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Effects of functional ingredients on gut inflammation in Atlantic salmon (*Salmo salar L*)

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ABSTRACT

Functional feed ingredients are frequently used in feeds for Atlantic salmon, often claimed to improve immune functions in the intestine and reduce severity of gut inflammation. However, documentation of such effects is, in most cases, only indicative. In the present study effects of two packages of functional feed ingredients commonly used in salmon production, were evaluated employing two inflammation models. One model employed soybean meal (SBM) as inducer of a severe inflammation, the other a mixture of corn gluten and pea meal (CoPea) inducing mild inflammation. The first model was used to evaluate effects of two packages of functional ingredients: P1 containing butyrate and arginine, and P2 containing β-glucan, butyrate, and nucleotides. In the second model only the P2 package was tested. A high marine diet was included in the study as a control (Contr). The six diets were fed to salmon (average weight of 177g) in saltwater tanks (57 fish per tank), in triplicate, for 69 days (754 ddg). Feed intake was recorded.

The growth rate of the fish was high, highest for the Contr (TGC: 3.9), lowest for SBM fed fish (TGC: 3.4). Fish fed the SBM diet showed severe symptoms of inflammation in the distal intestine as indicated by histological, biochemical, molecular, and physiological biomarkers. The number of differently expressed genes (DEG) between the SBM and Contr fed fish was 849 and comprised genes indicating alteration in immune functions, cellular and oxidative stress, and nutrient digestion, and transport functions. Neither P1 nor P2 altered the histological and functional symptoms of inflammation in the SBM fed fish importantly. Inclusion of P1 altered expression of 81 genes, inclusion of P2 altered 121 genes. Fish fed the CoPea diet showed minor signs of inflammation. Supplementation with P2 did not change these signs. Regarding composition of the microbiota in digesta from the distal intestine, clear differences regarding beta-diversity and taxonomy between Contr, SBM, and CoPea fed fish were observed. In the mucosa the microbiota differences were less clear. The two packages of functional ingredients altered microbiota composition of fish fed the SBM and the CoPea diet towards that of fish fed the Contr diet.

1. Introduction

A recent field survey regarding gut health of Atlantic salmon in commercial, salt water production sites along the coast of Norway revealed high frequency of symptoms of inflammation in the distal intestine (DI), symptoms which increased in severity throughout the production period [1]. Such inflammation may be related to the presence of antinutrients in some of the plant ingredients used in the diets. In Atlantic salmon, and some other fish species, inflammation can be induced by inclusion in the diet of standard soybean meal products (See review of relevant literature in Krogdahl et al. [2,3]), and in Siddiqui and Cresi [4]). However, diets used for salmon production in Norway today do not contain standard SBM products. Soy protein concentrate (SPC), on the other hand is the protein source included at the highest level, and was estimated to comprise 21% of the feed used in 2020 [5]. Studies of gut inflammation in Atlantic salmon indicate that SPC does

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not induce gut inflammation [3] at least not under relatively short term experimental conditions. However, some of the other commonly used plant ingredients have been in focus as possible causes of inflammatory processes in the intestine. Corn gluten is one of these which have been shown to induce inflammation in turbot [6,7]. Inflammation can also be induced by pea protein concentrate, another common ingredient in salmon diets [8]. On this background, it may be suggested that exposure to antinutrients from the such plant based ingredients may be the cause of the gradual progression of inflammation observed among salmon in farms along the coast of Norway [1].

In 2019, about 16% of Atlantic salmon transferred to sea in Norway were recorded as lost before slaughter [9]. In this situation, the use of functional ingredients in salmonid diets has become common practice intending to improve tolerance to environmental and handling stress and, not the least, strengthening resistance towards viruses, bacteria, and parasites. Prevention of gut inflammation is also an argument for the use of functional ingredients. They comprise prebiotics, probiotics and nucleotides, and some essential amino acids, vitamins and essential fatty acids [10]. Experiments addressing the ability of various functional ingredients to improve health and reduce mortality are many, as discussed in the review by Bharathi et al. [10]. However, very few studies have been reported from studies of use of functional ingredients in combination, as is often the commercial situation. In one recent study by Wang et al. [11] the most pronounced effects of inclusion of commercially relevant packages of functional ingredients, under commercial conditions, was increased consumption of feed per kilo of fish produced.

The aim of the present study was to investigate whether two mixtures of functional ingredients currently in commercial use, might mitigate gut inflammation induced by inclusion of ingredients known or suspected to induce inflammation in the distal intestine in Atlantic salmon, i.e. soybean meal, pea meal and corn gluten. The functional ingredients chosen for this study were butyrate, arginine, nucleotides, and β-glucan.

Butyrate, a monocarboxylic acid, is a product of microbial fermentation in the distal compartments of the intestine and serves as a substrate for enterocyte metabolism. According to a recent review by Siddiqui and Cresci [4], only a small amount reaches the blood circulation and other organs in an animal body. The review also states that butyrate, among many other roles, has anti-inflammatory properties due, in part, to its role in histone deacetylase (HDAC) inhibition in the intestinal epithelium and immune cells, as well as through inhibition of the activation of the transcription factor nuclear factor-κB (NF-κB) and thereby modulating proinflammatory cytokine production.

Arginine has gained a position as a functional ingredient in fish feed, due to its many roles in animals in addition to its role as a constituent in proteins. In the very comprehensive and detailed review of Andersen et al. [12] regarding functional amino acids, it is concluded that arginine appears to be an essential amino acids for fish, at least for some fish species. It serves as a precursor for production of biochemically and physiologically important components such as ornithine, polyamines, creatine, citrulline, glutamate, and nitric oxide, making this amino acid an essential molecule in an animal. If dietary supply or endogenous production of arginine becomes deficient, suboptimal conditions can be expected for many key functions in an animal body such as urea production, cellular stress responses, neurological processes, reproduction, smoltification, energy and lipid metabolism, digestive functions, as well as for the microbiota [12]. Arginine's position as a functional ingredient in fish diets is supported by results of challenge studies, e.g. in a study with channel catfish showing increased resistance towards *Edwardsiella ictaluri* [13], and with Senegalese sole showing increased resistance towards handling stress and infection by *Photobacterium damselae* subsp. piscicida [14]. It may, however, be argued that if supply of arginine above what is considered requirement improves health of an animal, the requirement estimate is too low and should be re-evaluated addressing biomarkers which are the most sensitive for indication of animal health and welfare.

Products of β-glucans, which contain natural cell wall polysaccharides from products such as cereal grains, in baker's yeast, mushrooms, seaweeds, and some bacteria species, are often used as functional ingredients in fish diets. Despite numerous experiments conducted to understand the basic mechanisms for the action of β-glucans there are many questions still to be answered as stated in several reviews, e.g., in the recent of Ching et al. [15]. What is clear, for example from the review of Ching et al. [15] is that β-glucans can affect many aspects of the immune system, such as components involved in activation of pattern recognition receptors (PRRs), in production of proinflammatory cytokines, tumour necrosis factor (TNF), and interleukin 8 (IL-8), Il-10 and IL-12 [16] with modulation of immune responses as a result.

Nucleotides are natural components of all plants and animals and present in most protein and carbohydrate rich feed ingredients used in aquaculture. The level of nucleotides in fish diets have decreased in parallel to the decrease in content of marine protein sources. Although nucleotides are not defined as essential for fish, some researchers argue that under demanding physiological periods and challenging environmental conditions, endogenous production may become insufficient for optimal function [17]. One possible factor may be a high energy cost for nucleotide biosynthesis. Producers of nucleotide ingredients claim that they increase growth and nutrient utilization, improve intestinal morphology and gut microbiota, reproduction, stress and disease resistance (Summarized in Hossain et al. [18]). However, negative effects may occur, in particular at high inclusion levels [19] possibly due to the high energy cost of catabolism and excretion. The mechanism of action of nucleotides has not been elucidated, as stated in the review by Hossain et al. [18].

2. Materials and methods

Typical signs of inflammation, which can be observed in fish, are loss of structure and function in affected tissues [20], as well as loss of appetite. Accordingly, the key observations in this study were effects in the intestine on tissue structure as indicated by histological observations, as well as on functions of the immune, digestion, and absorption systems, as indicated by alterations in molecular and biochemical biomarkers, and appetite as indicated by feed conversion and growth rate [21].

2.1. Fish, management and feeding

The feeding part of the experiment was conducted at Nofima's research facility at Sunndalsøra and lasted for 69 days. The research station is a facility approved by the Norwegian Animal Research Authority (NARA) and operates in accordance with Norwegian Regulations of June 17, 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). As no harmful procedures were forced upon the fish before euthanization, a specific permission was not needed for this experiment. The fish were Atlantic salmon (*Salmo salar* L) weighing on average 177g (SD of tank mean = 2.0) at start. Eighteen flow-through tanks with seawater, 1.5 m^2 surface area were used. The ambient water temperature averaged 11.2 °C, giving a total of 754 ddg, over the feeding period. Oxygen level in the water was kept between 80 and 100%. Each tank was stocked with 57 fish which were fed in 15–20% surplus of expected requirement. The amount of uneaten feed was recorded allowing estimation of feed intake [22], and mortality was observed.

2.2. Feed composition

The available resources allowed evaluation of six diets in triplicate, comprising one control diet (Contr) with high fish meal level and five experimental diets low in fish meal (Table 1). Three of the latter contained soybean meal, one of these (SBM) was made without functional ingredients, the other two were supplemented with each their packages

Table 1
Feed composition[a].

Diets	Contr	SBM	SBM + P1	SBM + P2	CoPea	CoPea + P2
Feed ingredients, %						
Fish meal	35.0	5.0	5.0	5.0	5.0	5.0
Soybean meal (HP)		30.0	30.0	30.0		
Corn gluten					25.0	25.0
Pea meal					7.5	7.5
Wheat gluten	20.0	21.0	21.0	21.0	20.0	20.0
Faba bean	5.0	5.0	5.0	5.0	5.0	5.0
Soy protein concentrate (SPC)	12.2	12.5	12.5	12.5	12.4	12.5
Wheat	10.6	6.8	5.3	6.6	4.0	4.1
Fish oil	8.5	9.4	9.3	9.4	10.6	10.4
Rapeseed oil	8.1	8.9	8.9	9.0	7.3	7.2
Astaxanthin	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin Mix	0.11	0.11	0.11	0.11	0.11	0.11
Mineral Mix	0.1	0.1	0.1	0.1	0.1	0.1
Yttrium premix	0.1	0.1	0.1	0.1	0.1	0.1
Inositol		0.124	0.124	0.124	0.124	0.124
Inorganic phosphate	0.602	1.529	1.505	1.529	1.794	1.781
Methionine		0.195	0.188	0.195	0.088	0.083
Lysine		0.551	0.523	0.551	1.246	1.228
Choline	0.229	0.443	0.443	0.443	0.443	0.443
Functional ingredients, %						
β-Glucans[b]				0.1		0.1
Butyrate			0.01	0.01		0.01
Nucleotides[b]				0.05		0.05
Arginine			1.5			
Analysed nutrient composition, %						
Dry matter	92.3	92.2	92.6	92.0	93.0	92.4
Crude lipid	19.4	18.1	18.3	19.1	21.2	19.7
Crude protein	52.4	45.7	47.7	46.1	47.9	49.0
Ash	6.1	4.5	4.4	4.5	4.1	4.1
Yttrium	0.008	0.007	0.008	0.007	0.007	0.007
Estimated values						
Gross Energy, MJ/kg	22	20	20	20	22	22
DP/DE, g/MJ	22	22	22	22	21	21

[a] Contr = control diet; SBM = diet containing soybean meal; CoPea = diet containing corn gluten and pea meal; P1 and P2: functional package 1 and 2; DP/DE: estimated ratio digestible protein/digestible energy.
[b] β-Glucans: Macrogard from Biorigin; Nucleotides from Lallemand.

of functional ingredients. The composition of the packages was chosen based on recommendations from the producers of the functional ingredients and supported by the feed industry partners involved in the project. Package 1 (P1) supplemented the diets with 0.01% butyrate and 1.5% arginine, and package 2 (P2) with 0.1% β-glucan, 0.01% butyrate and 0.05% nucleotides. Of the two last diets, both containing a mixture of corn gluten and pea protein concentrate, one was made without supplements (CoPea), the other with package P2. The diets were extruded and produced by Skretting AS at their experimental feed production unit.

