

Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar* L.)

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(Received 14 August 2007 – Revised 8 November 2007 – Accepted 8 November 2007 – First published online 2 January 2008)

Saponins are naturally occurring amphiphilic molecules and have been associated with many biological activities. The aim of the present study was to investigate whether soya saponins trigger the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar* L.), and to examine if dietary soya saponins increase the epithelial permeability of the distal intestine in Atlantic salmon. Seven experimental diets containing different levels of soya saponins were fed to seawater-adapted Atlantic salmon for 53 d. The diets included a fishmeal-based control diet, two fishmeal-based diets with different levels of added soya saponins, one diet containing 25 % lupin kernel meal, two diets based on 25 % lupin kernel meal with different levels of added soya saponins, and one diet containing 25 % defatted soyabean meal. The effect on intestinal morphology, intestinal epithelial permeability and faecal DM content was examined. Fish fed 25 % defatted soyabean meal displayed severe enteritis, whereas fish fed 25 % lupin kernel meal had normal intestinal morphology. The combination of soya saponins and fishmeal did not induce morphological changes but fish fed soya saponins in combination with lupin kernel meal displayed significant enteritis. Increased epithelial permeability was observed in fish fed 25 % defatted soyabean meal and in fish fed soya saponin concentrate independent of the protein source in the feed. The study demonstrates that soya saponins, in combination with one or several unidentified components present in legumes, induce an inflammatory reaction in the distal intestine of Atlantic salmon. Soya saponins increase the intestinal epithelial permeability but do not, *per se*, induce enteritis.

Saponins: Enteritis: Barrier function: Diarrhoea

Saponins are naturally occurring amphiphilic molecules consisting of a sugar moiety linked to a steroid or triterpenoid aglycone. They are widely distributed in wild plants and are also present in many cultivated crops. Saponins have an ability to disrupt biological membranes and are considered to be involved in the plant's defence system against microbial attack^(1–3). Saponins have also been reported to have several biological activities in animals, including cholesterol-lowering effects, immunostimulating effects, reproductive effects and glucocorticoid-like actions^(2,3). On the intestinal level, Gee *et al.*⁽⁴⁾ observed increased transmucosal uptake of the milk allergen β -lactoglobulin in rats *in vivo*, when isolated jejunal loops were exposed to *Gypsophila* saponins. Several other studies with saponins, including soya saponins, have demonstrated increased transepithelial uptake of macromolecules *in vitro*, probably due to enhanced permeability^(5–8). However, it has to our knowledge not yet been investigated whether orally delivered soya saponins can increase the epithelial permeability of the intestine.

Soya beans are one of the major dietary sources of saponins for humans and domestic animals, and also for farmed fish. Soya saponins are triterpene glycosides and can be divided into two major groups: A and B (Fig. 1)^(9–12). Studies in human subjects, chickens, mice and rats^(13–15) have previously demonstrated that the endogenous gut microflora is able to cleave off the sugar chain attached to carbon atom no. 3 of the triterpene (see Fig. 1), which effectively disrupts the surface-active properties of saponins. However, soya saponins were recently found to resist degradation during gut passage in Atlantic salmon⁽¹⁶⁾.

Previous studies have shown that high dietary levels of soya beans induce enteritis in the distal intestine of Atlantic salmon. The causative factor for the condition has still not been identified. van den Ingh *et al.*⁽¹⁷⁾ demonstrated that alcohol-extracted soyabean protein concentrate was free of the causative components, whereas soyabean molasses (the by-product from the alcohol extraction) caused the same inflammatory symptoms as observed in fish fed high levels of soyabean meal. Bureau *et al.*⁽¹⁸⁾ found

Abbreviations: DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one; P_{app} , apparent permeability; TEP, transepithelial potential; TER, transepithelial electrical resistance.

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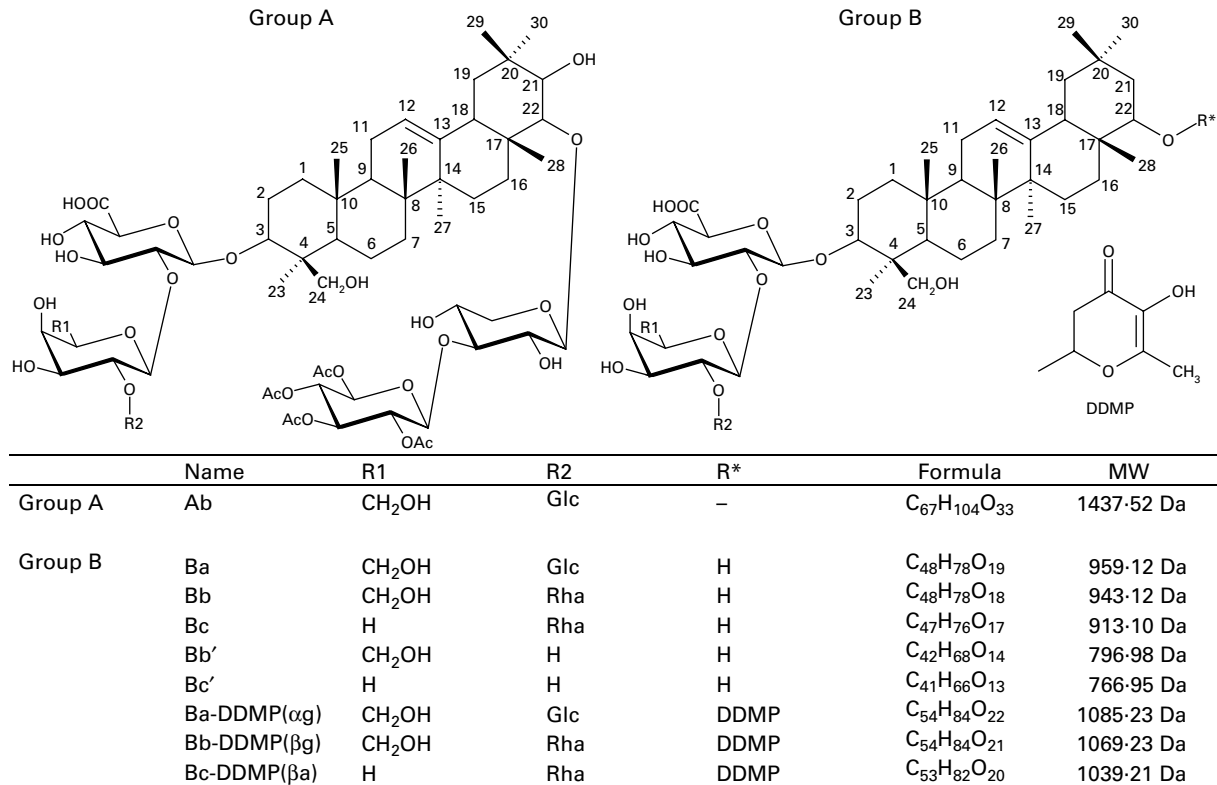


