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Replacing fishmeal with salmon hydrolysate reduces the expression of intestinal inflammatory markers and modulates the gut microbiota in Atlantic salmon (*Salmo salar*)

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The feed legislation allows the use of fish protein hydrolysates in feed for the same species in which it came from, since enzymatic hydrolysis degrades the proteins and eliminates potential prions, which have caused disease in mammals, but not in fish. In this trial, we investigated the effects of partially replacing dietary fishmeal (FM) with salmon protein hydrolysate (FPH) on the intestinal gene expression and microbiota. Atlantic salmon post smolts were either fed a control diet containing 30% fishmeal (FM), a 20% FM diet with 9% salmon hydrolysate (FPH-09) or a 10% FM diet with 18% salmon hydrolysate (FPH-18), until doubling of weight. Gene expression analysis by RNA sequencing of pyloric caeca (PC), midgut (MG) and hindgut (HG) revealed a downregulation of immunological genes involved in inflammation in the intestine of FPH-18 fed salmon compared to salmon fed the FM control. The gene expression of paralogous peptide transporters (PepT) was analyzed by real time quantitative PCR in PC, anterior midgut (AMG), posterior midgut (PMG) and HG of salmon fed all the three diets. The PepT1b paralog had highest relative expression levels in PC and AMG, suggesting that PepT1b is most important for peptide uptake in the anterior intestine. PepT1a was also mainly expressed in the PC and AMG, but at lower levels than PepT1b and PepT2b in the AMG. The PepT2b paralog had high levels of expression in AMG, PMG and HG indicating that it contributed significantly to peptide uptake in the posterior part of the gastrointestinal tract. The gut microbiota in the mucosa and digesta of the MG and HG, were dominated by the phyla Cyanobacteria and Proteobacteria, but also Firmicutes were present. The only dietary effect on the microbiota was the higher prevalence of the phyla Spirochaetes in the mucosa of FPH-18 fed salmon compared to the FM fed salmon. In conclusion, replacing FM with salmon

hydrolysate reduced the expression of inflammatory markers in the Atlantic salmon intestine suggesting improved health benefits. The reduced inflammation may be related to the reduced FM content, potentially bioactive peptides in the hydrolysate and/or the altered gut microbial composition.

KEYWORDS

Atlantic salmon (*Salmo salar*), feed, hydrolysate, gene expression, RNAseq, microbiota, peptide transporters

1 Introduction

The need for novel and sustainable feed ingredients for cultivated fish and land animals is increasing with the growing human population. By-products from marine food production are sustainable alternatives for production of feed ingredients (Rustad et al., 2011; Almås et al., 2020). In Norway a significant biomass of by-products is produced from farmed Atlantic salmon amounting to > 0.5 million tons in 2021 (Myhre et al., 2022). However, the feed legislation prohibits the reuse of proteins from Atlantic salmon into feed of the same species, unless it is hydrolyzed. Complete processing by enzymatic hydrolysis will degrade potential prions and is therefore an exception from the transmissible spongiform encephalopathy (TSE)-legislation (Regulation (EC) No 999/2001; Commission Regulation (EU) No 142/2011; Sandbakken et al., 2023).

Novel feed ingredients should be nutritious, palatable, digestible, and not have any negative impact on the immune response and health of the animal (Glencross, 2020). Protein hydrolysates are mixtures of peptides and free amino acids, which are nutritious, highly digestible and known to be palatable (Kristinsson and Rasco, 2000; Kousoulaki et al., 2013; Khosravi et al., 2015). Hydrolysates may also contain bioactive peptides (Zamora-Sillero et al., 2018; Gao et al., 2021; Siddik et al., 2021). Marine peptides in fish protein hydrolysates (FPH) have shown beneficial effects including antimicrobial, anti-inflammatory and immunomodulatory activities when consumed *in vivo* (Kang et al., 2019). Dietary inclusion of FPH in aquaculture has induced growth performance, digestibility and altered the immune response (Zheng et al., 2014; Siddik et al., 2018).

Optimized diets can improve the general health and immune functions of fish, thereby reduce losses due to diseases, as well as improving recovery after diseases in aquaculture (Waagbø, 1994; Dawood, 2020). Therefore, nutritional status has a major impact on the ability of fish to resist pathogens and cope with stress (Martin et al., 2016). Some feed additives have immunomodulatory properties, which either activates the immune system or downregulates immunological genes. Activating the innate immune system can be beneficial when the fish experiences pathogenic threats, but a general activation can be energy consuming and have negative impact on other processes like

growth (Tacchi et al., 2011). A downregulation of genes related to inflammation saves energy for the organism to grow and thrive.

Intestinal protein absorption is performed by different transporters in the brush border membrane of absorptive epithelial cells (Evans and Claiborne, 2006; Kiela and Ghishan, 2016). Peptide transporters (PepT) provide a more efficient uptake mechanism for protein building blocks than amino acid transporters since they transport di- and tripeptides instead of single amino acids (Wang et al., 2017). Due to the whole genome duplication event, Atlantic salmon has at least 4 genes encoding paralogous PepT which are part of the solute carrier 15a (Slc15a) family of transporter molecules. These 4 transporters are *slc15a1a*, *slc15a1b*, *slc15a2a* and *slc15a2b*, also known as PepT1a, PepT1b, PepT2a and PepT2b, respectively. PepT1 paralogs are low affinity/high-capacity transporters that serve a key role in peptide absorption, mainly in the proximal part of the intestine (Verri et al., 2003; Rønnestad et al., 2007; Ostaszewska et al., 2010; Wang et al., 2017). Most research available is on the PepT1b paralog, since PepT1a was identified and characterized in Atlantic salmon (*Salmo salar*) quite recently by Gomes et al. (2020). PepT2 paralogs are high affinity/low-capacity transporters which recently were characterized in Atlantic salmon by Vacca et al. (2022). Whereas PepT2b mainly is expressed in the kidney and midgut (MG) to hindgut (HG) (Del Vecchio et al., 2021; Vacca et al., 2022), PepT2a is more abundant in brain and gills, and appears to have a minor role in peptide uptake along the gastrointestinal tract (Del Vecchio et al., 2021). There is however little information on how the expression of peptide transporters is regulated by dietary proteins, and especially available peptides and free amino acids in Atlantic salmon.

Intestinal microbiota composition depends on the diet, environmental factors, and host selection. The microbiota affects the nutrient absorption, immune system, and disease resistance of the host (Luan et al., 2023). Mucosal microbiota is colonizing the mucus layer on the intestinal epithelial cells and is more stable and less affected by diet than the microbiota found in the digesta (feces) (Gajardo et al., 2016). This was clearly shown in Atlantic salmon fed diets with and without insect meal (Li et al., 2021), where the digesta-associated microbiota also showed the highest microbial richness and diversity. Dietary protein can affect the intestinal microbiota composition and function, and interactions between

proteins and microbiota can impact the host health (Bartlett and Kleiner, 2022). Furthermore, if the protein fraction is absorbed much faster than in normal diets, it is possible that remaining bacteria in midgut and hindgut sections will rely more on utilization of feed components reaching there in larger amounts such as fiber and carbohydrates. Such changes in available nutrients for the microbiota can have profound effect on fermentation patterns altering profiles of short chain fatty acids and other products of fermentation (Bartlett and Kleiner, 2022), and on production of inflammatory/anti-inflammatory components.

