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Low intestinal inflammation model (HP48) in Atlantic salmon (*Salmo salar*) and inflammatory mitigation by Bactocell

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ABSTRACT

Moderate levels of intestinal damage and inflammation are often seen in intensive fish aquaculture. The causes may be due to antinutrients from plant meals, stress or other causes. There is currently a lack of good models to explore these effects and so how to mitigate the consequences. Most studies have used full-fat soy or other compounds that cause intestinal damage that are likely not reversible. In this study we have explored the possibility of using soybean HP48, made from solvent extracted peeled soybeans, as a low-inflammation model in post-smolt Atlantic salmon, and then investigated whether supplementation of the probiotic *Pediococcus acidilactici* CNCM I-4622 – MA 18/5 M (Bactocell) could diminish this effect. The fish were fed triplicate diets. A Control diet containing 18.08% soy protein concentrate (SPC), a HP48 diet where most of the SPC was replaced by HP48 (5.00% SPC and 17.68% HP48), and a Bactocell diet that was identical to the HP48 diet but contained 0.03% Bactocell. After 10 weeks of feeding, the mid- and hind-intestinal health were assessed by histology, integrity (Ussing chamber) and gene expression (RNAseq). Transcriptomic and integrity data suggests that HP48 led to a disturbed mid-intestinal homeostasis with impaired cellular integrity and increased inflammation and cell turnover. Most of the transcriptomic effects were reversed with Bactocell including downregulation of immune genes and upregulation of transmembrane proteins such as type IV collagen, which is important in restoring epithelial homeostasis. In the hind-intestine, the HP48 diet led to deleterious morphological changes such as widening of lamina propria and stratum granulosum, disrupted mucosal folds, loss of absorptive vacuoles, and upregulation of several immune regulated genes and downregulation of genes involved in solute- and water transport. The intestinal integrity assessed by Ussing chamber was not affected. Bactocell supplementation did alleviate several of the morphological effects. However, it was not able to completely reverse the expression of immune- or transport related genes, suggesting a higher effect of probiotic supplement in the mid-intestine compared to the hind-intestine. This study demonstrates that the level of HP48 used here is sufficient to create low-level intestinal changes in Atlantic salmon, which is within range for functional feed ingredients to reverse.

1. Introduction

The production volume of farmed Atlantic salmon (*Salmo salar*) is continuously growing and is projected to increase. This extensive growth has led to a change in fish feed composition from being primarily based on marine-derived proteins to including higher quantities of terrestrial protein resources, such as soybean meal (SBM) and rapeseed meal (RSM) (Aas et al., 2019; Jobling, 2016). Soybean is an attractive protein source for substituting fishmeal (FM) due to its high protein content and availability, low cost, and a relatively well-balanced amino

Abbreviations: SBM, soybean meal; HP48, 48% high protein soy; SPC, soy protein concentrate; SGR, specific growth rate; TGC, thermal growth coefficient; TER, transepithelial electrical resistance; TEP, transepithelial potential; SCC, short circuit current/ion flux; P_{app}, apparent paracellular permeability.

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acid profile (Chakraborty et al., 2019). However, antinutritional factors present in soybean, such as trypsin inhibitor(s), saponins, lectins and oligosaccharides, have detrimental effects on the hind-intestinal epithelium of several carnivorous fish species including Atlantic salmon, chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) (Booman et al., 2018; Knudsen et al., 2007; Seibel et al., 2022). The morphological consequences have been widely studied and include increased permeability, loss of the normal supranuclear vacuolisation and widening of lamina propria and submucosa with infiltration of immune cells (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007; Knudsen et al., 2007). This SBM induced enteritis triggers transcriptional activation of several immune-related genes in the distal intestine, such as *CD99 antigen* (Sahlmann et al., 2013), *annexin A2-like*, (Kiron et al., 2020; Sahlmann et al., 2013) and *galectin-3* (Kiron et al., 2020), in addition to downregulation of cytochrome P-450s and other drug-metabolising enzymes and transporters (Morgan, 2009). Furthermore, studies have shown altered expression in genes related to transport mechanisms such as aquaporins (Hu et al., 2016), chloride channel proteins, solute carrier families and sodium-associated transporters (Kiron et al., 2020; Hu et al., 2016).

The intestinal epithelial barrier of fish is a semipermeable barrier that is not only important in absorbing nutrients, water and salts, but also serves as a protective layer to the surrounding external environment (Jutfelt, 2011; Sundh and Sundell, 2015). These challenging tasks are regulated by transcellular transport through the enterocytes (selective transporters and transporting proteins) and a paracellular pathway controlled by junctional complexes between the enterocytes (Sundh and Sundell, 2015). As the aqueous environment is a thriving habitat for microorganisms, fish are extremely vulnerable to changes in the internal and external environment that can challenge the intestinal epithelial integrity. In Atlantic salmon, factors such as dietary soya saponins, acute and chronic stress have been shown to increase the intestinal permeability (Knudsen et al., 2008; Sundh et al., 2019; Olsen et al., 2005). Such barrier dysfunction can lead to an increased inflow of foreign antigens and other potentially harmful substances, resulting in activation of the mucosal immune system, release of pro-inflammatory cytokines and eventually intestinal inflammation (Knudsen et al., 2008). Thus, the intestinal health status could be one of the defining factors in maintaining fish health and welfare.

Several functional feed supplements have the potential to improve salmon's intestinal health. One such group is probiotics (Tacchi et al., 2011). Probiotics are defined by the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Probiotics have shown positive effects on survival, health parameters, growth and gut microbiota in fish species such as sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), rainbow trout, tilapia (*Oreochromis niloticus*) and Atlantic salmon (Carnevali et al., 2004; Carnevali et al., 2006; Jaramillo-Torres et al., 2019; Panigrahi et al., 2004; Pirarat et al., 2006). One strain of probiotic is the single-strain, homo-fermentative lactic acid bacteria *Pediococcus acidilactici* CNCM I-4622 – MA 18/5 M (Bactocell, Lallemand Animal Nutrition, Blagnac, France), that has shown to limit the prevalence of vertebral column compression syndrome, improve intestinal microvilli morphology, and to modulate gut microbiota and the resulting immune response in rainbow trout (Al-Hisnawi et al., 2019; Aubin et al., 2005; Merrifield et al., 2010; Rasmussen et al., 2022). Bactocell also had positive effects on gut histomorphometry, immune parameters and performance parameters of European seabass, as well as improved the disease resistance of rainbow trout against *Yersinia ruckeri* infection (Eissa et al., 2022; Othani et al., 2020).

There have been many studies on the potential effect of probiotics and other compounds to modulate the pro-inflammatory effect of soybean. However, most trials have thus far used relatively large amounts of full fat and solvent extracted SBM which probably cause intestinal enteritis that is too severe to be mitigated by functional ingredients. Here we will use an alternative approach. High protein (HP) 48 is a product usually made from solvent-extracted peeled soybeans (low fibre) and it contains lower amount of antinutritional factors compared to SBM. Although it does lead to some enteritis in fish, the effect is much lower than that of full fat soy and is therefore widely used in many fish species. The study aims were twofold: a) to create a low-level inflammatory model for salmon using a diet supplemented with 48% of high protein soya containing a lower amount of antinutritional factors compared to the SBM diet; and b) to investigate whether inclusion of Bactocell can diminish this inflammatory response.

2. Material and methods

2.1. Experimental design

Atlantic salmon (*Salmo salar*) post-smolts averaging 80-90 g were transported from Lerøy Midt AS hatchery (Lensvik, Norway) to NTNU SeaLab (Department of Biology, Trondheim, Norway) on 15th of August 2016. The fish were fully vaccinated with AJ Micro 6 (Pharmaq, Overhalla, Norway). Upon arrival, the fish were distributed into 9 × 400 L tanks with flow-through seawater at a 24:0 h light:dark cycle and continuously fed the commercial diet previously fed in the hatchery. After approximately three weeks of acclimatisation, 270 fish were bulk weighted in pairs of 5 fish (106.8 ± 5.8 g) and randomly distributed into 9 × 400 L tanks (30 fish per tank; flow-through indoor system using natural seawater). The fish were allowed to adapt to the tanks for three days before being fed the experimental diets.

Triplicate tanks were randomly assigned to three feeding groups: the control diet (Control) and two experimental diets (HP48 and HP48 + Bactocell). The Control diet contained 18.08% soy protein concentrate (SPC, heated, alcohol extracted soybean meal) (Table 1). For the HP48 diets, 17.6% HP48 and only 5% SPC were added in order to induce a low level of intestinal inflammation. The Bactocell diet was identical to the HP48 diet, but was supplemented with 0.03% Bactocell. The level of Bactocell was chosen according to manufacturer's recommendation. Bactocell was provided by Lallemand Animal Nutrition, Blagnac, France and the expected and measured count of the probiotic bacteria in the feed before the onset of the trial was 3.00×10^6 and 5.23×10^5 CFU/g, respectively. The diets were made at BioMar Tech Center, Brande, Denmark. Diet gross chemical content is given in Table 2. The fish were fed to excess (20% feed spill), with feeding evenly distributed over 24 h by automatic feeders (Arvo-Tec T Drum 2000, Arvo-Tex Oy, Huutokoski, Finland). The seawater was kept at above 80–85% O_2 saturation, and the temperature, salinity and pH followed the natural variation of the sea (temperature: 11.4 ± 0.9 °C; salinity: 32–34 ‰; pH: 8.0–8.1). This trial

Table 1
Feed formulation (% of dry weight) of the three experimental diets Control, HP48 and Bactocell.

	Control	HP48	Bactocell
Fish Meal LT	12.45	14.16	14.14
Fish Meal SA Superprime	12.50	11.84	11.86
Soy HP48[a]		17.68	17.64
Soy SPC	18.05	5.00	5.00
Wheat gluten	7.70	14.10	14.10
Maize gluten	5.00		
Pea protein	10.00	5.00	5.00
Wheat	12.50	11.20	11.20
Fish oil, 18 EPA + DHA	9.71	9.82	9.82
Rapeseed oil, crude	9.44	9.91	9.91
Vitamin and minerals	2.14	2.00	2.00
AA-mix	0.82	0.97	0.97
Bactocell PA10			0.03
Yttrium	0.05	0.05	0.05
Lucantin Pink CWD 10%	0.05	0.05	0.05
Water change	−0.40	−1.76	−1.76
Total	100	100	100

[a] HP48; Protein 48.3%, Fat 2.4%, Crude fibre 3.9%, Water 11.8%, Ash 6.5%.

Table 2
Optimised diet composition (g/kg) of the three diets Control, HP48 and Bactocell.