Fig. 1. Structures of major saponins in soya beans. DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; MW, molecular weight.

that *Quillaja* saponins caused extensive damage to the intestinal mucosa of the hindgut in Chinook salmon and rainbow trout, and that soya saponin-rich extracts from soyabean meal and soyabean protein isolate had potent feeding-deterrent properties in Chinook salmon. They therefore suggested that soyabean-induced enteritis was caused by soya saponins. In a previous study⁽¹⁹⁾ it was demonstrated that neither oligosaccharides nor soyabean proteins larger than 8–10 kDa were able to induce the inflammatory reaction alone. Furthermore it was shown that the inflammatory response was triggered by soya saponins or unidentified components that followed the same solubility pattern.

In contrast to soya beans, lupins are well accepted by Atlantic salmon and do not induce enteritis in the distal intestine⁽²⁰⁾. Moreover, the saponin level in lupin seeds has been reported to be much lower (0.4–0.7 g/kg)⁽²¹⁾ than the saponin level typically found in defatted soyabean meal (5–7 g/kg)⁽¹⁶⁾.

The objective of the present study was to test whether soya saponins play a role in the onset of soyabean-induced enteritis in Atlantic salmon, and to further investigate the pathophysiological consequences of enteritis on the intestinal barrier function by assessment of mucosal permeability. The dietary effect of soya saponins was investigated both in combination with a fishmeal-based control diet and in combination with a diet containing 25% lupin kernel meal.

Materials and methods

Feed ingredients

The following saponin-containing test ingredients were obtained: hexane-extracted soyabean meal from Denofa (Fredrikstad,

Norway); lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia); soya saponin concentrate, 65% purity (Organic Technologies, Coshocton, OH, USA). The remaining 35% of the soya saponin concentrate was mainly oligosaccharide gums, according to the supplier.

Production of fish feed

Seven diets were produced as 3 mm pellets by twin-screw extrusion cooking at Skretting Feed Technology Plant, Stavanger, Norway. A preconditioner (DDC; Wenger Manufacturing, Inc., Kansas City, MO, USA) coupled to a twin-screw extruder (TX57; Wenger Manufacturing, Inc.) was used for diet production. The extruded pellets were dried for 25 min by warm air (75°C) in a single belt dryer (model 360; Wenger Manufacturing, Inc.). The dried pellets were finally coated with fish oil at 60°C under reduced pressure (VCC coater; Dinmussen BV, Sevenum, The Netherlands). The seven experimental diets comprised: a fishmeal-based control diet, two fishmeal-based diets with different levels of soya saponin concentrate, one diet containing 25% lupin kernel meal (lupin-based control diet), two diets based on 25% lupin kernel meal with different levels of soya saponin concentrate, and one diet containing 25% defatted soyabean meal. In order to obtain comparable growth, diets were formulated to be iso-energetic on a digestible energy basis (using table values on digestibility from Skretting's raw material database). The same quantity of vitamin and mineral premix was added to all diets to meet the National Research Council recommendation⁽²²⁾. The recipes are shown in Table 1.

Table 1. Recipes and proximate composition of experimental diets (g/kg as-is basis)

	Fishmeal control	Fishmeal + SSC (1.7 g/kg)	Fishmeal + SSC (2.6 g/kg)	Lupins	Lupins + SSC (1.7 g/kg)	Lupins + SSC (2.6 g/kg)	Soya beans
Fishmeal*	620.9	620.9	620.9	483.5	483.5	483.5	456.6
Lupin†	0.0	0.0	0.0	250.0	250.0	250.0	0.0
Soyabean meal‡	0.0	0.0	0.0	0.0	0.0	0.0	250.0
Wheat	80.0	78.3	77.4	80.0	78.3	77.4	80.0
Wheat starch	139.9	139.9	139.9	0.0	0.0	0.0	0.0
Minerals and vitamins§	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Pigment	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Fish oil¶	155.2	155.2	155.2	182.5	182.5	182.5	209.4
Soya saponin concentration**	0.0	1.7	2.6	0.0	1.7	2.6	0.0
Chemical composition							
Crude protein	454	449	454	455	444	450	467
Crude fat	228	231	225	291	283	269	264
Ash	91	90	90	80	77	79	84
DM	916	914	927	969	956	951	945
N-free extract	133	134	147	134	142	143	118
Gross energy (MJ/kg)††	22.1	22.1	22.2	24.5	24.1	23.7	23.5
Soya saponins‡‡	0.00	1.05	1.60	0.03	1.07	1.63	1.77

SSC, soya saponin concentrate.

* Scandinavian Fishmeal LT (Norsildmel, Fyllingsdalen, Norway).

† Lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia).

‡ Defatted soyabean meal (Denofa, Fredrikstad, Norway).