The current study aimed at assessing the effects of replacing 2/3 of the fishmeal (FM) in a standard diet, with salmon protein hydrolysate (FPH) on intestinal microbiota and health parameters by RNA sequencing analysis. Furthermore, we also report on the response of intestinal peptide transporter expression when the salmon were fed diets with 1/3 and 2/3 of the FM protein replaced with salmon hydrolysate.

2 Materials and methods

2.1 Feed and fish husbandry

A detailed description of the feed composition, experimental design and fish facilities have been reported in Sandbakken et al. (2023). In brief, Atlantic salmon (*Salmo salar*) post smolts were fed one of three diets with spray dried salmon hydrolysate (Nutrimar AS) replacing parts of the fish meal: a control diet with 30% fish meal (FM), a 9% hydrolysate diet with 20% fish meal (FPH-09) and a 18% hydrolysate diet with 10% fish meal (FPH-18) (Supplementary Table 1). The diets were designed to be iso-energetic, iso-nitrogenous, iso-lipidic as well as having the same amount of EPA, DHA and phospholipids. Salmon (mean start weight 141.5 ± 23.6 g (standard deviation)) were PIT-tagged and randomly distributed into 9 tanks (24 fish/tank, ~ 300 L, $10\text{--}14$ L min^{-1} water flow, $> 80\%$ O_2 saturation in the outlet water, $8.2\text{--}8.8^\circ\text{C}$ seawater, continuous light) and fed for 2 h twice a day until satiety. After 9 weeks of feeding, the mean salmon weight was 313.8 ± 50 g at sampling. The feeding trial was approved by the Norwegian Food Safety Authority (FOTS ID:23021) and was conducted at NTNU Sealab (Department of Biology, Trondheim, Norway).

2.2 Sampling procedures

Final samples were taken 60 and 62 days after the experimental start, giving RNA samples and microbiota samples, respectively. Before the first sampling day, the fish were not fed for 48 hours to ensure empty intestines for RNA analysis. Five fish from each tank were randomly sampled and euthanized in seawater with Finquel, MS-222 (800 mg/l). Weight, length and PIT-ID was registered before the 5 fish were put on ice and processed immediately. The sampling was performed one tank at a time.

The fish abdomen was opened, and fish were sexed by looking at the gonads. For each fish, 5 intestinal samples were collected for RNA analysis, by cutting ~ 2 mm of pyloric caeca, 3 segments of the midgut (anterior, mid, and posterior) and a middle segment of the hindgut (HG). Tissue samples for RNA analysis were put in 1 ml cold RNA later (RNA stabilization solution, Ambion Inc.), and stored for 24 h at 4°C before the samples were transferred to -20°C .

The remaining fish (19 per tank) were fed five 2-hour meals over the next 42 hours, before the next sampling two days later. Five random fish from the tanks fed FM control and FPH-18 were euthanized (MS-222) to collect microbiota from midgut (MG) and hindgut (HG) digesta and mucosa. The fish were sexed followed by aseptic removal of the intestine from the abdominal cavity. Sampling equipment was sterilized between each sample by burning 96% ethanol from the metal. The intestine was opened longitudinally with scissors, followed by digesta collection in cryotubes that were snap-frozen on dry ice. The remaining intestine was washed 3 times in cold sterile Phosphate Buffered Saline (PBS, P3813, Sigma-Aldrich) to remove traces of remaining digesta. Some of the intestinal mucosa was scratched off with the backside of a scalpel and snap frozen on dry ice. The same procedure was performed aseptically on both the MG and HG, resulting in four microbiota samples from each fish that were stored at -80°C until analysis.

An overview of analysis performed on the different intestinal segments from fish fed different diets is shown in Table 1.

2.3 RNA extraction and quantification

Intestinal samples were thawed on ice, dried off on a clean piece of paper and weighed (averagely 32 mg) in an Eppendorf tube

TABLE 1 Overview of analysis performed on the different intestinal segments in fish fed different diets: control diet with 30% fish meal (FM), 9% salmon hydrolysate diet with 20% fish meal (FPH-09) and 18% salmon hydrolysate with 10% fish meal (FPH-18).

Intestinal segment		Pyloric caeca (PC)	Anterior midgut (AMG)	Middle of midgut (MG)	Posterior midgut (PMG)	Hindgut (HG)
Analysis	Feed					
RNA seq.	FM FPH-18	X		X		X
RT-qPCR	FM FPH-09 FPH-18	X	X		X	X
Microbiota of digesta and mucosa	FM FPH-18			X		X

(1.5 ml). Samples were added 1 ml of an RLT Lysis buffer (RNeasy Mini kit) mixed with β -mercaptoethanol (1% in RLT) before homogenization using a rotor-stator homogenizer (Polytron Kinematica PT3000) for up to 10 seconds at max speed (28 100 rpm). All samples were kept on ice between homogenization, and frozen at -80°C before further processing. RNA was isolated as described in the RNeasy Mini kit (Qiagen) including a DNase treatment step with RNase-Free DNase set (ID:79256, Qiagen). RNA concentration was measured with Nanodrop 2000c Spectrophotometer (Thermo Scientific) and RNA quality was evaluated by 2100 Bioanalyzer using RNA 6000 Nano Kit (both from Agilent Technologies) following the manufacturer's protocol. All samples with RNA integrity number (RIN) of ≥ 7 were accepted for further use. RNA was kept at -80°C between analysis.

2.4 RNA sequencing and assembly

Extracted RNA samples (6 from each intestinal segment and diet, 36 in total) from pyloric caeca (PC), MG and HG from fish fed FM and FPH-18 diets were sent to The Beijing Genomics Institute (BGI, Beijing, China) for sequencing. RNA quality control, library construction and sequencing were done by BGI, including (i) mRNA enrichment and purification by using Oligo dT beads to enrich mRNA with a polyA tail, (ii) RNA fragment and reverse transcription by random N6-primed reverse transcription, followed by the second-strand cDNA synthesis, (iii) end repair of cDNA fragments, 3' adenylation and adaptor ligation, (iv) PCR amplification to enrich the purified cDNA template, (v) cyclization by heating PCR product, oligos and DNA ligase, (vi) DNA nanoball synthesis, and (vii) sequencing on DNBSec Technology platform.