2.3. Sampling

At termination of the feeding trial, six fish per tank were taken one by one from the tank and euthanized with an overdose of MS-222 (0.05–0.08 g/l). Blood samples were taken before weight and length were measured, and plasma collected. Thereafter, the fish were opened along the abdominal side and the organ package removed from the abdomen for further individual organ measurements and sampling. The liver was removed and weighed, and the digestive tract was gently stretched out, sectioned into the proximal intestine including the pyloric caeca (PI), mid (MI) and distal intestine (DI) and distal (PI2) half of the pyloric (PI), mid (MI) and proximal (DI1) and distal (DI2) half of the distal intestine (DI) [23]. Fig. 1 presents anatomical characteristic of the salmon intestine with indications of the sectioning used in the present study. External lipid was removed from these sections before they were separated and opened longitudinally. From fish which had eaten the same day, gut content samples, for enzyme, bile salt and microbiota analyses, were collected quantitatively from the main tract of all the sections as follows: from the proximal (PI1) and distal (PI2) half of the PI, the mid (MI) and the proximal (DI1) and distal (DI2) half of the DI. The weight of the cleaned tissues, i.e. PI including the caeca, MI, and DI, was recorded before tissue samples were taken for histology and molecular analyses in the middle of the sections. The remainder of the tissues were collected for enzyme analyses. Samples taken for histology were preserved on buffered formalin, samples of tissue and digesta for biochemistry and metabolomics were snap frozen in liquid N_2, whereas samples taken for molecular analyses were preserved on RNA later. Digesta and mucosa samples from the DI were collected under aseptic conditions from two fish per net pen for microbiota analyses as described by Li et al. [24].

2.4. Sample analyses

2.4.1. Nutrients in blood, biomarkers of digestive function, and histology

Details of the analytical procedures are presented by Wang et al. [11]. Standard methods were used for characterization of the feed and faeces, pooled per tank, regarding macronutrient, yttrium, and gross energy content. Plasma free fatty acids, cholesterol and total triacyl glycerides (TG) were analysed for six fish per tank, by standard procedures at the Central Laboratory of the Norwegian University of Life Sciences (NMBU). Trypsin activity was determined using benzoyl arginine p-nitroanilide as substrate whereas total bile acid concentration was quantified by the Enzabile test kit (No. 550101, BioStat Diagnostic Systems, Cheshire, U.K.) with taurocholic acid as standard. Leucine aminopeptidase (LAP) capacity in tissue homogenates of the DI was analysed using L-Leucine β-naphthylamide hydrochloride as substrate.

Histological evaluations of general structure were based on formalin-fixed, H&E-stained sections and were conducted for samples of the PC and DI. These assessments focused on the characteristic morphological changes of soybean meal-induced enteritis (SBMIE) in Atlantic salmon

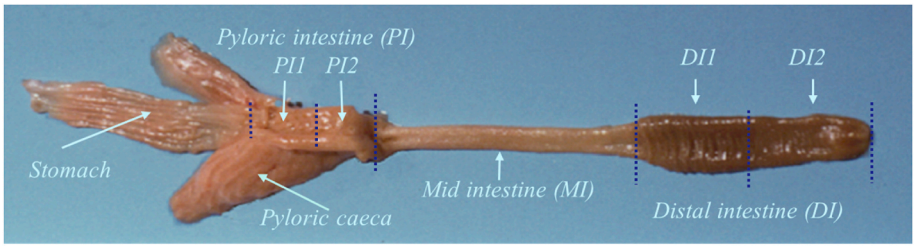

Fig. 1. Picture of opened salmon intestine with explanation of the sectioning used for sampling of digesta and tissue as explained in more detail in the text regarding sampling procedures.

DI, which consist of shortening of mucosal fold height, increase in width and cellularity of the submucosa and lamina propria compartments, and reduction in enterocyte supranuclear vacuolization [20]. For the pyloric caeca, increased vacuolization due to lipid retention in the enterocytes (enterocyte steatosis) was also assessed. All morphological characteristics evaluated for the PC and DI were graded on a scale of 0–4 where 0 represented normal; 1, mild changes; 2, moderate changes; 3, marked changes, and 4, severe changes.

2.4.2. Gene expression

Microarray analyses were performed on samples from the distal intestine, taken randomly from five fish per treatment, i.e. two fish from two cages and one from the third case used for each treatment. Nofima's 15k Atlantic salmon oligonucleotide DNA microarray SIQ6 (GPL30031) was used for the analyses. The slides were fabricated by Agilent Technologies (Santa Clara, CA, USA). Data were processed with Nofima's bioinformatics pipeline STARS (Krasnov et al., 2011). Differentially expressed genes (DEG) were selected at cut-off $\log_2$-Expression Ratios (ER) > 0.8 (1.75-fold) and $p < 0.05$ (*t*-test). The functional groups of genes (STARS annotation) were compared by mean $\log_2$ – ER of DEG. Data were submitted to NCBI Geo Omnibus under the accession number GSE221800.

2.4.3. Metabolome

Chemicals and reagents used in this study were purchased from Sigma-Aldrich (Søborg, Denmark) and included deuterium oxide (D_2O, 99.9 atom % D), sodium phosphate monobasic monohydrate (NaH_2PO_3, H_2O), sodium phosphate dibasic heptahydrate (Na_2HPO_3, $7H_2O$), sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP, 98 atom% D, ≥98.0%), and sodium azide (NaN_3, ≥99.5%). The water used throughout the study was purified using a Millipore lab water system (Merck KGaA, Darmstadt, Germany) equipped with a 0.22 μm filter membrane.

Each of the individual, plasma samples were measured by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. NMR analysis was performed as described by Aru et al. [25]. Briefly, equal amounts of plasma and phosphate buffer were gently mixed and, for each sample, 600 μl were transferred into an NMR tube. Proton (^{1}H) NMR spectra were recorded using a Bruker Advance III 600 MHz NMR spectrometer equipped with a 5 mm broadband inverse RT (BBI) probe. NMR spectra were measured in automation using the pulse program *cpmgpr1d* (BioSpin nomenclature). Further details on the instrument, instrument calibration and NMR measurements can be found in Aru et al. [25]. NMR spectra were imported into MATLAB 2020a (Mathworks Inc., Natick, MA, USA) where signals alignment was performed using *icoshi* [26]. Metabolite concentrations were calculated by row sum of the spectral intensities and were analysed by principal component analysis (PCA) [27].

2.4.4. Microbiome

For microbiota profiling, DNA was extracted from distal intestinal digesta and mucosa samples as previously described [28]. For quality control of the microbiota profiling protocol, along with the each of the DNA extraction batch, two 'blanks' (without any sampling materials) and two 'positive controls' i.e. mock (microbial community standard from Zymo-BIOMICS™, Zymo Research, California, USA) were included. Amplicon PCR followed by library preparation and sequencing was performed as described in Ref. [28]. As an extra measure to identify contaminating sequences, qPCR was performed separately to quantity 16S rRNA gene in the diluted DNA templates (samples, blanks, and mocks) used for the amplicon PCR.

Bioinformatics analysis of microbiota sequencing data was performed using QIIME2 version 2. The demultiplexed single-ended reads were denoised, trimmed and quality filtered using the DADA2 algorithm in QIIME2. The taxonomy was assigned to resulting amplicon sequence variants (ASVs) table by a Scikitlearn Naive Bayes machine-learning classifier, which was trained on the SILVA 132 99% ASVs that were trimmed to exclusively include the regions of 16S rRNA gene amplified by the primers used in the current study. ASVs table was filtered to remove chloroplast and mitochondria, low abundant ASVs with total abundance of less than 2 across all the samples and ASVs that are without a phylum-level taxonomic assignment or appeared in only one biological sample. In total 27 ASVs were detected as contaminant sequences following the procedure as described in our recent work [28].

To compute alpha and beta diversity indices, the ASVs tables were rarefied in 1837 reads for digesta samples and 526 reads for mucosa samples in order to have an even number of reads across all the samples. Alpha diversity was calculated using observed taxa and Shannon's diversity indices with Kruskal-Wallis test. Beta diversity was evaluated using Bray-Curtis and unweighted UniFrac distance metrics with PERMANOVA test. The MicrobiomeAnalyst package was used to analyses and graphical presentations of abundant taxa among the groups and visualization of diversity matrices, using ASVs table at feature level.

16S rRNA sequencing data is publicly available at the NCBI Sequence Read Archive (SRA) with the accession number SUB12035604 under the Bioproject PRJNA879257.

2.5. Calculations

Growth of the fish was calculated as Thermal growth coefficient: $TGC = 1000 * [\sqrt[3]{BW1}^{-} \sqrt[3]{BW0})^{/ddg}]$. BW0 and BW1 are the initial and final body weight (tank means) and ddg is daydegrees (no of feeding days x average temperature in °C). Condition factor (CF) = BW1 x 100/BL1, where BW is measured in g and BL = body length in cm. Organosomatic Indices (OSI) were calculated as: 100 x (organ weight/body weight). Apparent digestibilities (AD) of main nutrients was estimated by using Y_2O_3 (56) as an inert marker and calculated as follows: $ADN = 100 - (100 \text{ X } (M_{faeces}/M_{feed}) \text{ X } (N_{feed}/N_{faeces}))$, where M represents the percentage of the inert marker in feed and faeces and N represents the percentage of a nutrient in feed and faeces.

2.6. Statistical evaluation

For data evaluation regarding the results of gene expression, microbiota, and metabolome see detailed described under the relevant chapters above. Differences in histological scores for the various evaluated morphological characteristics of the PC, DI, and PC tissues were analysed for statistical significance using an ordinal logistic regression run in the R statistical package (version 3.6.2; 2019) within the RStudio interphase (version February 1, 5033; 2019). For other results one-way ANOVA was used employing the SAS 9.3 computer software (SAS 2017) with tank as the observational unit, i.e. n = 3.

