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Gut immune functions and health in Atlantic salmon (*Salmo salar*) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region

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ABSTRACT

The present study was conducted to strengthen the knowledge on gut immune functions and health in Atlantic salmon under large scale, commercial conditions in the Arctic region of Norway. Two groups of fish were monitored, one fed a series of diets without functional ingredients (Ref) and the other diets with functional ingredients (Test). The nutritional composition of the two diet series varied in parallel according to the nutrient requirements of the fish during the observation time. The content of functional ingredients in the Test diets, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, varied in accordance with a strategy developed by the feed company. The fish were observed at four sampling time points, the first (FW) in May 2016 two weeks before seawater transfer, the other three throughout the following seawater period until the fish reached a size of about 2 kg, i.e. in June, four weeks after seawater transfer (SW1); in November (SW2), and in April the following year (SW3). Gut health was assessed based on histopathological indicators of lipid malabsorption and gut inflammation, expression of gut immune, barrier and other health related genes, plasma biomarkers, somatic indices of intestinal sections, as well as biomarkers of digestive functions.

Seawater transfer of the fish (SW1 compared to FW) caused a marked lowering of expression of genes related to immune and barrier functions in the distal intestine, i.e. cytokines (*il1β*, *il10*, *tgfβ*, *ifnγ*), T-cell markers (*cd3γδ*), *myd88* and tight junction proteins (*zo-1*, *claudin-15*, *claudin-25b*), indicating suppressed immune and barrier functions. At SW2 and SW3, most of the immune biomarkers showed values similar to those observed at FW. The development of plasma cholesterol and triglyceride levels showed similar picture, with markedly lower levels after seawater transfer. Lipid malabsorption was observed in particular in fish from SW1 and SW2, as indicated by hyper-vacuolation of the pyloric caeca enterocytes with concurrently increased expression levels of *plin2*. Regarding effects of functional ingredients, significantly lower condition factor and plasma triglyceride level were observed for Test-fed fish at SW2, indicating a metabolic cost of use of a mixture of nucleotides, yeast cell walls and essential fatty acids. No clear effects of functional ingredients on expression of gut immune genes and other health indexes were observed through the observation period. The great, temporary lowering of expression of gut immune and barrier genes at SW1 is suggested to be an important factor underlying the increased vulnerability of the fish at this time point. Our findings regarding supplementation with functional ingredients raise questions whether some of these ingredients overall are beneficial or might come with a metabolic cost. Our results highlight the need for a better understanding of the cause and consequences of the suppression of gut immune functions of farmed Atlantic salmon just after seawater transfer, and the use of functional ingredients under commercial conditions.

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1. Introduction

Norwegian Atlantic salmon (*Salmo salar*) production has the potential to reach a level 4–5 times higher than today by 2050 [1,2]. Conditions for fulfilling such projected growth in production are considered to include expansion of aquaculture production in the Arctic region [3,4]. However, the environmental conditions are extreme in the north with very low average temperature and extreme variation in photoperiod. Use of diets formulated for fish in the south, may not be optimal for fish in the north. Adjustments to local conditions may be required to obtain optimal function and health. Literature searches reveal only limited information about nutrient requirement of fish produced under Arctic conditions. Therefore, as feeds used in the northern regions are quite similar to those in the south, it is not unlikely that the diets may be deficient and imbalanced for some nutrients.

It is well documented that suboptimal feed and feeding strategies may affect immune functions and health in fish [5–7]. In Atlantic salmon, the consequences may be most severe during the physiological transitions taking place in the fish in the period of transfer from freshwater to seawater [8–11]. Available information from studies conducted in the south of Norway show clear alterations in immune functions in Atlantic salmon upon seawater transfer with decreases in immune related plasma proteins and levels of lysozyme, IgM and leucocyte [12–14]. A study of expression of immune genes during smoltification and seawater transfer in tissue from pyloric region, employing microarrays, showed alteration in about 300 immune genes including cytokines and T cells marker genes [15]. Also in the distal intestine of Atlantic salmon, which is considered to play the most important role in immune-related gut functions [16] altered expression of immune-related genes has been observed [17].

In the present situation, with a weak knowledge basis for optimizing the nutrient content of the diet, preventative use of so-called functional ingredients is common. Such compounds are claimed to prevent or milden disease outbreaks and improve function and health during periods expected to be particularly challenging, for example the period just before and after seawater transfer [7,18]. The most commonly used functional ingredients for fish include certain nutrients (e.g. essential fatty acids and nucleotides), a range of polysaccharides (e.g. prebiotics) and microbial extracts (e.g. compounds from yeast cell walls) [7,18]. Such functional ingredients are claimed to have beneficial effects locally within the intestine with possible direct or indirect modulatory effects on gut immune responses [18,19], gut barrier functions [20] and gut microbiota [21]. Functional ingredients have different main functions. They are therefore used alone or in combination throughout the production cycle depending on the expected physiological and environmental challenges, to improve the robustness of the fish. Additionally, in the commercial production cycle, Atlantic salmon sense and respond to various changeable environmental factors (e.g. daylight, temperature, salinity and dissolved oxygen), aquaculture practices (e.g. netting and transferring of fish), as well as adjustments of macronutrient composition to meet fish requirement according to growth rate and body composition. Hence, long-term, lager scale feeding trials, conducted under commercial conditions, would represent an important validation step between small-scale feeding experiments of limited duration and the introduction of new diets into the commercial production [22].

The present study was therefore conducted to gain knowledge on fluctuations in gut immune functions and health of Atlantic salmon from late freshwater stage until one year in seawater farmed under large scale, commercial conditions in Arctic regions, and whether use of functional ingredients would benefit gut functions and health.

2. Materials and methods

2.1. Fish husbandry

This experiment was conducted following the Norwegian laws and regulations of the experimentation with live animals according to Norwegian Animal Research Authority. Atlantic salmon fry, from the same batch of eggs hatched in the spring of 2015, were raised at two smolt production tanks in the Bodø area of Norway (N67° – E14°), each tank containing about 180 000 fish. When ready for seawater transfer, the fish were transported to the sea location in Alta area (N70° – E22°) of Finnmark County by boat where each tank was split into triplicated net pens, each containing about 55 000 fish.

For water parameters, a vertically devise connected to an automatic winch (HF5000, Belitronics, Lunde, Sweden) was used to monitor water salinity, temperature and oxygen levels at 3 m depth throughout the experimental period. The temperature followed natural fluctuations in the water and averaged, 6.8 ± 2.6 °C (mean ± SD) for the entire period. Oxygen and salinity levels averaged 11.8 ± 1.3 mg/L and 29.8 ± 3.8‰ throughout the experimental period, respectively (Fig. S1).

2.2. Diets and feeding routines

Two groups of fish were monitored, one fed a series of diets without functional ingredients (Ref) and the other with functional ingredients (Test). The nutritional composition of the two diet series varied in parallel according to the development in nutrient requirements. As the production conditions in the north are unpredictable regarding attacks of pathogens and parasites, feeding with functional ingredients were chosen. The functional ingredients, i.e. nucleotides, yeast cell walls, a prebiotic and essential fatty acids, were supplemented to the Test diets either as single ingredient or as a mixture according to a strategy developed by the feed producer. Table 1 shows the composition of the diets fed during a period of several weeks before each of the four sampling time points. In freshwater, fish were fed continuously to satiation using automatic feeders. Also, during the first weeks in seawater, fish were fed according to appetite, by automatic feeders. Later, feed was supplied according to appetite in one to three meals depending on the length of daylight.

2.3. Sample collection

Samples were taken from the fish at four sampling time points as illustrated in Fig. 1 showing the experimental set-up. The samples were collected once during freshwater period, i.e. two weeks before seawater transfer in May 2016 (FW) and three times during the seawater period, i.e. four weeks after seawater transfer (SW1, June 2016) and two times thereafter (SW2 and SW3, November 2016 and April 2017). Only fish with content along the whole intestine were sampled to be sure that fish were exposed to the diets for several hours before the sampling. Fish were anesthetized with tricaine methanesulfonate (MS222, Argent Chemical Laboratories) and euthanized by cervical dislocation before tissue sampling. Three replicate sampling groups for each treatment, 12 fish per replicate were sampled, i.e. 36 fish per treatment. Length and body weight were measured for all fish, and blood were collected from the caudal vein using heparinized vacutainers for plasma biochemistry analyses. Thereafter, the abdominal cavity was opened, and the digestive organs were taken out and cleared of adipose tissue.

For each replicate, 6 of them were collected for the analysis of digestive functions, i.e. 18 fish per treatment. Specifically, the content from the proximal intestine (PI) divided in two (PI 1 and PI 2), mid intestine (MI) and distal intestine (DI) divided in two (DI 1 and DI 2) were collected and pooled for the analysis of bile acid concentration and trypsin activity. After removing the content, the tissues from PI, MI and DI were weighed for organosomatic indices, respectively, then collected for the evaluation of leucine aminopeptidase (LAP) capacity.

Another 6 fish per replicate were collected for the evaluations of histology and qPCR. More specifically, tissues from DI and pyloric caeca (PC) were rinsed in phosphate buffered saline (PBS) three times to remove traces of remaining chyme before cut into pieces for histological evaluation. These tissue were fixed in 4% phosphate-buffered

Table 1
Formulation and nutrient composition of the two diet series[a].