	Control	HP48	Bactocell
Moisture (%)	7.2	6.0	6.0
Energy – crude (MJ/kg)	23.1	23.4	23.4
Protein – crude (%)	19.2	19.2	19.2
Protein – DP salmon (%)	47.6	46.6	46.6
Fat – crude (%)	41.7	41.6	41.6
Ash (%)	23.8	24.2	24.2
Phosphorus – total (%)	7.5	7.7	7.8
Starch – crude (%)	1.2	1.2	1.2
NFE (%)	9.6	8.0	8.0
LA (C18:2 n-6)	201.4	201.4	201.4
ARA (C20:4 n-6)	21.4	23.7	23.7
EPA (C20:5 n-3)	0.2	0.2	0.2
DHA (C22:6 n-3)	10.4	10.6	10.6
EPA + DHA in feed (g/kg)	9.5	9.6	9.6
PUFA	19.9	20.1	20.1
Saturated FA	56.7	59.9	59.9

was approved by the Norwegian Experimental Animal committee (FOTS ID: 9067).

2.2. Sampling

Mean body weight was determined at day 0 (T_0); day 34–35 (T_1) and day 57 (T_2) based on total tank biomass measured in bulk of 5 fish. To do so, fish were sedated (60 mg L^{-1} tricainmesylat; Finquel.vet®, Scanvacc, Hvam, Norway) and then allowed to recover before being returned to their original housing. On day 57, 5 fish from each experimental tank were euthanised with an anesthetic overdose (100 mg L^{-1} tricainmesylat; Finquel.vet®) and the abdomen opened longitudinally. The mid-intestine (from the last pyloric caeca to the ileorectal valve) and the hind-intestine (from the ileorectal valve to the rectum), were then collected for Ussing chamber assays. Remaining fish were fed for a week and then fasted for 24 h and mid- and hind-intestine were sampled for analysis of histology and RNA sequencing. Samples for histology were fixed and stored in phosphate buffered formaldehyde (PBF) at 4 °C for 48 h until dehydration and embedded in paraffin wax using a tissue processor (TP1020, Leica Biosystems, Germany). Intestinal samples for RNA sequencing were stored in 2 ml Eppendorf tubes containing RNA-later® (Life technologies, Oslo, Norway) at 4 °C for 24 h before storage at −20 °C for further analysis.

2.3. Growth

Specific growth rate (SGR) was calculated based on the mean weight from each tank using eq. (1), where W_1 and W_2 are start and end weight (g), respectively, and t is the number of feeding days. Thermal growth coefficient (TGC) was calculated using eq. (2). SGR and TGC were calculated for the periods start to midpoint (day 0–34/35, SGR_1 and TGC_1), midpoint to end of trial (day 34/35–57, SGR_2 and TGC_2) and for the whole experimental period (day 0–57, SGR_{tot} and TGC_{tot}).

$$SGR = ((lnW_2 - lnW_1) * 100)/t \quad (1)$$

$$TGC = ((W_2^{1/3}\text{-}W_1^{1/3})/\text{sum day-degrees}) * 100 \quad (2)$$

2.4. Morphological evaluation of the intestine

For each diet, 6 individuals (2/tank) were assessed for histological evaluation. The embedded intestines were sectioned longitudinally with 4 μm thickness using a microtome (RM2255, Leica biosystems, Germany), and stained with Alcian Blue-PAS (ABPAS). The stained sections were scanned at 40× magnification using a digital slide scanner (NanoZoomer, Hamamatsu Photonics, Japan). Measurements were made using scanner software (NDP, Hamamatsu Photonics, Japan). For the mid-intestine, lamina propria width (μm), enterocyte height (μm), stratum granulosum height (μm) and mucosal fold height (μm) were measured in 6–10 randomly selected primary folds for each preparation. For the hind-intestine, lamina propria width (μm), enterocyte height (μm), stratum granulosum height (μm) were measured in 6–10 randomly selected folds for each preparation. Within each of these folds, mid area was marked, and the number of goblet cells were counted within this area to obtain the number of goblet cells per 0.1 mm^2. Hind-intestinal areas were also subjected to semi-quantitative scoring (modified from Knudsen et al., 2008; Løvmo et al., 2022) of enterocyte supranuclear vacuolisation and shape of the mucosal fold. These were given a severity score of 1, healthy; 2, mild; 3, moderate; 4, severe or 5, acute alterations. For vacuolisation, score 1 was given to folds showing large, supranuclear vacuoles while score 5 represents an absence of vacuolisation. For mucosal folds, score 1 was given when the fold was long and thin and score 5 to folds showing severe disruption with fused folds (Fig. 1). All scoring was done at the same magnification.

2.5. Intestinal barrier and transport function

Barrier and transport functions were assessed using the Ussing chamber system (Scientific Instruments, Simmerath, Germany) according to Sundell et al. (2003) with modifications described by Sundell and Sundh (2012). Intestines from 8 individuals per diet (distribution of 2, 2 and 4 from replicate tanks), were quickly dissected out, cleaned, opened longitudinally, and rinsed in ice-cold Ringer (saturated with air containing 0.3% CO_2) modified for seawater acclimated salmonids. Mid- and hind-intestine were separated, fatty tissue and serosa were gently peeled off under a stereo microscope. The sections were mounted onto Ussing chambers and 3 ml ringer solution was added to both the serosal and the mucosal sides of the chambers. After 60 min acclimatisation, the trial started by replacing the Ringer on the mucosal side with 3 ml Ringer containing 0.05 MBq ^{14}C-Mannitol (PerkinElmer, MA, USA) and the Ringer on the serosal side with fresh Ringer. Samples (100 μl) were collected from the serosal side after 20, 25, 30, 60, 80, 85 and 90 min, and at 20 and 90 min from the mucosal side. The samples were transferred to 20 ml counting vials, 10 ml Opti-Fluor (PerkinElmer, MA, USA) was added and then samples were counted (dmp) using a Packard TRI-CARB 2300 TR Liquid Scintillation Analyzer using a ^{14}C protocol. Apparent permeability (Papp) for ^{14}C-Mannitol was calculated using eq. (3). Transepithelial electrical resistance (TER) and transepithelial potential (TEP) were measured every fifth min. Data are given as mean values from the last 30 min of each incubation. The short circuit current (SCC) was calculated from the TER and TEP values (SCC = -TEP/TER) and represents the electrical current needed to short-circuit the epithelium and is equivalent to the sum of ion movement across the epithelium.

$$Papp = dQ/dt * 1/AC_0 \quad (3)$$

dQ/dt is the quantity of ^{14}C-Mannitol on the serosal side as a function of time (mol/s), C_0 the initial concentration of ^{14}C-Mannitol on the mucosal side (mol/ml), and A the surface area of exposed intestinal tissue in the chamber (0.80 cm^2).

2.6. Gene expression by mRNA sequencing

Total RNA was extracted from the mid- and hind-intestine ($n = 3$ or 4 per diet, a total of 23 samples). mRNA extraction was done using a RNeasy Plus Universal Mini kit (Qiagen, Hilden, Germany), including DNase treatment according to the associated protocol. RNA concentrations were measured using a Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and RNA quality was assessed using a Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). All 23 samples passed the quality check with an RNA integrity number (RIN) higher than 7. cDNA libraries were prepared using TruSeq Stranded mRNA prep HS protocol (Illumina, California, USA) according to instructions, with selection for 500 bp fragments. cDNA library was sent to Genomics Core Facility at the Norwegian University of Science and Technology (NTNU)

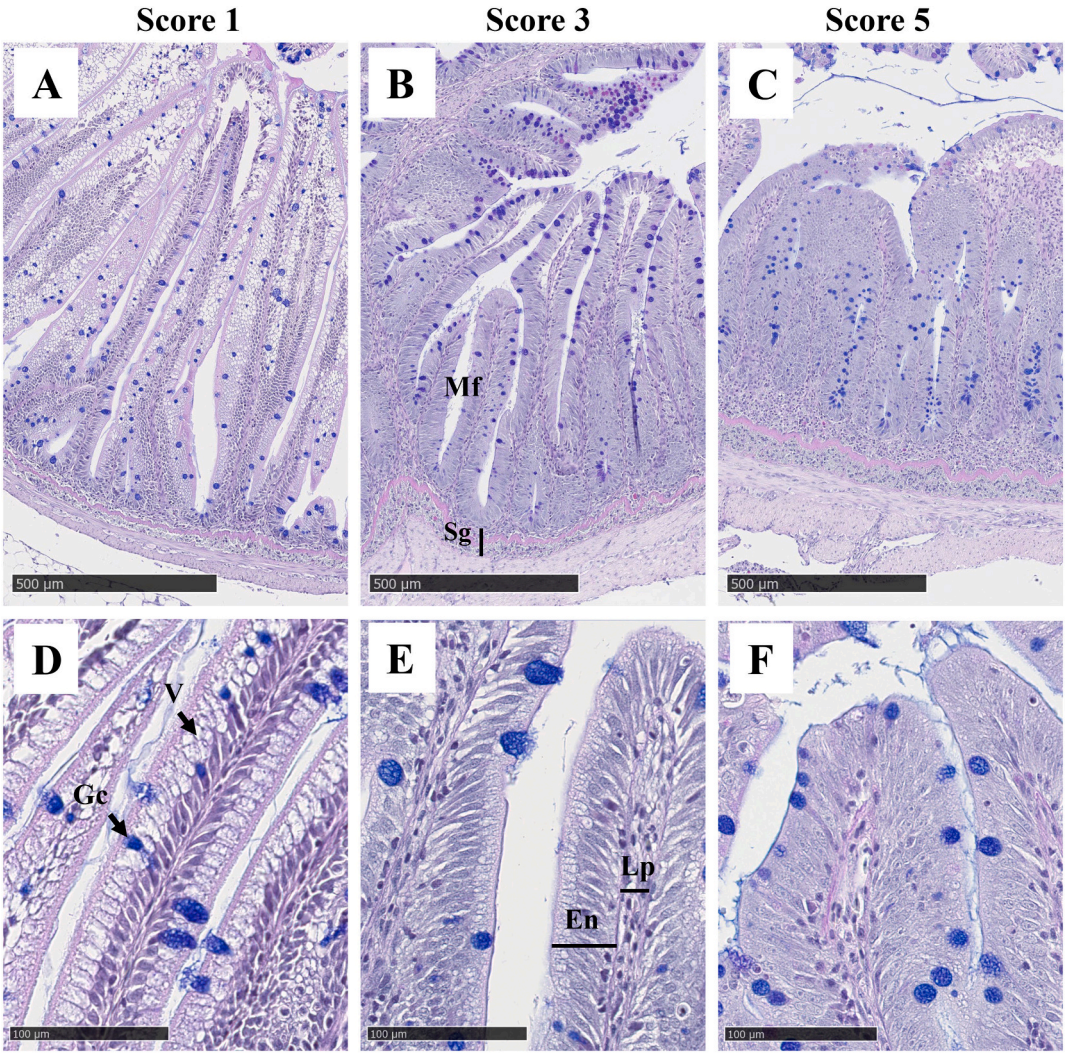

Fig. 1. Representative images of score 1, 3 and 5 of mucosal fold shape (A–C) (scale: 500 µm) and vacuolisation (D–F) (scale: 100 µm) in the hind-intestine. Score 1 of mucosal folds represent long and thin folds (A), whereas score 5 represent disrupted and fused folds (C). Score 1 of vacuolisation represent large supranuclear vacuoles (D), whereas score 5 represent absence of vacuoles (F). Stratum granulosum (Sg) and mucosal fold (Mf) is illustrated in B, vacuole (V) and goblet cell (Gc) in D, and enterocyte (En) and lamina propria (Lp) in E.

for sequencing. Libraries were quantified by qPCR using the KAPA SYBR FAST library quantification kit for Illumina Genome Analyzer (KAPA Biosystems, Woburn, MA, USA). A 2.5 nM solution of the sequencing library pool was subjected to cluster generation on a HiSeq4000 flow-cells by the cBot instrument (Illumina, Inc., San Diego, CA, USA). Double-end sequencing was performed for 2 × 75 cycles on a HiSeq4000 instrument (Illumina, Inc. San Diego, CA, USA), according to the manufacturer's instructions, producing 76 bp double-end reads. Base-calling was done on the HiSeq4000 instrument by RTA 2.7.7. FASTQ files were generated using bcl2fastq2 Conversion Software V2.20.0422 (Illumina, Inc. San Diego, CA, USA).

