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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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 transcriptive 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 soya-bean protein concentrate was free of the causative components, whereas soya bean 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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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 enteritis was caused by soya saponins. In a previous study(19) it was demonstrated that neither oligosaccharides nor soyabean protein isolate had potent feeding-deterrent properties in Chinook salmon and rainbow trout, and 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 trigger intestinal inflammation. 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 diet containing 25 % lupin kernel meal. In order to obtain comparable growth, diets were formulated to be isenergetic on a digestible energy basis (using table values on digestibility from Skretting’s raw material database). The remaining 35 % of the soya saponin concentrate was mainly oligosaccharide gums, according to the supplier.

### 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; Dinissen 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 isenergetic 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.
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
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Fishmeal* & 620.9 & 620.9 & 620.9 & 483.5 & 483.5 & 483.5 & 456.6
Lupin† & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0
Soya bean meal‡ & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0
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 off & 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‡‡ & 0.0 & 0.0 & 1.05 & 1.60 & 0.03 & 1.07 & 1.63 & 1.77

** 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).
§ Mineral and vitamin premix to meet the National Research Council recommendations(22).
‡ Defatted soyabean meal (Denofa, Fredrikstad, Norway).
† Lupin kernel meal from sweet lupins (Lupinus angustifolius; Agracorp, West Perth, Western Australia).
‡‡ 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*) 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 continued supply 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 methane-sulfonate, Finquel MS-222; Argent Chemical Laboratories, Redmond, WA, USA), for histological examination. The final sample was obtained per tank (three samples per treatment). 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, Hillerod, 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(10). 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-
Soyasaponins trigger intestinal inflammation

Extraction of soyasaponins from feed ingredients

Extraction was done at room temperature using 70 % aqueous ethanol. Soyasaponins 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 3000g 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 soyasaponin 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 15000g 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 (30 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 evaluated using light microscopy (Leica DM 5000B; Leica Microsystems GmbH, Wetzlar, Germany). One slide was prepared per fish. 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 (Papp) 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 Papp 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).

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

<table>
<thead>
<tr>
<th>Score</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Large vacuoles occupy almost the entire apical part of the enterocytes</td>
</tr>
<tr>
<td>2</td>
<td>Medium-sized vacuoles occupy less than half of the enterocytes</td>
</tr>
<tr>
<td>3</td>
<td>Small vacuoles are present near the apical membrane in most enterocytes</td>
</tr>
<tr>
<td>4</td>
<td>Scattered small vacuoles are still present in some enterocytes</td>
</tr>
<tr>
<td>5</td>
<td>No supranuclear vacuoles seem to be present</td>
</tr>
<tr>
<td></td>
<td>Lamina propria of simple folds</td>
</tr>
<tr>
<td>1</td>
<td>Lamina propria is a very thin and delicate core of connective tissue</td>
</tr>
<tr>
<td>2</td>
<td>Lamina propria appears slightly more distinct in some of the folds</td>
</tr>
<tr>
<td>3</td>
<td>Lamina propria has markedly increased in most of the folds</td>
</tr>
<tr>
<td>4</td>
<td>Lamina propria is thick in many folds</td>
</tr>
<tr>
<td>5</td>
<td>Lamina propria is very thick in many folds</td>
</tr>
<tr>
<td></td>
<td>Collagen fibrils</td>
</tr>
<tr>
<td>1</td>
<td>Almost no CT between base of folds and stratum compactum</td>
</tr>
<tr>
<td>2</td>
<td>Slightly increased amount of CT beneath some of the mucosal folds</td>
</tr>
<tr>
<td>3</td>
<td>Clear increase of CT beneath most of the mucosal folds</td>
</tr>
<tr>
<td>4</td>
<td>Thick layer of CT beneath many folds</td>
</tr>
<tr>
<td>5</td>
<td>Extreme thick layer of CT beneath some folds</td>
</tr>
</tbody>
</table>

CT, connective tissue; SF, simple folds; CF, complex folds.
Calculations of $P_{app}$ for mannitol was performed using:

$$P_{app} = \frac{dQ/dt}{AC_b},$$

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 $C_b$ 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 historical 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 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. 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. 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.

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

<table>
<thead>
<tr>
<th>Saponin</th>
<th>SSC*</th>
<th>Lupins†</th>
<th>Soya beans‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Ab</td>
<td>ND</td>
<td>ND</td>
<td>0.89</td>
</tr>
<tr>
<td>Ba</td>
<td>33.3</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Bb</td>
<td>355.8</td>
<td>18.4</td>
<td>2.54</td>
</tr>
<tr>
<td>Bc</td>
<td>168.2</td>
<td>10.4</td>
<td>0.93</td>
</tr>
<tr>
<td>Bb’</td>
<td>41.3</td>
<td>1.7</td>
<td>ND</td>
</tr>
<tr>
<td>Bc’</td>
<td>17.0</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Ba-DDMP</td>
<td>ND</td>
<td>ND</td>
<td>0.13</td>
</tr>
<tr>
<td>Bb-DDMP</td>
<td>ND</td>
<td>0.11</td>
<td>1.84</td>
</tr>
<tr>
<td>Bc-DDMP</td>
<td>ND</td>
<td>ND</td>
<td>0.74</td>
</tr>
<tr>
<td>Sum</td>
<td>615.6</td>
<td>31.4</td>
<td>7.06</td>
</tr>
</tbody>
</table>

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).
Fish fed 25% defatted soya bean 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, 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% soya bean 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 antigenic proteins and non-digestible carbohydrates30,31. However, fish fed 25% lupin kernel meal displayed normal control diet, however, had significantly lower faecal DM content than fish fed the fishmeal-based control diet.

