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Saponin-Containing Subfractions of Soybean Molasses Induce Enteritis in the Distal Intestine of Atlantic Salmon

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

KEYWORDS: Soybeans; Atlantic salmon; enteritis; molasses; saponins

INTRODUCTION

A limited supply of fishmeal could hamper future growth in the aquaculture industry, and much effort has therefore been made to find alternative protein sources that could replace fishmeal in feed formulations for carnivorous fish (*1*−*4*). From an ecological viewpoint, an ideal solution would be to find a suitable low-cost plant-derived protein. Soybean meal is one of the promising candidates due to its high protein content and steady supply. Several studies have revealed, however, that high inclusion levels of low-processed soybean products induce intestinal inflammation in the hindgut of Atlantic salmon (*5*−*9*). The inflammatory reaction is associated with several morphological changes, including loss of supranuclear vacuoles in the absorptive enterocytes, widening of the lamina propria of mucosal folds, increased amounts of connective tissue between the base of the mucosal folds and the stratum compactum, shortening of mucosal fold height, and infiltration of inflammatory cells in the lamina propria (*5*−*7*). The normal morphology of the distal intestine in Atlantic salmon is shown in **Figure 1A**, while **Figure 1B** displays typical soybean-induced enteritis. The enteritis associated with soybean meal currently limits its use in diets for Atlantic salmon.

Despite considerable work, the causative components for the condition remain unidentified. Important clues can, however, be found as to their identity. Van de Ingh et al. (*5*, *6*) demonstrated that they follow the protein fraction when the oil is extracted by hexane. Alcohol-extracted soybean protein

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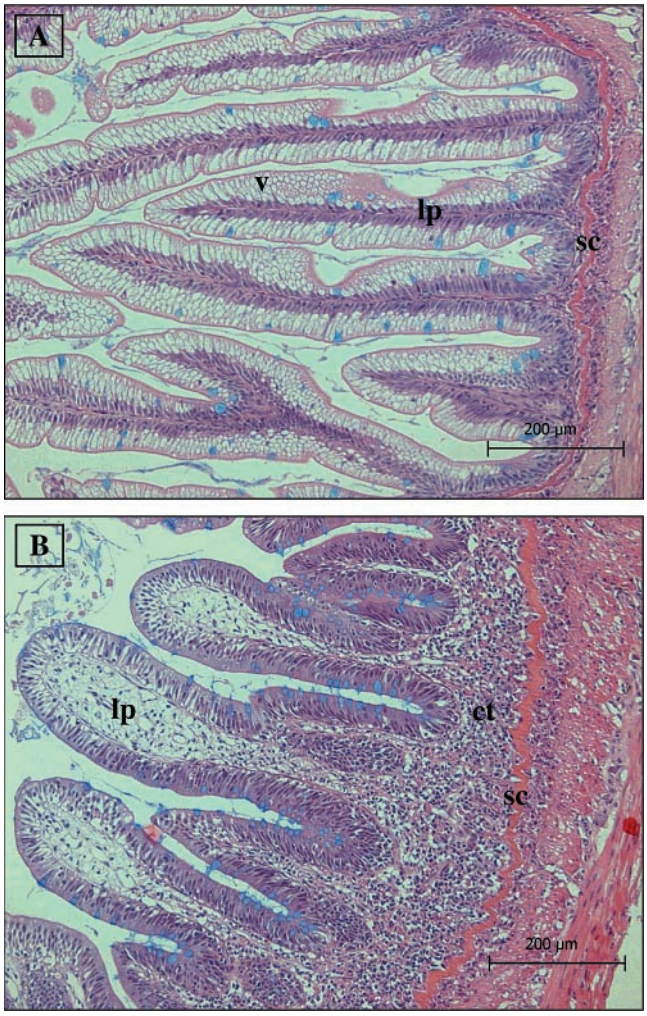

Figure 1. (**A**) Normal morphology of distal intestine in Atlantic salmon. (**B**) Typical signs of soybean-induced enteritis: Loss of vacuoles (v) in absorptive enterocytes, widening of lamina propria (lp) in mucosal folds, and increase of connective tissue (ct) between base of folds and stratum compactum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