3. Results

3.1. Growth, blood biomarkers, digestive physiology, and nutrient digestion

The fish grew well, and only one fish died during the feeding period. The thermal growth coefficient (TGC) varied between 3.4 and 3.9 (see Table 2), i.e. the average weight of the fish more than tripled during the feeding period. Fish fed the Contr diet showed the fastest growth. Fish fed the SBM and the SBM + P2 diet grew significantly less than the Contr fed fish, by 15% and 10%, respectively. Fish in other treatments showed intermediate growth rates not significantly different from those fed Contr and CoPea diets. None of the supplementation packages altered the growth rate significantly. The feed conversion ratios (FCR, Table 2) indicated high efficiency of nutrient utilization, between 0.55 and 0.61, in line with the high growth rates. The nutrient utilization was significantly higher for the Contr fed fish compared to the other fish, between which no significant differences were observed. The fish sampled at

Table 2
Results regarding body weight (BW), growth, expressed as thermal growth coefficient (TGC), feed conversion ratio (FCR), condition factor (CF), liver index (HSI, % of body weight), and digestibility (Dig.) of fat, crude protein (CP), and ash for all fish fed the six diets, and body characteristics for fish sampled at termination of the feeding period[a].

Variable	Diets						SEM	p(model)
	Contr	SBM	SBM + P1	SBM + P2	CoPea	CoPea + P2		
All fish								
BW, g	633^{a}	543^{c}	567^{bc}	569^{bc}	594^{b}	583^{b}	11	0.0011
TGC	4.0^{a}	3.4^{b}	3.6^{ab}	3.6^{b}	3.8^{ab}	3.7^{ab}	0.08	0.0071
CF	1.49^{a}	1.46^{a}	1.40^{b}	1.48^{a}	1.51^{a}	1.49^{a}	0.02	0.0097
FCR	0.55^{b}	0.61^{a}	0.61^{a}	0.61^{a}	0.59^{ab}	0.60^{a}	0.01	0.0121
HSI, %	1.17	1.19	1.16	1.18	1.27	1.24	0.03	0.1033
Dig. CP, %	89.6^{c}	90.1^{bc}	91.2^{ab}	90.9^{ab}	91.5^{a}	91.5^{a}	0.28	0.0006
Dig. Fat, %	96.9	97.0	97.2	96.7	96.4	96.4	0.24	0.3520
Dig. Ash, %	2^{a}	−21^{b}	−20^{b}	−22^{b}	−1^{a}	−6^{a}	2.9	0.0001
Sampled fish								
BW, g	644^{a}	598^{ab}	582^{b}	599^{ab}	630^{ab}	645^{a}	14.2	0.0453
Carcass weight, g	574^{a}	527^{b}	511^{b}	525^{b}	552^{b}	567^{b}	12.9	0.0367
Body length, cm	35.1	34.4	34.6	34.3	34.7	35.1	0.3	0.4370
CF	1.50^{a}	1.43^{b}	1.40^{b}	1.48^{a}	1.50^{a}	1.50^{a}	0.01	0.0004
HSI, %	1.13	1.2	1.17	1.17	1.27	1.2	0.02	0.0668

[a] Contr = control diet; SBM = diet containing soybean meal; CoPea = diet containing corn gluten and pea meal; P1=Package 1 of functional ingredients: 0.01% butyrate and 1.5% arginine; P2=Package 2 of functional ingredients: 0.1% β-glucan, 0.01% butyrate and 0.05% nucleotides. SEM = Pooled SEM. For ash the numbers are not indicating minerals absorbed from the diet, as minerals in drinking water is not accounted for in the input. The data indicate malfunction in the reabsorption of the minerals for the fish fed the SBM diets. Regarding the results for the sampled fish, six fish were sampled per tank. Tank mean was used as statistical unit.

termination for further investigations showed the same picture regarding body weight and carcass weight (Table 2), indicating that they represented the total population well.

Regarding nutrient digestibilities (Table 2), no significant diet effect was observed for lipid. For crude protein, the Contr fed fish showed the lowest values, a value significantly lower than that observed for the CoPea fed fish. The fish fed the unsupplemented SBM diet, showed intermediate protein digestibility, not significantly different from either the Contr or CoPea treatment. Compared to the corresponding basal diet, none of the supplementations affected protein digestibility significantly. The supplemented SBM diets, however, showed protein digestibilities significantly higher than observed for the Contr diet. Ash digestibility showed great differences, with very low and negative values for fish fed the SBM diets, irrespective of supplementation.

The plasma analyses showed significant diet effect for the cholesterol level ($p = 0.0093$; pooled SEM = 0.24) with the values in mM for Contr: 9.7^{a}, SBM: 8.4^{b}, SBM + P1: 8.3^{b}, SBM + P2: 8.5^{b}, CoPea: 9.5^{a}, CoPea + P2: 9.0^{ab} (No common asterix indicates significant difference). The diets with SBM, compared to the Contr diet, reduced plasma cholesterol significantly, independent of supplementation, whereas the CoPea diets did not affect the observed plasma variables. The diet effects were insignificant for plasma alanine transferase (total mean = 2.9 U/l; $p = 0.8953$, pooled SEM = 2.9), triglycerides (total mean = 0.34 mM; $p = 0.5270$, pooled SEM = 0.34), and glucose (total mean 4.8 mM; $p = 0.0912$; pooled SEM = 0.11).

The somatic indices of the intestinal sections (Table 3) showed, compared to fish fed the Contr diet, increased weight of the pyloric intestine (PI) for fish fed fish fed all the SBM diets, and for fish fed the CoPea diet without supplement. No significant diet effects were observed for the mid intestine (MI). The DI, on the other hand, showed great diet effects regarding the index. Fish fed the SBM diets showed reductions in the DI index of about 25%, whereas fish fed the CoPea diets showed a 30% increase.

Chyme bile salt concentration (Table 3) singled out the SBM diets which showed greatly reduced levels in the PI2 and in DI1 (not measured in PI1 and MI due to shortage of material). In DI2 most of the bile salt had, apparently, been reabsorbed, and the bile salt level did not show significant diet effects. Fish fed the CoPea diets did not differ significant from fish fed the Contr for any of the intestinal sections.

Chyme trypsin activity (Table 3) showed great tank variation in the proximal section of the intestine (PI2) and no significant diet effects. In the distal compartments the variation was much less. The results for DI1 were very different from those of the DI2. In DI1 fish fed the SBM diets showed similar values as the Contr fed fish, whereas the CoPea + P2 fed fish showed significantly lower activity than the Contr fed fish. Supplementation of the SBM basal diet with P1 and P2, did not affect the

Table 3
Results for sampled fish regarding relative weights of tissue (OSI, %), concentration of bile salts (BS, mg/g dry matter), and activity of trypsin (U/g dry matter) of pyloric (PI), mid (MI) and distal intestine (DI) of fish fed the six diets*.

Section	Variable	Diet							
		Contr	SBM	SBM + P1	SBM + P2	CoPea	CoPea + P2	SEM	*p(model)*
PI	OSI	1.99^{c}	2.41^{a}	2.24^{ab}	2.33^{a}	2.20^{ab}	2.13^{bc}	0.06	0.0067
	BS	207	106	133	123	193	218	11	<0.0001
	Trypsin	136	79	156	86	149	99	21	0.1777
MI	OSI	0.17	0.18	0.18	0.17	0.17	0.17	0.01	0.8156
	BS	104	36	35	40	105	111	6	<0.0001
	Trypsin	115	135	101	106	93	79	9	0.0358
DI	OSI	0.49b	0.38^{c}	0.37^{c}	0.38^{c}	0.65^{a}	0.65^{a}	0.01	<0.0001
	BS	20	13	12	17	15	13	7	0.7083
	Trypsin	12	121	95	85	8	7	7	<0.0001

*Different letters indicate significant difference between the different diet (ANOVA, n = 3. See Table 2 for explanation of diet symbols and SEM. The results are based on observations from six fish per tank. The statistical evaluations are based on tank means (n = 3).

Table 4
Results regarding specific activity (LAP_Prot, U/mg protein) and total capacity (LAP_Cap, U/kg fish) of leucine aminopeptidase (LAP) in tissue sampled from the pyloric (PI) and distal intestine (DI) of fish fed the six diets*.

Section	Variable	Diet							
		Contr	SBM	SBM + P1	SBM + P2	CoPea	CoPea + P2	SEM	*p(model)*
PI	LAP_Prot	713^{a}	706^{a}	688^{a}	767^{a}	597^{b}	524^{b}	29	0.0007
	LAP_Cap	569^{bc}	691^{a}	650^{ab}	746^{a}	552^{bc}	501^{c}	36	0.0036
DI	LAP_Prot	1126^{a}	155^{c}	208^{c}	242^{c}	621^{b}	525^{b}	35	<0.0001
	LAP_Cap	194^{a}	23^{c}	27^{c}	33^{c}	137^{b}	129^{b}	5	<0.0001

*Different letters indicate significant difference between the different diet (ANOVA, n = 3. See Table 2 for explanation of diet symbols and SEM. The results are based on tank pools of the tissues (n = 3).

trypsin activities in DI1 significantly. The same was observed for the supplementation of the CoPea diet with P2. However, in DI2 very low trypsin activities were observed for fish fed the Contr and those fed the CoPea diet, whereas for the SBM basal diet, values were 10 times higher than for the Contr and CoPea fed fish. For the SBM basal diet no effects were seen on trypsin activity upon supplementations with the P1 package. A small, but significant reduction was observed upon supplementation with P2, to a level somewhat less that observed for P1, i.e. 8 times higher than observed fish fed the other diets (Contr and CoPea).

In tissue samples from PI, specific activity of tissue leucine aminopeptidase (LAP) (Table 4) showed no difference between Contr and SBM fed fish, whereas fish fed the CoPea diets showed lower specific activity. The results for the two basal diets were independent of supplementation. A somewhat different pictures was seen when the activity was expressed as capacity, i.e. per body weight of the fish. Fish fed SBM diets showed elevated capacity, compared to the Contr fish, whereas the CoPea fed fish showed similar values as the Contr fish. No clear effect of supplementation was observed for any of the two basal diets. The tissue samples from DI showed a very different picture. Compared to fish fed the Contr diets, the fish fed any of the SBM diets showed an 80–90% reduction, independent of supplementation. Furthermore, fish fed the two CoPea diets showed reduced capacity, but the differences were less, about 50% compared to the Contr, again with no significant effect of supplementation.

Chyme dry matter decreased along the intestine of all fish (Supplementary Table 1). Fish fed the SBM diet, overall, showed significantly lower values than the Contr fed fish, whereas the chyme from CoPea fed fish did not differ from the Contr fed fish. The exception was chyme from the most proximal part of the intestine, PI1, in which the dry matter content was significantly higher in the CoPea fed fish. Supplementation of the SBM and CoPea diets with P1 and P2 did not change this pattern.

3.2. Histological observations

The histological investigations of tissue from the distal intestine (Fig. 2) showed healthy conditions in fish fed the Contr diet. Fish fed the SBM diets, showed all typical signs of inflammation, i.e. increased infiltration of immune cells in submucosa and lamina propria, shortening of mucosal folds and most markedly loss of supranuclear vacuolization.

