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

P.A. URÁN[1,2], J.W. SCHRAMA[1], S. JAAFARI[1], G. BAARDSEN[3], J.H.W.M. ROMBOUT[2], W. KOPPE[3] & J.A.J. VERRETH[1]

[1] *Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences, Wageningen, The Netherlands;* [2] *Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen, The Netherlands;* [3] *Skretting, Aquaculture Research Centre, Stavanger, Norway*

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 SBM-induced 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.

KEY WORDS: Atlantic salmon, enteritis, ingredient variability, soybean meal

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Correspondence: Johan W. Schrama, Aquaculture and Fisheries group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands. E-mail: Johan.Schrama@wur.nl

Introduction

Finding alternative protein sources to replace fishmeal (FM) 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 (including 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 (MF); 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 (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 number of goblet cells (GC) 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), because of 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 has the aim of clarifying whether different commercial sources of SBM can induce 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 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, Redmond, 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 (449 g kg^{-1}) than the other SBM batches, in which the crude protein content ranged from 482 to 492 g kg^{-1}. The amino acid profile was similar between the different SBM batches. The crude fibre content of the different SBM batches was not correlated with the crude protein content and ranged from 28 to 47 g kg^{-1}.

Seven experimental diets were formulated: one control diet, which contained FM and no SBM; and six SBM diets ('SBM1' to 'SBM6') each containing one of the six SBM batches (Table 2). The control diet was a mixture of FM, fish oil (FO), wheat starch and a standard vitamin and mineral premix. In the SBM diets, 200 g kg^{-1} SBM was included. Diets were formulated to have similar crude protein (i.e. iso-nitrogenous) and crude fat content (i.e. iso-lipidic). When compared with the FM diet, SBM was exchanged by both fish meal and wheat starch in the SBM diets. Furthermore, the FO content was slightly increased

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	NA	EU	SA	NA	NA	AU
Nutrient composition g kg^{-1}						
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 g kg^{-1}						
Arginine	33	36	36	37	35	36
Histidine	12	13	13	13	13	13
Isoleucine	19	21	21	21	20	19
Leucine	34	37	37	37	36	35
Lysine	28	29	29	30	30	29
Methionin	06	07	06	07	07	06
Cystin	07	07	07	08	08	07
Phenylalanine	22	25	25	25	24	23
Tyrosine	15	16	16	16	15	15
Threonine	18	19	19	20	19	19
Valine	20	21	22	22	21	21
Alanine	19	21	21	21	21	20
Aspartic acid	51	57	57	57	55	54
Glutamic acid	83	94	92	94	92	89
Glycine	18	20	20	21	20	20
Proline	23	25	25	25	25	24
Serine	24	26	26	26	25	25

NA, North America; EU, Europe; SA, South America; AU, Australia.

Table 2 Ingredient composition of the experimental diets

	FM[1]	SBM1[1]	SBM2–SBM6[1]
Ingredients g kg^{-1}			
Soybean meal[1]	0	200	200
Fishmeal[2]	587	464	453
Wheat starch	223	138	148
Nordic fish oil	189	197	198
Vitamin/mineral premix	2	2	2

[1] FM (fishmeal) is the control diet; SBM1 to SBM6 are the experimental diets containing solvent-extracted soybean meal, the number refers to the commercial SBM source described in Table 1. The SBM diets were formulated to contain similar amounts of crude protein (420 g kg^{-1}), crude fat (250 g kg^{-1}), ash (80 g kg^{-1}), starch (130 g kg^{-1}) and gross energy (23 MJ kg^{-1}).
[2] Scandinavian LT fishmeal.

in the SBM diets to keep the crude fat content equal between diets (Table 2). As a result of the lower crude protein content of the SBM1 batch (Table 1), a slightly smaller amount of fishmeal was replaced in the SBM1 diet when compared with other SBM diets (Table 2). For the SBM2–SBM6 diets, the ingredients exchanged were kept similar, ignoring the small differences in crude protein content. All SBM batches were processed by solvent extraction. For all SBM batches tested, SBs were dehulled or partially dehulled prior to the fat extraction. 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 (30 g kg^{-1}). 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 h. 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).

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. On the day of sampling, the morning feeding was skipped. Intestines were sampled between 18 and 22 h after the last feeding. The fish were killed by an overdose of anaesthetic. The intestines were removed immediately and rinsed in cold (4 °C) saline water. Samples for light microscopy (LM) 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 and thereafter mounted for each fish on glass slides. Each slide contained two non-serial transverse 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 haematoxylin and eosin. Slides were blindly evaluated after randomization.

