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Choline and phosphatidylcholine, but not methionine, cysteine, taurine and taurocholate, eliminate excessive gut mucosal lipid accumulation in Atlantic salmon (*Salmo salar* L)

Åshild Krogdahl[a], Anne Kristine Grostøl Hansen[a,b,*], Trond M. Kortner[a], Ingemar Björkhem[c], Aleksei Krasnov[d], Gerd M. Berge[e], Vegard Denstadli[b]

[a] Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), P.O. Box 369 Sentrum, N-0102 Oslo, Norway
[b] Biomar AS, Havnegata 9, 7010 Trondheim, Norway
[c] Karolinska University Hospital, Department of Laboratory Medicine, Division for Clinical Chemistry, 14152 Huddinge, Sweden
[d] Nofima AS, P.O. 210, N-1431 Ås, Norway
[e] Nofima AS, Sjølseng, N-6600 Sunndalsøra, Norway

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ABSTRACT

Excessive enterocyte lipid accumulation, with the suggested term lipid malabsorption syndrome (LMS), is frequently observed in Atlantic salmon (*Salmo salar* L), in small fish in fresh water as well as in large fish in seawater. The symptoms indicate insufficient supply of components involved in lipid assimilation. The questions addressed in the present work were whether dietary supply of components involved in phospholipid and sterol metabolism might prevent LMS. Atlantic salmon (35 fish, 330 g per 600 L tank) were fed a low fish meal diet (LF) as such or supplemented with taurocholate at two levels (3.5 and 6.9 g/kg), cholesterol (2.0 g/kg), taurine (0.8 g/kg), phosphatidylcholine (15.1 g/kg), choline (3.7 g/kg), cysteine (0.8 g/kg) or methionine (1.0 g/kg). A high fish meal diet (HF) was also included. The overall growth rate of the fish was high (TGC > 4.2) with no significant effects of diet. Fish fed the LF diet showed increased relative weight of the pyloric and mid intestine and excessive lipid accumulation in the enterocytes, characteristics which were nearly absent in fish fed the HF diet and the LF diet supplemented with choline and phosphatidylcholine. The phosphatidylcholine supplemented diet showed significantly higher lipid digestibility than the LF diet. None of the other supplements eliminated the signs of excessive enterocyte lipid accumulation. Phosphatidylcholine down-regulated *pcyt1a*, involved in the phosphatidylcholine synthesis, and both choline and phosphatidylcholine induced *apoaIV*, important in lipoprotein assembly, and markedly suppressed the lipid droplet marker *plin2*. Methionine supplementation did not stimulate endogenous synthesis of choline. Cholesterol supplementation suppressed sterol uptake and de novo cholesterol synthesis, and induced sterol efflux from the intestinal mucosa. Taurocholate and taurine induced their respective metabolic pathways. All feed supplements, in particular cholesterol and cysteine, down-regulated genes related to antiviral, chemokine, antigen presentation, immunoglobulinfunctions, as well as of extracellular proteases. The results of this study confirm the results from our previous study showing that choline or phosphatidylcholine is a necessary ingredient in low fish meal diets.

1. Introduction

Various gut health challenges, such as excessive enterocyte lipid accumulation, inflammation, neoplasia and ulcers are observed, seemingly, with increasing frequency in cultivated salmon. This development may be related to the change in content of nutrients, non-nutrients and antinutrients in fish diets resulting from the shift in proportion of fish meal and plant ingredients which has taken place over the last decades (Ytrestøyl et al., 2015; Aas et al., 2019). Reduced cholesterol and bile salt levels in digesta and blood are common findings in fish fed diets with high levels of plant components (Kortner et al., 2013; Romarheim et al., 2008; Romarheim et al., 2006).

* Corresponding author at: Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), P.O. Box 369, Sentrum N-0102, Oslo, Norway.
E-mail addresses: Ashild.Krogdahl@nmbu.no (Å. Krogdahl), annha@biomar.com (A.K.G. Hansen), Trond.Kortner@nmbu.no (T.M. Kortner), ingemar.bjorkhem@sll.se (I. Björkhem), Aleksei.Krasnov@Nofima.no (A. Krasnov), Gerd.Berge@Nofima.no (G.M. Berge), vegde@biomar.com (V. Denstadli).

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Moreover, diets high in plant ingredients contain low levels of phospholipids. The symptoms of excessive lipid accumulation in the pyloric caeca of Atlantic salmon, which are commonly observed in salmon in both fresh water and seawater, and in severe cases result in floating faeces around the sea cages indicate impaired absorption of lipids. The suggested term for the condition is lipid malabsorption syndrome (LMS) (Penn, 2011). The condition raises questions whether disturbances and deficiencies in sterol and phospholipid metabolism may cause LMS.

The work presented herein was conducted to follow up the results of a previous feeding study which aimed to reveal mechanisms underlying effects of dietary supplementation with components involved in lipid and sterol metabolism on gut function and health (Kortner et al., 2016; Kortner et al., 2014). In the former study, a high plant diet was supplemented with either taurocholate (1.8%), a crude mix of bovine bile salts (1.8%), taurine (0.4%), lecithin (1.5%) and cholesterol (1.5%), all key components in lipid and sterol metabolism. The results showed negative rather than positive effects of cholesterol and bile salt supplementation on gut inflammation (Kortner et al., 2016). The reason for this may have been that the levels chosen for bile salts and cholesterol were too high to be physiologically relevant for Atlantic salmon. The basis for the choices was levels used in former studies on rainbow trout by Japanese researchers giving results indicating beneficial effects of such levels (Iwashita et al., 2009; Iwashita et al., 2008). In our previous experiment we also included a crude preparation of phospholipids, i.e. soybean lecithin, observing no clear effect on enterocyte lipid accumulation. However, analyses of the lecithin showed that the content of phosphatidylcholine, the major phospholipid in the lipoproteins transporting lipids from the enterocytes, was very low (Kortner et al., 2016).

Our aim in the present work was therefore to gain more information on effects of various levels of pure taurocholate, the dominating bile salt in Atlantic salmon, and purified phosphatidylcholine, the dominating phospholipid involved in lipid transport across the intestinal mucosa, on gut inflammation and lipid transport. The role of free choline, supplemented as choline chloride, was also studied. Choline is the essential nutrient of phosphatidylcholine. Moreover, we wanted to find if supplementation with methionine, a key substrate in synthesis of choline from ethanolamine, might promote lipid gut mucosal transport. If so, also the level of cysteine, produced from methionine, may play a role for production of choline. Cysteine is also a key substrate/metabolite in the production of taurocholate, and was also included in the study (Schubert et al., 2003). Plant raw materials contains in general less phosphatidylcholine than fishmeal, and a high and low fishmeal diet was also studied. Fig. 1 illustrates the main pathways and components in the supply and metabolism of compounds important in production of phosphatidylcholine and indicates the position and role of the compounds studied in the present work (adapted from Harvey, 2011).

