < 0.05$, Fig.3).

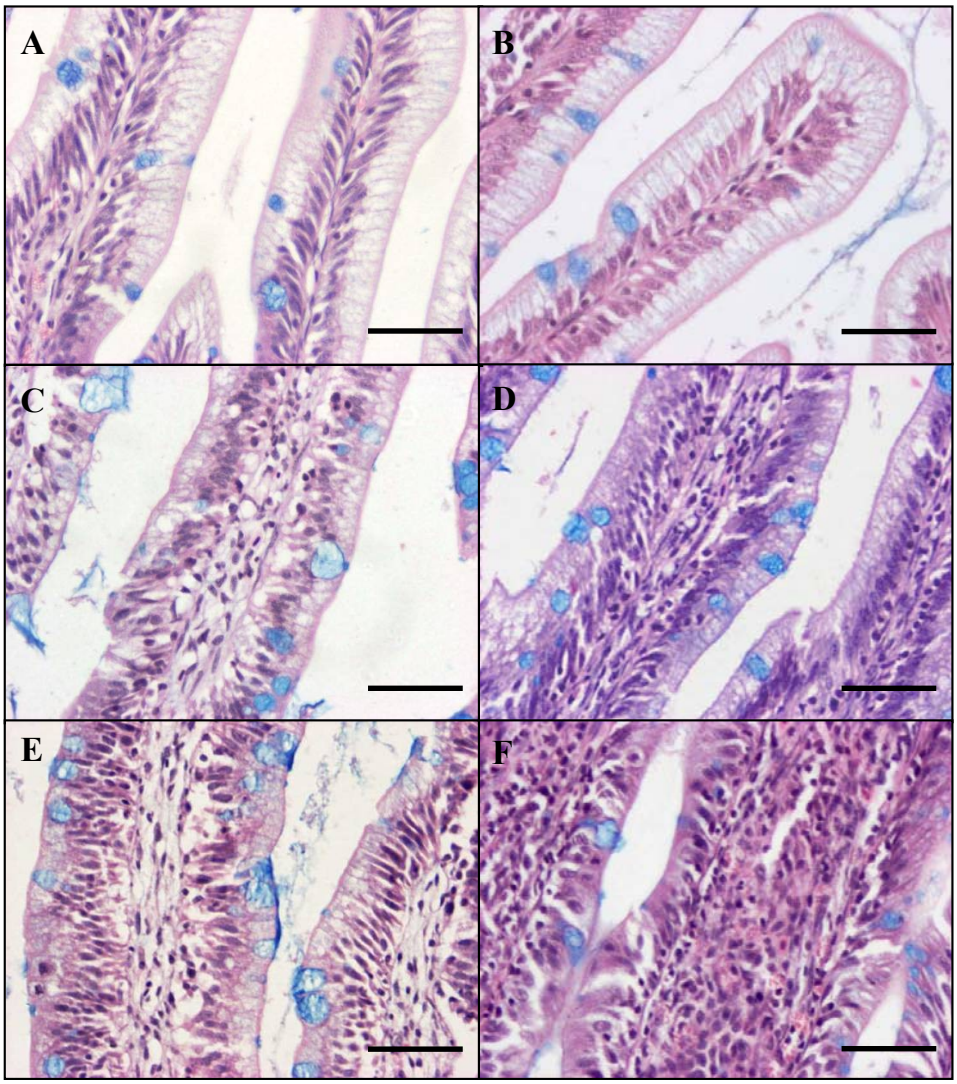

Figure 2 Morphological appearance of the distal intestine of fish reared at two different temperatures and fed either a fishmeal-based diet (0SBM) as control or a soybean meal-based (20SBM) diet. Distal Intestinal epithelium of fish fed the control diet that were kept at **A**) 8 °C and **B**) at 12 °C. Distal intestinal appearance after 7 days of SBM feeding of **C**) fish kept at 8 °C and **D**) fish kept at 12 °C. Distal intestinal appearance after 20 days of SBM feeding, **E**) fish kept at 8 °C and **F**) fish kept at 12 °C. (H & E, Alcian blue staining). Bar is 50 µm.

Table 3 Individual score and overall mean score at two sampling days and two temperatures for the different parameters used to assess the degree of enteritis developed by Atlantic salmon when they are fed a soybean meal-containing diet.

	Day 7				Day 20				Significance[1] (*P*)		
	8 °C	SE	12 °C	SE	8 °C	SE	12 °C	SE	Temp	Time	Interaction
MF	2.3	0.20	2.7	0.20	2.9	0.10	3.1	0.10	*	**	NS
SNV	1.9	0.10	2.3	0.12	3.3	0.25	3.8	0.20	**	***	NS
GC	2.1	0.10	3.2	0.12	2.8	0.12	3.5	0.16	***	**	NS
EG	2.3	0.20	2.8	0.12	2.9	0.19	3.5	0.23	*	**	NS
LP	3.0	0.23	3.1	0.19	3.4	0.19	3.9	0.10	NS	**	NS
SM	2.7	0.30	3.0	0.16	3.2	0.12	3.6	0.10	*	**	NS
Mean score	2.38	0.15	2.85	0.12	3.08	0.11	3.57	0.07	**	***	NS

MF, mucosal folds; SNV, supranuclear vacuoles; GC, goblet cells; EG, eosinophilic granulocytes; LP, lamina propria; SM, sub-epithelial mucosa.

[1] Statistical significance: $^{NS}P > 0.05$; $*P < 0.05$; $**P < 0.01$, $***P < 0.001$

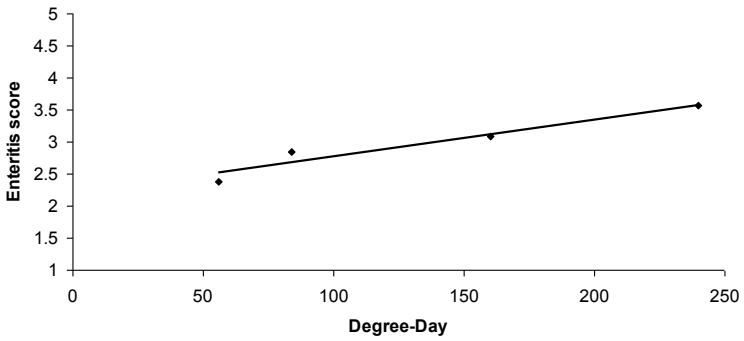

Figure 3 Mean enteritis scores as a function of time and temperature expressed as degree-day. The trend line indicates the speed of the enteritis development at different temperatures. Values are shown as mean scores of all parameters at two different temperatures (8 °C and 12 °C) and two different sampling points (Day 7 and Day 20). Estimated linear relationship between mean score (Y) and degree-day (X): Y= 2.19 (SE = 0.16) + 0.0058 (SE = 0.0110) X; R^2= 93.25 %, $P < 0.05$).

Discussion and Conclusions

The intestinal epithelium is an important site for the absorption of nutrients, immunity, osmotic balance, recycling of enzymes and macronutrients. Several authors have stated that the distal intestine of teleost fish is the principal site for the endocytosis of intact proteins, assuring its absorption and intracellular digestion (Stroband & van der Veen 1981; Rombout *et al*. 1985). This high endocytotic capacity possibly makes the distal intestine more sensitive to food-borne enteropathies. It is well known that quality and quantity of food are important factors in the development of the intestinal mass and the mucosal architecture (Buddington *et al*. 1997), but environmental conditions may also have a strong influence. Temperature is considered one of the most influential environmental factors on the development and growth of fish. In salmonids, it may affect physiological functions, feeding behaviour, stress responses and susceptibility to pathogenic organisms by affecting the innate immune system (Alcorn *et al*. 2002; Magnadóttir 2006).

The current study has shown that the severity of enteritis increased with water temperature, but the mechanism behind this increase remains unclear. Houpe *et al*. (1996) reported that environmental temperature influenced the functional demands of the intestine by altering the metabolism and the nutrient uptake. They suggested that fish exposed to different temperatures may adjust their absorptive capacities by influencing the activity of transporters when the absorptive tissue surface area is increased, the density of transporters is adapted or the physical and chemical characteristics of the apical membrane are adjusted, or perhaps, the combination of all processes. Due to the relatively higher metabolic activity of fish reared at higher temperatures and the uninterrupted exposure to noxious agents, the reaction to SBM feeding might have been stronger. Although there were no visual differences on feed intake between the two groups, a possible effect of feed intake in the observed different response cannot be neglected. The influence of feed intake on the development of enteritis needs to be further investigated.

Temperature could have affected the exposure time to noxious agents present in SBM by influencing the time digesta remains in contact with the intestinal epithelium. However, when the digesta passage rate increases at a higher temperature, the exposure time decreases. Therefore, the results of this study indicate that this is not the case since the highest degree of

enteritis was observed in fish reared at the higher temperature. Therefore, the stronger degree of enteritis seems to be more correlated to higher metabolism rather than to a lower digesta passage rate.

The influence of a higher metabolism could be reflected on the efficiency of the intestinal enzymatic activity. Any disruption on the normal activity of the enzymes linked to the brush-border membrane could have a severe impact on the uptake process and the loss of the regular supranuclear vacuolization. Krogdahl *et al*. (2003) showed a reduced enzymatic activity in the distal intestine with increasing SBM inclusion levels. The effect of temperature in the disappearance of the SNV due to reduced enzymatic activity is still an open question.

The current findings on time related changes of enteritis are in line with the study of Baeverfjord & Krogdahl (1996) in which the presence of all signs of the condition after day 7 of SBM feeding is described. After 20 days of SBM feeding, the condition was fully developed and the outline of the intestinal tissue was transformed. Furthermore, the effect of temperature is not equal for all measured parameters. SNV and GC seemed to be more severely affected by temperature constituting the fast responders during the development of the enteritis, whereas the structural parameters were less affected, especially in the case of LP where the difference among the two groups was not significant. Once the immune system is triggered, the cellular components activate the immune cascade, and consequently, the appearance of the intestinal epithelium starts to adapt and respond to those changes. Salmon recurrently exposed to the noxious agent contained in SBM showed no signs of recovery during the experimental period. On the contrary, an aggravation of the symptoms was noticed. Indeed, proof that the structural parameters are affected to a lesser extent after exposure to SBM at the two different temperatures, supports the fact that the time digesta is in contact with the intestinal epithelium, which, nonetheless, is not a main factor in explaining the higher degree of enteritis in fish kept at higher temperature.

From the present study, it can be concluded that temperature influences the enteritis process more concretely at higher temperatures, suggesting that the enteritis developed at a lower temperature seems to be a delay rather than other type or mechanism by which the aggravation of the symptoms is generally explained. Transit time is not the main factor causing the stronger reaction when Atlantic salmon are reared at higher temperatures. Instead an increase in the metabolic rate may well be the most suitable explanation of this

phenomenon. Membrane fluidity and membrane composition, altered over time, might partially explain the progressive condition developed by fish reared at the higher temperature. However, strong indications that the endocytosis process might be altered by changes in water temperature were outlined in this study. This fact constitutes an important feature to consider when studying the impact of SBM-feeding in the development of the enteritis condition in Atlantic salmon.

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Time-related changes of the intestinal morphology of Atlantic salmon *(Salmo salar L.)* at two different soybean meal inclusion levels

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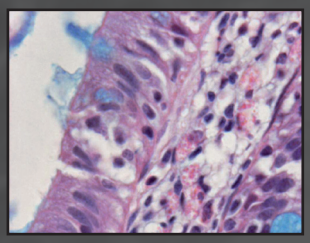
Chapter 3



Submitted for publication

Abstract

Soybean meal (SBM) induces enteritis in the distal intestine of Atlantic salmon. The present study assesses the effects of SBM doses on the kinetics of the enteritis process. Fish of 300g, kept at 12°C, were fed diets with different SBM inclusions: 0%, 10% and 20 % SBM for 57 days. Samples of the distal intestine of 5 fish per treatment were taken for histological and electron microscopic analysis. A semi-quantitative scoring system was used to assess the degree of the morphological changes induced by SBM feeding in the distal intestine epithelium. The first signs of enteritis appeared earlier in the salmon fed the 20SBM diet than those salmon fed the 10SBM diet. Thereafter, it increased steadily with time, displaying no signs of recovery. Furthermore, at the lower dose, the process marking the onset of enteritis began more gradually than at the higher dose and it displayed a tendency to level off after 13 to 20 days of continuous feeding. Electron microscopy indicated that the endocytosis process was hampered at day 3 of 20SBM and at 7 days of 10SBM. Furthermore, a strong reduction of microvilli was already evident after 7 days of 20SBM feeding, thus indicating a decreased uptake capacity of the distal enterocytes. In addition, transformation and migration of eosinophilic granulocytes was observed, which, in combination with the lysozyme C-immunoreactivity supports their protective role during the inflammatory process in the distal gut of Atlantic salmon. It can be concluded that the severity of enteritis and its kinetics are dose-dependent, showing no signs of recovery during feeding with diets containing SBM, which conversely, gives clear indications of an increased innate immunity.

Introduction

Soybean (SB) is widely known to contain adverse anti-nutritional compounds that may induce intestinal disorders in salmonids being especially harmful to Atlantic salmon (*Salmo salar* L.). Rainbow trout *Oncorhynchus mykiss* (Walbaum) seems to be less affected by the SB noxious factors that induce enteritis as was documented in previous studies (Nordrum *et al.* 2000; Refstie *et al.* 2000; Buttle *et al.* 2001). When Atlantic salmon is fed on soybean meal (SBM)-based diets, the morphology of the distal intestine is disturbed, which has been described by Baeverfjord & Krogdahl (1996) as "non- infectious sub-acute enteritis". The changes in the distal intestinal mucosa are described as: a deep shortening of the mucosal folds, a decreasing number of the supranuclear vacuoles in absorptive cells, a widening of the central stroma with a correspondingly high amount of connective tissue and an increased infiltration of inflammatory cells in the lamina propria (van den Ingh *et al.* 1991, 1996, Baeverfjord & Krogdahl, 1996), an increased number of goblet cells, and a shortening of the microvilli (van den Ingh *et al.* 1991). These pathological changes seem to be particularly present in the distal intestinal segment rather than on the proximal segment, as has already been reported in several studies on salmonids (van den Ingh *et al.* 1991; Burrells *et al.* 1999, Nordrum *et al.* 2000, Buttle *et al.* 2001).

Baeverfjord & Krogdahl (1996) established that after 3 weeks of SBM-feeding, the development of enteritis became critical for those Atlantic salmon, being fed a relatively high SBM-based diet (*c*. 33%) which induced all the above-mentioned characteristic signs of enteritis within the first week of experimental feeding. A more recent study (Krogdahl *et al.* 2003), using different solvent-extracted SBM inclusion levels, indicated that the degree of enteritis in the distal intestine, being measured at 60 days of SBM feeding, augmented with increasing SBM. Furthermore, even at lower SBM levels (15 to 25%), growth performance, feed conversion, apparent digestibility/utilization of macronutrients and energy were affected. Almost all studies on enteritis focused on the more advanced stages of the inflammation process (> 20 d after SBM feeding); with the exception of the kinetic study of Baeverfjord & Krogdahl (1996) which used a 33% dietary inclusion solvent extracted SBM. They found the initial signs of morphological changes at day 2, after feeding the SBM diet, at day 7 all typical characteristics of enteritis were present but the severity increased up until the last sampling

point at 21 days. Thus, morphological information on the onset of enteritis is limited as well as on the influence of the SBM level on the kinetics of enteritis. Information on the ultrastructural changes of the distal epithelium is lacking, both regarding the kinetics and the influence of SBM doses.

In this study the kinetics of morphological changes is assessed in fish fed diets containing 10% and 20% of SBM as part of the protein fraction. The main objective of this study is to describe the progressive morphological changes in the distal intestine of salmon that are fed diets containing different inclusion levels of a SBM variety positively selected to give a strong reaction. For this purpose the two mentioned diets and a control diet containing fishmeal as the sole protein source were fed to salmon. Morphological parameters characteristic for inflamed distal intestinal mucosa were assessed at seven different time points for the duration of the experimental period. A previously introduced semi-quantitative scoring system (Urán *et al.* 2008) was used in order to elucidate the impact on the intestinal morphology. In addition, as well as paying attention to ultrastructural changes during the enteritis process, a preliminary qualitative study on the role and contribution of the eosinophilic granulocytes to the inflammatory process is also presented.

Materials and Methods

Fish and rearing conditions

The experiment was carried out at Skretting Fish Trials Station, Lerang (Jørpeland, Norway). For this experiment, 300 Atlantic salmon (AquaGen strain) were sampled for gut histology and electron microscopy. The Atlantic salmon originated from a stock of fish already present at the research station. The experiment consisted of a two-week adaptation period and an eight-week experimental period. At the start of the adaptation period the fish weighed approximately 300 g.

For the experiment six indoor tanks with a diameter of 1 m each were used. The water volume in the tanks was 400 l. The stocking density was 50 fish per tank. Each tank was kept at flow through, at 12-15 l min^{-1} system. Seawater was pumped from a depth of 90m in the fjord, with a salinity of 34 ‰ and an oxygen concentration above 9 ppm. Prior the adaptation

period fish were kept at 8 °C. During the adaptation and the experimental period, water temperature was kept at 12 °C. The applied photoperiod was 18L : 6D.

Diets and feeding

Feed was produced at Skretting Feed Technology Plant, (Stavanger, Norway). Three diets were formulated: a control diet (0SBM) and two experimental diets (10SBM and 20SBM) (Table 1). The major ingredients in the 0SBM diets were: fishmeal (protein content above 70%), fish oil, and wheat. This control diet did not contain any SBM. For the experimental diets, fishmeal and wheat were exchanged, in the case of the 10SBM diet for 10% SBM, and in the case of the 20SBM diet for 20% SBM (solvent-extracted Hipro SBM) (Table 1). All diets were produced to be iso-energetic and iso-nitrogenic on a crude protein and a crude lipid basis. Diets were supplemented with a standard vitamin and mineral premix. Feed was produced as extruded 4 mm sinking pellets.

Prior to the experiment the fish were fed a commercial salmon diet (Skretting, Stavanger, Norway), which did not contain any SB products. During the adaptation period all tanks were fed with the control diet (0SBM). At the start of the experimental period (day 1), for each diet group, two tanks were changed to either a 10SBM or a 20SBM experimental diet. Two tanks reminded at the control diet. Fish were fed 20% in excess. Feed was divided into two meals per day and provided by automatic feeders.

Chemical analysis of diets

The nutrient composition of the experimental diets was determined using standard techniques for proximate analyses. Crude protein content was determined by the Kjeldahl Nitrogen measurement in accordance with the Nordic Committee on Food Analysis, Method No.6, 4th edition, 2003. Crude fat content was measured by low field nuclear magnetic resonance. Moisture content in the samples was measured by drying to constant weight at 102-105 °C for 16-18 h. Ash content was measured by combustion at 540 °C for 16-18 h, after which the remaining residues were weighed, both in accordance with the Nordic Committee on Food Analysis Method No.23, 3rd edition, 1991. The preceding analyses were carried out at the Skretting Aquaculture research centre (Stavanger, Norway). For chemical composition see Table 1.

Table 1 Formulation and chemical composition of the experimental diets

	Amount of protein replaced by soybean meal in percentage		
	0	**10**	**20**
Ingredients (g kg^{-1})			
Fishmeal[1]	564.3	519.8	475.3
Extracted soybean meal[2]	0	100	200
Wheat	210.6	140	70
Fish Oil [3]	222.6	237.3	252.1
Vitamin premix	1.3	1.3	1.3
Mineral premix	1.3	1.3	1.3
Pigment premix			
Yttrium oxide	0.1	0.1	0.1
Carophyll Pink	0.6	0.6	0.6
Total	1000.8	1000.4	1000.7
Chemical composition by analysis			
Crude Protein (g kg^{-1})	429.8	440.2	450.6
Crude Lipid (g kg^{-1})	277.1	289.2	301.4
Target dry matter (%)	95	95	95
Fat NMR (%)	30.5	31.5	32.8
Protein (%)	43.1	43.8	45.2
Moisture (%)	5.1	6.2	4.7
Ash (%)	7.2	7.0	7.3

[1] LT North Atlantic, Egersund, Norway.

[2] HiPro solvent-extracted soybean meal, protein content above 70%.

[3] Northern Hemisphere.

Sampling for intestinal morphology

During the experimental period, fish gut was sampled for histological measurements at seven different time points: day 3, 5, 7, 13, 20, 36, 57. At each sampling moment, five fish per treatment group were sampled (5 fish from the control group and 5 from each of the two experimental diets). The samples were taken from alternate tanks to avoid any drops in feed intake due to sampling stress. Directly after the morning meal, the fish were anaesthetized

using 0,05 g L^{-1} metacaine (Argent chemical laboratories, USA), and thereafter killed by a sharp blow to the head. The distal intestine was dissected from the place were the intestinal diameter increases, the mucosa becomes darker and annular rings are clearly noticeable.

Light Microscopy (LM)

For LM analysis a two-centimeter section of distal intestine of each fish was taken and gently rinsed with cold (4 ºC) saline. Samples were fixed in 4% phosphate-buffered formalin pH of 7.2 and stored at room temperature. After dehydration, in accordance with standard procedures, samples were embedded in paraffin. Transverse sections of 5 µm thickness were stained using a mixture of Haematoxylin & Eosin and Alcian blue pH 2.5. Alcian blue staining enhances the contrast between goblet cells and the supranuclear vacuoles. Slides were blindly evaluated after randomization.

Semi-Quantitative Scoring system

The LM sections were evaluated according to the semi-quantitative method developed at Wageningen University (Urán *et al.* 2008), which assesses the degree of SBM-induced enteritis on the Atlantic salmon distal intestine considering the following criteria: 1. the morphology of the mucosal folds (MF); 2. the presence and size of supranuclear vacuoles (SNV); 3. the abundance of goblet cells (GC); 4. the infiltration of eosinophilic granulocytes (EG) into the lamina propria and sub-epithelial mucosa; 5. the degree of widening of the lamina propria (LP); and 6. the degree of thickening of the sub-epithelial mucosa (SM). Sections were photographed with an Olympus DP 50 digital camera connected to a Nikon Microphot-FXA light microscope (Badhoevedorp, the Netherlands). The pictures were processed and analyzed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany).

Each of these parameters was scored on a scale from 1 to 5 (Table 2). An increasing scored value represents a more severe enteritis condition. (For illustrations of the different scores, see annex or check list of special publication at http://www.afi.wur.nl/UK/Publications/).

Table 2 Semi-quantitative scoring system for the different parameters used to assess the degree of enteritis developed by Atlantic salmon fed a soybean meal-based diet. From Urán *et al*. 2008.

	Score	Description
Mucosal Folds (MF)	1	Basal length
	2	Some shrinkage and bloating
	3	Diffused shrinkage and onset of tissue disruption
	4	Diffused tissue disruption
	5	Total tissue disruption
Supranuclear Vacuoles (SNV)	1	Basal SNV size
	2	Some size reduction
	3	Diffused size reduction
	4	Onset of extinction
	5	No SNV
Goblet Cells (GC)	1	Scattered cells
	2	Increased number and sparsely distributed
	3	Diffused number widely spread
	4	Densely grouped cells
	5	Highly abundant and tightly-packed cells
Eosinophilic Granulocytes (EG)	1	Few in SM basal small quantity
	2	Increased number in SM and some migration into LP
	3	Increased migration into LP
	4	Diffused number in LP and SM
	5	Dense EG in LP and SM
Lamina Propria (LP)	1	Normal size LP
	2	Increased size of LP
	3	Medium size LP
	4	Large LP
	5	Largest LP
Sub-epithelial Mucosa (SM)	1	Normal SM
	2	Increased size SM
	3	Medium size SM
	4	Large SM
	5	Largest SM

Electron microscopy (EM)

For the EM analyses smaller pieces of intestine were sampled and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 at 4 ºC for 4-6 h; then rinsed twice with

buffer 0.1 M phosphate buffer pH 7.2 plus 0.2 M sucrose and kept in the same buffer at 4 ºC for transport. Samples were post-fixed on 1% OsO_4 for 1-2 h and thereafter, embedded in Epon 812 (Electron microscopy Sciences, Fort Washington, USA). Semi-thin sections (1 µm) were mounted on glass slides and stained with toluidine blue. Ultra-thin sections were cut using a diamond knife installed on a Reichert Ultracut (Leica, Rijswijk, the Netherlands) and mounted on copper grids. Sections were counter-stained using uranyl acetate followed by lead citrate and examined in a Philips EM 208 electron microscope (Philips, Eindhoven, the Netherlands). Due to the limitations of this technique, two animals per treatment and one for the control were embedded in epon and further processed. In order to exclude local differences, two different parts of the same sample were analyzed. Pictures were made using a SIS Megaview III digital camera (Soft Imaging System GmbH, Münster, Germany) connected to the Philips EM 208. The pictures were processed and analyzed using the AnalySiS Extended Pro 3.1 software.

Immunohistochemistry (Lysozyme C)

Transverse section of distal intestine from control and SBM fed fish were deparaffinised and hydrated, in accordance with standard procedure. Sections were treated with Tris EDTA buffer pH 9 and kept at 95 °C for 20 min. Sections were then washed in PBS with 0.1 % Triton X-100 (PBS-t) and kept at room temperature. Afterwards, sections were incubated with 5% normal goat serum for 30 min, and then incubated with anti-human lysozyme C (1:50, Dako, Glostrup, Denmark) for 1 h. After washing twice with PBS-t, sections were incubated with horseradish peroxidase-labelled Goat-anti-rabbit-HRP (GAR-HRP, Dako, Glostrup, Denmark) for 1h. Slides were washed twice with PBS and kept on 0,05 M, pH 5 sodium acetate buffer for 10 min. Slides were incubated with 3-Amino-9-ethyl-carbazole substrate (AEC, Sigma-Aldrich, St-Louis, USA) until a reaction was observed and then they were counter-stained with Haematoxylin, rinsed in running tap water, and embedded in Kaiser's glycerine gelatine (Merck, Darmstadt, Germany). Sections were analyzed and photographed with an Olympus DP 50 digital camera connected to a Nikon Microphot-FXA light microscope. The pictures were processed and analysed using the AnalySiS Extended Pro 3.1 software.

Statistical analysis

All calculations were made using the SAS System (SAS 1999). The parameters used to score the degree of enteritis were analysed for the effect of diet using PROC GLM for each sampling point separately. The error terms of these analyses were tested for homogeneity of variances and normal distribution, using, respectively, the Levene's test and the Shapiro-Wilk test. Due to significant non-normality, data were analysed using a Kruskal-Wallis test. Pair-wise comparison was performed using the Mann-Whitney U-test. Herein, results are considered statistically significant when *P*-values are below 0.05.

Results

Morphological changes of the distal intestine

The control diet (0SBM) did not induce any sign of enteritis. The MF appeared as long finger-like structures, composed of enterocytes which were perfectly aligned along the mucosal epithelium. The nucleus of the enterocytes was located between the mid to basal part of the cell. The enterocytes presented normal round and SNV. The GC were present in a basal amount and they were scattered among the enterocytes. The LP was a delicate and single thin layer of cells underlying the epithelium. The SM found between the basal part of the folds and the stratum compactum was composed of a basal EG population forming the stratum granulosum. All control samples for the entire duration of the experimental period followed the same pattern described above. This is reflected in Fig.1 by the fact that all the semi-quantitative scores remained at the basal level of 1 for all the measured parameters, at all sampling points.

