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Removal of three proteinaceous antinutrients from soybean does not mitigate soybean-induced enteritis in Atlantic salmon (*Salmo salar*, L)

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ABSTRACT

A new non-GM cultivar of soybean, named *Triple Null* (TN), devoid of Kunitz trypsin inhibitor, lectin and the allergen P34/Gly m Bd 30 k has been developed from a commercial cultivar. Use of standard soybean meals in diets for Atlantic salmon most often induces distal intestinal inflammation whereas soy protein concentrate (SPC), in which several antinutrients are either removed or inactivated, does not. To find whether TN, devoid of three proteinaceous antinutrients, may lack the ability to trigger gut inflammation, an 8-week feeding experiment with Atlantic salmon (mean weight 41 g) was conducted comparing TN, its commercial counterpart (CSBM) and SPC at an inclusion level of 25% of crude protein, using SPC as a negative control. The diets were extruded at two levels of specific mechanical energy (SME). The results for TN and CSBM did not differ significantly regarding fish growth and body composition. For protein and amino acid digestibility, lower values were observed for TN than CSBM, but only at low SME. For protein retention, TN showed lower values than CSBM independent of SME treatment. Also, lipid digestibility was lower for TN than CSBM. Chyme bile salt concentration in proximal intestine was lower in fish fed TN than CSBM. Elevated trypsin activity in chyme from distal intestine was observed for both cultivars. The distal intestinal tissue regarding tissue weight, digestive enzyme activity, histological appearance and chyme microbiota were also similar for TN and CSBM. Both cultivars induced enteritis in the distal intestine. Expression of pro-inflammatory genes, as well as two stress related genes, were elevated for TN compared to CSBM. For most of the observed biomarkers, SPC showed improved values compared to TN and CSBM and no signs of enteritis was seen. SPC distinguished itself also regarding gut microbiota. Elevation of SME improved protein and amino acid digestibility, but only for TN and CSBM. The main conclusion is: The nutritional value of TN for Atlantic salmon is similar to that of CSBM. The explanation for the lack of effect of removal of the antinutrients is most likely that the extrusion process used for feed production is sufficient to inactivate proteinaceous antinutrients. Removal of these, therefore, does not affect nutritional value for Atlantic salmon.

1. Introduction

Cultivation of Atlantic salmon has grown rapidly since its beginning in the early 1970s. A limited supply of marine ingredients has stimulated the search for alternatives. Over the last three decades plant proteins and oil have been incorporated in salmon feed at increasing levels (Ytrestoyl et al., 2015). One of the main challenges associated with the use of plant ingredients is the presence of antinutritional factors (ANFs) that can negatively affect feed intake and fish health (Krogdahl et al., 2010). Standard soybean meal (SBM), the major protein rich ingredient in animal feeds worldwide, contains several ANFs, including trypsin inhibitors, lectins, goitrogenic factors, phytic acid, rachitogenic factors and saponins (Yasothai, 2016). Most domesticated animals seem to tolerate these, only showing reduced feed efficiency, when fed diets containing soybean meal. Atlantic salmon, however, when fed SBM at levels above 5–10%, show a pathologic condition

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Table 1a
Formulation of the experimental diets.

Formulation (g/kg)	Diet			Source
	TN	CSBM	SPC	
Fish meal, LT	230	235	236	NorsECO-LT, Norsildmel, Fyllingsdalen, Norway
Soy protein concentrate	0	0	190	68% protein, phytase treated, Soja protein, Becej, Serbia
Full fat soy, conventional	0	250	0	Minnesota Soybean, USA
Full fat soy, TN	270	0	0	*Triple Null*, Minnesota Soybean, USA
Vital wheat gluten	70	70	70	Gluvital 21TN, Cargill, Barby, Germany
Wheat	150	150	150	Feed grade wheat, Felleskjøpet, Kambo, Norway
Fish oil	220	236	240	NorSalmOil, Norsildmel, Fyllingsdalen, Norway
Soybean oil	0	0	50	Raw Soybean oil, Denofa, Fredrikstad, Norway
Choline chloride	0.3	0.3	0.3	Choline Chloride-70%, Indukern, S.A., Spain
Mono calcium phosphate	18.2	18.2	18.2	Mono calcium phosphate monohydrate-Feed Grade, Yara Animal Nutrition, Oslo, Norway
Limestone	4	4	4	Franzefoss Miljøkalk AS, Rud, Norway
L-Lysine	7	7	9	Lysine monoHCl, 99% feed grade, Cheil Jedang, Indonesia
DL-Methionine	13	13	15	Rhodimet® NP 99, *DL*-methionine, 99% feed grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil
L-Tryptophan	0.6	0.6	0.6	98% feed grade, PT. Cheil Jedang, Indonesia
L- Arginine	5	5	5	L-Arginine, 98.5%, Sigma-Aldrich Logistik GmbH, Steinheim, Germany
L- Threonine	1.8	1.8	1.8	98.5% feed grade, CJ (Shenyang) Biotech Co. LTD, Liaoning, China
L-Valine	4	3	4	96.5% feed grade, Ajinomoto Eurolysine, Paris, France
Stay C 35%	1	1	1	ROVIMIX, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland
Yttrium oxide	0.1	0.1	0.1	Metal Rare Earth Limited, Jiaxing, China
Premix	5	5	5	Vilomix Norway AS, Hønefoss, Norway; provides per kg feed, Vitamin A 2500 IU; Vitamin D_3 2400 IU; Vitamin E 0.2 IU; Vitamin K_3 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 mg; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.20 g; Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; total Ca: 0.915 g; total K: 1.38 g; total Na 0.001 g; total Cl 1.25 g.

referred to as soybean meal induced enteritis (SBMIE). This condition, observed in the distal intestine of both Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), is characterized by shortening of intestinal folds, decreased supranuclear vacuolization, thickening and increased immune cell infiltration in lamina propria and submucosa, reduced brush border enzymatic activity, elevated trypsin activity in the chyme and major alterations in immune, barrier and metabolic gene expression (Bakke-McKellep et al., 2007b; Bakke-McKellep et al., 2000b; Kortner et al., 2012; Marjara et al., 2012; Sahlmann et al., 2013; van den Ingh et al., 1991). Saponin has been found to be the ANF responsible for SBMIE, but interaction with other antinutrients, which can reinforce the condition, is likely (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2015). Alcohol-water washing, a costly step in production of soy protein concentrate (SPC), removes and inactivates several antinutrients and results in a product with do not induce SBMIE (van den Ingh et al., 1991). The content of ANFs in soybeans varies between cultivars (Becker-Ritt et al., 2004; Domagalski et al., 1992). By screening of a high number of cultivars and cross breeding of selected lines a new cultivar, *Triple Null*, has been developed, essentially devoid of three of the proteinaceous ANFs, namely the Kunitz trypsin inhibitor, the lectin and the immune-dominant allergen P34/Gly m Bd 30 k (Choi et al., 2016; Schmidt et al., 2015). The main objective of the work presented herein was to find whether removal of three proteinaceous soybean antinutrients from a standard soybean meal might prevent or reduce the severity of the inflammation most often observed in Atlantic salmon fed diets with standard soybean meal. As proteinaceous antinutrients are denatured to a large extent, but not completely, by the extrusion used in fish feed production, an additional aim was to find possible effects of varying conditions during feed processing.

2. Materials and methods

2.1. Study design

The study was designed according to a 3*2 factorial model. The factors were soy source and level of SME, i.e. specific mechanical energy (SME), applied during extrusion. Three Basal diets were made with three soybean products as protein source: *Triple Null* (TN), the commercial counterpart of *Triple Null* (CSBM) and soy protein concentrate (SPC), which does not induce SBMIE. The diets were processed at two levels of SME. Atlantic salmon, kept in fresh water, were fed these diets, for 8 weeks, in three replicate tanks per diet before collection of samples for observation of gut health and function. Remaining fish were continued on the experimental feeds for 4 weeks to collect feces for the estimation of nutrient digestibility.

2.2. Fish and experimental conditions

The feeding experiment was conducted following the institutional and national guidelines for the care and use of animal and approved by the National Animal Research Authority in Norway. The fish laboratory at Norwegian University of Life Sciences (NBMU), Ås, was chosen as experimental facility. It is based on a recirculation system (RAS) delivered by Sterner AS. One recirculation unit comprising a total of 24 tanks, with common particle filter, biofilter, CO_2 stripping and oxygenation, ozone disinfection, was allocated to the experiment. Before the feeding started, Atlantic salmon with an average weight of 41 g (SD for tank mean = 0.8) were sorted to obtain low weight variation, and randomly distributed to 18 cylindrical 250 L fiberglass tanks with 55 fish per tank. The fish were starved for 48 h to help adaptation to the tank environment. A 24-h light regime was employed. Each tank was supplied with freshwater and water temperature was between 13 and 14 °C during the experiment. To ensure optimal conditions, dissolved oxygen (kept between 80 and 90% saturation in the outlet water), pH (7.2–8.2), as well as ammonium (< 1 mg/L) and nitrite levels (< 0.1 mg/L) were measured either daily or weekly throughout the experimental period. The fish were fed three meals a day, in the morning, around noon and in the afternoon.