Firstly, reads mapped to rRNAs and low-quality reads (where $> 20\%$ of the bases had qualities < 10 in base quality score) were removed. Secondly, reads with adaptors and/or with unknown bases (N bases more than 5%) were filtered. Clean reads were mapped onto the reference genome assembly Ssal_v3.1 (GCF_905237065.1), followed by novel gene prediction. After novel transcript detection, coding transcripts were merged with reference transcripts to get complete reference, then clean reads were mapped to it using STAR (Dobin et al., 2013), then gene expression level was calculated for each sample with RSEM (Li and Dewey, 2011). RSEM is a software package for estimating gene and isoform expression levels from RNA-Seq data. Pearson correlation between all samples was calculated using *cor*. Hierarchical clustering between all samples was performed using *hclust*. PCA analysis with all samples was performed using *princomp*, and the diagrams were drawn with *ggplot2* in R. Circos software package (version 1.2.12.) was used to visualize data (Krzywinski et al., 2009). The clustering results were displayed with javaTreeview (Saldanha, 2004) using cluster (Eisen et al., 1998; De Hoon et al., 2004) software to analyze the expression genes and sample scheme at the same time by using the Euclidean distance matrix as the matrix formula.

In order to reflect the gene expression correlation between samples, all the samples were hierarchical clustered by the expression level of all genes, which can directly reflect the

relationship between each two samples. Sample variation was visualized by a principal component analysis (PCA) plot (Supplementary Figure 1A), which shows that all samples were divided into three parts according to intestinal segments, indicating the reliability of the sequenced samples. Differentially expression genes (DEGs) were detected between groups based on gene expression levels using DESeq2 algorithms. Summary of DEGs is shown in Supplementary Figure 1B. DEGs were detected including an average of 105 up-regulated genes and on average 61 down-regulated DEGs in the three intestinal segments.

Differentially expressed genes (DEGs) between the FM and FPH-18 group (log fold change ≥ 1 and adjusted p-value ≤ 0.05), were detected based on gene expression levels using DESeq2 algorithms. Clustering analysis and functional annotations including Gene Ontology (GO) (<http://www.geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>) enrichment were performed. The raw data has been uploaded to NCBI SRA database with BioProject No. PRJNA1072556.

2.5 Reverse transcription and real-time quantitative PCR

Reverse transcription was performed using LunaScript RT SuperMix Kit (New England BioLabs) with 1 μg RNA per 20 μl reaction volume, following the manufacturers protocol. A control without reverse transcriptase (no-RT) was added to each plate (Thermo Scientific, cat. No. AB0600) to evaluate the presence of genomic DNA in a sample. The plate was centrifuged (1 min, 900 rpm, Sigma 2-6 Compact Centrifuge) before incubation in a thermal cycler (Bio-Rad T100) for 2 min at 25°C for primer annealing, followed by cDNA synthesis at 55°C for 10 min and finally heat inactivation at 95°C for 1 min. The cDNA plates were stored at -20°C .

Primers were screened for specificity *in silico* by using BLAST (Ye et al., 2012) and this tool was also used to create new primers when the BLAST results of published primers were insufficient. The primers used for the analysis are listed in Table 2 (Sigma Aldrich). The primer efficiencies were tested by preparing dilution curves of the pooled samples and running a qPCR analysis as described below.

Quantitative real-time PCR (qPCR) was performed by using the LightCycler 480 SYBR Green I Master Kit (Life Science Roche, Cat. No. 04887352001) containing FastStart Taq DNA Polymerase. Primer specific master mixes were made by mixing SYBR Green (10 μl), primers (10 μM , 1 μl each) and nuclease free water (3 μl), multiplying the volumes with sample number and distributing 15 μl per well into 96 well qPCR plates (VWR, Cat. No. 732-1463). Diluted cDNA (1:6, 5 μl) from each sample, no-RT control from the cDNA reaction, no template control (NTC) and pooled cDNA samples of PC, MG and HG (all with the same dilution 1:6) were manually transferred into single wells in a plate and mixed by pipetting. The pooled samples were used as internal calibrators for the reference gene and gene of interest on each plate to facilitate comparisons between different plates. The plate was run in the

TABLE 2 Primers for RT-qPCR analysis of paralogous peptide transporters (PepT1a, -1b, -2a and -2b) and elongation factor 1 alpha (ELF1a).

Gene	GenBank Acc. No.	Primer sequences 5' - 3'	Amplicon (bp)	Efficiency (LinReg)	Source
PepT1a	XM_014172951	F: ATTGCACAGATCGAAGAACA	217	1.791	Own design
		R: CATCTTCATTATGATGGTCTACGAC			
PepT1b	NM_001146682	F: ACGGGCCGAGAGGAAGAGAGAT	124	1.876	Own design
		R: ACTTCTTGGTTAAGTTGCTCAACAACCGA			
PepT2a	XM_014165384	F: GGGGGACACAACAAGACCAT	198	1.891	(Del Vecchio et al., 2021)
		R: CCGCGTGTATGAACCTCA			
PepT2b	XM_014173652	F: AATGTCATTGTGCTGATCGTCGCA	225	1.892	Own design
		R: CTGGTCCATGTGGTATTCTGGTTC			
ELF1a	NM_001141909	F: CCTGTGGAAGTTGAGACTGG	173	1.903	(Irachi et al., 2020)
		R: GAGTCTGCCCGTCTTTGAG			

Lightcycler 480 qPCR instrument (Roche) with these cycling parameters: 1) Preincubation at 95°C for 10 min (1 cycle), 2a) denaturation at 95°C for 15 s, 2b) annealing at 60°C for 15 s, 2c) elongation at 72°C for 15 s (40 cycles of 2a-2c), followed by melt curve 3a) 95°C for 1 min, 3b) 65°C for 30 s, 3c) 97°C for 1 s and finally cooling at 37°C for 30 s.

The ΔC_t ($C_{t_{\text{gene}}} - C_{t_{\text{ref}}}$) values per sample were plotted by diet and intestinal segment in R (version 4.3.1) to reveal outliers, which were removed if they were deviating more than 1.5 interquartile ranges (IQR) above the 75% quartile or below the 25% quartile. After outlier removal, we had 8-15 (averagely 13.6) biological replicates per diet and intestinal segment. The NTC and no-RT controls showed C_t values ranges between 32.03-36.87 and 31.67-35.07, respectively. The choice of elongation factor 1 α (ELF1a) as reference gene was based on little variation in C_t values when testing effects of diets and different intestinal segments (Olsvik et al., 2005).

2.6 Bacterial microbiota analysis

DNA of mucosa and feces from MG and HG were extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Switzerland). For bacterial microbiota analysis, DNA samples were sequenced at BGI (China), using 16S rRNA gene amplicon sequencing.

DNA template (30 ng) and the 16S rRNA fusion primers (V3-V4 region) were added for PCR. All PCR products were purified by Agencourt AMPure XP beads, dissolved in elution buffer and eventually labeled to finish library construction. Library size and concentration were detected by Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on HiSeq platform according to their insert size.