2.6.1. *Quality control and genome alignment*

The following analysis was completed on a Linux server via command-line operations. The program Fastqc v0.11.8 (Babraham Bioinformatics) was used for quality assessment. The program multiqc was used to collate QC reports for all samples. QC assessment indicated sequences were of high quality, minimal adapters were present, and no adapter or quality trimming of reads was required. Mapping and gene annotation was based on the Atlantic salmon reference genome and annotation (ICSASG_v2. 6/10/2016) (Supplementary Tables 1 and 2). The genome was initially indexed and then reads were mapped to the indexed genome using HISAT2 (v2.2.1) (Kim et al., 2019). A count table of reads per gene region was generated using featureCounts (v2.0.1, 13.05.2020), using the RefSeq genome annotation information (GCF_000233375.1) for gene feature definitions. This count table was analysed in R version 4.0.5 (31.03.2021), where all subsequent analyses were performed, such as differential expression analysis and over-representation of significantly differential expressed genes (DEGs) in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

2.6.2. *Differential expression and pathway analysis*

Outliers and sample variation were assessed using base R tools (such as 'prcomp' and 'density') and visualised as density plots, PCA plots (Supplementary Fig. 1), heatmaps and dendrograms (Supplementary Fig. 2). Differential expression analysis was completed using the DeSeq2 package (Love et al., 2014). DESeq2 uses a negative binomial generalised linear model (GLM) to estimate fold change and expression variation between genes in treatment groups. The significance of gene expression differences is determined using a Wald test and then adjusted for false discovery (FDR) using Benjamini-Hochberg (Benjamini and Hochberg, 1995). Significantly DE genes were identified as having an FDR adjusted p value of <0.05. Assessment of KEGG pathways was completed using the ClusterProfiler package V3.9.1 (Yu et al., 2012).

KEGG pathway enrichment was examined using an overrepresentation test, to determine if the set of significantly differentially expressed genes per pathway were overrepresented in comparison to the total set of genes per pathway. Benjamini-Hochberg was again used to FDR test significance scores (i.e., p values) and if significantly enriched KEGG pathways were similarly to those with FDR-adjusted p values of <0.05. KEGG enrichment was also examined using Gene Set Enrichment Analysis (GSEA) which, rather than using just significantly DE genes, examines all genes in the transcriptome dataset, ranking them based on fold change. Enrichment in GSEA is based on proportions of highest ranked genes found in each pathway (Subramanian et al., 2005).

2.7. Statistics

Statistical analysis was performed in an R Studio environment, using R version 1.3.959 (©2009–2020, RStudio, PCB). Normal distribution of residuals was inspected using Q-Q plot and Shapiro-Wilks normality test, and homogeneity of variance was tested using Levene's test. Differences between dietary treatments were analysed using one-way ANOVA, and when F-test revealed a significant effect of treatment, the Tukey's Honest Significant Difference (HSD) post hoc test was used. Scoring data was treated with a non-parametric Kruskal-Wallis rank-sum test, and when significant, Dunn Kruskal-Wallis multiple comparison (Holm adjusted) post hoc test. Data are given as mean ± standard error of mean (SEM). Significance level was set to $p < 0.05$ and tendency level to $p < 0.1$.

3. Results

3.1. Growth

Initial growth rates (T_0 and T_1) were significantly higher in the Control group (1.63 ± 0.05) compared to those groups fed the HP48 and HP48 + Bactocell (SGR: 1.43 ± 0.05 and 1.45 ± 0.06, respectively) diet (Table 3). However, between T_1 and T_2 (SGR 2) growth was slightly higher, but not significantly in the HP48 groups (HP48 and HP48 + Bactocell) compared to the Control. Despite the tendency of higher growth in the HP48 groups in this period, SGR_{tot} still showed that the Control group had performed best overall (SGR: 1.66 ± 0.03). The HP48 + Bactocell group had an overall SGR of 1.58 ± 0.03 (similar to Control) while the SGR of fish fed the HP48 diet 1.55 ± 0.04 was lower ($p < 0.05$) than that of the Control.

Table 3
Growth performance of the three dietary groups Control, HP48 and Bactocell (n = 3) at sampling point T_0 (day 0), T_1 (day 34/35) and T_2 (day 57) of the experiment. Average body-weight (BW) per tank was determined based on total tank biomass by bulk-weighting 5 fish at a time. Specific growth rate per day (SGR, %) and thermal growth coefficient (TGC, %) were calculated between sampling points T_0 and T_1 (SGR 1/TGC 1), T_1 and T_2 (SGR 2/TGC 2) and T_0 and T_2 (SGR_{tot}/TGC_{tot}) ($n = 6$ ± SEM). Data was analysed using One-way ANOVA and Tukey HSD post hoc test when significant. ANOVA p-values denotes significant effect of diet (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, "tr" $p < 0.1$) and subscript letters mark statistically significant difference between diets ($p < 0.05$).

	Control	HP48	Bactocell	p-values
BW T_0 (g)	103.9 ± 5.3	108.5 ± 4.5	108.1 ± 6.2	
BW T_1 (g)	180.7 ± 13.2	178.9 ± 11.1	179.5 ± 16.1	
BW T_2 (g)	267.8 ± 19.3	262.5 ± 19.3	265.6 ± 21.0	
SGR 1 (%)	1.63 ± 0.05^{a}	1.43 ± 0.05^{b}	1.45 ± 0.06^{b}	**
SGR 2 (%)	1.71 ± 0.03	1.74 ± 0.07	1.78 ± 0.05	
SGR_{tot} (%)	1.66 ± 0.03^{a}	1.55 ± 0.04^{b}	1.58 ± 0.03^{ab}	*
TGC 1 (%)	2.35 ± 0.05^{a}	2.08 ± 0.06^{b}	2.11 ± 0.07^{b}	**
TGC 2 (%)	3.19 ± 0.35	3.23 ± 0.11	3.31 ± 0.06	
TGC_{tot} (%)	2.66 ± 0.04^{a}	2.49 ± 0.07^{b}	2.54 ± 0.05^{ab}	*

3.2. Morphological evaluation of the intestine

3.2.1. Mid-intestine

The histological assessment of the mid-intestine generally revealed few significant differences between fish fed the different diets (Table 4). There was, however, a tendency of higher enterocytes in the HP48+ Bactocell group (43.7 ± 5.77 μm) compared to the HP48 (38.0 ± 6.27 μm) and Control groups (35.6 ± 4.00 μm) ($p < 0.1$). The lamina propria width was found to be significantly (ANOVA) wider in the two diets supplemented with HP48 (HP48: 11.2 ± 6.27 μm, HP48 + Bactocell: 10.7 ± 5.77 μm) compared to the Control (8.4 ± 4.00 μm) ($p < 0.05$).

3.2.2. Hind-intestine

Diets clearly affected the hind-intestinal morphology of the fish (Fig. 2, Table 5). Feeding HP48 significantly increased lamina propria width (9.5 ± 0.39 μm) compared to the Control fed fish (5.3 ± 0.52 μm). Adding Bactocell to the HP48 diet significantly reduced lamina propria width (7.3 ± 0.33 μm) ($p < 0.05$), but it was still wider than the Control. The stratum granulosum height (μm) increased significantly from 51.3 ± 5.00 μm in fish fed the Control diet to 93.4 ± 8.04 μm in those maintained on the HP48 group ($p < 0.05$). While fish supplemented with Bactocell fell between the two other diet groups (72.9 ± 7.29 μm), with no significant difference to either the Control or HP48 groups. Goblet cell densities tended to be lower ($p < 0.1$), and the enterocyte height was higher in the Control fish compared to the two other diets ($p < 0.05$). Fig. 2 shows the relative frequency distribution of vacuolisation and mucosal fold scoring per diet group. In the Control group 50% of fish had a vacuolisation score of 1 and the other 50% a score of 2, which was significantly different from HP48 where 100% of the fish in HP48 scored 5 ($p < 0.05$). The scoring was lower in the HP48 + Bactocell group (score 3: 67%, score 4: 33%) compared to HP48, although not significantly. For mucosal folds, 83% of the Control group scored 1 and 17% scored 2. Feeding HP48 diet significantly increased the mucosal fold scores (score 3: 17%, score 4: 33%, score 5: 50%) compared to the Control ($p < 0.05$). The HP48 + Bactocell group was given intermediate scores (score 1: 17%, score 2: 33%, score 3: 33% and score 4: 17%) with no significant difference to either of the other groups.

3.3. Intestinal barrier and transport function

3.3.1. Mid-intestine

The paracellular transport of the alcohol sugar Mannitol (Papp ^{14}C-Mannitol) in the mid-intestine ranged from 0.03 to 0.31 10^{-6} cm/s with no clear difference between the dietary groups (Fig. 3). The electrical resistance (TER) was significantly higher in the Control group (231.0 ± 43.39 Ω cm^2) ($p < 0.05$). Feeding the HP48 diet reduced the TER to 181.6 ± 48.53 Ω cm^2, however the high variation in this group led to a non-significant result. Adding Bactocell to the HP48 diet gave lower

Table 4
Intestinal morphological assessment of the mid-intestine of fish fed the three diets Control, HP48 and Bactocell (mean ± SEM, $n = 3$, 2 fish/tank assessed). Data were analysed using One-way ANOVA and Tukey HSD post hoc test. ANOVA p-values denotes significant effect of diet (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, "tr" $p < 0.1$) and subscript letters mark statistically significant difference between diets ($p < 0.05$).