Feed composition	FW-Ref	FW-Test	SW1-Ref	SW1-Test	SW2-Ref	SW2-Test	SW3-Ref	SW3-Test
Ingredients (%)								
Marine protein sources[b]	40	40	30	30	19	19	19	19
Plant protein sources[c]	35	35	39	39	53	53	53	53
North Atlantic fish oil	9	9	24	24	10	10	10	10
Rapeseed oil	9	9	–	–	7	7	7	7
Binders & Micronutrients	7	7	7	7	11	11	11	11
Sum	100	100	100	100	100	100	100	100
Nutrient composition (%)								
Crude protein	44	44	44	44	46	46	46	46
Crude fat	22	22	28	28	22	22	22	22
Starch	7.5	7.5	8	8	10	10	10	10
Crude fiber	1.5	1.5	3	3	3	3	3	3
Ash	7	7	6	6	5	5	5	5
Functional ingredients[d]								
Essential fatty acids	–	–	–	✓	–	✓	–	✓
Nucleotides	–	✓	–	✓	–	✓	–	✓
Yeast cell walls	–	–	–	–	–	✓	–	–
One prebiotic	–	–	–	–	–	–	–	✓

[a] The composition of four different basic diets varied throughout the time of observation following the strategy developed for this commercial site according to the development and production and health of the fish in the farm. At each observation time, two diets were formulated, one without functional ingredients (Ref diet) and one with functional ingredients (Test diet). FW, sampling point in freshwater (May 2016); SW1, the first seawater sampling point (June 2016); SW2, the second seawater sampling point (November 2016); SW3, the final seawater sampling point (April 2017).
[b] Mix of Scandinavian origin fish meal and, fish protein concentrate (Norway).
[c] Mix of soy protein concentrate, wheat protein concentrate, wheat gluten, sunflower meal.
[d] Inclusion levels were determined according to recommendations from the producers and cannot be disclosed due to commercial interests and production of intellectual rights. These functional ingredients were added in the dry meal mix with other dry ingredients in a homogenous dry mix, then going to the preconditioner and extruder.

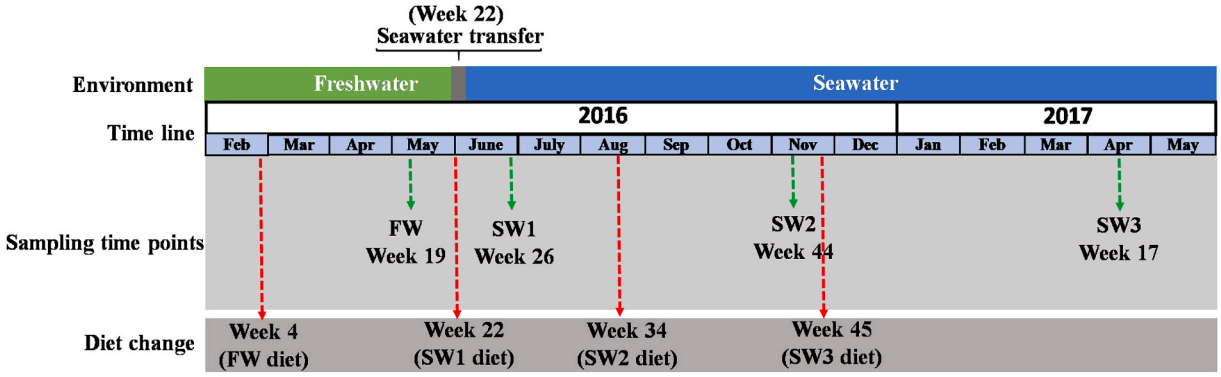

Fig. 1. Outline of the experimental setup. The time for sampling and diet change are shown in green and red dotted arrow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

formaldehyde solution for 24 h, and then transfer to 70% ethanol for storage. Regarding qPCR, tissues from DI and PC were preserved in RNAlater solution and incubated at 4 °C for 24 h, and then stored at −20 °C before RNA extraction.

3. Analytical procedures

3.1. Plasma biochemistry

Plasma triglyceride, free fatty acid, cholesterol, glucose, chloride and sodium were analyzed according to standard protocols at the Central Laboratory of the NMBU Faculty of Veterinary Medicine (Oslo, Norway).

3.2. Digesta trypsin activity and total bile acids concentration

Trypsin activity was determined according to the description of Kakade et al. [23], using benzoylarginine p-nitroanilide (No. B-4875, Sigma Chemical Co., St. Louis, MO) as the substrate. Bovine trypsin was used to make standard curve. The total bile acid concentration was quantified by the Enzabile test kit (No. 550101, BioStat Diagnostic Systems, Cheshire, U.K.). Taurocholic acid was used to make the standard curve.

3.3. Leucine aminopeptidase (LAP) capacity

The LAP capacity was analyzed according to the description of Bieth et al. [24] using tissue homogenates. The tissue homogenates were prepared using the ice-cold Tris−mannitol buffer (1:20, w/v). The four-(two-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (Pefabloc SC, Basel, Switzerland) was used as a serine proteinase inhibitor. The LAP capacity is expressed as mmol substrate hydrolysed per hour per kg fish.

3.4. Gut mucosa structure

Tissues from DI and PC were processed to produce 3-μm thickness sections that were stained with hematoxylin and eosin (H&E) according to standard histological techniques [25].

Evaluation of DI sections was conducted with light microscopy and focused on the morphological changes of soybean meal-induced enteritis

(SBMIE) in Atlantic salmon that consist of shortening of mucosal fold length, increase in width and cellularity of the submucosa and lamina propria, and reduction in enterocyte supranuclear vacuolization. For the PC, the degree of vacuolization of the enterocytes was evaluated. The morphological characteristics were scored on a scale of 0–4 where 0 represented normal; 1, mild; 2, moderate; 3, marked, and 4, severe changes as described by Bakke et al., 2007 [26].

3.5. Gut mucosa gene expression

Total RNA was extracted from DI and PC tissues using a FastPrep-24 homogenizer, Trizol® reagent and further purified with PureLink (Thermo Fisher Scientific) with an on-column DNase treatment. For PC, samples from FW, SW1 and SW2 were selected for analysis based on histological results, and equal amount of total RNA from three fish per replicate were pooled after RNA extraction. For DI, three individual fish per replicate were processed (i.e. 9 fish per treatment), and all four sampling points were included in the analysis. The integrity of the RNA samples was verified by the 2100 Bioanalyzer using RNA Nano Chips (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The average RNA integrity number (RIN) of all samples was 9.0. Total RNA was stored at −80 °C until use.

The synthesis of cDNA, DNase treatment and qPCR were conducted as previously described [27]. For DI, 32 target genes related to intestinal immune responses, barrier functions, osmoregulation and nutrient metabolism were selected. Primers and assay details are shown in Table 2. For PC, a panel of 16 target genes was profiled, targeting intestinal lipid and sterol metabolism, immune and barrier functions (Table 3). RNA polymerase II (*rnapolii*) and hypoxanthine phosphoribosyl transferase 1 (*hprt1*) for DI and beta-actin (*actb*) and gylceraldehyde-3-phosphate dehydrogenase (*gapdh*) for PC were used as reference genes for the target gene normalization based on their overall coefficient of variation (CV) and interspecific variance [28]. The geometric mean of the reference genes was used as the normalization factor. Mean normalized levels of target genes were calculated from raw quantification cycle (Cq) values [29].

3.6. Calculations

The thermal growth coefficient (TGC) was calculated as follows:

$$TGC = 1000 * [\text{sampling body weight (g)}^{1/3} - \text{initial body weight (g)}^{1/3}] * (\sum \text{day degree})^{-1}$$

Condition factor (CF) was calculated as follows:

$$CF = 100* \text{ sampling body weight (g) / sampling body length (cm)}^{3}$$

Organosomatic indices (OSI) was calculated as follows:

$$OSI\ (\%) = 100* \text{ organ weight / sampling body weight (g)}$$

3.7. Statistical analysis

In order to compare the mean values and clearly identify significant differences between sampling time points, as well as between different diets at each observation time, eight different treatments were defined by combining the sampling time points and diets, i.e. FW-Ref, FW-Test, SW1-Ref, SW1-Test, SW2-Ref, SW2-Test, SW3-Ref and SW3-Test. A two-

Table 2
Details of primer pairs used for qPCR assays in the distal intestine.