Regarding fish fed the CoPea diet, mild to moderate inflammatory changes in the submucosal compartment (Fig. 3) were observed in about two thirds of the fish. The morphological observations differed from those observed in the SBM fed fish. The inflammation in CoPea and CoPea + P2 was observed to be a multifocal infiltration of submucosa and lamina propria, with no accompanying loss in supranuclear vacuoles or shortening of the mucosal folds. Supplementation of CoPea with P2 did not significantly lessen occurrence of the inflammatory changes, although the scores decreased somewhat.

3.3. Gene expression

Comparison of the treatments showed the following numbers of DEGs: SBM vs Contr: 849; CoPea vs Contr: 26; SBM + P1 vs SBM: 81: SBM + P2 vs SBM: 121; SBM + P1 vs SBM + P2: 133; CoPea + P2 vs CoPea: 49; SBM vs CoPea: 698. The SBM diet induced gene expression related to cellular and protein stress (chaperones including *heat shock proteins* and *cognates*) and suppressed oxidative stress responses (Figs. 4

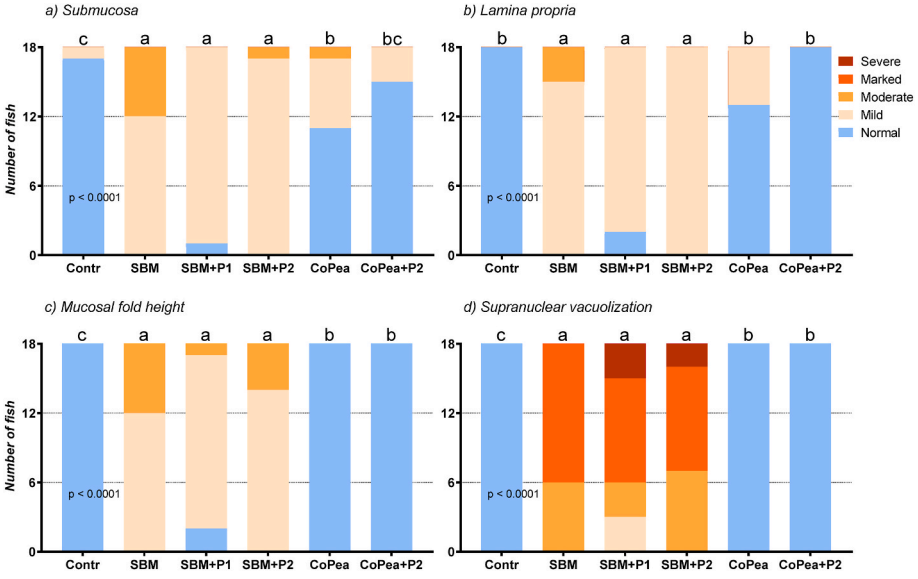

Fig. 2. Results of histological evaluation of tissue from the DI regarding width and cellularity of submucosa (a) and lamina propria (b), mucosal fold height (c) and supranuclear vacuolization (d) graded as normal, mild, moderate, marked, and severe. Six fish were observed per tank.

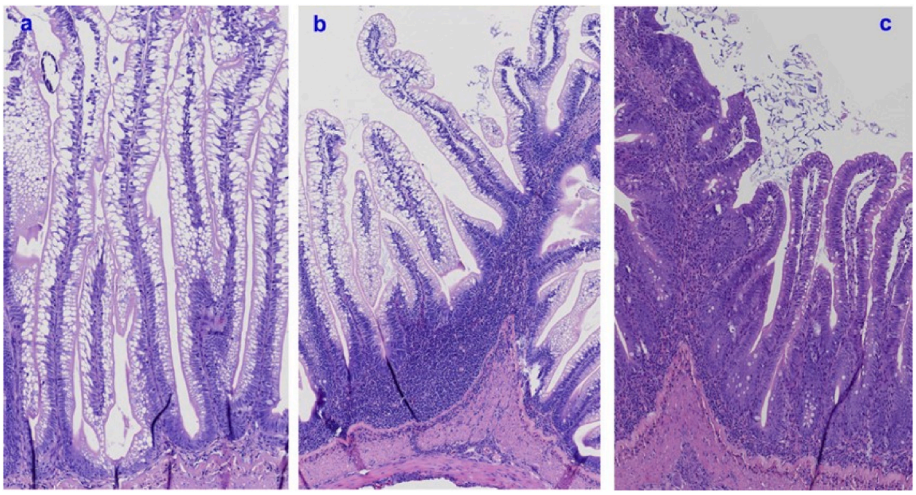

Fig. 3. Histology images from the distal intestine of fish from the GM-8 study showing the (a) normal and healthy DI mucosa. Image (b) is a representation of the appearance of the inflammation in the CoPea fed fish (localized area of marked inflammation with no loss in vacuoles and reduction in mucosal fold height) compared to Image (c) for SBM-fed fish showing classic SBM-induced enteritis.

and 5). *Immediate early response 5* and *2*, *junb1* and *dna damage inducible transcript 4* (Fig. 5) are generic stress markers revealed with meta-analyses of Atlantic salmon transcriptome [29]. Down-regulation of antigen presentation took place in parallel to stimulation of several functional groups of the immune system including a panel of genes identified as markers of acute inflammation [29]: *arginase ii*, *cathelicidin*, *tnf decoy receptor*, highly damaging matrix metalloproteinases (*mmp9* and *13*) and components of oxidative burst complex (*cytochrome b-245*). Up-regulation was observed in a number of chemokines and cytokines including *il11* and *il18,* while down-regulat was observed for *il6* known as a both pro and anti-inflammatory cytokine. Overall, of the 126 immune DEG, 91 genes were up regulated. Changes of metabolism in the SBM treatment were greater (345 DEG) and down-regulation prevailed (274 DEG). Decreased expression was observed for genes encoding transporters, proteases and protease inhibitors, proteins involved in metabolism of iron and heme, vitamins, sulfuric compounds, lipids, steroids, and xenobiotics (Fig. 6).

SBM increased 2.1-fold expression of *guanylin*, an intestine-specific peptide regulating transport of electrolytes and water.

Fish fed the SBM + P1 diet, compared to fish fed the SBM diet, showed 81 DEGS out of which 69 showed downregulation. In contrast, 68 of 121 genes that responded to P2 were upregulated. This difference is explained by the nature of the action with a predominance of immunosuppression (P1) and stimulation of metabolism (P2). Both packages reduced SBM-induced expression of several pro-inflammatory genes (Fig. 9). P1 downregulated 38 immune genes and only two immune genes were upregulated. Six immune genes were downregulated by both P1 and P2. Fourteen immune genes responded only to P2, and

Functional group	No	SBM vs Contr	SBM vs CoPea	SBM+P1 vs SBM	SBM+P2 vs SBM	SBM+P1 vs SBM+P2
Protein folding & modification	24	***1.5***	***1.5***	-1.0	***0.8***	***1.2***
Oxidative stress response	20	***-1.7***	***-2.1***	1.0	1.2	-1.2
Amino acid metabolism	34	-1.6	***-1.6***	1.1	***1.2***	-1.1
Transport	26	***-2.3***	***-2.1***	-1.0	***1.2***	***-1.3***
Mitochondria	32	***-1.8***	***-1.7***	1.1	***1.2***	-1.1
Proteases	46	***-1.5***	-1.3	-1.1	***1.1***	***-1.3***
Lysosomes	9	***-2.2***	***-2.1***	-1.0	***1.2***	***-1.2***
Retinoid metabolism	10	-1.4	-1.4	1.0	***1.3***	***-1.3***
Sulfur metabolism	5	***-5.1***	***-4.0***	1.1	***1.7***	***-1.5***
Cytochromes P450	10	-1.7	-1.7	1.1	***1.5***	***-1.4***
Lipid metabolism	60	***-2.0***	***-1.8***	-1.0	***1.3***	***-1.4***
Xenobiotic metabolism	45	***-1.9***	***-1.8***	1.1	***1.3***	***-1.3***
Antigen presentation	18	***-1.5***	***-2.7***	***-1.7***	1.2	***-2.0***
Chemokine	14	1.8	1.4	***-1.9***	***-1.6***	-1.1
Lectin	13	***1.7***	***2.0***	***-1.8***	***-1.6***	-1.2
Immune regulator	21	***1.9***	***1.7***	***-1.4***	-1.2	***-1.2***
TNF-related	10	***1.9***	***1.7***	***-1.3***	-1.1	-1.2

<-5 <-2.5 <-1.4 <-0.8 <-0.4 0 >0.4 >0.8 >1.4 >2.5 >5

Fig. 4. Effect of feeds on gene expression in distal intestine: functional groups. Data are folds; numbers of DEG are indicated. Significant expression differences ($p < 0.05$) are highlighted with underlined bold italics. The results are means of five fish per treatment, two from two of the tanks, one from the last.

Gene	SBM vs Contr	CoPea vs control	SBM vs CoPea	SBM+P1 vs SBM	SBM+P2 vs SBM	SBM+P1 vs SBM+P2
Heat shock cognate 70	***4.0***	1.0	***4.1***	-1.2	-1.5	1.2
Heat shock protein 90, alpha	***2.1***	1.0	***2.0***	-1.4	-1.7	1.2
Jun B-1	***4.0***	1.8	2.2	***-2.3***	-1.9	-1.2
Immediate early response 2-2	***2.0***	1.1	***1.8***	-1.5	1.0	-1.5
Immediate early response 5-1	***2.6***	1.2	***2.2***	-1.1	-1.0	-1.1
DNA damage-inducible 4 protein	***3.0***	2.1	1.4	-1.2	***2.0***	***-2.4***
Thioredoxin	***-2.8***	1.1	***-3.1***	1.4	***3.0***	***-2.1***
Superoxide dismutase [Mn]	***1.2***	0.9	1.6	1.3	1.4	-1.1
Selenoprotein P, plasma, 1	***-3.8***	0.7	***-2.7***	1.2	1.6	-1.3
Beta-2 microglobulin	***1.2***	1.2	***-2.2***	-1.3	1.2	-1.5
H-2 class I HC antigen, Q10 alpha chain	-7.7	2.9	***-22.2***	-1.3	1.9	-2.4
MHC class I antigen	***-3.6***	1.2	***-4.3***	-1.7	-1.1	-1.6
C-C motif chemokine 19-4	***11.7***	2.4	***4.8***	***-3.8***	***-4.8***	1.3
Chemokine CK-1 precursor	***4.1***	1.1	***3.7***	-1.6	-1.3	-1.3
Arachidonate 5-lipoxygenase-activatir	***2.0***	1.0	***2.1***	-1.6	-1.2	-1.3
Interleukin-6	***-2.7***	1.1	***-3.1***	2.5	1.2	-1.6
Interleukin-11	***4.2***	1.2	***3.4***	2.2	1.3	-1.8
Interleukin-18	***2.3***	1.0	***2.2***	-1.1	-1.2	1.1
TNF decoy receptor	***3.6***	1.3	***2.7***	-1.7	-1.7	1.0
C1q and TNF domains	***4.2***	1.1	***3.7***	-1.3	-2.5	1.9
Complement C1q protein 2	***3.3***	1.2	***2.9***	-2.0	-1.7	-1.2
Cathelicidin (cath)	***2.8***	1.0	***2.7***	-1.9	-2.1	1.1
E-selectin	***4.2***	1.5	***2.8***	***-2.8***	-2.9	1.0
Lectin, putative proteoglycan	2.6	0.8	***3.4***	***-3.9***	-2.5	-1.6
Cytochrome b-245 light chain	***4.8***	1.6	***3.0***	***-2.2***	***-2.1***	-1.1
Arginase, type II	***13.4***	1.0	***14.0***	-1.4	***-3.0***	2.1
Matrix metalloproteinase-9	1.5	0.5	***2.8***	***-2.1***	-1.6	-1.3
MMP 13 or Collagenase 3	***4.9***	0.9	***5.7***	1.1	-1.7	1.9

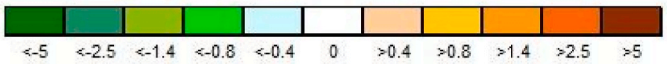

Fig. 5. Effect of feeds on gene expression in distal intestine: genes involved in stress and immune responses. Data are folds; differential expression is highlighted with underlined bold italics. Genes – markers of Atlantic salmon responses to stress, inflammation and bacterial infections [30] are in bold. The results are means of five fish per treatment, two from two of the tanks, one from the last.

six of them were activated. P2 stimulated the expression of 31 metabolic genes and only five were downregulated; with P1 those numbers were two and six. Package 2, but not Package 1, reversed the suppression of many metabolic genes, especially genes involved in the metabolism of lipophilic compounds (Fig. 10).