2. Materials and methods

2.1. Experimental diets

Ten experimental diets were formulated: a high fish meal diet (HF), a low fish meal diet (LF), and eight diets based on the LF diet with supplementation of taurocholate at two levels (LF_TC1 and LF_TC2), cholesterol (LF_CH), taurine (LF_TA), phosphatidylcholine (LF_PC), choline (LF_Cl), cysteine (LF_CY) and methionine (LF_ME). The receipts are shown in Table 1A. The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011) and BioMar standards to meet the requirements. Yttrium oxide (0.50 g/kg) was added as inert marker for estimation of nutrient apparent digestibility. The experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin screw extruder (Clextral, France).

2.2. Experimental animals, feeding and rearing conditions

The feeding trial was performed at Nofima's research facility at Sunndalsøra, Norway, a research facility approved by Norwegian Animal Research Authority (NARA), operating in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Atlantic salmon (*Salmo salar* L., post smolt, Sunndalsøra breed) with mean initial weight of 330 g ± 46 (mean ± SD) were pit tagged and randomly assigned to cylindrical fiberglass tanks (1m^3, 600 L), 35 fish per tank. The fish were weighed individually when allocated to the experimental units, to assure similar biomass in all tanks. The diets were allocated randomly to the tanks and two tanks were used per diet. The feeding period lasted 84 days. Each tank was supplied with flow through seawater at a rate of 6–7 L min^{-1} and constant light. During the feeding trial, water temperature decreased gradually from 11.5 to 8.0 °C. Dissolved oxygen in the outlet water was measured daily and was maintained above 80% saturation throughout the experiment. The fish were fed continuously using disc feeders aiming at an excess feeding of 20% (Helland et al., 1996). Feed intake was recorded by collection of spilled feed pellets in the outlet water.

2.3. Trial termination - sampling

After 84 days, feeding was terminated. From each tank 18 fish, randomly selected, were anaesthetized with tricaine methane-sulfonate (MS-222), followed by a sharp blow to the head. Weight and length were recorded for all fish and blood was sampled from the caudal vein in vacutainers with lithium heparin. The vacutainers were stored on ice prior to plasma preparation. Plasma was sampled in 2 mL aliquots and snap frozen in liquid nitrogen and stored at −80 °C. Following blood sampling the fish were opened ventrally. The gastro-intestinal tract was removed from the abdominal cavity, cleared of other organs and adipose tissue, and sectioned as follows: pyloric intestine (PI): the section from the pyloric sphincter to the most distal pyloric caeca; mid intestine (MI) from the distal end of PI and proximal to the increase in intestinal diameter; distal intestine (DI) section from the distal end of MI to the anus. From the first 10 fish,the tissue of the PI and DI, cleared of external fat, was collected and weighed, and tissue from pyloric caeca and PI and DI tissues were sampled for enzyme analyses. Digesta from PI and DI was collected and split in two samples, i.e. the proximal half (PI1 and DI1, respectively) and distal half (PI2 and DI2, respectively) for bile salt analyses. The intestinal samples were snap frozen in liquid nitrogen and stored at −80 °C.

The remaining eight fish per tank, were euthanized prior to sampling of tissue from the pyloric caeca, mid and distal intestines, and liver for histological examination and gene expression analysis. Tissues for histology were fixed in 10% neutral buffered formalin (4% formaldehyde) for 24 h and subsequently transferred to 70% EtOH for storage until processing. Samples for gene expression analyses were rinsed in sterile saline water, submerged in RNAlater®, incubated at 4 °C for 24 h and subsequently stored at −20 °C until analysis. The remaining fish in each tank were stripped for faeces and fed for one more week for an additional stripping in order to collect enough sample for digestibility analysis. Faecal samples were pooled, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Chemical analyses of feed and feaces

Diet and faecal samples were analyzed for dry matter (after heating at l05 °C for 16–18 h), ash (combusted at 550 °C to constant weight), nitrogen (crude protein) (by the semi-micro-Kjeldahl method, Kjeltec-Auto System, Tecator, Höganäs, Sweden), fat (diethyl ether extraction in a Fosstec analyzer (Tecator) after HCl-hydrolysis), starch (measured as glucose after hydrolysis by alpha-amylase (Novo Nordisk A/S,