2.3. Diet formulation and extrusion conditions

Diet formulations and nutrient compositions are shown in Tables 1a and 1b. The diets were produced at NMBU Centre for Feed Technology, Ås, Norway. Apparent digestibility coefficients from the USDA- Agricultural Research Services website were used to calculate the values for digestible protein and digestible energy. The diets were formulated to contain 354–393 g digestible protein, 281–315 g digestible fat, and 65–66 g digestible starch per kilogram. Each of the diets contained one

Table 1b
Chemical composition of the experimental diets (Kg^{-1}).

Soy source	TN		CSBM		SPC	
SME	Low	High	Low	High	Low	High
Dry matter (g)	965	957	972	962	971	969
Crude protein (g)	409	419	392	385	379	376
Crude fat (g)	250	267	267	264	267	259
Starch (g)	121	115	116	111	106	108
Gross energy (MJ)	23.5	23.7	23.9	23.8	24.0	24.0
Amino acids (g)						
Arg	25.8	25.4	22.4	22.3	21.6	22.1
His	9.2	9.1	8.1	8.0	7.8	8.0
Ile	18.5	18.3	16.0	15.9	15.4	15.8
Leu	31.3	31.0	27.9	27.7	27.3	27.6
Lys	33.7	33.4	29.0	28.8	28.3	28.8
Met	23.3	23.1	20.7	20.5	20.1	20.5
Phe	18.7	18.5	16.5	16.5	16.1	16.5
Thr	19.0	18.8	17.1	16.8	17.0	17.1
Val	24.4	24.4	22.0	21.7	20.2	20.5
Cys	5.0	5.0	4.4	4.4	4.5	4.5
Total AA	432	431	386	383	377	383
Minerals						
P (g)	9.54	9.35	9.66	8.97	9.24	9.36
Zn (mg)	161	155	153	144	160	157

source of soy protein, and similar proportions of amino acids, wheat gluten, wheat flour, fish meal and fish oil. Yttrium oxide was added in the formulation as a marker for digestibility assessment (Austreng et al., 2000). All dry ingredients were ground to 0.6 mm particle size. The full fat soybeans were ground twice, at 2.0 and 0.6 mm screens, as they blocked the 0.6 mm screen of the hammer mill when directly subjected to the 0.6 mm screen.

All dry ingredients were mixed using a 40 L twin shaft pedal mixer and extruded in a Bühler BCTG 62 mm twin screw extruder. The extrusion process was optimized to obtain a bulk density of 500–520 g/L in the extrudates before drying, to facilitate slow sinking in freshwater after drying and lipid coating. The differences in SME for the two diets with full-fat soy were obtained by increased feeder speed, temperature throughout the extruder and in the die, relative torque, and decreased water addition in the extruder. Higher SME in the SPC diet was achieved by increased temperature in sections 2–4, as well as increased drive power and screw speed. The pellets were dried to 10% moisture in a prototype fluid bed dryer with incoming and end temperatures declining from 150 to 55 °C, coated with fish oil in a prototype vacuum coater prior to cooling and bagging. The diets were extruded through two dies with a diameter of the holes of 3 mm. Pellets were cut by 3 rotating knives. Table S1 shows the extrusion conditions applied to the diets.

2.4. Sampling

Before the start of the experiment, 3 samples of 5 fish from the same population as the fish used in the feeding experiment, were euthanized with an overdose of tricanine methanesulfonate (MS-222, Finquel, Scan Aqua AS, Årnes, Norway) and stored at −20 °C for whole-body analysis. The fish were weighed in bulk per tank, anaesthetized with MS-222 (60 mg/L), at 28 days of feeding to assure that the experiment was running as expected. After 56 days of feeding, 12 fish per tank were anaesthetized with MS-222 (60 mg/L) and killed with a sharp blow to the head. Body length and weight of sampled individual fish were recorded. Blood was collected from the caudal vein using heparinized syringes. For digestive enzymes analysis, the intact intestinal tract of 6 fish per tank were removed, cleaned of all visceral fat and divided into 5 regions as previously described (Bakke-McKellep et al., 2000a): two halves of the pyloric intestine (PI1 and PI2), mid intestine (MI) and two halves of the distal intestine (DI1 and DI2). All the intestinal sections were opened longitudinally. Thereafter, the gut content from each individual region was collected, frozen in liquid nitrogen, and stored at −80 °C. The remaining 6 fish per tank were used for histomorphological analysis, gene expression and microbiota profiling. For histology, sections of DI tissue were taken, fixed in phosphate-buffered formalin (4% formaldehyde) for 24 h and then transferred to 70% ethanol until further analysis. For gene expression profiling, sections of DI tissue were collected in 1.5 mL Eppendorf tubes containing 1 mL RNAlater (Ambion, Thermo Fisher Scientific), incubated for 24 h at 4 °C and subsequently stored at −20 °C until further processing. For microbiota profiling, content of DI were collected in sterile 1.5 mL Eppendorf tubes, snap frozen in liquid nitrogen and subsequently stored at −80 °C until further processing.

The remaining fish in each tank were batch weighed and fed as earlier for an additional 28 days for feces collection. Once a week fish were anaesthetized by MS-222, and stripped of feces by the method of Austreng (1978). The fecal samples were pooled by tank and stored at −20 °C prior to analysis.

2.5. Plasma analysis

Blood samples were kept on ice until centrifuged at 3000*g* for 5 min. Then plasma was aliquoted into two separate Eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C until analysis. Plasma was analyzed for total triacylglycerides, non-esterified (free) fatty acids, cholesterol, glucose, sodium, alanine transaminase (ALT) and aspartate transaminase (AST) following standard procedures at the Central Laboratory under the Faculty of Veterinary Medicine, NMBU.

2.6. Chemical analyses of feed, feces and whole-body

The initial and final samples for whole-body analyses were homogenized in the frozen state in a meat mincer and lyophilized before analyses. Pooled fecal samples were lyophilized. The analysis of the whole-body content, feed and feces were performed as described by Refstie et al. (1997).

Amino acid contents were analyzed using a Biochrom 30 amino acid analyzer (Cambridge, U.K.) following the EC Commission Directive 98/64/EC (1999), after hydrolysis in 6 N HCl for 23 h at 110 °C. Tryptophan and tyrosine were analyzed after basic hydrolysis.

2.7. Analyses of enzyme activity and total bile salt concentration in intestinal contents

Fecal trypsin and bile salt analyses were performed on pooled freeze-dried gastrointestinal contents from PI1, PI2, MI, DI1, and DI2. Trypsin activity was determined colorimetrically, according to Kakade et al. (1973), using the substrate benzoyl-arginine–p-nitroanilide (BAPNA) (Sigma no. B-4875; Sigma Chemical Co., St. Louis, MO, USA).

Activity of the brush border membrane (BBM) enzyme leucine aminopeptidase (LAP; EC 3.4.11.1) was measured in intestinal tissue homogenates. The homogenates were prepared from tissues in ice-cold tris-mannitol buffer (1:20 *w/v*) containing the serine proteinase inhibitor 4-[2-Aminoethyl] benzenesulfonylfluoride HCl (Pefabloc® SC; Pentapharm Limited). Activity of LAP was determined colorimetrically using L-leucine-β-naphthylamide as the substrate as described by Krogdahl et al. (2003). Protein concentration of the homogenates was estimated using the BioRad® Protein Assay (BioRad Laboratories, Munich, Germany). Tissue protein concentration was used in the determination of LAP specific activity.

Bile salt concentration was analyzed using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory under Faculty of Veterinary Medicine, NMBU.

2.8. Evaluation of histological structure of the distal intestine

The DI tissue sections, 18 per treatment, were evaluated by light microscopy with focus on the characteristic morphological changes of diet-induced enteritis in Atlantic salmon DI that consist of changes in mucosal fold length, width and cellularity of the submucosa and lamina propria and degree of enterocyte supranuclear vacuolization. For representative images see Fig. S1. The degree of change of the DI morphological features was graded using a scoring system with a scale of 0–4 where 0 represented normal; 1, mild changes; 2, moderate changes; 3, marked changes and 4, severe changes. The sections were analyzed blind in a randomized order.