Fish fed diets containing SBM showed no visible morphological changes up until day 3. The first visible changes occurred on day 5 for the 20SBM diet. At this point, the SNV started to shrink and their regular alignment was disturbed. An increasing cell infiltration into the SM was also noticed. At day 5, for the 10SBM diet, there were no noticeably changes. At day 7 the above-mentioned morphological changes were detectable in both SBM diets but to a lesser degree for 10SBM diet (Fig. 1).

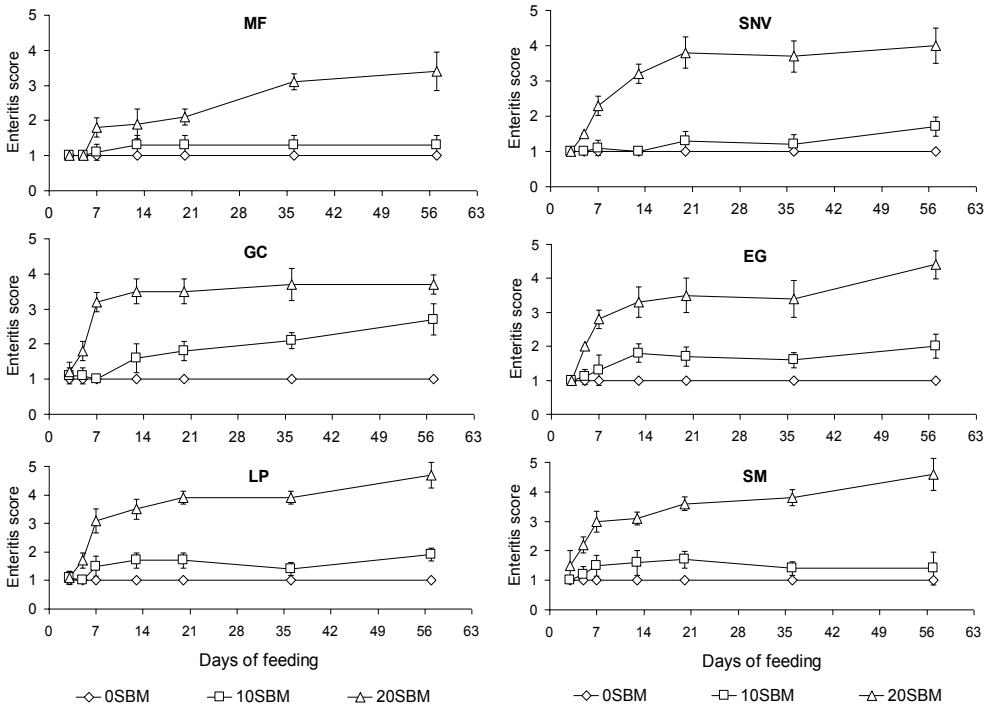


Figure 1 Enteritis parameters scored using the Wageningen semi-quantitative scoring system (Urán *et al.* 2008), where 1 indicates normal intestinal morphology and 5 indicates the highest degree of soybean meal (SBM) - induced enteritis in the distal intestine of Atlantic salmon. The changes on the morphology of the mucosal folds **MF**, the presence of the supranuclear vacuoles **SNV**, the abundance of goblet cells **GC**, the degree of infiltration of eosinophilic granulocytes **EG**, the widening of the lamina propria **LP**, and the thickening of the sub-epithelial mucosa **SM,** are shown. Three different diets, a control diet containing fishmeal as protein source and 0% of soybean meal (0SBM), a diet containing 10% and 20% of Soybean meal (10SBM and 20SBM respectively), both as replacements of fishmeal in the diet were fed for a period of 57 days.

The MF started to shrink with subsequent tissue disruption. The LP and SM had increased in size at both SBM inclusions. The SNV displayed a significant reduction and the number of GC increased steadily, particularly in the 20SBM diet. At day 13 major changes were distinctive between the two SBM diets. The fish fed the 20SBM diet presented a heavily changed intestinal mucosa, while the fish fed 10SBM showed only minor changes on MF, LP

and SM (Fig. 1). The SNV remained of a normal size and of regular alignment whilst the number of GC and EG increased. From day 13 onwards, all the scored parameters, except for the GC, tended to level off in fish fed 10SBM. However, for the 20SBM diet, most of the parameters measured showed aggravation during the experimental period (Fig. 1). The MF showed a mild increase during the first 20 days of feeding but further on diffused tissue disruption occurred until they appeared as bloated mucosal tissue. The SNV evidenced a fast reduction in size towards day 20 and reached the highest scores wherein vacuoles were completely absent. The GC became densely grouped cells from day 13 onwards. The number of EG increased from day 13 onwards during which there was a vast migratory increase and a dense number observed towards the LP and in SM respectively. At day 57, the symptoms observed in fish fed 10SBM had levelled off but the morphological structure of fish fed 20SBM seemed to have reached maximum scores for most of the parameters (Fig. 1). It was observed that the onset of the enteritis condition began at between 3 and 5 days and at day 7 (Table 3) all the parameters evaluated showed to be significantly different from those fish fed the control diet ($P < 0.05$).

Table 3 Significance of the enteritis symptoms developed by Atlantic salmon fed SBM-based diets expressed over a 57-day experimental period

	Days of SBM feeding						
	3	5	7	13	20	36	57
MF	NS	NS	**	*	***	***	**
SNV	NS	***	***	***	***	***	***
GC	NS	**	***	***	***	***	***
EG	NS	***	***	***	***	***	***
LP	NS	**	***	***	***	***	***
SM	NS	***	***	***	***	***	***

Statistical significance: $^{NS}P > 0.05$; $*P < 0.05$; $**P < 0.01$, $***P < 0.001$

Ultrastructural changes of the distal Intestine

The influence of SBM on the ultrastructure of the distal intestinal epithelia was observed on day 3 when the first evidence of a disturbed endocytosis activity was noticed particularly among fish fed the 20SBM diet. The pinocytotic invaginations at the basal part of

the Mv were less frequent than in controls. After 7 days of 20SBM feeding, these membrane-bound vesicles were not visible and there was no observation of any new engulfed material at the level of the terminal web, whereas in controls it still seemed to be filled with small pinocytotic vesicles pinching off from the apical membrane and moving towards the endosomes to finally fuse into the SNV (Fig. 2).

The disappearance of these structures was exacerbated towards the end of the experiment, specifically, on day 57 where virtually no SNV were observed. At the 10SBM diet, these changes all occurred at a lower velocity. The first evidence of the disappearance of the pinocytotic vesicles was noticed from day 7 onwards. After 57 days of continuous feeding, the disappearance of SNV did not reach the same levels as observed in the fish fed 20SBM. In addition, the microvilli (Mv) of fish fed 20SBM had dramatically decreased in size, measuring roughly one third of their normal length at day 7 when compared to the controls and this situation remained constant until the end of the experiment with no signs of recovery (Fig. 3). The brush border membrane of fish fed 10SBM also showed a decrease but to a much lesser extent. At day 7, less invaginations were observed but Mv were of normal size as compared to the controls and the SNV were reduced but still present until the end of the feeding trial.

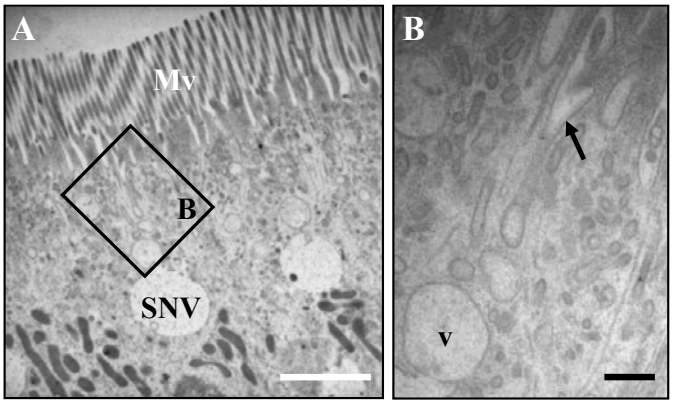


Figure 2 Ultrastructure of the distal intestine epithelium of Atlantic salmon fed the control diet. **A)** Normal absorptive cell shown as normally vacuolated enterocyte with high formation of pinocytotic vesicles (v) fusing into supranuclear vacuoles (SNV). The microvilli (Mv) are normal in size. Bar is 2µm. **B)** Detail of cytoplasmic invaginations (arrow) pinching off the apical membrane to fuse into bigger vesicles (v). Bar is 0.5µm.

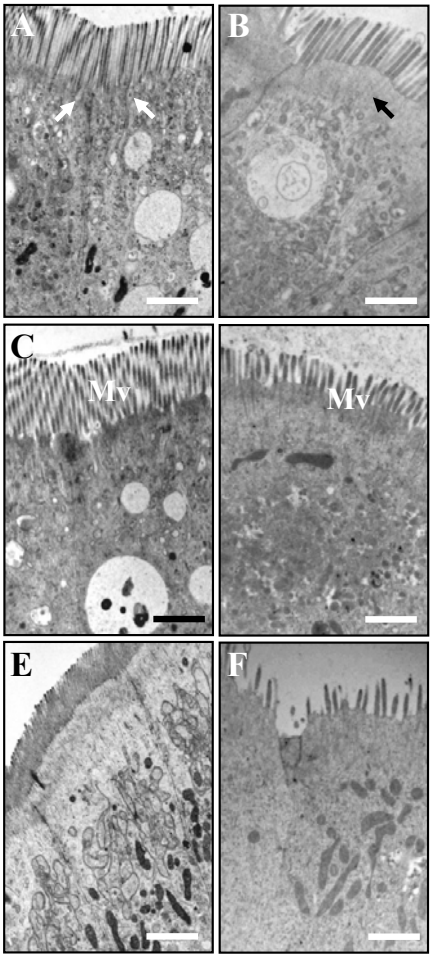


Figure 3 Ultrastructure of the distal intestine epithelium of Atlantic salmon fed soybean meal (SBM) based diet. **A**) As yet, no changes can be observed after 3 days of feeding a diet containing 10% SBM (10SBM). (The pinocytotic vesicles are indicated by the arrows), whereas **B**) shows the changes after 3 days of feeding a diet containing 20% SBM (20SBM). The pinocytotic vesicles started to diminish (arrow). **C**) After day 7 the first signs of a disrupted uptake process are observed in the 10SBM diet, indicated by the decreased formation of membrane-bound vesicles, however, microvilli (Mv) are still of normal size. **D**) After 7 days at the 20SBM diet, these invaginations disappeared and new engulfed material was not observed at the apical cytoplasm, Mv have reduced their size to nearly a third of their normal length. **E**) After day 57 at the 10% SBM feeding the changes at the distal intestine epithelium were not as severe but there still remains some size reduction of the brush border membrane and the absence of newly formed vesicles at the terminal web were observed. **F**) After 57 days at the 20% SBM feeding, a severe reduction of the Mv size and the total absence of the new vesicles and SNV were noticed. Bar is 2 μm.

There was a clear difference in the appearance of the granular content of the EG observed under the electron microscope. During the first 7 days of 20SBM feeding, the granules began changing their density and this process continued until day 20 with some of them appearing as electron lucent in comparison to the electron dense granules observed in the controls (Fig. 4). After 20 days, the amount increased steadily with a strong presence of intermediate electron dense granules. In fish fed 10SBM the transformation was milder and electron dense or intermediate dense granules were observed less frequently.

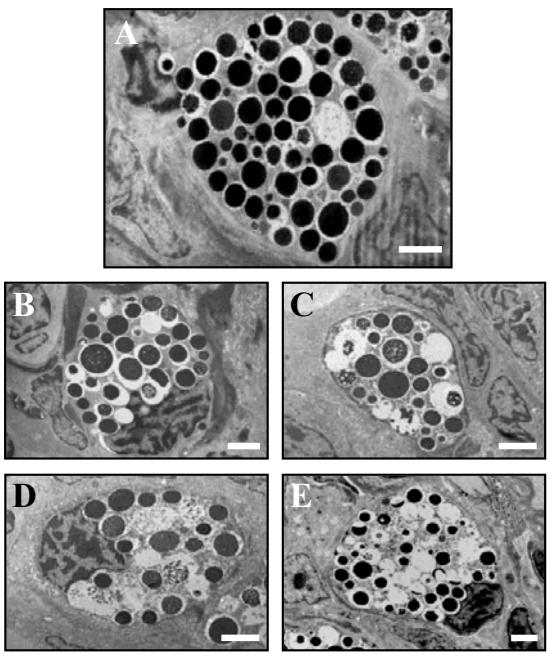


Figure 4 Eosinophilic granulocytes (EG) of the distal intestine epithelium of Atlantic salmon. **A)** Appearance of intestinal EG of fish fed a control diet (no inclusion of soybean meal); note the predominant electron dense granules. **B**) Predominant type of EG with intermediate and electron lucent granules, present in lamina propria and sub-epithelial mucosa after 7 days of feeding a diet containing 10% SBM (10SBM). **C**) Day 7 after feeding a diet containing 20% SBM (20SBM), note the tendency of more intermediate and electron lucent granules. **D**) Appearance of EG after 57 days of feeding a 10SBM diet, with electron lucent granules as the most abundant type. **E**) EG after 57 days of feeding a 20SBM diet, showing predominantly electron lucent granules. Bar is 2 μm.

Detection of Lysozyme C in EG

Using immunohistochemistry, the Lysozyme C immunoreactivity was observed within the granules of the EG present in the LP and SM of the distal gut (Fig. 5).

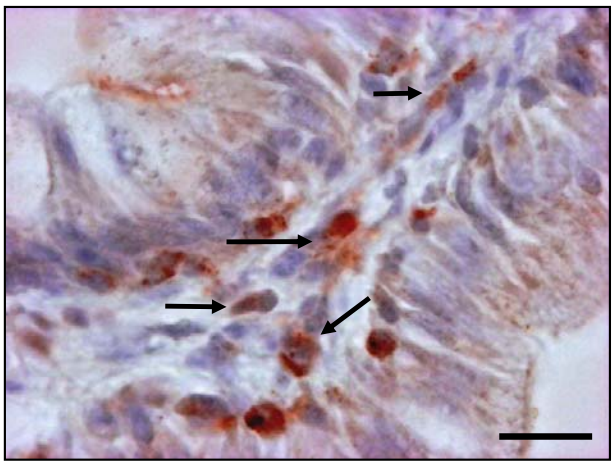


Figure 5 Immunohistochemistry using an anti-lysosyme C serum on the distal intestinal segment of Atlantic salmon fed with a SBM-based diet. Immunoreactive lysozyme C cells (arrows) indicate the presence of lysozyme in EG in the lamina propria of the distal intestine. Bar is 20 µm.

Discussion

The main objective of this study was to describe the progressive morphological changes in the intestine of Atlantic salmon fed different SBM inclusion levels. The findings from the present study suggest that a 20% SBM inclusion level leads to a severe inflammation of the distal intestinal epithelium, whereas a 10% SBM inclusion level induces a slow and mild reaction. The increased inflammation with increasing dietary SBM levels is in line with the study of Krogdahl *et al.* (2003) who measured morphological changes at 60 days of SBM feeding. The present study further demonstrated that the kinetics of the different clinical signs

differ depending on the inclusion level. The 20SBM inclusion level boosts the inflammatory process, leading to a faster and steadily increasing reaction. This dose gives a more severe response that progressively worsens for most of the parameters assessed. At the lower dose (10SBM), the same symptoms appear to be quite stable with the tendency to level off after 13 to 20 days of continuous SBM feeding. Therefore, the results obtained from this study showed that SBM-induced enteritis is a dose-dependent process with no clear signs of recovery.

It was only after 7 days of SBM feeding that all morphological signs of enteritis became visible, an observation that is conducive with the light microscopy observations of Baeverfjord & Krogdahl (1996). Nonetheless, in the current study, electron microscopy revealed the first signs of distal epithelial disturbances after 3 days of 20SBM-feeding. These findings suggest that the endocytosis process might be hampered resulting in the absence of small vesicles that finally fuse into the SNV. Rombout *et al.* (1985) described this process as the normal transport mechanism in the distal gut of common carp. King *et al.* (1982) observed changes of the intestinal Mv, disruption of the terminal web and swelling of the apical cytoplasm possibly explained by the disturbances in the membrane-associated transport processes in rats fed kidney bean lectins. It remains to be investigated as to whether the reduction in microvillous surface area at the brush border membrane and the decreased pinocytotic uptake in this study are correlated to the disappearance of the SNV. Krogdahl & Bakke-McKellep (2005) mentioned that changes in enzyme activities may also be the result of changes in tissue mass, cell numbers, morphology and enzyme synthesis in the absorptive cells. So, it may also be speculated that the atrophy of the Mv and probably the hampered uptake process observed in this study could be the consequence of an unbalanced enzymatic profile caused by contact with noxious factors present in SB fractions. Knudsen *et al.* (2007) postulated soyasaponins or a combination with factors like antigenic SB proteins or even with opportunistic intestinal microflora to be the causal factors that trigger the inflammatory reaction. Some components were suggested to hamper vacuolization most likely by disturbing the endocytosis process.

An interesting feature observed in this study was the ultrastructural change in the EG. Although their real function is not completely understood, it is suggested that they are important contributors to the inflammation process and therefore to the innate immunity (Kodama *et al.* 2002). In salmonids these EG got their name due to the acidophilic nature,

staining the granules red after the use of eosin (Ezeasor & Stokoe, 1980). These cells are widely distributed in connective tissue, especially in the gastro-intestinal tissue and gills (Sveinbjørnsson *et al.* 1996), particularly the ones located at the intestinal tract can form a considerable basal cell population arranged as a continuous layer, the so-called stratum granulosum (cf. Paulsen *et al.* 2001). According to the present observations, it is suggested that these cells undergo an activation process, changing the granular content from electron dense to electron lucent, in this case as a response to the SBM-induced inflammation. According to Reite (1996), the EG are part of a protective barrier against certain substances, in our case the SBM. Due to their similar morphology and enzymatic profile they are suggested to be mammalian mast cell analogues (Ellis 1985), sharing the presence of basic proteins and acidic glycosaminoglycans in the cytoplasmic granules, but differing in their absence of histamine, leukotrienes C_4 and B_4 and prostaglandin D_2 (cf. Powell *et al.* 1991) but also IgE-receptors. Like mammalian mast cells, fish EG appear to be abundantly present at sites of persistent inflammation (cf. Reite & Evensen, 2006). Their granules seem to contain antimicrobial substances which are involved in the fish non-specific defence mechanism (Sveinbjørnsson *et al.* 1996). This study supports the presence of lysozyme in the EG granules and suggests, at least, an antimicrobial function for the EG. In combination with a strong increase in GC and probably mucus production, the secretion of antimicrobial substances by EG may be a very powerful innate immune reaction during the inflammation process. In any case this function is supported by the infiltration of the EG inwards the LP of the MF. However, via the use of electron microscopy it is still not clear whether the change in granular content (from dense to lucent) is related to the secretion of the granular content. A possible explanation to this observation could be the alteration of the physicochemical properties of the granules or simply a difference on the emptying rates of the granules forming a mixed population of heterogeneous maturation stages where certain types are predominant. In this case, the more advanced the condition is the more electron-lucent granules are present. Previous studies have suggested the presence of other antimicrobial peptides (AMP) like Piscidin in striped bass hybrid skin, gill and gut mast cells (Silphaduang & Noga, 2001); pleurocidin in winter flounder gill EG (Murray *et al.* 2007). Nevertheless, the mechanism through which these substances are released, whether this is an activation or a degranulation process, and the way in which it may be related to changes in the ultrastructure of the

granules, remains to be clarified.

In conclusion, SBM-induced enteritis is a dose-dependent process that increases over time with no clear signs of recovery. Nonetheless, this process does provide clear indications of an increased innate immunity. The morphological changes observed in SNV and Mv are indications of a hampered endocytosis process that, in the long term, might affect the resistance against commensals and pathogens present in the hind gut. This study, related to the kinetics of the inflammatory process, constitutes a useful tool for the diagnosis of the early development of SBM-induced enteritis. As soon as the substances inducing the aforementioned changes are known, optimal sources of proteins can be selected to solve the entanglements of the fish meal replacement strategies for the optimization of plant-based feed formulations for Atlantic salmon.

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Soybean meal-induced enteritis in common carp *(Cyprinus carpio L.)* and the gene expression of inflammatory mediators in intestinal leukocytes

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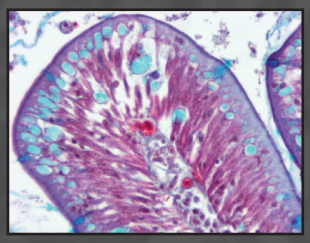
Chapter 4



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Abstract

The development of soybean meal (SBM) induced enteritis, in the hindgut of the omnivorous common carp (*Cyprinus carpio* L.). The developed condition was assessed when carp, continuously fed on animal protein, were transferred to a diet in which 20% of the protein was replaced by SBM. After week one most of the inflammation parameters were already present, but at week three, a strong aggravation of the condition was observed which included: a shortening of the mucosal folds, the disappearance of the supranuclear vacuoles, an increased number of goblet cells, a thickened lamina propria and sub-epithelial mucosa with increased numbers of basophilic granulocytes as well as a decreased uptake capacity of enterocytes (impaired endocytosis and microvilli). Contrary to previous observations made with respect to Atlantic salmon, common carp start to recover from the forth to the fifth week after switching to SBM feeding. At this stage, the supranuclear vacuoles refill and most of the parameters revert to basal levels. During the enteritis process, real time quantitative PCR analyses were conducted to measure the expression of inflammatory and anti-inflammatory cytokine genes in the isolated intestinal epithelial leukocytes. The pro-inflammatory interleukin 1β (IL-1β) and tumour necrosis factor α1 (TNF-α1) genes were up-regulated during the inflammation process while the anti-inflammatory interleukin 10 (IL-10) was down-regulated after an initial up-regulation at week one. Transforming growth factor β (TGFβ) expression showed an up-regulation from week three onwards despite the high Ct value and the low primer efficiency shown. This study confirms the contribution of intraepithelial lymphocytes IEL (mainly T-like cells) and basophils in the enteritis process. In addition, the results show a clear involvement of up- and down-regulated cytokine genes in both the onset and recovery of the SBM-induced enteritis in the hindgut of carp.

Introduction

Soybean meal (SBM)-containing diets are known to induce an inflammatory response in the distal intestinal epithelium of certain carnivorous species. This condition is well described for salmonid fish (Baeverfjord & Krogdahl, 1996) when fish are transferred to a SBM-containing diet. Buddington *et al*. (1997) stated that carnivorous fish are less adapted to diets containing ingredients of plant origin. The typical signs of SBM-induced enteritis in the distal intestinal mucosa of Atlantic salmon are: a shortening of the mucosal folds with reduced absorptive capacity of the enterocytes lining the epithelium (Urán 2008) with subsequent loss of the normal supranuclear vacuolisation; a thickening of both lamina propria and sub-epithelial mucosa with a severe infiltration of inflammatory cells, particularly of macrophages and eosinophilic granulocytes; and, increased numbers of goblet cells in the epithelium (van den Ingh *et al*. 1991, 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001).

The severity of SBM-induced enteritis differs between species. The reaction is less strong in rainbow trout (*Oncorhynchus mykiss*) than that found in Atlantic salmon (*Salmo salar* L.; Nordrum *et al*. 2000). Only limited information on the occurrence of SBM-induced enteritis is available for non-salmonid fish species. In Atlantic halibut (*Hippoglossus hippoglossus*) (Grisdalle-Helland *et al*. 2002), Atlantic cod (*Gadus morhua*) (Refstie *et al*. 2006; Hansen *et al*. 2006), channel catfish (*Ictalurus punctatus*) fingerlings (Evans *et al*. 2005), and Egyptian sole (*Solea aegyptiaca*) (Bonaldo *et al*. 2006), no histopathological differences were observed in the intestine after feeding a SBM-based diet, however, sampling in all these experiments on non-salmonids was conducted only from 9 weeks onwards. Therefore, it cannot be ruled out that these species did react but were able to adapt to the SBM-diet.

Soyasaponins are probably the trigger to initiate the inflammation response observed in Atlantic salmon (Knudsen *et al*. 2007). Therefore, more attention needs to be paid to the earlier stages of the enteritis process and the mechanisms behind it. In order to answer the question as to whether SBM-feeding has an impact on omnivorous fish, common carp (*Cyprinus carpio* L.) has been used in this study to describe the time-related changes in intestinal morphology. Due to the fact that a recent study in Atlantic salmon (Bakke-

McKellep *et al*. 2007) demonstrated an infiltration and up-regulation of T cell specific genes during SBM-enteropathy, special attention has been paid to the expression of inflammatory and anti-inflammatory cytokine genes in intestinal T-like cells.

Carp were kept on an animal protein-based diet (fishmeal) from the time of hatching and for 3, 6 or 9 months, when they were switched to a SBM-diet for a 5-week period. This diet showed to induce severe enteritis in Atlantic salmon (Urán *et al*. 2008).. Fish were part of 3 different experimental set-up and only the results from the 9 month old fish are presented in this current study. Samples for light and electron microscopy were collected together with samples for real-time quantitative Polymerase Chain Reaction, which was used to study the gene expression of interleukin 1β (IL-1β), tumour necrosis factor α1 (TNF-α1) , interleukin 10 (IL-10), and transforming growth factor β (TGFβ).

Animals, materials and methods

Fish and rearing conditions

Common carp (*Cyprinus carpio* L.) of the R8R3 strain were bred and kept at "De Haar-Vissen" facilities of the Animal Sciences Group of Wageningen University (The Netherlands). Carp larvae were kept in recirculating, filtered, UV-sterilised water at 25 ± 1ºC. From 4 dpf until 4 weeks of age, the larvae were fed *Artemia salina* nauplii. Thereafter, the carp were transferred to another system and kept in recirculating, filtered, UV-sterilised water at 23 ºC. Prior to the experimental period, carp were fed a fishmeal-based diet (0SBM; Table 1). At 9 months of age, 60 carps weighing 40.0 ± 2 g were randomly distributed over two tanks (30 fish per tank). During a 2 week adaptation period all fish were fed the control diet (0SBM). After the adaptation period, the fish in one tank were fed the experimental diet (20SBM) for 5 weeks, while the other fish remained on the control diet. In the 20SBM diet (Table 1), fishmeal, fish oil, and wheat were exchanged for 20% SBM compared to the control diet. Diets were formulated to be iso-nitrogenous and iso-energetic on a crude protein and a crude lipid basis. Diets were supplemented with a standard vitamin and mineral premix. Feed was produced as extruded sinking pellets (Skretting, Aquaculture Research Centre,

Stavanger, Norway). The fish were fed 4% of their body weight per day, which was divided into two equal servings and given by hand.

Table 1 Ingredients and chemical composition of the experimental diets.

	Diets[1]	
	0SBM	**20SBM**
Ingredients (g kg^{-1})		
Fishmeal[2]	564.3	475.3
Soybean meal[3]	-	200.0
Wheat	210.6	70.0
Fish Oil[4]	222.6	252.1
Vitamin premix	1.3	1.3
Mineral premix	1.3	1.3
Pigment premix		
Yttrium oxide	0.1	0.1
Carophyll Pink	0.6	0.6
Chemical composition (by analysis) (g kg^{-1})		
Crude Protein	429.0	450.0
Crude Lipid	277.0	301.0
Moisture	51.0	47.0
Ash	72.0	73.0

[1] Amount of fish meal replaced by soybean meal (SBM) expressed in percentage (0 and 20 % respectively).