Raw data were filtered to obtain high-quality clean data, and overlapping clean reads were merged to tags and further clustered to operational taxonomic units (OTU). Taxonomic classifications were assigned to OTU representative sequence using Ribosomal Project Database. Analysis like alpha diversity, beta diversity,

differential species analysis, network and model prediction were carried out on the OTU profile table and taxonomic annotation results. Taxonomic analysis of OTU representative sequences was carried out by RDP classifier Bayesian algorithm to identify the composition of microbial structure. Abundances of species on seven levels (Phylum, Class, Order, Family, Genus, Species) was calculated after annotation (Cole et al., 2014). In the present study, only phylum level is presented.

2.7 Statistical analysis

Statistical analysis of RT-qPCR results was performed in R Studio (version R-4.3.1) (R Core Team, 2023). Homogeneity of variance in the residuals after removing outliers was tested using Levene's test. Normal distribution of the residuals was tested by using Q-Q plot and Shapiro-Wilk's normality test. Dietary effects were tested by One-way ANOVA of ΔC_t ($C_{t_{\text{gene}}} - C_{t_{\text{ref}}}$) values for each gene and intestinal segment separately. In cases where ANOVA revealed significant difference of diet, Tukey's Honest Significant Difference (HSD) *post hoc* test was used to find where the differences were. The significance level was set to $p < 0.05$.

3 Results

3.1 Fish performance

The specific growth rate, digestibility and intestinal histology results from this specific trial have been presented elsewhere (Sandbakken et al., 2023). In summary, salmon fed the hydrolysate diets showed significantly higher specific growth rate over the first 25 feeding days, which may indicate feed stimulatory properties of the hydrolysate. The FPH-18 diet had significantly higher protein and amino acid digestibility than the other diets, and the ash digestibility increased linearly with the FPH content, indicating increased mineral uptake from the hydrolysate diets.

Histological analysis of midgut (MG) and hindgut (HG) of the FM and FPH-18 diets did not reveal any significant differences, and the intestines appeared normal and healthy.

3.2 RNA sequencing

Gene expression analysis by RNA sequencing in pyloric caeca (PC), midgut (MG) and hindgut (HG) generally revealed few differentially expressed genes (DEGs) between the salmon fed FM and FPH-18 diets (Supplementary Figure 1B). Gene ontology (GO) enrichments and KEGG (Kyoto Encyclopedia of Genes and Genomes) enriched pathways mostly grouped the downregulated genes, even though more upregulated DEGs (69-129), than downregulated DEGs (44-78) were detected in all intestinal segments of salmon fed FPH-18 compared to FM (Supplementary Figure 1B).

GO biological process enrichment revealed a downregulation of antimicrobial and antiviral immunity in PC, MG and HG of salmon fed the FPH-18 diet compared to the FM diet (Figure 1). In the PC, a

downregulation of several immune-related GO biological processes was seen (Figure 1A), like interferon stimulated gene (ISG)15-protein conjugation (GO:0032020), cellular defense response (GO:0006968), positive regulation of T cell cytokine production (GO:0002726) and positive regulation of cytokine production involved in immune response (GO:0002720), which are part of cellular responses to viral and bacterial infections. In the MG, few downregulated GO biological processes were found, and they were linked to triglyceride biosynthetic process (GO:0019432) and superoxide metabolic process (GO:0006801), which are related to lipid metabolism and prevention of oxidative damage (Figure 1B).

In the HG, a downregulation of several GO biological processes related to viral immune response was prominent in salmon fed FPH-18 compared to FM (Figure 1C). The processes ISG15-protein conjugation (GO:0032020), type I interferon-mediated signaling pathway (GO:0060337), cellular response to type I interferon (GO:0071357), viral genome replication (GO:0019079), regulation of defense response to virus (GO:0050688), positive regulation of pattern recognition receptor signaling pathway (GO:0062208),

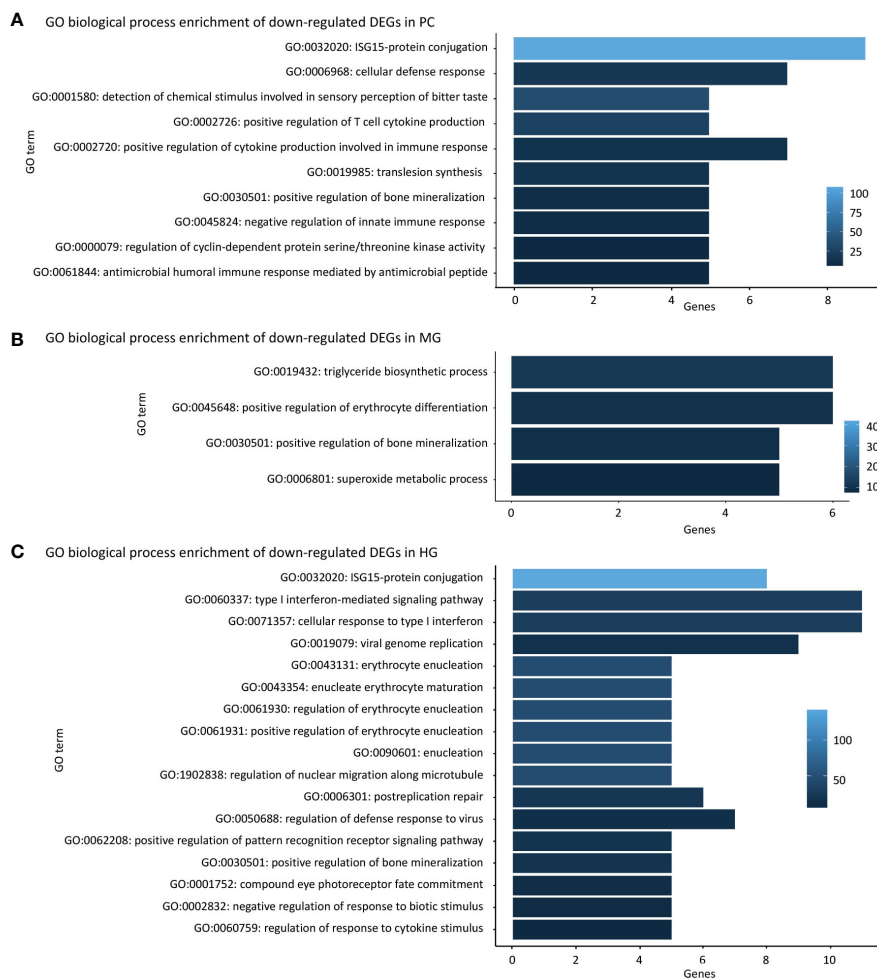


FIGURE 1

FPH-18 reduces inflammatory responses in salmon intestinal segments. Gene ontology (GO) enrichments of differentially expressed genes (DEGs) in 18% salmon protein hydrolysate diet (FPH-18) compared to the fishmeal control diet (FM). GO biological process enrichment of down-regulated DEGs in pyloric caeca (PC), midgut (MG) and hindgut (HG) are shown in (A–C) respectively. Only DEGs ≥ 5 genes are displayed, and the color intensity represents fold enrichment.

negative regulation of response to biotic stimulus (GO:0002832) and regulation of response to cytokine stimulus (GO:0060759) are related to pathogen recognition and antimicrobial immunity. This indicates that the immune system did not identify any threats and could therefore downregulate expression of immune-response genes to save energy.