2.9. Quantitative real-time PCR

Quantitative PCR (qPCR) assays of DI samples were performed according to the MIQE standards (Bustin et al., 2009), on 3 animals from each of 3 tank replicates of the 6 diet groups ($n = 9$ animals per diet). Total RNA was extracted in a randomized order using a custom made Reliaprep simplyRNA HT protocol (Promega) and a Biomek 4000 laboratory automation workstation (Beckman Coulter). The RNA extraction included a DNase treatment according to the manufacturer's protocol. RNA integrity was evaluated by the 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies). RNA purity and concentration were measured using Take3 micro-volume plates and Epoch microplate spectrophotometer (BioTek Instruments). All samples had RNA integrity numbers (RIN) > 8.6, with a mean RIN value of 9.7. After extraction, total RNA was stored at −80 °C until use.

First strand cDNA was synthesized from 1.0 µg total RNA in 20 µL reactions using Superscript IV VILO Mastermix (Thermo Fisher Scientific), and a mixture of Oligo(dT)$_{20}$ and random hexamer primers according to the manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at −20 °C. A panel consisting of 13 target genes with key roles in intestinal immune, metabolic and stress/antioxidant function were profiled (Table 2). Expression of individual gene targets was analyzed using the LightCycler 96 (Roche Diagnostics). Each 10 µL DNA amplification reaction contained 2 µL PCR-grade water, 2 µL of 1:10 diluted cDNA template (corresponding to 8 ng total RNA), 5 µL of Lightcycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µL (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC) and an inter-plate calibrator. Pipetting was performed using the Biomek 4000 automation workstation. A three-step qPCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (10 s), 60 °C (10 s) and 72 °C (15 s). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of PCR products after each run by agarose gel electrophoresis. For target gene normalization, *gapdh*, *rnapol2* and *hprt1* were evaluated for use as reference genes by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variance (Kortner et al., 2011). The geometric mean of *gapdh*, *rnapol2* and *hprt1* was used as the internal normalization factor. Mean normalized levels (MNE) of target genes were calculated from raw quantification cycle (Cq) values (Muller et al., 2002).

2.10. High-throughput sequencing of gut microbiota

Since the high SME is the most relevant for the feed industry and the resources were limited, we only analyzed the bacterial population in fish fed high SME diets.

Total genomic DNA was extracted from 100 mg of DI content using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the standard procedure provided by the manufacturer with the exception of adding a bead beating step followed by heating at 95 °C for

Table 2
Primer sequences, efficiency, amplicon size, annealing temperature and function for the genes profiled in qPCR.

Genes	Accession no.	Primer sequence (5′-3′)		Amplicon size (bp)	Annealing temp. (°C)	PCR efficiency	Gene function	Reference
		Forward	Reverse					
myd88	NM_001136545	GACAAAGTTTGCCCTCAGTCTCT	CCGTCAGGAACCTCAGGATACT	87	60	1.80	NF-κB activation	Marjara et al. (2012)
cd3γδ	NM_001123621/NM_001123721	AAAGGCGCATGGACAGATCT	GCCCGCACAACATTAAAGCT	160	60	1.99	T cell marker	Li et al. (2019)
il1β	NM_001123582	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCGCTCGTAG	73	60	1.80	Pro-inflammatory cytokine	Marjara et al. (2012)
ifnγ	FJ263446	CTAAAGAAGGACAACCGCAG	CACCGTTAGAGGGAGAAATG	159	60	1.90	Pro-inflammatory cytokine	Marjara et al. (2012)
il17a	GW574233	AGGGGACAAAGGAGAGGTGT	GGTGACAGAGAGCGTGTGTG	114	60	2.00	Pro-inflammatory cytokine	Marjara et al. (2012)
tgfβ1	EU082211	AGTTGCCTTGTGATTGTTGTCG	CTCTTCAGTAGTGGTTTGTCG	191	60	1.90	Anti-inflammatory cytokine	Lilleeng et al. (2009)
mmp13	NM_001140524	ATTGTTCACGGCTGCTTCTT	CCAGAAGACAGTTCCGTGTG	94	60	2.00	Tissue remodeling	Sahlmann et al. (2013)
pcna	BT056931	TGAGCTCGTCGGGTATCTCT	GTCCTCATTCCCAGCACACT	170	55	2.00	Cell proliferation	Kortner et al. (2013)
fabp2b	G||209,731,517	TGCCTTCCCCTCATTCTCTA	GGTGATACGGTCTTCATCCAA	82	60	2.00	Fatty acid transporter	Venold et al. (2013)
aqp8ab	KC626879	GTTGGCATAGTTCTCCTTTGATG	TTTCAACCCTCCCTTCACC	148	60	2.00	Water channel	Kortner et al. (2012)
sod1	BG936553	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	141	60	1.94/1.95	Antioxidant defense	Hamre et al. (2016)
cat	BG935638	CCCAAGTCTTCATCCAGAAACG	CGTGGGCTCAGTGTTGTTGA	101	60	2.00	Antioxidant defense	Hamre et al. (2016)
hsp70	BG933934	CCCCTGTCCCTGGGTAATTG	CACCAGGCTGGTTGTCTGAGT	121	60	1.96	Stress response	Sagstad et al. (2008)
gapdh	BT050045	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	96	60	1.90	Reference gene	Kortner et al. (2011)
rnapo2	BG936649	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGCATCTTCCTGC	157	60	1.80	Reference gene	Kortner et al. (2011)
hprt1	BT043501	CGGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	255	60	2.00	Reference gene	Kortner et al. (2011)

Gene full names: myeloid differentiation factor 88 (*myd88*); cluster of differentiation 3 γδ (*cd3γδ*); interleukin 1β (*il1β*); interferon γ (*ifnγ*); interleukin 17A (*il17a*); transforming growth factor β1 (*tgfβ1*); matrix metalloproteinase 13 (*mmp13*); proliferation cell nuclear antigen (*pcna*); fatty acid binding protein 2b (*fabp2b*); aquaporin 8ab (*aqp8ab*); Cu/Zn superoxide dismutase (*sod1*); catalase (*cat*); heat shock protein 70 (*hsp70*); glyceraldehyde-3-phosphate dehydrogenase (*gapdh*); RNA polymerase II (*rnapo2*); hypoxanthine phosphoribosyl transferase 1 (*hprt1*).

7 min at the beginning as suggested by Knudsen et al. (2016). DNA extraction controls i.e. a blank negative control and a positive mock control (ZymoBIOMICS Mock Community Standard, Zymo Research Corp, Irvine, CA, USA) were included in the DNA extraction protocol. Following the extraction, PCR amplification of the V1-V2 region of the 16S rRNA gene using 27F and 338R primers (Roeselers et al., 2011), with the Illumina overhang adapters was performed for all the extracted DNA in duplicate including a PCR negative control (molecular grade water instead of DNA template). The PCRs were carried out as described previously (Gajardo et al., 2017) in 25 μL reactions with 12.5 μL of Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA), 11 μL molecular grade PCR water, 0.25 μL each of the forward and reverse primers (1 μM final concentration) and 1 μL DNA. After the PCR amplification, all the duplicate amplicons were pooled and run on 1.5% agarose gel. Samples with bright bands between 350 and 400 bp were considered suitable for library preparation.

PCR products clean-up, library quantification, normalization, and pooling were performed as outlined in the protocol by Illumina (Illumina, 2013). Briefly, the PCR products were cleaned using AMPure beads followed by index PCR using the Nextera XT Index kit and subsequently another round of purification with the AMPure beads. Prior to library normalization and pooling, cleaned PCR products were run on a Bioanalyzer using the Agilent DNA 1000 kit to assess the amplicon size and quantified using the Qubit® dsDNA HS assay kit (Thermo Scientific). The pooled library was then denatured, diluted to 6 pM, and the PhiX control was spiked into the final pool at 15% [v/v], before 2 × 300 bp paired-end sequencing on the MiSeq platform using the MiSeq v3 reagent kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

2.11. Sequence data analysis

The demultiplexed, pair-ended reads were analyzed using the QIIME2 (version 2017.10) (Bolyen et al., 2018). Reads were trimmed off the primer sequence (forward reads, first 20 bps; reverse reads, first 18 bps), truncated where the sequence quality drops (forward reads, at position 250 bp; reverse reads, at position 190 bp) and denoised using the DADA2 algorithm (Callahan et al., 2016). After the sequence denoising, the taxonomy was assigned to representative sequences by a naive Bayes machine-learning classifier (Bokulich et al., 2018), using the Greengenes 13.8 as the reference database. Mitochondria and chloroplast sequences were removed from the analysis. In addition, contaminant sequences were removed based on their prevalence and abundance in the samples and controls, and our prior knowledge of common contaminant sequences found in our lab. The majority of removed sequences were classified as *Pseudomonas veronii*, *Halomonas*, an unclassified member of Halomonaceae family and *Shewanella* algae.