[2] LT North Atlantic, from Egersund, Norway.

[3] HiPro solvent-extracted soybean meal, protein content above 70%.

[4] Northern hemisphere.

Chemical analysis of diets

The nutrient composition of the experimental diets was determined using standard techniques for proximate analyses. Crude protein content was determined by the Kjeldahl Nitrogen measurement in accordance with the Nordic Committee on Food Analysis, Method No.6, 4th edition, 2003. Crude fat content was measured by low field nuclear magnetic resonance. Moisture content in the samples was measured by drying to constant weight at 102-105 °C for 16-18 hours. Ash content was measured by combustion at 540 °C for 16-18

hours, after which the remaining residues were weighed, both in accordance with the Nordic Committee on Food Analysis Method No.23, 3rd edition, 1991. The preceding analyses were carried out at the Skretting aquaculture research centre (Stavanger, Norway). For chemical composition see Table 1.

Fish sampling

Fish fed the control diet were sampled at the start of the experiment at week 0, (all controls are expected to have the same trend according to previous observations) and fish fed the 20SBM diet were sampled at weeks 1, 2, 3, 4, and 5. At each sampling moment 5 fish were sampled, 2 fish were used for light and electron microscopy and 3 fish for cytokine gene expression analysis. Fish were sacrificed with a 0.03 % of tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA), buffered with 0.06 % sodium bicarbonate to a pH of 7.2.

Light microscopy (LM)

The second gut segment from 2 fish per time point sampled at each treatment was dissected. A two-centimeter section of the distal intestine was taken and fixed in a mix of methanol, formalin and acetic acid (MFAA) at a ratio of 85:10:5. After dehydration, in accordance with standard procedures, samples were embedded in paraffin. Three to four cross-sections of 5 µm thickness were stained using Alcian blue pH 2.5 followed by Crossman staining to enhance both the contrast between goblet cells and the supranuclear vacuoles and the presence of basophilic granulocytes, respectively. The intestinal morphology was evaluated semi-quantitatively according to the severity of the main characteristics of the SBM-induced enteritis: the morphology of the mucosal folds (MF); the presence and size of supranuclear vacuoles (SNV); the abundance of goblet cells (GC); the infiltration of basophilic granulocytes (BG) into the lamina propria (LP) and sub-epithelial mucosa; the degree of widening of the LP; and the degree of thickening of the sub-epithelial mucosa (SM). Sections were photographed with an Olympus DP 50 digital camera (Olympus, Japan) connected to a Nikon Microphot-FXA light microscope (Nikon, Badhoevedorp, the Netherlands). The pictures were processed and analyzed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany).

Electron microscopy (EM)

For the EM analyses, smaller pieces of the second gut segment were collected from fish fed the 20SBM diet. The samples were fixed in 1% OsO_4, 2% glutaraldehyde, 1% $K_2Cr_2O_7$ in 0.1 M sodium cacodylate buffer, pH 7.2 at 4 ºC for 1 hour and were then rinsed twice in aqua dest. Samples were dehydrated in ethanol and propylene oxide and thereafter, embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, USA). Ultra-thin sections were cut using a diamond knife installed on a Reichert Ultracut (Leica, Rijswijk, the Netherlands) and mounted on copper grids. Sections were counter-stained using uranyl acetate followed by lead citrate and examined in a Philips EM 208 electron microscope (Philips, Eindhoven, the Netherlands). Pictures were taken using a Mega View III digital camera connected to the Philips EM 208. The pictures were processed and analyzed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany).

Immunohistochemical detection of T-like cells

The second gut segment of 3 fish was collected from 0SBM and 20SBM fed fish. Samples were fixed and dehydrated and embedded in paraffin, as was previously described for LM. Transverse section of 5 µm thickness were mounted on poly-L-lysine coated slides (Menzel Gläser, Braunschweig, Germany), then sections were deparaffinised using xylene at 40 °C for 5 minutes and hydrated in accordance with standard procedure. Sections were heated for 30 minutes at 95 ºC in a 0.01 M citrate buffer (pH 6). To inhibit endogenous protein digestion, sections were treated with 5 µg/ml Proteinase K at 37 °C for 10 minutes and then incubated with 5 % Normal Goat serum (NGS) at room temperature for 30 minutes. Slides were incubated for 1 hour with Rabbit-anti-human CD3ε serum (N° A0452, Dako Glostrup, Denmark; dilution 1:50). After being washed twice with PBS-tween (PBS-t), sections were incubated with polyclonal horseradish peroxidase-labelled Goat-anti-rabbit-HRP (GAR-HRP, N° P0448, Dako, Glostrup, Denmark) as a secondary antibody, for 1 hour. Slides were washed twice with PBS-t and kept on 0,05 M, pH 5 sodium acetate buffer for 10 minutes. Slides were incubated with 3-Amino-9-ethyl-carbazole substrate (AEC, Sigma-Aldrich, St-Louis, USA) until a reaction was observed and then they were counter-stained with Haematoxylin, rinsed in running tap water, and embedded in Kaiser's glycerine gelatine

(Merck, Darmstadt, Germany). All necessary controls, such as omission of the first and/or second antibody and reaction with a non-immune serum showed negative results.

Sections were analyzed and photographed with an Olympus DP 50 digital camera connected to a Nikon Microphot-FXA light microscope (Badhoevedorp, The Netherlands). The pictures were processed and analysed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany).

RNA isolation and cDNA synthesis of second gut segment leukocytes

Cell suspensions were prepared from the second gut segment of 3 fish at all sampling point. The epithelium was scraped off through a 50 µm mesh in c-RPMI containing 0.1% sodium azide, and then washed for 10 minutes at 370 x g at 4 °C in c-RPMI and resuspended in 2 ml of c-RPMI. The cell suspensions were centrifuged in a discontinuous Percoll gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and then diluted in c-RPMI to yield densities of 1.02, 1.07 and 1.08 g ml^{-1}. After centrifugation for 30 minutes at 450 x g at 4 °C, the cells layered at the 1.02/1.07 and 1.07/1.08 interfaces were collected, mixed in the same collection tube and washed in cRPMI for 10 minutes at 450 x g and washed in 1 ml PBS buffer and centrifuged again. Thereafter, the cells were lysed by adding 350 µl of 0.1 M β-mercaptoethanol, RLT buffer (1:100) mix. Total RNA extraction was carried out using the RNeasy mini kit (Qiagen), strictly in accordance with the manufacturer's instructions. RNA concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden) at a 260 nm wavelength. RNA was stored at -80°C until further use. An aliquot of 2 µg RNA of each sample was treated with 2 µl 10x DNase I reaction buffer and 2 µl DNase amplification grade (Invitrogen, Carlsbad, USA) adding nuclease free water to a total volume of 16 µl, and incubated for 15 minutes at room temperature. DNase was inactivated by adding 2 µl 25 mM EDTA and incubated for 10 minutes at 65 °C. cDNA was synthesised using reverse transcriptase superscript™ (Invitrogen), strictly in accordance with the manufacturer's instructions. Aliquots of RNA from each sample were treated without RT superscript™, hence, they were considered as non-RT controls.

Real time quantitative-polymerase chain reaction (RQ-PCR)

cDNA samples were first diluted 1:10 in RNase-free water. To aliquots of 5 µl of diluted cDNA, 9 µl of master mix (7 µl 2x QuantiTec SYBR Green Master Mix (Qiagen), 0.84 µL 5 µM forward and reverse primers (300 nM each) and RNase-free water to complete the volume) were added. Quantitative PCR was performed in a 72-well Rotor-Gene™ (Corbett Research, Sydney, Australia). The primers corresponding to the genes of interest including their efficiency (E) are listed in Table 2. Cycling conditions were: denaturation at 95ºC for 15 minutes, 40 cycles of RQ-PCR with a three-step: amplification denaturation at 95ºC for 15 seconds; annealing at 60ºC for 30 seconds; elongation at 72ºC for 30 seconds; and a final hold at 60ºC for 1 minute. Melting was performed with continuous fluorescence acquisition from slow heating starting at 60ºC and reaching up to 90ºC with a rate of 1ºC increase for every 5 seconds. For each gene, the threshold, E and melting temperature were determined. Fluorescence values were obtained using a Rotor-gene analysis software V6.0.2 The fluorescence emitted by SYBR-green attached to the double stranded DNA defined the cycle threshold (Ct) or take-off value of the reaction when the threshold of the most efficient amplification for the primer combination in question was reached (Huttenhuis *et al*., 2006). Data were analyzed using the Pfaffl method (Pfaffl, 2001) to obtain the relative expression ratio (R) of a target gene given by:

$R = (E^{GOI^{\wedge}\ (Ctreference-\ Ctsample)})/(E^{HKG^{\wedge}\ (Ctreference-Ctsample)})$ where E is the efficiency of primer combination, GOI is the gene of interest, reference is the control fish at the start of the experiment (week 0) of SBM and HKG is the house-keeping gene. The average E value per run was calculated for each of the primers and for the counterpart sample. The relative gene expression ratio R was calculated for a target gene relative to the HKG. It was further calculated based on the E and the Ct value of the sample against week 0, which is considered the control sample. For both the target and the house-keeping gene an average of the $E^{C_T\ CONTROL}$ was considered for the calculations. 40S and β-actin were used as the HKG, both with comparable results. Only 40S was used as the final HKG and the results are presented based on its expression.

Table 2 Primers sequences used for amplification of specific gene production with RQ-PCR technique with efficiencies (E) calculated according to the equation E = 10[-1/slope].
IL-1β: interleukin 1 beta, TNF-α1: tumor necrosis factor alfa 1, IL-10: interleukin 10. TGFβ: transforming growth factor beta. FW: forward and RV: reverse primers.

Primer		Sequence (5'→3')	Product length (bp)	Genbank Accession Ref.	Primer efficiency (E)
40S	FW	CCGTGGGTGACATCGTTACA	69	AB012087	1.76
	RV	TCAGGACATTGAACCTCACTGTCT			
IL-1β	FW	AAGGAGGCCAGTGGCTCTGT	69	AB010701	1.80
	RV	CCTGAAGAAGAGGAGGCTGTCA			
TNF-α1	FW	GCTGTCTGCTTCACGCTCAA	106	AJ311800	1.82
	RV	CCTTGGAAGTGACATTTGCTTTT			
IL-10	FW	GCTGTCACGTCATGAACGAGAT	132	AB110780	1.81
	RV	CCCGCTTGAGATCCTGAAATAT			
TGFβ	FW	ACGCTTTATTCCCAACCAAA	97	AF136947	1.61
	RV	GAAATCCTTGCTCTGCCTCA			

Statistical analysis for RQ-PCR results

The results on the different cytokine gene expression are presented as the average value of 3 fish ± SE of the mean. The effect of the sampling time after being transferred to the SBM-diet (week 0, 1, 3 and 5) were tested by a one way ANOVA. This analysis was done using the general linear model procedure of SAS (SAS/v8 1999). The level of significance was established at $P < 0.05$.

Results

The results from 3 different experiments conducted in 3, 6 and 9 month old common carp respectively, were closely observed for a 5 week period with LM, EM and RQ-PCR. Due to the fact that all 3 experiments produced similar results in all the techniques used, only the data obtained from 9 month old carp are described in this study.

Morphological changes of the distal gut segment

Fish fed 0SBM did not show any sign of enteritis (Fig. 1A). The MF were of normal length and shape, and were composed of absorptive cells with SNV aligned lengthwise. The nucleus of the enterocytes was located between the mid to basal regions of the cell. A basal amount of GC was observed scattered among the enterocytes. The LP was a thin layer of cells. The SM, found between the basal part of the folds and muscularis mucosa, contained numerous BG.

The distal intestine of fish fed continuously on 20SBM for 7 days revealed the first evidence of enteritis (Fig. 1B). The SNV started to shrink and were less regularly aligned. An increasing cell infiltration, particularly BG and lymphocytes into the LP and SM, was also noticed (not shown). The MF did not show any shrinkage compared to the control. Both the LP and SM had increased in size. After 14 days of 20SBM feeding (Fig. 1C), the SNV are considerably reduced and the number of GC has increased. The MF remained of the same length but with a stumpy appearance and a heavily infiltrated LP. After 3 weeks (Fig. 1D), there was a strong reduction in the size of SNV and the GC was more abundant. The number of BG had increased and they appeared to have invaded the LP. At week 4 (Fig. 1E), the reduced vacuolization is even stronger and the LP remained infiltrated with BG and were not showing any further increase in thickness. The number of GC appeared to have declined. At week 5 (Fig. 1F), most of the assessed parameters appeared to be in the process of recovery to the normal situation but the SNV do not yet have the size and the appearance of SNV in control samples and the LP is still thicker compared with control.

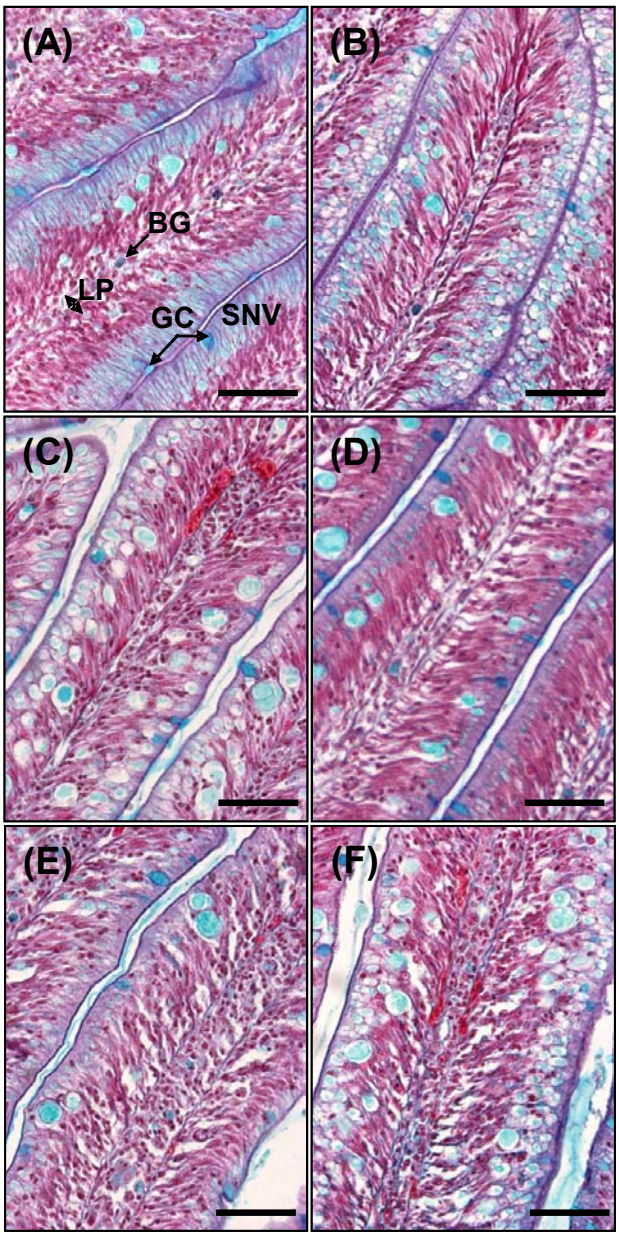

Figure 1 Intestinal morphology of common carp. **A**) Control fish fed the control diet (0SBM). Appearance of the distal intestinal morphology after feeding a diet containing 20% soybean meal (20SBM) **B**) 1 week. **C**) 2 weeks, **D**) 3 weeks, **E**) 4 weeks and **F**) 5 weeks after continuous feeding. SNV: supranuclear vacuoles, GC: goblet cells, BG: basophilic granulocytes, LP: lamina propria. Alcian Blue and Crossman staining. Bar is 50µm.

BG could easily be recognized because they displayed a blue stained cytoplasm with numerous red to blue stained round granules (Fig. 2). During the enteritis process they seem to migrate into the LP.

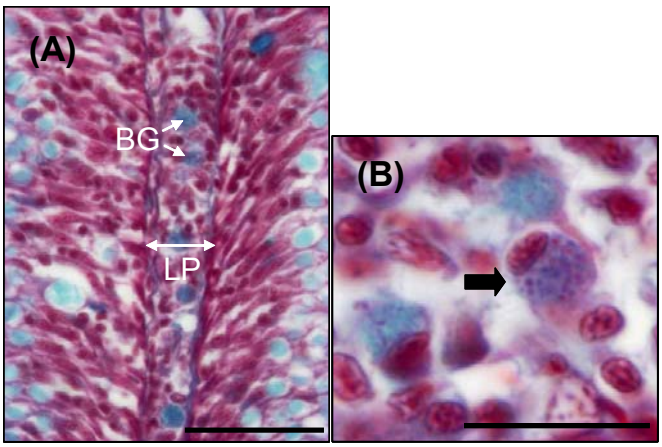

Figure 2 Appearance of an inflamed distal gut epithelium of common carp after exposure to 20 SBM. **A**) High amount of basophilic granulocytes (BG) in lamina propria (LP). Bar is 20 µm. **B**) Detailed basophilic granulocyte (arrow). Alcian Blue and Crossman staining. Bar is 10 µm.

Clear ultrastructural changes were observed after the 20SBM feeding. These observations partly match the strongest degree of enteritis previously observed under the LM at the same time point. In addition, the invaginations at the basal part of the microvilli (Mv) were much less abundant after 1 week of 20SBM, displaying less frequent membrane-bound vesicles pinching off and fusing into larger vesicles (Fig. 3A). After 3 weeks of 20SBM feeding, these membrane-bound vesicles were totally absent and no new pinocytotic vesicles at the level of the terminal web were observed (Fig. 3B). In addition, the Mv displayed a strong decrease in size compared to the control.

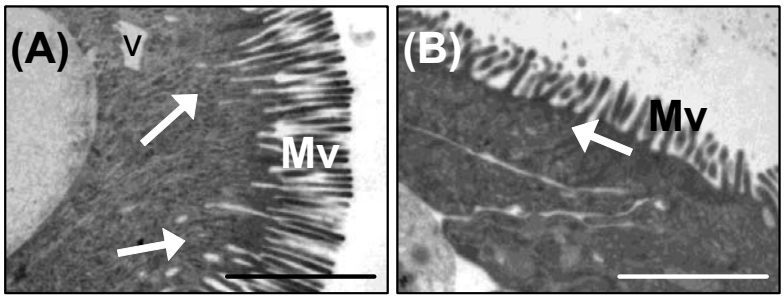

Figure 3 Ultrastructure of the distal intestinal enterocytes of common carp **A)** after 1 week of 20SBM feeding. Arrows indicate the membrane-bound vesicles fusing into larger vesicles (v). Microvilli (Mv) are seen as large, tightly packed finger-like structures. **B)** distal intestinal enterocyte after 3 weeks of 20SBM. A vesicle-free terminal web (arrow). Note the shorter and more disperse Mv. Bar is 2 μm.

Immnohistochemical detection of T-like cells

The described methodology using anti-CD3ε antibody to detect T-like cells in the second gut segment of common carp gave clear indications of the presence of $CD3\varepsilon^+$ intraepithelial lymphocytes (IEL) located at the basal part of the epithelium lining the LP at week 0 and 1 (Fig. 4A). After 3 and 5 weeks of continuous 20SBM feeding, a migration of these positive cells was observed towards the apical part of the epithelial cells (Fig. 4B).

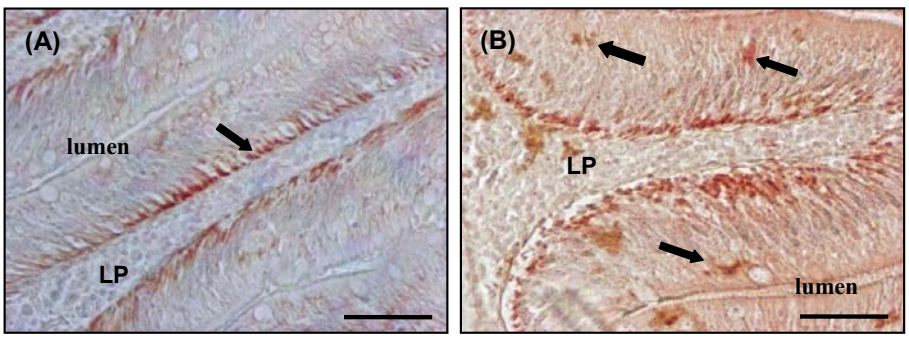

Figure 4 Migration of $CD3\varepsilon^+$T-like cells in distal intestinal epithelium of common carp fed 20SBM. **A)** $CD3\varepsilon^+$ cells in the basal part of the epithelial lining in contact with lamina propria (LP) at 1 week after feeding (arrow). **B)** $CD3\varepsilon^+$ cells migrating towards the apical part of the epithelium (arrows) at 3 weeks after feeding. Bar is 50μm.

Gene expression during the SBM-induced enteritis

To understand the possible implications of the IEL during the enteritis process, the expression of pro-inflammatory IL-1β, TNF-α1, and anti-inflammatory IL-10 and TGFβ genes were monitored at weeks 0, 1, 3 and 5 (Fig. 5). Although only 3 animals per sampling point were measured, the results presented here are similar to two other trials, and hence, the results can be considered as representative. The different cytokine genes measured at these time points and their expression relative to the house-keeping gene 40S (which except for TGFβ, all values are above the HKG), are described as follows (average expression levels are given in Fig. 5):

Pro-inflammatory cytokines (IL-1β and TNF- α1)

Numerical differences between sampling points on the pro-inflammatory cytokines IL-1 β and TNF-α1 expression were found. In comparison with control fish fed 0SBM and sampled at week 0, expression of IL-1β showed a peak at week 1. The expression of IL-1β was at all time points above the control levels. TNF-α1 gene expression was up-regulated at all time points relative to control fish. Its expression increased from week 1 until week 5.

Anti-inflammatory cytokines (IL-10 and TGFβ)

IL-10 gene expression was different between sampling points (P<0.03) Strong up-regulation of IL-10 gene expression was observed after week 1 of SBM feeding but at week 3 and 5, the expression level was down-regulated again to values either lower (week 3) or similar (week 5) than the control level. TGFβ gene expression was up-regulated at all time points relative to control fish. However, at week 3 and 5 the levels were higher although there was a high variation among the triplicates and the difference between weeks was not significant.

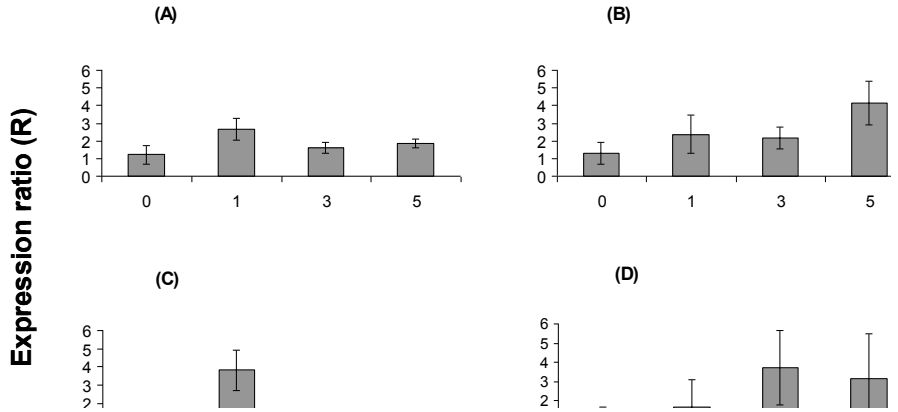

Figure 5 Kinetics of cytokine gene expression measured with real time quantitative PCR in IEL of the distal gut of carp fed on 20SBM compared with the control (0SBM). Average Ct value (number of cycles to pass the threshold) at week 0: IL-1β: 26.6, TNF-α1: 25.7, IL-10 24.0, TGFβ: 29.2. **A)** IL-1β, **B)** TNF-α1, **C)** IL-10 and **D)** TGFβ. Amplification is related to the house keeping gene 40S. Bars represent average values of 3 fish per sampling point ± SE.

Discussion

Soybean meal (SBM) fed to carp, *Cyprinus carpio* L., has been shown to cause an inflammatory response in the second gut segment of this omnivorous species similar to that earlier described SBM-induced enteritis in carnivorous Atlantic salmon (Urán *et al*. 2008), feeding exactly the same formulation as used in this study. This SBM-induced inflammation is a well described phenomenon in Atlantic salmon and has been reported by Baeverfjord & Krogdahl (1996) as "non-infectious sub-acute enteritis of the distal epithelial mucosa". The typical signs of the distal intestinal mucosa are described as: a shortening of the MF; a loss of the normal supranuclear vacuolisation of the enterocytes; a widening of the lamina propria (LP); a profound infiltration of inflammatory cells into the LP (van den Ingh *et al*. 1991, 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*.

2001); an increased presence of eosinophils (Urán *et al*. 2008) and monocytes/macrophages and neutrophils in the LP; an increased amount of GC in the epithelium (Bakke-McKellep *et al*. 2000); as well as a shortening of the Mv and a strongly decreased endocytotic activity of the enterocytes (van den Ingh *et al*. 1991; Urán 2008). In addition, more recently increased T-cell like reactivity was shown in the inflamed intestine of Atlantic salmon (Bakke-McKellep *et al*. 2007).

These morphological and cellular indicators of enteritis reported for Atlantic salmon were also observed in common carp, including the migration of basophils (morphological homologues of salmon eosinophils) into the LP and a slight migration of $CD3\varepsilon^+$ T-like cells towards the apical part of the epithelium. The main difference observed between both species was a slower start of the enteritis process in carp and after 4 weeks a recovery of the enteritis is observed in common carp, while in Atlantic salmon the condition tends to worsen unless SBM is withdrawn from its dietary regime. Baeverfjord & Krogdahl (1996) reported a full recovery of the distal intestinal epithelium of Atlantic salmon after only 21 days of the switch to a soybean-free diet.

A reduced endocytotic capacity was observed in common carp as it was previously reported for Atlantic salmon (Urán 2008). After 3 weeks of SBM feeding, the endocytotic capacity of the enterocytes is reduced coinciding with the disappearance of SNV as was observed in their salmon counterparts. The recovery of common carp along with the re-appearance of SNV could be attributed to the omnivorous nature of species which have a higher ability than carnivorous species to modulate digestive functions to digest plant protein (Buddington *et al*. 1997).

As far as we know, this is the first time that such a temporary enteritis effect has been reported for common carp, a species that usually receives a plant-based diet. Even though such feeds are effective for raising omnivorous and herbivorous species, they are less successful for carnivores, because of the assumed digestive limitations (Buddington *et al*. 1997). Despite the fact that in the present study a SB variety was selected which is known to induce enteritis, these phenomena may occur as well in aquaculture when young fish start to ingest food pellets containing SBM. Even though carp seems to adapt to SBM and completely recovers from the enteritis, more attention should be paid to the vulnerability of these animals during this inflammation process. Our studies on Atlantic salmon showed a diminished

endocytotic capacity of the second segment, and reduced Mv and a strongly reduced uptake of anally intubated ferritin in 20SBM fed Atlantic salmon (Urán 2008).