§ Mineral and vitamin premix to meet the National Research Council recommendations⁽²²⁾.

|| Carophyll Pink (DSM Nutritional Products, Basel, Switzerland).

¶ South American oil containing 30% SFA of total fatty acids.

** Soya saponin concentrate, 69% purity (Organic Technologies, OH, USA).

†† Calculated by using physiological fuel values of 24, 39 and 17 kJ/g for protein, fat and carbohydrate, respectively, according to Jobling⁽²⁴⁾.

‡‡ Total level of soya saponins calculated based on the soya saponin analyses of dehulled lupins, defatted soyabean meal and soya saponin concentrate (see Table 3).

Fish trial

The feeding trial, which complied with the guidelines from the Norwegian National Animal Research Authority, was conducted at Skretting Fish Trials Station (Lerang, Jørpeland, Norway). Seawater-adapted Atlantic salmon (*Salmo salar* L.) with an initial average weight of 81 g were fed the seven different experimental diets for 53 d. The fish were randomly distributed into twenty-one circular 400 litre fibreglass tanks at a density of seventy fish per tank. The tanks were equipped with waste feed collection and continuously supplied with seawater (15 litres/min per tank). Water was pumped from 90 m depth and held at a temperature between 8.5°C and 8.7°C during the experiment. The light–dark schedule was 24 h light. The seven different diets were fed to triplicate tanks (three tanks per treatment), twice per d, aiming at 20% overfeeding, and waste feed was collected. An intermediate sampling was done after 26 d feeding. Two fish from each tank (six per treatment) were killed with an overdose of anaesthesia (tricaine methanesulfonate, Finquel MS-222; Argent Chemical Laboratories, Redmond, WA, USA), for histological examination. The final sampling was done after 53 d feeding. Five fish per tank (fifteen per treatment) were killed with an overdose of anaesthesia, for histological evaluation. Four of the dietary groups were picked out for gut permeability studies. From these groups four additional fish were killed per tank (twelve fish per treatment) with a blow to the head. The remaining fish in the tanks were stripped to collect faeces according to Austreng⁽²³⁾. One pooled faeces sample was obtained per tank (three samples per treatment).

Proximate analyses

DM measurements were done by drying to constant weight at 102–105°C. Ash was measured by burning samples at 550°C

for 16–18 h. Crude protein was quantified as N × 6.25 using a Kjeltac Auto Sampler-System (Tecator AB, Hoganas, 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, Hillerød, Denmark) according to Nordic Committee on Food Analysis, method no. 160, 1998. Gross energy was calculated using physiological fuel values of 24, 39 and 17 kJ/g for protein, fat and carbohydrate, respectively, according to Jobling⁽²⁴⁾.

Quantification of soya saponins

Separation and quantification of soya saponins were performed using reverse-phase HPLC with diode array detection (HPLC-DAD) as described previously⁽¹⁶⁾. The separation was achieved using a Hewlett-Packard series 1050 HPLC-DAD system with a 250 mm × 4.6 mm internal diameter, 5 mm, Supercosil ABZ +Plus, C18 reverse-phase column (Supelco, Sigma-Aldrich). The mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and 0.05% trifluoroacetic 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 soya saponins was confirmed by HPLC retention time, UV absorption spectra recorded at 200–350 nm and liquid chromatography–MS using positive electrospray ionisation. The following soya saponins were detected and quantified: Ab, Ba, Bb, Bc, Bb', Bc', Ba-2,3-dihydro-2,5-dihydroxy-6-

methyl-4H-pyran-4-one (DDMP), Bb-DDMP and Bc-DDMP (for molecular structure, see Fig. 1).

Extraction of soya saponins from feed ingredients

Extraction was done at room temperature using 70% aqueous ethanol. Soya saponins were extracted from soyabean meal by mixing 100 mg finely ground soyabean meal with 3 ml solvent for 15 min on a vibrator (IKA-VIBRAX type VXR; Janke & Kunkel, Staufen, Germany). After extraction the suspension was centrifuged at 3000 g for 5 min. The extraction procedure was repeated three times by re-suspending the pellet. The four collected supernatant fractions were pooled to get a total extraction volume of 12 ml. The same extraction procedure was used for lupins but starting with 500 mg finely ground lupin kernel meal. The commercial soya saponin concentrate was prepared for quantification in the following way: 15 mg of the product was dissolved in 10 ml 70% aqueous ethanol and subjected to 10 min sonification in an ultrasound water-bath at 30°C. All extracts were centrifuged at 15 000 g for 5 min before injection in the HPLC system.

Histological examinations

A 2 cm section of the anterior part of the distal intestine (from just posterior to the ileorectal valve) was carefully removed, cut open longitudinally, and fixed in phosphate-buffered formaldehyde (4%, pH 7.2). Samples were then dehydrated, embedded in paraffin and cut with a rotary microtome (3.0 µm thickness, Leica RM 2155; Leica Microsystems GmbH, Wetzlar, Germany). One slide was prepared per fish. Slides were stained with a combination of haematoxylin-eosin and alcian blue 8 GX, pH 2.5. The latter stain 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; Leica Microsystems GmbH, Wetzlar, Germany) according to the scoring criteria given in Table 2, as previously described⁽¹⁹⁾. A score of '1–2' represented normal morphology, a score of '3' was given to distinct morphological signs of inflammation, while a score of '5' was given to very severe symptoms of enteritis. Histological samples were randomised and blindly evaluated.

