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Clues from the intestinal mucus proteome of Atlantic salmon to counter inflammation

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ABSTRACT

Intestinal inflammation in Atlantic salmon was studied by profiling the intestine mucus proteome, employing iTRAQ and 2D LC-MS/MS approach. Two fish groups were fed soy saponin-containing (inflammation inducer) diets (SO and SP) and two control fish groups were fed diets devoid of soy saponin (CO and CP) for 36 days. The CP and SP diets contained a health additive. Inflammation characteristics in the intestine were milder in the SP-fed fish compared to the SO-fed fish. The SO group was characterised by alterations of many proteins. KEGG pathways such as phagosome and lipid binding were possibly affected in the SO group due to the higher abundant proteins like Integrin beta 2 precursor, Coronin 1A, Cathepsin S precursor, Vesicle-trafficking protein, and Neutrophil cytosol factors. On the other hand, the SP group had fewer altered proteins and inflammation characteristics; aminoacyl-tRNA biosynthesis and ribosome in the fish group were plausibly changed due to the higher abundance of many large and small subunit of ribosomes. Elevation of the abundance of ribosomal proteins, aminoacyl-tRNA ligases, and appropriate abundance of Glycogen phosphorylase and Glutamine synthetase could possibly alleviate intestinal inflammation.

Data are available via ProteomeXchange with identifier PXD027922 and PXD029849.

Significance: Intestinal inflammation, caused by dietary factors, can be considered as a non-infectious disease. Hence, researchers are gathering clues to avert the associated health issues. The present study was conducted to infer the alterations in the intestine mucus proteome induced by a dietary health additive to counter intestinal inflammation in farmed Atlantic salmon. The reduction in the number of affected proteins and their alterations point to mechanisms evoked by the premix. Our knowledge on inflammation associated proteome in fish is limited and the present study not only highlights the changes, but also opens the possibility to avert the dysfunction of the organ through a dietary approach.

1. Introduction

Intestinal inflammation in humans is a serious disease that has been previously regarded as a malady linked to high-income countries, where the risk factors include strict hygiene, diets containing low fibre, high fat and protein, and low exposure levels to common childhood infectious agents [1,2]. Now Kaplan and Windsor [3] have indicated that nations all over the world can be categorized according to the different epidemiological stages of development of intestinal bowel diseases (IBD). A recent study from low, medium and high-income countries have identified ultra-processed food as the culprit [4]. Biological agents such as anti-TNF agents and anti-integrin molecules and immunomodulators that affect different immune pathways were found to be effective in treating certain patients, but not all who are suffering from the disease [5,6]. Although such clinical therapies have decreased IBD-related deaths [7], it would be ideal to find dietary alternatives to alleviate the inflammatory responses at the intestinal mucosa.

Intestinal mucosa is comprised of i) an outer mucus layer that is

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home to the commensal microbiota, and contains antimicrobial proteins and secretory immunoglobulins, and ii) a single layer of epithelial cells and lamina propria that houses the innate and adaptive immune cells [8]. Although intestinal mucus is an important barrier of the organ, there are not many studies that have investigated the association between the mucus proteins and intestinal inflammation. Nevertheless, mucosal biomarkers of IBD and the differential responses in the intestinal compartments are already reported [9,10]. The differentially expressed proteins in a non-human primate model of colitis were similar to those observed in the proteomes of ulcerative colitis patients [11]. Since barrier functions are flawed in patients with intestinal disorder, mucus can be considered as a target for therapy aimed at alleviating inflammation. Subtle changes in the components that regulate the intestinal integrity and immune homeostasis of the ecosystem can weaken the intestinal barrier [12]. Furthermore, loss of barrier component strength and compromised immune system initiate inflammatory mechanisms in the gut of humans and animals [13].

Atlantic salmon, a carnivorous fish, is prone to intestinal inflammation when they are offered feeds containing antinutritional factors like soy saponins [14–16]. It is a widely studied fish species and is considered as a good model for evaluating the effect of fishmeal alternatives in feeds of carnivorous fish. Several studies have examined intestinal inflammation in this fish due to its known allergic response to dietary soybean meal compared to adults of the model organism zebrafish [17]. We observed only subdued inflammatory responses in adult zebrafish fed 50% soybean meal [18]. Nevertheless, zebrafish is recognized as a powerful model to investigate conserved pathways that mediate gut epithelial homeostasis and prevent inflammation [19]. Larval zebrafish develop diet-induced intestinal inflammation characteristics similar to those observed in humans and mouse models [20]. On the other hand, several studies have examined the efficacy of specific additives that may provide protection against intestinal inflammation. For example, dietary nucleotides were shown to influence zebrafish growth by reducing the inflammatory response [21]. Refstie, Baeverfjord, Seim and Elvebø [22] demonstrated potential beneficial effects of beta-glucans and mannan oligosaccharide in minimising the impact of plant proteins such as soybean meal on the growth, nutrient digestibility, and disease resistance of Atlantic salmon. A review about the requirement of antioxidant vitamins like C and E in salmon fed high plant diets has concluded that higher levels of the vitamins can ensure normal growth and physiological functions that are prerequisites for protection from oxidative stress which could lead to chronic inflammation [23]. Beneficial effects of vitamin C on fish health have been reviewed by Trichet, Santigosa, Cochin and Gabaudan [24] and Dawood and Koshio [25].

Many studies have revealed that inflammation-linked disturbances can jeopardise the intestine homeostasis in fishes [14,26–28]. However, there are only few reports on the mucus proteome of fish that developed intestinal inflammation. We have previously reported the changes in the distal intestinal proteome of Atlantic salmon caused by feeds or by single dietary component or chemical allergens [29–31]. In the present study, we targeted changes in the Atlantic salmon gut mucus proteome caused by soy saponin in feed because protein abundance alterations can reflect host health. In addition, the efficacy of a dietary health additive in reducing the intestinal inflammation characteristics is described through the differences in mucus proteome profiles in the fish.

2. Materials and methods

The feeding trial was approved by the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-10050).