Knudsen *et al*. (2007) suggested that SBM molasses, mainly soyasaponins may be the inducers of inflammation. Saponins are well studied as adjuvants on animal immune responses and enhance cell mediated and antibody responses (Rajput *et al*. 2007). In addition, they are also effective via oral use, although saponins do not enter the intestinal epithelial barrier (Pickering *et al*. 2006). In trout fed SBM, saponins can enhance the specific plasma antibody responses after an intraperioneal injection with formalin fixed *Aeromonas salmonicida* bacterin (Penn 2005). In addition, saponins appear to also be involved in non-specific immune reactions, such as inflammation and monocyte proliferation (cf. Rajput *et al*. 2007). Finally, saponins also induce the production of cytokines, such as interleukins and interferons, which may explain their immunostimulation effects (Rajput *et al*. 2007). In mice an orally given purified *Quillaja saponaria* saponin derivative (QS-21) combined with a protein tetanus toxoid vaccine, could give Th-1 responses after high doses and Th-2 responses after low doses of QS-21 (Boyaka *et al*. 2001), again strongly indicating the interference of saponins with cytokines. Savan & Sakai 2006, described cytokines as important mediators in the immune response through the regulation of local and systemic immune inflammatory and regulatory events. In addition, the involvement of T-cell like reactivity has been suggested in the intestinal inflammatory response of Atlantic salmon because of the up-regulation of gene expression of T cell-specific molecules: CD3, CD4, CD8α and CD8β (Bakke-Mckellep *et al*. 2007). As far as we know, this study has, for the first time, reported the expression of cytokine genes in a fish enteritis process, in particular the expression of IL-1β and TNF-α1 as pro-inflammatory and IL-10 and TGFβ as anti-inflammatory cytokine. The basal expressions of all the four genes studied are given in Fig. 5 in which the order of expression is IL-10>>TNF-α1 ≥ IL-1β >>TGFβ. As far as we know, the expression of these cytokines (excluding IL-10) is only described for the trout gut (Mulder *et al*. 2007), however, it has been measured with RT-PCR and whole extracts of the gut, instead of the IEL which has been used in this current study. The main difference observed is the low expression of TGFβ in carp IEL compared to a relatively high expression of TGFβ in the distal gut of trout. Whether this difference is related to the other isolation method used, or to species differences, or even due to the different methodology used (semi-qualitative vs. quantitative) remains to be seen.

The expression of the IL-1β and TNF-α1 genes are both above the control levels at week 1, 3 and 5 after the start of 20SBM feeding, strongly suggesting that these genes are both involved in the enteritis process. However, it is remarkable that the expression of both genes seem to peak, showing a trend to be up-regulated at different weeks: IL-1β at week 1 and TNF-α1 at week 5, when fish had already started to recover. On the other hand, it has to be mentioned that the IL-1β was reproducible in the three comparable experiments while the peak of TNF-α1 was only found in this experiment. The expression of the IL-10 gene shows a tendency to be up-regulated with values above the control level at week 1, while this elevation was more expected before or during the recovery phase of enteritis. In mice it has been demonstrated that saponin can induce IL-10 production after a subcutaneous injection combined with antigen (Tadokoro *et al*. 1996). The relation between IL-10 and enteritis can be better visualized through the well studied inflammatory bowel diseases (IBD) in humans, although these intestinal disorders, such as ulcerative colitis and Crohn's disease, are more related to the intolerance to the resident microbial population of the gut. (Frogsberg 2006; cf. Bakke-McKellep *et al*. 2007). Up-regulation of IL-10 mRNA levels but also IL-10 production was measured in the gut tissue of active ulcerative colitis and Crohn's disease, as well as elevated levels of IL-10 in serum (cf. Autschbach *et al*. 1998). These authors describe that IL-10 is not generally down-regulated in active IBD and is even more strongly expressed by the mononuclear cells in the submucosa. Their results suggest that the elevated production of IL-10 in the submucosa is insufficient to down-regulate the pro-inflammatory action of inflammatory cytokines as IL-1β in the LP. It has to be mentioned that the present cytokine expression results are obtained using isolated cell fractions that are supposed to contain mainly intraepithelial leukocytes (Rombout *et al*. 1993). During recovery, IL-10 may be up-regulated elsewhere in the 2nd segment and hence not visible in this study. Nevertheless, no explanation can yet be given for the short term, up-regulation of IL-10 around the 1st week of inflammation when the inflammation process is still far from clear.

The up-regulation trend shown by TGFβ at weeks 3 and 5, also corresponds with the recovery of the inflamed carp gut. Hence, TGFβ can be considered as a general down-regulator of cell-mediated immune responses (Mulder *et al*. 2007) and as such can be seen as the inducer for the recovery phase.

Due to such factors, one could possibly conclude that the down-regulation of IL-10 after week 1 and up-regulation of IL-1β and TNF-α1 and TGFβ from week 1 onwards may play an inducing role in the SBM-induced enteritis process but these observations need more attention and additional measurements in other intestinal compartments to explain their significance.

The migration of basophils into the LP of inflamed carp gut is similar to the eosinophil reaction in the second segment of Atlantic salmon (Urán 2008). The latter study and others (Sveinbjørnsson *et al*. 1996; Silphaduang & Noga 2001; Murray *et al*. 2007) have shown the production of lysozyme by these granulocytes and hence an anti-microbial function for these cells. Although the used anti-lysozyme antibody did not react with carp basophils there is enough evidence (unpublished RQ-PCR results on isolated basophils) to state that they produce lysozyme C. Therefore, the reaction of the basophils may be dedicated as one of the defence mechanisms to protect the inflamed gut against microbial invasion.

In conclusion, in the present study we presented the kinetics of SBM-induced enteritis for common carp along with the cytokine regulation profile after a continuous exposure to antigens present in the diet. The onset of the condition was set at week 1 with the initiation of a profound alteration of the distal intestinal architecture. This study also confirms the contribution of IEL, basophils, T-like cells, GC and enterocytes to the onset and regulation of the inflammatory process. In contrast to Atlantic salmon, common carp starts to recover its endocytotic capacity towards the end of the experiment but a persistently higher expression value of pro-inflammatory cytokine genes is still observed. It suggests a unique adaptation of the intestinal tissue of common carp to poor quality protein sources while a constant surveillance of the innate immune system ensures the integrity and health of the intestinal epithelium.

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Soybean meal-induced uptake block in the distal enterocytes of Atlantic salmon *(Salmo salar L.)*

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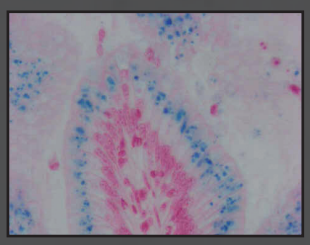
Chapter 5



Submitted for publication

Abstract

The replacement of fishmeal by soybean meal (SBM) induces enteritis in the distal intestine of Atlantic salmon. The enterocytes at the distal intestinal mucosa are heavily hampered and the disappearance of supranuclear vacuoles (SNV) seems to be the most suitable indicator for the onset of enteritis. In this study ferritin was used to evaluate the changes in uptake capacity of the distal enterocytes under the influence of a SBM-based diet. After one week of SBM-feeding, ferritin uptake was rather diminished and the SNV decreased in size compared to the control. At three weeks ferritin uptake could not be detected anymore and the SNV were invisible in the enterocytes. The linkage between the disappearance of SNV and endocytosis block will be discussed.

Introduction

Soybean meal (SBM) is a potential alternative among the plant materials to reduce the use of fishmeal (FM) for aquafeeds. However, the presence of some anti-nutritional factors hampers its utilization for salmonid diets. It is known that SBM induces a "sub-acute inflammation of the distal intestine" in Atlantic salmon (Baeverfjord & Krogdahl 1996), characterised by the loss of the normal supranuclear vacuolisation, a shortening of the mucosal folds (MF), a thickening of both lamina propria (LP) and sub-epithelial mucosa (SM) with a severe infiltration of macrophages and eosinophilic granulocytes (EG), an increased numbers of goblet cells (GC) in the epithelium (van den Ingh *et al*. 1991; van den Ingh *et al*. 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001; Urán *et al*. 2008).

The impact of SBM on the distal intestine of Atlantic salmon is striking but not yet well understood, despite the interest and the commercial importance of the species (Bakke-McKellep *et al*. 2000). It has been generally accepted that the second gut segment (main part of the distal intestine), is the principle site of endocytosis of intact proteins in teleost fish (Stroband *et al*. 1979, Stroband & van der Veen 1981; Rombout *et al*. 1985; Sire & Vernier 1992). Although the high endocytotic capacity of the distal enterocytes can have absorptive as well as immunological impact, it possibly makes the distal intestine also more sensitive to food-borne enteropathies (Bakke-McKellep *et al.,* 2000). Indeed enterocytes of the distal intestine are the first cells affected when Atlantic salmon is fed SBM diets (Urán *et al*. 2008). Previous ultrastructural studies conducted on old Atlantic salmon and young common carp, have shown a severe decrease in endocytosis by the distal enterocytes, although carp showed a slower impairment and a subsequent recovery after 5 weeks (Urán 2008).

The aim of this study was to evaluate the impact of SBM-feeding on the enterocyte uptake capacity of Atlantic salmon. Young salmon were used to establish whether age and freshwater conditions had any influence on the severity of SBM-induced enteritis described for old fish kept in seawater. Therefore the same experimental diet was used as in previous experiments on old fish (Urán *et al*. 2008). Special attention is paid to the correlation between impaired uptake and the disappearance of the supranuclear vacuoles (SNV). Therefore ferritin is used as macromolecule because it can be detected easily at the light microscopical level.

Materials and Methods

Fish rearing conditions

The experiment was carried out at 'De Haar Vissen' facilities, Animal Science group, Wageningen University, The Netherlands. The Atlantic salmon (Irish strain) originated from the fish farm Forellenzucht Hischquellen, schloβ Holte-Stukenbrock, Germany. The experiment consisted of a two-week adaptation period and three-week experimental period. At the start of the adaptation period the fish weighted approximately 30 g.

For the experiment four circular indoor tanks with a diameter of 1 m each were used. The water volume in the tanks was 600 L. Two tanks were stocked with 28 fish each and the other two tanks with 36 fish each, according to the treatment. Each tank was kept at a flow through system. Freshwater was pumped to the tanks at a temperature of 13 ± 2°C and an oxygen concentration above 9 ppm (saturated), pH of 8.2 and NH_4^+, NO_2^-, NO_3^- levels kept at 0. The water conductivity was 180 µs. The photoperiod was approximately 10L : 14D (natural light regime during winter time).

Diets and feeding

Feed was produced at Skretting Feed Technology Plant, (Stavanger, Norway). Two diets were formulated: a control diet (0SBM) and a experimental diets (20SBM) (Table 1). The major ingredients in the 0SBM diets were: FM, fish oil (FO), and wheat. This control diet did not contain any SBM. For the experimental diet, FM and wheat were exchanged for 20% SBM (solvent-extracted Hipro SBM). The diets were produced to be iso-energetic and iso-nitrogenic on a crude protein and a crude lipid basis. Diets were supplemented with a standard vitamin and mineral premix. Feed was produced as extruded 3 mm sinking pellets.

Prior to the experiment and during the adaptation period all tanks were fed with the control diet (0SBM). At the start of the experimental period, two of the fours tanks were changed to the 20SBM experimental diet. The other two tanks remained at the control diet. Fish were fed 20% in excess. Feed was divided into two meals per day and provided by automatic belt feeders.

Table 1 Chemical composition of the experimental diets.

	Diets[1]	
	0 SBM	**20 SBM**
Ingredients (g kg^{-1})		
Fishmeal[2]	562.4	470.3
Soybean meal[3]	0.0	200.0
Wheat	201.1	84.7
Fish Oil[4]	234.5	243.0
Vitamin premix	1.0	1.0
Mineral premix	1.0	1.0
Analyzed chemical composition (g kg^{-1})		
Dry matter	914	941
Crude Protein	415	446
Crude fat	268	287
Ash	88	89

[1] Amount of protein replaced by Soybean meal in percent.

[2] LT north Atlantic, Egersund, Norway.

[3] Extracted solvent HiPro Soybean Meal.

[4] Northern hemisphere.

Chemical analysis of diets

Feed samples were ground using a 1 mm screen before analysing dry matter, ash, crude protein and crude fat content. Dry matter content was determined by drying samples for 4 hours at 103°C until constant weight (ISO 6496, 1983) and ash content by incineration in a muffle furnace for 4 hours at 550°C (ISO 5984, 1978). Crude fat content was determined by petroleum ether extraction in a Soxhlet apparatus (ISO/DIS, 1996). Crude protein (N x 6.25) was measured by the Kjeldahl method after acid digestion (ISO 5983, 1979). The analysed chemical composition of the diets is given in Table 1.

Administration of ferritin

A solution containing 1% horse spleen ferritin (Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS), was anally intubated directly into the second gut segment.

Fish were starved 24 hours before the intubation procedure to ensure an empty intestine for the better delivery of the markers. Before the procedure, fish were anaesthetised using 0.05% tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) TMS buffered with 0.1 % sodium bicarbonate, in tank water.

Sampling scheme

Sampling for distal intestine was performed at various time points after ferritin intubation. At the week 1, samples were taken at 90min, 6 and 12 h. and at week 3 at 30, 60, 90 min and 6 h after intubation for both dietary groups (n=8 per time point/diet). To check for background ferritin staining and to discard the induction of inflammation due to intubation, PBS intubated fish (n=4) and no intubated fish (n=2) were sampled after 90 min, each at week 1 and 3 from both 0SBM and 20SBM diet groups. After intubation fish were kept in smaller tanks (120 L), under the same conditions as in the original ones, and were individually tagged to control the exact time after intubation.

Light Microscopy (LM)

At each time point after intubation, fish were sacrificed with an overdose of anaesthesia. For LM analysis a two-centimetre section of distal intestine (thicker region of the intestinal tract characterized by more prominent and darker annular rings) of each fish was dissected out and fixed in a mix of methanol, formalin, acetic acid (85:10:5). After 24 hours the samples were transferred to 70% ethanol and stored until further processing. The samples were dehydrated using standard procedures and embedded in paraffin. Transverse sections of 5 μm thickness were made and stained with Haematoxylin/Eosin and Alcian blue (pH 2.5), to enhance the contrast between GC and SNV.

Scoring enteritis

The LM sections were evaluated according to the semi-quantitative method developed at Wageningen University (Urán *et al*. 2008), which assesses the degree of SBM-induced enteritis on the Atlantic salmon distal intestine considering the criteria fully described in Chapter 2. (For illustrations of the different scores, see annex or check the list of special

publication attachments at http://www.afi.wur.nl/UK/Publications/). Three out of the eight sampled fish per time point and diet, were scored and included in the analysis.

Assessment of the presence of ferritin in tissue

For the detection of ferritin in tissue, slides were stained with Perls' staining procedure described by Romeis (1968), to detect the presence of iron components in tissue. All sampling points after intubation were analyzed at week 1 and week 3 after SBM-feeding. Sections were photographed with an Olympus DP 50 digital camera (Olympus, Japan) connected to a Nikon Microphot-FXA light microscope (Badhoeverdorp, the Netherlands). The pictures were processed and analyzed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Münster, Germany).

Statistical analysis

All calculations were made using the SAS System (SAS 1999). The parameters used to score the degree of enteritis were analysed for the effect of diet using PROC GLM for each sampling point (week 1 or week 3) separately. The results are considered statistically significant when *P*-values are below 0.05.

Results

Morphological changes of the distal intestine

The control diet (0SBM) did not induce any sign of enteritis. The MF appeared as long finger-like structures, composed of enterocytes which were perfectly aligned along the mucosal epithelium. The nucleus of the enterocytes was located between the mid to basal part of the cell. The enterocytes presented normal round to oval SNV. The GC were present in a basal number and were scattered among the enterocytes. The LP was a delicate and single thin layer of cells underlying the epithelium. In the SM a basal number of EG was present as reflected in Fig.1. All semi-quantitative scores remained at the basal level for the measured parameters at all sampling points, indicating the absence of any sign of enteritis.

Fish fed 20SBM showed morphological changes at week 1. At this sampling moment, fish fed the 20SBM diet compared to fish fed the control diet had different scores for MF, SNV, LP and SM ($P < 0.05$) but not for GC ($P = 0.14$) and EG ($P = 0.06$). The MF become shorter with subsequent tissue disruption. The SNV start to shrink and their regular alignment was disturbed. A tendency for an increased infiltration of EG into the LP and SM was noticed. The amount of GC is numerically higher and the LP and SM had increased in size. At week 3 after 20SBM-feeding, heavily changed intestinal mucosal architecture could be detected. Most of the parameters measured were more affected worsen along the experimental period (Fig. 1) and at this sampling moment all score parameters were different between fish fed the control and 20SBM diet ($P < 0.001$). Diffused tissue disruption occurred at the level of the MF, appearing as bloated mucosal tissue. The SNV were completely absent. The GC appeared densely packed especially at the tip of the folds. The number of EG increased in SM, also with abundant presence along the LP.

In Table 2, the inflammatory response observed in young animals of an initial average weight of 30 g kept in freshwater was compared to the response observed in a previous experiment using old fish of an average weight of 300 g kept in seawater during the experimental period (Urán 2008). The results of this comparison suggest that both young and old fish experience a similar SBM-induced enteritis with an onset of the condition at week 1 and an aggravation at week 3. Some of the parameters tended to be higher in younger fish but for GC and EG the effect was slightly lower than in their old counterparts. In summary, the inflammation process was more or less similar at both ages.

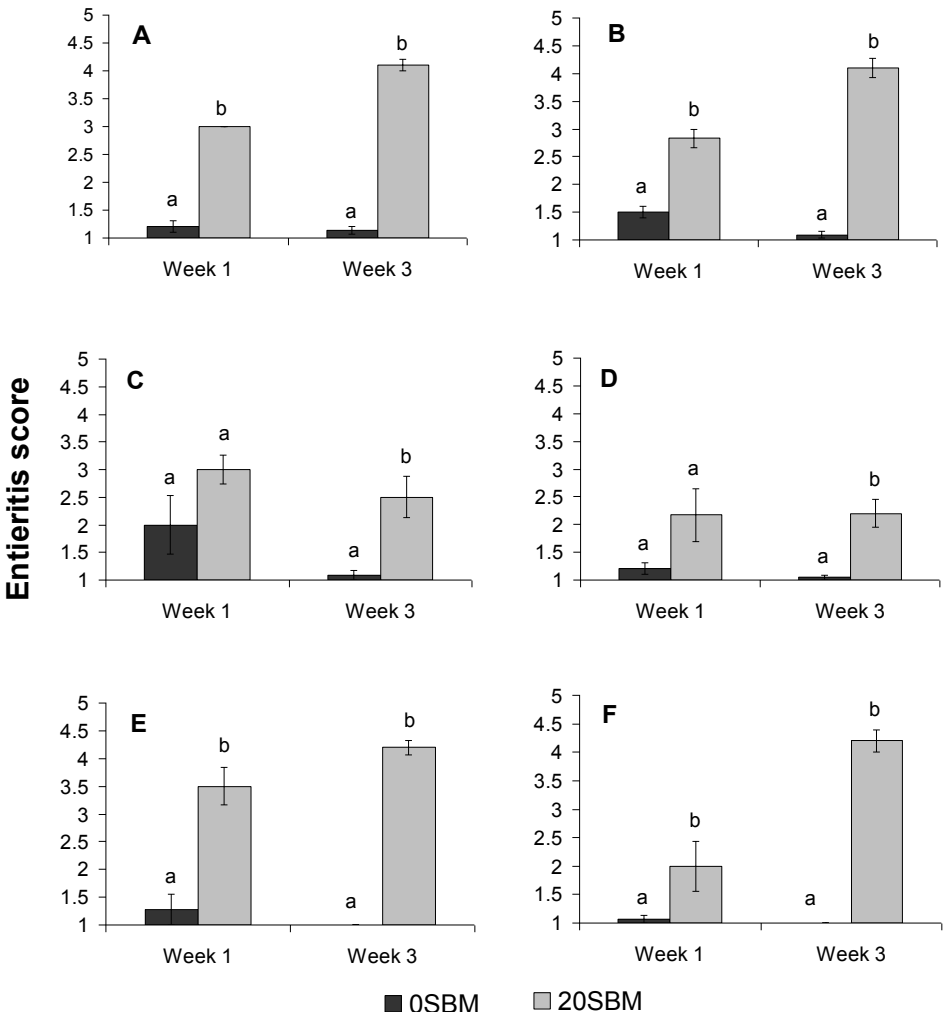


Figure 1 Parameters used to assess the degree of enteritis of the distal intestinal mucosa of Atlantic salmon after feeding a soybean meal-containing diet (20SBM) compared to a control diet (0SBM) at 1 and 3 weeks of continuous feeding, results are presented as mean values ± SE. The degree of enteritis has been assessed using the semi-quantitative scoring system (Urán *et al*., 2008). **A**. Mucosa folds (MF) , **B**. Supranuclear vacuoles (SNV), **C**. Goblet cells (GC), **D**. Eosinophilic granulocytes (EG), **E**. Lamina propria (LP), **F**. Sub-epithelial mucosa (SM). Means at each sampling point (week) having a different letter (ab) differ significantly ($P < 0.05$).

Table 2 Enteritis degree response from fish fed a soybean meal-based diet in two different experimental settings. The current study used young Atlantic salmon kept in freshwater of 30 g initial average weight, compared to old Atlantic salmon kept in seawater of 300 g initial average weight, used in a previous study (Urán 2008).

	Week 1			Week 3		
	30 g	300 g	% similarity	30 g	300g	% similarity
MF	3.0	2.7	111	4.1	3.1	132
SNV	2.8	2.3	123	4.1	3.8	108
GC	3.0	3.2	94	2.5	3.5	71
EG	2.2	2.8	78	2.2	3.5	63
LP	3.5	3.1	113	4.2	3.9	108
SM	2.0	3.0	67	4.2	3.6	117
Total	2.8	2.85	96	3.55	3.57	99

Presence of ferritin in tissue

Fish either not intubated or intubated with PBS did not show a background staining for ferritin, neither any sign of inflammation.

Anally intubated ferritin at week 1 and week 3 showed clear differences in the uptake process related to fish intubated with PBS. Fish fed the control diet, showed a normal uptake of ferritin with abundant presence of Perls' stained blue vacuoles within the enterocytes (Fig. 2). These observations hold for all controls throughout the experimental period.

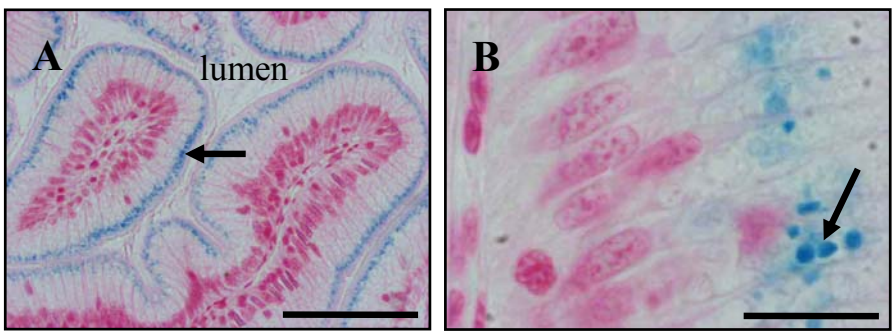


Figure 2 Detection of ferritin in distal intestinal enterocytes of young Atlantic salmon fed a fishmeal-based diet (0SBM) which did not contain any soybean meal. **A.** Ferritin uptake 90 min post-intubation. Ferritin molecules are stained at the apical part of the enterocytes, just under the brush border membrane; Bar is 100 µm. **B**. Ferritin in a lower position throughout the supranuclear vacuoles 6 hours post-intubation. Arrows are pointing at ferritin accumulations in the enterocytes. Bar is 20 µm.

In 20SBM fed fish, the tendency on the observed ferritin uptake at both week 1 and week 3 was impaired. At week 1, Perls' stained ferritin was detected within the enterocytes but the amount is substantially decreased compared to the control diet 0SBM (Fig. 3A). There were no clear differences between intubation points however after 6 hours Perls' stained ferritin, was also detected basally towards the LP (Fig. 3B). At week 3, all fish analysed did not show any ferritin uptake, although, ferritin was clearly present in the lumen at all time points (Fig. 3C and 3D).

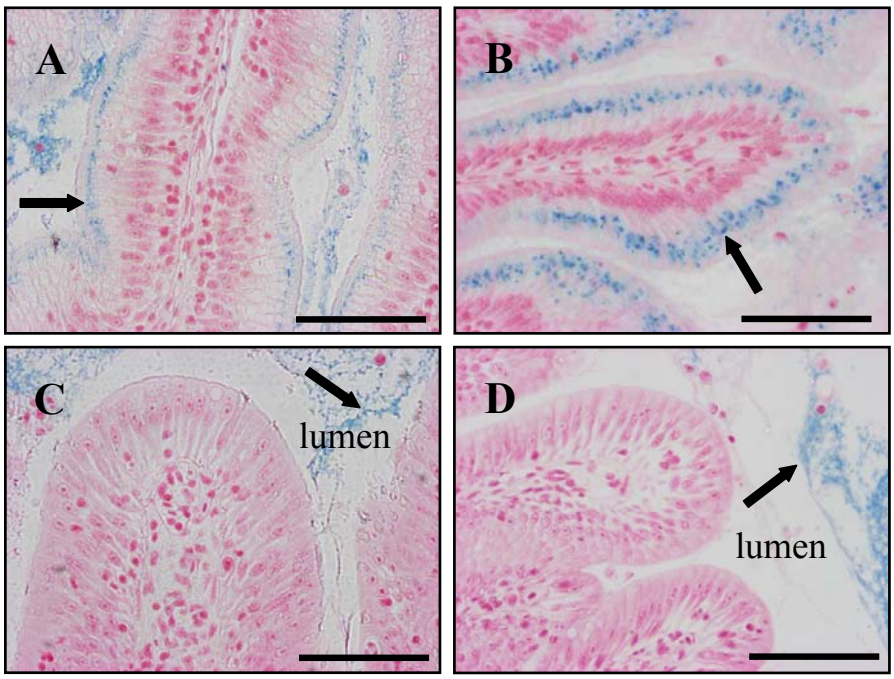


Figure 3 Ferritin uptake after 1 and 3 weeks of SBM feeding. **A)** Ferritin uptake at week 1 after 90 min post-intubation. **B)** Ferritin uptake at week 1 after 6 hours post-intubation. Perls' stained ferritin in enterocytes is indicated with arrows. **C)** No uptake at week 3 after 90 min post-intubation. **D)** No uptake at week 3 after 6 hours post-intubation. Perls' stained ferritin is only present in the lumen (arrows). Bar is 100 µm.

Discussion and Conclusions

In the current study, young salmon kept in freshwater were used to assess the typical morphological changes characteristic of a SBM-induced enteritis and its impact on the uptake capacity of the inflamed distal gut. At week 1 the symptoms of the enteritis condition were already present but at week 3 they became more severe. The symptoms were similar to previous observations on old Atlantic salmon kept in sea water and fed the same type of SBM variety (Urán 2008). These results at least suggest that the severity of enteritis is not related to age or smoltification of the fish. Only the numbers of GC and EG tended to be higher in old fish, but this may be related to development and hence an increased immunity at later ages. Old Atlantic salmon kept in freshwater also develop SBM-induced enteritis (Bakke-McKellep *et al*. 2000). More recently the influence of SBM feeding on the parr to smolt transformation was studied (Bakke-McKellep *et al*. 2006) and despite the inflamed intestine and apparent reduction in digestive functions augmented effects on osmoregulatory capacity was not found in SBM-fed fish. In conclusion salinity seems not to have a remarkable influence on the severity of SBM-induced enteritis.