In order to compute alpha and beta diversity, the feature table was rarefied at 10,000 sequences in order to have an even number of reads across all the samples. The alpha diversity was evaluated using the Shannon index. To evaluate beta diversity, Bray-Curtis distances were calculated to account for abundances of individual taxa (weighted) or based solely on presence/absence (unweighted). Kruskal-Wallis-pairwise test was performed to compare the differences in alpha diversity within QIIME 2. In addition, PERMANOVA was performed based on Bray-Curtis distance using the PRIMER v7 software (PRIMER-E Ltd., Luton, UK). For graphical presentation of beta diversity (nMDS plots), the taxa table at genus level provided by QIIME 2 was exported to PRIMER v7 software. Graphs for taxa distribution were generated using QIIME2 and EXCEL. The heat map was generated using the heatmapper online software (Babicki et al., 2016).

2.12. Other calculations and statistical analyses

Feed conversion ratio (FCR) for the two feeding periods was calculated separately as: FI * (FBW -IBW)$^{-1}$, where FI is feed intake, and FBW and IBW represent final and initial body weights, respectively. FCR for the whole experiment was calculated as: (FI_1 + FI_2) * $G_F{}^{-1}$, where FI_1 and FI_2 represent feed intake during period 1 and 2 respectively and G_F represents gain per fish. G_F was calculated as: (W_{end1} - W_{01}) + (W_{end2} - W_{02}), where W_{end1} is final fish weight at end of period 1, W_{01} is initial fish weight at start of feeding period 1, W_{end2} is final fish weight at end of period 2 and W_{02} is initial fish weight at start of feeding period 2.

Apparent digestibility coefficients (ADC) of individual nutrients and energy were calculated as: ADC = 100 * (1 - M_d * $M_f{}^{-1}$ * N_f * $N_d{}^{-1}$), where M_d and M_f represent the concentration of inert marker in the diet and feces, N_d and N_f represent the concentration of individual nutrients or energy in the diets and feces respectively.

Retention of specific nutrients and energy were calculated as: R_N = 100 * (FBW * N_F – IBW * N_I) * (FI * N_d)$^{-1}$, where N_F and N_I represent the nutrient or energy content in the final and initial whole fish samples (pooled samples of 5 fish per tank) respectively.

The results, except those from histological and molecular analyses, were analyzed statistically by two-way analysis of variance using the SAS STAT software ver 9.4. The class variables in the analysis were Basal diet named after the soybean product used in the diet (BasalD) (n = 3) and High or Low SME (Temp) applied during feed processing (n = 2). Significant ($p < .05$) differences in least-square means were ranked by the *P*-diff routine in SAS. Tank means were used as the experimental unit for the production and nutrition related observations.

For the qPCR results, statistical analyses were performed using GraphPad Prism 6.05. Diet group and SME treatment were evaluated as class variables in a two-way ANOVA with interaction. Multiple comparisons between groups were further analyzed using Fischer's LSD test. The level of significance was set to $p < .05$.

For the histological observations, the scores generated were categorical variables and the differences between the diets were explored by contingency analysis using the chi-squared test.

3. Results

3.1. Effects on growth, feed utilization, nutrient digestibility and retention

Growth rate did not show clear differences between fish fed the various diets, although the group fed TN diet showed slightly higher weight gain compared to the CSBM at the 28-day sampling. (Fig. 1 and Supplementary table S2). The fish fed SPC diet showed slightly higher weight gain at 56 days relative to fish fed with the other diets. No difference was detected between diets treated at high and low SME at any of the sampling time points.

Body composition was similar for fish fed the TN and the CSBM diets. Fish fed the diet with SPC showed significantly lower dry matter than the former two regardless of SME (Table 3). A similar trend was seen for body lipid content, but only in fish fed low SME diets, as indicated by the significant interaction. Regarding protein retention, the TN showed significantly lower values than the CSBM (Table 3), while the highest retention was observed in fish fed the SPC diets. The fish did not show significant SME effects regarding body composition.

Fecal dry matter was similar for fish fed the TN and CSBM diet, significantly lower than for SPC-fed fish. High SME tended (p = .069) to cause lower fecal dry matter than low SME (Table 4).

Regarding protein digestibility (Table 4), the statistical analyses revealed a significant interaction between Basal diet and SME. At low SME protein digestibility was lower for TN (85.6%) than CSBM (87.1%). The diet with SPC showed the highest protein digestibility (90.3). At high SME the difference between TN (89.9), CSBM (90.1) and SPC (90.3%) was only marginal. High SME gave higher protein digestibility. Overall, the results for amino acid digestibility gave a similar picture regarding diet and SME effects as protein digestibility. Fig. 2 illustrates of the main effects and the interaction between diets and SME effects.

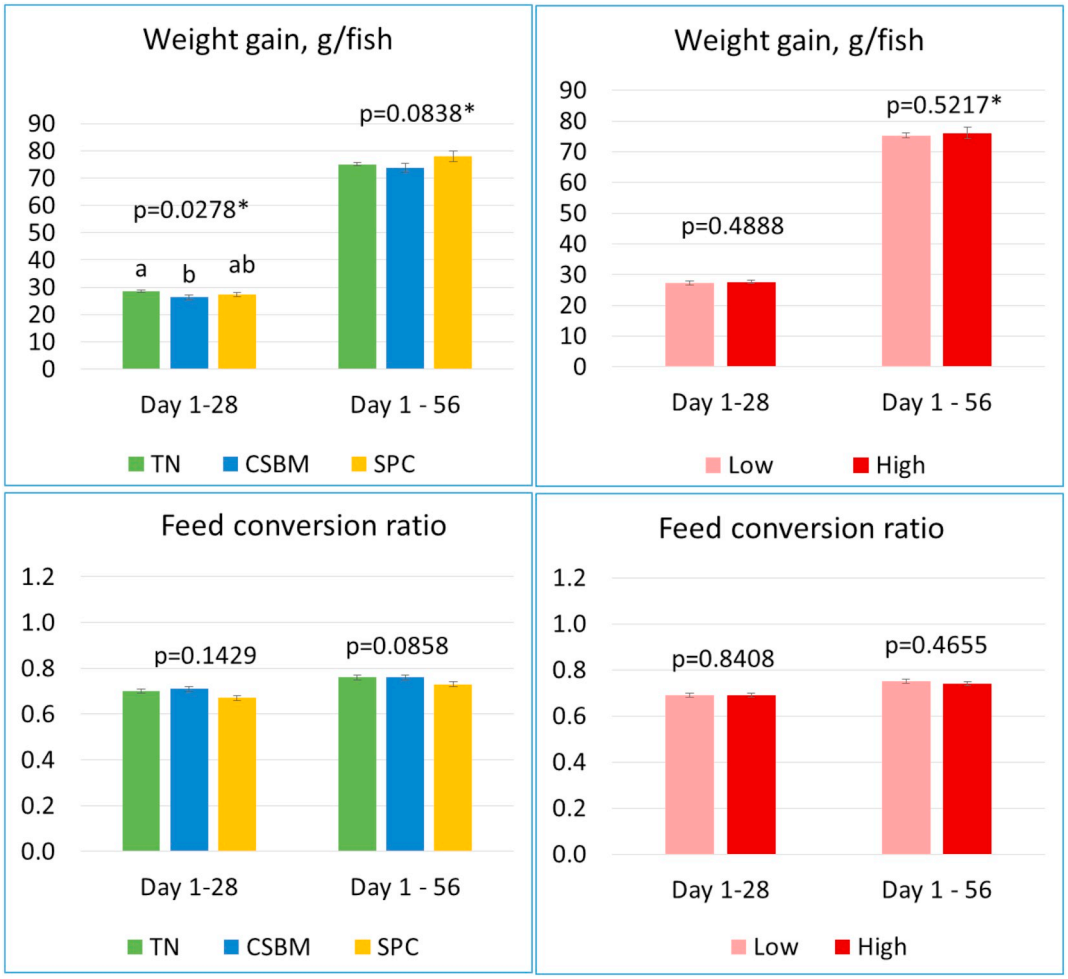

Fig. 1. Weight gain and feed conversion ratio in Atlantic salmon after 28 and 56 days of feeding of diets with different soybean products, TN, CSBM or SPC, treated at low or high SME. *significant interaction; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate; SME, specific mechanical energy.

The three diets showed significant differences regarding lipid digestibility (Table 4). The TN fed fish exhibited lower lipid digestibility than the CSBM. However, the SPC showed the lowest value. Total energy digestibility showed a similar pattern for the Diet and SME effects as lipid digestibility.

3.2. Organ weight, brush border enzyme activity and chyme dry matter

Relative weight of the distal intestinal section was similar for fish fed the TN and CSBM basal diets (Table 5). Fish fed SPC showed significantly higher values than fish fed the two soybean meals. Also for SME, significant effect on organ weight was observed, with higher values for high SME than low.

Activity of LAP in DI mucosa, whether expressed as specific activity or activity per kg fish of LAP, showed similar values for TN and CSBM, whereas SPC showed higher values (Fig. 3). High SME resulted in an increase in LAP activity compared to low SME, but only when expressed as specific activity.