GO molecular function enrichment showed that some downregulated DEGs were related to molecular binding, such as protein tag activity (GO:0031386) in the PC and HG, dynein heavy chain binding (GO:0045504), nuclear androgen receptor binding (GO:0050681) and protein self-association (GO:0043621) in HG (Figure 2). This indicates that the salmon hydrolysate diet (FPH-18) may reduce molecular interactions and protein binding compared to the FM diet.

The comparison between FPH-18 and FM fed salmon had 5 KEGG enriched pathways, where 4 were downregulated and 1 upregulated (Table 3). The downregulated pathways in the PC are related to viral, microbial and pathogen sensing and include the RIG-I-like receptor, NOD-like receptor and toll-like receptor signaling pathways as well as the cytosolic DNA sensing pathway.

3.3 Expression of specifically interesting genes

Some specifically interesting genes were picked for in depth analysis of immune response and ion transport. Heatmap of gene expression in the three intestinal segments related to immune

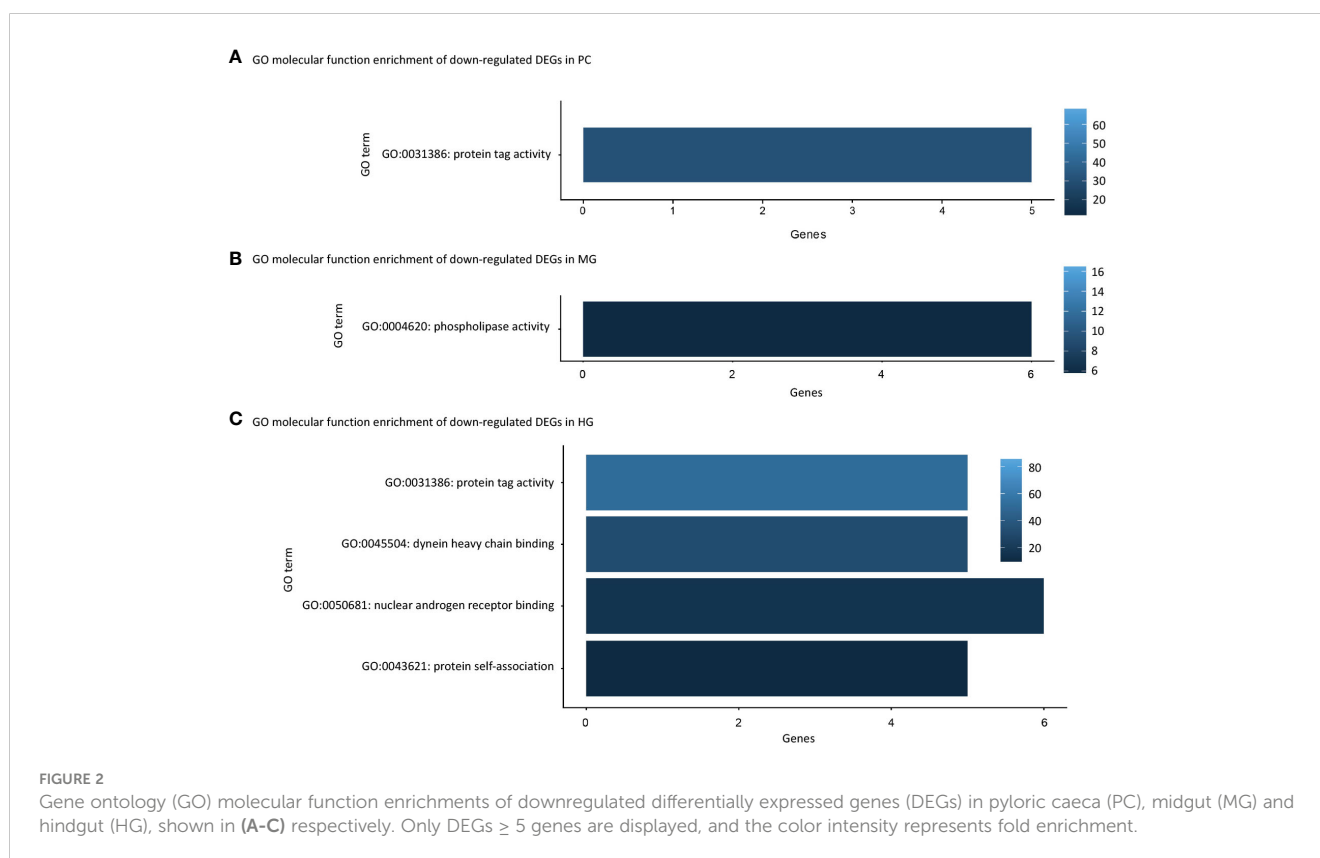
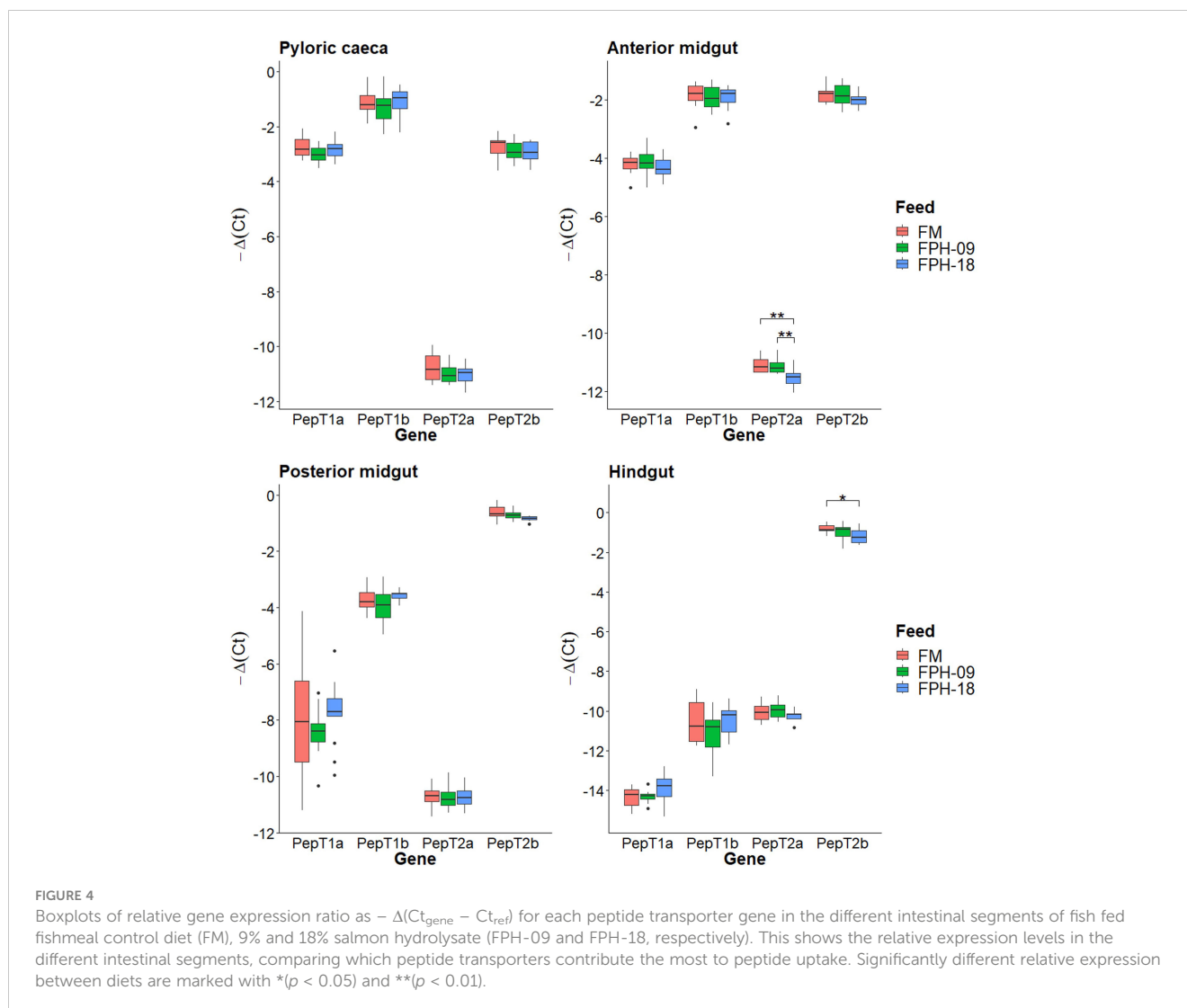