Function	Acronym[a]	Primer sequence (5'→3')	Primer sequence (5'→3')	Annealing temp	Product size (bp)	Acc.no
		Forward	Reverse			
Pro-inflammatory	*il17a*	TGGTTGTGTGCTGTGTGTCTATGC	TTTCCCTCTGATTCCTCTGTGGG	60	136	GW574233
	il8	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	60	136	NM_001140710
	il1β	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	60	73	NM_001123582
	ifnγ	CTAAAGAAGGACAACCGCAG	CACCGTTAGAGGGAGAAATG	60	159	FJ263446
Anti-inflammatory	*tgfβ*	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	60	191	EU082211
	il10	CGCTATGGACAGCATCCT	AAGTGGTTGTTCTGCGTT	62	59	EF165028
Adaptive immunity	*myd88*	GACAAAGTTTGCCCTCAGTCTCT	CCGTCAGGAACCTCAGGATACT	60	110	NM_001136545
	gilt	ACGGAAATGCACACGAATCT	GCCTCCATGCAGTAGACGAT	60	148	BT047766
T-cell markers	*cd4α*	GAGTACACCTGCGCTGTGGAAT	GGTTGACCTCCTGACCTACAAAGG	60	121	NM_001123611
	cd8β	CGCACACACCTCAACAACTC	ATTGATGCGCAGTGTGAAAG	59	153	AY693394
	cd3γδ	AAAGGCGCATGGACAGATCT	GCCCGCACAACATTAAAGCT	60	160	NM_001123621
Goblet cell markers	*muc13*	ATTGTCGGCACTGTTCTTGG	GGAGCTCTTCTTGGACGTCT	60	88	DY723445
	muc2	TCTGTCCTGATGGGATGAAAC	GGACTCCAAACAAACGCAAT	60	143	CK885177
Stress response	*cat*	CCCAAGTCTTCATCCAGAAACG	CGTGGGCTCAGTGTTGTTGA	60	101	BG935638
	hsp70	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	60	121	BG933934
	sod	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	55	140	BG936553
Tissue remodeling	*pcna*	TGAGCTCGTCGGGTATCTCT	GTCCTCATTCCCAGCACACT	55	170	BT056931
Mucosal barrier function	*zo-1*	CAAAGCCAGTGTATGCCCAG	CAGCTTCATACTCGGCCTGA	60	119	XM_014175464.1
	e-cadherin	ACTATGACGAGGAGGGAGGT	TGGAGCGATGTCATTACGGA	60	107	BT058864.1
	claudin-15	GGCACGTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	60	92	BK006395
	claudin-25b	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	60	101	BK006399
	occludin	GACAGTGAGTTCCCCACCAT	ATCTCTCCCTGCAGGTCCTT	60	101	NM_001173656.1
Nutrient transport	*slc6a6*	GGAGGTGGAAGGACAGATCA	ACATGCCACCTTTCGTTACC	60	143	NM_001139800
	fabp2b	TGCCTTCCCCTCATTCTCTA	GGTGATACGGTCTTCATCCAA	60	152	BT046827
	pept	GGCTTTCTGCTCTGTGAAGG	TAGGGGGACACAACAAGACC	55	89	NM_001146682
	slc10a2	CCCTGGGAATCTACGTCAAA	GGTCCAGGAGGACTGGTACA	60	134	XP_014019465
Osmoregulation	*ecc*	GCAGTGTTGCTGCTGGTTTA	TCAGGCACCACTGGGTTAAT	60	104	C081R050
	aqp10	GGTGTTGGTGATCGGAGTCT	CGCCCTAAACACCTCATCC	62	121	DW569041
	aqp1a	CTACCTTCCAGCTGGTCCTG	TGATACCGCAGCCTGTGTAG	62	141	BT046625
	aqp1b	CTGTGGGTCTGGGACATCTT	TAAGGGCTGCTGCTACACCT	62	153	NM_001140000.1
	nkaα1b	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	60	81	NM_001124460
	aqp8 ab	GTTGGCATAGTTCTCCTTTGATG	TTTCAACCCTCCCTTCACC	60	148	KC626879.1
Reference genes	*rnapoii*	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGGATCTTCCTGC	60	157	BG936649
	hprt1	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	60	255	BT043501

[a] For explanation of gene abbreviations see Tables S1–3.

Table 3
Details of primer pairs used for qPCR assay in the pyloric caeca.

Function	Acronym[a]	Primer sequence (5'→3')	Primer sequence (5'→3')	Annealing temp	Product size (bp)	Acc.no
		Forward	Reverse			
Immune	*il1β*	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	60	73	NM_001123582
	tgfβ	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	60	191	EU082211
	cd3γδ	AAAGGCGCATGGACAGATCT	GCCCGCACAACATTAAAGCT	60	160	NM_001123621
Mucosal barrier function	*Zo-1*	CAAAGCCAGTGTATGCCCAG	CAGCTTCATACTCGGCCTGA	60	119	XM_014175464.1
	claudin-15	GGCACGTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	60	92	BK006395
	claudin-25b	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	60	101	BK006399
Nutrient transport	*pept*	GGCTTTCTGCTCTGTGAAGG	TAGGGGGACACAACAAGACC	55	89	NM_001146682
Osmoregulation	*nka-α1b*	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	60	81	NM_001124460
	aqp8 ab	GTTGGCATAGTTCTCCTTTGATG	TTTCAACCCTCCCTTCACC	60	148	KC626879
Stress response	*hsp70*	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	60	121	BG933934
Lipid metabolism	*fabp2b*	TGCCTTCCCCTCATTCTCTA	GGTGATACGGTCTTCATCCAA	60	152	BT046827
	hmgcr	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	60	224	NM_001173919
	plin2	CCCAGGTCTACTCCAGCTTC	CAGCGACTCCTTCATCTTGC	60	104	BT072598
	apoa1	CTGGTCCTCGCACTAACCAT	TGGACCTCTGTGCAGTCAAC	60	144	NM_001123663
	cyp51	TGCATTGGGGAGAACTTTGC	ATCTGATGACGGGGTTGTGT	60	148	DY731118
	npc1l1	CCAAAGACCTGATCCTGGAA	CGAAGCACACATCCTTCAGA	60	108	CB505644
Reference genes	*gapdh*	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	60	84	BT050045
	actb	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	60	88	AF012125

[a] For explanation of gene abbreviations see Tables S4–5.

way ANOVA was considered unsuitable as diet composition varied throughout the observation time both regarding macronutrient and functional ingredients composition. Differences in histological scores were analyzed for statistical significance using the Fisher exact test. Post hoc analysis for significant Fisher Exact Test results was conducted using the Chisq.post.hoc test. Both statistical tests were run in the R statistical package (version 3.4.2; 2017) within the RStudio interphase (version 1.1.383; 2017; RStudio Inc.). Other statistical analyses were performed with JMP Pro 13.0.0 (SAS Institute, United States). One-way ANOVA and Tukey-Kramer HSD multiple comparisons were performed to interpret and compare the mean values and identify significant differences between these eight different treatments. Data were evaluated for homogeneity of variance and normality of residuals using the "residual by predicted" plot and histogram, respectively. When necessary, data were transformed by box-cox power transformation to meet the statistical assumptions, and then refitted for a second evaluation. The level of significance in all analyses was set at $P < 0.05$. In the tables and figures, values with the same superscript letter are not significantly different. Figures were made using GraphPad Prism 8 (GraphPad Software, La Jolla, California, United States).

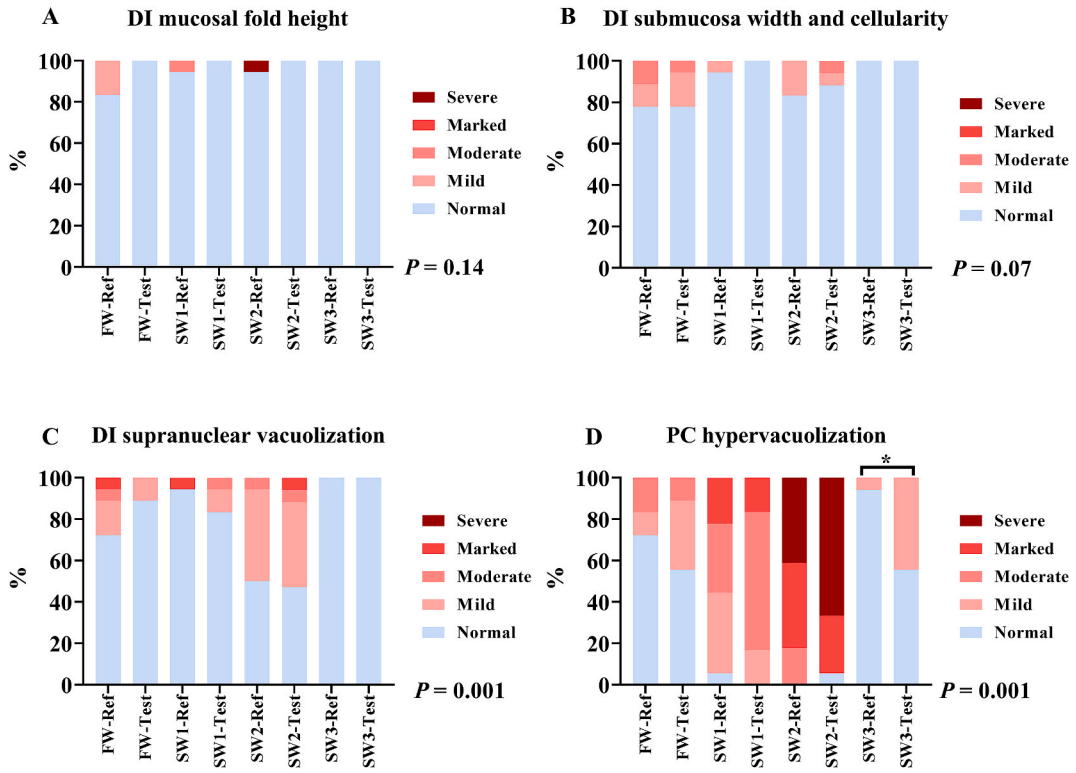

Fig. 2. Contingency charts showing proportions of sampled fish that scored normal, mild, moderate, marked and severe for the selected distal intestine and pyloric caeca morphological characteristics among treatments. *P* values of the Fisher's exact test are shown on the lower right corner of the subplot. Asterisk indicates significant effect of diet ($P < 0.05$, n = 18). DI: distal intestine, SM: submucosa, PC: pyloric caeca.