Table 3
Whole-body composition[1] and nutrient retentions of fish fed diets with TN, CSBM or SPC treated at low or high SME.

	Soy source			SME		P > F (Model)	P > F (Source)	P > F (SME)	P > F (Interaction)
	TN	CSBM	SPC	Low	High				
Body composition (g/kg wet weight)									
Dry matter	322[a]	322[a]	305[b]	315	317	0.025	0.009	0.610	0.130
Crude protein	168	168	165	168	166	0.013	0.120	0.060	0.009
Crude lipid	122	111	110	115	121	0.160	0.150	0.340	0.160
Ash	23	22	22	22	23	0.520	0.330	0.650	0.420
Retention, 0–56 d, % of intake									
Crude protein	57.6[c]	61.7[b]	64.8[a]	60.9	61.8	< 0.0001	< 0.0001	0.260	0.091

[a,b,c] Significant ($p < .05$) differences between sources of soy protein, ranked by PDIFF under LSmeans in SAS. SME, specific mechanical energy; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate.

[1] Initial body composition values (g/kg wet weight) (mean ± SEM, $n = 3$): dry matter, 289.9 ± 3.5; crude protein, 155.0 ± 0.8; crude fat, 101.6 ± 3.2; ash, 20.6 ± 0.2.

Table 4
Fecal dry matter and apparent digestibility of macronutrients and energy in fish fed diets with TN, CSBM or SPC treated at low or high SME.

	Soy source			SME		$P > F$ (Model)	$P > F$ (Source)	$P > F$ (SME)	$P > F$ (Interaction)
	TN	CSBM	SPC	Low	High				
Fecal dry matter[1] (%)	9.2^{b}	9.5^{b}	10.5^{a}	9.9^{x}	9.6^{y}	< 0.0001	< 0.0001	0.0690	0.2800
Apparent digestibility (%)									
Crude protein	88.6^{b}	88.5^{b}	90.3^{a}	87.7^{x}	90.1^{y}	< 0.0001	0.0002	< 0.0001	0.0004
Crude lipid	93.0^{b}	94.3^{a}	88.1^{c}	91.0^{x}	92.6^{y}	< 0.0001	< 0.0001	0.0007	0.2100
Energy	81.1^{b}	82.3^{a}	80.1^{c}	79.4^{x}	82.6^{y}	< 0.0001	0.0007	< 0.0001	0.4400
Arg	93.6^{b}	93.5^{b}	95.5^{a}	92.8^{y}	92.5^{x}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
His	88.8^{b}	89.1^{b}	92.0^{a}	88.4^{x}	91.0^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Ile	90.8^{b}	90.8^{b}	93.3^{a}	90.0^{x}	92.7^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Leu	91.6^{b}	93.4^{a}	93.5^{a}	90.8^{x}	93.2^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lys	93.4^{b}	92.8^{b}	95.6^{a}	93.0^{x}	94.8^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Met	96.4^{b}	96.4^{b}	97.2^{a}	96.1^{x}	97.0^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Phe	90.9^{b}	90.9^{b}	93.4^{a}	90.0^{x}	92.8^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Thr	87.6^{b}	88.2^{b}	91.3^{a}	87.3^{x}	90.2^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Val	91.7^{b}	91.3^{b}	94.1^{a}	91.0^{x}	93.3^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cys	78.4^{b}	77.6^{c}	83.9^{a}	77.6^{x}	82.4^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Total AA	91.1^{b}	90.4^{b}	92.9^{a}	90.8^{x}	92.5^{y}	< 0.0001	< 0.0001	< 0.0001	< 0.0001

[a,b,c]Significant ($p < .05$) differences between sources of soy protein and [x,y] SME levels, ranked by PDIFF under LSmeans in SAS. SME, specific mechanical energy; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate.
[1] Assessed by freeze-drying.

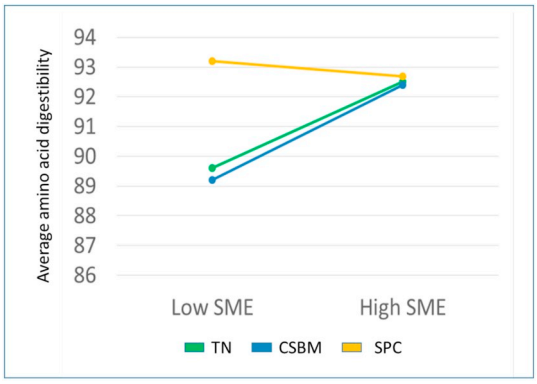

Fig. 2. Pattern of interaction between soy type (TN, CSBM and SPC) and SME (Low vs High) for apparent digestibility (%) of total (excluding Trp) amino acids. All statistically significant interactions in apparent nutrient digestibility followed the same pattern. See Fig. 1 for explanation of abbreviations.

3.3. Chyme bile salt concentration and trypsin activity along the intestine

Regarding chyme bile salt concentration significant differences were found in PI1 and DI1 (Fig. 4). Chyme in PI1 showed significantly lower values for fish fed TN than fish fed CSBM, whereas SPC fed fish showed higher values than both, significantly higher only compared to TN. In DI1, TN and CSBM showed similar values, significantly lower than SPC. Variation in SME did not cause a significant effect on chyme bile salt concentration (data not shown).

Trypsin activity in distal intestinal chyme, for both D1 and DI2, showed similar values for TN and CSBM fed fish, values which were significantly higher than those for SPC (Table 6). High SME treatment resulted in higher trypsin activity in the DI (Table 6).

3.4. Blood plasma metabolites

Among the observed plasma indicators of nutrient metabolism, significant effects of basal diet were observed for all but plasma free fatty acids (Table 7). Fish fed TN and CSBM differed significantly only regarding plasma triglycerides for which those fed the CSBM showed lower values than those fed TN. In fish fed SPC, both plasma glucose and cholesterol were lower than the fish fed TN and CSBM. For plasma free fatty acids no significant effects were observed. There was no SME effect on these plasma variables.

Regarding indicators of liver function, no clear diet effect was seen. However, lower values were observed for fish fed high SME diets than low for both ALT and AST.

3.5. Histological appearance of the wall of the distal intestine

The morphological changes in DI in fish fed the different diets are shown in Fig. 5. Fish fed a diet with TN or CSBM showed similar DI morphology. Both diets induced mild to moderate inflammatory changes in most of the fish evaluated. DI sections from fish fed the SPC diet were predominantly normal and healthy in appearance. Level of SME did not affect any of the observed intestinal morphological characteristics.

Table 5
Relative weight of distal intestine and leucine aminopeptidase (LAP) activity in fish fed diets with TN, CSBM or SPC treated at low or high SME.

	Soy source			SME		$P > F$ (Model)	$P > F$ (Source)	$P > F$ (SME)	$P > F$ (Interaction)
	TN	CSBM	SPC	Low	High				
DISI (%)	0.41^{b}	0.44^{b}	0.61^{a}	0.49	0.48	< 0.0001	< 0.0001	0.5358	0.2577
LAPprot	169^{b}	185^{b}	274^{a}	$197^{\times}$	223^{y}	< 0.0001	< 0.0001	0.0021	0.1004
LAPkg	29^{b}	32^{b}	77^{a}	45	47	< 0.0001	< 0.0001	0.4282	0.0073[1]

[a,b]Significant ($p < .05$) differences between sources of soy protein and [x,y] SME levels, ranked by PDIFF under LSmeans in SAS. SME, specific mechanical energy; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate; DISI, distal intestine somatic index; LAPprot (μmol/h/mg protein), tissue specific activity of leucine aminopeptidase; LAPkg (mmol/h/Kg fish), total capacity of leucine aminopeptidase.
[1] Reducing effect of high SME on soy source CSBM, increasing effects on TN and SPC.