Gene expression in DI in the CoPea fed fish was not very different from that in the Contr fed fish, as changes were found in only 26 genes (Table 5). However, most of the affected genes belong to the functional groups of the immune system, such as antigen presentation, chemokine, lectin immune regulator and TNF-related (Fig. 8). Only marginal effects were observed upon supplementation of the CoPea diet with P2, inducing differences in only 49 genes, most of which are involved in general metabolism and most likely not in inflammation.

3.4. Results of metabolome analyses

The result of the metabolomics analyses of salmon blood plasma revealed that individual variability was by far the highest variation source in the metabolite concentrations (up to 83% of the total metabolite variability). Such a high individual variability may have masked weak dietary effects.

The ^{1}H NMR spectral landscape was dominated by the intense resonances from the methylene (–CH_2–), methyl (-CH_3), and ethylene (-CH═CH-) moieties of lipids in lipoproteins followed by the lactate signal. The bulk metabolome of the plasma also included cholesterol and cholesterol ester, the organic acids acetate and formate, the amino acids alanine, glutamine, glycine, methionine, lysine, tyrosine, tryptophan, phenylalanine, the branched-chain amino acids leucine, isoleucine, and valine as well as choline and glucose. A total of 65 ^{1}H NMR resonances were assigned as metabolites or functional groups from lipids in lipoproteins (level 2 assignment). PCA was performed to scrutinize the variability of the metabolite concentrations. A total of three comparison models were made: 1) comparison of the Contr and the SBM and CoPea basal diets (Fig. 7) presented as PCA biplots of scores (samples) and loadings (variables, metabolite relative concentrations), 2) comparison of the CoPea diets (Fig. 8A), and 3) comparison of the SBM diets (Fig. 8B). In Fig. 7, the biplot of PC1 vs PC2 (approx. 46% of the explained variance) for model 1 (comparison of SBM and CoPea basal diets with Contr) is shown. The figure reveals minor separations between Contr and the basal diets (SBM and CoPea) along PC2, with the majority of the SBM samples clustering at negative PC1 values. The individual fish distribution along PC2 was more driven by the higher choline, phosphocholine and cholesterol concentrations, which were higher in samples clustering at positive PC2 values (mainly fish fed the

Gene	SBM vs Contr	CoPea vs Contr	SBM+P1 vs SBM	SBM+P2 vs SBM	CoPea+P2 vs CoPea	SBM vs CoPea	SBM+P1 vs SBM+P2
Solute carrier family 13	***-1.9***	-1.1	1.3	1.7	-1.3	***-1.8***	-1.4
Aquaporin 8b	***-8.1***	1.2	-1.8	1.1	1.2	***-9.8***	-2.1
Transcobalamin-2	***-33.8***	***-1.8***	1.7	2.1	1.1	***-19.1***	-1.2
Solute carrier family 6	***-3.1***	-1.1	-1.0	1.4	1.1	***-2.8***	-1.3
4-aminobutyrate aminotransferase	***-12.8***	-1.1	-1.1	2.3	1.3	***-11.3***	-2.7
Cysteine dioxygenase type 1	***-4.1***	-1.2	-1.0	***1.8***	1.2	***-3.5***	***-1.8***
Heme-binding protein 2	***-97.0***	-1.7	1.5	1.2	1.6	***-57.0***	1.3
Heme oxygenase	***-3.5***	-1.4	-1.1	1.2	1.3	***-2.4***	-1.3
Tissue inhibitor of metalloproteinase 2a	***-4.7***	1.3	1.1	-1.0	1.2	***-6.1***	1.1
Meprin A subunit alpha	***-5.6***	1.1	-1.4	1.3	1.1	***-6.0***	***-1.9***
Serine carboxypeptidase CPVL	***-3.4***	-1.1	-1.1	1.5	1.1	***-3.1***	-1.6
Angiotensin-converting enzyme	***-3.0***	-1.2	1.1	1.2	-1.2	***-2.6***	-1.1
7-dehydrocholesterol reductase (DHCR7)	***-10.4***	-1.4	-1.3	1.8	-1.0	***-7.7***	-2.4
Vitamin D3 hydroxylase-associated protein	***-2.6***	1.0	-1.0	1.3	-1.2	***-2.6***	-1.3
3-oxo-5-beta-steroid 4-dehydrogenase	***-2.3***	-1.1	1.1	1.2	-1.2	***-2.1***	-1.1
Solute carrier family 26 member 6	***-2.4***	-1.2	1.3	1.6	-1.0	***-2.0***	-1.2
Sulfotransferase	***-12.4***	-1.1	1.5	***1.9***	1.2	***-11.8***	-1.3
Apolipoprotein A-II	***-2.8***	1.0	1.4	1.8	-1.1	***-2.9***	-1.3
Acyl-coenzyme A thioesterase 11	***-11.5***	-1.4	-2.2	1.6	***2.6***	***-8.1***	-3.5
Long-chain-fatty-acid--CoA ligase ACSBG1	***-6.7***	-1.3	1.1	***2.4***	1.7	***-5.1***	***-2.2***
Fatty acid-binding protein, intestinal	***-3.7***	-1.2	-1.5	***2.0***	1.1	***-3.2***	***-2.9***
Fatty acid desaturase 6	***-11.5***	-1.5	1.3	***2.7***	1.4	***-7.9***	-2.0
Cytochrome P450 2F3	***-3.2***	-1.2	1.1	1.5	-1.0	***-2.8***	-1.4
Cytochrome P450 2D15	***-2.7***	1.1	1.3	***2.0***	-1.1	***-3.0***	-1.6
Cytochrome P450 XXVIA1	***-3.5***	1.0	1.2	1.7	-1.0	***-3.4***	-1.4
Cytochrome P450 1A1	-2.0	1.1	-1.0	1.2	1.3	***-2.1***	-1.2
Carboxylesterase	***-3.4***	-1.2	1.6	***1.9***	1.4	***-2.9***	-1.2
UDP-glucuronosyltransferase 2A2	***-3.4***	-1.2	-1.2	1.5	1.7	***-2.9***	***-1.8***
Guanylin precursor	***-4.0***	-1.4	1.1	1.5	-1.3	***-2.9***	-1.4

Fig. 6. Effect of feeds on gene expression in distal intestine: genes involved in metabolism. Data are folds; differential expression is highlighted with underlined bold italics (Krasnov et al., 2021). The results are means of five fish per treatment, two from two of the tanks, one from the last.

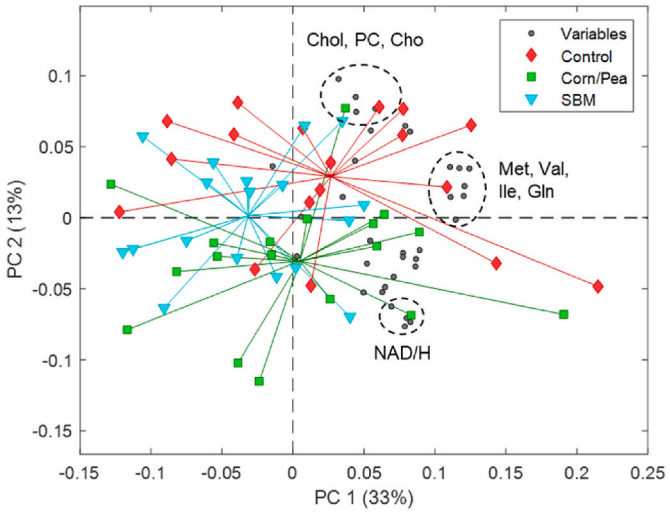

Fig. 7. Biplots of the PCA performed on the plasma metabolite concentrations as measured by ^{1}H NMR. Plasma samples from individual salmon (N = 54) fed the basal diets (Control, Corn/Pea, and SBM, see legend for color-coding explanation) were included in the PCA. Keys: Cho: cholesterol; Chol: choline; PC: phosphocholine; Met: methionine; Val: valine; Ile: isoleucine; Gln: glutamine; NAD/H: NAD^{+} and NADH. The plots are based on all observations, i.e. 6 fish from each of the 18 tanks.

Contr diet). The individual fish distribution along PC1 mostly reflects the high interindividual variability and was driven by the amino acid methionine, valine, isoleucine, and glutamine, which were higher in the fish samples at higher PC1 values.

Fig. 8 show the biplots for comparison 2 (8A) and 3 (8B). No clear separation was revealed between the CoPea diets and sample distribution in the PC1 vs PC2 biplot (approx. 47% of the explained variance) was dominated by the great interindividual differences (Fig. 8A). In Fig. 8A, the biplot showing the comparison amongst the SBM diets is illustrated (approx. 38% of the explained variance). A weak trend can be observed along PC2, with samples fed the SBM + P1 diet and SBM + P2 diet clustering in opposite PC2 quadrants. Choline, phosphocholine, and cholesterol were more abundant in the SBM + P1 samples, while plasma samples from salmon fed the SBM + P2 diet had higher amounts of the coenzyme nicotinamide adenine dinucleotide. As before, sample distribution along PC1 (approx. 26% explained variance) mostly reflected the high interindividual variability and was driven by the amino acid methionine, leucine, isoleucine, histidine, and glutamine.

The concentration of the most discriminant metabolites are shown in Table 5.