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 MF; (2) the presence and size of supranuclear vacuoles (SNV); (3) the number of GC; (4) the degree of infiltration abundance and of eosinophilic granulocytes (EG) into the LP and into the sub-epithelial mucosa; (5) the degree of widening of the 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 (Olympus, Tokyo, Japan) connected to a Nikon Microphot-FXA light microscope (Nikon Instruments Europe BV, Amstelveen, 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 3. For illustration of the different scores, see 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).

Table 3 Description of the semi-quantitative scoring system according to Urán *et al*. (2008), using different parameters to assess the degree of enteritis developed by Atlantic salmon fed with 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	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

Statistical analysis

Individual fish were taken as the experimental unit in the statistical analysis (nine replicates per dietary treatment). 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 mean values between diets was carried out using the Tukey's 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 with the control diet (FM) did not show any sign of SBM-induced enteritis. The overall score of fish fed with the FM diet was different from fish fed with other experimental diets, all of which contained SBM (SBM1–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 with the SBM1, SBM3 and SBM4 diets attained the highest overall enteritis score, which differed significantly ($P < 0.05$) from fish fed with the SBM6 diet, which showed the mildest enteritis response. The SBM2 and SBM5 diets gave an intermediate response when compared with the other SBM diets.

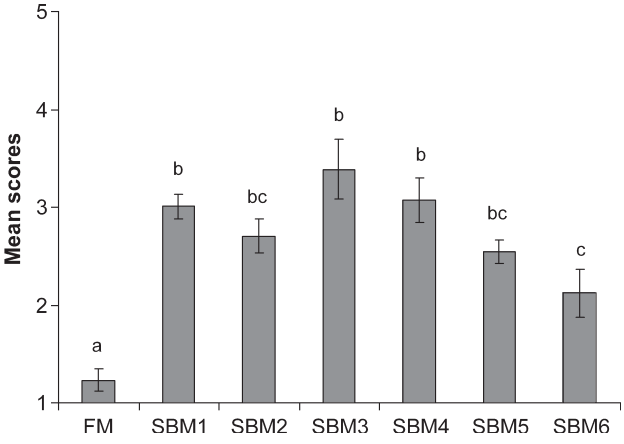

Figure 1 Mean values of the overall enteritis score per experimental diet (effect of diet, $P < 0.001$). FM is the fishmeal (control) diet, SBM1 to SBM6 are the diets containing 200 g kg^{-1} soybean meal of commercial source 1–6, respectively, as described in Table 1. Mean values per diet having no 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 these enteritis parameters were influenced by the diet ($P < 0.001$). The separate parameters in fish fed with the FM diet did not increase and were generally different from fish fed with the SBM diets, except for MF, SNV and LP. Regarding the parameters MF, SNV and LP, fish fed with the FM diet did not significantly differ from fish fed with the SBM diets, which gave a mild enteritis response (especially the SBM6 diet).