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

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

MATERIALS AND METHODS

Separation of Soybean Molasses. Soybean molasses was kindly provided by Solae Denmark A/S (Århus, Denmark). The molasses was separated into three subfractions by phase separation using *n*-butanol (product 33065, Sigma-Aldrich). Molasses and water-saturated *n*-butanol were mixed 1:1 (v/v) and allowed to separate overnight in a separation funnel. A dense layer of yellow precipitate formed between the two phases. The mixture was separated into three fractions (butanol phase, precipitate, and water phase) and evaporated to dryness at 70 °C in a rotary evaporator under reduced pressure. The residues were resuspended in water and evaporated to dryness again several times in order to remove butanol completely. Each subfraction was finally resuspended in water to reach the initial volume of molasses. The batch of soybean molasses used in the present work contained 62% dry matter (w/w). Preliminary analyses of the obtained fractions revealed that 15% of the total dry matter was recovered in the butanol phase, 35% in the precipitate, and 50% in the water phase. Molasses and the obtained subfractions were analyzed for protein, fat, ash, sucrose, raffinose, stachyose, and soyasaponins. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the size distribution of the proteins present within each fraction.

Dry Matter and Ash. Dry matter measurements were done by drying to constant weight at 102−105 °C. Ash was measured by burning samples at 550 °C for 16−18 h.

Protein and Fat. Crude protein was quantified as $N \times 6.25$ using a Kjeltec autosampler system (Tecator AB, Sweden) according to Nordic Committee on Food Analysis (Method no. 6, 4th ed., 2003). Total fat was measured by acid hydrolysis using a Soxtec 2050 extraction system (Foss Analytical, Denmark) according to Nordic Committee on Food Analysis (Method no. 160, 1998).

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

Quantification of Soyasaponins. Separation and quantification of soyasaponins were performed using reverse-phase high-performance liquid chromatography with diode array detection (HPLC-DAD) as described previously (*10*). Briefly, the separation was achieved using a Hewlett-Packard series 1050 HPLC-DAD system with a 250 mm × 4.6 mm i.d., 5 μm, Supercosil ABZ + Plus, C_{18} reverse-phase column (Supelco). The mobile phases were 0.05% trifluroacetic acid in water (solvent A) and 0.05% trifluroacetic acid in acetonitrile (solvent B). The gradient elution was linear from 25 to 50% B, 0−65 min; linear from 50 to 60% B, 65−70 min; linear from 60 to 100% B, 70−75 min; isocratic at 100% B, 75−85 min; then linear from 100 to 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 soybean saponins was confirmed by HPLC retention time, UV absorption spectra recorded at 200−350 nm, and liquid chromatography−mass spectrometry using positive electrospray ionization. Molasses and molasses subfractions were diluted 1:10 in 70% aqueous ethanol and centrifuged at 15000*g* for 5 min before injection on the HPLC system. Detected and quantified were the following soyasaponins: Ab, Ac, Af, Ba, Bb, Bc, Ba-DDMP, Bb-DDMP, and Bc-DDMP (see ref *10* for molecular structures).

SDS-PAGE. The size distribution of the proteins present in the different molasses fractions was evaluated by SDS-PAGE according to ref *11*. The electrophoresis was done using 10−20% tricin gradient gels (Novex, Invitrogen, Groningen, The Netherlands). The resuspended molasses fractions were diluted 1:10 in H_2O and mixed 1:1 with sample buffer [0.1 M Tris buffer, 8% (w/v) SDS, 24% (v/v) glycerol, 0.025% (w/v) Coomassie blue, and 0.04 M 1,4-dithiothreitol, pH 6.8]. The mixtures were boiled for 5 min before they were loaded on the gel (10 μL per well). A standard protein mixture was included on the gel for

Table 1. Formulation of Diets for Fish Trial 1

		diet					
		A	B	C	D	E	F
fish meal (Scandinavian LT)	g/kg	490	625	625	625	625	625
wheat	g/kg	108	120	120	120	120	120
wheat starch	g/kg	0	53	9	0	0	62
minerals, vitamins, pigment	g/kg	3	3	3	3	3	3
defatted soybean meal[a]	g/kg	200	0	0	0	0	0
butanol phase[b]	g/kg	0	9	0	0	0	0
precipitate and water phase[b]	g/kg	0	0	53	0	0	0
butanol-treated molasses[b]	g/kg	0	0	0	62	0	0
untreated molasses[b]	g/kg	0	0	0	0	62	0
fish oil[c]	g/kg	199	190	190	190	190	190
total	g/kg	1000	1000	1000	1000	1000	1000
molasses equivalents[d]			10%	10%	10%	10%	

[a] Denofa, Norway. [b] Dry matter. [c] Northern hemisphere. [d] Wet matter basis.

molecular weight estimation (Mark12, product LC5677, Noves, Invitrogen, the Nederlands). Electrophoresis was carried out for 1 h and 20 min at 125 V (constant). Finally, the gel was stained with Coomassie blue.