Phenylalanine level was highest in the all the experimental diets, while tyrosine was found to be highest in the plasma samples collected from individuals fed the SBM diets, with plasma from individuals fed the CoPea diets having values in-between the Contr and SBM samples. Leucine and glutamine exhibited a similar distribution, with plasma samples from individuals fed the CoPea + P2 diet having the highest plasma with values comparable to the Contr diet levels, while SBM showed values comparable to the Contr diet, regardless of supplementation. The only exception was SMB + P1, for which glutamine was

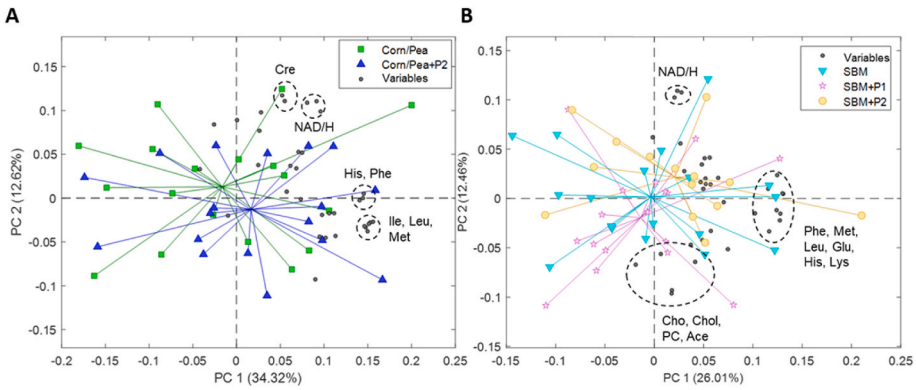

Fig. 8. PCA biplots for model 2 (CoPea (Corn/Pea) vs CoPea + P2 (Corn/Pea + P2), N = 36) and model 3 (SBM vs SBM + P1 vs SBM + P2, N = 52, 2 outliers removed - 109 and 161). See legend for color-coding explanation. Keys: Ace: acetate; Cho: cholesterol; Chol: choline; PC: phosphocholine; Met: methionine; Val: valine; Ile: isoleucine; Gln: glutamine; His: histidine; Phe: phenylalanine; Leu: leucine; Cre: creatine. The plots are based on all observations, i.e. 6 fish from each of the 18 tanks.

found to be slightly lower than the Contr, while leucine was slightly higher. Choline and glycine exhibited the opposite trend observed for phenylalanine and tyrosine, and their concentration decreased in all the experimental diets. Individuals fed the CoPea and SBM basal diets had the lowest amount of plasma choline, while all the supplemented diets showed values between the Contr and the basal diets. Regarding glycine, the lowest amounts were found in plasma from salmon fed the SBM diets, with the plasma from individuals fed the SBM + P1 diet showing the lowest level. Plasma from fish fed the CoPea diet had values in-between the Contr and SBM diets.

3.5. Results of microbiota analyses

Alpha diversity, measured as observed taxa and Shannon indices, are presented in Supplementary Figs. 1a and 1b for digesta and 1c and 1d for mucosa. Observed taxa measures taxa richness (i.e. number of different taxa), whereas Shannon index measures richness and evenness (i.e. number of different taxa and their relative abundance) in a sample.

In the digesta, significant difference in alpha diversity matrices was observed between the fish fed Contr diet and the SBM diet only for Shannon Index. For the Contr and the CoPea fed fish, however, the difference was significant for both indices. Between SBM and CoPea diets the results showed no significant difference. Supplementation with P1 and P2 to the SBM diet did not change the observed taxa significantly, neither did supplementation with P2 to the CoPea diet. However, supplementation of P1 and P2 to the SBM diet significantly changed Shannon index.

In the mucosa, alpha diversity did not significantly differ between SBM and Contr diet fed fish or between CoPea and Contr diet fed fish. Supplementation with P1 to the SBM diet significantly increased Shannon index in the mucosa microbiota. On the other hand, P2 supplementation to the SBM or CoPea diet did not significantly change alpha diversity.

Beta diversity indicating differences in microbial composition, was assessed by Bray-Curtis dissimilarity (Supplementary Fig. 2a digesta and 2b for mucosa) and unweighted Unifrac distance (figures are not shown). Bray-Curtis index measures the presence and absence of the taxa in each group while UniFrac distance is based on branches in a phylogenetic tree that are either shared or unique amongst the samples.

Microbiota in the digesta from the six treatments clustered separately from each other (Supplementary Fig. 2a). Pairwise PERMANOVA analysis indicated significant differences in microbial composition between the SBM and the Contr as well as between the CoPea and the Contr treatments based on both the beta diversity matrices. Further, bacterial composition in SBM and CoPea groups were also significantly different. Supplementation of P1 and P2 to SBM diet caused a significant shift in microbial composition. Addition of P2 to CoPea diet also changed microbial composition significantly. In the mucosa, however, microbiota of the six treatments did not cluster separately (Supplementary Fig. 2b), but the microbiota from SBM group clustered distinctly from all the other groups. Pairwise PERMANOVA analysis found significant differences in microbial composition between CoPea and Contr as well as between SBM and CoPea treatments based on both matrices. Supplementation with P1, but not P2, to SBM diet showed a significant shift in microbial composition in mucosa as indicated by Bray-Curtis dissimilarity index as well as unweighted UniFrac distance. Addition of P2 to CoPea diet did not show any significant change in microbiota composition in mucosa.

Taxonomic analysis revealed that the phylum *Firmicutes* was predominant in digesta (Supplementary Fig. 3a) of all treatments (79%–98%), except of the SBM treatment (46%). In the SBM fed fish *Proteobacteria* dominated comprising 52% of the total abundance. Supplementation with P1 and P2 reduced its abundance to 5% and 6%, respectively and increased abundance of *Firmicutes*, P1 to 93% and P2 to 91%). Contr and CoPea diet fed fish contained 13 and 21% of *Proteobacteria* of the total abundance, respectively. Supplementation of P2 to CoPea diet reduced *Proteobacteria* abundance to 1%. In mucosa as well, the phylum *Firmicutes* was predominant (Supplementary Fig. 3b) in most of the groups (Contr, 52%, CoPea, 57%, SBM + P1, 76%, SBM + P2,55%, CoPea + P2, 90%) except SBM group (14%). In the SBM group, *Proteobacteria* predominated with 80% of total abundance, while in fish fed Contr and CoPea diets the *Proteobacteria* comprised 29% and 41% of total abundance. In mucosa as well, supplementation to the SBM diet reduced *Proteobacteria,* for P1, 10% and P2, 37%, and increased *Firmicutes*. Supplementation with P2 to CoPea diet also reduced *Proteobacteria* abundance in mucosa to 5%.

The most predominant genera in digesta were quite different among the six groups (Fig. 9). In Contr fed fish, *Leuconostoc* (31%) and *Weissella* (23%) were predominant genera followed by *Lactobacillus* (12%). In SBM fed fish, the genera, *Aliivibrio* (50%) *Leuconostoc* (18.4%) and *Weissella* (14%) predominated, while in CoPea fed fish, *Lactobacillus* (67%) and *Photobacterium* (20%) dominated. Supplementation with P1 and P2 to SBM changed the predominant genera to become more similar to those in the Contr group. Both SBM + P1 and SBM + P2 groups had

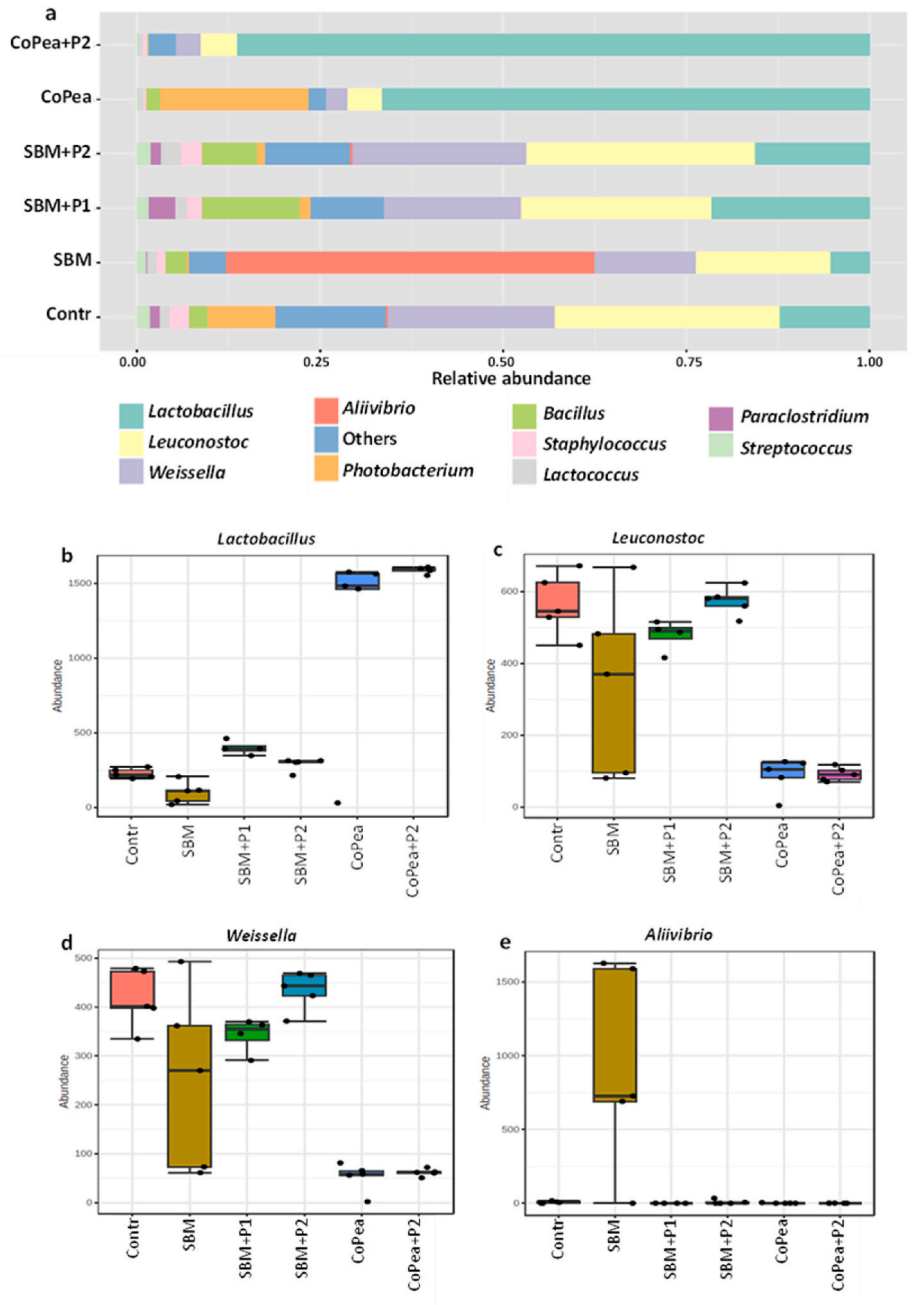

Fig. 9. Ten most abundant genera of digesta from distal intestine of the Atlantic salmon fed with six diets.

Leuconostoc (26% and 31%), *Lactobacillus* (22% and 16%) and *Weissella* (19% and 24%) as predominant genera. In CoPea group, supplementation of P2 further increased the presence of *Lactobacillus* to 86% of total abundance while *Photobacterium* diminished.

In mucosa as well, the treatments differed regarding predominating genera (Fig. 10).