In this study the impact of SBM on the endocytosis process has been examined, using ferritin as a large and easy detectable macromolecule. At week 1, ferritin uptake was reduced, however still present into a certain extent in some of the fish analysed. At week 3 ferritin uptake in the vacuoles was not observed, coinciding with a fully developed enteritis condition. These results are in line with earlier described disappearance of endocytosis vesicles and invaginations at the ultrastructural level (Urán 2008). Previous LM and EM studies using ferritin as marker molecule (Stroband & van der Veen, 1981; Rombout *et al*. 1985; Rombout & van der Berg 1989) have evidenced that endocytotic vesicles fuse with each other and with lysosomes, finally resulting in an accumulation and digestion of ferritin in the SNV. The present results also show the correlation between endocytotic uptake and SNV formation. A complete block of endocytosis even results in the disappearance of SNV, one of the most obvious signs of SBM-induced enteritis. In this study the complete block occurs between 1 and 3 weeks of SBM feeding, while in earlier ultrastructural observations it was reported at 1 week (Urán 2008). Although, electron microscopy, allowing only minor parts to be studied, is not the most suitable technique to estimate the onset of the endocytosis block, a

complete uptake block will probably be obtained short after the 1st week of SBM-feeding. It has been well documented that ferritin uptake is the result of fluid phase uptake in a process that seems to be receptor driven; in any case horseradish peroxidase (HRP; Rombout *et al*. 1985) and LTB (Companjen *et al*. 2006) have been shown to be taken up by receptors. However, the exact function of this endocytosis process has never been elucidated. Which substances in SBM are responsible for the endocytosis block is still unclear, although it has been suggested that SB lectins may block receptor sites in the distal intestine (cf. Nordrum *et al*. 2000). More recently it has been suggested that the disappearance of SNV might be caused by an unbalanced enzymatic activity (Krogdahl & Bakke-McKellep 2005). However, this study strongly suggest that endocytosis block is the first step, followed by changes in and disappearance of the SNV. More attention has to be paid to the subsequent reactions of enterocytes (i.e. production of danger signals like heat shock proteins) and the onset of inflammation reactions in the connective tissue. It has been demonstrated that T cells and EG play an important role in the SBM-induced enteritis process of the distal gut (Bakke-McKellep *et al*. 2007), resulting in elevated levels of inflammatory cytokines (Urán 2008).

In conclusion, more research has to be dedicated to the SBM substances responsible for the endocytosis block, for a better selection of suitable soybeans to be included in the diet formulation of Atlantic salmon. Strong variations in severity of enteritis between different SB batches are described in this thesis and may be used to find the most important causative agent. In addition, more research is needed to translate the endocytosis block and subsequent disappearance of SNV into the severe enteritis process observed in the distal gut.

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Variation in commercial sources of soybean meal influences the severity of enteritis in Atlantic salmon *(Salmo salar L.)*

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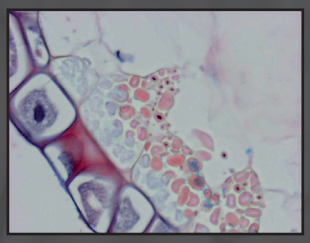
Chapter 6



Submitted for publication

Abstract

Soybean meal (SBM) is a potential alternative for the replacement of fishmeal in aquafeeds. In Atlantic salmon, however, dietary SBM causes an inflammation of the distal intestine, known as enteritis. The objective of the present study is to verify whether different (geographically spread) commercial sources of SBM yield contrasting inflammatory responses. To do so, six SBM batches, from different origins, were included in the Atlantic salmon diets at the level of 20%. After 4 weeks of feeding the distal intestine of the salmon was sampled and scored by a semi-quantitative scoring system, which assessed six separated parameters, characterizing the extent of enteritis. The overall mean score as well as the score of the separate parameters varied between the different commercial sources of SBM included in the diet. The variation in SBM caused different degrees of disparity in the score of the separate parameters. The parameter that was most affected by the variation in the source of SBM was the disappearance of supranuclear vacuoles in enterocytes. In contrast, the increase in goblet cells showed the smallest variation between the different SBM sources. This study shows that different commercial sources of SBM can result in differences in the severity of SBM-induced enteritis in Atlantic salmon.

Introduction

Finding alternative protein sources to replace fishmeal in fish feed is important if the growth of the aquaculture industry is to be sustained (Francis *et al*. 2001; Tacon 2003). Soybean meal (SBM) is one such potential alternative (Gatlin *et al*. 2007), and consequently, it has already been used for several fish species. However, most plant-derived nutrient sources contain various anti-nutritional substances (Francis *et al*. 2001). Low-processed soybean (SB) products (incl. SBM) induce a non-infectious intestinal inflammation in the second gut segment (distal intestine) of Atlantic salmon (Baeverfjord & Krogdahl, 1996). This SB-induced enteritis is characterized by: a shortening of the mucosal folds; loss of the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium; a widening of the central stroma within the mucosal folding, with increased amounts of connective tissue; a profound infiltration of the inflammatory cells in the lamina propria (van den Ingh *et al*. 1991; 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001); an increased number of goblet cells in the epithelium, a shortening of the microvilli (van den Ingh *et al*. 1991) and finally a strongly decreased endocytotic activity of the enterocytes (Urán 2008). Immunological mechanisms are probably involved in the pathogenesis, but the precise causes of the inflammatory process have not, as yet, been identified. Nonetheless, some authors suggest that alcohol-soluble components of the SBM may induce the inflammatory process (van den Ingh *et al*. 1996; Krogdahl *et al*. 2000, Knudsen *et al*. 2007).

Considerable variation can exist in the nutritional value (nutrient content, anti-nutritional factors, etc.) among sources (batches) of the same plant ingredient (Jiang 2001), due to factors such as: genetics, growing conditions, harvesting, processing, storage, etc. Numerous studies have compared the severity of the enteritis induced by different types of SB products such as: full-fat SB (raw or toasted), solvent-extracted SBM, soy protein concentrates, SB molasses (Olli & Krogdahl 1995; Bjerkeng *et al*. 1997; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Grisdale-Helland *et al*. 2002; Knudsen *et al*. 2007). However, it is not known whether the severity of SB-induced enteritis is also affected by the variation between different commercial sources of SB products (e.g., SBM). This study wants to clarify whether different commercial

sources of SBM can result in differences in the severity of SB-induced enteritis in Atlantic salmon.

Materials and Methods

Fish and rearing conditions

The experiment was carried out at the Skretting Fish Trials Station, Lerang, Jørpeland, Norway. The experiment consisted of a 3-week adaptation and a 4-week experimental period. Seawater-adapted Atlantic salmon (*Salmo salar* L.; AquaGen strain), which originated from a stock of fish present at the research station, were fed one of seven experimental diets during the experimental period. The average fish weight was 241 g and 396 g, respectively, at the start of the adaptation period and the end of the experimental period. Fish were randomly assigned to one of seven circular (1 m diameter), 400 L, fibreglass tanks at a stocking density of 25 fish per tank. Tanks were equipped with feed-waste collectors and were continuously supplied with seawater (15 L min^{-1}), which was pumped from a 100 m depth in the fjord. Inlet seawater had a salinity of 34 ‰ and an oxygen concentration of about 9 ppm. The inlet water temperature remained constant at 12°C. Tanks were stationed indoors, where the photoperiod was 18L: 6D. At the end of the experimental period, after having been fed one of the experimental diets for 4 weeks, nine fish per treatment (63 fish in total) were sacrificed with an overdose of anaesthetic (Finquel MS-222, Argent Chemical Laboratories, United States) for gut histological measurements.

Diets and feeding

The current study aims at assessing whether different commercial sources (batches) of solvent extracted SBM cause differences in the degree of enteritis. To create a large variation between the different SBM batches, six SBM batches ("SBM1" to "SBM6") were purchased, all of which originated from different commercial sources (SBM producing plants) from around the world: three from North America and one from South America, Europe and Australia, respectively (Table 1). The SBM1 batch had a lower crude protein content (44.9%) than the other SBM batches, in which the crude protein content ranged from between 48.2 to

49.2%. The amino acid profile was similar between the different SBM batches. The crude fibre content of the different SMB batches was not correlated with the crude protein content and ranged from between 2.9 to 4.7%.

Seven experimental diets were formulated: one control diet (FM), which contained fishmeal and no SBM; and six SBM diets ("SBM1" to "SBM6") each containing one of the six SBM batches (Table 2). The control diet (FM) was a mixture of fishmeal, fish oil, wheat starch and a standard vitamin and mineral premix. In the SBM diets, 20% SBM was included. Diets were formulated to have similar crude protein (i.e., iso-nitrogenous) and crude fat content (i.e., iso-lipidic). Compared to the FM diets, SBM was exchanged by both fish meal and wheat starch in the SBM diets. Furthermore, the fish oil content was slightly increased in the SBM diets to keep the crude fat content equal between diets (Table 2). Due to the lower crude protein content of the SBM1 batch (Table 1), a slightly smaller amount of fishmeal was replaced in the SBM1 diet compared to other SBM diets (Table 2). For the SBM2 to SBM6 diets, the ingredients exchanged were kept similar, ignoring the small differences in crude protein content. The diets were produced at Skretting Feed Technology Plant (Stavanger, Norway), in the form of extruded 4 mm sinking pellets.

The experimental diets were randomly assigned to one of the seven tanks and were fed to the salmon during the 4-week experimental period. During the adaptation period, fish were fed a commercial salmon diet containing a low amount of SBM (3%). During both periods, fish were fed twice a day using automatic feeders. The experimental protocol aimed at 10% overfeeding of the fish, which was checked by the feed-waste collection.

Chemical composition analysis

The chemical composition of the different SBM sources was determined using standard techniques for proximate analyses. Samples were analysed for crude protein, moisture, crude fibre and amino acid profile. Crude protein content was determined by the Kjeldahl nitrogen measurement in accordance with the Nordic Committee on Food Analysis, Method No.6, 4th edition, 2003. Moisture content in the samples was measured by drying to constant weight at 102-105 °C for 16-18 hours. Crude fibre was calculated according to EEG L344/35-37, 1992. The amino acid profile was determined according to the EU-method "commission directive 98/64/EC, 1998 (Table 1).

Table 1 Chemical composition and background information on the different commercial sources of solvent-extracted soybean meal (SBM) tested

	SBM1	SBM2	SBM3	SBM4	SBM5	SBM6
Background on source of SBM:						
Production plant	A	B	C	D	E	F
Location of production plant[a]	NA	EU	SA	NA	NA	AU
Nutrient composition g/kg						
Crude protein	449	492	492	492	484	482
Moisture	119	115	123	113	115	113
Crude fibre	35	29	37	28	36	47
Amino Acids %						
Arginine	3.3	3.6	3.6	3.7	3.5	3.6
Histidine	1.2	1.3	1.3	1.3	1.3	1.3
Isoleucine	1.9	2.1	2.1	2.1	2.0	1.9
Leucine	3.4	3.7	3.7	3.7	3.6	3.5
Lysine	2.8	2.9	2.9	3.0	3.0	2.9
Methionin	0.6	0.7	0.6	0.7	0.7	0.6
Cystin	0.7	0.7	0.7	0.8	0.8	0.7
Phenylalanine	2.2	2.5	2.5	2.5	2.4	2.3
Tyrosine	1.5	1.6	1.6	1.6	1.5	1.5
Threonine	1.8	1.9	1.9	2.0	1.9	1.9
Valine	2.0	2.1	2.2	2.2	2.1	2.1
Alanine	1.9	2.1	2.1	2.1	2.1	2.0
Aspartic acid	5.1	5.7	5.7	5.7	5.5	5.4
Glutamic acid	8.3	9.4	9.2	9.4	9.2	8.9
Glycine	1.8	2.0	2.0	2.1	2.0	2.0
Proline	2.3	2.5	2.5	2.5	2.5	2.4
Serine	2.4	2.6	2.6	2.6	2.5	2.5

[a] NA = North America; EU = Europe; SA = South America; AU = Australia.

Table 2 Ingredient composition of the experimental diets

	FM[a]	SBM1[a]	SBM2 to SBM6[a]
Ingredients g/kg			
Soybean meal[a]	0	200	200
Fishmeal[b]	587	464	453
Wheat starch	223	138	148
Nordic fish oil	189	197	198
Vitamin/mineral premix	2	2	2

[a] FM (fishmeal) is the control diet; SBM1 to SBM6 are the experimental diets containing solvent extracted soybean meal, the number refer to the commercial SBM source described in Table 1. The SBM diets were formulated to contain similar amounts of crude protein (42%), crude fat (25%) , ash (8%), starch (13%) and gross energy (23 MJ/kg). [b] Scandinavian LT fishmeal.

Sampling and assessment of the degree of enteritis

After the 4-week experimental period, samples of the distal intestine (considered as the section from the distal end of the mid intestine to the anus) were collected from nine fish per tank. The fish were killed by an overdose of anaesthetic. The intestines were removed immediately and rinsed in cold (4 °C) saline. Samples for light microscopy (LM) were placed in 4% neutral phosphate-buffered formalin with a pH of 7.2. After dehydration, samples were embedded in paraffin following standard histological procedures. Transverse sections of approximately 5 µm were cut and thereafter mounted on glass slides and stained using haematoxylin and eosin. The LM sections were evaluated according to the semi-quantitative method developed at Wageningen University (Urán *et al*. 2008), which assesses the degree of SB-induced enteritis in the distal intestine of Atlantic salmon in accordance with the following criteria: 1. the appearance and length of the mucosal folds (MF); 2. the presence and size of supranuclear vacuoles (SNV); 3. the number of goblet cells (GC); 4. the degree of infiltration abundance and of eosinophilic granulocytes (EG) into the lamina propria and into the sub-epithelial mucosa; 5. the degree of widening of the lamina propria (LP); and, 6. the degree of thickening of the sub-epithelial mucosa (SM). Each of these parameters was scored on a scale from 1 to 5. A score of increasing value represents a more severe enteritis condition. For illustration of the different scores, see annex or check list of special publications at http://www.afi.wur.nl/UK/Publications/. Additionally, an overall enteritis score was calculated per fish as the average score of the six parameters scored per fish (MF, SNV, GC, EG, LP and SM).

Statistical analysis

The effect of the experimental diet on the separate, scored enteritis parameters (MF, SNV, GC, EG, LP and SM) and the overall enteritis score were analysed by a one-way ANOVA, using PROC GLM of SAS (SAS 1999). Error term analysis using PROC UNIVARIATE (SAS 1999) showed that all enteritis parameters were normally distributed. A post hoc comparison of means between diets was done using the Tukey test. The level of significance was set at $P < 0.05$.

Results

The overall enteritis score at the different experimental diets is presented in Fig. 1. It was affected by the diet ($P < 0.001$). Fish fed the control diet (FM) did not show any sign of SBM-induced enteritis. The overall score of fish fed the FM diet was different from fish fed other experimental diets, all of which contained SBM (SBM1 to SBM6) ($P < 0.05$). However, the degree of enteritis varied among the SBM diets, being dependent upon the commercial source of SBM included. Fish fed the SBM1, SBM3, and SBM4 diets attained the highest overall enteritis score, which differed significantly ($P < 0.05$) from fish fed the SBM6 diet, which showed the mildest enteritis response. The SBM2 and SBM5 diets gave an intermediate response compared to the other SBM diets.

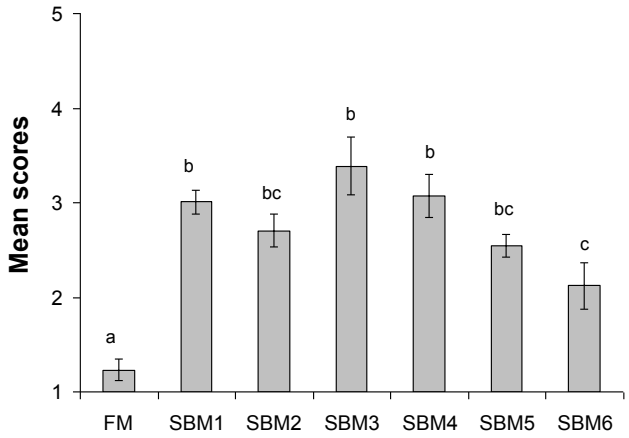


Figure 1 Mean values of the overall enteritis score per experimental diet (effect of diet, $P < 0.001$). FM is the fishmeal (control) diet, SBM1 to SBM6 are the diets containing 20% soybean meal of commercial source 1 to 6 respectively as described in Table 1. Mean values per diet having no common letter (abc) differ significantly ($P<0.05$).

The scores of the separate enteritis parameters, MF, SNV, GC, EG, LP and SM, are shown in Fig. 2. All of these enteritis parameters were influenced by the diet ($P < 0.001$). The separate parameters in fish fed the FM diet did not increase and were generally different from

fish fed the SBM diets, except for MF, SNV and LP. Regarding the parameters MF, SNV and LP, fish fed the FM diet did not significantly differ from fish fed the SBM diets, which gave a mild enteritis response (especially the SBM6 diet).

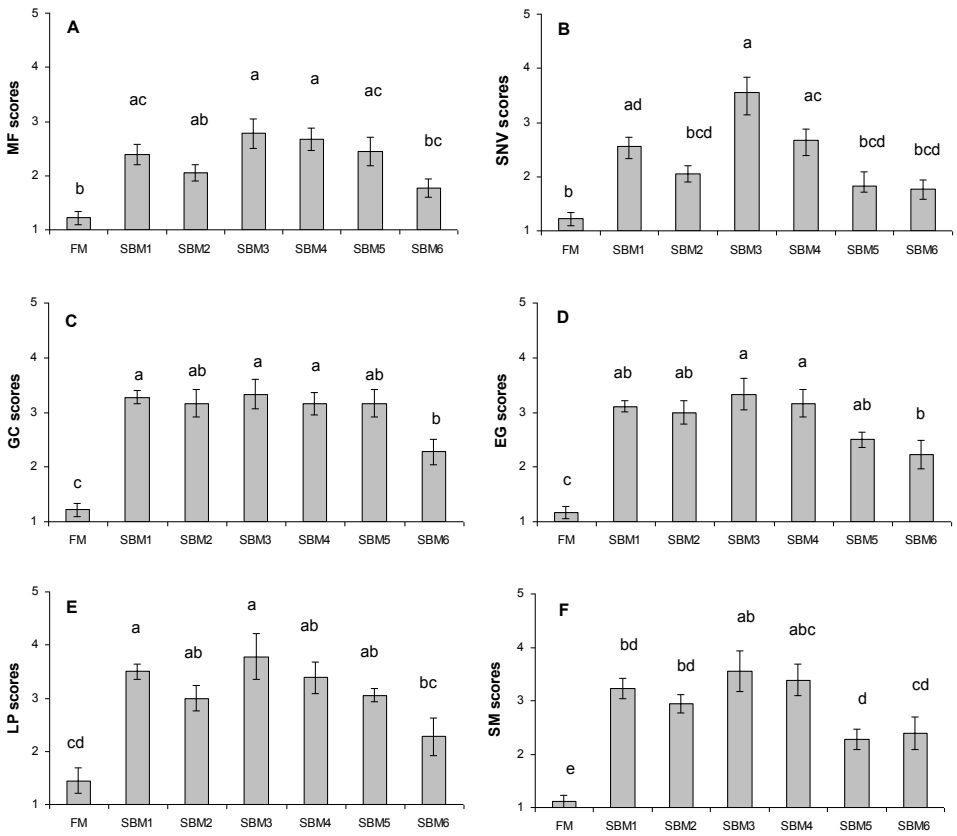


Figure 2 Mean values of the enteritis parameters scored per experimental diet: **A)** mucosal folds (MF); **B)** supranuclear vacuoles (SNV); **C)** goblet cells (GC); **D)** eosinophilic granulocytes (EG); **E)** lamina propria (LP); **F)** sub-epithelial mucosa (SM); (diet effect, $P < 0.001$ for all parameters). FM is the fish meal (control) diet, SBM1 to SBM6 are the diets containing 20% soybean meal of commercial source 1 to 6 as described in Table 1. Mean values per diet having no common letter (abcde) differ significantly ($P < 0.05$).

Similarly as for the overall score, the separate parameters revealed a SBM batch variation in the degree of enteritis. However, the extent of the variation between the SBM diets differed between the different parameters (Fig. 2). The largest contrasts in scores among the SBM diets were observed for the disappearance and disturbances of the enterocytes' supranuclear vacuoles (SNV score; Fig. 2B). The contrasts among diets for the MF, EG, LP and SM were lower compared to the SNV, but the general pattern among diets for these parameters was comparable to that of SNV, showing the highest response at the SBM3 diet and the lowest at the SBM6 diet. The increase in the number of GC gave the smallest contrasts between the SBM diets (GC score, Fig. 2C). The GC score was only lower for fish fed the SBM6 diets, whereas no differences were present between the other SBM diets (SBM1 to SBM5). The smaller contrasts in GC among the SBM diets compared to the other parameters are also reflected by the calculated correlation coefficients between different parameters using only the fish fed with the SBM diets (Table 3). The correlation between the GC score and the other scores ranged from between 0.44 and 0.70 (mean = 0.58) and was lower than the correlations between the other enteritis parameter (MF, SNV, EG, LP and SM), which ranged from between 0.66 and 0.88 (mean = 0.78).

Table 3 Correlation coefficients among the different scores of enteritis parameters (MF, SNV, GC, EG, LP and SM[a]) for fish fed the diets containing soybean meal (thus excluding the control diet) (n = 54; for all correlations $P < 0.001$)

	SNV	GC	EG	LP	SM
MF	0.66	0.44	0.70	0.79	0.73
SNV		0.57	0.79	0.80	0.80
GC			0.70	0.63	0.58
EG				0.81	0.88
LP					0.84

[a] MF = mucosal folds; SNV = supranuclear vacuoles; GC = goblet cells; EG = eosinophilic granulocytes; LP = lamina propria; SM = sub-epithelial mucosa.

Discussion and Conclusions

In this study, the inclusion of soybean meal (SBM) in the diet of Atlantic salmon induced the classical signs of enteritis in the distal intestine, affecting the intestinal epithelium at a structural and cellular level, as had been previously described in various studies (van den Ingh *et al*. 1991, 1996; Baeverfjord & Krogdahl 1996; Krogdahl *et al*. 2000; Refstie *et al*. 2000; Buttle *et al*. 2001). In literature, several studies show that the type of soybean (SB) product (e.g., full-fat SB, solvent-extracted SB, SB molasses), can result in differences in enteritis response (Krogdahl *et al*. 2000; Refstie *et al*. 2000, 2001, 2005; Sanden *et al*. 2005; Knudsen *et al*. 2007). The current study demonstrates that the severity of enteritis also varies between different commercial sources (batches) of the same SB product, e.g., SBM. This finding raises the question: what is the cause of this variation in enteritis' responses between the commercial sources of SBM? However, this is difficult to answer because the causative agent has not yet been identified. In the current study, the differences in enteritis' responses between the experimental diets were not related to the crude protein or crude fibre content of the SBM sources. The correlation coefficient between the mean enteritis score per diet and crude protein content of the SBM batch was 0.001 (P = 0.997; n=6), and crude fibre content was -0.562 (P = 0.246; n=6). Nonetheless, it should be made clear that the absence of significant correlation might be due to the small number of tested SBM batches (n=6) in combination with the small differences in composition between the SBM batches (Table 1). The absence of a relation between the degree of enteritis with crude protein as well as crude fibre is in line with the hypothesis that one or more alcohol-soluble components of SB might induce an inflammatory response (Olli *et al*. 1995, van den Ingh *et al*. 1996, Krogdahl *et al*. 2000). The study of Knudsen *et al*. (2007) suggests that soyasaponins may be involved in triggering the enteritis response.

In the current study, a semi-quantitative scoring system was used to assess the degree of enteritis, which scores six separate indicative parameters of the inflammation response: mucosal folds (MF); supranuclear vacuoles (SNV); goblet cells (GC); eosinophilic granulocytes (EG); lamina propria (LM); and sub-epithelial mucosa (SM). The degree of variation between the commercial SBM sources varied among the different parameters scored (Fig. 2). The observation that the disappearance of SNV in enterocytes displayed the largest

differences between SBM sources suggests that the appearance of SNV in enterocytes is the most sensitive parameter to detect (small) differences in the causative agent(s) within SBM sources. This is supported by the statement of Krogdahl *et al*. (2003), that the manifested enteritis condition is characterized by the absence of SNV, being the first organelles affected. The smaller variation in scoring of the other parameters might be an indication that the shifts in these parameters occur during a more secondary stage of the enteritis cascade. GC are known to be involved in the innate defence system through the production of mucus that gives protection and which acts as a lubricant of the alimentary tract against chemical and mechanical damage (Marchetti *et al*. 2006). The smallest differences between SBM sources were visible in the abundance of GC in the MF. Only at one SBM batch (SBM6), which gave the lowest enteritis response for all other parameters, the number of GC was lower compared to the other SBM batches. These data suggest that GC respond more to a threshold value than to a proportional dose response, while other enteritis parameters are more likely to respond depending on dose.

The current observation between SBM source variation in the enteritis response has both scientific and practical implications. When studies on SB-induced enteritis are compared, it must be realized that differences among studies might be due to source (batch) variation in the SB ingredients used. For a proper comparison of the impact of different SB products (e.g., full-fat SB, solvent-extracted SBM, soy protein concentrates etc.), differences in the origin of the SB might bias the contrasts displayed in the enteritis response. Furthermore, the general applicability of results from studies on the dose response of the dietary inclusion of SBM is hampered by the existence of inter-source (batch) variations. For practical feed formulation, the observed presence of SBM batch variations necessitates extra safety margins for the inclusion of SBM if no specific information is present on the type of SBM batch. Obtaining indicative parameters (requiring additional research) for estimating the potential of specific batches of SBM on the enteritis response might facilitate higher inclusion levels of SBM in Atlantic salmon diets without compromising the health status of the fish.

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Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon

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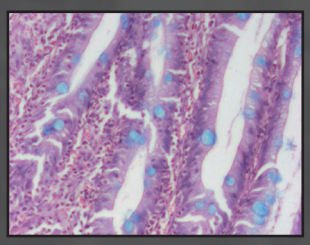
Chapter 7



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Abstract

The current work aimed at tracing the causative components for soybean-induced enteritis in Atlantic salmon (*Salmo salar* L.). Soybean molasses was subjected to phase-separation using n-Butanol. Three sub-fractions were obtained: butanol phase, precipitate and water phase. The biochemical composition of soybean molasses and the obtained sub-fractions were analyzed in detail: Protein, fat and ash were quantified according to standard methods. Sucrose, raffinose and stachyose were quantified using high-performance anion-exchange chromatography. Soyasaponins were quantified using reverse phase high-performance liquid chromatography. Finally, sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to evaluate the size distribution of the proteins present in each fraction. Molasses and the different sub-fractions were thereafter fed to Atlantic salmon in two successive fish trials. The level of intestinal inflammation was evaluated by light microscopy using a semi-quantitative scoring system. Histological assessments revealed that Atlantic salmon fed a combination of butanol phase and precipitate displayed significant enteritis. Atlantic salmon fed the water phase displayed normal intestinal morphology. Conclusions: The causative components for soybean-induced enteritis withstand butanol treatment and prolonged heating at 70°C. Sucrose, raffinose, stachyose, nor soybean proteins larger than 10 kDa induce enteritis alone. Soyasaponins, or components that follow the same solubility pattern, trigger the inflammatory reaction. We therefore suggest that soybean-induced enteritis in Atlantic salmon is induced by soyasaponins alone, or by soyasaponins in combination with other factors e.g. antigenic soybean proteins or the intestinal microflora.