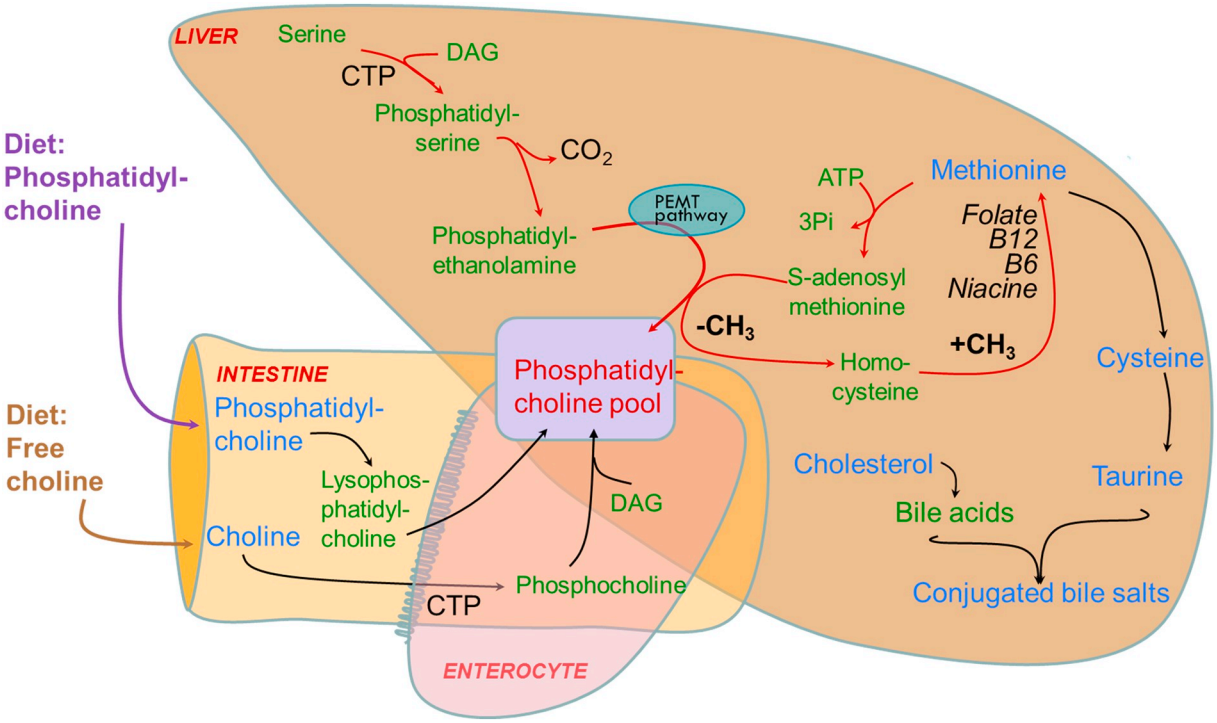


Fig. 1. Main pathways of compounds important for supply of phosphatidylcholine. Phosphatidylcholine supplied by the diet is hydrolyzed to lysophosphatidylcholine in the intestine, absorbed and reesterified to phosphatidylcholine in the enterocyte. Dietary free choline is transported as such through the brush border into the enterocytes, followed by activation via the Kennedy pathway, i.e. by use of both ATP and cytidine triphosphate (CTP) and fusion with diacylglycerol (DAG), producing phosphatidylcholine. Phosphatidylcholine circulating in the blood and bile is also part of the phosphatidylcholine pool. Endogenous synthesis is possible if the necessary substrates are available, i.e. phosphatidylethanolamine which can be produced from serine by incorporation of serine into phosphatidylserine, through the Kennedy pathway, and subsequent decarboxylation to generate phosphatidylethanolamine. However, the Kennedy pathway from serine appears to be insufficiently developed in many animal species, in particular in young individuals. Ethanolamine is converted to phosphatidylcholine after three methylation steps catalyzed by phosphatidylethanolamine methyl transferase (PEMT). The condition for the methylations, is sufficient supply of methionine as methyl donor as well as of the B-vitamins folic acid, cobalamine (B12), pyridoxine (B6) and niacin, necessary for remethylation of the donor molecules. Dietary supply of taurine may also be of importance for the size of the phosphatidylcholine pool, as taurine is needed for conjugation of bile acids, and is produced from methionine, via cysteine. Low supply may reduce availability of methyl groups for formation of choline. Phosphatidylethanolamine can also be supplied from the diet either as such, or as free ethanolamine, and follows the same pathways of absorption as indicated for dietary phosphatidylcholine and choline. In the figure, blue colored compound indicate those investigated in the present study regarding importance for phosphatidylcholine availability in an animal. (Adopted from Harvey R.A., 2011). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bagsvaerd, Denmark) and amylo-glucosidase (Bohringer Mannheim GmbH, Mannheim, Germany), followed by glucose determination by the 'Glut-DH method' (Merck, Darmstadt, Germany)), gross energy (using the Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA), and yttrium (by inductivity coupled plasma (ICP) mass-spectroscopy as described by (Refstie et al., 1997)).

2.5. Plasma analysis

Plasma was analyzed for non-esterified (free) fatty acids (NEFA), total triglycerides, cholesterol and total bile acids following standard procedures at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo.

2.6. Intestinal histology

Evaluation of histological appearance of tissues from PI and DI was performed at the Norwegian University of Life Sciences (NMBU) using standard histological methods. Slides were randomized to ensure blinded examination and evaluated using a light microscope. Proximal intestine (pyloric caeca) tissue samples from four individuals per tank (i.e. eight per diet) were evaluated. Enterocyte hypervacuolation was assessed semi quantitatively, indicating the proportion of total mucosa affected: Score 1 = no hypervacuolation (normal) (≤ 10%); Score 2 = Mild to moderate hypervacuolation, some areas appear normal; (10–25%); Score 3 = Moderate hypervacuolation in almost all areas (25–50%) or Score 4 = Moderate to severe hypervacuolation in almost all areas (clearly abnormal) (≥ 50%). Fig. 2 shows representative pictures of pyloric caeca samples given scores 1 and 4.

Histological appearance of the DI, focusing on indications of processes corresponding to soybean induced enteritis, a scoring system with a scale of 0–10 was used where 0–2.5 represented normal, > 2.5 to 4.5 mild changes, > 4.5 to 6.5 moderate changes, > 6.5–8 marked changes, and > 8–10 severe changes. The scores were categorical variables and the differences between the diets were explored by contingency analysis using the chi-squared test. The following variables were observed: changes in mucosal fold length, width and cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolation, and frequency of goblet cells, intra-epithelial lymphocytes, mitotic figures and apoptotic bodies within the epithelial layer.

2.7. RNA extraction

Based on the results of the histological examination, showing clear effects of diet in the pyloric caeca but hardly any in the distal intestine, we chose to focus the gene expression analyses on the pyloric caeca tissue.

Total RNA was extracted using a Precellys® homogenizer, Trizol® reagent and further purified with PureLink RNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) including an on-column DNase

Table 1A
Feed ingredient composition*.

Ingredients	HF	LF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LF_CI	LF_CY	LF_ME
NA LT fishmeal[a], %	14.78	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
SA SP Sara Rousing[b], %	14.78	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
Soy protein concentrate, %	8.31	19.80	19.80	19.80	19.80	19.80	19.80	19.80	19.80	19.80
Corn gluten, %	4.93	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
Pea protein, %	9.14	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87
Wheat gluten, %	0.00	7.84	7.84	7.84	7.84	7.84	7.84	7.84	7.84	7.84
Beans dehulled, %	12.81	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87
Sunflower exp., %	9.05	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76
Fish oil (Standard)[c], %	7.17	7.63	7.63	7.63	7.63	7.63	7.63	7.63	7.63	7.63
Rapeseed oil, %	16.72	17.80	17.11	17.45	17.60	17.72	16.29	17.43	17.72	17.70
DL-Methionine[d], %	0.21	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
L-Lysine[d], %	0.01	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66
L-Threonine[d], %	0.07	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
L-Histidine[d], %	0.27	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Vit/Min mix, %	0.37	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Mono calcium phosphate, %	1.27	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41
Barox, %	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Yttriuim, %	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Taurocholate (sodium)[d], g/kg			6.9	3.5						
Cholesterol[d], g/kg					2.0					
Taurine (≥99%)[d], g/kg						0.8				
Soy phosphatidylcholine (95%)[e], g/kg							15.1			
Choline chloride (70%)[f], g/kg								3.7		
L-Cysteine[d], g/kg									0.8	
DL-Methionine[d], g/kg										1.0

* HF, high fishmeal diet; LF, low fishmeal diet; LF_TC1 and TC2, supplemented with taurocholate; LF_CH, supplemented with cholesterol, LF_TA, supplemented with taurine; LF_PC, supplemented with phosphatidylcholine; LF_Cl, supplemented with choline chloride, LF_CY, supplemented with cysteine; LF_ME, supplemented with methionine.