In the Contr fed fish *Photobacterium* (12.8%) and Staphylococcus (13%) dominated, in SBM fed fish *Aliivibrio* (72%), and in CoPea *Lactobacillus* (42%), *Photobacterium* (40%) and *Staphylococcus* (10%) dominated. Supplementation with P1 and P2 to the SBM diet changed the same genera in mucosa of both groups but in different proportions. Supplementation with P1 greatly decreased *Aliivibrio* (1.6%) and increased the abundance of *Staphylococcus* (27%), *Lactobacillus* (15.5%), *Leuconostoc* (13%) and *Weissella* (9%). P2 also decreased *Aliivibrio* (32.6%) to some extent and mostly increased *Leuconostoc* (18%), *Staphylococcus* (10.4%), *Weissella* (10%) and *Lactobacillus* (7%). Similar to the digesta, in mucosa supplementation with P2 to CoPea increased the abundance of *Lactobacillus* (69%) and diminished *Photobacterium*. *Staphylococcus* also decreased in fish fed theCoPea + P2 diet (5%) compared to the CoPea group.

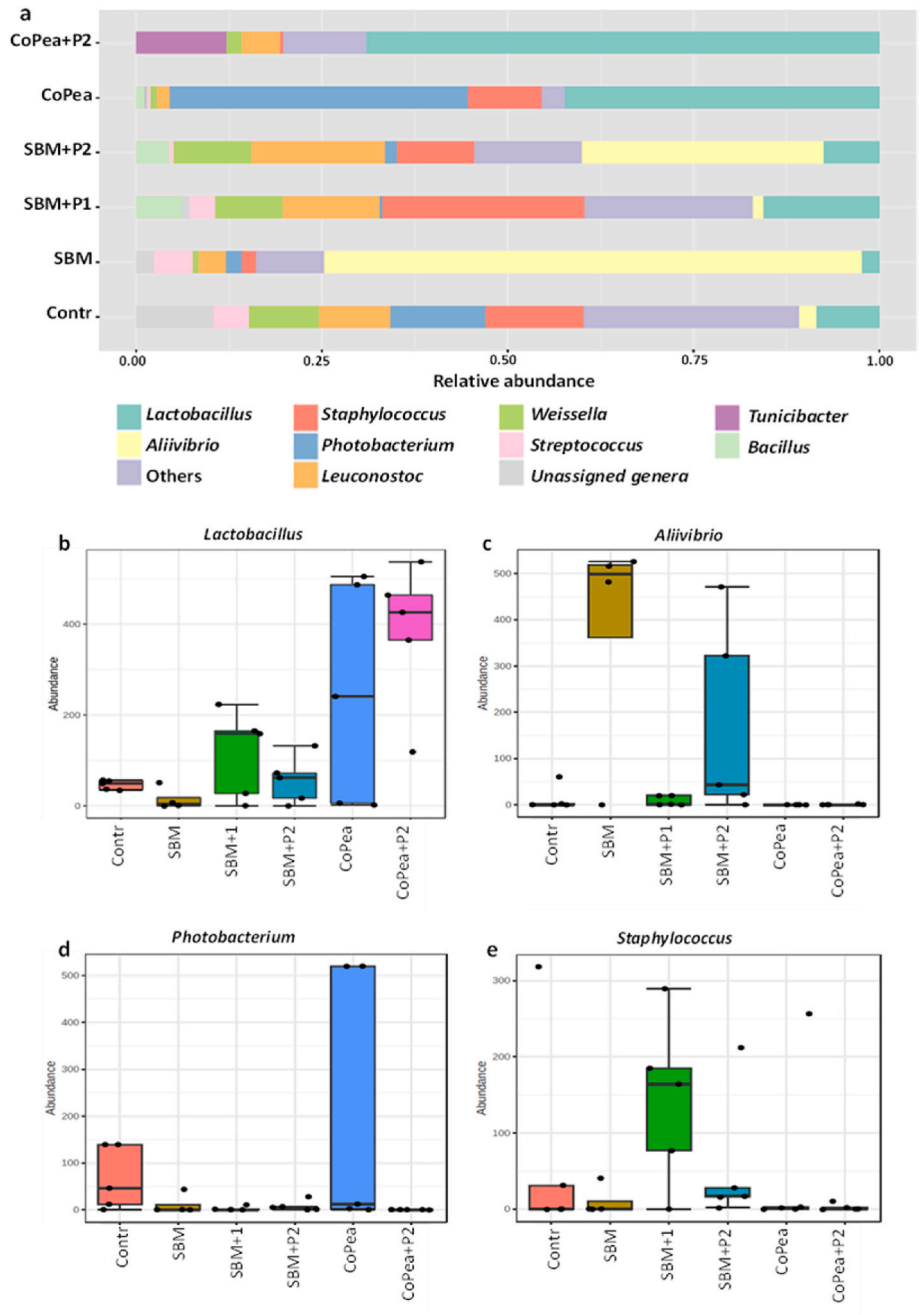

Fig. 10. Ten most abundant genera of mucosa from distal intestine of the Atlantic salmon fed with six diets.

Table 5
Concentration of choline, glutamine, glycine, leucine, phenylalanine, and tyrosine in the plasma samples from salmon fed the different diets*.

	Diets							P(model)
	Control	SBM	SBM + P1	SBM + P2	Corn/Pea	Corn/Pea + P2	Pooled SEM	
Choline	0.9[a]	0.6[b]	0.7[b]	0.7[b]	0.6[b]	0.7[b]	0.17	0.0002
Glutamine	0.59[bc]	0.58[bc]	0.50[bc]	0.58[c]	0.67[a]	0.72[a]	0.03	0.0117
Glycine	1.40[a]	1.00[b]	0.78[c]	1.05[b]	1.07[b]	1.10[b]	0.04	0.0024
Leucine	2.28[b]	2.23[b]	2.45[b]	2.33[b]	3.63[a]	3.91[a]	0.05	0.0003
Phenylalanine	0.30[b]	0.41[a]	0.43[a]	0.44[a]	0.39a[ba]	0.43[a]	0.03	0.0004
Tyrosine	0.19	0.41	0.39	0.42	0.32	0.32	0.03	0.0619

*Different letters indicate significant difference between the different diet (ANOVA, n = 3).

4. Discussion

4.1. SBM and CoPea diets vs the contr diet

4.1.1. Indicators of growth, feed utilization, nutrient metabolism, and gut health

The differences between fish fed the SBM or the CoPea diet compared to the Contr diet regarding apparent nutrient digestibility, i.e. positive for protein and insignificant for lipid, are in line with a previous study in our laboratory [31]. The explanation for the relatively low apparent protein digestibility of the Contr diet was supposedly that the harvesting and processing conditions used for production of fish meal reduced amino acid digestibility. The cause of the negative values for apparent digestibility of ash in fish fed the SBM diets was most likely increased faecal output of endogenous components due to loss of absorptive functions and diarrhoea related to the inflammation in the DI, as discussed below.

Although apparent protein digestibility was high for the diets with high content of plant ingredients compared to the Contr diet, growth rates were lower, significantly so for the SBM diet showing about 15% reduction, and FCR was correspondingly higher. This result is in line with earlier observations [32]. Soybean meal contains a number of antinutrients which may interfere with growth and feed utilization [33]. It contains high level of indigestible fibre which necessarily, increase FCR. The elevation in weight of the pyloric region may be related to the content of antinutrients challenging the digestive capacity and inducing tissue and capacity expansion. One example of such responses is induction of trypsin secretion triggered by the presence of soybean protease inhibitors in salmon diets [34]. Another, and may be the major reason for the growth retardation, is the SBM diet's content of saponins, the key component for induction of inflammation in the distal intestine, as shown in many studies [35]. The fish in the SBM treatment showed typical signs of soybean induced enteritis in the distal intestine, i.e. diarrhoea, reduced tissue weight, histological alterations, alterations in tissue and chyme enzyme activities. The histology results show that our intention to induce an inflammation situation to be used as a model for evaluation of remediating effects of functional ingredients on symptoms of inflammation was reached.

The inflammatory changes observed in the fish fed the CoPea were milder and quite different compared to the typical SBM-induced changes showing more focal lesions characterized by marked infiltration of the submucosa and lamina propria but with no accompanying loss in the epithelial vacuolization and shortening of mucosal folds, with the rest of the mucosa appearing normal and healthy. The differences in the enteritis symptoms could suggest different mechanisms of induction of the inflammation, such as localized mechanical damage to the mucosa. It has been suggested that the enteritis induced by standard qualities of SBM may be too severe to serve as model for the study of possible beneficial effects of functional ingredients on gut health. An alternative model in which soybean products with lower content of antinutrient are immune triggers, has been found useful by Nordvi et al. [36], and has shown health promoting effects of a probiotic. The present CoPea diet may serve a similar purpose.

The clear effects of the SBM diet on chyme bile salt concentration, which were visible all along the intestinal tract, was most likely due to formation of insoluble complexes between saponins, cholesterol and bile salt, which restricts uptake and increase excretion (See review in Ref. [37]. As bile salts are produced from cholesterol, the low plasma cholesterol level revealed by both the classical biochemical and the metabolome analyses, indicate increased conversion to bile salts and accordingly supports this consideration. Several previous studies in salmon and rainbow trout have documented draining of faecal bile salts and reduced cholesterol levels after feeding SBM-based diets (See review in Ref. [37].

The diet effects indicated by the metabolome analyses on plasma amino acid levels of the basal diets were most likely due to differences in nutrient composition of the diets. However, as diet effects on amino acid metabolism were not a goal of the present study, the amino acid composition of the diets was not analysed. These results are, therefore, not further discussed herein. The choline level of the diets, on the other hand, was formulated to be similar for all diets, allowing discussion of the effects of the basal diets compared to the Contr diet on plasma choline level. The lower level observed for plasma choline and its metabolite phosphocholine in fish fed the SBM and CoPea diets compared to those fed the Contr diet, may be suggested to have been due to lower absorption, or to higher catabolism in the organism possibly due to higher demand. As choline digestibility recently was found to be high in Atlantic salmon, in particular for choline supplemented as choline chloride [28], and only marginally dependent on diet composition, a lower absorption for the SBM and CoPea diets is less likely to be the explanation than higher demand. Choline is the sole source of methyl groups for many important processes, not at least epigenetic modification of nucleic acids and histones, which are dependent on several B-vitamins [38]. It is therefore suggested that the lower plasma levels of choline, was due to increased demand in fish fed the SBM and CoPea diets.

The CoPea diet resulted in fewer and less clear differences compared to the Contr diet than the SBM diet. The slight, significant increase in the relative weight of the PI, and a larger increase in DI, may be related to the higher content of fibre in the CoPea diet, as discussed above for the SBM diet. The significantly lower LAP capacity of the DI, loss of function in spite of marked increase in tissue weight, may have been due to the mild inflammation indicated by the histological results for fish in this treatment.