4. Results

4.1. Histological characteristics of the gut mucosa

The distal intestine of the fish showed normal morphological characteristics for most of the samples based on observations of mucosal fold height, submucosa width and cellularity and enterocyte supranuclear vacuolization. The exception was for samples taken at SW2 for which about 50% of the fish from both Ref and Test diets showed mild to marked reduction in supranuclear vacuolization of the enterocytes (Fig. 2 A, B and C). In pyloric caeca, enterocyte hyper-vacuolization, interpreted as steatosis and indication of lipid malabsorption, was observed (Fig. 2 D). The severity of this hyper-vacuolization increased gradually from FW through SW1, culminated at SW2 and decreased at SW3 (Fig. 2 D). A diet effect was observed for fish sampled at SW3, i.e. a higher proportion of mild steatosis for fish fed the Test diet compared to fish fed Ref diet (Fig. 2 D).

4.2. Gene expression

4.2.1. Gene expression in distal intestine

Overall, expression of genes related to gut immune functions and other health related functions showed clear differences between sampling time points. The results observed at SW1, i.e. just after seawater transfer, revealed substantially suppressed expression of most immune, barrier, stress, goblet cell marker genes as well as some other gut function related gene expressions. However, thereafter, i.e. at SW2 and SW3, most of gene expression levels returned back to the levels observed at FW (Fig. 3 and Tables S1–3).

Also, goblet cell marker mucin-13 (*muc13*) showed lower expression levels in fish at SW1 compared to FW fish, followed by an increase at the later seawater sampling points ($P < 0.05$, Fig. 4 A). Regarding diet effects, only negligible differences were observed.

In detail: Pro-inflammatory cytokines (interleukin-1 beta, *il1β* and interferon gamma, *ifnγ*), showed high expression at FW, a drop after seawater transfer, followed by an increase or no change throughout the rest of the observation period ($P < 0.05$, Fig. 4 B and C). A similar pattern was seen for anti-inflammatory cytokines (transforming growth factor-beta, *tgfβ* and interleukin 10, *il10*) (Fig. 4 D and E), T-cell markers (e.g. cluster of differentiation 3 γδ, *cd3γδ*) (Fig. 4 G) and myeloid differentiation factor 88 (*myd88*) (Fig. 4 I) showing high expression in fish from FW, followed by lower expression of fish from SW1 and higher expression thereafter. For cluster of differentiation 8 beta (*cd8β*), fish showed the lower expression at FW and SW1 but increased at later sampling points (Fig. 4 F). Gamma-interferon inducible lysosomal thiol reductase (*gilt*) showed higher expression at SW3 compared to those from other sampling time points (Fig. 4 H).

Similar to the observations for the immune related genes, genes related to tight junction barrier function (zo-*1*, *claudin-15* and *claudin-25b*) showed lower expression levels in fish from SW1 compared to FW fish, followed by an increase throughout the later seawater sampling points (Fig. 5).

For the intestinal water channel aquaporin-8ab (*aqp8ab*), gene expression levels increased progressively from the first to the last sampling point ($P < 0.05$, Fig. 6 A). The gene expression levels of aquaporin-10 (*aqp-10*) showed a similar trend regarding differences between sampling points but except for a decreased expression at SW2 (Fig. 6 B). Compared to the expression of *aqp-10* and *8adb*, very low expression

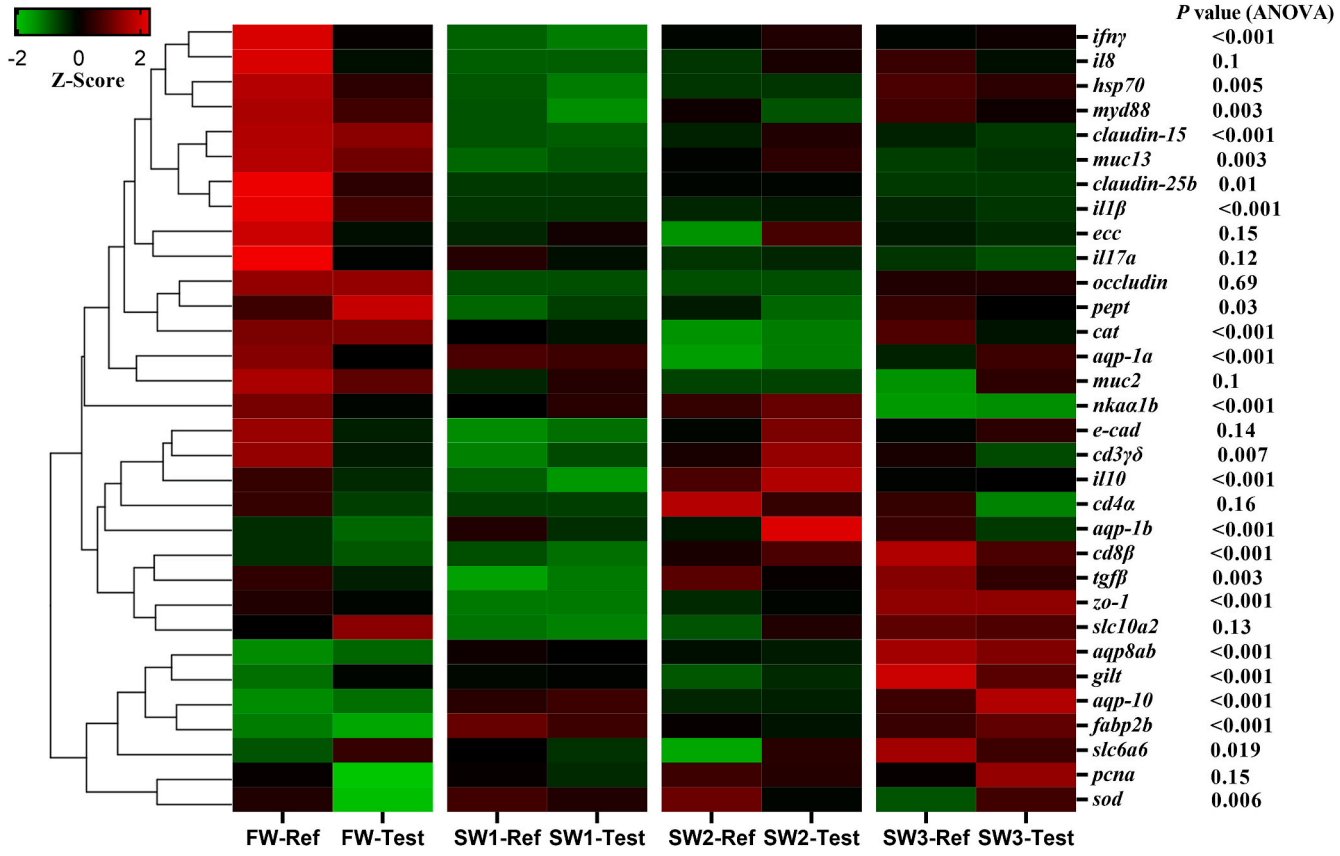

Fig. 3. Heatmap showing the hierarchical clustering of differentially expressed genes in distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. Colors correspond to Z-score (z = (relative expression – mean)/standard deviation) based on the relative gene expression, i.e., red (high positive value) indicates higher relative expressions; Green (low negative value) indicates the lower relative expressions. The level of significance in all analyses was set at $P < 0.05$ between treatments (n = 9). An alternative representation of the data and the explanation of gene abbreviations can be found in Tables S1–3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