Ussing chamber technique

The four fish groups that were subjected to gut permeability studies were: fish fed the fishmeal-based control diet, fish fed the fishmeal-based diet combined with soya saponin concentrate (2.6 g/kg), fish fed the lupin kernel meal combined with soya saponin concentrate (2.6 g/kg), and finally fish fed the soyabean meal diet. The permeability of the distal intestine was studied using the Ussing chamber technique. Four fish per tank (twelve per treatment) were killed by a blow to the head, and the anterior part of the distal intestine (from just posterior to the ileorectal valve) was carefully removed, rinsed and placed in salmon Ringer solution (150 mM-NaCl, 2.5 mM-KCl, 2.5 mM-CaCl₂, 1.0 mM-MgCl₂, 10 mM-glucose and 5 mM-HEPES buffer; pH was set to 7.8 with 1 M-NaOH), continuously gassed with air and kept on ice. The tissues were mounted into modified Ussing chambers as described by

Table 2. Histological scoring system for morphological changes induced by soya beans in the distal intestine of Atlantic salmon (*Salmo salar* L.)

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

CT, connective tissue; SF, simple folds; CF, complex folds.

Sundell *et al.*⁽²⁵⁾, exposing 0.75 cm² of the epithelium. The half-chambers each contained 4 ml Ringer solution kept at 10°C. The solution was continuously mixed and oxygenated by gas-lift with air. One pair of Pt electrodes supplied current and one pair of Ag/AgCl electrodes (Radiometer, Copenhagen, Denmark) were used for the measurement of transepithelial potential (TEP) differences. The transepithelial resistance (TER) and short-circuit current were measured and calculated every 5 min as described by Sundell *et al.*⁽²⁵⁾. The electrical parameters function as a continuous measure of the epithelial viability and integrity. In leaky epithelia, as fish intestinal epithelia are, TER mainly reflects the paracellular permeability⁽²⁶⁾. The apparent permeability (P_{app}) for mannitol (described below) is similarly used for assessing paracellular permeability. The TEP measures active ion-transporting activity that is maintained through ion transport ATPases, and can thus be used for viability monitoring⁽²⁷⁾.

The intestinal segments were kept in the chambers for a total time of 150 min. At time equals 60 min, the mucosal Ringer solution was replaced by a Ringer solution with 0.03–0.04 MBq [¹⁴C]mannitol/ml (Amersham, St Louis, MO, USA) in order to assess the paracellular permeability by measuring the P_{app} of the small hydrophilic marker molecule. Every 10 min for 90 min, 50 µl samples were withdrawn from the serosal compartment. One sample was also collected from the mucosal half chamber immediately following the change of mucosal Ringer solution. To each sample, 4 ml of Optiphase High Safe II (Wallac, Turku, Finland) was added and the radioactivity was assessed using a liquid scintillation counter (Beckman LS 1801; Beckman, Fullerton, CA, USA).

Calculations of P_{app} for mannitol was performed using:

$$P_{app} = dQ/dt \ 1/AC_0,$$

where dQ/dt is the appearance rate of mannitol in the serosal Ringer solution, A is the exposed intestinal surface area in the Ussing chamber and C_0 is the initial mannitol concentration in the mucosal Ringer solution.

Statistics

The study was performed as a completely randomised single-factor experiment investigating the impact of seven independent diets on the intestinal health of Atlantic salmon. As an additional experiment, the dietary effect on gut permeability was examined for four of these diets.

Faecal DM results were analysed by one-way ANOVA. The Student–Newman–Keuls multiple comparisons test ($\alpha = 0.05$) was used to compare all pairs of means. The histological scoring results were treated as non-parametric data. Kruskal–Wallis one-way ANOVA was therefore applied to test for equality of treatment means. A multiple comparisons test with mean ranks (Student–Newman–Keuls, $\alpha = 0.05$) was used as a *post hoc* test to compare all pairs of mean ranks. These statistical analyses were performed using UNISTAT[®] Statistical package version 5.5 (Unistat Ltd, London, UK).

Intestinal paracellular permeability was measured as TER and as P_{app} for mannitol in parallel. Data on P_{app} and TER were subjected to a Levene F test to check for equal variances. When appropriate, the dataset was subjected to log 10-transformations to obtain equal variances. Subsequent ANOVA and Student–Newman–Keuls *post hoc* tests were conducted on the transformed data. The TEP results were still non-homogeneous after log 10-transformation and were thus analysed by a non-parametric Kruskal–Wallis test. These statistical analyses were performed using SPSS version 13 (SPSS, Inc., Chicago, IL, USA).

Results

All fish groups grew well and had good appetite during the feeding trial. The average growth rate was 1.66 % per d, and the average daily feed intake was 1.18 % of body weight. This is slightly better than the performance Austreng *et al.* (28) reported for Atlantic salmon at this size and temperature. The feeding period was too short (53 d) to find significant differences in growth between dietary groups. However, the trial was not designed to study growth effects but to investigate dietary effects on gut health.

The level of soya saponins was quantified in the three saponin-containing test ingredients. The total content of soya saponins in lupin kernel meal, defatted soyabean meal and soya saponin concentrate was 0.1, 7.1 and 615.6 g/kg, respectively. The distribution of the individual types of soya saponins in these three ingredients is shown in Table 3. The soya saponin level found in defatted soyabean meal was consistent with our previous findings⁽¹⁶⁾. Only very low levels of soya saponins were found in lupin kernel meal, which is in agreement with earlier reports^(21,29). The total soya saponin level in the soya saponin concentrate was fairly consistent with supplier data. Based on the level of soya saponins in the three

saponin-containing test ingredients, the total soya saponin level in the different diets was calculated (Table 1). The total level of soya saponins in the soyabean diet corresponded approximately to the saponin level in the lupin and fishmeal diets with the high (2.6 g/kg) level of added soya saponins.

After 26 d feeding, an intermediate sampling was done to evaluate morphological changes in the distal intestine. The semi-quantitative scoring system shown in Table 2 was used to evaluate the level of inflammation. Results are given in Table 4. Fish fed the fishmeal-based control diet displayed normal morphology, while significant enteritis was observed in fish fed 25 % defatted soyabean meal. None of the other dietary groups differed significantly from the fishmeal-based control diet due to the low number of evaluated fish per group. However, distinct signs of inflammation (average enteritis score above 3) were seen in four out of six fish fed the highest level of soya saponins in combination with lupin kernel meal. Fish fed soya saponins in combination with fishmeal displayed normal morphology.