2.1. Experimental fish and facility

Atlantic salmon (*Salmo salar*) post-smolts, all immature fish of both sexes, that originated from Sundsfjord Smolt (Nygårdsjøen, Norway) were maintained for 5 months at the Research Station, Nord University, for the feeding trial. For the experiment, 240 fish of average initial weight 129.35 ± 13.48 g were randomly distributed into replicate tanks of the four study groups. After a 2-week acclimation period on a commercial feed (Ewos Micro, Ewos AS, Bergen, Norway), the fish were fed on the experimental feeds. The water in the 800 l rearing tanks originated from a depth of 250 m in the Saltenfjorden and the rearing tanks were part of a flow-through seawater system at the research station. The water flow rate was maintained at 1000 l per h, and the average temperature and salinity of the rearing water were 7.6 ± 0.5 °C and 33 g per l, respectively. The dissolved oxygen, measured at the water outlet of the tanks was in the saturation range 87–92%, and we employed a photoperiod regimen of 24 h throughout the experimental period.

2.2. Experimental feeds

Four feeds were prepared by Sparos Lda, Olhão, Portugal; the basal ingredients are presented in Table 1. CO and CP were the control feeds, without and with a health additive (nucleotides, antioxidant vitamins C and E, beta-glucan), respectively. On the other hand, SO and SP were the inflammation-causing feeds, without and with the health additive. The ingredients causing inflammation, that were included in the SO and SP feeds, were: soybean meal 48 (5%), full-fat soybean meal and (5%) and soy saponin of purity 40% (2%). The health additive was included as a premix at 0.41% to the CP and SP feeds, at the expense of wheat meal. The feed formulations were adjusted using wheat meal and were isoproteic and isolipidic with 45% crude protein and 26% crude fat, respectively.

These feeds were offered twice a day (08:00–09:00 and 14:00–15:00) at 1.2% of body weight per day to the respective fish groups, using computer-controlled automatic feeders (Arvo Tech, Huutokoshi, Finland), for a period of 36 days. During this period, left over feeds were collected daily, 1 h after the end of a feeding cycle, by removing the uneaten feed pellets from the feed collectors of each rearing tank.

2.3. Sampling

At the end of the 36-day trial, the fish were euthanized after exposing them to an overdose (300 mg/l) of MS222 tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA).

The mucus from the distal intestine was collected from the different groups of fish. The fish were dissected, the intestine segment was clamped, and the mucus was gently squeezed out into a sterile Petri dish containing 2 ml of PBS (spiked with Halt Protease inhibitor cocktail 100×, 10 μl/ml PBS). The mucus samples were transferred to cryotubes, snap-frozen in liquid nitrogen and stored at −80 °C. Thereafter, the

Table 1
Formulation of the basal experimental feeds.

Ingredients, %	CO/CP	SO/SP
Fishmeal	15.00	15.00
Soy protein concentrate	20.00	20.00
Wheat gluten	16.50	12.60
Corn gluten	5.00	5.00
Soybean meal 48	0.00	5.00
Soybean meal full fat	0.00	5.00
Wheat meal	15.68/15.27	8.08/7.67
Fish oil	9.60	9.40
Rapeseed oil	14.40	14.10
Soy lecithin	0.50	0.50
Choline chloride	0.20	0.20
Betaine HCl	0.50	0.50
Monoammonium phosphate	0.60	0.60
Amino acids (His, Lys, Thre, Tryp, Meth)	1.45	1.45
Yttrium oxide	0.02	0.02
Vitamin and Mineral premix - DSM	0.50	0.50
Astaxanthin, mg/kg	0.05	0.05
Soy saponins 40%	0.00	2.00

mucus samples were freeze-dried (VirTis BenchTop™ K, Warminster, USA) for 10 h, and then shipped to National University of Singapore (NUS), for the protein analyses.

In addition to mucus samples, we collected distal intestine samples to understand the changes in the micromorphology. The histological procedures employed to generate the images are described in our previous publication [32].

2.4. Protein study

2.4.1. Protein extraction and quality check

The protein studies were performed at the Protein and Proteomics Centre, Department of Biological Sciences, NUS, Singapore. For protein extraction, the lyophilized mucus samples in the cryotubes were first treated with 100 µl of sodium dodecyl sulphate (SDS) lysis buffer [4% SDS (Sigma-Aldrich, St. Louis, MO, USA), 0.5 M triethylammonium bicarbonate buffer pH 8.5 (TEAB; Sigma-Aldrich), and 1 × Protease and Phosphatase Inhibitor cocktail (Thermo Scientific, Rockford, IL, USA)]. The tubes containing the samples were mixed adequately using a vortex and incubated for 5 min in a block heater at 95 °C, after which they were allowed to cool down on an ice bath at 4 °C. Samples were sonicated for 20 s using an ultrasonic probe. Thereafter the tubes with the lysed samples were loaded on a temperature-controlled (4 °C) microcentrifuge (Centrifuge 5424 R, Eppendorf, Hamburg, Germany) to centrifuge at 13000 rpm for 20 min. The obtained supernatant was transferred into a new microtube that was kept for 5 min on ice. An aliquot from this supernatant was used for determining the protein content in the sample employing the Qubit™ Protein Assay Kit (Invitrogen, Eugene, OR, USA) and the Qubit® 3.0 Fluorometer (Invitrogen). We also performed RC DC™ protein assay (Bio-Rad, catalog # 500–0120, California, USA) for protein quantification according to the manufacturer's protocol.

One dimensional gel electrophoresis was used to confirm the quantity and check the quality of the protein samples for further downstream work. For this, 4 µg of protein samples from each replicate was first mixed with 4× SDS loading dye. Next, the samples were denatured by incubating for 10 min at 95 °C. For the denaturing electrophoresis, the samples were loaded in 12% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad, catalog # #4561045) and run for 1 h in a Mini-PROTEAN Electrophoresis System (Bio-Rad) containing SDS Tris-glycine as the running buffer. After that, the gel was washed with deionized water for 10 min, stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, catalog # 1610436) for 20 min and destained overnight at room temperature employing a solution of 40% methanol and 10% acetic acid and 50% water.