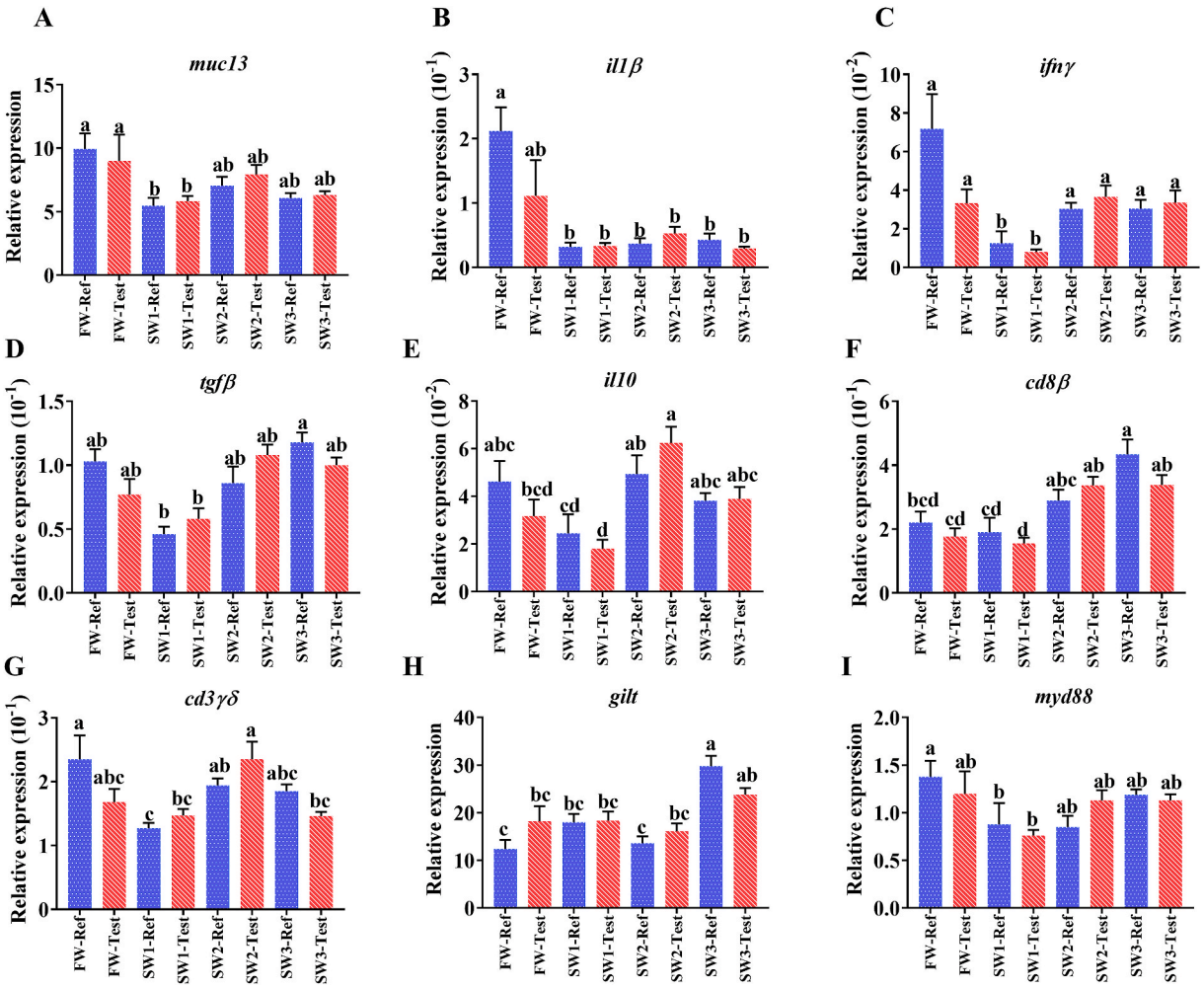

Fig. 4. Expression levels of genes related to immune responses in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For explanation of gene abbreviations, see Table S1. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

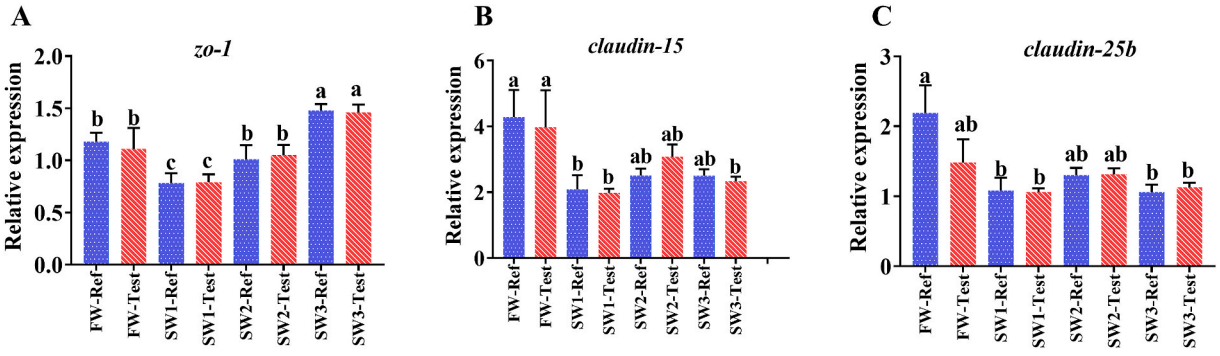

Fig. 5. Expression levels of genes related to barrier function in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Table S1. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

levels of aquaporin-1a and 1b (*aqp-1a* and *1b*) were found for all fish without clear difference between sampling points (Table S2).

The peptide transporter (*pept*) showed decreasing expression levels after sea water transfer (Fig. 6 C), while a significant increase was found for fatty acid binding protein 2b (*fabp2b*) ($P < 0.05$, Fig. 6 D).

Regarding the expression profile of other gut health related genes, such as interleukin 17A (*il17a*), interleukin 8 (*il8*), cluster of differentiation 4α (*cd4α*), epithelial chloride channel protein (*ecc*), solute carrier family 10-member 2 a (*slc10a2*), e-cadherin (*e-cad*), occluding, proliferating cell nuclear antigen (*pcna*) and mucin-2 (*muc2*), no significant effects of life stage were found ($P > 0.05$, Tables S1–3). No clear and consistent diet effects were found on the expression level of genes related to intestinal immune responses, barrier functions, osmoregulation and nutrient metabolism at any of the sampling points

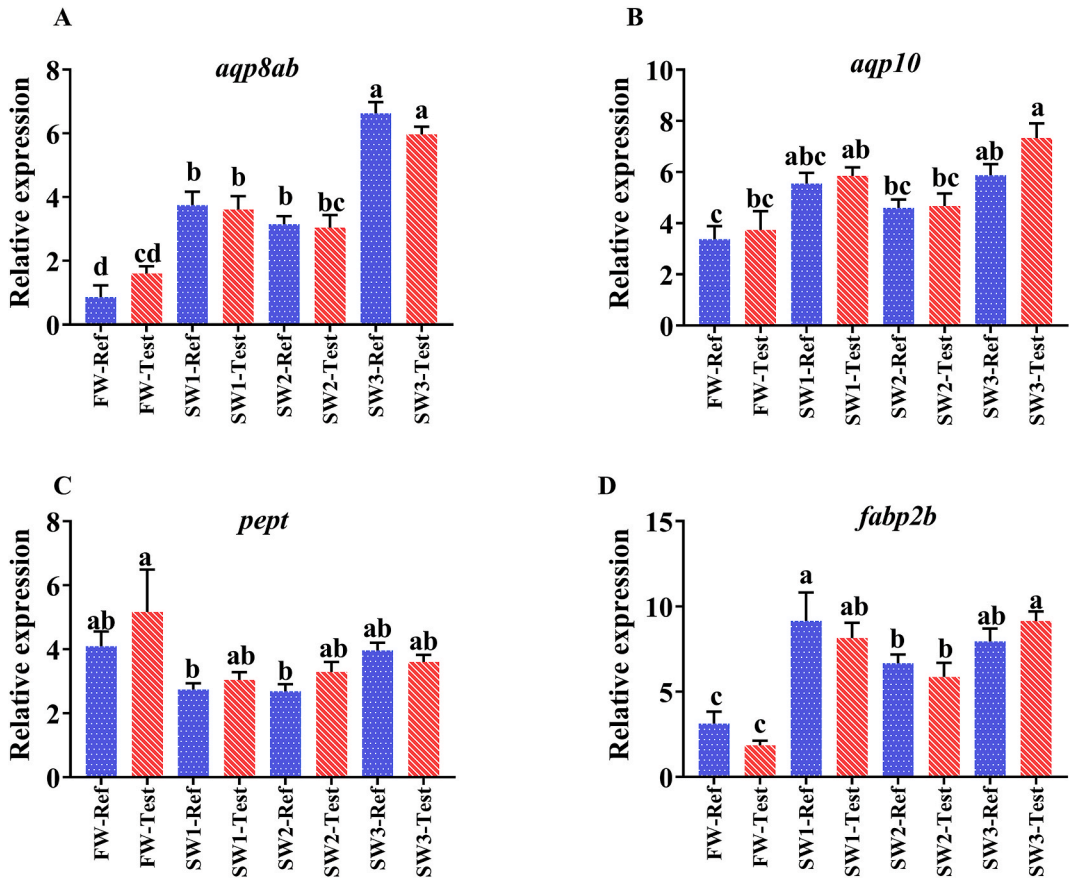

Fig. 6. Expression levels of genes related to water and nutrient transport in the distal intestine of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Tables S1–3. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 9) and values sharing the same letters are not significantly different.

(Tables S1–3).

4.2.2. Gene expression in pyloric caeca

The tissue of PC showed increased expression levels of *aqp8ab* in fish from SW1, followed by a suppression at SW2 (Fig. 7 A). Expression of *pept* decreased from FW throughout the seawater stages ($P < 0.05$, Fig. 7 B). The opposite picture was found for lipid droplet marker perilipin-2 (*plin2*) with higher expression levels observed after seawater transfer ($P < 0.05$, Fig. 7 C). The expression level of cholesterol synthesis gene cytochrome P450 51 (*cyp51*) increased and stabilized after sea transfer ($P < 0.05$, Fig. 7 D). The diet effect was insignificant for these variables at all sampling points. Regarding the gene profile expression of other immune, barrier function and metabolism related genes in PC, no significant effects of life stage nor of diet were found ($P > 0.05$, Tables S4 and 5).

4.3. Growth and body indices

Growth rate, estimated as TGC, was based on the initial weights of the whole population 6 months before FW sampling time point, as well as on the weights of the individual sampled fish. The results showed large differences between the sampling points. In the period of FW and SW1, fish showed the lowest TGC ($P < 0.05$, Fig. 8). In the next period, the growth rate increased significantly to a value about twice as high and stabilized thereafter. In the period between SW2 and SW3, fish fed Test diet tended to have lower growth than the fish fed the Ref diet, but no statistical difference was found (Fig. 8).