Introduction

A limited supply of fishmeal could hamper future growth in the aquaculture industry and much effort has therefore been made to find alternative protein sources that could replace fishmeal in feed formulations for carnivorous fish (Tacon 2003; FAO 2002; Naylor *et al*. 2000; Francis *et al*. 2001). From an ecological viewpoint an ideal solution would be to find a suitable low-cost plant-derived protein. Soybean meal (SBM) is one of the promising candidates due to its high protein content and steady supply. Several studies have revealed, however, that high inclusion levels of low-processed soybean (SB) products induce intestinal inflammation in the hindgut of Atlantic salmon (van den Ingh *et al*. 1991,1996; Baeverfjord & Krogdahl 1996; Bakke-McKellep *et al*. 2000). The inflammatory reaction is associated with several morphological changes, including: loss of supranuclear vacuoles in the absorptive enterocytes, widening of the lamina propria of mucosal folds, increased amounts of connective tissue between the base of the mucosal folds and stratum compactum, shortening of mucosal fold height, and infiltration of inflammatory cells in the lamina propria (van den Ingh *et al*. 1991, 1996; Baeverfjord & Krogdahl 1996). The normal morphology of the distal intestine in Atlantic salmon can be seen in figure 1A while figure 1B displays typical soybean-induced enteritis. The enteritis associated with soybean meal currently limits its use in diets for Atlantic salmon.

Despite considerable work, the causative components for the condition remain unidentified. Important clues can, however, be found as to their identity. Van de Ingh *et al*. (1991, 1996) demonstrated that they follow the protein fraction when the oil is extracted by hexane.

Alcohol extracted SB protein concentrate did not, however, induce inflammation. In addition, it was found that fish fed soybean molasses (the by-product of alcohol extraction) displayed the same signs of inflammation as fish fed SBM. It can thus be concluded that the causative components are soluble in aqueous alcohol and resist alcohol treatment at elevated temperatures. SB molasses is a brown liquid composed of 60% dry matter, 5% protein, 5% lipids, 5% ash and 45% nitrogen free extracts. Sucrose, raffinose and stachyose constitute for approximately 35% of molasses. The remaining 10% of the nitrogen free extracts includes other sugars, isoflavones and saponins (supplier data, Solae Europe, S.A., Switzerland).

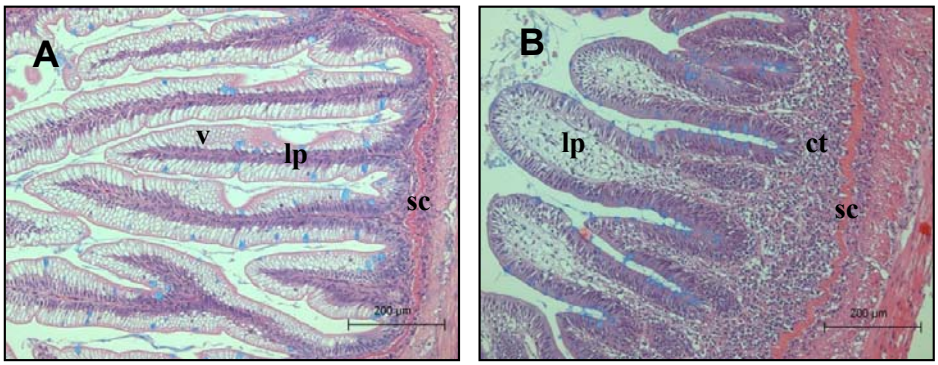

Figure 1 A) Normal morphology of distal intestine in Atlantic salmon. **B)** Typical signs of soybean-induced enteritis: Loss of vacuoles (v) in absorptive enterocytes; widening of lamina propria (lp) in mucosal folds; and increase of connective tissue (ct) between base of folds and stratum compactum (sc). Staining: Hematoxylin & Eosin and Alcian blue 8 GX.

The current work aimed at tracing the causative components for soybean-induced enteritis in Atlantic salmon. Soybean molasses was subjected to phase-separation and the biochemical composition of the sub-fractions was investigated in detail. Two fish trials were conducted to evaluate the physiological impact of the sub-fractions on intestinal morphology.

Materials and Methods

Separation of soybean molasses

SB molasses was kindly provided by Solae Denmark A/S, Århus, Denmark. The molasses was separated into 3 sub-fractions by phase separation using n-Butanol (product no. 33065, Sigma-Aldrich). Molasses and water saturated n-Butanol were mixed 1:1 (v/v) and allowed to separate overnight in a separation funnel. A dense layer of yellow precipitate formed between the two phases. The mixture was separated in 3 fractions (butanol phase, precipitate and water phase) and evaporated to dryness at 70°C in a rotary evaporator under reduced pressure. The residues were re-suspended in water and evaporated to dryness again several times in order to remove butanol completely. Each sub-fraction was finally re-suspended in water to reach the initial volume of molasses. The batch of soybean molasses

used in the present work contained 62% dry matter (w/w). Preliminary analyses of the obtained fractions revealed that 15% of the total dry matter was recovered in the butanol phase, 35% in the precipitate and 50% in the water phase. Molasses and the obtained sub-fractions were analyzed for protein, fat, ash, sucrose, raffinose, stachyose and soyasaponins. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was used to evaluate the size distribution of the proteins present within each fraction.

Chemical analysis of diets

Dry matter measurements were done by drying to constant weight at 102-105°C. Ash was measured by burning samples at 550°C for 16-18 hours. Crude protein was quantified as N x 6.25 using a Kjeltec Auto Sampler-System (Tecator AB, Sweden) according to Nordic Committee on Food Analysis, Method No. 6, 4th edition 2003. Total fat was measured by acid hydrolysis using a Soxtec 2050 extraction system (Foss Analytical, Denmark) according to Nordic Committee on Food Analysis, Method No. 160, 1998.

Quantification of oligosaccharides

Quantification was done using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Standards of sucrose, raffinose and stachyose were purchased from Sigma-Aldrich, Inc., MO, USA (product # S1174, R0250 and S4001, respectively). Separation was done on a Dionex HPAEC-PAD system using a Dionex CarboPac PA-1 column. The injection volume was 10 µl and the compounds were eluted isocratically with 200 mM NaOH for 15 minutes at a flow rate of 1.0 mL/min. The oligosaccharides were identified by comparing their retention times to the authentic standards. Quantification of the oligosaccharide was accomplished by reference to standard curves made for each of the three oligosaccharides. The molasses fractions were diluted 1:3000 in distilled H_2O, centrifuged at 14000*g* for 5 minutes, and filtered through a 0.22 µm GHP membrane filter prior to injection into the HPAEC-PAD system.

Quantification of soyasaponins

Separation and quantification of soyasaponins was performed using reverse phase high-performance liquid chromatography with diode array detection (HPLC-DAD) as described

previously (Knudsen *et al.* 2006). Briefly, the separation was achieved using a Hewlett-Packard series 1050 HPLC-DAD system with a 250 mm x 4.6 mm i.d., 5 µm, Supercosil ABZ +Plus, C_{18} reverse phase column (SUPELCO). The mobile phases were 0.05% trifluroacetic acid in water (solvent A) and 0.05% trifluroacetic acid in acetonitrile (solvent B). The gradient elution was linear from 25 to 50% B, 0-65 min; linear from 50 to 60% B, 65-70 min; linear from 60-100% B, 70-75 min; isocratic at 100% B, 75-85 min; then linear from 100-25% B, 85-90 min and finally isocratic at 25% B, 90-100 min. The flow rate was 0.5 ml/min, the injection volume was 50 µl and the column temperature was 30°C. Identification of SB saponins was confirmed by HPLC retention time, UV absorption spectra recorded at 200-350 nm and LC-MS using positive electrospray ionization. Molasses and molasses sub-fractions were diluted 1:10 in 70% aqueous ethanol and centrifuged at 15000*g* for 5 minutes before injection on the HPLC system. The following soyasaponins were detected and quantified; Ab, Ac, Af, Ba, Bb, Bc, Ba-DDMP, Bb-DDMP and Bc-DDMP (see Knudsen *et al*. 2006 for molecular structure).

SDS-PAGE

The size distribution of the proteins present in the different molasses fractions was evaluated by SDS-PAGE according to (Schägger & von Jagow, 1987). The electrophoresis was done using 10-20% tricin gradient gels (Novex, Invitrogen, Groningen, The Netherlands). The re-suspended molasses fractions were diluted 1:10 in H_2O and mixed 1:1 with sample buffer (0.1 M Tris buffer, 8% (w/v) SDS, 24% (v/v) glycerol, 0.025% (w/v) Coomassie blue, 0.04 M 1,4-Dithiothreitol, pH 6.8). The mixtures were boiled for 5 minutes before they were loaded on the gel (10 µL per well). A standard protein mixture was included on the gel for molecular weight estimation (Mark12^{TM}, Product no. LC5677, Noves, Invitrogen, the Netherlands). Electrophoresis was carried out for 1 h and 20 minutes at 125V (constant). Finally, the gel was stained with Coomassie blue.

Production of feed for Fish Trial 1

Six diets were produced as 4 mm pellets by twin-screw extrusion cooking (TX57, Wenger Manufacturing, Inc., United States) at Skretting Feed Technology Plant, Stavanger, Norway. Molasses and molasses fractions were mixed with the other ingredients before

extrusion. The molasses batch contained 620 g dry matter per kg and 15% of this dry matter was recovered in the butanol phase, 35% in the precipitate and 50% in the water phase. By knowing these ratios it was possible to calculate how much soybean molasses the added amounts of sub-fractions corresponded to. The different sub-fractions were included at a level that corresponded to approximately 10% (w/w, wet basis) soybean molasses. The recipes are shown in Table 1.

Table 1 Formulation of diets for Fish Trial 1

	Diet						
		A	B	C	D	E	F
Fish meal (Scandinavian LT)	g/kg	490	625	625	625	625	625
Wheat	g/kg	108	120	120	120	120	120
Wheat Starch	g/kg	0	53	9	0	0	62
Minerals, vitamins, pigment	g/kg	3	3	3	3	3	3
Defatted soybean meal [a]	g/kg	200	0	0	0	0	0
Butanol phase [b]	g/kg	0	9	0	0	0	0
Precipitate & Water phase [b]	g/kg	0	0	53	0	0	0
Butanol treated molasses [b]	g/kg	0	0	0	62	0	0
Untreated molasses [b]	g/kg	0	0	0	0	62	0
Fish Oil [c]	g/kg	199	190	190	190	190	190
Total	g/kg	1000	1000	1000	1000	1000	1000
Molasses equivalents [d]			10%	10%	10%	10%	

[a] Denofa, Norway

[b] Dry matter

[c] Northern Hemisphere

[d] Wet matter basis

Production of feed for Fish Trial 2

Dry pellets (4 mm) were produced by twin-screw extrusion cooking (TX57, Wenger) at Skretting Feed Technology Plant, Stavanger, Norway. The pellets had the following composition; 80% fishmeal, 15.5% wheat, 4% wheat starch and 0.5% premix (minerals, vitamins and pigment). These dry pellets were used as a carrier matrix to test the impact of all different combinations of sub-fractions. Instead of adding the molasses fractions before extrusion the molasses fractions were coated on the dry pellets using a specially designed lab-scale vacuum coater. A known amount of dry molasses sub-fraction was re-suspended in water and sprayed onto the dry pellets under reduced pressure. The coated pellets were then

dried at 102°C to achieve a moisture content of 10%. Using the same lab-scale coater, pellets were thereafter coated with sufficient fish oil to ensure they would sink. The different sub-fractions were again included at a level that corresponded to approximately 10% (w/w, wet basis) soybean molasses. The quantity of oil required to ensure pellets would sink differed between diets. Consequently, the control diet (not containing any molasses), and the diet containing the butanol phase only were relatively high in fish oil content compared to the other diets (Table 2). In summary, all diets contained the same carrier matrix but were coated with different sub-fractions of molasses and quantities of oil. Recipes for all diets are shown in Table 2.

Table 2 Formulation of diets for Fish Trial 2 [a]

		Diet							
		A	B	C	D	E	F	G	H
Fish meal (Scandinavian LT)	g/kg	574	591	570	587	574	581	583	540
Wheat	g/kg	111	114	110	113	111	112	113	104
Wheat Starch	g/kg	29	29	28	29	28	29	29	27
Minerals, vitamins, pigment	g/kg	3	3	3	3	3	3	3	3
Butanol phase (DM)	g/kg	10	0	0	0	0	0	0	0
Precipitate (DM)	g/kg	0	23	0	0	0	0	0	0
Water phase (DM)	g/kg	0	0	31	0	0	0	0	0
Butanol phase & Precipitate (DM)	g/kg	0	0	0	41	0	0	0	0
Butanol phase & Water phase (DM)	g/kg	0	0	0	0	44	0	0	0
Precipitate & Water phase (DM)	g/kg	0	0	0	0	0	57	0	0
Untreated molasses (DM)	g/kg	0	0	0	0	0	0	66	0
Fish Oil (Northern Hemisphere)	g/kg	273	240	258	227	240	218	213	326
Total	g/kg	1000	1000	1000	1000	1000	1000	1000	1000
Molasses equivalents (wet matter basis):		11.0%	10.7%	10.1%	13.1%	10.9%	10.8%	10.6%	-

[a] DM, dry matter. All diets contained the same carrier matrix but were coated with different sub-fractions of molasses and different quantities of fish oil

Fish Trial 1

The trial was conducted at Skretting Fish Trials Station, Lerang, Jørpeland, Norway. Seawater adapted Atlantic salmon (*Salmo salar* L.) with an initial average weight of 213 g, were fed 6 different experimental diets for 62 days. Fish were randomly distributed to 18 circular 400 L fiberglass tanks at a stocking density of 30 fish per tank. The tanks were equipped with waste feed collection and continuously supplied with seawater (15 L min^{-1} per tank). Water was pumped from 90 m depth and held a constant temperature of 8.3°C during the experiment. The 6 different diets were fed to triplicate tanks (3 tanks per treatment) twice a day, aiming at 20% overfeeding, and waste feed was collected. At the end of the feeding period 4 fish from each tank (12 fish in total per treatment) were sacrificed with an overdose of anesthetic (Tricaine Methanesulfonate, Finquel MS-222, Argent Chemical Laboratories, USA), for histological examination.

Fish Trial 2

The trial was conducted at Skretting Fish Trials Station, Lerang, Jørpeland, Norway. Seawater adapted Atlantic salmon (*Salmo salar* L.) with an initial average weight of 202 g, were fed 8 different experimental diets for 44 days. The fish were randomly distributed to 8 circular 100 L fiberglass tanks at a stocking density of 20 fish per tank. The tanks were equipped with waste feed collection and continuously supplied with seawater (4 L min^{-1} per tank). Water was pumped from 90 m depth and held a constant temperature of 9.0°C during the experiment. The 8 different diets were fed to the 8 different tanks (1 tank per treatment) twice a day, aiming at 20% overfeeding, and waste feed was collected. At the end of the feeding period 10 fish from each tank were sacrificed with an overdose of anesthetic (Tricaine Methanesulfonate, Finquel MS-222, Argent Chemical Laboratories, USA), for histological examination.

Histological examination

A two-centimeter section of the distal intestine was carefully removed, rinsed in saline water and fixed in phosphate buffered formaldehyde (4%, pH 7.2). Samples were then dehydrated, embedded in paraffin and cut according to standard histological procedures.

Slides were then stained with a combination of Hematoxylin & Eosin and Alcian blue 8 GX. The latter was included in order to increase the contrast between goblet cells and vacuoles.

Four different morphological parameters were evaluated using light microscopy (Leica DM 5000B) according to the scoring criteria given in Table 3. A score of "1-2" represented normal morphology while a score of "5" was given to morphological symptoms of severe enteritis. The semi-quantitative scoring system was adapted from Urán *et al*. (2004). Histological samples were randomized and evaluated blind.

Table 3 Histological scoring system for morphological changes induced by soybeans in the distal intestine of Atlantic salmon [a].

Score	Appearance
	Supranuclear vacuoles
1	Large vacuoles occupy almost the entire apical part of the enterocytes.
2	Medium sized vacuoles, which occupy less than half of the enterocytes.
3	Small sized vacuoles near the apical membrane in most enterocytes.
4	Scattered small vacuoles are still present in some enterocytes.
5	No supranuclear vacuoles are present.
	Lamina propria of simple folds
1	Very thin and delicate core of connective tissue in all simple folds.
2	Lamina propria appears slightly more distinct and robust in some of the folds.
3	Clear increase of lamina propria in most of the simple folds.
4	Thick lamina propria in many folds.
5	Very thick lamina propria in many folds.
	Connective tissue (between base of folds and stratum compactum)
1	Very thin layer of connective tissue between base of folds and stratum compactum
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds.
4	Thick layer of connective tissue beneath many folds.
5	Extreme thick layer of connective tissue beneath some folds.
	Mucosal folds
1	Simple and complex folds (CF) appear long and thin. Thin side-branches on CF.
2	Simple mucosal folds have medium length. CF are still long but appear thicker.
3	Simple folds have short to medium length. Side-branches on CF are stubby.
4	Thick CF are prevalent. Simple folds are short. Almost no side-branches on CF.
5	Both complex and simple folds appear very stubby.

[a] Adapted from Urán *et al*. 2004.

Statistics

The histological scoring results were treated as non-parametric data. Kruskal-Wallis One-Way ANOVA was therefore applied for testing equality of score medians among treatment groups. A multiple comparisons test with mean ranks (Student-Newman-Keuls, $\alpha = 0.05$) was used as post hoc test to compare all pairs of mean ranks.

Results

Soybean molasses and obtained sub-fractions were subjected to several biochemical analyses. Protein, fat and ash were analyzed according to standard methods. Sucrose, raffinose and stachyose were quantified using HPAEC-PAD. Soyasaponins were quantified using HPLC-DAD. The composition of soybean molasses and the 3 different sub-fractions are shown in Table 4.

Table 4 Composition of molasses and distribution of the different components after phase-separation [a]

	Molasses (g/kg) (Wet matter basis)	Relative distribution between phases		
		Butanol phase (%)	Precipitate (%)	Water phase (%)
Dry matter	620 ± 5	15	35	50
Ash	43 ± 6	5	20	75
Protein (N x 6.25)	52 ± 1	13	51	35
Fat	96 ± 8	68	32	0
Sucrose	219 ±22	4	34	62
Raffinose	23 ± 1	2	30	67
Stachyose	117 ± 8	1	31	67
Soyasaponins [b]	20 ± 1	60	39	1
Unidentified residue [c]	50			

[a] The reported composition of molasses is the average value of 3 measurements ± SD. [b] Composition: 3.1, 0.8 and 0.8 g/kg of soyasaponin Ab, Ac, and Af respectively; 0.2, 3.2 and 1.0 g/kg of soyasaponin Ba, Bb and Bc respectively; 0.6, 7.4 and 2.8 g/kg of saponins Ba-DDMP, Bb-DDMP and Bc-DDMP respectively. [c] Includes isoflavons and soluble non-starch polysaccharides.

The composition of molasses was in good agreement with the supplier data. Sucrose and stachyose were the main oligosaccharides present in molasses. Approximately two-thirds of the oligosaccharides were recovered in the water phase, one-third in the precipitate, while the butanol phase was almost free of oligosaccharides. The soyasaponins were separated in a ratio of approximately 60:40 between the butanol phase and the precipitate, while the water phase contained only trace amounts.

Analysis for crude protein suggested that most of the proteins were recovered in the precipitate. SDS-PAGE was used to evaluate the size distribution of the proteins present in each fraction. The Coomassie stained gel is shown in Figure 2. This analysis revealed that the precipitate in fact contained almost all proteins. Only very weak bands of proteins could be seen in the water phase. Hence, the water phase contained mainly non-protein nitrogen. The butanol phase was free of proteins, with the exception of a small protein of 8-10 kDa.

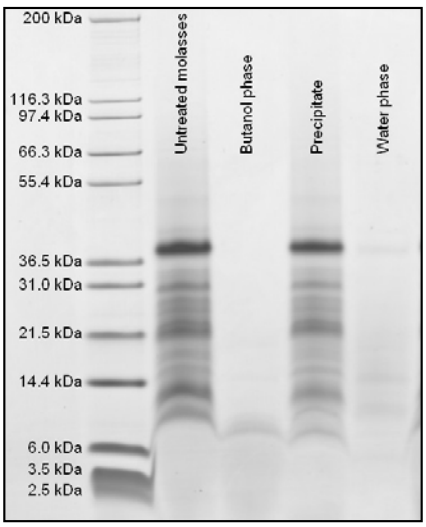

Figure 2 SDS-PAGE of molasses fractions using a 10-20% tricin gradient gel. First lane from left, Mark12^{TM}.

Two separate fish trials were carried out in order to test the effect of the obtained sub-fractions on intestinal morphology. The objective of trial 1 was to investigate whether the butanol treatment, and the subsequent evaporation at 70°C, would inactivate the causative component/s and if the isolated butanol phase could induce enteritis independently. A diet

containing regular defatted soybean meal was included for the purposes of comparison. Results from the histological evaluation are given in Table 5. Fish fed the control diet (without soybeans) displayed normal morphology while significant enteritis was observed in fish fed 20% defatted soybean meal. Diets containing 10% untreated molasses and 10% butanol treated molasses induced severe morphological changes similar to the 20% soybean meal diet. The diet containing a combination of precipitate and water phase had only a weak effect on all 4 morphological parameters. Interestingly, the butanol-phase containing diet had a strong impact on vacuoles, lamina propria and connective tissue but did not provoke the stubby appearance of mucosal folds typically associated with soybean-induced enteritis.

Table 5 Histological evaluation of distal intestine [a]

Fish Trial 1	Diet A: Soybean meal	Diet B: Butanol phase	Diet C: Precipitate & Water phase	Diet D: Butanol treated molasses	Diet E: Untreated molasses	Diet F: Control
Vacuoles	2.92 ± 0.90^{bc}	3.96 ± 1.01^{c}	2.46 ± 1.23^{ab}	3.63 ± 0.61^{bc}	3.08 ± 0.60^{bc}	1.50 ± 0.60^{a}
Lamina Propria	3.96 ± 0.86^{c}	3.04 ± 0.84^{bc}	2.33 ± 1.21^{ab}	3.75 ± 0.78^{c}	3.63 ± 0.57^{c}	1.29 ± 0.69^{a}
Connective tissue	3.71 ± 0.92^{b}	3.46 ± 0.99^{b}	2.17 ± 0.94^{a}	3.92 ± 0.82^{b}	3.96 ± 0.86^{b}	2.00 ± 1.07^{a}
Mucosal Folds	4.13 ± 0.68^{b}	2.33 ± 0.49^{a}	2.42 ± 1.06^{a}	3.88 ± 0.68^{b}	3.71 ± 0.69^{b}	1.42 ± 0.47^{a}

[a] Intestinal cuts were scored according to the criteria listed in Table 3. A score of "1-2" represent normal morphology while a score of "5" represent severe enteritis. Reported data are mean values from 12 fish ± SD. Means followed by different letters are significantly different (Multiple comparisons test with mean ranks, Student-Newman-Keuls, $\alpha = 0.05$).

Trial 2 was initiated to investigate why neither of the two sub-fractions tested in trial 1 had a strong effect on mucosal folds. Results from the histological evaluation are shown in Table 6. Fish fed the water phase displayed normal morphology while fish fed the combination of butanol-phase and precipitate showed the same morphological changes as fish fed soybean molasses. Results from trial 2 were very consistent with those from trial 1, demonstrating clearly that the trigger component was split between the butanol phase and the precipitate whereas this component was absent from the water phase. Soyasaponins were the only quantified components that were separated poorly between the butanol phase and

precipitate (Table 4). Fish fed the butanol phase alone showed the same morphological changes as observed in trial 1; vacuoles, lamina propria and connective tissue were significantly affected but only a weak impact was observed on mucosal folds. Interestingly, the combination of water and butanol phases had a significantly greater impact on mucosal folds than the butanol phase alone.

Table 6 Histological evaluation of distal intestine[a]

Fish Trial 2	Diet A: Butanol phase	Diet B: Precipitate	Diet C: Water phase	Diet D: Butanol phase & Precipitate	Diet E: Butanol & Water phase	Diet F: Precipitate & Water phase	Diet G: Untreated molasses	Diet H: Control
Vacuoles	4.20 ±0.86 ^{cd}	2.75 ±1.21 ^{abc}	1.70 ±0.59 ^{a}	4.40 ±0.94 ^{d}	3.60 ±0.70 ^{bcd}	2.05 ±1.04 ^{ab}	4.35 ±0.58 ^{d}	2.05 ±0.50 ^{ab}
Lamina Propria	3.15 ±0.47 ^{b}	3.00 ±0.82 ^{b}	1.90 ±0.74 ^{ab}	3.15 ±0.75 ^{b}	2.85 ±0.75 ^{b}	1.80 ±0.82 ^{ab}	3.10 ±1.13 ^{b}	1.00 ±0.00 ^{a}
Connective tissue	2.55 ±0.55 ^{ab}	2.30 ±0.75 ^{ab}	1.80 ±0.67 ^{ab}	2.95 ±0.93 ^{b}	2.50 ±0.58 ^{ab}	1.75 ±0.75 ^{a}	3.05 ±1.19 ^{b}	1.65 ±0.47 ^{a}
Mucosal Folds	2.25 ±0.54 ^{abc}	2.00 ±0.53 ^{ab}	1.50 ±0.33 ^{a}	3.50 ±0.91 ^{cd}	3.15 ±0.53 ^{bcd}	2.35 ±1.00 ^{abc}	3.75 ±0.75 ^{d}	1.25 ±0.26 ^{a}

[a] Intestinal cuts were scored according to the criteria listed in Table 3. A score of "1-2" represent normal morphology while a score of "5" represent severe enteritis. Reported data are mean values from 10 fish ± SD. Means followed by different letters are significantly different (Multiple comparisons test with mean ranks, Student-Newman-Keuls, $\alpha = 0.05$).

Discussion

SB molasses has previously been shown to contain components that cause SBM-induced enteritis in the distal intestine of Atlantic salmon (van den Ingh *et al*. 1996, Krogdahl *et al*. 2000). In the present study soybean molasses were separated into three sub-fractions by phase separation and both molasses and the obtained sub-fractions were subjected to extensive biochemical analyses. All possible combinations of the three sub-fractions were fed to Atlantic salmon and the impact on intestinal morphology was evaluated. Results revealed that fish fed the water phase displayed normal morphology while fish fed a combination of butanol-phase and precipitate showed the same morphological changes as fish fed soybean

molasses. Soyasaponins were the only quantified components that were separated poorly between the butanol phase and precipitate. It can thus be concluded that soyasaponins, or components that follow the same solubility pattern, need to be present to induce the inflammatory reaction. Bureau et al. (1998) demonstrated that *Quillaja* saponins cause extensive damage to the intestinal mucosa of the hindgut in Chinook salmon and Rainbow trout. This supports the hypothesis that soyasaponins play a key role in soybean-induced enteritis in Atlantic salmon.