Based on visual inspection and the quality (less degradation) of the gel images, prepared from 5 mucus samples of each group, 4 samples were selected for further analyses (Fig. 1). Bovine serum albumin was employed as the reference protein in the gel. Two isobaric tags for relative and absolute quantitation (iTRAQ) sample sets were prepared; set 1 was comprised of the samples from CP and SP groups while set 2 contained the samples from CO and SO groups.

2.4.2. Tube-Gel digestion and iTRAQ labelling

The tube-gel digestion of each sample was performed according to the description of Purushothaman, Das, Presslauer, Lim, Johansen, Lin and Babiak [33]. Briefly, 100 µg protein from each sample was taken for tube-gel preparation, after which 4% SDS, 10% acrylamide solution, 0.25% ammonium persulfate (APS) and 0.25% tetramethylethylenediamine (TEMED) were added to form the gel. After gel polymerization, a fixing reagent (50% methanol, 12% acetic acid) was employed to fix the gel. Thereafter, the gel was cut into 1 mm^3 pieces, cleansed with 50 mM TEAB, and dehydrated with 100% acetonitrile (ACN); these steps were repeated thrice. The gels were then reduced with 5 mM Tris (2-carboxyethyl) phosphine at 57 °C for 60 min and alkylated with 10 mM methyl methanethiosulfonate (MMTS) for 60 min at room temperature. In the following washing step (× 2), the gel slices were washed with 500 µl of 50 mM TEAB and dehydrated with 500 µl of 100% ACN. The gels were digested with trypsin (1 µg per 20 µg of proteins) at 37 °C for 16 h. The gel pieces containing solution was transferred to a tube, subjected to centrifugation at 6000 x*g* for 10 min and the supernatant containing the digested peptides was collected. The gel pieces in the centrifuge tube were once again mixed with 200 µL each of 50 mM TEAB and 100% ACN and the centrifugation step was repeated to collect the supernatant. The supernatant containing the digested peptides was collected and dried by vacuum dryer.

Isobaric tags were employed for relative and absolute quantification (iTRAQ) of proteins. The dried peptide samples were re-dissolved in 30 µl of dissolution buffer (0.5 M TEAB, pH 8.5). Two sets of iTRAQ Reagents 8-plex kit (SCIEX, Foster City, CA) were used to label the digested peptides according to the manufacture's protocol. All the labelled peptides of one set of 8-plex iTRAQ were pooled and desalted using Sep-Pak C18 cartridge (Waters, Milford, MA). In the final step, the two sample sets were lyophilized before proceeding to RP-HPLC and 2D LC-MS/MS.

2.4.3. 2D LC-MS/MS analysis

After lyophilization of peptide samples, 2D LC-MS/MS was performed as described below [34,35]. A high pH reversed-phase high-performance liquid chromatography unit (RP-HPLC; 1290 Infinity LC system; Agilent, Santa Clara, CA, US) equipped with a C18 column (WATERS Xbridge C18, 3.5 µm, 3.0 mm × 150 mm; Waters Milford,

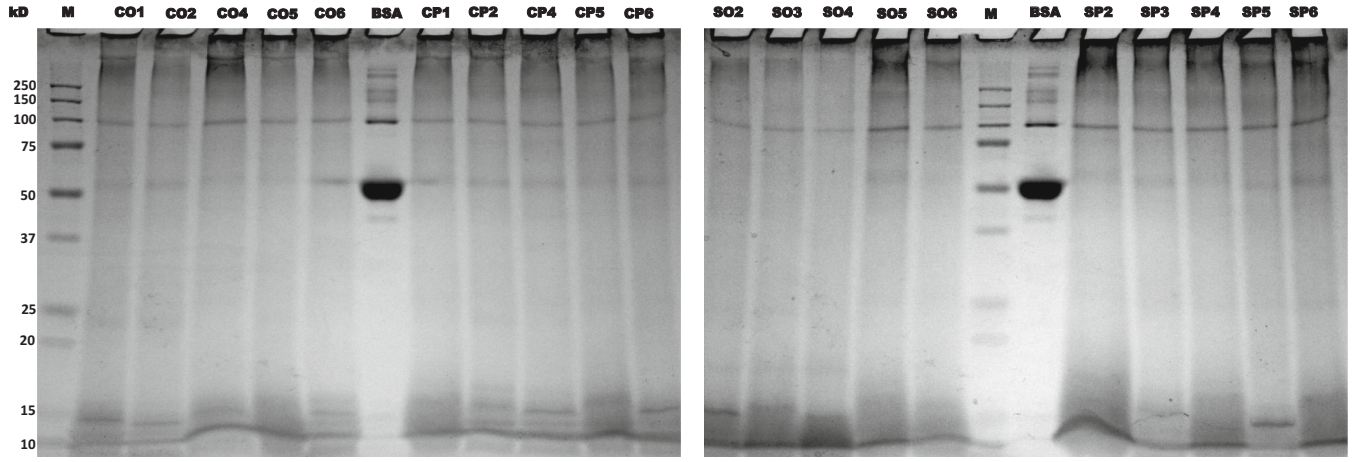

Fig. 1. SDS-PAGE gels of the mucus protein samples from the 4 study groups. Each lane was loaded with 4 µg of each protein samples and BSA (4 µg) was included in the SDS- PAGE as the reference protein.

Massachusetts, US) was employed to perform the first-dimension separation of the proteins. Mobile phase A (20 mM ammonium formate in water, pH 10) and mobile phase B (20 mM ammonium formate in 80% ACN, pH 10) were used for gradient preparation. The elution gradient was formed as follows: mobile phase B 0–0% for 5 min, 0–60% for 65 min, held at 60% for 5 min, 60–100% for 1 min, continued at 100% for 5 min and 100–0% for 1 min and subsequently sustained for 10 min at 0%. The eluted fractions (192) were collected in two 96-well v-bottom plates at a flow rate of 0.5 ml/min and combined into 20 concatenated fractions and lyophilized.