Production of Feed for Fish Trial 1. Six diets were produced as 4 mm pellets by twin-screw extrusion cooking (TX57, Wenger Manufacturing, Inc., United States) at Skretting Feed Technology Plant (Stavanger, Norway). Molasses and molasses fractions were mixed with the other ingredients before extrusion. The molasses batch contained 620 g dry matter per kg, and 15% of this dry matter was recovered in the butanol phase, 35% in the precipitate, and 50% in the water phase. By knowing these ratios, it was possible to calculate how much soybean molasses corresponded to the added amounts of subfractions. The different subfractions were included at a level that corresponded to approximately 10% (w/w, wet basis) soybean molasses. The recipes are shown in **Table 1**.

Production of Feed for Fish Trial 2. Dry pellets (4 mm) were produced by twin-screw extrusion cooking (TX57, Wenger) at Skretting Feed Technology Plant. The pellets had the following composition: 80% fishmeal, 15.5% wheat, 4% wheat starch, and 0.5% premix (minerals, vitamins, and pigment). These dry pellets were used as a carrier matrix to test the impact of all different combinations of subfractions. Instead of adding the molasses fractions before extrusion, the molasses fractions were coated on the dry pellets using a specially designed lab-scale vacuum coater. A known amount of dry molasses subfraction was resuspended in water and sprayed onto the dry pellets under reduced pressure. The coated pellets were then dried at 102 °C to achieve a moisture content of 10%. Using the same lab-scale coater, pellets were thereafter coated with sufficient fish oil to ensure that they would sink. The different subfractions were again included at a level that corresponded to approximately 10% (w/w, wet basis) soybean molasses. The quantity of oil required to ensure pellets that would sink differed between diets. Consequently, the control diet (not containing any molasses) and the diet containing the butanol phase only were relatively high in fish oil content as compared to the other diets (**Table 2**). In summary, all diets contained the same carrier matrix but were coated with different subfractions of molasses and quantities of oil. Recipes for all diets are shown in **Table 2**.

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

Fish Trial 2. The trial was conducted at Skretting Fish Trials Station. Seawater-adapted Atlantic salmon (*S. salar* L.) with an initial average weight of 202 g were fed eight different experimental diets for 44 days. The fish were randomly distributed to eight circular 100 L fiberglass tanks at a stocking density of 20 fish per tank. The tanks were equipped with waste feed collection and continuously supplied with seawater (4 L/min per tank). Water was pumped from 90 m depth and held a constant temperature of 9.0 °C during the experiment. The eight different diets were fed to the eight different tanks (one tank per treatment) twice a day, aiming at 20% overfeeding, and waste feed was collected. At the end of the feeding period, 10 fish from each tank were sacrificed with an overdose of anesthetic (tricaine methanesulfonate, Finquel MS-222, Argent Chemical Laboratories), for histological examination.

Histological Examination. A 2 cm section of the distal intestine was carefully removed, rinsed in saline water, and fixed in phosphate-buffered formaldehyde (4%, pH 7.2). Samples were then dehydrated, embedded in paraffin, and cut according to standard histological procedures. Slides were then stained with a combination of hematoxylin and eosin and Alcian blue 8 GX. The latter was included in order to increase the contrast between goblet cells and vacuoles. Four different morphological parameters were evaluated using light microscopy (Leica DM 5000B) according to the scoring criteria given in **Table 3**. A score of "1−2" represented normal morphology, while a score of "5" was given to morphological symptoms of severe enteritis. The semiquantitative scoring system was adapted from Urán et al. (*12*). Histological samples were randomized and blindly evaluated.