	Control	HP48	Bactocell	p-values
Stratum granulosum height (μm)	71.07 ± 11.15	69.48 ± 5.45	71.39 ± 9.46	
Lamina propria width (μm)	8.37 ± 4.00	11.22 ± 6.27	10.69 ± 5.77	*
Enterocyte height (μm)	35.61 ± 4.00	38.01 ± 6.27	43.72 ± 5.77	tr
Mucosal fold height (μm)	574.19 ± 93.47	661.92 ± 108.97	644.78 ± 43.08	

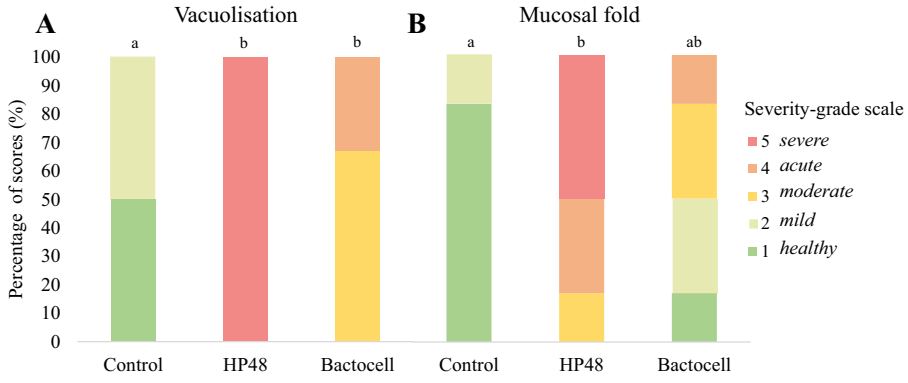

Fig. 2. Relative frequency distribution of (A) vacuolization and (B) mucosal fold scores in the hind-intestine per diet group. Scoring was performed on 6 fish/diet with 6 folds assessed per fish. Score 1 (green) for vacuolisation represents large, supranuclear vacuoles while score 5 (red) represents absence of vacuoles. Score 1 (green) for mucosal folds represents thin, long folds whereas a score of 5 (red) represents tissue disruption with fused mucosal folds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Intestinal morphological assessment (mean ± SEM) of the hind-intestine of fish fed the three diets Control, HP48 and Bactocell (mean ± SEM, n = 3, 2 fish/tank assessed). Data was analysed using One-way ANOVA and Tukey HSD post hoc test, except scoring data that was analysed using non-parametric Kruskal-Wallis rank sum test and Holm adjusted Dunn Kruskal-Wallis multiple comparison post hoc test. ANOVA *p*-values denotes significant effect of diet (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, "tr" $p < 0.1$) and subscript letters mark statistically significant difference between diets ($p < 0.05$).

	Control	HP48	Bactocell	*p*-values
Goblet cell density (n / 0.1 mm^2)	39.09 ± 3.76	59.12 ± 7.79	49.52 ± 5.11	tr
Lamina propria width (μm)	5.28 ± 0.52^a	9.45 ± 0.39^b	7.25 ± 0.33^c	***
Stratum granulosum height (μm)	51.25 ± 5.00^a	93.35 ± 8.04^b	72.94 ± 7.29^{ab}	**
Enterocyte height (μm)	42.21 ± 2.53^a	37.68 ± 2.16^b	34.80 ± 0.92^b	tr
Vacuolization score	1.28 ± 0.070^a	4.94 ± 0.036^b	3.39 ± 0.20^b	***
Mucosal fold score	1.11 ± 0.08^a	4.25 ± 0.35^b	2.57 ± 0.37^{ab}	***

variance with a TER of 145.0 ± 39.55 Ω cm^2, significantly lower than that of the Control group ($p < 0.01$) but similar to the HP48 group. The transepithelial potential (TEP) and short circuit current (SCC) were similar in all dietary groups.

3.3.2. Hind-intestine

The paracellular diffusion of ^{14}C-Mannitol in the hind-intestine ranged from 0.01 to 0.11 10^{-6} cm/s with no significant difference between the feeding groups (Fig. 4). The TER was higher in the hind-intestine compared to the mid-intestine (averaging 298 Ω cm^2 and 182 Ω cm^2 in the hind- and mid- intestine, respectively). Fish fed the Control diet had a TER of 326.8 ± 31.98 Ω cm^2. Supplementing feed with HP48 tended to reduce the TER (295.1 ± 86.14 Ω cm^2 in HP48 and 273.9 ± 47.53 Ω cm^2 in HP48 + Bactocell), although the high variation in especially the fish fed the HP48 diet led to non-significant results.

3.4. Gene expression

Results were analysed to determine the effects of HP48 compared to the Control (Control vs HP48) and then the possible mitigating effects of Bactocell on the HP48 diet (HP48 vs HP48 + Bactocell). There were no comparisons made between the Control and the HP48 + Bactocell groups. The transcriptional profiles are given as significantly DEGs and over-representation of significantly DEGs in KEGG pathways.

3.4.1. Mid-intestine

A total of 48 genes (5 upregulated and 43 downregulated) were differentially expressed in the mid-intestine of the HP48 group compared to the Control group (Supplementary Table 3). Among the strongest upregulated genes were T-cell surface antigen CD2-like and haemoglobin subunit beta-3-like (7.29 and 7.02 log2fc, respectively). The downregulated genes included genes related to calcium channels, apolipoprotein B synthesis, aquaporin-8-like and solute carrier family 35 member F3b. In this comparison, one KEGG pathway was significantly enriched (Benjamini-Hochberg adjusted *p*-values <0.05). This pathway, ECM-receptor interaction, had 4 DEGs that were all downregulated in the HP48 group. These were Integrin 1β-like (CD29), collagen alpha-2 (VI) chain-like (2 LOC hits), and thrombospondin 1-like (THBS) (Supplementary Table 4).

Comparing the gene expression of fish fed the HP48 + Bactocell diet to those fed the HP48 diet revealed a total of 497 DEGs (456 upregulated and 41 downregulated) (Supplementary Table 5). Among these, 37 DEG reversed the modulation induced by HP48 when compared to the Control group (Fig. 5, Table 6). For instance, while the gene T-cell surface antigen CD2-like was strongly upregulated in the HP48 compared to the Control, Bactocell supplementation of the HP48 diet caused a significant downregulation (7.29 and − 3.69 log2fc, respectively). The same applied to glycoprotein-*N*-acetylgalactosamine 3-beta-galactosyltransferase 1-B-like which was downregulated by HP48 compared to the Control group, while it was upregulated with the addition of Bactocell (−4.11 and 3.33 log2fc, respectively).

Seven enriched KEGG pathways were detected in the Bactocell group compared to the HP48 group, including ECM-receptor interaction (19 DEGs), cell adhesion molecules (12 DEGs), phagosome (12 DEGs), neomycin, kanamycin and gentamicin biosynthesis (3 DEGs), starch and sucrose metabolism (4 DEGs), fructose and mannose metabolism (5 DEGs) and galactose metabolism (4 DEGs) (Fig. 6, Supplementary Table 6). The ECM-receptor interaction pathway was generally upregulated in the Bactocell group compared to the HP48 group. This included genes such as collagen alpha-2 (VI) chain-like, laminin β-2, Integrin 1β-like and thrombospondin-1-like. The cell adhesion molecule pathway included downregulation of two genes, T-cell surface antigen CD2-like and major histocompatibility complex class I-related gene protein-like and upregulation of integrins and genes related to neural cell adhesion. The phagosome pathway was also, in general, upregulated including collectin-12-like, thrombospondin 4a and tubulin β-1-like.

3.4.2. Hind-intestine

A total of 1268 genes (531 upregulated and 737 downregulated) were differentially expressed in the hind-intestine of HP48 fed fish compared to the Control (Supplementary Table 7). Several solute carrier families and sodium-associated transporters such as solute carrier family 25 member 48 (*slc25a48*), solute carrier family 26 member 6 (*slc26a6*)