To perform the second-dimension separation, the lyophilized samples were dissolved in 30 μl of 2% ACN in water. For MS/MS analysis, 5 μl of each of the 20 fractions were injected independently into a 200 μm × 0.5 mm trap column and eluted on an analytical 75 μm × 15 cm RP column (ChromXP C18-CL, 3 μm) using an Eksigent nanoLC Ultra and ChiPLC-nanoflex system (Eksigent, Dublin, CA) in Trap-Elute configuration. The solvents employed for the RP LC were mobile phase A (2% ACN and 0.1% formic acid) and mobile phase B (98% ACN and 0.1% formic acid) to separate peptides with a flow rate of 300 nL/min. The elution gradient was formed with a flowrate of 300 nL/min as follows: mobile phase B 0–10% in 1 min, 10–20% in 79 min, 20–28% in 30 min, 28–45% in 10 min, 45–80% in 5 min, held at 80% in 5 min, 80–5% in 13 min and finally held at 5% of mobile phase B for 14 min. The LC fractions were analysed with a TripleTOF® 5600^{+}system (AB SCIEX, Framingham, Massachusetts, US) under the positive ionization mode. The conditions employed for acquiring the MS spectra were mass range of 350–1250 *m/z* at high-resolution mode (resolution >30,000) with the accumulation time of 250 ms per spectrum. The 30 most abundant precursors were selected between charge range + 2 to +4 (accumulation time: 100 ms) per duty cycle. In each duty cycle, 15 s dynamic exclusion was employed for MS/MS analysis in high sensitivity mode (resolution >15,000) with rolling collision energy and iTRAQ reagent collision adjustment settings turned-on.

2.4.4. Peptide and protein identification and bioinformatics analysis

Identification of iTRAQ-labelled proteins was performed with ProteinPilot™ 5.0 software Revision 4769 (AB SCIEX); using the Paragon database search algorithm (5.0.0.0.4767) and false discovery rate (FDR) cut-off [36]. The search parameters for identifying the proteins were: Sample Type: iTRAQ 8plex (Peptide Labelled); Cysteine Alkylation: MMTS; Digestion: Trypsin; Instrument: TripleTOF5600; Special Factors: None; Species: None; ID Focus: Biological Modification; Database: 190316_Salmo_Salar_NCBI_refseq.fasta (Total search entries 195,272); Search Effort: Thorough; FDR Analysis: Yes; Background correction: Yes; and User modified parameter files: Yes. For protein identification, 1% FDR was used as the cut-off. Differential abundances of proteins (DAPs) were determined using *t*-test. The iTRAQ ratios that were > 1.3 or < 0.77 (*p*-value <0.05) were categorized as proteins with increased and decreased abundance, respectively [37].

The data obtained from NUS was further analysed at Nord University. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [38] partner repository with the dataset identifier PXD027922. R packages were employed to perform the enrichment analyses. Functions of AnnotationHub version 2.18.0 and clusterProfiler version 3.14.0 were employed to annotate the differentially abundant proteins. KEGG enrichment and GO enrichment analyses were performed using the functions of clusterProfiler and we employed a *p*-value cut-off <0.05. Functions of ggplot2 version 3.2.1 and ggraph version 2.0.0 were employed to prepare and format the graphs and networks.

2.4.5. Sequential window acquisition of all theoretical fragment ion spectra mass-spectrometry (SWATH-MS) data acquisition and data processing

SWATH-MS was performed to validate the differential abundance of certain proteins (detected from the iTRAQ data) that belonged to the enriched KEGG pathways. Aliquots of 100 μg total protein from each mucus sample were digested using the S-Trap mini column (Protifi, Farmingdale NY, US) according to the manufacturer's recommendations. Synthetic iRT peptides (Biognosys AG, Switzerland) were spiked in the digested peptide samples at 10% final concentration for retention time alignment. Online reversed-phase LCMS analysis was performed on 1 μg of peptides from each sample using the ekspert nanoLC-425 (Eksigent) coupled to the TripleTOF 6600 system (SCIEX). The peptides were first loaded onto a ProteoCol C18P trap column (3 μm 120 Å 300 μm × 10 mm, Trajan, Australia) before separation on an Acclaim PepMap100 C18 analytical column (3 μm 100 Å 75 μm × 250 mm, Thermo Scientific). Peptide elution was performed on a linear gradient of 5–30% solvent B for 120 min, at a flow rate of 300 nL/min. Eluted peptides were analysed on the MS in SWATH mode. Precursor ions were acquired across 400–1600 *m/z* at 50 ms accumulation time per spectrum. Fragmentation information was collected using a setup of 100 variable SWATH windows across 400–1200 m/z precursor mass range. Each SWATH window was acquired in high sensitivity mode for 30 ms across 100–1800 m/z. Rolling collision energy was enabled with 5 eV spread. Acquired SWATH data were analysed using the DirectDIA workflow in Spectronaut 15 (Biognosys). Full analysis parameters were included in the deposited raw data (to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD029849). Briefly, Pulsar library search was performed using Trypsin/P with specific digestion against the database described above, allowing for up to two missed cleavages. MMTS was specified as a fixed modification, while acetyl (protein N-term) and oxidation (Met) were allowed as variable modifications. FDR was controlled at 1% based on peptide-to-spectrum match. Data independent acquisition-based data extraction was performed at maximum intensity with dynamic mass tolerance and chromatography window extraction. For protein and peptide identifications, we employed a q-value cut-off 0.01, while at least two and up to 10 proteotypic peptides for each protein were used for quantification. A global normalization strategy on median was employed. Differential abundance was determined using unpaired *t*-tests.

3. Results

3.1. Summary of iTRAQ quantitative analysis and SWATH validation of differentially abundant proteins

We found that in total 5770 and 3074 proteins were present in the two analysis sets (SO vs CO and SP vs CP), respectively. Among them, 5334 and 2578 proteins were identified and quantified employing an FDR of 1% and with minimum one peptide (confidence interval ≥ 95%) in SO vs CO and SP vs CP comparisons, respectively. Among them, 1752 and 610 proteins had a fold change ≥1.3 or ≤ −1.3 and *P*-value <0.05: differentially abundant proteins (DAPs). Of these DAPs, 249 and 219 had higher abundance and 1503 and 391 had lower abundance based on the SO vs CO and SP vs CP comparisons, respectively (Supplementary Tables 1, 2, 3, 4). We also performed SWATH validation, 23 and 27 higher abundant proteins, as well as 142 and 79 lower abundant proteins (corresponding to the iTRAQ results) in both SO vs CO and SP vs CP, respectively (Supplementary Tables 5, 6, 7, 8).