[a] NA, North Atlantic, supplied by Norsildmel AS.

[b] SA SP, South American Superprime, supplied by Köster Marine Proteins GmbH.

[c] Supplied by FF Skagen.

[d] Supplied by Sigma-Aldrich.

[e] Supplied by Avanti-INstruchemie.

[f] Dry on vegetable carries, supplied by Balchem.

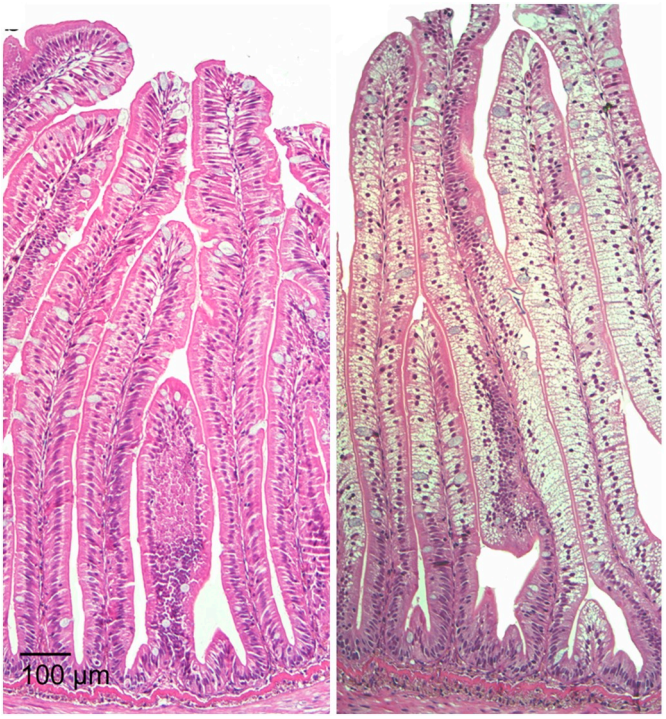


Fig. 2. Histological severity of vacuolation of the pyloric caeca tissue. Pyloric caeca with enterocyte hypervacuolation graded as 1 = no vacuolation/normal (left image) and 4 = moderate to severe hypervacuolation (right image).

treatment. The integrity of the RNA samples was verified by the 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). RNA integrity number (RIN) was > 8 for all samples and average RIN was 9.1, indicative of excellent RNA quality. Total RNA was stored at −80 °C until use.

2.8. Microarrays

A two-colour microarray design was used, where individual fish samples (five in each study group, two to three individuals from each tank duplicate) were labeled with fluorescent Cy3 and hybridized against a common reference sample labeled with fluorescent Cy5. The common reference sample consisted of a pool of equal amounts of RNA from all individual fish included in the analysis. Nofima's Atlantic salmon 15 k oligonucleotide microarray SIQ-6 (GEO accession GPL16555) was manufactured by Agilent Technologies and unless indicated otherwise, the reagents and equipment were from the same source. RNA amplification and labelling were performed with a Two-Colour Quick Amp Labelling Kit and a Gene Expression Hybridization kit was used for fragmentation of labeled RNA. The input of total RNA used in each reaction was 200 ng. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 10 rpm), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix Pro 6.0 was used for spot to grid alignment, assessment of spot quality, feature extraction and quantification. Subsequent data analyses were performed with the bioinformatic system STARS (Krasnov et al., 2011). After filtration of low-quality spots flagged by GenePix, Lowess normalization of $\log_2$-expression ratios (ER) was performed. Genes that

Table 1B
Macronutrient (%), cholesterol, choline and bile salt content of the diets (mg/kg feed), as analyzed.

Diet	HF	LF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LF_Cl	LF_CY	LF_ME
Dry matter	92.9	94.3	95.1	95.7	93.4	95.5	95.4	94.8	95.6	95.6
Ash	7.1	5.9	6.1	6.0	5.9	6.0	6.0	5.8	6.0	5.9
Lipid	29.0	26.0	26.9	26.1	26.6	27.3	28.3	26.4	27.0	27.7
Protein	39.8	41.2	40.0	41.1	40.6	40.7	41.3	40.9	39.2	40.5
Starch	5.3	6.9	6.8	7.0	6.8	6.8	6.5	7.0	7.1	7.0
Choline, free	410	411	403	407	419	408	401	2180	423	393
Choline, total	1860	1190	1180	1170	1190	1180	2870	2980	1200	1160
Cholesterol	1.42	0.70	0.75	0.65	4.45	0.66	0.74	0.63	0.61	0.67
Tot bile salt	274	86	4840	2534	86	86	86	86	86	86

Choline, free = choline not bound to phosphatidylcholine.
Choline, total = both choline bound to phosphatidylcholine and free.

passed quality control in at least four samples per group were included in subsequent analyses. The HF diet and all LF supplemented diets were compared against the LF reference diet group. Differentially expressed genes (DEG) were selected by criteria: $\log_2$ fold difference > 0.8 and $p < .05$ (*t*-test). STARS annotated genes by GO, KEGG and custom vocabulary. Groups of functionally related genes were compared by mean $\log_2$-FC and difference from LF was assessed (t-test, $p < .05$). Complete data files were deposited in NCBI's Gene Expression Omnibus with accession no. xx. (will be published after article acceptance).

2.9. Quantitative real-time PCR

Quantification of pyloric caeca gene expression by quantitative real-time PCR (qPCR) was conducted to validate the microarray results, and to examine selected genes related to lipid and sterol metabolism. Totally, 24 genes involved in metabolism of lipids and bile acids were analyzed (Table S1). Assays were performed according to MIQE standards (Bustin et al., 2009) on eight animals from each diet group (four individuals from each tank duplicate). First strand cDNA synthesis was performed using 0.8 μg total RNA from all samples using Superscript III (Invitrogen, Thermo Fisher Scientific, USA) in 20 μL reactions, and primed with a mixture of Oligo$(dT)_{20}$ and random hexamer primers according to the manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at −20 °C. PCR primers were obtained from the literature or designed using Primer3web software version 4.0.0 (http://primer3.ut.ee/). Detailed information of the primers is shown in Table S1. All primer pairs gave a single band pattern for the expected amplicon of interest in all reactions. PCR reaction efficiency (E) for each gene assay was determined using 2-fold serial dilutions of randomly pooled cDNA. Expression of individual gene targets was analyzed using the LightCycler 480 (Roche Diagnostics). Each 10 μL DNA amplification reaction contained 2 μL PCR-grade water, 2 μL of 1:10 diluted cDNA template (corresponding to 8 ng total RNA), 5 μL of LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 μL (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC). The three-step qPCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (10 s), 60 °C (10 s) and 72 °C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The PCR products were evaluated by analysis of melting curve and by agarose gel electrophoresis to confirm amplification specificity. For target gene normalization, *actb, ef1a, gapdh* and *rps20* were evaluated for use as reference genes by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variance (Kortner et al., 2011). The graph showed a stable expression pattern and was therefore used as normalization factor. Relative expression of target genes was calculated using the $^{\Delta\Delta}C_q$ method (Livak and Schmittgen, 2001).