The final sampling was done after 53 d feeding. The morphological changes in the distal intestine were evaluated for fifteen fish per diet. Results are given in Table 5. The results were consistent with the histological evaluation from the intermediate sampling. Fish fed the fishmeal-based control diet displayed normal morphology, while significant enteritis was observed in fish fed 25 % defatted soyabean meal. No morphological changes could be seen in the distal intestine of fish fed soya saponin concentrate in combination with fishmeal. However, the two diets containing soya saponin concentrate in combination with lupin kernel meal had a significant and dose-dependent effect on all four histological parameters: The size of the absorptive vacuoles was reduced, thickness of *lamina propria* had increased, the mucosal folds had become shorter and the amount of connective tissue between the base of folds and *stratum compactum* had increased. Representative light microscopy images of the morphological changes are shown in Fig. 2. Distinct signs of enteritis (average enteritis score above 3) were seen in nine out of fifteen fish in the group fed the highest level of soya saponins in combination with lupin kernel meal.

Table 3. Level of soya saponins in tested feed ingredients (g/kg 'as is') (Mean values and standard deviations)

Saponin	SSC*		Lupins†		Soya beans‡	
	Mean	SD	Mean	SD	Mean	SD
Ab		ND		ND	0.89	0.02
Ba	33.3	0.3		ND		ND
Bb	355.8	18.4		ND	2.54	0.09
Bc	168.2	10.4		ND	0.93	0.08
Bb'	41.3	1.7		ND		ND
Bc'	17.0	0.7		ND		ND
Ba-DDMP		ND		ND	0.13	0.02
Bb-DDMP		ND	0.11	0.01	1.84	0.02
Bc-DDMP		ND		ND	0.74	0.01
Sum	615.6	31.4	0.11	0.01	7.06	0.16

SSC, soya saponin concentrate; ND, not detectable; DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one.

* 65 % purity (Organic Technologies, Coshocton, OH, USA).

† Lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia).

‡ Defatted soyabean meal (Denofa, Fredrikstad, Norway).

Table 4. Histological evaluation of distal intestine*
(Mean values and standard deviations from six fish)

	Fishmeal control		Fishmeal + SSC (1.7 g/kg)†		Fish meal + SSC (2.6 g/kg)†		Lupins‡		Lupins + SSC (1.7 g/kg)†		Lupins + SSC (2.6 g/kg)†		Soya beans§	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Intermediate sampling														
Vacuoles	2.0 ^{a,b}	1.4	1.3 ^a	0.6	1.6 ^a	0.7	1.8 ^{a,b}	0.9	2.6 ^{a,b}	1.5	3.3 ^{a,b}	0.9	4.3 ^b	0.8
Lamina propria	1.5 ^{a,b}	0.6	1.1 ^a	0.2	1.5 ^{a,b}	0.5	2.0 ^{a,b}	0.7	2.7 ^{a,b,c}	0.6	2.5 ^{b,c}	0.6	4.3 ^c	0.6
Submucosa	1.3 ^a	0.6	1.8 ^{a,b}	0.5	1.9 ^{a,b}	0.4	2.5 ^{a,b}	1.3	2.3 ^{a,b}	1.3	2.5 ^{a,b}	1.3	3.8 ^b	1.0
Mucosal folds	1.4 ^{a,b}	0.7	1.2 ^a	0.3	1.3 ^{a,b}	0.6	2.1 ^{a,b,c}	1.2	2.7 ^{a,b,c}	1.0	3.2 ^{b,c}	0.8	4.4 ^c	0.7
Average score	1.6 ^a	0.7	1.3 ^a	0.2	1.6 ^a	0.5	2.1 ^{a,b}	1.0	2.6 ^{a,b}	1.0	2.9 ^{a,b}	0.7	4.2 ^c	0.7
Fish with an average score >3 (n)	0		0		0		1		1		4		6	

SSC, soya saponin concentrate.

^{a,b,c} Mean values with unlike superscript letters are significantly different (multiple comparisons test with mean ranks, Student–Newman–Keuls, $\alpha = 0.05$).

* Intestinal cuts were scored according to the criteria listed in Table 2. A score of 1–2 represents normal morphology, a score of 3 was given to distinct morphological signs of inflammation, while a score of 5 represents severe symptoms of enteritis.

† 65% purity (Organic Technologies, Coshocton, OH, USA).

‡ Lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia).

§ Defatted soyabean meal (Denofa, Fredrikstad, Norway).

Fish fed 25% defatted soyabean meal and fish fed soya saponin concentrate in combination with lupin kernel meal had significantly higher epithelial permeability than fish fed the fishmeal-based control diet, as determined by both P_{app} and TER. The epithelial permeability was also significantly higher in fish fed soya saponins in combination with fishmeal although no visible inflammation was observed in this dietary group (Fig. 3). TEP was significantly affected by the diet. The group fed soya beans had the lowest TEP at 0.46 mV, the fishmeal control group 0.96 mV, and the other two groups had TEP similar to the fishmeal control diet (fishmeal + soya saponins 0.88 mV; lupins + soya saponins 1.15 mV). The short circuit current did not differ significantly between the groups.

The fish that were not killed for histology or gut permeability studies were stripped for faeces. Pooled faeces samples were collected from each fish tank. The average faecal DM content from the three replicated tanks within each dietary group is shown in Table 6. Addition of soya saponin concentrate to the fishmeal-based control diet had no effect on faecal DM content. Fish fed the lupin-based

control diet, however, had significantly lower faecal DM content than fish fed the fishmeal-based control diet. The diets containing soya saponin concentrate in addition to lupin kernel meal caused the faecal DM to decrease further compared with the lupin-based control diet. Fish fed the diet containing 25% soyabean meal displayed the lowest faecal DM content.