3.2. Differences in enrichment of the proteins with increased abundance in fish with the inflamed intestine

The proteins that had increased abundance (Supplementary Tables 1, 3, 5, 7) in either the SO or SP groups were compared to those in the CO or CP groups, respectively, to understand the enriched KEGG pathways and GO terms.

3.2.1. Pathways

In the SO vs CO comparison, only phagosome pathway (under transport and catabolism) was enriched, whereas 2 pathways (ribosome

and aminoacyl-tRNA biosynthesis; under translation) were enriched for the SP vs CP comparison (Figs. 2A, B, Supplementary Table 9, 10).

3.2.2. GO terms

Enrichment of the GO terms also followed the same trend; more terms (15 vs 1) were enriched in the SP vs CP comparison (Fig. 3A, B).

Fig. 2. The proteins that had higher abundance in the SO and SP groups were in enriched KEGG pathways. A. The KEGG pathway phagosome associated proteins of the SO group. B. The KEGG pathways ribosome and aminoacyl-tRNA biosynthesis associated with the proteins of the SP group.

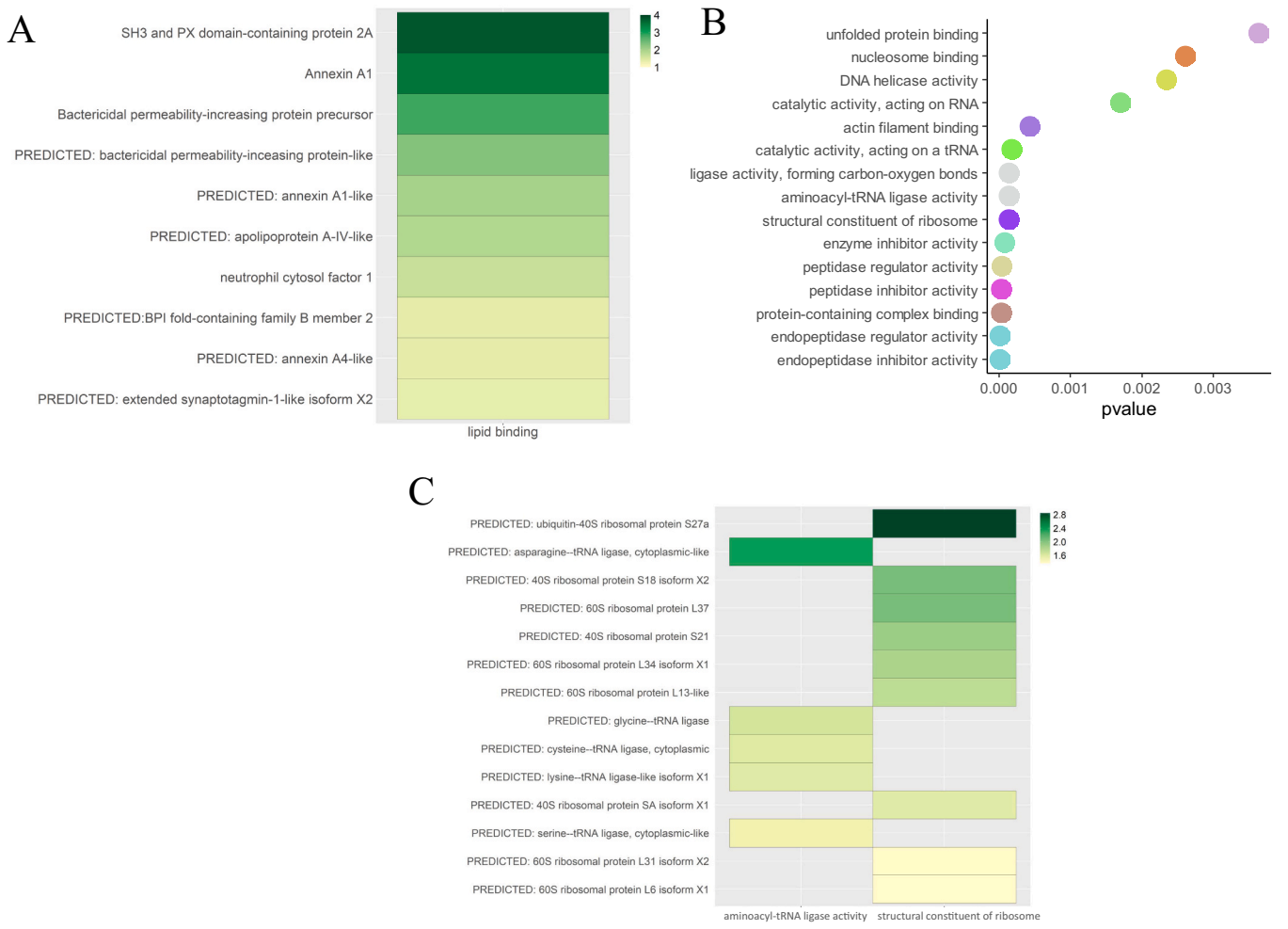

Fig. 3. The proteins that had higher abundance in the SO and SP groups were in enriched gene ontology terms. A. The GO term lipid binding related proteins of the SO group. B. The GO terms ($p < 0.05$) associated with the proteins that had higher abundance in the SP group. C. The GO terms structural constituent of ribosome and aminoacyl-tRNA ligase activity linked proteins of the SP group.

3.2.3. Associated proteins

The phagosome pathway (KEGG) was enriched because of the increased abundance of Integrin beta 2 precursor, Coronin 1A, Cathepsin S precursor, Vesicle-trafficking protein, and Neutrophil cytosol factors, among others (Fig. 2A). The altered GO term, namely lipid binding of the SO vs CO comparison included Bactericidal permeability-increasing protein, Apolipoprotein A-IV and Annexins (Fig. 3A). The enriched KEGG and GO terms of the SP vs CP comparison, namely ribosome and aminoacyl-tRNA biosynthesis were connected to many large and small subunit of ribosomes (Figs. 2B, 3C, Supplementary Table 10).