2.10. Calculations

Crude protein (CP) was calculated as N x 6.25. Thermal-unit growth coefficient (TGC) was calculated as: $TGC = (FBW^{1/3} - IBW^{1/3}) \times (\Sigma D^{\circ})^{-1}$, where IBW and FBW are the initial and final body weights (tank means) and ΣD° is the thermal sum (feeding days x average temperature in °C). Feed efficiency ratio (FERdm) was calculated as: (FBW-IBW)/FIdm where FIdm represents feed intake per dry matter. Organosomatic indices were calculated as percentages of the weight of the organ in relation to body weight. Apparent digestibility (AD) was estimated by the indirect method using Y_2O_3 as an inert marker (Austreng et al., 2000) and calculated as: $AD_N = 100 - [100 \times (M_{feed}/M_{faeces}) \times (N_{faeces}/N_{feed})]$ where M_{feed} and M_{faeces} are percent concentration of the inert marker (Y_2O_3) in feed and faeces, respectively and N_{feed} and N_{faeces} represent percent concentration of a nutrient in feed and faeces, respectively.

2.11. Statistical analyses

Data was analyzed using one-way ANOVA followed by Duncan's multiple range test for post hoc comparison, unless otherwise noted. Tank means (i.e. the mean of all individuals per tank) were used as the statistical unit. For histological results the scores generated were categorical variables and the differences between the diets were explored by contingency analysis using the chi-squared test.

3. Results

The fish appeared healthy throughout the feeding period, and no mortality was recorded.

3.1. Diet composition, including cholesterol and bile salt level

The analyzed content of nutrient in the diets showed results close to the intended composition (Table 1B). As expected, diet cholesterol level was higher in the HF (1.4 g/kg) and the LF_CH (4.4 g/kg) diet, than the LF basal diet (0.7 g/kg). The HF diet contained much more bile salts than the LF diet, and the T_CA supplemented diet clearly deviated from the others as intended. The diet differences reflected the level of cholesterol and bile salts of the ingredients and the supplementation.

3.2. Effects on feed growth and nutrient digestibility

Fish growth rates and feed efficiencies are shown in Table 2. Growth, as indicated by TGC, was in general very high for all treatments. The differences between the treatments did not reach significance. The highest growth was observed for fish fed the LF_PC diet.

Apparent digestibility of lipid is shown in Table 2. Lipid digestibility was in general high for all diets. Phosphatidylcholine supplementation to the LF diet (LF_PC) increased lipid digestibility significantly

Table 2
Growth (TGC), feed efficiency ratio based on feed dry matter (FEdm) and apparent digestibilities.

Diet	TGC	FERdm	Apparent digestibility, %		
			Crude lipid	Crude protein	Starch
HF	4.39	1.29	96.9^{abc}	85.9^{d}	73.8^{a}
LF	4.22	1.35	95.7^{bcd}	89.4^{bc}	63.2^{bcde}
LF_TC1	4.18	1.31	97.4^{ab}	89.9^{abc}	68.2^{b}
LF_TC2	4.15	1.30	97.7^{ab}	90.4^{a}	65.7^{bcd}
LF_CH	4.31	1.36	96.8^{abc}	89.2^{c}	59.6^{ef}
LF_TA	4.25	1.30	94.3^{d}	90.2^{a}	67.5^{bc}
LF_PC	4.53	1.30	98.1^{a}	90.1^{ab}	57.0^{f}
LF_CI	4.21	1.28	96.8^{abc}	90.3^{abc}	60.9^{def}
LF_CY	4.15	1.37	97.3^{abc}	89.8^{abc}	67.5^{bc}
LF_ME	4.08	1.36	96.6^{abc}	89.9^{abc}	62.4^{cde}
P(model)	0.0768	0.3397	0.0412	< 0.0001	0.0005
Pooled SEM	0.081	0.040	0.64	0.23	1.56

TGC (thermal-unit growth coefficient) = $(FBW^{1/3} - IBW^{1/3}) \times (\Sigma D^{\circ})^{-1}$; FER (feed efficiency ratio) = (FBW-IBW)/FIdm. For explanation of diet codes see Tables 1A and 1B.

compared to the LF reference and showed a result similar to the HF diet. All diets, except the LF diet, showed significantly higher lipid digestibility than the diet supplemented with taurine (LF_TA). None of the other treatments differed significantly regarding lipid digestibility. Compared to the LF treatment, crude protein digestibility increased significantly by dietary supplementation with the lower level of taurocholate (LF_TC2), and with taurine (LF_TA) (Table 2). None of the other supplementations caused significant differences compared to the LF. The lowest protein digestibility was observed for the HF diet and the result was significantly lower than for all other treatments. Among fish fed the LF based diets, significant reduction in starch digestibility was observed for LF_PC treated fish (Table 2). No other significant differences were observed between these treatments. For the HF fed fish, starch digestibility was significantly higher than for all the other treatments, supposedly due to the lower starch level in the HF diet.

3.3. Organosomatic indices

Relative organ weights (organosomatic indices, OSI) of the pyloric (PI), mid (MI) and distal (DI) intestines, as well as liver are shown in Fig. 3. Somatic indices for PI and MI differed significantly between diet groups. The OSI for PI was significantly higher in fish fed LF in comparison with the HF diet. The tissues with high somatic indices also had a whitish and foamy appearance. Among the fish groups feda supplemented LF diet, those fed choline and phosphatidylcholine showed significantly lower OSI for PI, as well as normal colouration and texture compared to the groups fed the other LF diets. Also compared to fish fed the HF diet, the LF_PC and LF_Cl groups showed lower OSI for PI. The OSI for MI showed a similar effect of diet as OSI for PI, whereas no dietary effect was observed for OSI of the DI. The greatest difference in liver OSI was observed between fish fed LF diet and those fed the LF_PC and LF_CL diets, and the difference was close to significant ($p = .0530$).