The lowest, and similar CF values for the two diets, were observed in fish from FW and SW1, whereas those from SW2 and SW3 showed significantly higher values. At SW2 and SW3, lower CF values were observed for the Test diet compared to the Ref diet (Table 4).

Regarding organo-somatic indices for PI, significant differences were seen between all sampling points ($P < 0.05$, Table 4) with increasing values from FW to SW1 and SW2. At SW3, the values had decreased to levels falling between those of SW1 and SW2. Regarding diet effects, no clear differences between the diets were observed at FW, SW1 or SW2. At the last sampling point (SW3) however, higher PI somatic indices were shown for fish fed Test diet ($P < 0.05$, Table 4). The results for MI and DI somatic indices showed the same trends regarding differences between sampling points, but the differences were less pronounced (Table 4). A significant diet effect was observed in DI at SW3, i.e. higher value in fish fed the Test diet (Table 4).

4.4. Plasma biomarkers

Plasma cholesterol and triglyceride decreased substantially after seawater transfer ($P < 0.05$, Fig. 9 A and B). Thereafter the values returned to a level not significantly different from that of the fish sampled at FW and remained at this level. Regarding plasma free fatty acid, the values decreased slightly after the seawater transfer, for then to increase to values at about the same level as observed in fish in the fresh water. Thereafter the values decreased significantly at SW3 ($P < 0.05$, Fig. 9 C). Fish from the FW sampling point showed markedly higher glucose levels compared to fish from seawater samplings ($P > 0.05$, Fig. 9 D). No significant diet effects were observed for plasma cholesterol, triglyceride, free fatty acid or glucose levels at any of the sampling time points, except for a significantly lower plasma triglyceride level in fish fed the Test diet at the SW2 sampling point ($P < 0.05$, Fig. 9 B).

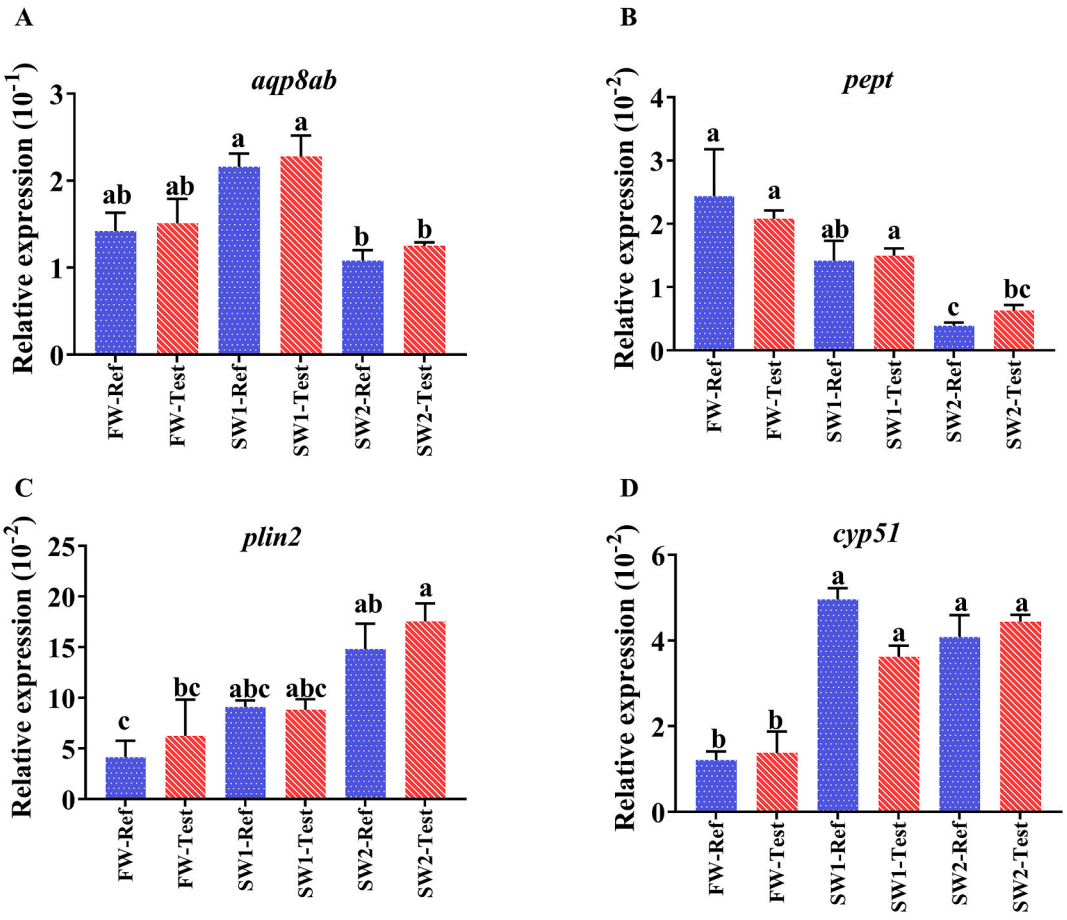

Fig. 7. Expression profile of selected genes in the pyloric caeca of Atlantic salmon fed Ref and Test diets during the observation period. For the explanation of gene abbreviations, see Tables S4–5. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 3) and values sharing the same letters are not significantly different.

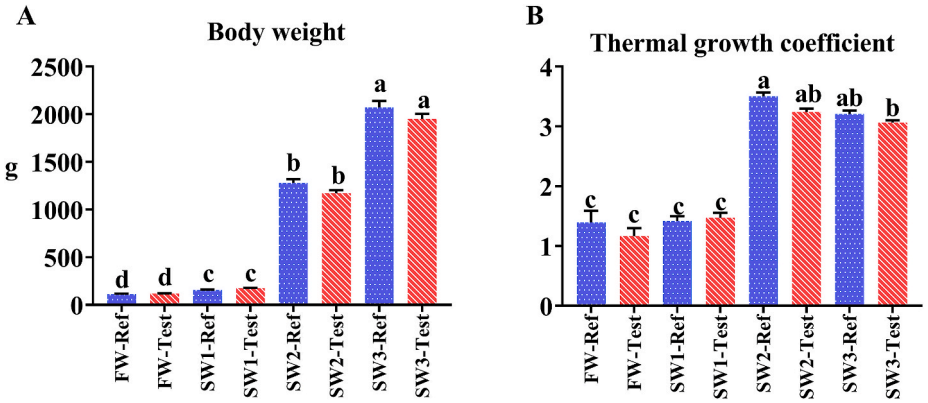

Fig. 8. Growth performance of Atlantic salmon fed Ref and Test diets during the observation period. Data were presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 36) and values sharing the same letters are not significantly different.

Compared to fish from the FW sampling point, fish from the SW had higher plasma chloride and sodium levels ($P < 0.05$, Fig. 9 E and F). A diet effect was only observed at SW2, i.e. higher plasma chloride levels in fish fed the Test diet compared to those fed Ref diet ($P < 0.05$, Fig. 9 E).

4.5. Digesta bile acid concentration and trypsin activity

Compared to fish from other sampling points, fish from SW3 had higher digesta bile salts concentrations in PI 1, PI 2, MI and DI 1 (Table 5). For PI 1 and PI 2, the lowest values were observed in freshwater, while for DI the lowest values were observed right after seawater transfer. In samples from DI 2, no significant differences were observed between life stages. Moreover, no clear diet effect was observed at any of the sampling points (Table 5).

The trypsin activities in intestinal digesta showed no significant differences between sampling points nor between the two diets ($P > 0.05$, Table 5).

Table 4
Condition factor and indices of gut sections in Atlantic salmon fed Ref and Test diets during the observation period[a].

	Condition factor	PI	MI	DI
One-Way ANOVA				
P value	<0.001	<0.001	<0.001	<0.001
Pooled SEM	0.02	0.12	0.01	0.02
Mean values -Tukey-Kramer HSD				
FW-Ref	1.03^{d}	1.6^{e}	0.17^{de}	0.37^{de}
FW-Test	1.04^{d}	1.6^{e}	0.16^{e}	0.32^{e}
SW1-Ref	1.02^{d}	2.4^{d}	0.22^{bcd}	0.49^{bc}
SW1-Test	1.03^{d}	2.2^{d}	0.21^{cde}	0.44^{cd}
SW2-Ref	1.42^{a}	4.8^{a}	0.40^{a}	0.54^{ab}
SW2-Test	1.33^{b}	4.9^{a}	0.38^{a}	0.52^{abc}
SW3-Ref	1.30^{bc}	3.1^{c}	0.25^{bc}	0.49^{bc}
SW3-Test	1.24^{c}	3.7^{b}	0.28^{b}	0.58^{a}

[a] For explanation of sampling point and diet abbreviations see Table 1. PI, proximal intestine; MI, mid intestine; DI, distal intestine. Values with same superscript in a column are not significantly different ($P < 0.05$, condition factor n = 36, organosomatic indices n = 18).