3.3. Differences in enrichment of the proteins with decreased abundance in fish with the inflamed intestine

The proteins of decreased abundance (Supplementary Tables 2, 4, 6, 8) in either the SO or SP groups compared to the respective control groups, CO or CP were employed to understand the enriched KEGG pathways and GO terms. The proteins that were unique in a comparison is also described to point out the target proteins in the SO and SP groups.

While 47 KEGG pathways were enriched in the SO vs CO comparison, 36 were enriched in the SP vs CP comparison (Fig. 4A, B, Supplementary Tables 9, 10). In the latter comparison, only 50 GO terms were enriched compared to 111 in the former comparison (Supplementary Figs. 1, 2).

3.3.1. Pathways

KEGG pathways that were unique for the SO vs CO comparison were 2-oxocarboxylic acid metabolism, sulfur metabolism, protein processing in endoplasmic reticulum, ferroptosis, phagosome, ABC transporters, butanoate metabolism, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, lysine degradation, selenocompound metabolism, folate biosynthesis, drug metabolism – other enzymes, sulfur relay system (Supplementary Table 9). Thus, folding, sorting and degradation, metabolism of cofactors and vitamins, membrane transport, cell growth and death pathways were unique for the enrichment of abundance-decreased proteins associated with the SO group (Fig. 4A). The unique ones for the SP vs CP comparison were arginine biosynthesis, biosynthesis of unsaturated fatty acids, starch, and sucrose metabolism (Supplementary Table 10). The abundance-decreased proteins-related enrichment of both comparisons included, among others, common pathways such as carbohydrate, amino acid and lipid metabolisms, xenobiotics biodegradation and metabolism pathways, endocrine system, other glycan degradation (Fig. 4A, B). However, only fewer components were present under these classifications for the CP vs SP comparison.

3.3.2. GO terms

As for the SO vs CO comparison, among other GO terms, there were many related to transmembrane movement or transport, ATPase, oxidoreductase activity, enzyme activities, binding of magnesium ion,

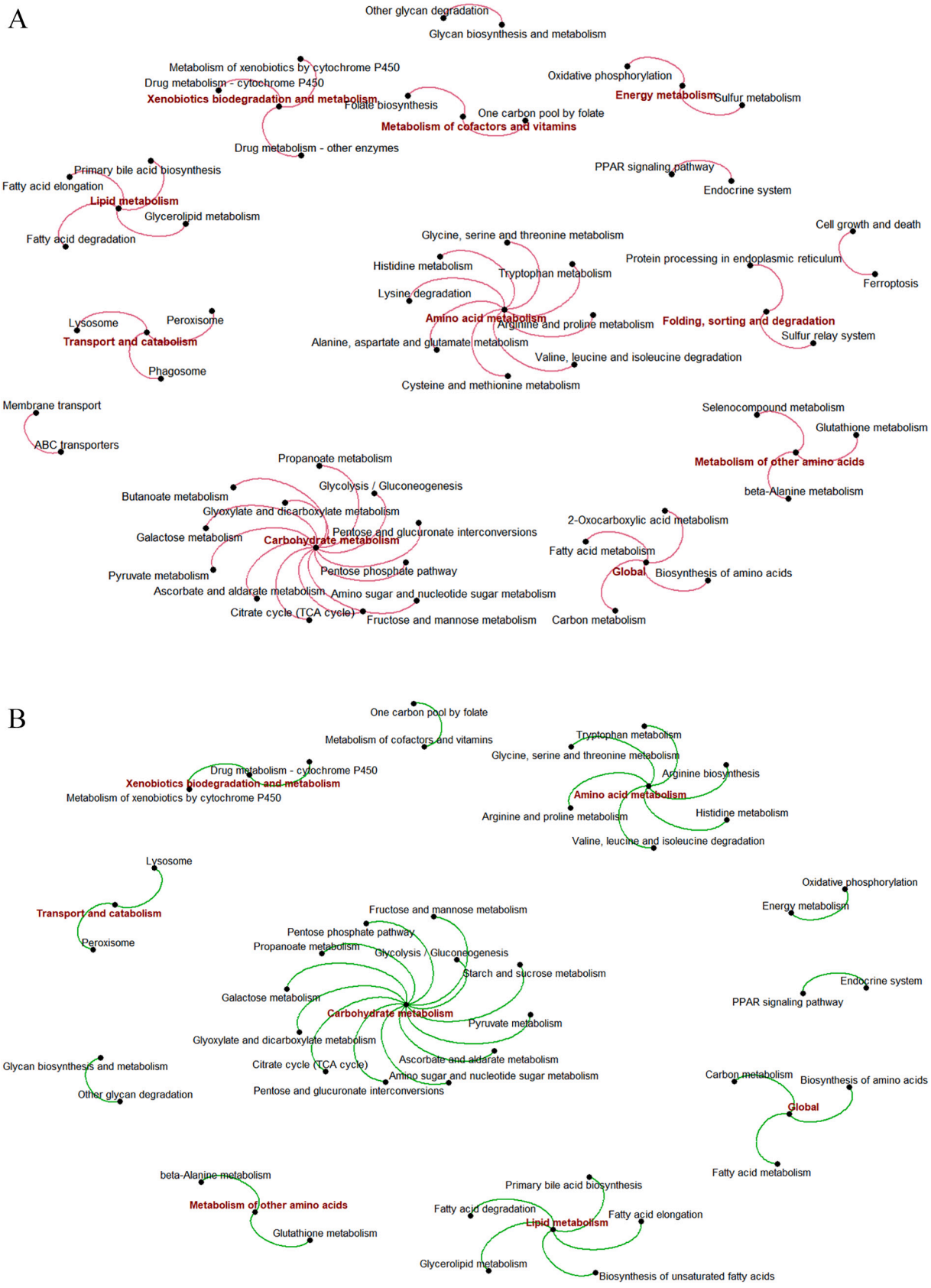

Fig. 4. Networks showing the association of the lower abundant proteins-enriched KEGG pathways to the divisions recognized by the KEGG database. A. Network of the SO group. B. Network of the SP group.

sulfur compound, NAD, lipid, and unfolded protein (Supplementary Fig. 1). The unique GO terms for the SP vs CP comparison were, among others, phosphorylase activity, carboxypeptidase activity, manganese ion binding, protein-containing complex binding and metal-loaminopeptidase activity (Supplementary Fig. 2). The GO term 'lipid binding' was also enriched based on the abundance-decreased proteins in the SO group; the associated proteins included sorting nexin proteins and different annexins, and retinol binding proteins (Supplementary Fig. 3).