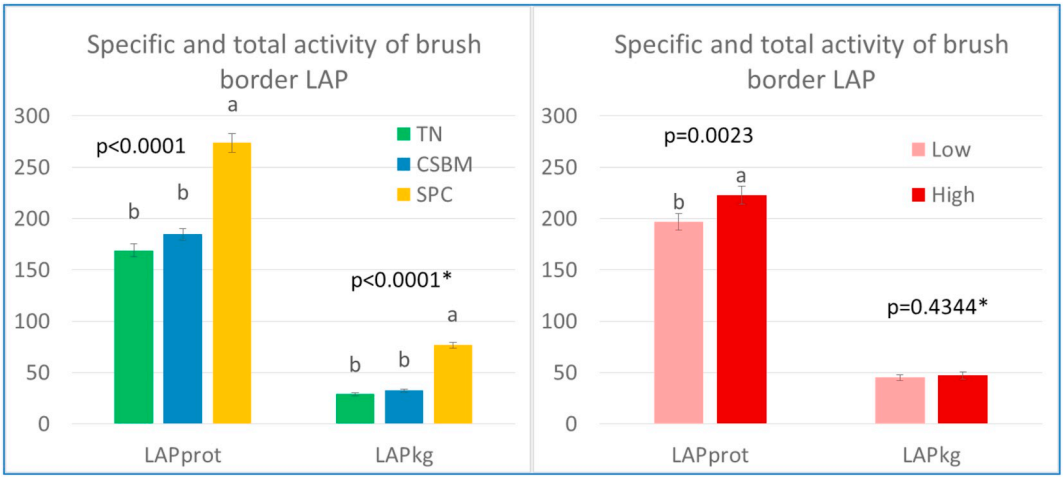

Fig. 3. Specific activity (LAPprot, μmol/h/mg protein) and total activity (LAPkg, mmol/h/Kg fish) of leucine aminopeptidase in distal intestine of fish fed diets with different soybean products, TN, CSBM or SPC, treated at low or high SME. *significant interaction. See Fig. 1 for explanation of abbreviations.

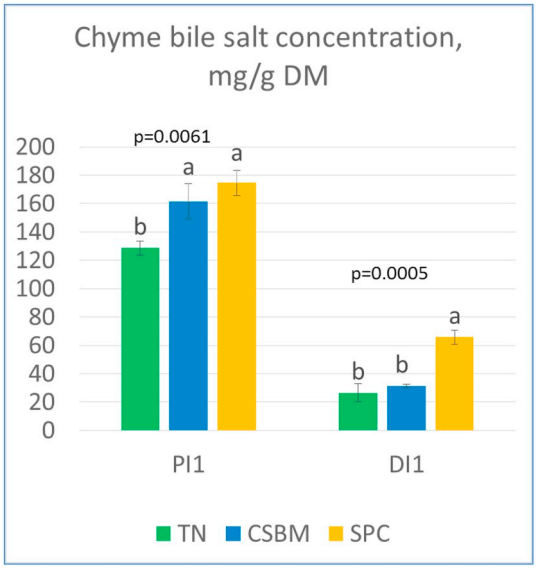

Fig. 4. Bile salt concentration in PI1 and DI1 of fish fed diets with TN, CSBM or SPC. The figure shows means of low and high SME. No significant interactions were identified. PI1, proximal half of the pyloric intestine; DI1, proximal half of distal intestine. See Fig. 1 for explanation of other abbreviations.

3.6. Distal intestinal gene expression

An overview of the gene expression results showing diet and SME effects are presented in Table 8. Details are shown in supplementary Fig. S2 and S3. Fish fed TN compared to CSBM, showed higher expression of seven of the 13 observed genes. For the other genes, the two diets did not show a significant difference. These differences were only seen for diets processed at low SME. Fish fed diets treated at high SME did not show significant differences in gene expression between TN and CSBM. The observed differences between TN and CSBM regarded immune functions, i.e. expression of *il1β* and *il17a*, both coding for pro-inflammatory cytokines, *tgfβ1* coding for an anti-inflammatory cytokine, and *cd3γδ*, a T-cell marker; markers of tissue remodeling and cell proliferation: *mmp13* and *pcna*, respectively; as well as stress related genes, i.e. *cat* and *hsp70*.

Gene expression in fish fed TN and CSBM deviated from those fed SPC in a similar pattern, but the differences were generally more pronounced for TN fed fish with more significant differences. For TN vs SPC the significant differences regarded both low and high SME showing largely the same pattern for both, i.e. with no significant effect or significant upregulation of immune related genes: *il1β, il17a, tgfβ1* and *myd88,* downregulation of *ifnγ*; upregulation of tissue remodeling and cell proliferation genes *mmp13* and *pcna;* marked downregulation of the lipid and water transporters *fabp2* and *aqp8ab*; upregulation of the stress genes *cat* and *hsp70*.

The two SME levels employed did, overall, not affect gene expression. The exception regarded *il1β,* showing the lowest value among the diets at low SME, the highest at high SME, and *ifnγ* showing lower expression at high SME.

3.7. Distal intestinal microbiota composition

After bioinformatic analysis a total number of 1.6 million reads were included in this study. The minimum read per sample was 12,368 reads and the maximum was 131,359 and a total average of 61,110 read/sample. The sequencing depth was sufficient, as indicated by the rarefaction curves (see Fig. S6 in the supplemental material).

Overall, the differences in taxonomic composition, alpha and beta diversity between samples from TN and CSBM fed fish were minor, whereas SPC fed fish distinguished themselves from the TN and CSM

Table 6
Trypsin activity in DI content of fish fed diets with TN, CSBM or SPC treated at low or high SME.

	Soy source			SME		$P > F$ (Model)	$P > F$ (Source)	$P > F$ (SME)	$P > F$ (Interaction)
	TN	CSBM	SPC	Low	High				
DI1	45^{a}	44^{a}	26^{b}	30^{x}	46^{y}	0.0058	0.0100	0.0047	0.2187
DI2	40^{a}	38^{a}	12^{b}	21^{x}	38^{y}	0.0006	0.0003	0.0028	0.2250

^{a,b}Significant ($p < .05$) differences between sources of soy protein and ^{x,y} SME levels, ranked by PDIFF under LSmeans in SAS. SME, specific mechanical energy; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate; DI1, content of the proximal half of distal intestine; DI2, content from the distal half of the distal intestine.

Table 7
Plasma indicators of metabolism and liver function in fish fed diets with TN, CSBM or SPC treated at low or high SME.

	Soy source			SME		P > F (Model)	P > F (Source)	P > F (SME)	P > F (Interaction)
	TN	CSBM	SPC	Low	High				
Free fatty acids	0.65	0.62	0.71	0.70	0.62	0.1643	0.3670	0.0980	0.2080
Glucose	6.9[a]	6.8[a]	5.6[b]	6.5	6.4	< 0.0001	< 0.0001	0.4032	0.0100[1]
Cholesterol	10.3[a]	9.9[a]	8.5[b]	9.6	9.6	< 0.0001	< 0.0001	0.8899	0.9466
Triglycerides	4.5[a]	3.9[b]	4.5[a]	4.5	4.1	0.0121	0.0343	0.1252	0.0570
Na	159	160	160	159	159	0.0087	0.3373	0.5359	0.0014[2]
ALT	108	85	110	112[y]	89[x]	0.0681	0.1350	0.0421	0.3322
AST	2513	2039	2612	2645[y]	2131[x]	0.0313	0.1213	0.0345	0.1558

[a,b]Significant (p < .05) differences between sources of soy protein and [x,y] SME levels, ranked by PDIFF under LSmeans in SAS. SME, specific mechanical energy; TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate; ALT, alanine transaminase; AST, aspartate transaminase.
[1] Greater reducing effect of high SME for soy source CSBM than TN and SPC.
[2] Opposite direction of difference between low and high SME for soy source TN (158 vs 160 mM) than CSBM (159 vs 158 mM) and SPC (160 vs 159 mM).

fish. Alpha diversity as measured by Shannon index (Table 9), showed no significant difference between the TN and CSBM fed fish (p = .76) whereas the SPC fed group was significantly higher from the TN and CSBM (Kruskal-Wallis test, FDR adjusted p < .05). Similarly, both weighted and unweighted (presence/absence) measures of beta diversity using Bray-Curtis metric, showed that the TN and CSBM groups were quite similar whereas, the SPC group was significantly different from the TN and CSBM groups (Fig. 6 and Table 9).

At phylum level, twenty-one different phyla were identified among the fish fed the different soybean products. Three phyla, i.e. Firmicutes, Proteobacteria and Actinobacteria, accounted for > 90% of total abundance in all the groups. Among these, the main contributors to the total relative abundance were Firmicutes and Proteobacteria. No clear differences were observed between TN and CSBM. Fish fed SPC showed higher values of Firmicutes and Fusobacteria and lower proportions of Proteobacteria than fish fed CSBM and TN (Fig. 7A, Table S3a).

At genus level, there were no clear differences between the CSBM and TN groups although some differences in the relative abundance of *Trichococcus*, *Bacillus* as well as undefined members of family Enetrobacteriaceae and Rhodobacteriaceae were detected (Fig. 7B, S4). Bacteria belonging to genus *Lactobacillus*, *Photobacterium, Weisella, Streptococcus* and *Psychrilobacter* were significantly more abundant in the SPC group compared to the CSBM and TN groups. On the other hand, genus *Acinetobacter,* and undefined group of Gammaproteobacteria were relatively less abundant in samples belonging to the SPC group compared to CSBM and TN groups (Fig. 7B, S4; Table S3b).

Further analysis of the lactic acid producing bacteria (LAB), whose presence is often considered beneficial to the host, revealed that the SPC group had a relatively higher proportion of these bacteria (26.2%) compared to the CSBM (14.4%) and the TN (12.5%) groups (Fig. 8).