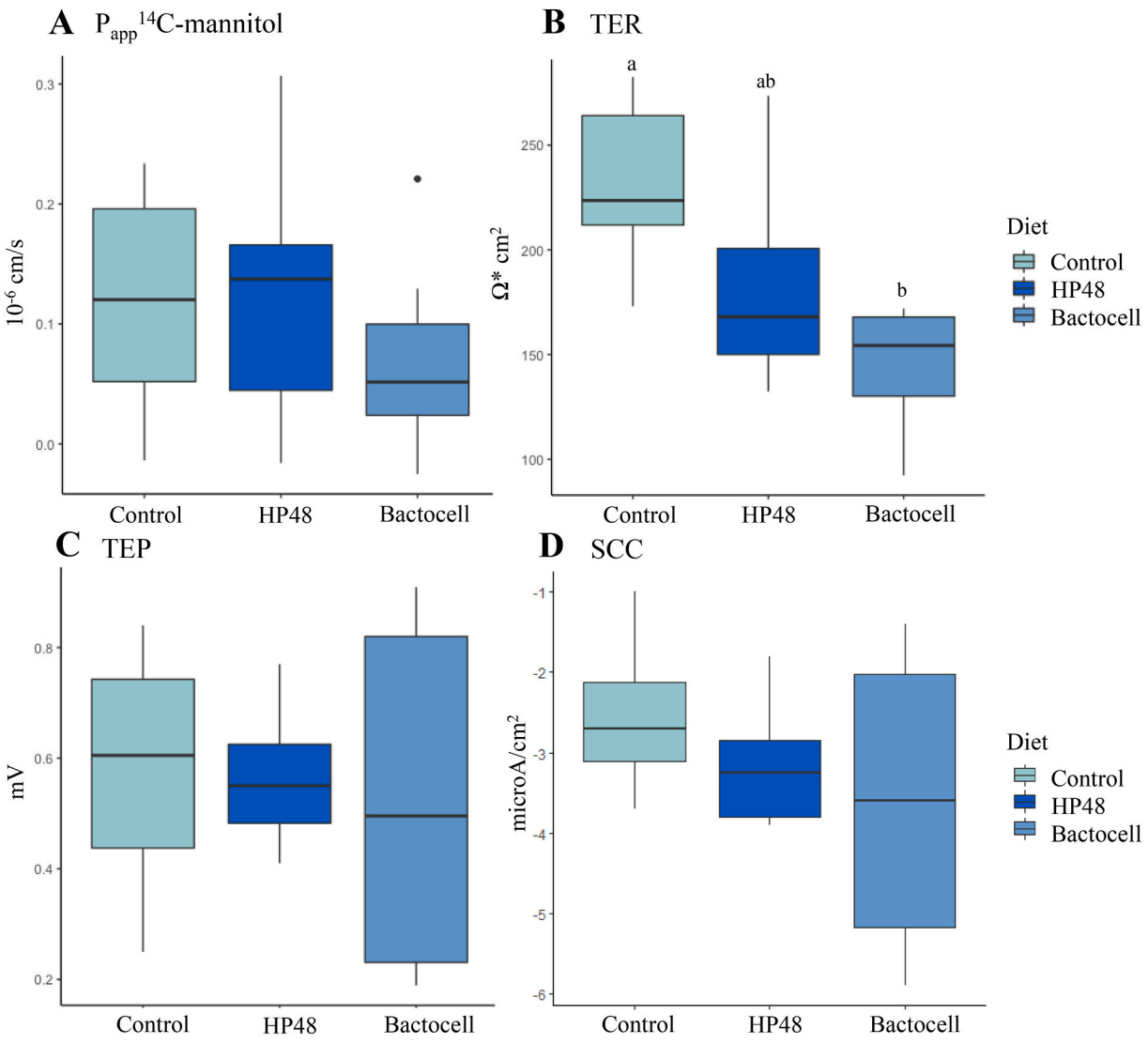

Fig. 3. Intestinal barrier transport function assessed by P_{app} for ^{14}C- Mannitol (A), TER (B), TEP (C) and SCC (D) over the mid-intestinal mucosa of fish fed Control-, HP48- and Bactocell diet ($n = 7$ or 8 per diet). Data were analysed using One-way ANOVA and TukeyHSD post hoc test and letters denotes significant differences between diets ($p < 0.05$).

and potassium channel, subfamily K, member 5a (*kcnk5a*) were downregulated in the HP48 group. The HP48 diet also downregulated genes related to chloride channels (e.g., chloride channel protein 2-like and chloride channel protein 1-like), protein degradation (trypsin I-P1-like), aquaporins and tight junction transmembrane proteins such as claudin-14 and cadherin-2-like. Several inflammatory genes were upregulated in the HP48 compared to the Control group, such as Annexin A2-A, CD99 molecule, Galectin-3 and interleukin 17F-like. Other genes altered in the HP48 group were cytochrome P450 enzymes and UDP-glucoronosyltransferases.

KEGG pathway analysis of the Control compared to HP48 showed a total of 18 enriched pathways (See Fig. 7 for the 10 most upregulated pathways and Supplementary Table 8 for the full list of enriched pathways). The 10 most enriched pathways in the HP48 group included biosynthesis of cofactors (21 DEGs), drug metabolism – cytochrome P450 (14 DEGs), glycerophospholipid metabolism (14 DEGs), retinol metabolism (12 DEGs), fructose and mannose metabolism (11 DEGs), porphyrin and chlorophyll metabolism (9 DEGs), neomycin, kanamycin and gentamicin biosynthesis (4 DEGs), metabolism xenobiotics by cytochrome P450 (8 DEGs) and steroid hormone biosynthesis (8 DEGs).

A total of 34 DEGs were found when the groups HP48 and HP48 + Bactocell were compared (Table 7) and no enriched pathways were identified. One of the two downregulated genes were cytopsin-A which is involved in cytokinesis and spindle organisation. The upregulated genes consisted of several genes involved in transcription, such as zinc finger protein 177-like, histone-lysine *N*-methyltransferase, H3 lysine-36 and H4 lysine-20 specific-like. Another group of upregulated genes in the HP48 + Bactocell group (compared to HP48 only) was proteases such as chymotrypsins, elastases, trypsins and carboxypeptidases.

Among the 34 DEGs in the comparison of HP48 vs HP48 + Bactocell, 8 DEGs were also regulated in HP48 compared to the Control, but in opposite direction (Fig. 8, Table 8), indicating some mitigation by Bactocell. Two cytochromes P450s (cytochrome P450 2K1 and cytochrome P450 2 K3-like) were downregulated by HP48 compared to the Control (−3.23 and −3.17 log2fc, respectively) while they were upregulated in the HP48 + Bactocell vs the HP48 group (3.34 and 3.35 log2fc, respectively). Apolipoprotein Da, duplicate 2 and microfibril-associated glycoprotein 4-like were also strongly downregulated in HP48 compared to the Control (−5.79 and −7.38 log2fc) and strongly upregulated in HP48 + Bactocell compared to HP48 (7.9 and 8.72 log2fc).

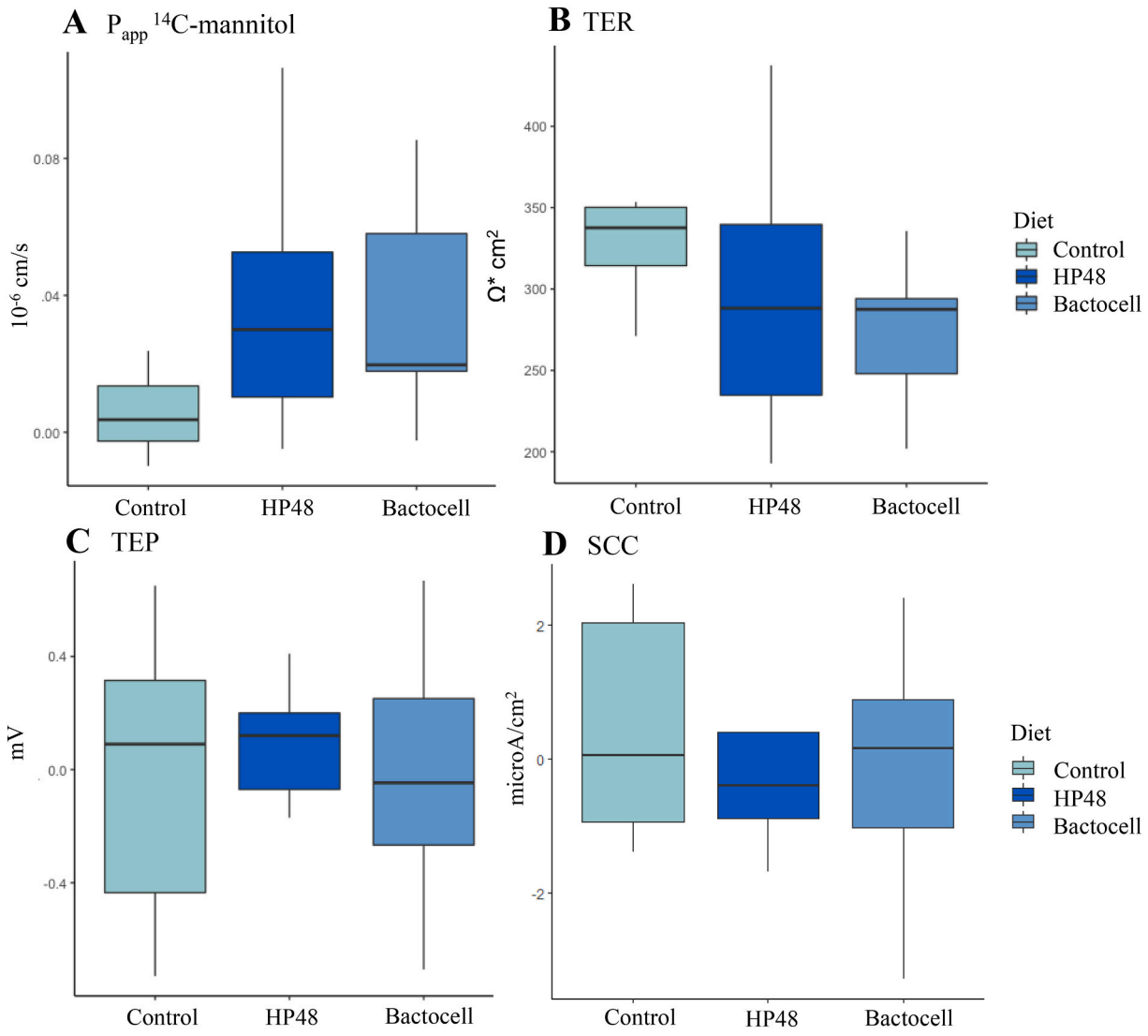

Fig. 4. Intestinal barrier transport function assessed by P_{app} for ^{14}C- Mannitol (A), TER (B), TEP (C), and SCC (D) over the hind-intestinal mucosa of fish fed Control-, HP48- and Bactocell diet ($n = 7$ or 8 per diet). Data were analysed using One-way ANOVA and TukeyHSD post hoc test and letters denotes significant differences between diets ($p < 0.05$).

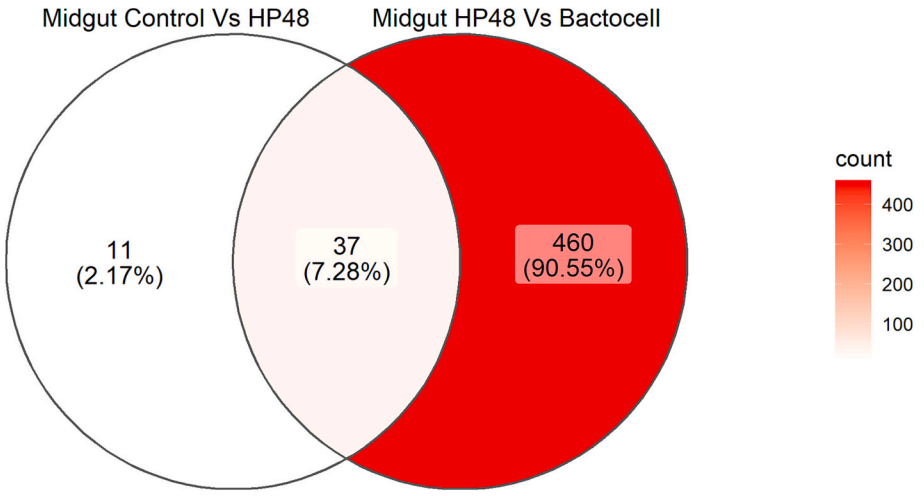

Fig. 5. Venn-diagram with the number of $\log_2$ fold change DEGs in the mid-intestine of Control vs HP48 and HP48 vs HP48 + Bactocell. Overlaps show DEGs shared by the groups. Complete list of DEGs can be found in Appendix 1.

Table 6
Comparison between DEGs in Control vs HP48 and HP48 vs HP48 + Bactocell included 37 common genes in the mid-intestine. Upregulated genes (LFC > 1) are highlighted in green and downregulated genes in red (LFC < −1). All expression values are reported as Log_2 fold change and *p* values are Benjamini-Hochberg adjusted ($p < 0.05$).