TABLE 3 Results from KEGG enrichment of RNA sequencing data of intestinal samples from salmon fed FPH-18 compared to FM fed salmon.

Enrichment	KEGG ID	Description	DEG ratio*	P value adjusted
PC up-regulated DEGs	sasa04512	ECM-receptor interaction	5/198 (2.53%)	0.01419
PC down-regulated DEGs	sasa04622	RIG-I-like receptor signaling pathway	7/167 (4.19%)	0.00064
	sasa04623	Cytosolic DNA-sensing pathway	6/126 (4.76%)	0.00070
	sasa04620	Toll-like receptor signaling pathway	6/236 (2.54%)	0.01440
	sasa04621	NOD-like receptor signaling pathway	7/380 (1.84%)	0.02543
HG down-regulated DEGs	sasa04622	RIG-I-like receptor signaling pathway	5/167 (2.99%)	0.00349

*DEG ratio, DEG number/total gene number in the signaling pathway.
Differentially expressed genes (DEG) count ≥ 5 .



3.5 Bacterial microbiota

In the present study, numerous bacterial phyla were identified. [Figure 5](#) shows the relative abundance of the predominant gut phyla levels in mucosa (A) and digesta (B) from MG and HG of salmon fed a FM or FPH-18 diet. At the phylum level, Cyanobacteria, Proteobacteria, Firmicutes, Spirochaetes and Actinobacteria were predominant in mucosa from MG and HG of salmon fed the two diets, but some dietary effects were noticed ([Figure 5A](#); [Supplementary Table 2](#)). The relative abundance of Cyanobacteria, Proteobacteria and Firmicutes in HG mucosa decreased by FPH-18 feeding, in contrast to the relative abundance of Spirochaetes which increased. In the MG, the relative abundance of Cyanobacteria and Spirochaetes increased in the mucosa of salmon fed FPH-18. The relative abundance of Actinobacteria decreased while Firmicutes were unaffected by dietary treatment. The only dietary effect seen in mucosa was the increase in Spirochaetes in the MG and especially the HG of salmon fed FPH-18. Preliminary results on genus level, revealed a high abundance of *Brevinema*, and especially *Brevinema andersonii* on a species level, in mucosa of fed fish FPH-18 in contrast to the FM fed fish.

In the digesta, the relative abundance of phyla was predominated by Cyanobacteria, Proteobacteria, Firmicutes, Spirochaetes, Actinobacteria and Bacteroides ([Figure 5B](#); [Supplementary Table 3](#)). The highest relative abundance of Cyanobacteria (43.4%) was noticed in HG digesta from fish fed the FPH-18 diet, and Proteobacteria (44.4%) in MG digesta from fish fed FM. Firmicutes abundance was relatively stable (values between 9.7–15.3%) in digesta samples, while the abundance of Spirochaetes (19.6%) was highest in the digesta from fish fed the FPH-18 diet. The only clear dietary effect in digesta composition was the higher abundance of Spirochaetes in MG of FPH-18 fed salmon.

4 Discussion

4.1 Gene expression by RNA sequencing

The results from GO and KEGG clearly shows that replacing fishmeal with salmon hydrolysate reduced the inflammatory load as viewed by the reduction of pro-inflammatory markers in the

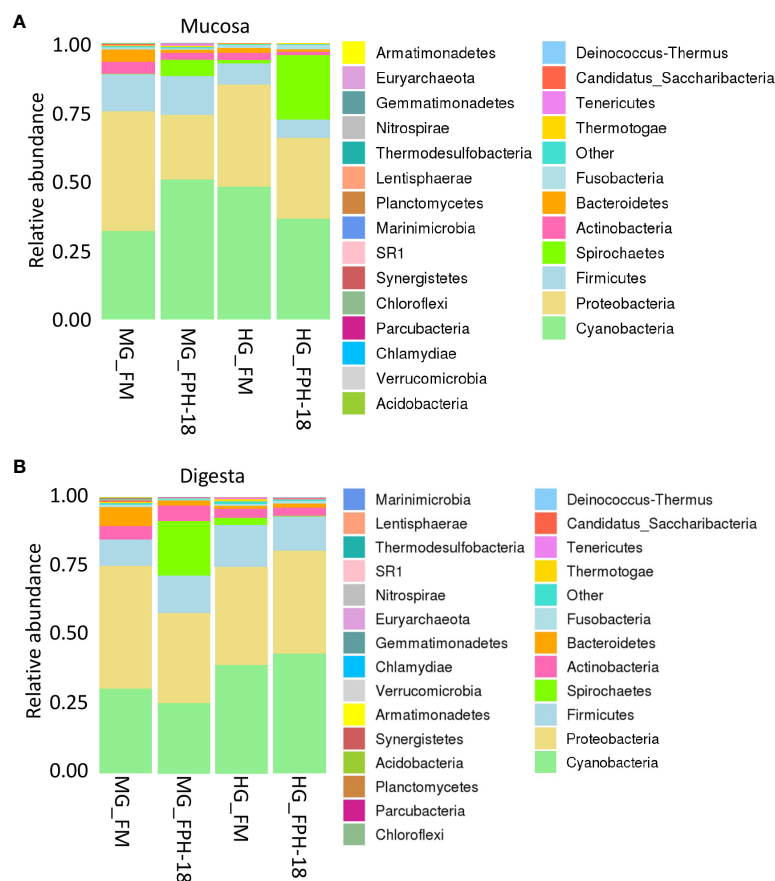


FIGURE 5

The composition of bacterial gut microbiota at phylum level in mucosa (A) and digesta (B) from midgut (MG) and hindgut (HG) of salmon fed the fishmeal (FM) diets or 18% salmon hydrolysate (FPH-18) diet ($n = 6$).