3.3.3. Associated proteins

The enrichment of pathways such as folding, sorting and degradation, membrane transport, cell growth and death pathways were associated with an array of proteins (Supplementary Figs. 4, 5). The associated proteins included Heat shock proteins, Protein transport proteins, Calpain, Calreticulin, Ribosome-binding protein, Calnexin, Ubiquitin proteins, ATP-binding cassette sub-families, Multidrug resistance proteins, Serotransferrin, Cell surface antigen and Anion channel proteins. The oxidative phosphorylation pathway was enriched based on the proteins that reduced abundance in both SO and SP groups (Supplementary Fig. 6, 7). However, not many proteins were associated with the SP groups (Supplementary Fig. 7); those altered included Cytochrome c oxidase and ATP synthase subunits and V-type protein ATPase subunits. Similarly, PPAR signalling pathway associated with the SO group (not with the SP group) was based on many proteins, and among them were Retinoic acid receptor, Platelet glycoprotein 4, Perilipin-2 and Carnitine O-palmitoyl transferase 1 and 2 (Supplementary Fig. 8). Likewise, in the case of peroxisome, SO group (not in the SP group) had Superoxide dismutase, Xanthine dehydrogenase/oxidase, D-amino acid oxidase isoform, Hydroxyoxidase 1, Phytanoyl-CoA and peroxisome associated enzymes (Supplementary Fig. 9). The abundance-decreased Vacuolar protein pump subunit H, V-type proton ATPase subunits, Ganglioside GM2 activator, CD63 antigen were linked to the enrichment of lysosome pathway in the SO group (not in the SP group; Supplementary Figs. 10, 11). The abundance-decreased proteins other than these led to the enrichment of lysosome pathway in the SP group (Supplementary Fig. 11). Based on the abundance-decreased proteins, the KEGG pathway phagosome was enriched only in the SO group; the associated proteins were Calnexins, Ras-related proteins, MHCI-related proteins, Integrin beta-1, Calreticulin, Macrophage-mannose receptor 1, Tubulins, Antigen-peptide transporter, Cathepsin L1 and V-type proton ATPases (Supplementary Fig. 12).

3.4. Micromorphogical changes were not prominent in the intestine of fish fed the additive

Histology of the distal intestine indicated that inflammation characteristics were milder in the fish on the SP diet compared to the fish on the SO diet (Supplementary Figs. 13, 14).

4. Discussion

Proteins are integral components of the mucus layer, and this barrier that is rich in specific proteins [39] also contains other proteins, which are imperative for guarding the underlying epithelial layer. Changes in the expression of the mucus proteins can affect the health of the host. Not many studies have explored the alterations in the mucus proteins of hosts that have developed intestinal inflammation, a chronic disease that is associated with many dietary components. Hence in the present study, we employed intestinal inflammation model in Atlantic salmon to study the changes in the mucus proteins of the fish. The differences in the mucus proteome linked to an additive is also explained to describe its inflammation-alleviating potential, besides identifying protein markers associated with intestine inflammation. The present baseline information will enable in-depth studies on target proteins to treat intestinal inflammation.

As in mammals, fishes have phagocytes, namely neutrophils, dendritic cells (DCs), monocytes, macrophages, epithelial cells and certain T and B lymphocyte subsets that can recognize, engulf, and kill pathogens [40]. Phagosomes are involved in not only pathogen killing but also antigen presentation [40]. Many proteins that are associated with phagosomes significantly increased their abundance in fish with intestinal inflammation (SO group). For example, Beta 2 integrins, the cell surface receptors specific to leukocytes [41], which are essential for recruiting immune cells to inflammatory sites [42] had higher abundance in the SO group. The other proteins that had higher abundance and were associated with the phagosome pathway included Coronin-1, the overexpression of which indicates neutrophil survival [43], Neutrophil cytosol factor 1 (SH3 and PX domain-containing protein 1A) and SH3 and PX domain-containing protein 2A that are connected to NADPH oxidase and generation of reactive oxygen species (ROS) [44]. Cathepsin S, another protein that had higher abundance in the SO group, is activated in macrophages, and it is an inflammation driver and vesicle-trafficking protein [45]. ER resident Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein Sec22b, also a vesicle-trafficking protein, is required for the fusion of endoplasmic reticulum with plasma membrane during phagocytosis [46], and this protein had higher abundance in the fish group with intestinal inflammation. Furthermore, eosinophil peroxidase, a molecule associated with gastrointestinal dysfunction in experimental ulcerative colitis [47] had higher abundance in the SO group. These findings indicate the degree of inflammation in the SO group. In a related communication, we have presented the inflammatory characteristics observed in the intestine of Atlantic salmon [32].

Ribosomal proteins, namely 40S ribosomal proteins S18, SA, S27, S21 and 60S ribosomal proteins L34, 6, 37, 31, 13 had higher abundance in the fish that received the dietary health additive (SP group), but not in the fish group affected by inflammation. High levels of these ribosomal proteins are known to decrease the p53-mediated DNA damage response [48] or the changes in the small and large ribosomal subunits is linked to cell proliferation or apoptosis [48–52]. The higher abundance of both ribosome subunits 40S and 60S and proteins of aminoacyl-biosynthesis in the SP group is interesting. In contrast, phagosome-associated proteins had higher abundance in the SO group. The histology of the intestine suggests milder inflammatory features in the SP group, probably pointing to the efficacy of elevating the abundance of the ribosomal proteins and the associated amino acid ligase at the sites of inflammation in the intestine.