Discussion

In the present study, it was demonstrated that soya saponins, together with one or several unidentified legume components, trigger an inflammatory reaction in the distal intestine of Atlantic salmon. Soya saponins alone, however, do not cause intestinal inflammation. Furthermore it was shown that dietary soya saponins compromise gut integrity in the distal intestine of Atlantic salmon by increasing the intestinal epithelial permeability.

Both soya beans and lupins contain high amounts of anti-genic proteins and non-digestible carbohydrates^(30,31). However, fish fed 25% lupin kernel meal displayed normal

Table 5. Histological evaluation of distal intestine*
(Mean values and standard deviations for fifteen fish)

	Fish meal control		Fish meal + SSC (1.7 g/kg)†		Fish meal + SSC (2.6 g/kg)†		Lupins‡		Lupins + SSC (1.7 g/kg)†		Lupins + SSC (2.6 g/kg)†		Soya beans§	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Final sampling														
Vacuoles	1.8 ^a	1.1	1.6 ^a	0.9	1.5 ^a	0.7	1.3 ^a	0.4	2.3 ^{a,b}	1.0	3.3 ^{b,c}	1.1	4.6 ^c	0.5
Lamina propria	1.5 ^a	0.6	2.0 ^{a,b}	0.4	1.4 ^a	0.5	1.9 ^{a,b}	0.6	2.2 ^{a,b}	0.7	2.7 ^b	0.8	4.9 ^c	0.2
Submucosa	1.6 ^a	0.5	1.9 ^{a,b}	0.6	2.1 ^{a,b}	1.2	2.2 ^{a,b}	0.7	2.2 ^{a,b}	0.6	2.9 ^b	1.0	4.7 ^c	0.5
Mucosal folds	1.5 ^a	0.4	1.5 ^a	0.4	1.5 ^a	0.5	1.7 ^a	0.4	2.5 ^b	0.6	3.0 ^{b,c}	0.7	4.8 ^c	0.4
Average score	1.6 ^a	0.4	1.7 ^a	0.4	1.7 ^a	0.6	1.8 ^a	0.4	2.3 ^{a,b}	0.6	3.0 ^b	0.7	4.7 ^c	0.3
Fish with an average score >3 (n)	0		0		1		0		1		9		15	

SSC, soya saponin concentrate.

^{a,b,c} Mean values with unlike superscript letters are significantly different (multiple comparisons test with mean ranks, Student–Newman–Keuls, $\alpha = 0.05$).

* Intestinal cuts were scored according to the criteria listed in Table 2. A score of 1–2 represents normal morphology, a score of 3 was given to distinct morphological signs of inflammation, while a score of 5 represents severe symptoms of enteritis.

† 65% purity (Organic Technologies, Coshocton, OH, USA).

‡ Lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia).

§ Defatted soyabean meal (Denofa, Fredrikstad, Norway).

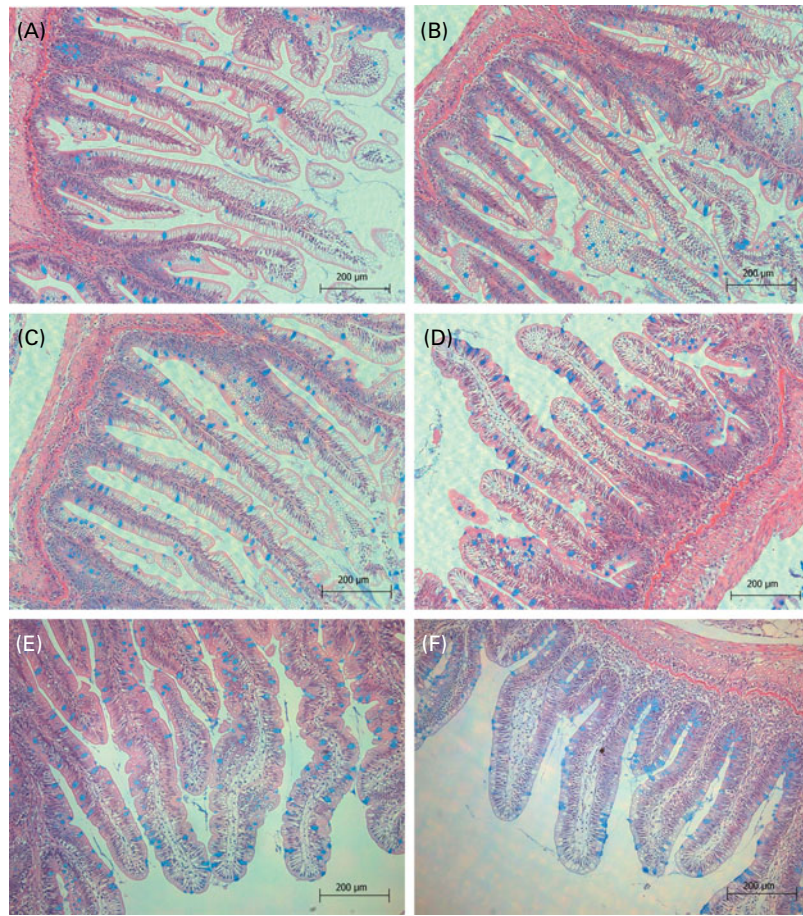


Fig. 2. Intestinal morphology of the distal intestine in Atlantic salmon (*Salmo salar* L.) fed different diets: (A) fishmeal-based control diet; (B) fishmeal-based control diet + soya saponin concentrate (2.6 g/kg); (C) diet containing 25% lupin kernel meal; (D) and (E) diet containing 25% lupin kernel meal + soya saponin concentrate (2.6 g/kg); (F) diet containing 25% defatted soyabean meal. Intestinal cuts were stained with a combination of haematoxylin-eosin and alcian blue 8 GX. Images were recorded in bright field (100 \times magnification) using a Leica digital microscope (model DM 5000B; Leica Microsystems GmbH, Wetzlar, Germany).