intestinal tissue, particularly towards pathogens like viruses. Along with the downregulation of RIG-I-like receptors, NOD-like receptors and toll-like receptors, which are intracellular pattern recognition receptors involved in the recognition of pathogen-associated molecular patterns (PAMPs) by the innate immune system (Barton and Medzhitov, 2003; Kanneganti et al., 2007; Loo and Gale, 2011). It therefore appears likely that the immune system does not sense immediate threats when salmon hydrolysate is partially replaced by FM in the diet. This could suggest that there are components in FM that cause a general upregulation of inflammatory and immune pathways, and that reduction of these would reduce the need to mount a response. Downregulation of genes associated with the immune system has also been seen in the liver of Atlantic salmon that were starved for 28 days. It was also interesting to note that when these fish were challenged with a pathogen, they showed a higher upregulation of immune genes than the non-starved fish (Martin et al., 2010), thus having a potentially better response to a real threat. Downregulation of the immune-related genes is beneficial for saving energy and may still be activated as necessary upon pathogenic threats. It can be speculated that the improved growth response during early phases of the feeding trial these fishes were analyzed from (Sandbakken et al., 2023) can be related to reduced need for energy to mount a continuous low-level inflammatory response.

Another possibility for the downregulation of immune parameters, may lie in a direct effect of protein hydrolysates containing potentially bioactive peptides. Some hydrolysates have been shown to have immunomodulatory functions, which may activate or downregulate the immune system, depending on the hydrolysate source and composition (Kiewiet et al., 2018). Bioactive peptides have been found in salmon protein hydrolysates made by enzymatic hydrolysis of salmon rest raw materials (Opheim et al., 2015). A peptide fraction from salmon hydrolysate showed anti-inflammatory activity *in vitro* by reducing the expression level of pro-inflammatory interleukins in macrophage cells upon lipopolysaccharide (LPS) stimulation (Ahn et al., 2012). Feeding 5% bioactive peptides from farmed Atlantic salmon to European sea bass also downregulated inflammatory responses in the intestine, but the anti-inflammatory effect was not seen at a 10% dietary inclusion level (Parma et al., 2023). Protein hydrolysates from shrimp and tilapia fed to European sea bass also showed a downregulation of interferon-related genes (Leduc et al., 2018) as seen in this study with salmon hydrolysate. Interferons are signaling molecules produced by immune cells during viral infections, and a down-regulation of these indicate a reduction in viral threats (Ellis, 2001). Furthermore, Leduc et al. (2018) detected positive effects on immune responses and gut health by including 5%

hydrolysate from shrimp, tilapia, or a mix of those to a 5% FM diet, and the European sea bass showed similar growth performance as the positive control fed a 20% FM diet. This indicates that also low inclusion levels of hydrolysates can have beneficial effects in restoring immune function and gut health of fish fed low FM diets, without compromising the growth. In this trial, we only compared the FPH-18 diet with the FM control which had positive effects on growth, digestibility, and gut health (Sandbakken et al., 2023). However, it would be interesting to explore a possible dose-response relationship of salmon hydrolysate to find the optimal inclusion level for maintaining a functional immune system and a good general gut health.

Peptides can also exert immunomodulatory effects by directly stimulating receptors, or by interfering with inflammatory signaling pathways after cellular uptake via a peptide transporter or via endocytosis (Kiewiet et al., 2018). There is also an interesting connection to PepT1 where studies have shown that PepT1 is upregulated in the colon during intestinal inflammation in mammals, and that specific soy peptides transported through PepT1 reduce the inflammatory response *in vitro*. After transport of peptides through PepT1 to the cytosol, some bioactive peptides resist degradation by peptidases and may inhibit the main inflammatory signaling pathways in human intestinal Caco2 cells (Dalmasso et al., 2008; Kovacs-Nolan et al., 2012; Kiewiet et al., 2018).

Some marine peptides have also shown antimicrobial effects (Kang et al., 2019), which may affect the mucosal microbiota. Thus, the immunomodulatory effects of hydrolysates may be due to a direct effect by bioactive peptides, an indirect effect through modulation of the bacterial microbiota or a combination of these.

4.2 Intestinal expression of peptide transporter genes

Based on previous literature from vertebrates (Chen et al., 2005; Gilbert et al., 2008; Bakke et al., 2010), our initial hypothesis was that high dietary peptide content would increase expression of intestinal peptide transporter (PepT) in the anterior part of the intestine, and perhaps reduce the expression in posterior regions since these components are readily absorbed in the anterior part. However, the only significant differences were a slight reduction of PepT2a in the anterior midgut and PepT2b in the hindgut (HG) of FPH-18 fed fish. The difference in the HG could be explained by rapid absorption of the hydrolysate peptides and free amino acids in the proximal part of the intestine, while the FM proteins had to be enzymatically degraded before absorption. The small differences observed may be due to the same protein content in all experimental diets and efficient protein degradation by endogenous enzymes in the digestive tract of Atlantic salmon.

In this trial, the fish were not fed for 48 hours prior to collection of RNA samples. Luminal content can affect PepT expression, but 4 days of fasting had minimal effect on expression of PepT paralogs in intestinal segments of Atlantic salmon (Del Vecchio et al., 2021).

The relative expression of PepT1b was highest in the anterior part of the intestine, followed by a decreasing expression level

further down the gastrointestinal tract as previously observed in zebrafish (Verri et al., 2003), Atlantic cod (Rønnestad et al., 2007), European sea bass (Terova et al., 2009) and Atlantic salmon (Gomes et al., 2020). PepT1b had about 4 times (2^{-2}) higher expression levels than PepT1a in PC and AMG, which is in line with what was reported by (Gomes et al., 2020). The relative expression of PepT1b and PepT2b was similar in the anterior midgut (AMG), while PepT2b had the highest mRNA concentration of all paralogs in posterior midgut (PMG) and HG. These results are comparable to previous data on Atlantic salmon (Del Vecchio et al., 2021). Expression of PepT2a was generally negligibly low in all intestinal segments, and 500-1000x lower than PepT2b in PMG and HG, as previously reported (Del Vecchio et al., 2021; Vacca et al., 2022).