Analysis of GO terms have also given clues on the intensity of inflammation in the fish that were offered soy saponin (SO group). For example, terms linked to lipid binding for the SO vs CO comparison included Bactericidal permeability-increasing protein, Apolipoprotein A-IV and Annexins. The higher abundance of the Bactericidal permeability-increasing protein that is mainly expressed in neutrophils could be pointing to the possible anti-microbial activity against Gram-negative bacteria in the inflamed intestine [53]. Both endogenous anti-inflammatory and protective proteins such as Apolipoprotein A-IV and Annexin A1 that help in repair of intestinal mucosal epithelium and Annexin A4 that also stimulates epithelial cell proliferation [54–56] had higher abundance in the SO group. The barrier breach observed in the intestine of the SO group may correspond to the increased antimicrobial responses at the mucosal surfaces.

Enrichment of many KEGG pathways and GO terms connected to the proteins that had lower abundance clearly points to fewer alterations in the SP group compared to the group that magnified the impact of inflammation i.e., in the SO group. Heat shock proteins, Protein transport proteins, Calreticulin, Ribosome-binding protein, Calnexin, Ubiquitin proteins, ATP-binding cassette sub-families, Serotransferrin, Cell surface antigen and Anion channel proteins that are associated with pathways such as folding, sorting and degradation, membrane transport, cell growth and death could indicate disturbances to transport and secretion of nascent proteins, cargo protein vesicle transport, presence

of activated macrophages, flow of metabolites and ions via mitochondrial membrane, and iron availability to pathogens [57–63]. The lower abundant proteins (Calnexins, Ras-related proteins, MHCI-related proteins, Integrin beta-1, Calreticulin, Macrophage-mannose receptor 1, Tubulins, Antigen-peptide transporter, Cathepsin L1, V-type proton ATPases) that were enriched in the phagosome pathway could indicate the reduction in activities like clearance of viable or apoptotic cells by immune cells, antimicrobial activity and ER stress sensing [40,64,65]. The decreased abundance of Cytochrome c and ATP synthase subunits and V-type protein ATPase subunits associated with the group that received the additive (SP) could be an indication of the milder inflammatory signs. Reduction in peroxisome-associated proteins such as Phytanoyl-CoA α-hydroxylase, D-amino acid oxidase, and Hydroxyoxidase 1, Xanthine oxidase in the SO group may be pointing to the lowering of ROS production during oxidative stress and aggravation of the inflammation [66–68]. Peroxisome proliferator-activated receptor pathway-associated proteins that had lower abundance in the SO group could indicate aberrations in, among others, the rate-limiting step of fatty acid oxidation, lipid droplet biogenesis, intracellular triacylglycerol accumulation and long chain fatty acid signalling [69–75]. The decreased abundance of Vacuolar protein pump subunit H, V-type proton ATPase subunits, Ganglioside GM2 activator, CathepsinZ, CD63 antigen under the inflammatory condition (SO group) could have an impact on the degradation of glycogen to glucose, degradation of Ganglioside GM2 by β-hexosaminidase A, allergic inflammation and activities in the acidic compartments [76–78]. The abundance-decreased proteins (many Sorting nexins, Annexins and Retinol-binding proteins) of the SO group that were enriched in the GO term lipid binding indicate the effects on membrane trafficking and protein sorting, Na^{+}/Ca^{2+} exchanger, vesicle aggregation, transport of retinol from liver and vitamin A, intracellular fatty acid-binding and activation of PPARγ targets [79–83].

Interestingly some of the proteins that are known to reduce inflammation were unique to the group that received the additive (SP). This includes Glycogen phosphorylase, and a decrease of this rate-limiting enzyme of glycogenolysis has been reported to cause deficits in inflammatory cytokine expression, nitric oxide production, IL-1β secretion and NLRP3 inflammasome priming in response to microbial stimuli [84]. Although pharmacological approaches that inhibit glycogenolysis could disturb physiologic processes, this enzyme can be used to modulate the metabolism of innate immune cells such as macrophages as a therapeutic approach to reduce acute inflammation [85]. Furthermore, inflammatory cytokines are suppressed by glutamine synthetase inhibitor [86], and Glutamine synthetase had lower abundance in both the SO and SP groups compared to their respective controls. However, the reduction was lower in the case of the SP group (iTRAQ and SWATH) that had milder inflammation. It has been reported that antibacterial and inflammatory capacity of monocytes could be revived by inhibiting the cell Glutamine synthetase [87]. On the other hand, the inhibition of this enzyme in brain-resident macrophages can release inflammatory mediators [88]. With respect to the enrichment of the biosynthesis of unsaturated fatty acids pathway based on the proteins that had lower abundance in the SP group, it is known that some of such fatty acids can inhibit inflammation and reduce the secretion of proinflammatory cytokines [89]. Moreover, n-3 polyunsaturated fatty acids can alleviate intestinal inflammation [90]. The SP feed in the present study enabled in counteracting inflammation in Atlantic salmon.

5. Conclusions

Intestinal inflammation model in Atlantic salmon had the characteristic inflammation features and the proteins that had increased/decreased abundance could be pointing to the broad impact on the organism caused by intestinal dysfunction. The enriched KEGG pathways and GO terms could indicate recruitment of more immune cells, strong anti-microbial defence, aberrations related to formation, sorting, transport and secretion of proteins, issues with transport of metabolites, iron availability to pathogens and aberrations in lipid metabolism.

On the other hand, the application of an additive helped limit the inflammation, marked by fewer altered proteins. It should be noted that probably other inflammation strategies such as probiotic application can also alleviate the inflammation in Atlantic salmon [30] albeit through a different mode of action. The ribosomal proteins and amino acyl biosynthesis-associated proteins in this group of fish could be effective in reducing inflammation-induced damages. The appropriate decrease in abundance of Glycogen phosphorylase and Glutamine synthetase could point to the possible mechanism that alleviates intestinal inflammation. The key proteins that were altered in the inflamed intestine proteome of the model fish could be the clues to decipher ways to combat the disease through dietary intervention.

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Declaration of Competing Interest

JD and VVT are from SPAROS Lda. Olhão, Portugal and DSM Nutritional Products, Kaiseraugst, Switzerland, respectively. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from DSM Nutritional Products. The funder had the following involvement in the study: research design and feed formulation.

Data availability

The sequences are submitted in a public repository (PRIDE).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104487.

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