4. Discussion

Most of the physiological and growth related parameters were similar for the TN and the CSBM fed fish indicating that the nutritional values of these two soybean products were similar. These findings, particularly those related to gut health and function, inflammatory responses and microbial population indicate that the effects on

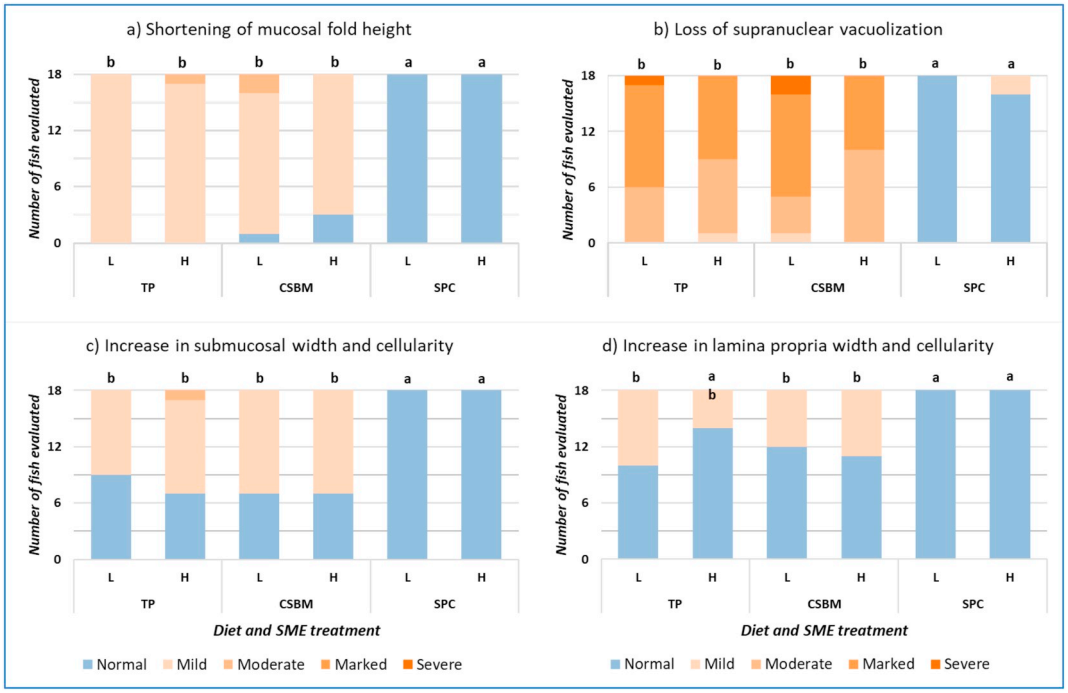

Fig. 5. The number of sampled individuals that were scored as "normal", "mild", "moderate", "marked", or "severe" for selected morphological features of the DI during the histological evaluation. The x-axis represents the diet groups 1, 2 and 3. Chart columns not sharing similar letters on top are statistically distinct according to methods and criteria in the 'Statistics' section above. See Fig. 1 for explanation of abbreviations.

Table 8
Comparisons of diet and SME effects on the gene expression profile[a].

Gene name	TN vs CSBM		TN vs SPC		CSBM vs SPC		Low vs High SME
	Low	High	Low	High	Low	High	
il1bβ	↑	ns	ns	↑	ns	↑	si[b]
il17a	↑	ns	↑	ns	ns	ns	ns
ifnγ	ns	ns	ns	↓	ns	↓	si[c]
igfβ	↑	ns	↑	ns	ns	ns	ns
myd88	ns	ns	↑	↑	ns	↑	ns
cd3γδ	↑	ns	ns	ns	ns	ns	ns
mmp13	↑	ns	↑	↑	↑	↑	ns
pcna	ns	ns	ns	↑	ns	↑	ns
fabp2b	ns	ns	↓	↓	↓	↓	ns
aqp8ab	ns	ns	↓	↓	↓	↓	ns
sod1	ns	ns	ns	ns	↓	ns	ns
cat	↑	ns	↑	↑	↑	↑	ns
hsp70	↑	ns	↑	↑	↑	↑	ns

TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate; SME, specific mechanical energy; ns, non-significant difference; si, significant interaction.

[a] See Table 2 for information on genes. Upward arrows indicate higher values in the comparison for TN, CSBM, and High SME, and vice versa.

[b] CSBM showed lowest value at low SME, highest value at high SME.

[c] Similar pattern at high and low SME, but more pronounced at High SME.

Table 9
Alpha and beta diversity of gut microbiota in fish fed diets with TN, CSBM or SPC treated at high SME.

Alpha diversity, Shannon index[1]			TN	CSBM	SPC
			4.28[a]	4.35[a]	6.75[b]
Beta diversity[2]			Pairwise comparisons		
			TN vs CSBM	CSBM vs SPC	SPC vs TN
	Weighted	pseudo-*t*	0.44	2.1	2.03
		p	0.943	0.008	0.007
	Unweighted	pseudo-*t*	1.06	1.5	1.3
		p	0.24	0.003	0.008

TN, *Triple Null* soybean meal; CSBM, commercial soybean meal; SPC, soy protein concentrate.

[1] Different letters denote significant differences in Shannon index between groups with different sources of soy protein as reported by QIIME2 (Kruskal-Wallis test, FDR-adjusted $p < .05$).

[2] Significant differences in beta diversity between groups with different sources of soy protein as reported by PRIMER v7 (PERMANOVA test based on Bray-Curtis distance metrics, $p < .05$).

inflammatory responses and microbial populations were similar whether the fish were fed the TN or CSBM. The results confirm that proteinaceous antinutrients are not active in soybean meal when used in salmon diets which have been subject to an extrusion process, employing higher SME than normal for other animal diets. Our results confirm that extrusion, whether at low or high SME, inactivates proteins such as the Kunitz trypsin inhibitor, lectin and allergens in the soybeans. This means that neither the TN nor the CSBM diets contained these proteins in active form. These results are in line with the general understanding which has accumulated over the last decade, that the soy saponins are the key inducer of SBMIE in Atlantic salmon (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2015).

One of the few variations detected between TN and CSBM diets was the induction of some of the inflammation biomarkers, which was higher for the TN than the CSBM diet. This was seen for both the pro-inflammatory responses, represented by *il1bβ*, and T_H17 responses, represented by *il17a*, as well as the transcription factors involved in generating these responses (*myd88* and *tgfβ1*). Notably, these differences were only present in the low SME treated group indicating that the causal factor, or interaction between compounds, causing these differences is labile to variation in SME. It is difficult to speculate about the factors causing these differences especially with the possible complex interaction between the different diet components. One possibility, however, is that the relative amounts of other ANFs, that stimulate inflammatory responses, has changed in the TN cultivar leading to a higher concentration in the TN diet, but this remains to be documented.

For both the TN and the CSBM diets, most of the measured parameters were different from the reference SPC diet, which seemed to be a superior feed ingredient in this experiment. The detected changes, particularly the increase in trypsin activity, decreased LAP and bile salts concentration, suppression of *aqp8* and *fabp2* gene expression as well as histo-morphological changes in the DI corresponded with the characteristic features of the SBMIE as previously established (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007a; Bakke-McKellep et al., 2000b; Krogdahl et al., 2015; van den Ingh et al., 1991; Venold et al., 2013). The superior nutritional value of SPC was an expected result, as the production of SPC includes steps which remove saponins and low molecular carbohydrates, and also inactivate antigens (Berk, 1992).

Targeted high-throughput sequencing of the 16S rRNA gene in bacteria gives an overview of the relative abundance of the populations. One interesting but also intriguing finding in this study is the higher presence of genus *Acenitobacter* in fish fed TN and CSBM diets. This bacteria has usually been considered as saprophytic and are found in water, soil, vegetables as well as fresh water and marine fish species (Baumann, 1968; Berlau et al., 1999; Čož-Rakovac et al., 2002; Jaafar et al., 2013). Recently, however, it was shown that some species such as *A. johnsonii* and *A. lwofii* can cause clinical diseases in rainbow trout and common carp (Kozinska et al., 2014). Since the increase in these bacteria coincided with inflammatory responses and the development of SBMIE, it is possible that there is an interplay between the amount of *Acinetobacteria* and the development of SBMIE. Whether the increase of

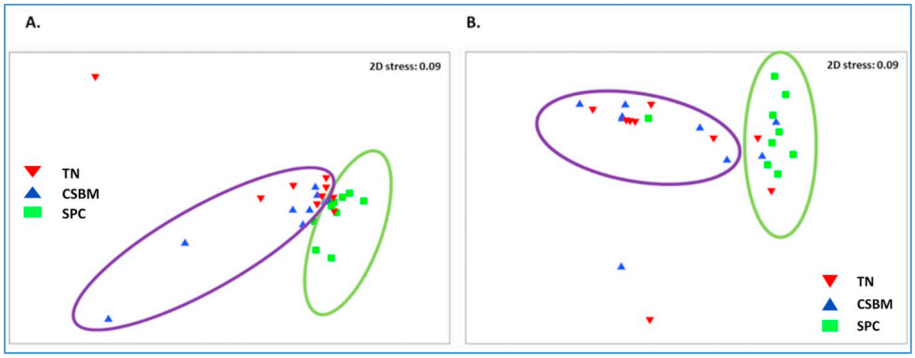

Fig. 6. Non-metric MDS analysis of microbiota present in digesta samples belonging to different diet groups using Bray-Curtis distance metric. The plots represent beta diversity using unweighted (presence/absence) (A) and weighted (B) measures.