Statistics. The histological scoring results were treated as nonparametric data. Kruskal−Wallis one-way analysis of variance was therefore

Table 2. Formulation of Diets for Fish Trial 2[a]

		diet							
		A	B	C	D	E	F	G	H
fish meal (Scandinavian LT)	g/kg	574	591	570	587	574	581	583	540
wheat	g/kg	111	114	110	113	111	112	113	104
wheat starch	g/kg	29	29	28	29	28	29	29	27
minerals, vitamins, pigment	g/kg	3	3	3	3	3	3	3	3
butanol phase (DM)	g/kg	10	0	0	0	0	0	0	0
precipitate (DM)	g/kg	0	23	0	0	0	0	0	0
water phase (DM)	g/kg	0	0	31	0	0	0	0	0
butanol phase and precipitate (DM)	g/kg	0	0	0	41	0	0	0	0
butanol phase and water phase (DM)	g/kg	0	0	0	0	44	0	0	0
precipitate and water phase (DM)	g/kg	0	0	0	0	0	57	0	0
untreated molasses (DM)	g/kg	0	0	0	0	0	0	66	0
fish oil (Northern hemisphere)	g/kg	273	240	258	227	240	218	213	326
total	g/kg	1000	1000	1000	1000	1000	1000	1000	1000
molasses equivalents (wet matter basis)		11.0%	10.7%	10.1%	13.1%	10.9%	10.8%	10.6%	

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

Table 3. Histological Scoring System for Morphological Changes Induced by Soybeans in the Distal Intestine of Atlantic Salmon[a]

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

[a] Adapted from ref *12*.

applied for testing equality of score medians among treatment groups. A multiple comparisons test with mean ranks (Student−Newman−Keuls, $\alpha = 0.05$) was used as a posthoc test to compare all pairs of mean ranks.

RESULTS

Soybean molasses and obtained subfractions were subjected to several biochemical analyses. Protein, fat, and ash were analyzed according to standard methods. Sucrose, raffinose, and stachyose were quantified using HPAEC-PAD. Soyasaponins were quantified using HPLC-DAD. The compositions of soybean molasses and the three different subfractions are shown in **Table 4**. The composition of molasses was in good agreement with the supplier data. Sucrose and stachyose were the main oligosaccharides present in molasses. Approximately two-thirds of the oligosaccharides was recovered in the water phase and one-third was recovered in the precipitate, while the butanol phase was almost free of oligosaccharides. The soyasaponins were separated in a ratio of approximately 60:40 between the butanol phase and the precipitate, while the water phase contained only trace amounts.

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

Two separate fish trials were carried out in order to test the effect of the obtained subfractions on intestinal morphology. The objective of trial 1 was to investigate whether the butanol treatment, and the subsequent evaporation at 70 °C, would inactivate the causative component(s) and if the isolated butanol phase could independently induce enteritis. A diet containing regular defatted soybean meal was included for the purposes of comparison. Results from the histological evaluation are given in **Table 5**. Fish fed the control diet (without soybeans)

Table 4. Composition of Molasses and Distribution of the Different Components after Phase Separation[a]

	molasses (g/kg) (wet matter basis)	relative distribution between phases		
		butanol phase (%)	precipitate (%)	water phase (%)
dry matter	620 ± 5	15	35	50
ash	43 ± 6	5	20	75
protein ($N \times 6.25$)	52 ± 1	13	51	35
fat	96 ± 8	68	32	0
sucrose	219 ±22	4	34	62
raffinose	23 ± 1	2	30	67
stachyose	117 ± 8	1	31	67
soyasaponins[b]	20 ± 1	60	39	1
unidentified residue[c]	50			

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

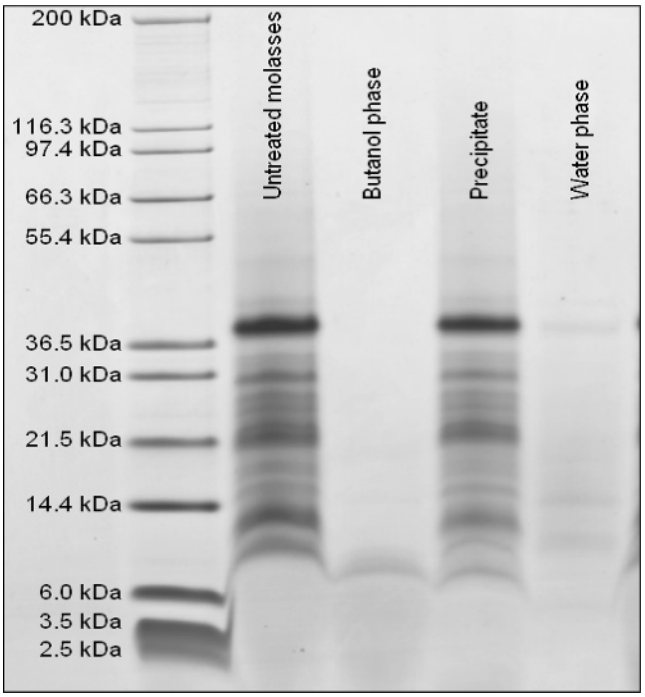

Figure 2. SDS-PAGE of molasses fractions using a 10–20% tricin gradient gel. First lane from left, Mark12.