4.6. Brush border leucine aminopeptidase (LAP) capacity

In the PI and DI, the LAP capacity increased significantly after the seawater transfer ($P < 0.05$) and tended to stabilize at a higher level thereafter (Fig. 10). In the MI, the LAP capacity increased slightly and tended to stabilize after the seawater transfer. No clear diet effects on intestinal LAP capacity were observed at any of the sampling points ($P > 0.05$, Fig. 10).

4.7. Management observations

Over the 15-week observation period in freshwater, the body weight in both the fish fed the Ref and Test diet showed average weight of 122 g. The mortality during the freshwater phase was similar for fish fed Ref and Test diets, averaging 9%. The main part of the loss (about 80%) was due to hemorrhagic smolt syndrome (HSS), not uncommonly observed in fish within the smoltification period. At slaughter time, TGC, calculated for all fish in the farm over the whole saltwater phase, showed high values and a trend ($P = 0.085$) towards higher growth for fish fed the Ref diet compared to the Test diet, 3.7 and 3.6, respectively. There was no significant difference between diets regarding FCR ($P = 1.00$). In the seawater period, recorded mortality did not differ significantly ($P = 0.09$) and averaged 8.6% for fish fed the Ref and the Test diet. There were two main events of increased mortality in this period. The first during transport from the freshwater to the seawater site, the second right after seawater transfer which is a frequent observation. Some of this mortality might partly be due to complications from the pre-existing HSS condition. In December, the first winter in seawater, the population was diagnosed with heart and skeletal muscle inflammation (HSMI) which temporarily increased mortality. No outbreaks of parvicapsulosis or teacibaculosis, commonly challenging salmon in the northern regions, occurred in the studied population.

5. Discussion

The main findings of the present work were as follows:

Major, temporary drops were observed in expression of gut immune and barrier function genes four weeks after seawater transfer. Apparent enterocyte hyper-vacuolation in pyloric caeca was observed in samples from FW to SW2 with concurrent increased expression levels of the lipid droplet surface marker *plin2*. No clear indication of beneficial effects was observed throughout the observation period. However, an increased metabolic cost was indicated by lower CF and plasma triglyceride levels, and a tendency to lower growth at SW2 due to the mixture of nucleotides, a prebiotic and essential fatty acids used at this time point.

5.1. Gut immune and barrier functions

Firm conclusions regarding consequences for the fish health, performance of the many alterations in the expression of gut immune and barrier related genes two weeks before seawater transfer and four weeks after, cannot be made. However, existing literature indicates that most

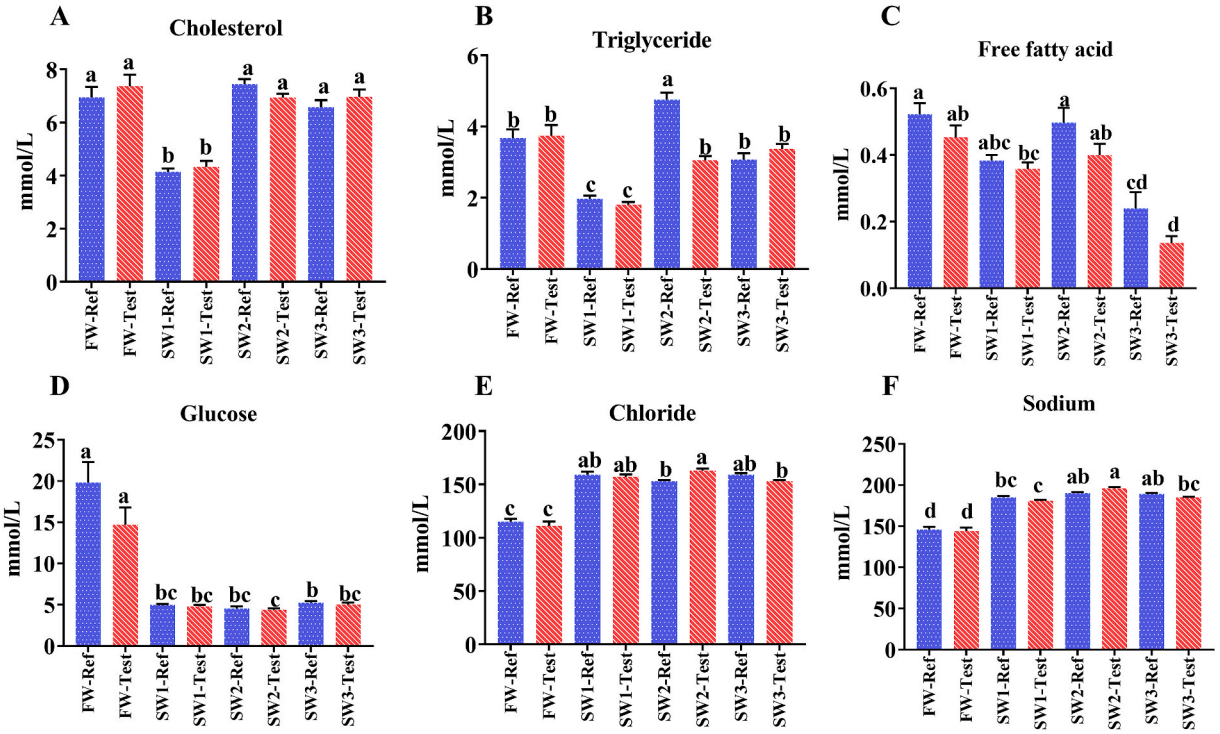

Fig. 9. Plasma biomarkers of Atlantic salmon fed Ref and Test diets during the observation period. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 36) and values sharing the same letters are not significantly different.

Table 5
The total bile acid concentration and trypsin activity in the digesta of Atlantic salmon fed the Ref and Test diets during the observation period[a].

	Total bile acid levels (mg/g dry matter)					Trypsin activity (U/mg dry matter)				
	PI 1	PI 2	MI	DI 1	DI 2	PI 1	PI 2	MI	DI 1	DI 2
One-Way ANOVA										
P value	<0.001	<0.001	0.002	0.002	0.15	0.05	0.073	0.18	0.46	0.38
Pooled SEM	15.4	12.5	6.9	4.1	2.0	44.8	31.1	24.7	16.6	10.6
Mean values-Tukey-Kramer HSD										
FW-Ref	142^{c}	131^{b}	94^{c}	28^{bc}	9	196	203	172	94	27
FW-Test	152^{c}	126^{b}	106^{bc}	21^{bc}	8	201	203	166	113	49
SW1-Ref	187^{bc}	167^{ab}	108^{abc}	16^{c}	5	252	328	180	61	14
SW1-Test	171^{c}	177^{ab}	107^{bc}	16^{c}	5	334	338	228	74	23
SW2-Ref	174^{c}	144^{b}	97^{c}	29^{b}	14	316	238	107	70	46
SW2-Test	194^{bc}	155^{b}	98^{c}	30^{b}	13	340	246	126	74	46
SW3-Ref	292^{a}	233^{a}	157^{a}	47^{a}	9	401	235	164	105	41
SW3-Test	265^{ab}	229^{a}	133^{ab}	41^{a}	9	373	267	142	100	27

[a] The explanation of sampling point and diet abbreviations see Table 1. PI 1, the proximal half of the proximal intestine; PI 2, the distal half of the proximal intestine; MI, mid intestine; DI 1, the proximal half of the distal intestine; DI 2, the distal half of the distal intestine. Values with same superscript in a column are not significantly different ($P < 0.05$, n = 3).

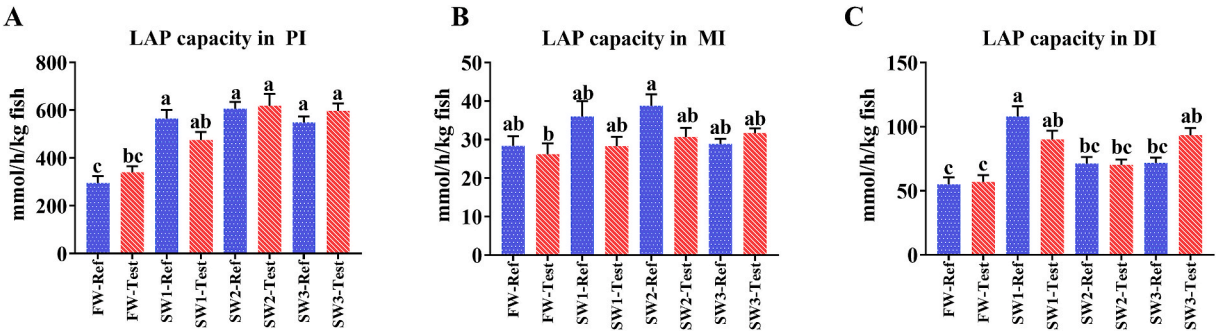

Fig. 10. Leucine aminopeptidase (LAP) capacity of Atlantic salmon fed Ref and Test diets during the observation period. PI, proximal intestine; MI, mid intestine; DI, distal intestine. Data are presented as mean ± SEM. Different letters among values denote significant differences ($P < 0.05$, n = 18) and values sharing the same letters are not significantly different.

of the alternations seen after seawater transfer weaken disease resistance and production [15,30,31]. The mucus layer, with its mucins, acts as the first immune barrier [32]. Downregulation of *muc13* in DI just after seawater transfer, as observed in our study indicates a reduction of goblet cells, since the gene *muc13* codes for the main sialomucin and its downregulation expression has been observed to be accompanied by reduced goblet cells [33,34]. Also, the downregulation of *muc13* might render the tissue more prone to inflammation, as supported by the study in mice that *muc13* prevents intestinal inflammation by inhibiting epithelial cell apoptosis [35].