Biochemical analyses of molasses revealed that the main oligosaccharides present were sucrose and stachyose, which is in accordance with Berg (1992). The separation of soyasaponins was surprisingly poor and only 60% of the total amount was found in the butanol phase, even though n-Butanol is a suitable solvent for extraction of soyasaponins (Kitagawa *et al*. 1985). The remaining 40% was found in the precipitate while the water phase contained only trace amounts of soyasaponins. It is known that soyasaponins follow the protein fraction during production of SB protein isolates (Ireland *et al*. 1986). The high amount of retained saponins in the precipitate might therefore be due to protein-saponin interactions.

The histological evaluation in fish trial 1 confirmed that soybean molasses contains the causative factors for soybean-induced enteritis. Moreover, the components proved to be extremely stable since they were able to withstand both butanol treatment and evaporation to dryness at 70°C. The butanol phase contained components that effectively disrupted vacuolization, probably by interfering with endocytosis. The butanol phase also caused an intermediate increase of lamina propria and connective tissue. The mean score for mucosal folds, however, was only slightly affected. The fact that the butanol phase affected some, but not all, of the evaluated parameters could indicate that the causative factor was poorly separated between the two sub-fractions (1: butanol phase and 2: precipitate & water phase). Fish trial 2 revealed that the causative component was split between butanol phase and precipitate while the water phase was free from this component. The diet that included both precipitate and water phase had only a weak effect on all four histological parameters in both trials. The precipitate and water phase in combination contained more than 95% of all sucrose, raffinose and stachyose in molasses. Hence, it can be concluded that the oligosaccharides alone do not trigger the inflammatory reaction. The same argument holds for

soybean proteins larger than approximately 10 kDa since the precipitate and water phase together contained all larger proteins in molasses. It can therefore be concluded that the major antigenic soybean proteins (including glycinin, β-conglycinin, and lectins) do not induce intestinal inflammation alone.

Gypsophila saponins have been shown to increase the transmucosal uptake of the milk allergen β-lactoglobulin in the small intestine of rats *in vivo* (Gee *et al*. 1997), and several *in vitro* studies with saponins have demonstrated increased trans-epithelial uptake of macromolecules (Alvarez & Torres-Pinedo, 1982; Onning *et al*. 1996; Chao *et al*. 1998; Sim *et al*. 2005). In contrast to earlier findings in endothermic animals, soyasaponins were recently found to resist degradation during gut passage in Atlantic salmon (Knudsen *et al*. 2006). The observed inflammatory reaction might therefore be a secondary effect of increased intestinal permeability facilitated by soyasaponins. Increased intestinal permeability could expose the underlying mucosa to antigenic soybean proteins or perhaps to intestinal microflora. The gut microflora is known to be involved in inflammatory bowel diseases in humans (Guarner 2006), and translocation of bacterial cells and bacterial antigens across the mucosal barrier has also been reported in fish (Olafsen & Hansen, 1992; Ringø *et al*. 2001, 2003). In general, translocation of bacteria is favored by bacterial overgrowth, reduced immunity of the host, or increased permeability of the gut lining (Ringø *et al*. 2003). A recent study by Ringø *et al*. (2006) has demonstrated that non-digestible carbohydrates also affect fish gut microflora. A shift in the microbial population, caused by high levels of non-digestible carbohydrates in the feed, might therefore explain why the water phase, which was high in carbohydrates but very low in both proteins and soyasaponins, seemed to increase the impact of the butanol phase on the mucosal folds.

In summary, the current work demonstrates that the causative components for soybean-induced enteritis resist butanol treatment and prolonged heating at 70°C. Sucrose, raffinose, stachyose, nor soybean proteins larger than 10 kDa induce enteritis alone. Soyasaponins, or components that follow the same solubility pattern, trigger the inflammatory reaction. We therefore suggest that soybean-induced enteritis in Atlantic salmon is induced by soyasaponins alone, or by soyasaponins in combination with antigenic soybean proteins or the intestinal gut microflora.

The present work examined the effect of crude sub-fractions of soybean molasses on intestinal morphology in Atlantic salmon. Biochemical analyses of the sub-fractions made it possible to rule out several of the components that could be suspected for causing soybean-induced enteritis. However, in order to demonstrate which soybean components that are causing enteritis in Atlantic salmon, feeding trials with purified components are required. Further studies to investigate the effect of isolated and well-characterized soyasaponins on intestinal morphology in Atlantic salmon are presently being done.

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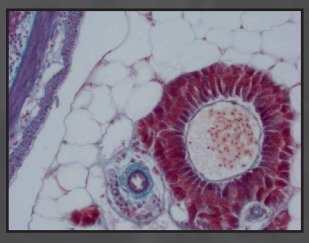
GENERAL DISCUSSION

Chapter 8



The general aim of this study was to gather more information on the mechanisms behind soybean meal (SBM)-induced enteritis in fish. In order to do so, the following steps were necessary:

- First, a reliable scoring system to compare the kinetics of the disorder needed to be developed (Chapter 2).

- Second, a more thorough understanding of the influence of SBM on the kinetics of the disorder with special emphasis on the early development needed to be acquired (Chapter 3).

- Third, the impact of dietary and non-dietary factors on the development of enteritis were studied. Examples of the studied factors are inclusion levels and commercial sources of SBM, the potential causative components in SBM, water temperature and salinity (Chapters 2, 3, 6 and 7).

- Fourth, a comparison of the etiology of enteritis in a carnivorous salmonid (Atlantic salmon) and an omnivorous non-salmonid species (common carp) was made with special emphasis on the changes in enterocytes, such as endocytotic activity, as well as the presence of supranuclear vacuoles and the decrease of microvilli (Chapters 4 and 5).

Scoring Enteritis

At the start of this research project, most studies assessed the degree of SB-induced enteritis in qualitative terms by describing histological alterations of the second gut segment (e.g. Refstie *et al*. 2000, 2001; Sanden *et al*. 2005; Bakke-Mckellep *et al*. 2007a). However, such an approach does not facilitate a comparison between studies with regard to the impact of environmental factors or to the impact of different SB products and components.

Therefore, in the present study we decided to develop a more quantitative method to measure the degree of enteritis. Two approaches were tested:

1. A "semi- quantitative" method (Urán *et al*. 2004) that quantifies the degree of enteritis by applying a scoring system using five classes for six separated enteritis parameters. This method is fully described in Chapter 2 (Urán *et al*. 2008).
2. A "quantitative method" that scores two enteritis parameters (Urán *et al*. 2005). By applying a morphometrical analysis of light photomicrographs, two aspects were determined: 1) the ratio between width of the mucosal fold and the width lamina propria (MF/LP), and 2) the number of goblet cells per 100µm of mucosal fold.

Both methods were applied to the experiment described in Chapter 3. In Figure 1, the relationship between comparable parameters of both methods is outlined. The lamina propria (LP) score, given by the semi-quantitative method, coincided (r= 0.80; $P<0.05$; n=39) with the measured MF/LP ratio. Similarly, the semi-quantitative score of goblet cells (GC) showed a good correlation to the counted number of GC/100µm (r=0.78; $P<0.05$; n=39).

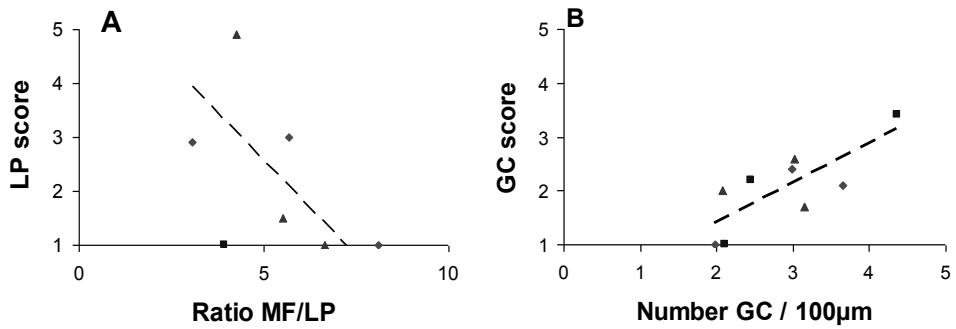


Figure 1 The relationship between the semi-quantitative and the quantitative analysis of: **A)** the ratio between the mucosal fold (MF) the width of the lamina propria (LP) measurement and **B)** the number of goblet cells (GC). The triangles, squares, and diamonds represent mean values per treatment on days 7, 20 and 57, respectively. (Adapted from Urán *et al*. 2005).

Despite these positive results, the quantitative method yielded a large individual/sample variation. As a result, a large number of observations per sample are required, making these measurements more labour intensive. Therefore, it was ultimately decided to further develop and use the semi-quantitative method.

The accuracy of the semi-quantitative scoring method was tested by comparing the scoring skills of two different observers, analyzing the same samples of the experiment described in Chapter 3. This method demonstrated a strong correlation between observers. In Figure 2, the relationship between the mean scores of two separate observers is shown (r=0.87; $P< .0001$; n=77.

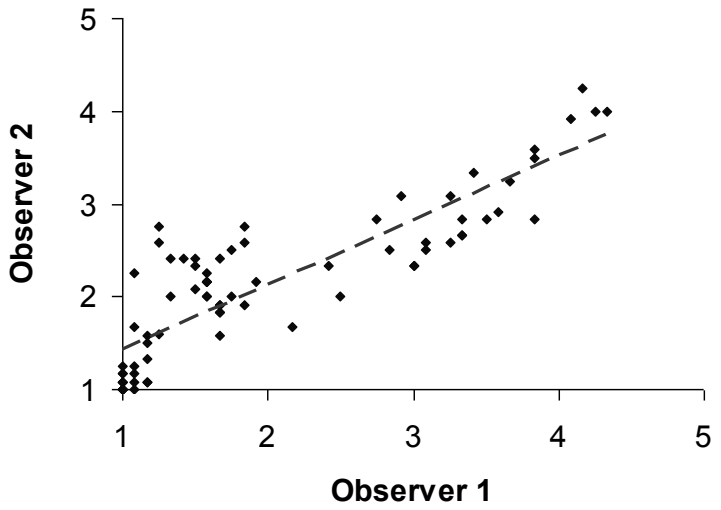


Figure 2 The relationship between the semi-quantitative results from two different observers. The evaluation of the histological slides was a blind test. The values represent the overall mean of six separated parameters per fish.

This semi-quantitative method is an advancement on the more simplified semi-quantitative method, presented in Morris *et al.* (2005), in which all the parameters were grouped in a unique category. When their approach is used, the probability of identifying slight changes in the enteritis response is reduced due to the low resolution of their method.

Our semi-quantitative method offers the possibility of acquiring detailed information at different cellular and structural levels. In doing so, the etiology of the disorder, as well as the different dietary and non-dietary factors involved in its development, can be more thoroughly illustrated and examined.

In conclusion, our method proved to be reliable, accurate and more precise than the hitherto known, comparable scoring methods.

The impact of different factors on the development and severity of enteritis

The present study had the objective of examining whether, and if so how, dietary and non-dietary factors influence the inflammatory process which leads to enteritis through SBM feeding.

With regard to the dietary factors, the occurrence of enteritis is clearly related to feeding diets containing SBM. Several studies have attempted to replace SBM by other soy products particularly for salmonid diets. However, most SB products contain high levels of anti-nutritional factors, and this limits their use in aquafeeds (Alexis & Nengas 2001; Francis *et al*. 2001).

Several aspects involved in the production process of different SB products may affect the quality and the composition of the final SB product and may be responsible for variations in the response when fed to fish. Chapter 3 illustrates an experiment in which different inclusion levels of dehulled solvent-extracted SBM (10 and 20% of the protein fraction) were tested in time. The SBM used was a commercial source known to induce a strong inflammatory reaction. The lower inclusion level resulted in a mild response in contrast to the response at the higher level which continued over time, displaying no signs of recovery. The severity of enteritis was clearly dose-dependent. The diet containing the highest inclusion level was also applied to the omnivorous common carp (Chapter 4).

Upon switching to SBM diets, common carp, developed similar symptoms of enteritis as the Atlantic salmon. In contrast to the Atlantic salmon, common carp appears to recover from this disorder after a few weeks of SBM feeding. This recovery starts with the re-appearance of the supranuclear vacuoles (SNV) in the absorptive cells. Therefore, the

condition of SNV might be important in revealing the mechanisms behind SBM-induced enteritis.

Based on the fact that enteritis appears to be dose-dependent, SBM from different soy processing plants were tested in Chapter 6. This study showed clear dissimilar enteritis responses between the SBM from different commercial sources. Once again, the most affected parameter was the disappearing SNV. In contrast, the goblet cells (GC) were only slightly affected by the dietary differences. Anyway, it is suggested that most parameters indicating enteritis react proportionally to the inclusion level of the SB product rather than to a threshold value above which enteritis is developed and below not (yes/no response).

To date, it is not clear which component in soy is the causative agent for inducing enteritis. The alcohol-soluble components (namely SB molasses) which result from the extraction of SB protein concentrates are often considered as being the inducers of this inflammatory process (Olli & Krogdahl 1995; van den Ingh *et al*. 1996; Krogdahl *et al*. 2000; Knudsen *et al*. 2007). Chapter 7 demonstrates that the inflammation is induced by molecules smaller than 10 kDa. Based on the morphological differences induced by the different fractions used in the study we argue that glycin, β-conglycinin and lectins, proteins which are commonly presumed to be the main causative factors of enteritis, should be ruled out. Nonetheless, when combined with soyasaponins, and perhaps with the intestinal microbiota, they may have an influence. Saponins have been used as potent adjuvants due to their ability to increase cell membrane permeability (Ronnberg *et al*. 1995; Oda *et al*. 2000, 2003). They are believed to enhance small molecules and/or macromolecules to cross the cell membrane (Alvarez & Torres-Pinedo 1982, Mick *et al*. 1988). However, the ferritin experiment (Chapter 5) did not show such an increased permeability when SBM was fed. As such, the concentration and composition of soyasaponins in the SB and in the final SB product may depend on the variety and processing conditions, as is suggested by Hu *et al*. (2002). However, more research is needed to verify this hypothesis.

On the other hand, it is also possible that non-dietary factors are involved in the etiology of the disorder. Husbandry conditions, such as temperature, may influence the severity of enteritis by acting directly on feed intake or by affecting the metabolic rate at the enterocyte level. In the present study (Chapter 2), water temperature influenced the speed at which enteritis developed in Atlantic salmon. At 12 °C, enteritis developed faster and more

acute than at 8 °C. This was in contrast to the general experience in the industry (W. Koppe, personal communication). At 8 °C a number of factors were observed to be less affected: the disappearance of the supranuclear vacuoles (SNV), the shortening of the mucosal folds (MF); the widening of the lamina propria (LP) and of the sub-epithelial mucosa (SM). The different responses between the two temperatures may be explained by a difference in the metabolic rate. Temperature could have influenced the rate at which food was ingested, or transported in the intestinal tract, and/or absorbed by the gut. At higher temperatures, all these processes proceed at a higher rate, increasing the contact between causative agent and animal per unit of time.

Another non-dietary factor which was briefly addressed in this thesis was the possible effect of salinity and age on the development of SBM-induced enteritis. In Chapter 5, young fish, kept in freshwater, were compared with older fish (from a different experiment, Chapter 3) kept in seawater. Our results indicate that enteritis is not dependent on age or smoltification. Usher *et al*. (1990) proposed an osmoregulatory role of the gut in seawater-adapted smolts, affecting its digestive role by altering the physico-chemical conditions, the activity of digestive enzymes, and the active transport of ions in the gut lumen. It was expected that the changes mentioned by Usher *et al*. (1990) could have increased the severity of SBM-induced enteritis of the Atlantic salmon exposed to seawater. Apparently, feeding of SBM did not impair their osmoregulatory ability. This concurs with the previous results of Bakke-McKellep *et al*. (2006) who proved that the osmoregulatory capacity of fish displaying the typical signs of SBM-induced enteritis was maintained. These findings are in accordance with the results of the present study. However, more detailed research is required to exclude the influence of salinity on the enteritis process.

In conclusion, this thesis shows that dietary factors (inclusion level and different soy sources) and temperature are probably more important than age and salinity in the severity of enteritis in Atlantic salmon. Consequently, more attention must to be paid to the responsible SB factors. The differences in the described plant sources may be useful to take into consideration. A further factor derived from the present study is that in addition to Atlantic salmon, another species (common carp), can also temporarily develop enteritis. Nonetheless, for reasons presently unknown, common carp seems to recover from enteritis or to adapt to the SBM.

Mechanisms behind the development of SBM-induced enteritis

As discussed earlier, limited information is known about the SBM components that cause enteritis and the mechanisms behind the disorder. In our study, we describe how endocytosis in the second gut segment is blocked and that the consequent fast decrease in SNV number and size seems to be the first symptom observed. This phenomenon occurs in two completely different species (carp and salmon) studied in this thesis (Chapters 4 and 5 respectively). This observation suggests that the mechanism has to do with blocking a receptor mediated uptake. Unfortunately, at present we cannot relate this to one of the SB factors that have so far been studied. The disturbance of such an essential basic mechanism as endocytosis must have a great impact on the functioning of enterocytes, rapidly resulting in the disappearance of SNV, and finally, also in the decrease of the height of the microvilli. It could be that these disturbances of the enterocytes result in the release of danger signals, such as heat shock proteins (HSP), which may be responsible for the recruitment of leucocytes and, as a culminating point, in the increasing inflammation of the connective tissue. The role of HSP70 in SBM-induced enteritis has been evidenced already in Atlantic salmon by Bakke-McKellep *et al*. (2007b). In addition, the up- or down-regulation of inflammatory and/or anti-inflammatory cytokines produced by intestinal cells may also play a crucial role. In any case, migratory reactions of intra-epithelial T cells and the up-regulation of T cell specific responses have been described during the onset of enteritis in Atlantic salmon (Bakke-McKellep *et al*. 2007c). Furthermore, there was a strong invasion of the eosinophilic granulocytes (EG) from the sub-epithelial mucosa inwards to the lamina propria. This observation and the change of the morphology of the EG granules indicates an activation of these cells. EG in fish are known to produce lysozyme (Sveinbjørnsson *et al*. 1996; Silphaduang & Noga 2001; Reite & Evensen 2006; Murray *et al*. 2007) which may have a protective role in the inflamed intestinal tissue. In the studies conducted by Refstie *et al.* (2006) and Bakke-McKellep *et al* (2007b), it was proposed that SBM inclusion in diets of Atlantic salmon may influence the intestinal microbiota. An increase in the number and diversity of the microbial community has been described. The observed reaction of EG may be an important defence against it. Nonetheless, this is not a sufficient explanatory factor, as shown by Krogdahl *et al*. (2000), who proposed a higher susceptibility to bacterial diseases

during SBM induced enteritis. Although no direct evidence is presented in this thesis, some authors suggest that an increase in intestinal permeability facilitates the entry of microbes or their products into the mucosal compartment (Braat *et al*. 2006). In line with such a hypothesis, the resemblance of SBM-induced enteritis and inflammatory bowel disease (IBD, such as Coeliac disease) has been suggested (Baeverfjord & Krogdahl 1996). As discussed earlier, soyasaponins could facilitate intestinal permeability, exposing the underlying mucosa to a variety of antigens and microbiota. This may be the reason as to why the Atlantic salmon gut becomes seriously harmed by the SBM. However, it does not explain why common carp seems to react similarly upon the SBM noxious substances, but appears to recover from enteritis from week 4 onwards. It is worthwhile to investigate which mechanisms are involved in the recovery/adaptation of common carp. This knowledge may increase the understanding of SBM-induced enteritis also in Atlantic salmon. Moreover, the results found in common carp also emphasize that aquaculture must be aware of the possibility of a temporary enteritis response in many other fish species. This temporary response may have been overlooked up until now, due to the (too) late sampling after switching to the SBM diets. Such a temporary enteritis response may result in a higher susceptibility for diseases when fish switch to a SBM-containing diet.

Final conclusions

The work presented in this thesis contributes to the understanding of the etiology of the enteritis disorder. Knowing that etiology deals with the cause or origin of a disorder and the factors which influence its development, it can be said that SBM-induced enteritis is related more to dietary factors than non-dietary factors (i.e. temperature). Nevertheless, non-dietary factors do have an impact on the speed at which the disorder is developed. The inclusion level of SBM into the diet formulation has a great impact on the severity at which all the typical symptoms of the disorder are expressed, affecting the endocytosis process and initiating a cascade of events in the immune system. The actual causative components present in SB are still matters for future research, especially in terms of clarifying their mechanisms of action. It is suggested in this thesis that the decreased nutrient uptake capacity of the

enterocytes is directly related to the disappearance of the SNV as seen in Atlantic salmon. The reasons why the Atlantic salmon is not capable of recovering from the enteritis, while common carp does, remain unknown.

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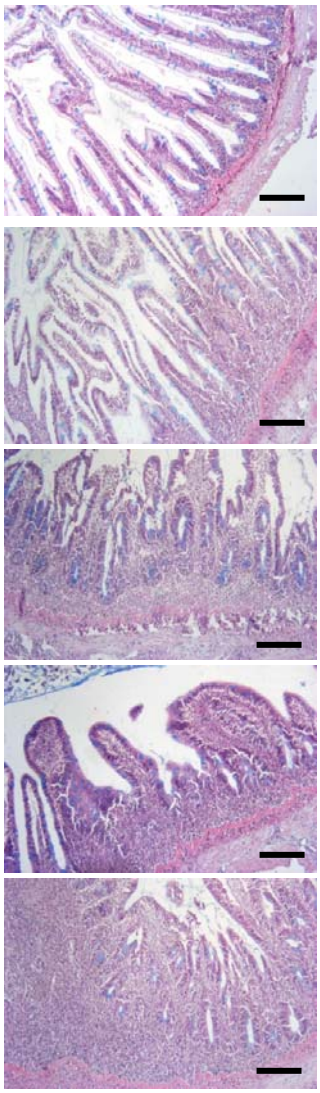
SEMI-QUANTITATIVE SCORING SYSTEM

Also available at:
http://www.afi.wur.nl/UK/Publications/

Mucosal folds (MF)



1
Basal length

2
Some shrinkage and bloating

3
Diffused shrinkage and onset of tissue disruption

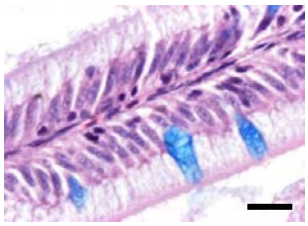
4
Diffused tissue disruption

5
Total tissue disruption

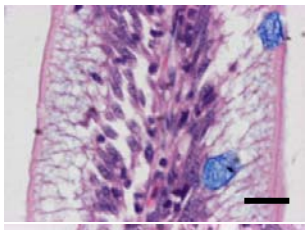
Bar is 200 µm

Supranuclear vacuoles (SNV)

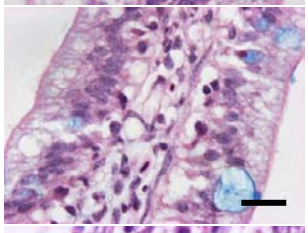
1
Basal SNV size



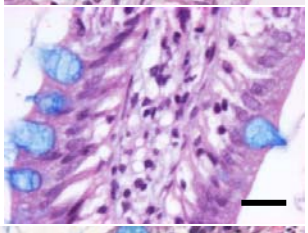
2
Some size reduction



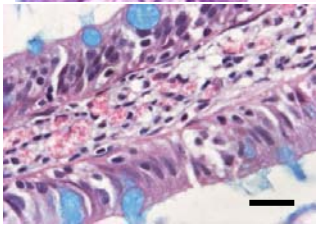
3
Diffused size reduction



4
Onset of extinction

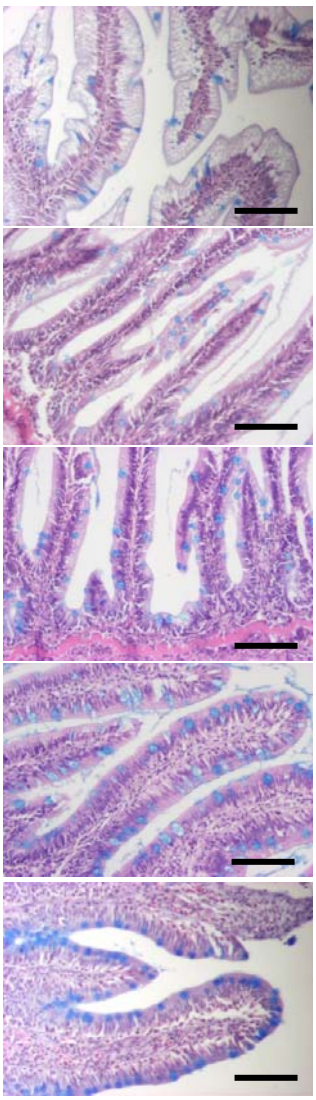


5
No SNV



Bar is 20 µm

Goblet cells (GC)



1

Scattered cells

2

Increased number and sparsely distributed

3

Diffused number widely spread

4

Densely grouped cells

5

Highly abundant and tightly-packed cells

Bar is 100 µm

Eosinophilic granulocytes (EG)

1

Few in sub-epithelial mucosa (SM) basal quantity

2

Increased number in SM and some migration into lamina propria (LP)

3

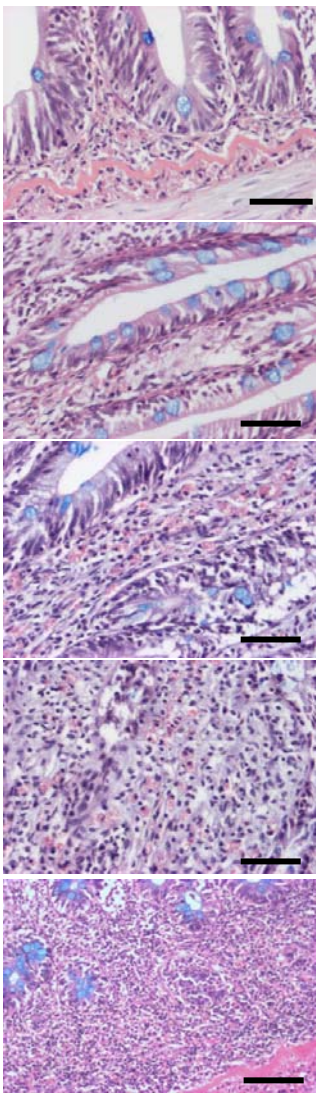
Increased migration into LP

4

Diffused number in LP and SM

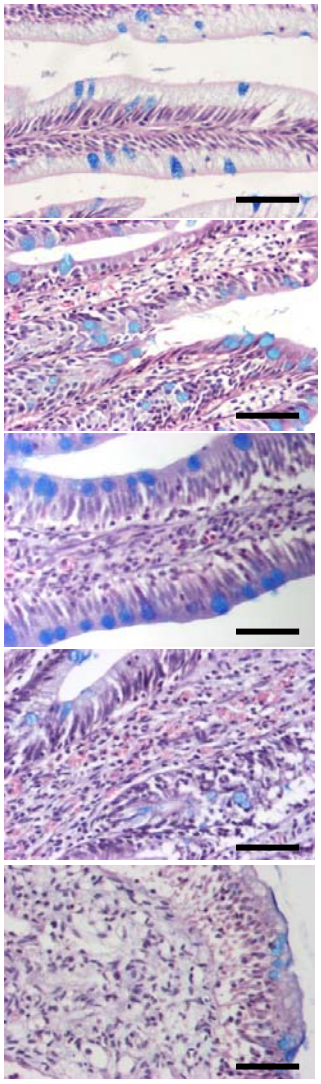
5

Dense EG in LP and SM



Bar is 50 µm

Lamina propria (LP)



1
Normal size

2
Increased size

3
Medium size

4
Large LP

5
Largest LP

Bar is 50 µm

Sub-epithelial mucosa (SM)

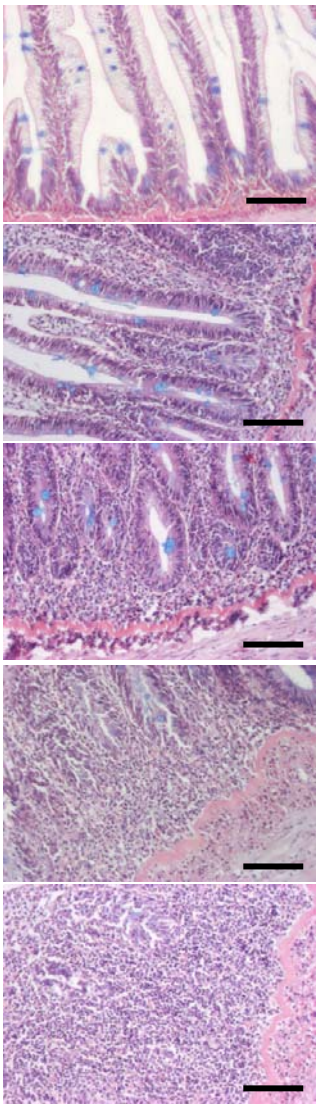
1
Normal SM

2
Increased size SM

3
Medium size SM

4
Large SM

5
Largest SM



Bar is 100 µm

SUMMARY

Presently, most efforts to substitute fishmeal by soybean meal (SBM) in aquafeeds are being confronted by numerous problems and constraints. The inclusion of SBM, especially in the diet of salmonids, induces an inflammatory response of the distal intestinal mucosa, known as enteritis. The general aim of this study was to understand the etiology and the underlying mechanisms of this disorder. To do so, numerous factors (both dietary and non-dietary) were evaluated and the possible mechanisms behind the inflammatory process were studied in both Atlantic salmon (*Salmo salar* L.) and common carp (*Cyprinus carpio* L).