Table 5. Histological Evaluation of Distal Intestine[a]

fish trial 1	diet A: soybean meal	diet B: butanol phase	diet C: precipitate and water phase	diet D: butanol-treated molasses	diet E: untreated molasses	diet F: control
vacuoles	2.92 ± 0.90 bc	3.96 ± 1.01 c	2.46 ± 1.23 ab	3.63 ± 0.61 bc	3.08 ± 0.60 bc	1.50 ± 0.60 a
lamina propria	3.96 ± 0.86 c	3.04 ± 0.84 bc	2.33 ± 1.21 ab	3.75 ± 0.78 c	3.63 ± 0.57 c	1.29 ± 0.69 a
connective tissue	3.71 ± 0.92 b	3.46 ± 0.99 b	2.17 ± 0.94 a	3.92 ± 0.82 b	3.96 ± 0.86 b	2.00 ± 1.07 a
mucosal folds	4.13 ± 0.68 b	2.33 ± 0.49 a	2.42 ± 1.06 a	3.88 ± 0.68 b	3.71 ± 0.69 b	1.42 ± 0.47 a

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

Table 6. Histological Evaluation of Distal Intestine[a]

fish trial 2	diet A: butanol phase	diet B: precipitate	diet C: water phase	diet D: butanol phase and precipitate	diet E: butanol and water phase	diet F: precipitate and water phase	diet G: untreated molasses	diet H: control
vacuoles	4.20 ± 0.86 cd	2.75 ± 1.21 abc	1.70 ± 0.59 a	4.40 ± 0.94 d	3.60 ± 0.70 bcd	2.05 ± 1.04 ab	4.35 ± 0.58 d	2.05 ± 0.50 ab
LP	3.15 ± 0.47 b	3.00 ± 0.82 b	1.90 ± 0.74 ab	3.15 ± 0.75 b	2.85 ± 0.75 b	1.80 ± 0.82 ab	3.10 ± 1.13 b	1.00 ± 0.00 a
CT	2.55 ± 0.55 ab	2.30 ± 0.75 ab	1.80 ± 0.67 ab	2.95 ± 0.93 b	2.50 ± 0.58 ab	1.75 ± 0.75 a	3.05 ± 1.19 b	1.65 ± 0.47 a
MF	2.25 ± 0.54 abc	2.00 ± 0.53 ab	1.50 ± 0.33 a	3.50 ± 0.91 cd	3.15 ± 0.53 bcd	2.35 ± 1.00 abc	3.75 ± 0.75 d	1.25 ± 0.26 a

[a] Intestinal cuts were scored according to the criteria listed in **Table 3**. A score of "1–2" represents normal morphology while a score of "5" represents severe enteritis. Reported data are mean values from 10 fish ± SD. Means followed by different letters are significantly different (multiple comparisons test with mean ranks, Student–Newman–Keuls, $\alpha = 0.05$). LP, lamina propria; CT, connective tissue; and MF, mucosal folds.

displayed normal morphology, while significant enteritis was observed in fish fed 20% defatted soybean meal. Diets containing 10% untreated molasses and 10% butanol-treated molasses induced severe morphological changes similar to the 20% soybean meal diet. The diet containing a combination of precipitate and water phase had only a weak effect on all four morphological parameters. Interestingly, the butanol phase-containing diet had a strong impact on vacuoles, lamina propria, and connective tissue but did not provoke the stubby appearance of mucosal folds typically associated with soybean-induced enteritis.

Trial 2 was initiated to investigate why neither of the two subfractions tested in trial 1 had a strong effect on mucosal folds. Results from the histological evaluation are shown in **Table 6**. Fish fed the water phase displayed normal morphology, while fish fed the combination of butanol phase and precipitate showed the same morphological changes as fish fed soybean molasses. Results from trial 2 were very consistent with those from trial 1, clearly demonstrating that the trigger component was split between the butanol phase and the precipitate whereas this component was absent from the water phase. Soyasaponins were the only quantified components that were poorly separated between the butanol phase and the precipitate (**Table 4**). Fish fed the butanol phase alone showed the same morphological changes as observed in trial 1; vacuoles, lamina propria, and connective tissue were significantly affected, but only a weak impact was observed on mucosal folds. Interestingly, the combination of water and butanol phases had a significantly greater impact on mucosal folds than the butanol phase alone.