In this trial, intestinal expression of PepT paralogs were comparable to previous data in Atlantic salmon, and minimal effects of increased level of dietary peptide were observed. PepT1b showed highest expression levels in the anterior part of the intestine, while PepT2b was most highly expressed in the posterior part of the intestine, thus they both contribute to intestinal peptide absorption in different parts of the gastrointestinal (GI) tract.

4.3 Bacterial microbiota

The present study revealed dietary effect on phyla level of the gut microbiota, an effect well known in fish (Cahill, 1990; Ringø et al., 1995; Egerton et al., 2018; 2020; Kokou et al., 2022; Luan et al., 2023). In addition, the bacterial microbiota in the digesta differed to some extent from the mucosal microbiota (Ringø et al., 2016; Li et al., 2021; Nyholm et al., 2022). The present study investigated the mucosal gut microbiota, but most fish studies have focused only on the digesta microbiota. Since the mucosal microbiota might influence fish health, more focus should be directed towards the mucosal community and furthermore on the importance of the gut microbiota on lipid-, carbohydrate-, protein- and amino acid metabolism of aquatic animals (Ringø et al., 2022).

Different segments of the teleost GI tract must be investigated separately, as the colonization patterns between beneficial bacteria and pathogens may vary along the intestine (Ringø et al., 2003). The difference in colonization of mucosal phyla between MG and HG of Atlantic salmon fed the two diets in the present study was revealed, and the findings are in accordance with the results described in previous studies evaluating the gut microbiota (Ringø et al., 1995; Gajardo et al., 2016). As evaluation of gut microbiota is relatively new, it is important to analyze the different GI tract segments to fully see the relationship between these communities. Furthermore, outcompeting beneficial intestinal microorganisms may lead to dysbiosis and increased gut permeability (Zhang et al., 2020).

The gut microbiota of marine fish of economic interest for aquaculture are being dominated by the phyla Proteobacteria (Egerton et al., 2018) and the results of the present study agrees with the above mentioned studies. Regarding Cyanobacteria, the phyla is seldomly reported as a part of the fish gut microbiota and not at the relative abundance level as revealed in the present study. The reason for this observation is not known, and merits further investigations.

Spirochaetes, Gram negative bacteria with long corkscrew or spiraled morphology are reported to be a part of the intestinal microbiota in several fish species (Egerton et al., 2020; Iwatsuki et al., 2021; Li et al., 2021). A dietary effect was noticed on the relative high abundance of Spirochaetes in mucosa of MG (6%) and HG (23%) of FPH-18 fed Atlantic salmon, while Egerton et al. (2020) revealed significantly lower abundance of intestinal Spirochaetes in Atlantic salmon fed 80% plant protein. The abundance of Spirochaetes in the mucosa of FPH-18 fed salmon were however considerably lower than the 50 and 70% Spirochaetes found in the hindgut mucosa of Atlantic salmon fed a commercial and insect meal diet, respectively (Li et al., 2021). The Spirochaetes detected in this trial were dominated by the genera *Brevinema*, more specifically the species *Brevinema andersonii*, but the prevalence varied between the individual fish. Among the Phyla Spirochaetes, the species *B. andersonii* has been associated with altered expression of pro- and anti-inflammatory genes, and *Spirochaetaceae* with genes related to intestinal barrier function, when found in the hindgut mucosa (Li et al., 2021). High abundance of Spirochaetes including *B. andersonii* has also been detected in the hindgut mucosa of Atlantic salmon fed a prebiotic alginate oligosaccharide derived from brown macroalgae (Gupta et al., 2019). The Spirochaete *B. andersonii* has genes associated with butyrate production, which is favorable for the gut health, since butyrate is one of the main short chain fatty acids (SCFAs) that provide energy for enterocytes and maintain mucosal integrity and immune homeostasis (O'Keefe, 2016). Based on our results, the downregulation of immune responses seen in the present study could be linked to the mucosa associated bacteria, especially Spirochaetes, and their fermentation products. However, as the interactions between immune system and intestinal bacteria are more investigated in mammals than in fish (Maynard et al., 2012; Daniel et al., 2021; Schlechte et al., 2022), so this topic merits further research. It is important to increase the knowledge on how dietary composition influences the immunity and health of farmed aquaculture species.

5 Conclusion

The first paper from this trial (Sandbakken et al., 2023) clearly showed that salmon protein hydrolysate can partially replace fishmeal in diets for Atlantic salmon giving good growth, increased digestibility of protein and ash and good intestinal health as viewed by histology. Intestinal gene expression analysis of the current manuscript showed a downregulation of several inflammatory pathways particularly towards virus and to some extent bacteria when salmon post-smolts were fed 18% salmon hydrolysate compared to a FM control diet. This could reduce energy expenditure by the immune system and free energy available for improved growth. The cause for this downregulation is not known at present but is being studied further. On one hand, it is possible that FM contain molecules that activates the immune system. On the other hand, the salmon hydrolysate with its high load of potentially bioactive peptides may have modulated the immune response through several possible pathways. It is also possible that bacteria selected by salmon hydrolysate could have contributed to

reduce the presence of potential pathogens. The gut microbiota in the digesta and mucosa of the MG and HG, were dominated by the phyla Cyanobacteria and Proteobacteria in both diets. However, the phyla Spirochaetes were more prevalent in the mucosa of FPH-18 fed salmon, compared to the FM fed salmon. This dietary difference on mucosal microbiota and the microbial fermentation products may have influenced the inflammatory responses as seen in the gene expression analysis. Further research is needed to fully understand the relationship between fish nutrition, intestinal microbiome, and the immune system. Furthermore, it is well known that dietary manipulation modulates the intestinal microbiota which may improve the resistance towards bacterial infection, this topic must be investigated in future studies when evaluating the effect of salmon protein hydrolysate.

Peptide transporter gene expression was minimally affected by different dietary peptide content, and mostly varied between the intestinal segments. PepT1b contributed most to peptide uptake in the PC, while in AMG PepT1b and PepT2b showed similar relative expression levels. From PMG to HG, PepT2b showed the highest relative expression levels, indicating that PepT2b contributed the most to peptide uptake in the posterior part of the gastrointestinal tract.

Data availability statement

The raw data has been uploaded to NCBI SRA database with BioProject No. PRJNA1072556.

Ethics statement

The animal study was approved by Norwegian Food Safety Authority (FOTS ID:23021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

IS: Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. HS: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. LJ: Writing – review & editing, Data curation. YZ: Writing – review & editing, Visualization, Formal analysis, Data curation. ER: Writing – original draft. RR: Writing – review & editing, Supervision, Data curation. IY: Writing – review & editing, Formal analysis, Data curation. KF: Writing – review & editing, Data curation. RO: Writing – original draft, Supervision, Conceptualization.

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Conflict of interest

Authors IS and KF were employed by the company Nutrimar AS.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2024.1376516/full#supplementary-material>

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