intestinal morphology whereas fish fed 25% soyabean meal developed severe enteritis. These observations are in agreement with a recent study by Refstie *et al.* (20). Interestingly, the present study shows that enteritis can be induced by 25% lupin kernel meal if soya saponins are added to the diet. The low level of soya saponins in lupin kernel meal therefore explains why lupins are well tolerated by Atlantic salmon whereas soyabean meal causes enteritis. Even though the combination of soya saponin concentrate and lupin kernel meal gave significant inflammation, the impact of 25% soyabean meal was stronger, despite a comparable total level of soya saponins. An explanation could be that the commercial soya saponin concentrate did not contain any DDMP-conjugated group B soya saponins or group A soya saponins. The glycosylation pattern of saponins is known to have a major impact on adjuvant activity, critical micelle concentration and the ability of saponins to disrupt cell membranes^(32–34). Another explanation could be that the activity of the saponins is lower when they are added in their purified forms compared with when they are naturally present within the soyabean matrix. Finally, it is possible that the unidentified components, that need to be present together with soya saponins, are more abundant in defatted soyabean meal than in lupin kernel meal.

The Ussing chamber results demonstrate that dietary soya saponins increase the intestinal permeability of the distal intestine in Atlantic salmon, as determined by both reduced transepithelial resistance and increased apparent permeability of [¹⁴C]mannitol. To our knowledge, this is the first time that orally delivered saponins have been demonstrated to increase the permeability of the intestine. Increased paracellular permeability can occur through increased tight junction permeability, or through disruption of the epithelial monolayer^(35,36). Both saponins⁽⁴⁾ and inflammation⁽³⁷⁾ are factors that may increase epithelial permeability. Fish fed soya saponin concentrate in combination with fishmeal had increased epithelial permeability but no signs of inflammation. The increased permeability observed in this group should therefore be ascribed to the effect of soya saponins alone whereas the increased epithelial permeability in fish fed 25% soyabean meal probably reflects a combined effect of soya saponins and the inflammatory reaction.

An impaired barrier function of the intestinal epithelium will make the underlying mucosa more exposed to foreign antigens from the gut lumen. We therefore suggest that soyabean-induced intestinal inflammation is a secondary effect of the membrane-disturbing properties of soya saponins. The foreign antigens, which induce the inflammation, could be

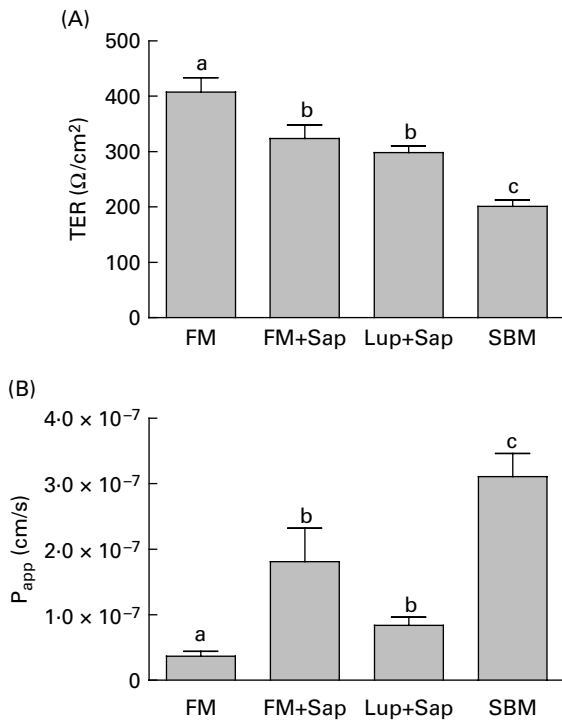


Fig. 3. Ussing chamber measurements of (A) transepithelial electrical resistance (TER) and (B) apparent permeability (P_{app}) for [^{14}C]mannitol in the anterior part of the distal intestine in seawater-adapted Atlantic salmon (*Salmo salar* L.). Values are means (n 12), with standard errors represented by vertical bars. ^{a,b,c}Mean values with unlike letters are significantly different (multiple comparisons test, Student–Newman–Keuls on log 10-transformed data, $\alpha = 0.05$). FM, fishmeal-based control diet; FM + Sap, fishmeal-based control diet + soya saponin concentrate (2.6 g/kg); Lup + Sap, diet containing 25% lupin kernel meal + soya saponin concentrate (2.6 g/kg); SBM, diet containing 25% defatted soyabean meal.

antigenic legume proteins or antigens from the intestinal microflora.

In a previous study it was demonstrated that 10% dietary soyabean molasses, which contain less than 5% protein, still induce enteritis after being processed in 1:1 (v/v) *n*-butanol at 70°C for more than 1 h⁽¹⁹⁾. In addition to the denaturing effect of the butanol treatment, these soyabean proteins had previously been exposed to ethanol extraction during production of soyabean protein concentrate. These considerations weaken the hypothesis that the inflammation should be a

response to native antigenic legume proteins. However, it cannot be ruled out that smaller peptides are involved.