DISCUSSION

Soybean molasses has previously been shown to contain components that cause soybean-induced enteritis in the distal intestine of Atlantic salmon (*6*, *13*). In the present study, soybean molasses was separated into three subfractions by phase separation and both the molasses and the obtained subfractions were subjected to extensive biochemical analyses. All possible combinations of the three subfractions were fed to Atlantic salmon, and the impact on intestinal morphology was evaluated. Results revealed that fish fed the water phase displayed normal morphology while fish fed a combination of butanol phase and precipitate showed the same morphological changes as fish fed soybean molasses. Soyasaponins were the only quantified components that were poorly separated between the butanol phase and the precipitate. It can thus be concluded that soyasaponins, or components that follow the same solubility pattern, need to be present to induce the inflammatory reaction. Bureau et al. (*14*) demonstrated that Quillaja saponins cause extensive damage to the intestinal mucosa of the hindgut in Chinook salmon and rainbow trout. This supports the hypothesis that soyasaponins play a key role in soybean-induced enteritis in Atlantic salmon.

Biochemical analyses of molasses revealed that the main oligosaccharides present were sucrose and stachyose, which is in accordance with ref *15*. The separation of soyasaponins was surprisingly poor, and only 60% of the total amount was found in the butanol phase, even though *n*-butanol is a suitable solvent for the extraction of soyasaponins (*16*). The remaining 40% was found in the precipitate while the water phase contained only trace amounts of soyasaponins. It is known that soyasaponins follow the protein fraction during production of soybean protein isolates (*17*). The high amount of retained saponins in the precipitate might therefore be due to protein−saponin interactions.

The histological evaluation in fish trial 1 confirmed that soybean molasses contains the causative factors for soybean-induced enteritis. Moreover, the components proved to be extremely stable since they were able to withstand both butanol treatment and evaporation to dryness at 70 °C. The butanol phase contained components that effectively disrupted vacuolization, probably by interfering with endocytosis. The butanol phase also caused an intermediate increase of lamina propria and connective tissue. The mean score for mucosal folds, however, was only slightly affected. The fact that the butanol phase affected some, but not all, of the evaluated parameters could indicate that the causative factor was poorly separated between the two subfractions (1, butanol phase; and 2, precipitate and water phase). Fish trial 2 revealed that the causative component

was split between butanol phase and precipitate, while the water phase was free from this component. The diet that included both precipitate and water phase had only a weak effect on all four histological parameters in both trials. The precipitate and water phase in combination contained more than 95% of all sucrose, raffinose, and stachyose in molasses. Hence, it can be concluded that the oligosaccharides alone do not trigger the inflammatory reaction. The same argument holds for soybean proteins larger than approximately 10 kDa since the precipitate and water phase together contained all larger proteins in molasses. It can therefore be concluded that the major antigenic soybean proteins (including glycinin, β-conglycinin, and lectins) do not induce intestinal inflammation alone.

Gypsophila saponins have been shown to increase the transmucosal uptake of the milk allergen β-lactoglobulin in the small intestine of rats in vivo (*18*), and several in vitro studies with saponins have demonstrated increased trans-epithelial uptake of macromolecules (*19*−*22*). In contrast to earlier findings in endothermic animals, soyasaponins were recently found to resist degradation during gut passage in Atlantic salmon (*10*). The observed inflammatory reaction might therefore be a secondary effect of increased intestinal permeability facilitated by soyasaponins. Increased intestinal permeability could expose the underlying mucosa to antigenic soybean proteins or perhaps to intestinal microflora. The gut microflora are known to be involved in inflammatory bowel diseases in humans (*23*), and translocation of bacterial cells and bacterial antigens across the mucosal barrier has also been reported in fish (*24*−*26*). In general, translocation of bacteria is favored by bacterial overgrowth, reduced immunity of the host, or increased permeability of the gut lining (*26*). A recent study by Ringø et al. (*27*) has demonstrated that nondigestible carbohydrates also affect fish gut microflora. A shift in the microbial population, caused by high levels of nondigestible carbohydrates in the feed, might therefore explain why the water phase, which was high in carbohydrates but very low in both proteins and soyasaponins, seemed to increase the impact of the butanol phase on the mucosal folds.

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

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

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