		Control vs HP48		HP48 vs Bactocell	
Gene_symbol	Description	Adjusted *p*	Log_2FC	Adjusted *p*	Log_2FC
LOC106567039	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1-B-like	0.00747	-4.11	3.0e-02	3.33
LOC106566791	semaphorin-3ab-like	0.00091	-3.04	4.9e-03	2.08
LOC106562968	microtubule-associated protein 1B-like	0.00055	-2.60	4.2e-02	3.18
LOC106579284	uncharacterized LOC106579284	0.00014	-2.48	1.2e-11	3.24
nos1	nitric oxide synthase 1 (neuronal)	0.00055	-2.06	1.2e-07	2.70
LOC106569416	dynamin-3-like	0.03129	-2.06	5.8e-05	2.52
LOC106595963	fibrillin-1-like	0.01163	-1.91	2.8e-03	1.99
LOC106565729	neural cell adhesion molecule L1-like protein	0.02172	-1.86	1.2e-02	1.75
LOC106589493	myosin-16-like	0.02801	-1.83	8.8e-04	2.10
LOC106588819	stathmin-2-like	0.01905	-1.80	4.0e-04	1.96
LOC106572978	neural cell adhesion molecule L1.1-like	0.03947	-1.78	4.9e-03	1.85
slc35f3b	solute carrier family 35 member F3b	0.01163	-1.76	1.1e-05	1.94
LOC106564733	hexokinase-1-like	0.02801	-1.74	3.8e-04	1.97
LOC106573568	cortexin-1-like	0.03947	-1.73	1.4e-02	1.78
LOC106569208	collagen alpha-1(XVIII) chain-like	0.00857	-1.73	1.5e-04	1.77
LOC106608297	transforming growth factor beta-3-like	0.02266	-1.71	3.4e-02	1.72
LOC106561029	hyaluronidase-4-like	0.02447	-1.67	4.8e-02	1.58
LOC106591024	alpha-actinin-1-like	0.03207	-1.64	3.5e-03	1.57
LOC106582941	calcium-dependent secretion activator 1-like	0.00857	-1.61	6.6e-06	2.02
prph	peripherin	0.04738	-1.57	4.1e-03	1.85
LOC106601645	tumor necrosis factor receptor superfamily member 16-like	0.0066	-1.43	5.0e-05	1.57
dpysl3	dihydropyrimidinase like 3	0.01905	-1.37	3.6e-03	1.42
LOC106605431	thrombospondin-3a-like	0.00321	-1.36	1.3e-03	1.42
LOC106582581	collagen alpha-2(VI) chain-like	0.00228	-1.35	1.5e-07	1.81
synm	synemin, intermediate filament protein	0.00089	-1.35	1.3e-06	1.32
LOC106583501	pleckstrin homology domain-containing family A member 6-like	0.02801	-1.34	3.1e-04	1.58
LOC106599515	integrin beta-1-like	0.00092	-1.33	2.0e-07	1.50
LOC106611061	latent-transforming growth factor beta-binding protein 2-like	0.00324	-1.32	2.3e-10	1.85
LOC106612894	neurobeachin-like	0.01163	-1.31	3.9e-03	1.55
LOC106608590	mitogen-activated protein kinase kinase kinase kinase 2-like	0.03001	-1.29	2.5e-03	1.35
LOC106591280	fibrillin-1-like	0.01844	-1.21	1.5e-04	1.51
LOC106561616	anoctamin-1-like	0.03926	-1.18	2.1e-02	1.03
LOC106572620	rab GDP dissociation inhibitor alpha-like	0.01844	-1.05	1.8e-03	1.18
LOC100136494	decorin	0.02801	-1.04	3.2e-04	1.26
LOC106592181	collagen alpha-2(VI) chain-like	0.00747	-1.04	1.5e-03	1.14
si:ch211-173a9.6	olfactomedin-4-like	0.01533	1.67	1.5e-02	-1.61
LOC106575468	T-cell surface antigen CD2-like	0.00115	7.29	4.2e-02	-3.69

4. Discussion

Increasing the inclusion level of vegetable ingredients into Atlantic salmon (*Salmo salar*) feed is usually considered to increase the risk of epithelial barrier dysfunctions and intestinal inflammation (Gatlin et al., 2007). Supplementing functional feed ingredients that can diminish these inflammatory responses would allow for higher inclusion of plant ingredients and improved intestinal health and fish welfare. The inflammatory effect of soybean meal is widely studied (Booman et al., 2018, De Santis et al., 2015, Knudsen et al., 2008, Krogdahl et al., 2003., Zhou et al., 2018). However, trials often include SBM with high levels of antinutrients causing acute intestinal damage, and it is expected that functional ingredients cannot provide sufficient benefits against such severe damage. One aim of this study was therefore, to create a model for low-level intestinal inflammation using HP48. The second aim was then to explore if the probiotic bacteria *Pediococcus acidilactici* CNCM I-4622 – MA 18/5 M would have an affect on the induced low-level inflammation.

4.1. Dietary effect on growth

In general, detrimental effects related to growth and intestinal health

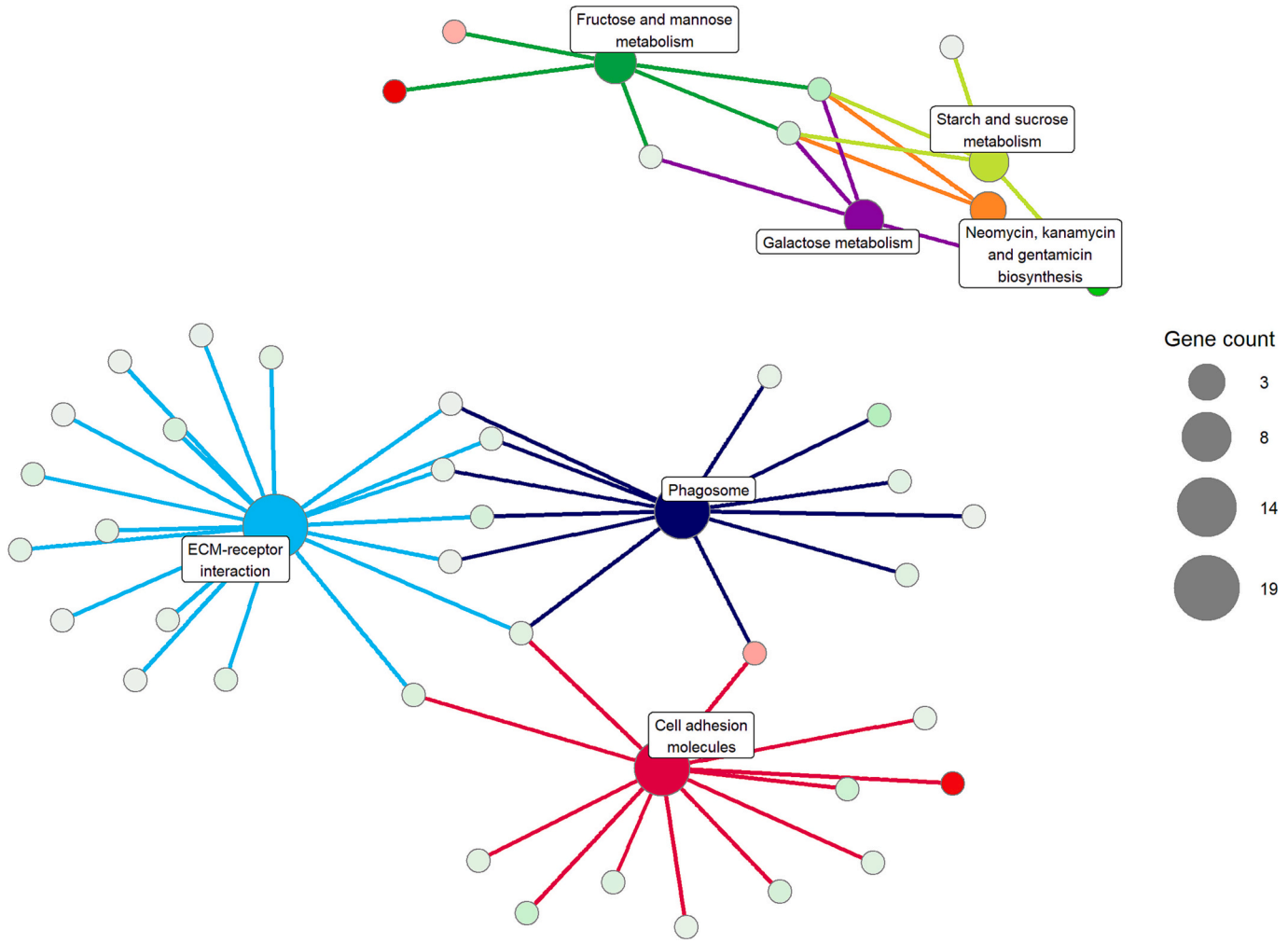

Fig. 6. Concept network of the seven enriched KEGG pathways in the mid-intestine of HP48 vs HP48 + Bactocell. The point size of each pathway indicates the number of DEGs in that pathway. Smaller points represent individual DE genes found in each pathway and are coloured by intensity of $\log_2$ fold change (green = upregulated and red = downregulated). Links between pathways and their associated DE genes are coloured by pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is dependent on the source and inclusion rate of soybean and its anti-nutritional factors (Zhou et al., 2018). Krogdahl et al., 2003 observed that increasing the inclusion of SBM correlated with reduced growth and decreased activity of mucosal enzymes in the hind-intestine. This effect is mostly thought to be caused by antinutritional factors, such as saponins and lectins (Knudsen et al., 2007; Krogdahl et al., 2015). In the present study, the SGR of fish fed the HP48 diet was significantly lower than that of Control fish. The total SGR over the trial duration for the HP48 + Bactocell group fell between that of the Control and HP48, indicating that Bactocell supplementation reduced the detrimental effects of HP48 on somatic growth. The difference in SGR between fish fed HP48 diets and the Control appeared to be greatest in the first period of the experiment (day 0–34/35) and levelled off in the last period (day 34/35–57). This suggests a gradual adaption to the feed and compensatory growth; a mechanism seen in fish after a period of growth depression (Ali et al., 2003). The same acclimatisation towards the end of the experiment was not seen in severely affected Atlantic salmon fed solvent-extracted SBM (Refstie et al., 1998) containing much higher levels of antinutritional factors. Taken together, it appears that the fish were able to compensate for the negative effects of HP48 at the level tested here which induced only mild and reversible growth penalties. In that sense, the amount of HP48 used in this study can be deemed appropriate to assess the potential alleviating effects of functional ingredients in the current and future trials.