4.1.2. Gene expression indicators

The expression differences between fish fed the Contr diet and the basal SBM diet are in correspondence with the results of previous studies by both character and scale (See review in Ref. [39]) comprising up-regulations indicting increased cellular and protein stress, stimulation of several functional groups of the immune system including acute inflammation and oxidative burst complex, and a number of chemokines and cytokines, and down-regulation of antigen presentation, and several metabolism related genes, of particular interest are those involved in metabolism of lipids. The results strongly indicate that the SBM diet contained compounds which require activation of specific defence mechanisms, possibly at the expense of others. Trade-off between inflammation and metabolism is typical for SBM-induced enteritis. As an example, the inflammatory response was associated with proliferative and apoptotic actions in the intestinal mucosa, resulting in reduction in the number of mature, developed enterocytes and, consequently, loss of metabolic functions [40]. Suppression of multiple metabolic pathways including proteolysis and transport may affect an overall activity of cells as well as specific functions related to digestion and absorption, and this might have contributed to a decrease in growth rate. The observed downregulation of genes involved in xenobiotic metabolism indicate weakened ability of the intestine to neutralize harmful compounds thus increasing load on the liver. These results are in accordance with the histological findings of severe inflammation in DI. Of note is also the up-regulation of *guanylin* which controls ion and water absorption. Combined with down-regulation of the water channel *aquaporin 8b*, this may be a mechanism underlying the high water content observed for the chyme in the DI. The association between SBM-induced enteritis and reduced expression levels of the aquaporin gene, *aqp8,* was first reported by Kortner et al. [41] and *aqp8* has in later studies been among the more robust molecular biomarkers of diet-induced inflammation (See review in Ref. [36]. The observed effects of the CoPea diet on gene expression, compared to the Contr diet were small and there were no signs of inflammation.

4.1.3. Microbiome indicators

The alteration in digesta and mucosa microbiota composition

induced by the SBM diet compared to the Contr diet, differed from observations in an earlier shorter term (3 weeks) feeding trial with Atlantic salmon fed a diet containing 20% SBM in which no clear change in the microbial composition was observed [42]. This may indicate that microbial effects of diet changes take time to develop. Even though phylum *Proteobacteria* predominated in SBM treatment with significantly higher levels compared to other treatments, this phylum has previously been reported to belong to the Atlantic salmon core microbiota [24,43]. Therefore, it is very difficult to relate its high abundance to the severe inflammation observed in SBM fed fish. A recent study of gut microbiota in six commercially salmon farms, found that *Aliivibrio* was among the predominant microbiota after about ten months in sea water (Midtlyng, personal communication) when fed standard commercial diets without standard soybean meal. Several other studies have reported the same genus among the core microbiota in Atlantic salmon [24]. High predominance of *Aliivibrio* in both the mucosa and digesta exclusively in SBM group along with enteritis in the present study need further clarification.

Lactic acid bacteria are among the core bacteria in Atlantic salmon [43,44], and they are presumed to have beneficial effects on salmon health and function through improvement of digestive process and immune regulation [45]. Lactic acid bacteria including *Lactobacillus* are known to produce SCFAs, and mammalian studies indicate that intestinal epithelial cells obtain most of the energy requirements from the SCFAs [46]. Therefore, increased energy supply from increased *Lactobacillus* levels as a result of high fiber content in CoPea diet could have enhanced the DI growth. Further, dietary administration of Lactobacillus are generally shown to increase overall growth in finfish. The unchanged growth rate observed in CoPea group with the increased *Lactobacillus* abundance may be due to the several confounding factors in diet composition, e.g. effects of antinutrients in pea. Moreover in the present study, SCFAs acetate and formate were identified and quantified from the ^{1}H NMR spectra of salmon plasma. However, no significant diet-related changes were observed (data not shown).

4.2. Remediating effects of the P1 package containing butyrate and arginine in SBM fed fish

4.2.1. Indicators of growth, feed utilization, nutrient metabolism, and gut health

The absence of effects of dietary supplementation with P1 on growth, other production biomarkers, and on the status of the inflammation in the DI, indicates that the arginine level in the basal SBM diet was sufficient to cover the needs of the fish, including needs for immune functions. Arginine is essential in fish feeds and play many key roles in an animal body besides being a part of all proteins. Estimates of arginine requirement should be set at levels covering all needs, including those related to immune functions [47]. Moreover, the absence of such effects also indicates that neither the butyrate supplementation modulated the immune responses to the extent that the severe inflammation induced by the soybean meal in the diet showed significant signs of improvement. Absence of butyrate in plasma metabolome was expected as butyrate is metabolized by the enterocytes and would normally not reach the blood. The results regarding effects of butyrate are in line with the results of Gao et al. [48] investigating effects of a mixture of acetate and butyrate on growth and immune responses in rainbow trout. However, other fish species, such as gilthead sea bream (*Sparus aurata*) [49], have shown positive effects on growth as well as on biomarkers of immune functions. The possible causes of the differences observed between experiments employing different fish species regarding effects of butyrate on growth and immune functions are many, first of all species differences, differences in developmental stages and environment, and not at least differences in diet composition. The latter may be related to differences in the diets regarding coverage of nutrient requirement for which present knowledge is highly insufficient for most species. A recent review [50] clearly underlines the present situation regarding knowledge on vitamin requirement in Atlantic salmon, and the situation is no better for the other essential micronutrients. For some fish species the situation appears somewhat better, e.g. for grass carp (*Ctenopharyngodon idella*), but for most fish species the situation is even worse. Likely consequences of this situation are that the actual causes of differences between results of feeding experiments with functional ingredients, which often are impure and may contain essential nutrients and other bioactive compounds than those in focus, are hidden variation in degree of nutrient deficiencies or excesses in the diets used in the studies. There is a chance that the absence of beneficial effects of P1 in the present study was due to negative interactions between the two compounds in the package. It is, however, no relevant information in the scientific literature supplying basis for a discussion of such possible relationships.

4.2.2. Gene expression indicators

The anti-inflammatory effect of P1 reversed the expression changes of a number of inflammatory mediators activated with SBM including such emblematic indicators as *c-c motive chemokine 19*, *cytochrome b-245,* and *matrix metalloproteinase 9*. However, the histological and biochemical observations show that this regulation of gene expression did not diminish the symptoms of SBM-induced enteritis.

4.2.3. Microbiome indicators

The supplementation with the P1 package to SBM diet greatly changed the microbial composition in the DI and seemed to eliminate most of the difference observed between the SBM and the Contr group. A possible mechanism underlying this is enforcement of the anaerobic environment, which could have eliminated the presence of aerobic bacteria *Aliivibrio* and increased the presence of facultative anaerobic/anaerobic bacteria *Leuconostoc*, *Lactobacillus* and *Weissella* in the intestine of fish fed the SBM + P1 diet. Addition of butyrate to a commercial diet has also been found to increase lactic acid bacteria counts in Nile tilapia distal intestine [51]. However, these changes did not affect the severity of the gut inflammation.

4.3. Remediating effects of the P2 package containing β-glucan, butyrate and nucleotides in fish fed the SBM diet

4.3.1. Indicators of growth, feed utilization, nutrient metabolism, and gut histology

As the only effect of P2 supplementation to the SBM diet was a slight reduction in trypsin activity in the chyme of DI2, P2 effects appeared marginal. This effect in a biomarker for which high level represents high rate of repair activities in the gut wall, indicate that tissue repair processes slowed down somewhat in the fish fed the SBM + P2 diet [52], but was still high. The effect may have been a result of the β-glucan or the nucleotides, or the combination of the two. As no information has been found regarding this biomarker which can through light on these observations, further discussion must await further studies.

4.3.2. Gene expression indicators

With regard to gene expression, the hallmark of P2 effect was the reduction of the SBM-induced suppression of metabolism in the distal intestine: 31 genes with metabolic functions and which were down-regulated in SBM, increased expression in SBM + P2. These genes have diverse roles. The increased expression of genes involved in lipid and xenobiotic metabolism indicates stimulation of some key processes.

4.3.3. Microbiome indicators

Supplementation of P2 package to SBM changed the gut microbial compositions significantly, making it similar to the Contr group. Also in this treatment, probably due to the influence of butyrate, aerobic bacteria, *Aliivibrio* may have decreased in abundance and facultative anaerobic/anaerobic bacteria *Leuconostoc*, *Lactobacillus* and *Weissella* may have increased in abundance in the intestine. However, the overall composition of the microbiota in fish fed the SBM + P1 and SBM + P2

diets differed significantly, which mean that a mixture of arginine and butyrate, and of butyrate, β-glucan, and nucleotides, affected the microbiota differently.

4.4. Remediating effects of the P2 package containing β-glucan, butyrate and nucleotides in fish fed the CoPea diet

4.4.1. Indicators of growth, feed utilization, nutrient metabolism, and gut histology

When P2 was added to the CoPea diet, the results were, overall, similar to those observed when P2 was added to the SBM diet, i.e. no significant effects on growth, feed utilization, or on biochemical, metabolic, and histological indicators of gut inflammation. As the CoPea diet, in contrast to the SBM diet, did not alter trypsin activity in the digesta of the DI, the lack of reducing effect was as expected.

4.4.2. Gene expression indicators

The observed changes in DEG response to P2 supplementation to the CoPea diet were few and rather scattered. The results therefore do not supply sufficient information for a discussion.

4.4.3. Microbiome indicators

Increased abundance of *Lactobacillus* along with the other lactic acid bacteria *Leuconostoc* and *Weissella* both in the digesta and mucosa with the supplementation of P2 might have resulted from the change in the anaerobicity of the environment facilitated by butyrate as well as the influence of prebiotic β-glucan. However, lack of information on such relationships prevent further discussion of underlying mechanisms.

5. Conclusions

- Fish fed SBM, compared to fish fed Contr, showed severe inflammation in the DI with structural and functional losses in line with earlier observations of effects of inclusion of soybean meal in salmon diets. Marked effects on gut microbiota were observed.
- Fish fed CoPea, compared to fish fed Contr, showed minor but clear histological signs of inflammation in the DI as well as functional losses. Marked effects on gut microbiota were observed.
- Supplementation of the SBM diet with the P1 package of functional ingredients did not significantly affect structural symptoms of inflammation but modulated expression of some of the observed inflammation related genes. Gut microbiota was affected in a direction towards that of fish fed the Contr diet.
- Supplementation of the SBM diet with the P2 package of functional ingredients did not significantly affect either structural or other symptoms of inflammation in the DI, but modulated expression of some genes involved in metabolic processes in the DI. Gut microbiota was affected in a direction towards that of fish fed the Contr diet.
- Supplementation of the CoPea diet with P2 did not significantly affect any of the symptoms of inflammation or alter other important functional aspects. Gut microbiota was affected in a direction towards that of fish fed the Contr diet.

CRediT authorship contribution statement

Åshild Krogdahl: Conceptualization, Data curation, Validation, Visualization, Writing – original draft. **Anusha K.S. Dhanasiri:** Data curation, Visualization, Validation, Writing – review & editing. **Aleksei Krasnov:** Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. **Violetta Aru:** Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. **Elvis M. Chikwati:** Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. **Gerd M. Berge:** Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. **Søren Balling Engelsen:** Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. **Trond M. Kortner:** Conceptualization, Data curation, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

None of the co-authors or any of the others involved have conflicts of interest regarding the conductance and presentation of the experiment.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108618.

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