Soyabean meal, lupin kernel meal and soyabean molasses contain high levels of non-digestible oligosaccharides^(19,31). Ringø *et al.*⁽³⁸⁾ have recently demonstrated that non-digestible carbohydrates also affect the gut microflora in fish. The intestinal inflammation might therefore be a response to a shift in the microbial population caused by high levels of non-digestible carbohydrates in the feed, in combination with increased epithelial permeability caused by soya saponins. The gut microflora is known to be involved in inflammatory bowel diseases in humans⁽³⁹⁾, and translocation of bacterial cells and bacterial antigens across the mucosal barrier has also been reported in fish^(40–43). In general, translocation of bacteria is favoured by bacterial overgrowth, reduced immunity of the host, or increased gut lining permeability⁽⁴⁴⁾. This hypothesis, however, seems to be weakened by a study of Refstie *et al.*⁽⁴⁵⁾, in which administration of the broad-spectrum antibiotic, oxytetracycline, did not suppress soyabean-induced enteritis. Recently, Bakke-McKellep *et al.*⁽⁴⁶⁾ confirmed these findings but they also demonstrated that the gut microflora changed dramatically when soya beans were introduced in the diet. Certain strains of bacteria, for example, *Enterococcus* spp., were found adherent to the distal intestine in fish fed soyabean meal but not in fish fed the fishmeal control diet. Adherent *Enterococcus* spp. in the distal intestine of fish fed soyabean meal was reduced, but not eliminated, by administration of oxytetracycline. Thus, it cannot be ruled out that the microflora plays a role in soyabean-induced enteritis.

Previous studies^(47,48) have reported soyabean-induced enteritis in Atlantic salmon to coincide with reduced faecal DM content. However, it has not been confirmed whether soyabean-induced diarrhoea is directly linked to intestinal inflammation or not. In general, diarrhoea can have several causes, including intestinal irritation, osmotic changes, psychogenic stimuli, and intestinal motility disorders⁽⁴⁹⁾. Lallès *et al.*⁽⁵⁰⁾ reported hypermotility of the colon, mediated by hypersensitivity, to be the main cause of soyabean-induced diarrhoea in preruminant calves, while soluble NSP in soya beans have been suspected to be major contributors to soyabean-induced diarrhoea in Atlantic salmon through osmotic mechanisms⁽⁵¹⁾. The faecal DM measurements in the present study revealed that soya saponins in combination with lupin kernel meal caused a significant drop in faecal DM content compared with the lupin-based control diet. No effect was seen of soya saponins combined with fishmeal compared

Table 6. Dry matter content in pooled faeces samples (Mean values and standard deviations for three tanks per diet)

	Fishmeal control		Fishmeal + SSC (1.7 g/kg)*		Fishmeal + SSC (2.6 g/kg)*		Lupins†		Lupins + SSC (1.7 g/kg)*		Lupins + SSC (2.6 g/kg)*		Soya beans‡	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Faecal DM content (%)	13.7 ^a	0.1	14.0 ^a	0.4	13.5 ^a	0.4	12.5 ^b	0.2	11.0 ^c	0.5	11.1 ^c	0.3	9.8 ^d	0.3

SSC, soya saponin concentrate.

^{a,b,c,d}Mean values with unlike superscript letters are significantly different (multiple comparisons test, Student–Newman–Keuls, $\alpha = 0.05$).

* 65% purity (Organic Technologies, Coshocton, OH, USA).

† Lupin kernel meal from sweet lupins (*Lupinus angustifolius*; Agracorp, West Perth, Western Australia).

‡ Defatted soyabean meal (Denofa, Fredrikstad, Norway).

with the fishmeal-based control diet. It can thus be concluded that enteritis is a major contributor to soyabean-induced diarrhoea. However, the present work also demonstrated that the lupin-based control diet caused significantly lower faecal DM than the fishmeal-based control diet, even though no signs of enteritis were observed. This might be due to osmotically driven diarrhoea caused by the high amounts of oligosaccharides and soluble NSP, which are found in most legumes, including lupins and soya beans⁽³¹⁾. In summary, soyabean-induced diarrhoea in Atlantic salmon should be considered as the net result of several diarrhoea-inducing factors of which enteritis and probably also osmotic mechanisms are the major contributors.

The present study has shown that orally delivered soya saponins increase the intestinal permeability in Atlantic salmon. A few studies have previously shown that soya saponins are degraded by the gut microflora in endothermic animals^(13–15). The study of Gestetner *et al.*⁽¹³⁾ demonstrated, however, that the degradation takes place in the caecum of chicks, rats, and mice, and that soya saponins pass intact through the small intestine in these species. Since *in vitro* studies have demonstrated that saponins increase the permeability of the small intestine in rats and rabbits^(4,5,7), it is not unlikely that orally delivered soya saponins could impair the barrier function of the small intestine in mammals. This could have important implications for infant nutrition since soya protein-based formulas, which are often used for infants already predisposed to food allergies, are made from soyabean protein isolate, which contains even higher levels of soya saponins than defatted soyabean meal used for animal feed^(18,52). Permeability enhancement caused by orally delivered soya saponins could, however, also have beneficial effects in pharmaceutical applications. The results from the present study make it therefore relevant to investigate the effect of orally delivered soya saponins on the mammalian intestine.

The present study demonstrates that soya saponins trigger the onset of an inflammatory reaction in the distal intestine of Atlantic salmon. For inflammation to occur, however, one or several other components also need to be present in the diet. These unidentified components are present in both soyabean meal and lupin kernel meal. Furthermore it is demonstrated that orally delivered soya saponins increase the epithelial permeability of the distal intestine in Atlantic salmon. The mechanism by which soya saponins trigger the onset of enteritis is suggested to be through increased epithelial permeability, which exposes the local immune system to foreign antigens from the gut lumen, leading to an inflammation reaction.

Acknowledgements

The study was funded by Skretting Aquaculture Research Centre, Stavanger, Norway. There are no conflicts of interest. D. K. designed the experiment, interpreted the results and wrote the article. D. K. was responsible for the fish trial and carried out the saponin analyses and the histological assessments. F. J. interpreted the gut permeability results and wrote the paragraphs in the article related to the Ussing chamber analyses. F. J. carefully read and edited the manuscript. H. S. installed the Ussing chambers, conducted all the Ussing chamber experiments and analysed the raw data. H. S. also commented on the manuscript. K. S. designed the

gut permeability part of the study and installed the Ussing chambers. K. S. also helped to edit the manuscript. W. K. and H. F. contributed equally to the study by helping to design the study and interpreting the results. W. K. and H. F. also helped to edit the manuscript.

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