4.2. Dietary effect on mid-intestine

The pyloric caecae and the mid-intestine are the main sites for nutrient uptake. These regions are not regarded as sensitive to soybean derivatives as that of the hind-intestine (van den Ingh et al., 1991). It is suggested that since salmon are unable to degrade the harmful compounds in soy (Knudsen et al., 2006), they will accumulate in the hind-intestine resulting in a more pronounced effect in this region (Olsen et al., 2007). Here we found that HP48 did affect the mid-intestine, particularly through reduced TER and a widening of the lamina propria. The widening of the lamina propria by soybean antinutrients is often linked to inflammation and increased migration of immune cells to the inflamed tissue (Booman et al., 2018). We found clear indications of this by the strong upregulation of T-cell surface antigen CD2-like gene, as has also been observed in the hind-intestine of juvenile grass carp in response to SBM (Wu et al., 2018). Likewise, an activation of T-cells was observed in the lamina propria and submucosa in the hind-intestine of Atlantic salmon with SBM-induced enteritis, although it is not certain whether this reaction is involved in inducing inflammation or is a part of a defence mechanism towards foreign antigens entering the epithelium (Bakke-McKellep et al., 2007; Romarheim et al., 2013). Upregulation of olfactomedin-4 could also be an indication of activated infiltrating immune cells as this glycoprotein was upregulated in patients with *Helicobacter pylori* infected gastric mucosa (Liu et al., 2010). Immune cell

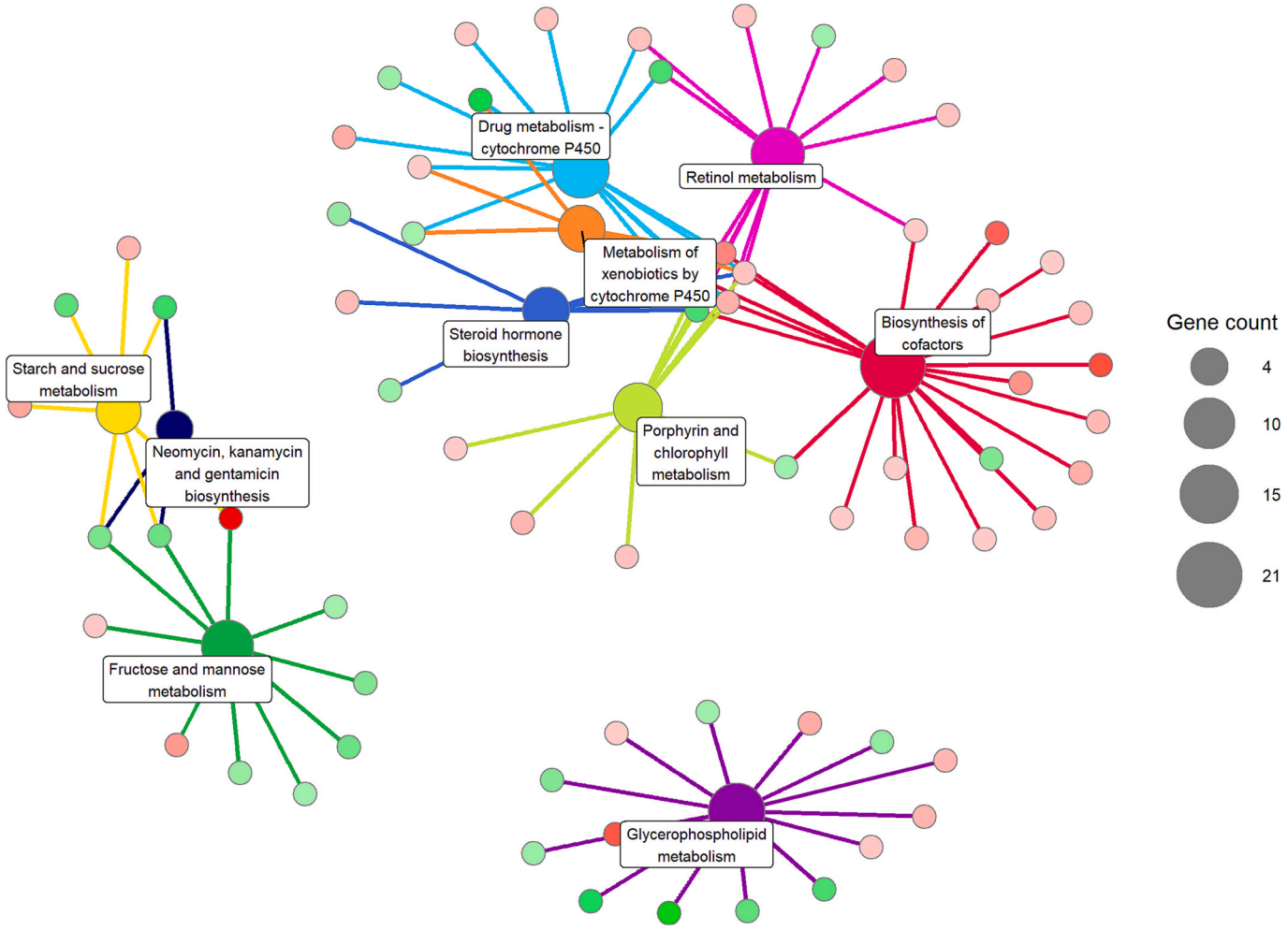

Fig. 7. Concept network of the 10 most enriched KEGG pathways in the hind-intestine of Control vs HP48. The point size of each pathway indicates the number of DEGs in that pathway. Smaller points represent individual DE genes found in each pathway and are coloured by intensity of $\log_2$ fold change (green = upregulated and red = downregulated). Links between pathways and their associated DE genes are coloured by pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflow with consequent cytokine production also tends to reduce barrier functions (Neurath, 2014), which can potentially lead to increased antigen and bacterial uptake making the fish more susceptible to diseases (Krogdahl et al., 2000). The reduced TER can also be a direct cause of saponins and other membrane permeabilizers impairing control of ionic and water flux (Knudsen et al., 2008). It is possible that such loss of fluid balance can result in downregulation of aquaporin-8 (*aqp-8*) as seen in fish fed HP48. Loss of aquaporins is often seen in fish with hind-intestinal enteritis and has been linked to impaired fluid transport (Hu et al., 2016; Krogdahl et al., 2015; Kortner et al., 2012; Tipsmark et al., 2010). The downregulation of genes related to collagen synthesis and cellular maturation (such as Integrin 1β-like and collagen α2(VI) chain-like) was rather striking. Collagen VI is an essential component of the basement of the epithelial layer, and loss of collagen VI synthesis would result in increased sloughing and migration of intestinal epithelial cells (Groulx et al., 2011). Furthermore, downregulation of nitric oxide synthase 1 (*nos 1*) indicates oxidative stress, probably caused by the anti-nutritional factors as seen in Zebrafish fed with SBM (Micheloni et al., 2022). Thus, these results may suggest that HP48 does disturb intestinal homeostasis in mid-intestine of salmon, impairing cellular integrity, and increasing inflammation and cell turnover.

Adding Bactocell to the HP48 diet had little effect on mid-intestine physiological parameters (Ussing chamber and histomorphometry), and while the histological values tended to clearly improve towards the values found in the Control group, these results were not significant. However, interestingly, Bactocell appeared to restore most of the transcriptional effects caused by the HP48 diet. This included upregulation of the collagen related genes and *nos1* in addition to downregulation of the T-cell surface antigen CD2-like and olfactomedin-4. These findings have similarities to that found in rats where a probiotic mix (*Lactobacillus paracasei*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus and Lactobacillus acidophilus*) increased skin wound contraction compared to the Control group partly by reducing the inflammatory phase, and accelerating the fibrosis process and deposition of collagen (Tagliari et al., 2019). The HP48 + Bactocell diet also upregulated several basement membrane proteins such as type IV collagen, laminins and other transmembrane collagens that are thought to play an important role in mucosal healing and restoring epithelial homeostasis (Mortensen et al., 2019). Thus, these results suggest that this probiotic supplementation has a distinctive protective role on the mid-intestinal epithelium, dampening the mild inflammation induced by HP48 and promoting regeneration of epithelial cells, including increasing the collagen synthesis.

4.3. Dietary effect on hind-intestine

Feeding diets with antinutrients to salmon often cause severe damage to the hind-intestine (Krogdahl et al., 2015). These pathological

Table 7
DEGs in comparison of HP48 vs HP48 + Bactocell hind-intestine. Upregulated genes (LFC > 1) are highlighted in green and downregulated genes in red (LFC < −1). All expressions values are reported as Log_2 fold change, and p values are Benjamini-Hochberg adjusted ($p < 0.05$).

Gene_symbol	Description	Adjusted p	Log_2FC
LOC106582081	uncharacterized LOC106582081	1.7e-02	-4.51
LOC106571627	cytospin-A-like	1.2e-03	-3.24
LOC106571736	uncharacterized LOC106571736	4.9e-02	1.54
LOC106600650	zinc finger protein 177-like	5.7e-03	1.63
igfbp1a	insulin-like growth factor binding protein 1a	2.8e-02	1.65
LOC106568016	histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific-like	6.3e-03	1.91
LOC106590666	somatomedin-B and thrombospondin type-1 domain-containing protein-like	5.7e-03	2.16
LOC106578611	limb region 1 protein homolog	1.9e-02	2.51
LOC106569843	BOLA class I histocompatibility antigen, alpha chain BL3-7-like	4.9e-02	2.62
LOC106585383	N-acylethanolamine-hydrolysing acid amidase-like	3.1e-03	2.81
LOC106594728	cytochrome P450 2K1	2.7e-02	3.34
LOC106606455	cytochrome P450 2K3-like	2.9e-03	3.35
LOC106577871	uncharacterized LOC106577871	1.3e-05	3.99
LOC106577950	sterile alpha motif domain-containing protein 9-like	1.1e-03	5.61
prss1	serine protease 1	3.4e-02	6.02
LOC106588135	chymotrypsin A-like	4.9e-02	6.03
LOC106566159	elastase-1	1.6e-02	6.42
LOC106577461	trypsin-2-like	3.3e-03	6.50
trp-i	trypsin I	1.6e-02	6.58
LOC106561547	high choriolytic enzyme 2-like	6.6e-03	6.66
LOC106562747	uncharacterized LOC106562747	1.3e-05	6.73
ela2l	elastase 2 like	1.2e-02	6.73
endou	endonuclease, polyU-specific	1.3e-02	6.93
ela2	elastase 2	6.3e-03	7.07
tryp	Trypsin	1.2e-03	7.26
cpb1	carboxypeptidase B1 (tissue)	3.1e-03	7.39
cbpa1	Carboxypeptidase A1	3.2e-03	7.41
trp-ii	trypsin II	1.2e-03	7.42
LOC106577430	trypsin-2-like	3.1e-03	7.58
LOC106570835	perforin-1-like	3.7e-02	7.67
apoda.2	apolipoprotein Da, duplicate 2	3.8e-02	7.90
LOC106577462	trypsin-like	6.3e-03	7.95
LOC106594252	microfibril-associated glycoprotein 4-like	2.7e-02	8.72
LOC106576254	hemagglutinin/amebocyte aggregation factor-like	1.5e-02	9.21