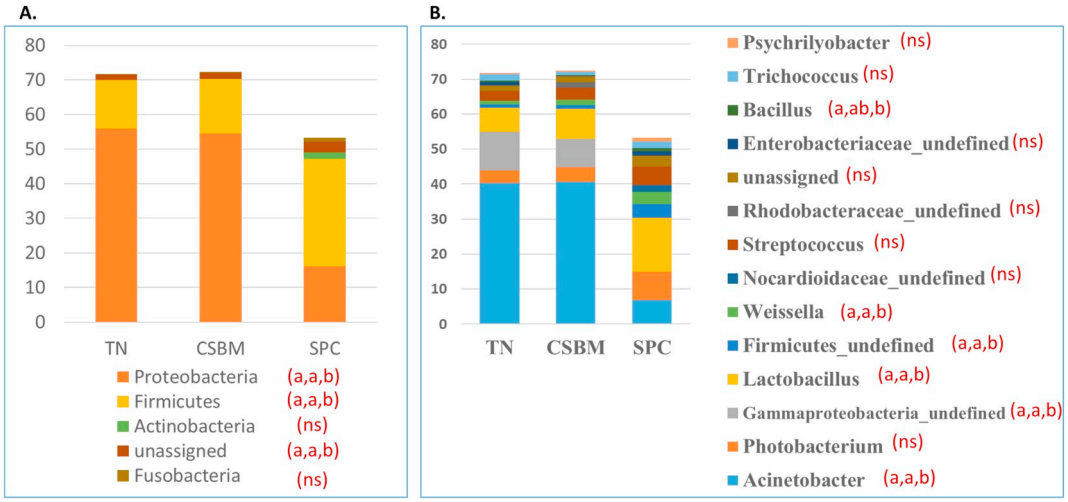

Fig. 7. Summary of the most abundant bacterial taxa. The graphs are plotted using the relative abundance of dominant taxa which account for > 50% of the total relative abundance. A, at phylum level; B, at genus level. Significant differences are indicated in parenthesis. Results with different letters are significantly different according to Wilcoxon signed rank test (ns = no significant differences).

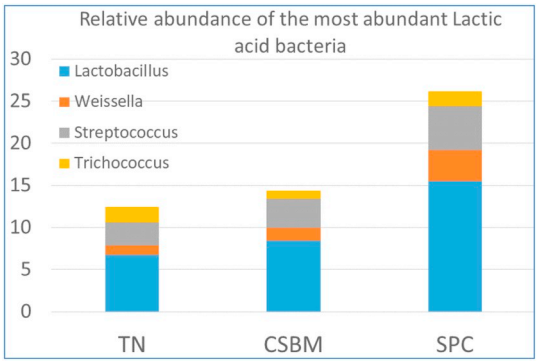

Fig. 8. Distribution of lactic acid producing bacteria in the fish fed with the different diets. The data represent the relative abundance of these bacteria as a percentage.

these bacteria is a result of their involvement in inducing the inflammatory response or is just a reflection of changed gut environment due to SBMIE remains unknown and requires further investigation.

Another notifiable finding from the microbiota profiling was the lower relative abundance of LAB in fish fed TN and CSBM diets relative to SPC fed group which may appear surprising as SBMs contain high levels of easily fermentable carbohydrates. These observations should be considered in light of the results of studies comparing fish meal-based diets without or with SBM, in which higher proportion of LAB bacteria was shown in both trout (Desai et al., 2012; Reveco et al., 2014) and salmon (Gajardo et al., 2017) when fed SBM. In another study, using conventional culturing method, no change in LAB was detected between fish fed diets containing SBM and fishmeal (Navarrete et al., 2013). These different findings indicate that the change in LAB bacteria is related to diet formulations and is influenced by other components in the diet. Together these results indicate that the quality of the carbohydrates greatly influences the microbial population, and that choice of degree of processing, and the composition of reference diet, may greatly influence the outcome. In our previous study we observed that LAB increased in the fish with inflamed guts (Gajardo et al., 2017). The present study suggests that the variation in presence of LAB, is not necessarily related to gut inflammation. This brings to the fore the challenge of comparing the outcomes of the different studies when different formulations of the reference diets have been used.

Bile salt concentration in intestinal chyme differed between the different diet groups with the TN fed fish showing the lowest concentrations. The most likely explanation for this effect is the high content of fibres in soybeans, standard as well as SPC, with the ability to bind bile salts draining them from the body (Kortner et al., 2013). The differences observed between treatments in the present study were small compared to those observed earlier (Romarheim et al., 2008). A diet without soybean meal and with high fish meal content, would most likely have given fish with chyme bile salt levels up to twice as high as observed in the present study.

Blood glucose levels were higher in fish fed TN and CSBM than in those fed SPC. Moreover, high SME reduced plasma glucose level compared to low, and the effect was greater for CSBM fed fish than those fed TN. The cause of the elevated glucose level in fish fed the TN and CSBM diets was most likely the content of sucrose in these soy products. The processing procedures used for SPC effectively removes all compounds soluble in the employed mix of alcohol and water, including sucrose while sucrose may represent about 5% of weight in dried, full fat soybeans (Berk, 1992). The reason for the decreasing effect of high SME on plasma glucose may be covalent binding at higher SME of the sucrose constituents, glucose and fructose, to molecules such as amino acids, rendering them indigestible and therefore less prone to elevate blood glucose, due to a Maillard reaction (Wei et al., 2018).

Blood cholesterol level was higher in fish fed the TN and CSBM than those fed SPC, independent of SME level. The mechanism underlying this difference is not obvious. However, the level of total fibre is higher in SPCs than in standard SBMs, as the removal of soluble carbohydrates up-concentrates all other nutrients. It is well known that many fibres bind cholesterol in the intestine and thereby reduce blood cholesterol (Romarheim et al., 2006; Romarheim et al., 2008). The difference in fibre content of the soy products may, therefore, be the cause of the difference in the resulting plasma cholesterol. On the other hand, the diet with TN caused higher plasma triglyceride level than that with the CSBM, which showed similar levels as observed for fish fed the SPC diet. The cause of this difference may be related to the absence of the antinutrients in the TN variety. It is, however, difficult to suggest a mechanism for an effect of removal of the lectin, Kunitz soybean inhibitor, or an allergen, or the combined effects of their removal.

Plasma Na showed significant effect of SME level which differed depending on the type of soy product in the diet. However, all observed plasma Na levels were well within the normal range (Sandnes et al., 1988). A mechanism for the differences is difficult to suggest. Moreover, SME treatment affected trypsin activity in the both sections of DI. The fish fed the diets which had been subject to low SME treatment, showed the lowest trypsin activity. The explanation for this difference

might be that the trypsin inhibitors in the soybeans treated at low SME was inactivated to a lower degree than those in the soybeans treated at higher SME. The consequence was, hence, a higher degree of inactivation of the trypsin level in the intestine of the fish fed the diets treated at low SME. It is well known that inactivation of trypsin inhibitors increase with increasing heat treatment up to a level where all is inactivated (van der Ven et al., 2005). Full inactivation is not aimed at, as this will reduce the protein quality of the soybean meal to very low levels due to processes such as Maillard reactions (Wei et al., 2018).

5. Conclusions

The main finding of the present study was the great similarity of TN and CSBM regarding nutritional value and induction of SBMIE by both, independent of SME. The few observed differences between fish fed TN and CSBM, seen only at low SME, indicated that TN stimulates rather than diminish some of the immunological and stress related processes. At high SME these effects were reduced to levels not significantly different from those of CSBM. The SPC deviated from the TN and CSBM, as expected, showing higher protein digestibility and feed utilization, and no induction of inflammation in the DI. High SME resulted in higher protein and amino acid digestibility, but only for TN and CSBM, not for SPC.

Data availability

The raw sequence data are available: SRA accession number SRP193955.

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Declaration of Competing Interest

There are no conflicts of interest related to the performance of this study or regarding publishing of the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734495.

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