**Table 4. Histological evaluation of distal intestine***

(Mean values and standard deviations from six fish)

<table>
<thead>
<tr>
<th>Intermediate sampling</th>
<th>Fishmeal control (1·7 g/kg)†</th>
<th>Fishmeal + SSC (2·6 g/kg)†</th>
<th>Fishmeal + SSC (2·6 g/kg)†</th>
<th>Lupins‡</th>
<th>Lupins + SSC (1·7 g/kg)†</th>
<th>Lupins + SSC (2·6 g/kg)†</th>
<th>Soyabean§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuoles</td>
<td>Mean  1·4</td>
<td>Mean  2·0</td>
<td>Mean  3·0</td>
<td>Mean  0·9</td>
<td>Mean  2·6</td>
<td>Mean  3·4</td>
<td>Mean  1·7</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>1·5</td>
<td>2·0</td>
<td>3·0</td>
<td>0·9</td>
<td>2·6</td>
<td>3·4</td>
<td>0·8</td>
</tr>
<tr>
<td>Submucosa</td>
<td>1·9</td>
<td>2·6</td>
<td>3·7</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
<td>0·6</td>
</tr>
<tr>
<td>Mucosal folds</td>
<td>2·0</td>
<td>2·6</td>
<td>3·7</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
<td>0·6</td>
</tr>
<tr>
<td>Average score</td>
<td>1·7</td>
<td>2·5</td>
<td>3·6</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
<td>0·6</td>
</tr>
</tbody>
</table>

**Table 5. Histological evaluation of distal intestine***

(Mean values and standard deviations for fifteen fish)

<table>
<thead>
<tr>
<th>Final sampling</th>
<th>Fish meal control (1·7 g/kg)†</th>
<th>Fish meal + SSC (2·6 g/kg)†</th>
<th>Lupins‡</th>
<th>Lupins + SSC (1·7 g/kg)†</th>
<th>Lupins + SSC (2·6 g/kg)†</th>
<th>Soyabean§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuoles</td>
<td>Mean  2·0</td>
<td>Mean  3·0</td>
<td>Mean  4·0</td>
<td>Mean  0·9</td>
<td>Mean  2·6</td>
<td>Mean  3·4</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>1·5</td>
<td>2·0</td>
<td>3·0</td>
<td>0·9</td>
<td>2·6</td>
<td>3·4</td>
</tr>
<tr>
<td>Submucosa</td>
<td>1·9</td>
<td>2·6</td>
<td>3·7</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
</tr>
<tr>
<td>Mucosal folds</td>
<td>2·0</td>
<td>2·6</td>
<td>3·7</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
</tr>
<tr>
<td>Average score</td>
<td>1·7</td>
<td>2·5</td>
<td>3·6</td>
<td>0·8</td>
<td>2·9</td>
<td>4·0</td>
</tr>
</tbody>
</table>

**SSC, soya saponin concentrate.**

* Mean values with unlike superscript letters are significantly different (multiple comparisons test with mean ranks, Student–Newman–Keuls, α = 0.05).
* Mean values with unlike superscript letters are significantly different (multiple comparisons test with mean ranks, Student–Newman–Keuls, α = 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 soya bean meal (Denola, Fredrikstad, Norway).
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. 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 x magnification) using a Leica digital microscope (model DM 5000B; Leica Microsystems GmbH, Wetzlar, Germany).
Soya saponins trigger intestinal inflammation

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\(^*\). Rings\textit{ et al.}\(^{38}\) have recently demonstrated that non-digestible carbohydrates also affect the gut microbiota 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 microbiota 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 \textit{et al.}\(^{45}\), in which administration of the broad-spectrum antibiotic, oxytetracycline, did not suppress soyabean-induced enteritis. Recently, Bakke-McKellep \textit{et al.}\(^{46}\) confirmed these findings but they also demonstrated that the gut microbiota changed dramatically when soya beans were introduced in the diet.

Certain strains of bacteria, for example, \textit{Enterococcus} spp., were found adherent to the distal intestine in fish fed soyabean meal but not in fish fed the fishmeal control diet. Adherent \textit{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 microbiota 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\(^{49}\). Lalles \textit{et al.}\(^{50}\) reported hypermotility of the colon, mediated by hypersensitivity, to be the main cause of soyabean-induced diarrhoea in prminorant 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

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 process 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

Table 6. Dry matter content in pooled faeces samples

<table>
<thead>
<tr>
<th></th>
<th>Fishmeal control</th>
<th>Fishmeal + SSC (1.7 g/kg)(^*)</th>
<th>Fishmeal + SSC (2.6 g/kg)(^*)</th>
<th>Lupins†</th>
<th>Lupins + SSC (1.7 g/kg)(^*)</th>
<th>Lupins + SSC (2.6 g/kg)(^*)</th>
<th>Soya beans‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>Faecal DM content (%)</td>
<td>13·7(^a)</td>
<td>14·0(^b)</td>
<td>13·5(^b)</td>
<td>12·5(^b)</td>
<td>11·0(^c)</td>
<td>11·1(^e)</td>
<td>9·8(^d)</td>
</tr>
</tbody>
</table>
with the fishmeal-based control diet. It can thus be concluded that enteritis is a major contributor to soybean-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\(^{(51)}\). In summary, soybean-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 \textit{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 soya bean protein isolate, which contains even higher levels of soya saponins than defatted soya bean 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

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