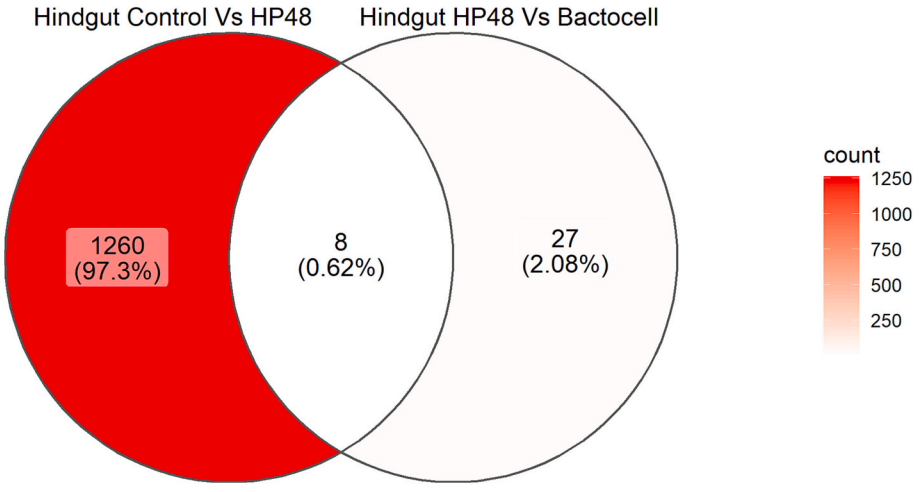

Fig. 8. Venn-diagram with the number of log_2 fold change DEGs in the hind-intestine of Control vs HP48 and HP48 vs HP48 + Bactocell. Overlaps show DEGs shared by the groups. Complete list of DEGs can be found in Appendix 1.

symptoms include increased paracellular permeability, severe histological changes including increased lamina propria width, increased infiltration of immune cells and disrupted mucosal folds in addition to altered expression of immune related genes (Knudsen et al., 2008; Krogdahl et al., 2015; Marjara et al., 2012). In the present study, the lack of clear effect on epithelial integrity, but clear effects on histology

Table 8
Comparison between DEGs in Control vs HP48 and HP48 vs HP48 + Bactocell included 8 common genes in the hind-intestine. Upregulated genes (LFC > 1) are highlighted in green and downregulated genes in red (LFC < −1). All expressions values are reported as Log_2 fold change, and p values are Benjamini-Hochberg adjusted ($p < 0.05$).

		Control vs HP48		HP48 vs Bactocell	
Gene_symbol	Description	Adjusted *p*	Log_2FC	Adjusted *p*	Log_2FC
LOC106582081	uncharacterized LOC106582081	4.0e-06	6.62	1.7e-02	-4.51
LOC106571736	uncharacterized LOC106571736	4.1e-02	-1.55	4.9e-02	1.54
LOC106590666	somatomedin-B and thrombospondin type-1 domain-containing protein-like	4.6e-07	-2.71	5.7e-03	2.16
LOC106594728	cytochrome P450 2K1	1.4e-02	-3.23	2.7e-02	3.34
LOC106606455	cytochrome P450 2K3-like	6.0e-03	-3.17	2.9e-03	3.35
LOC106577871	uncharacterized LOC106577871	4.2e-02	-3.49	1.3e-05	3.99
apoda.2	apolipoprotein Da, duplicate 2	4.3e-03	-5.79	3.8e-02	7.90
LOC106594252	microfibril-associated glycoprotein 4-like	1.9e-02	-7.38	2.7e-02	8.72

parameters proves that the enteritis-model induced a low-level inflammation targeted to mimic the mild condition experienced in a commercial setting. As expected, most of the morphological damages observed, including widening of lamina propria, loss of absorptive vacuoles, reduction of enterocyte height and disrupted mucosal folds suggest inflammatory damages and increased rate of cell turnover, migration and apoptosis (Chikwati et al., 2013; Knudsen et al., 2007; Krogdahl et al., 2015; van den Ingh et al., 1991). The modulation of inflammation observed by histopathological diagnostic was, to a large extent, corroborated by the molecular upregulation of several genes involved in soybean generated intestinal inflammation (Kiron et al., 2020; Kortner et al., 2011; Marjara et al., 2012). Among these were the cell-surface protein, *CD99*, that has been reported to induce T-cell activation (Kwon et al., 2007) and *IL-17F* that together with *IL-17A* are key cytokines for recruitment and activation of neutrophils to the tissue (Korn et al., 2009). Upregulation of IL-17s has been associated with both inflammatory diseases such as inflammatory bowel disease in humans (Fujino et al., 2003; Seiderer et al., 2008) and SBM enteritis in fish (Marjara et al., 2012; Miao et al., 2018). The upregulation of galectin-3 and annexin A2 agrees with Kiron et al., 2020, who found similar results in salmon fed another model containing HP48 and full-fat soybean meal. Galectin-3 has been suggested to increase the production of pro-inflammatory cytokines by macrophages and increase in migration of other immune cells in mice with colitis (Simovic Markovic et al., 2016), while the actin-binding protein annexin A2 is involved in migrating intestinal epithelial cells (Rankin et al., 2013).

The transcriptional profile of the hind-intestine of fish fed the HP48 group also showed downregulation of several genes involved in intestinal transport mechanisms compared to the Control group. This included genes associated with chloride channel proteins, potassium transport (*kcnk5a*) and solute carrier families such as *slc26a6*, important in Cl^- / HCO_3^- exchange at the apical membrane (Grosell, 2010). These results agree with Kiron et al., 2020, that showed comparable alterations of the intestinal transport mechanisms in Atlantic salmon hind-intestine fed soybean products. Moreover, we observed a downregulation of *aqp-8* in the hind-intestine of fish fed HP48, corresponding to several other studies involving fish fed soybean derivatives (Hu et al., 2016; Krogdahl et al., 2015; Kortner et al., 2012). This water channel protein is one of the most important aquaporins in the Atlantic salmon intestine and is located both at the brush border membrane and the basolateral part of the enterocytes ensuring sufficient uptake and transport of water from the lumen to the blood vessels (Engelund et al., 2013; Sundh et al., 2014; Tipsmark et al., 2010). Such disturbed ion and fluid uptake can lead to diarrhoea-like conditions, that could have a negative effect on nutrient transport and reduce fish growth (Baeverfjord and Krogdahl, 1996; Hu et al., 2016; Krogdahl et al., 2003; van den Ingh et al., 1991).

Bactocell supplemented to the HP48 diet appeared in many aspects to diminish some of the negative effects of HP48. While the effect of HP48 on barrier functions appeared unaffected, both the lamina propria width and stratum granulosum height were reduced and there were larger absorptive vacuoles and less disrupted mucosal folds in the HP48 + Bactocell supplemented group. This agrees with Vasanth et al. (2015) who found that salmon fed Bactocell displayed decreased expression of inflammatory genes, less disrupted mucosal folds, and increased expression of trypsin-1 in the hind-intestine compared to the Control when exposed to oxazolone. Although we did not specifically observe reduced expression of inflammatory genes by Bactocell supplementation in the present study, there was an upregulation of trypsin-1 in this group compared to HP48 as well as several other genes encoding for protein digestion, such as trypsin-2, carboxypeptidase A and —B, chymotrypsin, and elastase. An increased digestive enzymatic activity was similarly found in olive flounder (*Paralichthys olivaceus*) after dietary probiotic supplementation (of *Bacillus* sp. and *Lactobacillus plantarum*) compared to the SBM group (Jang et al., 2019). Although upregulation of trypsin-like activity in the hind-intestinal wall of SBM fed Atlantic salmon has been suggested to contribute to increased disease severity (Lilleeng et al., 2007), this did not appear to be the case in the present study. Furthermore, the Bactocell group strongly upregulated apolipoprotein Da 2 (*apoda.2*) compared to the HP48, which reversed the down-regulation induced by HP48 compared to the Control group. In addition to being important for lipid transport and uptake, apolipoproteins display antimicrobial and anti-inflammatory roles in humans (Gomaraschi et al., 2005). Increasing evidence suggest that apolipoproteins also play important roles in the primary defence barriers in fish, such as the intestinal mucosa (Villarroel et al., 2007). For instance, apolipoproteins showed antimicrobial activity against Gram-positive and Gram-negative bacteria, including some fish pathogens in carp (*Cyprinus carpio*) and rainbow trout (Concha et al., 2004; Villarroel et al., 2007). Although this could suggest increased protection to foreign antigens, the transcriptional effects were not as strong in the hind-intestine compared to the mid-intestine. This could indicate that Bactocell has a more beneficial effect in the mid-intestine or that there is an increased colonisation of probiotic bacteria in this intestinal segment compared to the hind-intestine. Thus, a dose-response trial would be interesting to investigate whether a higher inclusion of Bactocell would lead to more effects lower down in the intestine.

5. Conclusion

In conclusion, feeding a HP48 supplemented diet to Atlantic salmon triggered transcriptional changes in the mid-intestine and generated a low-level inflammation in the hind-intestine which was associated with

a significant yet moderate reduction in growth performance. Thus, this study validates a mild and naturally (orally) induced enteritis model using a widely used raw ingredient and is therefore both commercially relevant and ideal to study the potential beneficial roles of feed additives on the intestinal health of salmon. Bactocell appeared to have major beneficial effects in the mid-intestinal epithelium, diminishing the effect of HP48, and promoting cellular regeneration. In the hind-intestine, the probiotic supplementation tended to improve the morphological effects of HP48 which was generally not associated with reduced expression of inflammatory genes, but with marked upregulation of the apolipoprotein gene. This could reinforce the putative role of apolipoproteins in the defence barrier of fish and their involvement in the promotion of gut health by probiotic lactic acid bacteria. Hence, addition of Bactocell to Atlantic salmon feed could offer protection against the low-level intestinal inflammation created by vegetable ingredients.

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Availability of supporting data

Transcriptome metadata have been submitted to the NCBI Sequence Read Archive (SRA) under accession number PRJNA844085.

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CRediT authorship contribution statement

Malene Fosse Nordvi: Writing – original draft, Data curation, Formal analysis. **Signe Dille Løvmo:** Writing – review & editing, Data curation, Formal analysis. **Paul Whatmore:** Writing – review & editing, Data curation, Formal analysis. **Henrik Sundh:** Writing – review & editing, Data curation. **Trygve Sigholt:** Writing – review & editing, Funding acquisition. **Rolf Erik Olsen:** Supervision, Conceptualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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