In **Chapter 2**, water temperature was addressed as an important husbandry condition which could influence the speed at which the disorder develops. The study demonstrated that the onset of enteritis is delayed at a lower temperature (8 °C vs. 12 °C) A semi-quantitative scoring system was developed to assess the extent of the morphological changes observed in this study. This tool was further used in all the subsequent studies of this thesis.

Chapter 3 describes the influence of SBM on the kinetics of the disorder. This was studied at two different inclusion levels over a period of 57 days. At the higher dose (20% SBM), the signs of enteritis seemed to increase steadily whereas at the lower dose (10% SBM) the disorder displayed a tendency to level off, a process starting from two weeks onwards. It was concluded that the severity of enteritis and its kinetics are dose-dependent, showing no signs of recovery during feeding SBM-containing diets. Electron microscopy studies suggested a blocking of the endocytosis process and a strong decrease of microvilli length. Endocytosis is an important, basic and generic process in animal physiology. As such, if SBM or any compound in it can be shown to induce enteritis (i.e. through the possible blocking of endocytosis), it would appear very remarkable that only salmonids develop this disorder. Therefore, a comparative study was done in another fish species with different (more herbivorous) feeding habits (i.e. common carp).

In **Chapter 4,** SBM-induced enteritis in the omnivorous common carp was studied and compared to the SBM-induced intestinal inflammation in the carnivorous Atlantic salmon. For the first time ever reported, the results suggested that the symptoms of enteritis also occur in common carp. Contrary to the observations in studies with Atlantic salmon, the common carp started to recover from week four onwards. This recovery was explicitly noticeable by the re-appearance of the supranuclear vacuoles (SNV). The morphological changes and the modulation of the inflammatory process were defined and correlated to the

up- and down-regulation of several cytokines that were presumed to influence this process. Again in this study, electron microscopy suggested that endocytosis blocking is directly linked to the disappearance of the SNV and the onset of enteritis symptoms.

The potential role of endocytosis in the development of the disorder was further investigated in **Chapter 5**. Ferritin was used to prove the eventual blocking of endocytosis. After one week of SBM-feeding, ferritin uptake had diminished and the SNV had decreased in size. At week 3, ferritin uptake could not be detected and the SNV were not present in the enterocytes. This indicates that endocytosis block is indeed the first step in the disappearance of SNV. In addition, the results of this study on young Atlantic salmon (30 g) kept in freshwater, facilitated a comparison with older salmon (300 g) kept in seawater, regarding the influence of salinity on the severity of enteritis. It was suggested that SBM-induced enteritis was not strongly influenced by either salinity or age, but further studies are needed to support this observation.

In **Chapter 6**, SBM obtained from different commercial sources and production plants world-wide were compared. The goal was to determine whether the extent of enteritis in Atlantic salmon depends on the origin and/or the processing of the soybeans. The most affected parameter was again the disappearance of SNV in enterocytes. In contrast, the increase in goblet cells showed the smallest variation between the different SBM sources. This study showed that different commercial sources of SBM can result in different severities of SBM-induced enteritis.

Literature suggests that soyasaponins, possibly in combination with other factors, are responsible for the initiation of the enteritis process. Therefore, **Chapter 7** studies if different sub-fractions, obtained through phase-separation of molasses with n-Butanol, had an effect on the development of enteritis. The results indicate that enteritis was only present in fish fed with the butonol phase and the precipitate sub-fractions. This suggests that soyasaponines, possibly in combination with other factors (e.g. intestinal microflora) do indeed influence the onset and development of the enteritis process.

The results of this thesis are discussed in **Chapter 8**. It is concluded that the etiology and further development of SBM-induced enteritis is related to the dietary factors rather than to the non-dietary factors. SBM inclusion levels and the commercial source used for the diet formulation have a great impact on the severity of the disorder, mainly affecting the

endocytosis process. This thesis indicates that the endocytosis block and subsequent dissapearence of the SNV seem to be crucial for the onset of the inflammation process. Nonetheless, the actual causative components present in SB are still issues that require further research. In addition to Atlantic salmon, another species like common carp can also temporarily develop enteritis but after 4 weeks of SBM feeding, they start to recover. The reasons for this recovery remain unknown.

SAMENVATTING

Bij het vervangen van vismeel in visvoer door sojaschroot (SBM) stuit men op verscheidene problemen en beperkingen. Toevoeging van SBM in zalmvoer, veroorzaakt een ontsteking (enteritis) in het distale deel van de darm. Het algemene doel van dit onderzoek was meer inzicht te verkrijgen over de ontwikkeling, de oorzaken en de onderliggende mechanismen betrokken bij deze ontsteking van de darm. Hiervoor zijn verschillende factoren die deels verband houden met de voeding, deels met andere factoren, onderzocht in zowel Atlantische zalm (*Salmo salar* L.) als gewone karper (*Cyprinus carpio* L.).

De watertemperatuur, een belangrijke houderijfactor, werd bestudeerd in **hoofdstuk 2** vanwege de mogelijke invloed op de snelheid waarmee de enteritis zich ontwikkelt. Uit het onderzoek bleek een lage (8 ºC) watertemperatuur de ontwikkeling van de enteritis te vertragen in vergelijking tot een hoge temperatuur (12 ºC). Om dit onderzoek te kunnen uitvoeren werd een semi-kwantitatief scoringssysteem ontwikkeld, waarmee het mogelijk was de mate van de morfologische veranderingen in de darm vast te stellen. Deze scoringsmethode is ook toegepast op alle verdere onderzoeken in dit proefschrift.

In **hoofdstuk 3** is de invloed van SBM op het verloop van de ontsteking in de tijd (e.g. de kinetiek) beschreven. Dit werd onderzocht door in het voer van de zalm verschillende percentages SBM (10% versus 20%) in te brengen en de eventuele ontwikkeling van enteritis te meten gedurende een periode van 57 dagen. Bij het dieet met 20% SBM bleef de ernst van de enteritis toenemen in de tijd, terwijl bij het dieet met 10% SBM de enteritis na 2 weken stabiliseerde. Er kan geconcludeerd worden dat de mate van enteritis dosis-afhankelijk is en dat er geen herstel optreedt zolang er SBM gevoerd wordt. Elektronen microscopisch onderzoek toonde aan dat er geen endocytose plaats vond en dat de lengte van de microvilli sterk afnam. Endocytose is een belangrijk basaal en generiek proces in de dierlijke fysiologie. Dit leidde tot de hypothese dat de bestudeerde enteritis waarschijnlijk niet alleen in zalm plaats vindt.

Daarom werd in **hoofdstuk 4** onderzocht wat de invloed is van het voeren van SBM houdende diëten aan de karper, een omnivore vis. De resultaten zijn vergeleken met die van de (carnivore) Atlantische zalm. Ook in de karper ontstaan symptomen van enteritis waarneer SBM houdende diëten gevoerd worden. Echter, in tegenstelling tot de Atlantische zalm, trad in de enterocyten van de karper na 4 weken herstel op, waarbij het weer verschijnen van de supranucleaire vacuoles (SNV) het meest opvallend was. De morfologische veranderingen en

de mate van ontsteking werden gedefiniëerd en gekoppeld aan de up- and down-regulatie van verschillende cytokines die verondersteld worden het ontstekingsproces te reguleren. Elektronen microscopisch onderzoek toonde aan dat ook hier eerst de endocytose geblokkeerd werd, gevolgd door het verdwijnen van de SNV, alvorens het herstel optrad.

De mogelijke rol van de endocytose bij de ontwikkeling van de ontsteking is verder onderzocht in **hoofdstuk 5**. Om aan te tonen dat er werkelijk geen endocytose meer optrad, werd ferritine toegediend, op verschillende tijdstippen na de overschakeling op een dieet dat SBM bevatte. Na 1 week was de opname van ferritine sterk verminderd en waren de SNV kleiner geworden. Na 3 weken werd er geen opname van ferritine meer waargenomen en waren de SNV verdwenen. Deze resultaten tonen aan dat de endocytose-blokkade een rol speelt bij het verdwijnen van de SNV. Een vergelijking van de resultaten verkregen met jonge Atlantische zalm (30 g) gehouden in zoet water en deze verkregen bij oudere dieren (300 g) gehouden in zout water, suggereerde dat de saliniteit van het water en de leeftijd van de zalm nauwelijks van invloed waren op de ontwikkeling van enteritis. Verder onderzoek is echter nodig om deze waarneming te ondersteunen.

In **hoofdstuk 6** werden verschillende partijen SBM, die afkomstig waren van verschillende commerciële sojaverwerkingsbedrijven, met elkaar vergeleken om te onderzoeken of het ontstaan van enteritis bij de Atlantische zalm afhankelijk is van herkomst van het SBM en/of het productieproces. De meest beïnvloedde parameter was opnieuw de verdwijning van de SNV. Uit dit onderzoek is gebleken dat de herkomst van het SBM van belang is voor de ernst/mate van enteritis in de Atlantische zalm.

In de literatuur wordt verondersteld dat sojasaponines alleen of in combinatie met andere factoren verantwoordelijk zijn voor de initiatie van het enteritis proces. Daarom werd in hoofdstuk 7 onderzocht of verschillende subfracties die verkregen werden bij fasescheiding van sojamelasse met n-Butanol, een effect hadden op de ontwikkeling van enteritis. De resultaten tonen aan dat enteritis alleen voorkwam in vis die gevoerd werd met de subfractie uit de butanol-fase en het precipitaat. Daarmee wordt gesuggereerd dat sojasaponines, mogelijk in combinatie met andere factoren zoals darmflora of anderszins, inderdaad van invloed zijn op het ontstaan en de ontwikkeling van het enteritis proces.

In **hoofdstuk 8** worden de resultaten van dit proefschrift bediscussiëerd. De SBM-geïnduceerde enteritis is duidelijk dieet afhankelijk. De herkomst van het SBM en het

percentage SBM in het voer zijn erg belangrijk bij het ontstaan en het verloop van de enteritis. Met name de blokkade van het endocytose proces en de daarop volgende verdwijning van de SNV lijken cruciaal in het op gang komen van het ontstekingproces. Echter verder onderzoek naar de bij dit proces betrokken componenten is noodzakelijk. In vergelijking met de Atlantische zalm kunnen ook andere vissoorten tijdelijk enteritis ontwikkelen na toediening van SBM, echter in het geval van de karper trad na 4 weken herstel op. De reden waarom karper wel en zalm niet herstelt is nog onbekend.

RESUMEN

Actualmente, la mayoría de esfuerzos en la substitución de la harina de pescado por harina de soya para alimento de peces, ha encontrado un gran número de obstáculos y restricciones. Se sabe que la inclusión de esta harina de soya (SBM, por su sigla en inglés), particularmente para dietas de salmónidos induce un proceso inflamatorio a nivel de la mucosa del intestino posterior. Dicho proceso se conoce como enteritis. El objetivo principal de esta investigación es entender la etiología y los mecanismos responsables de este trastorno. Para este efecto, se evaluan numerosos factores (tanto alimentarios como no-alimentarios) y se estudian los posibles mecanismos responsables de dicho proceso inflamatorio, tanto en salmón del Atlántico (*Salmo salar* L) como en carpa común (*Cyprinus carpio* L.).

En el **capítulo 2**, se evalúa la influencia de la temperatura del agua como un factor fundamental que puede afectar la velocidad con la que este trastorno intestinal se desarrolla. Con este estudio se demuestra que la fase inicial de este trastorno se retrasa en los peces mantenidos a la temperatura más baja (8 vs. 12 °C). Para poder evaluar el grado de intensidad de los cambios ocurridos a nivel morfológico se desarrolla un sistema de clasificación semi-cuantitativo. Esta herramienta se utiliza en este estudio y en los estudios posteriormente mencionados en esta tesis.

El **capítulo 3**, describe la influencia de SBM en la cinética de dicho trastorno intestinal. Se estudian dos niveles diferentes de inclusión a lo largo de un período de 57 días. Los síntomas característicos de la enteritis se incrementan constantemente especialmente en peces alimentados con la dosis más alta empleada en este estudio (20% SBM) con respecto a la dosis más baja (10% SBM), bajo la cual los peces muestran una tendencia a estabilizar dichos síntomas, tendencia que se acentúa a partir de la segunda semana después de iniciado el esquema de alimentación experimental. De este estudio se concluye que la severidad de este trastorno y su cinética, son dosis-dependientes, que no muestran ningún signo de mejoría mientras que se continúa con este tipo de alimentación. Con base en estudios realizados usando microscopia electrónica, se puede sugerir claramente que el proceso de endocitosis se bloquea junto con una fuerte disminución en el tamaño de las microvellosidades que bordean la superficie intestinal. Se sabe que la endocitosis es un importante proceso básico y genérico en la fisiología del animal. Si se pudiera comprobar que la SBM o cualquiera de sus componentes está induciendo la enteritis (por ejemplo, a través del bloqueo del proceso de endocitosis), sería muy sorprendente que sólo los salmónidos fueran los únicos peces en

padecer dicho trastorno. Por lo tanto, se realiza un estudio comparativo en otra especie con diferentes hábitos (más herbívora) como por ejemplo la carpa común.

En el **capítulo 4**, la enteritis inducida por la SBM, se estudia en la carpa común. Dicho trastorno en esta especie considerada omnívora, se compara con el mismo tipo de inflamación intestinal observado en salmón del Atlántico, especie considerada como carnívora. Los resultados indican que la carpa común también desarrolla dicha inflamación debido a la alimentación con dietas a base de harina de soya. Este reporte constituye el primero de este tipo en la literatura científica. Opuesto a lo observado anteriormente en el salmón del Atlántico, la carpa común, empieza a recuperarse a partir de la cuarta semana de alimentación a base de soya. Esta recuperación se indica explícitamente por la reaparición de las vacuolas supra nucleares (SNV por su sigla en inglés). Todos los cambios morfológicos y la modulación del proceso inflamatorio además de definirse, se relacionan con el incremento o disminución de los niveles de citocinas involucradas aparentemente en la regulación del proceso inflamatorio. Nuevamente, las observaciones realizadas en el microscopio electrónico, revelan que el bloqueo del proceso de endocitosis está directamente ligado a la desaparición de las vacuolas supra nucleares y a la primera aparición de los síntomas de la enteritis.

En el **capítulo 5** se investiga el papel que el proceso de endocytosis podría jugar en la aparición de la enteritis,. Por medio del uso de la molécula ferritina, se logra demostrar el posible bloqueo del proceso de endocitosis. Después de una semana de alimentación con soya, rebaja la absorción de ferritina y las vacuolas supra nucleares disminuyen su tamaño. A la tercera semana, no se puede detectar ninguna absorción de ferritina; además dichas vacuolas ya no están presentes. Esto indica que el bloqueo de la endocitosis es efectivamente el primer paso en la desaparición de las vacuolas. Adicionalmente, los resultados de este estudio en juveniles de salmón del Atlántico (30 g) mantenidos en agua dulce, facilitan la comparación con salmones más adultos (300 g) mantenidos en agua de mar, con respecto a la severidad de la enteritis inducida por la salinidad del agua. Estos resultados sugirieren que ni la salinidad ni la edad de los peces son factores definitivos en la acentuación de este trastorno intestinal, lo cual requiere de mayores estudios para corroborar los resultados obtenidos.

En el **capítulo 6**, se comparan harinas de soya obtenidas de diferentes fuentes comerciales y diferentes plantas de producción. El objetivo principal es determinar si el grado

de enteritis desarrollado por el salmón depende del origen y/o del sistema de procesamiento de la soya. El parámetro indicador de enteritis más afectado es nuevamente el de las vacuolas supra nucleares a nivel de los enterocitos. Por otro lado, el incremento de las células caliciformes presenta el grado de variación más bajo entre las diferentes fuentes de SBM. Este estudio demuestra que las diferentes fuentes comerciales de SBM pueden presentar diferentes grados de enteritis.

En la literatura, se sugiere que las saponinas de soya y posiblemente en combinación con otros factores, son las responsables de la iniciación del proceso inflamatorio. Por lo tanto, en el **capítulo 7,** se estudia si las diferentes fracciones de molazas de soya obtenidas por medio de la separación de fases usando n-butanol, tienen algún efecto en el desarrollo de la enteritis. Los resultados indican que dicho proceso se presenta solo cuando los peces son alimentados con la combinación de la fase de butanol y el precipitado. Lo cual sugiere que las saponinas de soya tanto solas como en combinación con otros factores como por ejemplo la microflora intestinal entre otros, en efecto influencian la aparición y el desarrollo de la enteritis.

En el **capítulo 8**, se presentan los resultados del presente trabajo de investigación. Se concluye que la etiología y el desarrollo de la enteritis debido a la presencia de SBM en la dieta, estan relacionados más con los factores alimentarios que con los no alimentarios. Los niveles de inclusión en la dieta y las fuentes comerciales tienen un gran impacto en la severidad de este trastorno, principalmente al influenciar el proceso de endocitosis. En esta tesis se indica que el bloqueo del proceso de endocitosis relacionado con la desaparición de las vacuolas supra nucleares parecen ser los aspectos cruciales en la aparición del proceso inflamatorio. Sin embargo, los agentes inductores presentes el la soya, siguen aun desconocidos y se requiere de una investigación más exhaustiva. Además se puede demostrar que la enteritis se desarrolla temporalmente en otra especie diferente al salmón como lo es la carpa común, que muestra indicios de recuperación a partir de la cuarta semana de alimentación continua con SBM. Sin embargo, la razón a la que se atribuye esta recuperación permanece sin elucidarse.

LIST OF PUBLICATIONS

ACKNOWLEDGMENTS

TRAINING AND SUPERVISION PROGRAM

Peer-reviewed articles

Urán, P.A.; Gonçalves, A, Taverne-Thiele, J.J.; Schrama, J.W.; Verreth, J.A.J.; Rombout, J.H.W.M. **2008**. Soybean meal induces enteritis in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology*. Available as OnlineEarly article. DOI:10.1016/j.fsi.2008.02.013

Urán, P.A.; Schrama, J.W.; Rombout, J.H.W.M.; Koppe, W.; Obach, A.; Jensen, L.; Koppe W.; Verreth, J.A.J. **2008.** Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar* L.) at different temperatures. *Aquaculture Nutrition*. Available as OnlineEarly article. DOI:10.1111/j.1365-2095.2007.00534.x

Knudsen, D.; **Urán, P.A.**; Arnous A.; Koppe W.; Frøkiær, H. **2007**. Saponin containing sub-fractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. *Journal of Agricultural and Food Chemistry*, **55**, 6, 2261-2267. DOI: 10.1021/jf0626967.

Urán, P.A.; Schrama, J.W.; Rombout, J.H.W.M.; Taverne-Thiele, J.J.; Obach, A.; Koppe W.; Verreth, J.A.J. **2008.** Time-related changes of the intestinal morphology of Atlantic salmon (*Salmo salar* L.) at two different soybean meal inclusion levels. (Submitted).

Urán, P.A.; Aydin, R.; Rombout, J.H.W.M.; Schrama, J.W.; Verreth, J.A.J. Alterations on the endocytosis process when Atlantic salmon smolts (*Salmo salar* L.) are fed a soybean meal-containing diet. (Submitted).

Urán, P.A.; Jaafari, S. Baardsen, G.; Schrama, J.W.; Rombout, J.H.W.M.; Verreth, J.A.J. Soybean meal sources influence the severity of enteritis in Atlantic salmon (*Salmo salar* L.). (Submitted).

Proceedings

Urán, P.A.; Rombout, J.H.W.M.; Schrama, J.W.; Koppe, W.; Obach, A.; Jensen, L.; Verreth, J.A.J. **2006**. Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar* L.): an electron microscopic study. In: *Abstracts XII International symposium of fish nutrition and feeding. Biarritz, France*. INRA. pg. 134.

Urán, P.A.; Van Houcke, J.; Rombout, J.H.W.M.; Koppe, W.; Fontanillas, R.; Schrama, J.W.; Verreth, J.A.J. **2005**. Comparison of methodologies for measuring the degree of soybean induced enteritis in Atlantic salmon (*Salmo salar* L.). In: *Abstracts World Aquaculture*, *Bali, Indonesia*. Special publication. pg. 666.

Urán, P.A.; Rombout, J.H.W.M.; Koppe, W.; Obach, A.; Jensen, L.; Schrama, J.W.; Verreth, J.A.J. **2004**. Effects of soybean meal on intestinal morphology of Atlantic salmon (*Salmo salar* L.) In: *Abstracts Aquaculture Europe, Barcelona, Spain.* European Aquaculture Society. Special publications No. 34, 803-804.

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Esta tesis esta dedicada a todos aquellos
a quienes las oportunidades les son esquivas

Training and Supervision Program	**Graduate School WIAS**
Name PhD student	**Paula Andrea Urán Carmona**
Project title	Etiology of soybean-induced enteritis in fish
Group	Aquaculture and Fisheries
Daily supervisor(s)	Dr. Johan Schrama and Dr. Jan Rombout
Supervisor	Prof. Dr. Johan Verreth

EDUCATION AND TRAINING		
The Basic Package	**year**	**ECTS***
WIAS Introduction Course (mandatory, 1.5 credits)	2003	1.5
Course on philosophy of science and/or ethics (mandatory, 1.5 credits)	2005	1.5
Subtotal Basic Package		**3**
Scientific Exposure		
International conferences		
Aquaculture Europe "Biotechnology for Quality", Barcelona, October 20-23	2004	1.2
World aquaculture, Bali, May 9-14	2005	1.5
XII International Symposium Fish Nutrition and Feeding, Biarritz, May 28-June 1	2006	1.5
Seminars and workshops		
Darmen Dag, October 31, Wageningen Centre for Food Sciences and Numico	2003	0.3
Seminar plus "Fats and Seafood for Health"	2004	0.3
WIAS Science Day	2004-7	1.2
PhD Retreat "Unity in diversity" May 13-14	2004	0.6
Seminar plus "Dietary Protein" Stable Isotopes, October 6-7	2004	0.6
Workshop "Challenges for Mediterranean Aquaculture", Barcelona, October 19	2004	0.3
ELISA: Basic understanding and trouble shooting, October 17 and 23	2007	0.3
Presentations		
Oral: In Aquaculture Europe, Barcelona, October 22	2004	1.0
Poster: WIAS Science day, Wageningen, February 17	2005	1.0
Oral: In World Aquaculture, Bali, May 11	2005	1.0
Poster: WIAS Science day, Wageningen, March 9 (Prize at Best Poster)	2006	1.0
Poster: XII International Symposium Fish Nutrition and Feeding, Biarritz	2006	1.0
Subtotal International Exposure		**13**
In-Depth Studies		
Disciplinary and interdisciplinary courses		
Fish Immunology, Wageningen, April 10-14	2005	1.5
Ecophysiology of the gastrointestinal tract, Wageningen, February 28-March 3	2005	1.2
Fish Workshop Immunology and Vaccination, Wageningen, April 18 - 22	2006	1.5
Advanced statistics courses		
WIAS Course Design of Animal Experiments, September 21-23	2005	1.0
Basic and Advanced Statistics, Wageningen, June 12- 30	2006	3.0
Subtotal In-Depth Studies		**8**

Professional Skills Support Courses	**year**	**ECTS***
Use of Laboratory Animals	2004	4.0
Techniques for Scientific Writing	2003	1.2
Supervising MSc thesis work	2005	1.0
Subtotal Professional Skills Support Courses		**6**
Research Skills Training		
Preparing own PhD research proposal	2003-4	6.0
Subtotal Research Skills Training		**6**
Didactic Skills Training		
Supervising practicals and excursions		
Practicals: Fish Anatomy and Shrimp Defence System	2004	0.6
Practical: Gut Histology and Fishmeal Replacement	2005-8	1.2
Practical: Cell Biology and Health	2007	3.0
Supervising Theses		
MSc Thesis Jasper van Houcke, Wageningen University/Ghent University	2004	2.0
MSc Thesis Setareh Jaafari, Wageningen University	2005	2.0
Erasmus Thesis Adriana Gonçalves, Wageningen University/Porto University	2005	1.5
MSc Thesis Rozelin Aydin, Wageningen University/Cukurova University	2006	1.5
Erasmus Thesis Angel Cánovas, Wageningen University/Murcia University	2006	1.5
Tutorship		
BSc Project Karin Fieten and Willem van den Veen, Wageningen University	2004	1.5
Preparing course material		
FCF-30304: Fish anatomy and Shrimp defence system	2004	1.5
AFI-32306: Gut Histology and Fishmeal Replacement	2004-5	4.0
Subtotal Didactic Skills Training		**20**
Management Skills Training		
Organisation of seminars and courses		
Organising Committee for WIAS Science Day 2006	2005-6	1.5
Membership of boards and committees		
Aquarius Board	2003-4	1.0
Subtotal Management Skills Training		**3**
Education and Training Total		**59**