3.4. Chyme bile salt concentration along the intestine

No difference was observed in chyme bile salt concentration along the intestine between the HF and LF fed fish (Table 3). Fish fed the LF diets supplemented with bile salt showed elevated chyme bile salt concentration. Compared to the LF diet, the LF_TC1 diet caused significantly elevated levels in PI2, whereas both these bile salt supplemented diets produced elevated bile salt levels in DI1 and DI2. In the two distal most intestinal sections, cholesterol supplementation, LF_CH, elevated chyme bile salt concentration significantly. Moreover, phosphatidylcholinesupplementation increased bile salt concentration in DI1, and the same trend was observed in DI2.

3.5. Histology

The degree of vacuolation of the pyloric caeca were significantly lower in fish fed the HF, LF_PC and LF_Cl diets compared to all the other treatments (Fig. 4). No significant differences were observed between the HF, LF_PC and LF_Cl groups. The fish fed the other diets showed moderate to severe vacuolation, with no significant difference between the groups. The DI sections of all fish showed morphological appearance typical of healthy DI mucosa, except for one individual.

3.6. Blood plasma biochemistry

Blood plasma was analyzed for free fatty acids, triglycerides, cholesterol and total bile salts (Table 4). The plasma cholesterol was significantly higher in fish fed the LF_CH diet compared to all the other treatments except for LF_PC. No significant differences were observed for the other indicators analyzed.

3.7. Pyloric caeca microarray

The LF diet was used as a reference for comparison with other diets. The numbers of DEG, which reflect the magnitude of transcriptome responses to additives ranged from 25 (LF_TC1) to 171 (CF_CH) (Table 5) (See Table S2 for the list of all DEGs). The difference between the HF and the LF diet control was small – only 42 DEG. Several functional groups of immune genes showed coordinated expression changes being down-regulated in fish given the supplemented diets (Fig. 5). Of note is that in HF fed fish expression of these genes was also significantly lower than in LF fed fish. The largest group (50 DEG, Fig. 5) was innate antiviral immunity related, which included a number of emblematic markers of viral infections, such as *mx, viperin, ifn-induced protein 44* and *very large inducible GTPases*. The metabolic responses were relatively small but some of them might have functional consequences. All LF diets except LF_TA caused down-regulation of a small set of extracellular proteases (*chymotrypsin b*, *carboxypeptidase a2*, *proproteinase e*, *duodenase* and *elastase*) and their expression further decreased for diets with additives (Fig. 6A). Nine genes with the key roles in terpenoid and steroid biosynthesis were down-regulated by LF_CH (Fig. 6B) suggesting suppression of the entire pathway.

3.8. Pyloric caeca qPCR

Gene expression profiles were further studied with PCR (qPCR) focusing on genes involved in biosynthesis and transport of fatty acids, cholesterol, bile acids and phospholipids. Results are presented in Fig. 1A–C. In general, the qPCR results reflected alterations in lipid and sterol metabolic pathways. The strongest responses were seen in the LF_CH, LF_PC and LF_Cl groups. In accordance with the microarray data, a clear transcriptional suppression of cholesterol uptake and biosynthesis was observed for fish fed the LF_CH diet (Fig. 7A). The cholesterol influx transporter *npc1l1* was down-regulated, whereas the apical efflux transporter *abcg5* was induced, indicative of possible reduction of cholesterol uptake from the gut. The marked down-regulation of cholesterol biosynthesis was confirmed by reduced levels of the enzymes IPP synthase (*idi1*) and *cyp51* as well as the controlling transcription factor *srebp2*. On the other hand, cholesterol supplementation produced increased expression levels of the fatty acid synthesis transcription factor *srebp1*.

Alterations of genes involved in lipid metabolism were also observed for the two choline supplemented groups (LF_PC and LF_Cl) (Fig. 7B and C). The rate-limiting enzyme in the phosphatidylcholine synthesis (*pcyt1a)* was down regulated by phosphatidylcholine inclusion, and a similar trend was observed for choline kinase (*chk*) and *pemt*. An interesting finding was the clear down-regulation of a proposed marker for lipid load of non-adipogenic cells, *adipophilin/perilipin 2* (*plin2*) in both choline supplemented groups and the HF group.