The observation in our study of down-regulation of expression of pro-inflammatory (*ifnγ* and *il1β*) and anti-inflammatory cytokines genes (*tgfβ* and *il10*) after seawater transfer may have important implications for the disease resistance of fish, since inflammatory cytokines play critical roles in intestinal immune homeostasis [36,37]. Decreased expression of inflammatory cytokines genes may be expected to induce tissue damage due to overreaction of inflammatory response [38–41]. However, this did not appear to be the case in our situation as no clear inflammation signs was observed in the present study. The suppression of pro-inflammatory cytokines genes may be due to the modulation of the *myd88*-independent pathway [42], observed as lower expression of *myd88* in DI after seawater transfer in our study. Regarding anti-inflammatory cytokines, decreased expression of *tgfβ* usually corroborates with *il10* downregulation, also observed in our study, working as a regulatory cytokine in T-cells [43]. T-cell barrier-disrupting effect can be mediated through decreased counteraction of anti-inflammatory cytokines, e.g. *tgfβ* [44,45], as found in our study in which T cell markers, i.e. *cd3γδ* and *cd8β*, revealed lower expression levels at SW1. The activation of T cells may be suppressed by *gilt* indicating that lower *gilt* expression possibly takes place in the course of sensitization of T cells [45,46]. These results are suggested to support the hypothesis that the decreasing gene expression of T cell markers may related to the reduction of T cells or intestinal epithelial cell loss [45]. The lower expression levels of immune-related genes at SW1 may be an unavoidable effect of all the physiological challenges related to stress due to transporting, handling and exposure to new pathogens, as well as great changes in environmental conditions, such as alteration in water temperature and salinity [47–49]. It is not unlikely that the observed alteration in expression of immune genes are important factors in the mechanisms underlying the increased mortality observed soon after seawater transfer [50,51].

The decrease in expression of *zo-1* and claudins after seawater transfer is line with earlier studies [52,53]. Also, the work of [54,55] showed rapidly changing intestinal permeability and translocation rates after seawater transfer which may cause disturbances in barrier functions and suppressed immune defense mechanisms. As mentioned above, the triggers of the changes in immune and barrier functions just after seawater transfer may be both physiological, e.g. related to the smoltification processes, and farming conditions, such as feed deprivation, handling stress, rapidly changing water temperature, water salinity and other environmental factors [55–57]. The dominating claudins, i.e. *claudin-15* and *claudin-25b*, in the intestine of Atlantic salmon are involved in the reorganization of the intestinal epithelium and may affect paracellular permeability during seawater acclimation [58,59]. Hence, the decreased *claudin-15* and *claudin-25b* expression levels after seawater transfer, which is accompanied by increase in paracellular permeability, might also be explained by natural physiological responses for osmoregulation during seawater adaption rather than the

loss of the intestinal immune function with implications for the immune apparatus.

As the fish developed towards SW2 and SW3, most of the biomarkers which were down regulated just after sea transfer, increased to the level observed in fish from FW. This observation suggests that immunological adaption to seawater environment was reached at a stage between SW1 and SW2, i.e. more than four weeks after seawater transfer, as supported by an earlier study [15] that recovery time of the immune-related gene expression to pre-transfer levels should take more than three weeks.

5.2. Variation in PC hyper-vacuolization

The hyper-vacuolization in the PC observed in our study at SW1 and SW2, indicates insufficient lipid transport capacity across the mucosa at these time points [60,61]. These observations corresponded with the increases in *plin2* (adipophilin) expression, a marker for lipid droplets and lipid accumulation, and shunting of lipid into storage vacuoles [62]. Similar correspondence between pyloric caeca hyper-vacuolization and *plin2* expression has been observed in salmon previously [43]. In the current study, we also observed an increased expression of *cyp51*, which is involved in de novo synthesis of cholesterol, another component involved in lipid transport. The apparent hyper-vacuolization of PC observed at the two sampling time points may have different causes. At SW1, the lipid accumulation may be related to the generally slower level of several biomarkers and functions, e.g. the low plasma cholesterol levels, which might lead to reduced capacity for lipid transport, and possibly other factors of importance for lipid transport. Deficiency of nutrients, due to low feed intake, may also be a factor. The hyper-vacuolization observed at the SW2 sampling, one the other hand, may be due to high feed intake and therefore high fat intake concomitant with insufficient supply of components essential for lipid transport, e.g. choline [63]. Until recently, and probably still, commercial salmon feeds, mainly based on plant ingredients, have been deficient in choline for efficient transport of lipid across the mucosa when feed intake is high [63,64].

5.3. Effects of functional ingredients

Several reviews [7,65,66] conclude that inclusion of single or mixtures of functional ingredients, for example, nucleotides, prebiotics and immunostimulants, will improve growth or health of fish during stressful farming conditions, such as seawater transfer and critical life stages. In our study, fish fed the Test diet, with selected functional ingredients, tended to grow slower at SW2 compared to those fed Ref diet. At this sampling point, fish were fed diet with nucleotides, yeast cell walls and essential fatty acids and showed reduced condition factor and plasma triglyceride level, indicating lower lipid content in the body, which would be expected to be due to increased energy demand and consequently reduction in energy efficiency. Overall, the present results indicate that these selected functional ingredients, used during the production cycle, may represent a metabolic cost for the fish. Our findings are in line with previous reports of decreased efficacy of immune-stimulants, including yeast cell walls, after long-term oral administration to fish [67].

The lack of effects of functional ingredients on expression of immune genes and other health indices in our study, was unexpected in light of the results of a multitude of controlled feeding experiments, typically demonstrating strong immune-modulating effects of such components when included in animal feed. It is, however, known that, such effects depend on several factors, including the characteristics of the functional ingredient itself, timing and duration of administration, species and life stage [7,65,66]. One explanation for the apparent discrepancy between our observations and the majority of available scientific literature may therefore be related to the fact that the present experiment was conducted under Arctic conditions, which have never been assessed in controlled laboratory experiments. Another factor may be that the accuracy of the estimates of our study was not as high as in controlled feeding experiments, which in turn may be able to detect smaller differences than the present experiment. The long-term use of functional ingredients throughout the production cycle, is another possible reason for the lack effects on the immune biomarkers [68]. The present experiment reveals a great need for further studies of effects of functional ingredients under commercial conditions in the Arctic region to find whether they are useful or not, and how they should be used if useful.

5.4. General performance

Our observations of low growth performance and body indices at FW and SW1 are in line with previous reports of poor growth performances in shorter time periods before [69,70] and after [71,72] seawater transfer. The poor growth performance is assumed to be related to the demanding smoltification process that takes place in this period as well as stress due to handling and the exposure to new seawater environment, which affect osmoregulation and may reduce feed intake [73,74]. Overall, these adaptation processes imply a higher energy requirement during this developmental stage, for which the fish do not seem to be able to compensate by adjusting their feed intake [10,75,76]. Our observations of lower plasma nutrient concentrations, such as triglyceride and cholesterol, in fish at SW1, support this consideration.

The observation of stable plasma chloride and sodium levels at all the SW sampling time points, at higher levels than observed for FW, are in agreement with the results of previous studies [9,10,77,78]. The results indicate that the fish were well adapted to new seawater environment four weeks after seawater transfer. Aquaporins, especially *aqp8ab*, also play a central role in osmoregulation through trans-epithelial water transport [8,79,80]. One previous study found the expression levels of *aqp8ab* in the intestine was elevated after seawater transfer [8]. These observations were in line with our study that increasing expression levels of *aqp8ab* in DI were observed throughout the production cycle, from the lowest level at FW, to the highest level at SW3, strongly suggesting the importance of *aqp8ab* in regulation of intestinal transcellular uptake of water during seawater acclimation, as also supported by previous studies in Atlantic salmon [81] and Japanese eels [82].

6. Conclusion

The processes taking place from two weeks before to four weeks after seawater transfer caused large reductions in plasma nutrient content and intestinal immune and barrier functions. Apparent enterocyte hyper-vacuolization in pyloric caeca was observed in samples from SW1 to SW2, while there were no clear signs of inflammation of the distal intestine which looked healthy throughout the observation period. Functional ingredients, used throughout the observation period, did not show beneficial effects, but seemed to represent a metabolic cost for the fish.

CRediT authorship contribution statement

Jie Wang: Formal analysis, Experiment design, Sampling, Analyses, Writing, Writing - original draft. **Trond M. Kortner:** Formal analysis, Experiment design, Sampling, Analyses, Writing - review & editing, Supervision. **Elvis M. Chikwati:** Formal analysis, Analyses, Writing - review & editing. **Yanxian Li:** Sampling, Writing - review & editing. **Alexander Jaramillo-Torres:** Sampling, Writing - review & editing. **Jan Vidar Jakobsen:** Writing - review & editing, Funding acquisition. **Jarle Ravndal:** Writing - review & editing, Funding acquisition. **Øyvind Jakobsen Brevik:** Experiment design, Writing - review & editing, Funding acquisition. **Olai Einen:** Writing - review & editing, Funding acquisition. **Åshild Krogdahl:** Experiment design, Sampling, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.09.019.

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