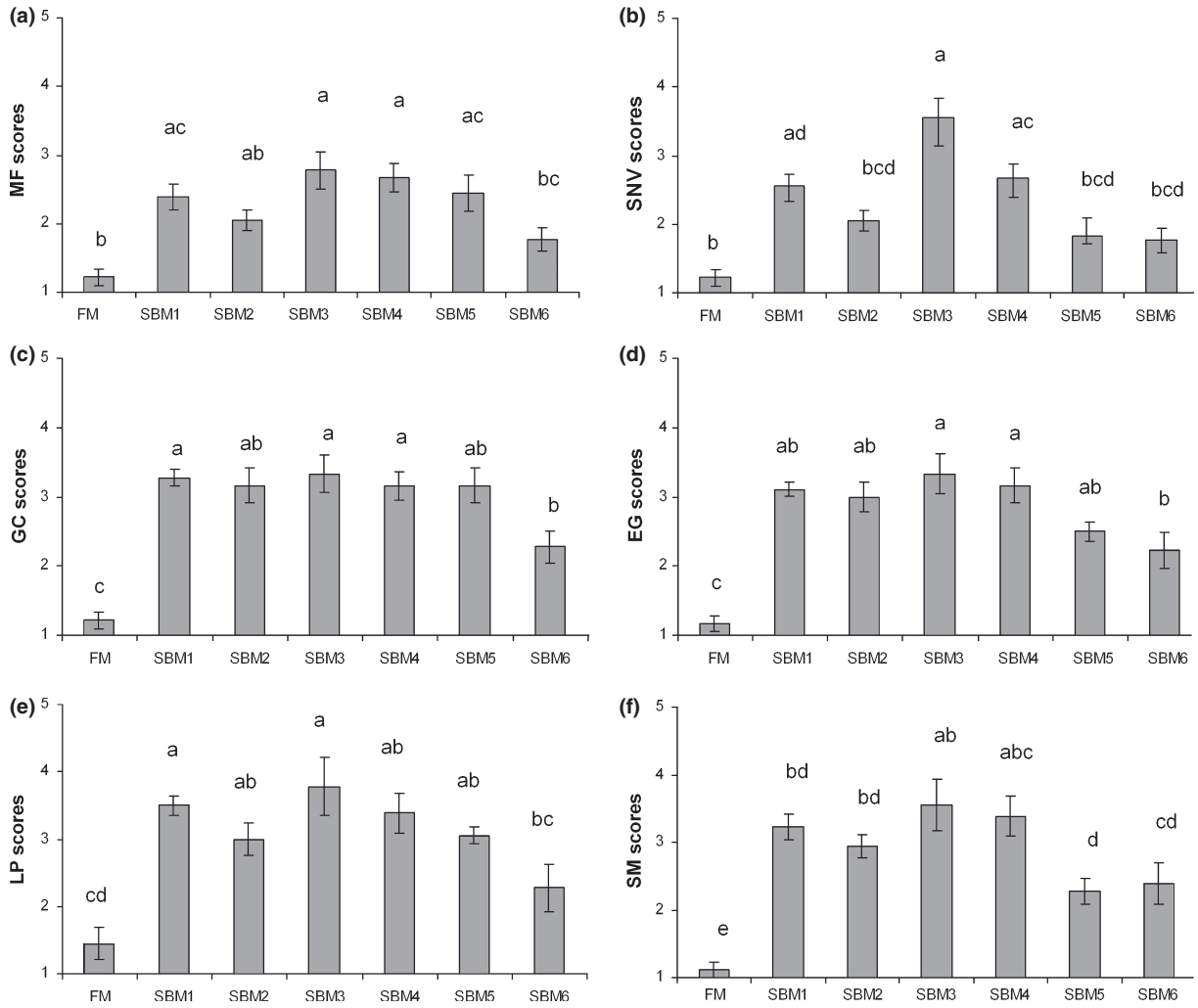

Figure 2 Mean values of the enteritis parameters scored per experimental diet: a, mucosal folds (MF); b, supranuclear vacuoles (SNV); c, goblet cells (GC); d, eosinophilic granulocytes (EG); e, lamina propria (LP); f, sub-epithelial mucosa (SM); (diet effect, $P < 0.001$ for all parameters). FM is the fishmeal (control) diet, SBM1 to SBM6 are the diets containing 200 g kg^{-1} soybean meal of commercial source 1–6, respectively, as described in Table 1. Mean values per diet having no common letter (abcde) differ significantly ($P < 0.05$).

Similarly to 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' SNV score (Fig. 2b). The contrasts among diets for the MF, EG, LP and SM were lower compared with 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 with the SBM6 diets, whereas no differences were present between the other SBM diets (SBM1–SBM5). The smaller contrasts in GC among the SBM diets when compared with the other parameters are also reflected by the calculated correlation coefficients between different parameters using only the fish fed with the SBM diets (Table 4). The correlation between the GC score and the other scores ranged 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 between 0.66 and 0.88 (mean = 0.78).

Discussion and conclusions

Composition variability of feed ingredients is an important issue, of which practical feed formulators should be aware

Table 4 Correlation coefficients among the different scores of enteritis parameters (MF, SNV, GC, EG, LP and SM) for fish fed with 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

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

of (Jiang 2001). Variability between sources (batches) of feed ingredient is not only related to differences in nutrient composition, but this difference can also be reflected in variation in the availability of nutrients. Glencross *et al.* (2008) showed in rainbow trout that in addition to composition variability of lupin meals, considerable variation in nutrient digestibility was also present. In rainbow trout, the bioavailability of lysine varies between blood meals of various origins and especially between different technological treatments (El-Haroun & Bureau 2007). In the current study, it was assessed whether different sources (batches) of SBM resulted also in variation in the degree of the SBM-induced enteritis response in Atlantic salmon. The inclusion of SBM in the diet of Atlantic salmon in this study, 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 the existing literature, several studies show that the type of 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. The difference in response between the SBM sources might be due to differences in the amount of causative agent consumed by the fish, as the SBM-enteritis severity is dose-dependent (Baeverfjord & Krogdahl 1996; Urán 2008). Differences in causative agent intake might be caused by (1) differences in feed intake (Atlantic salmon were fed to satiation in the current experiment) and (2) differences in causative agent concentration between the SBM sources. The later might be due to various factors such as SB variety, growing conditions of the SBs, harvesting moment, storage conditions and/or processing conditions. 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: MF; SNV; GC; EG; LP; 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 when compared with 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 the dose.

The current observation between SBM source variations 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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