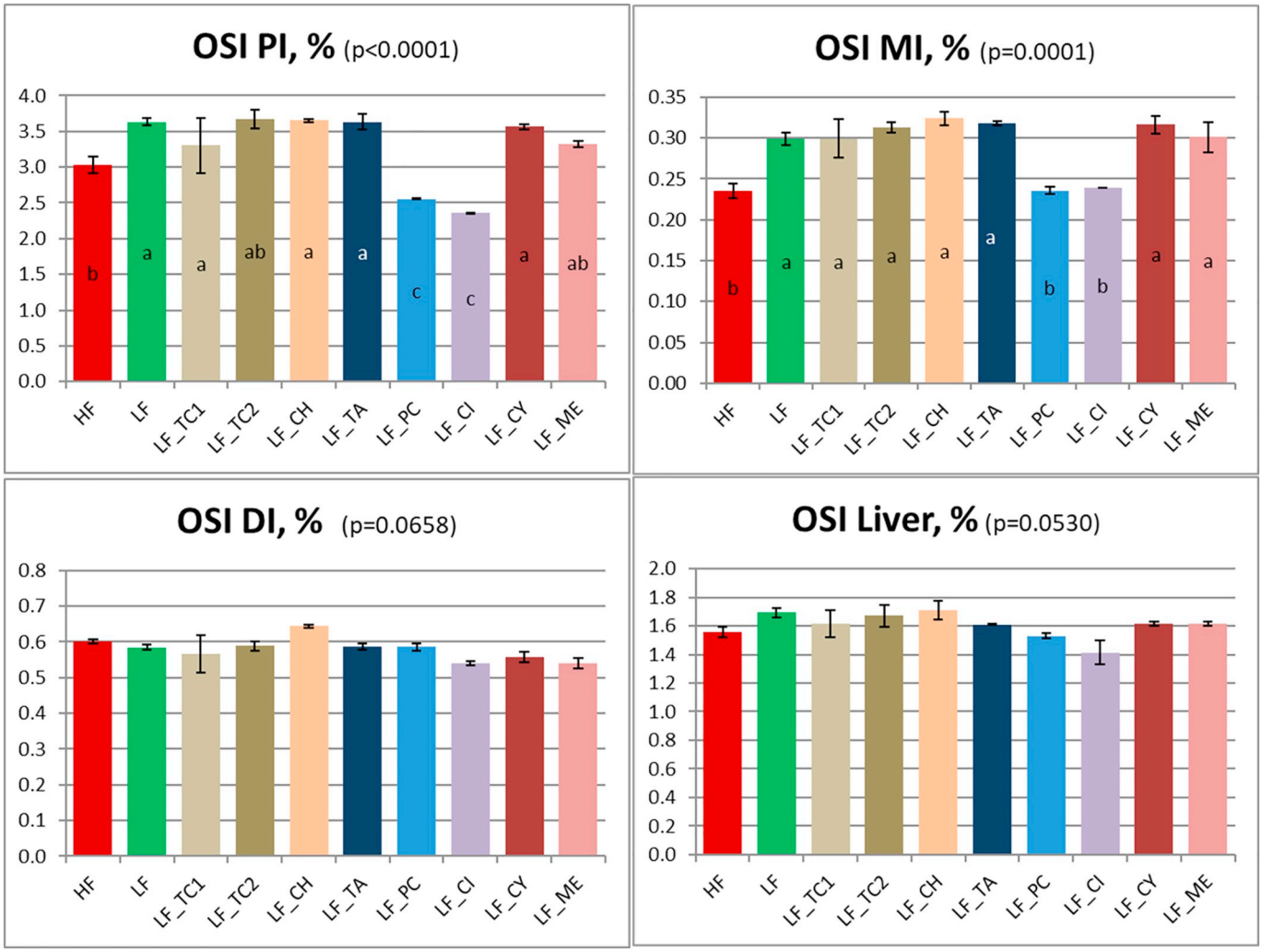


Fig. 3. Gut and liver somatic indices (OSI). PI = pyloric intestine; MI = mid intestine, DI = distal intestine. Different letters denote significant differences between diet groups. Error bars indicate SEM.

Table 3
Bile salt concentration in chyme along the intestinal tract, mg/g dry matter.

Diet	PI1	PI2	MI	DI1	DI2
HF	228	170^{b}	127	52de	11^{b}
LF	212	149^{b}	121	39^{e}	5^{b}
LF_TC1	259	234^{a}	154	77^{a}	19^{a}
LF_TC2	249	190^{b}	154	74^{ab}	18^{a}
LF_CH	219	165^{b}	132	71^{abc}	18^{a}
LF_TA	219	187^{b}	132	53de	8^{b}
LF_PC	212	182^{b}	155	58^{bcd}	11^{b}
LF_CI	208	190^{b}	156	56^{cde}	10^{b}
LF_CY	188	160^{b}	143	44de	6^{b}
LF_ME	251	177^{b}	129	56^{cde}	12^{ab}
P (model)	0.1923	0.0313	0.7225	0.0039	0.0041
Pooled SEM	12.5	9.4	13.0	4.7	1.6

PI = pyloric intestine; MI = mid intestine, DI = distal intestine. For explanation of diet codes see Tables 1A and 1B.

Phosphatidylcholine inclusion also significantly up regulated both *apoaIV* and *apoB*, involved in lipoprotein assembly. A similar trend was seen for *mgat2a*, the fatty acid transporter *fabp2* and the connected transcription factors *ppara* and *pparγ*. Choline significantly induced the expression of *apoaIV*. Genes involved in cholesterol metabolism were

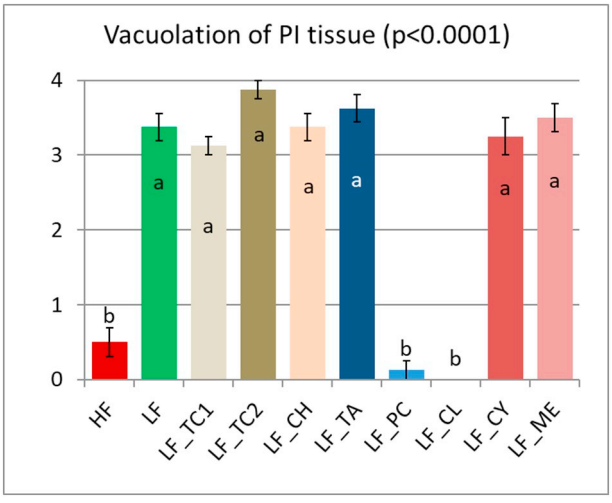


Fig. 4. Degree of vacuolation of PI tissue. The columns indicate average score for fish fed the different diets. Score 1 = Normal, Score 4 = Moderate to high vacuolation. Different letters denote significant differences between diet groups. Error bars indicate SEM.

Table 4
Blood indicators of lipid and sterol metabolism.

Diet	FFA, mM	Cholesterol, mM	TG, mM	Bile salts, uM
HF	0.30	10.3[b]	3.5	14
LF	0.27	8.8[b]	2.9	20
LF_TC1	0.42	9.8[b]	2.9	35
LF_TC2	0.36	9.2[b]	3.2	34
LF_CH	0.41	13.6[a]	3.2	30
LF_TA	0.41	9.0 [b]	3.2	34
LF_PC	0.26	11.7[ab]	4.1	20
LF_CI	0.36	10.6[b]	3.5	26
LF_CY	0.31	8.8[b]	3.1	20
LF_ME	0.38	8.8[b]	3.2	25
P(model)	0.8764	0.0255	0.7433	0.4438
Pooled SEM	0.08	0.86	0.40	6.54

FFA = free fatty acids. TG = triglycerides. For explanation of diet codes see Tables 1A and 1B.

also significantly affected in fish fed the choline enriched diets, indicative of increased cholesterol uptake and/or synthesis. Phosphatidylcholine significantly induced the cholesterol transporter *abcg5* and the master regulator *srebp2*. Choline induced the expression of the two cholesterol biosynthetic enzymes *idi1* and *cyp51* in addition to *srebp2* (Fig. 7A).

For the other supplemented diets few significant changes in gene expression were seen. The two taurocholate groups, LF_TC1 and LF_TC2 showed stable transcript profiles for the selected genes, in accordance with microarray data. Induced expression of *idi1* and *cyp51* was seen for the LF_CY diet (Fig. 7A), which could indicate an increased capacity for cholesterol biosynthesis. Some differences between the LF and the HF basal diets were seen, which could reflect the different degree of lipid accumulation between these two diet groups. As previously noted, the lipid load marker *plin2* was clearly induced in the LF diet as compared to the HF diet. In contrast, reduced levels of expression of apolipoproteins (*apoaI, apoaIV, apoB*) were observed (Fig. 7B).

4. Discussion

The discussion below is organized as follows. Firstly, the results for the fish fed the LF diet are compared to the results for those fed the HF diet. Thereafter effects of the individual supplements are discussed.

4.1. LF versus HF diet

The 50% lower cholesterol and 30% lower bile salt content of the LF diet compared to the HF diet did not induce significant differences in plasma cholesterol, plasma bile salt level or sterol and bile-related gene expression, indicating that the body's endogenous cholesterol synthesis and further conversion to bile acids compensated for the lower supply to fish fed the LF diet. These findings are in line with the results of an earlier study comparing diets varying in plant ingredients (Kortner et al., 2016). The absence of effects on plasma free fatty acids, triglycerides and bile salts are in line with the results of the study by Kortner et al. (2016). However, in earlier feeding experiments with salmon, major drops in plasma cholesterol and bile salt have been observed in fish fed diets with high inclusion of plant ingredients compared fish fed diets high in fish meal, in particular when soybean meal has been included (Kortner et al., 2013; Romarheim et al., 2008; Romarheim et al., 2006). Varying dietary levels of compounds with the ability to compete with cholesterol for absorption, such as phytosterols and saponins, may be the explanation for the difference between experiments (Krogdahl et al., 2015). Another point to keep in mind is that fish meal level in commercial salmon diets has dropped significantly the last 10–20 years. In earlier studies the fishmeal control diets contained typically around 50–60% of fish meal. Today diets with around 30% of fishmeal represents a high fishmeal control diets, based on today's practical salmon diets.

The macroscopically whitish and foamy appearance of the pyloric

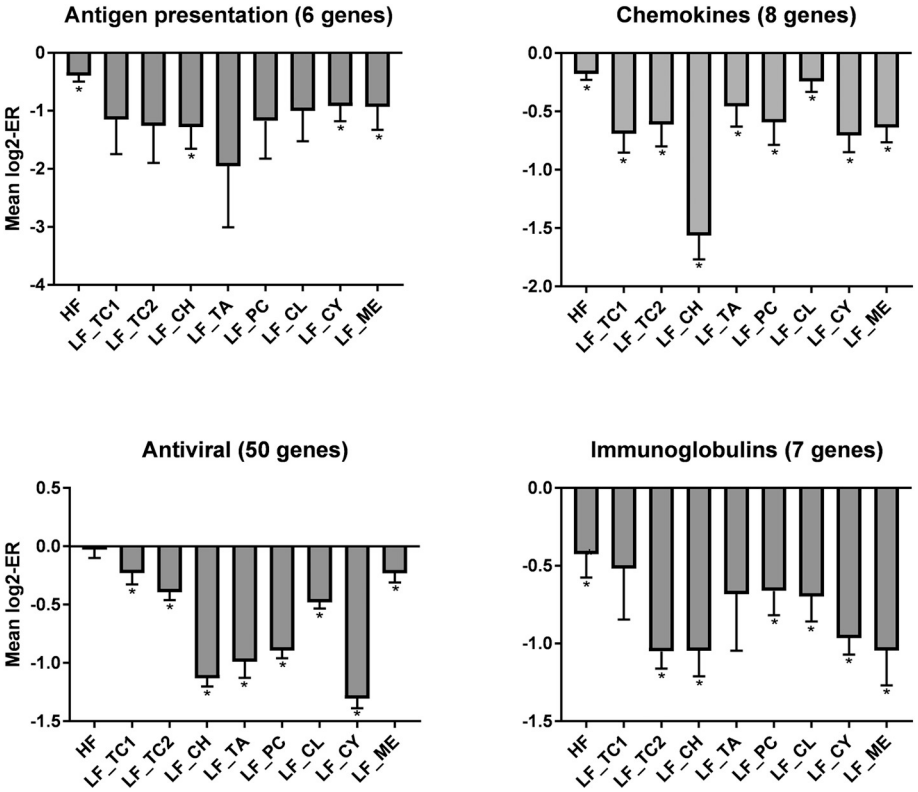
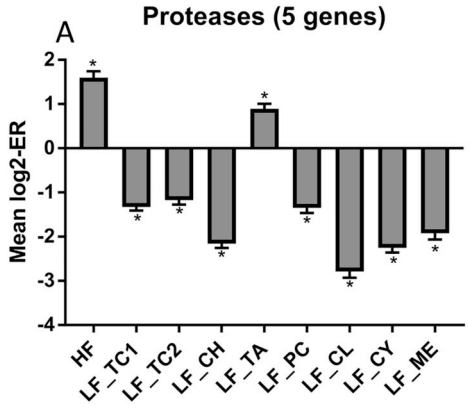


Fig. 5. Groups of immune genes with correlated expression profiles (microarray analyses). The numbers of genes are indicated in parentheses. Data are mean log^2-Expression Ratios ± SE, significant differences from LF are indicated with asterisks.



B

Genes	CH/LF Fold
3-keto-steroid reductase	-2.26
7-dehydrocholesterol reductase	-2.43
Diphosphomevalonate decarboxylase	-4.05
Farnesyl pyrophosphate synthetase	-3.10
Hydroxymethylglutaryl-CoA synthase	-2.47
Isopentenyl-diphosphate Delta-isomerase	-2.88
Lanosterol 14-alpha demethylase	-4.18
Squalene monooxygenase	-4.49
Squalene synthase	-2.87

Fig. 6. Differentially expressed genes with metabolic functions (microarray analyses). A: extracellular proteases. Data are mean log2-Expression Ratios ± SE, significant differences from LF are indicated with asterisks. B: genes of steroid biosynthesis pathways, data are LF_CF to LF ratio (folds), all differences are significant.

intestine and the histological observations of excessive lipid droplet accumulation in the fish fed the LF diet is in accordance with our previous study (Gu et al., 2014). The increased relative weight of the PI and MI observed in LF fed fish were most likely a result of an increase in lipid content due to increased lipid vacuolation (Hansen et al., 2020). This lipid accumulation was presumablya result of reduced lipid transport from the intestinal mucosa to the circulatory system. The observed symptoms were similar to those described in detail for Arctic charr (200–250 g) by Olsen and co-workers (Olsen et al., 2000; Olsen et al., 1999). The charr were fed semi-purified diets with linseed oil as the only lipid source. The authors suggested deficiency of certain fatty acids as the plausible explanation for the lipid accumulation. Excessive lipid accumulation in the enterocytes due to deficiency of essential fatty acids has recently been documented also for Atlantic salmon (Bou et al., 2017). Enterocyte lipid accumulation has been observed in PI of rainbow trout (500 g) fed a diet containing either fish oil, soybean oil or soybean lecithin as the only fat source (Olsen et al., 2003), whereas only minor accumulation was observed in fish fed diets with fish oil and soybean lecithin. Based on these results, the authors concluded as follows: fish may require exogenous phospholipids in order to sustain a sufficient rate of lipoprotein synthesis and phosphatidylcholine was suggested to be the key compound in this context. A study of development in fish of expression of genes involved in the pathways for the production of phospholipids have shown low values at the early stages (Carmona-Antonanzas et al., 2015; De Santis et al., 2015). However, phosphatidylcholine requirement of Atlantic salmon after juvenile stage appears not to have been investigated so far (NRC, 2011).

Despite clear differences in gut mucosa structure and enterocyte hypervacuolation, changes in gene expression were small or moderate by magnitude, as evaluated by a combination of microarray analyses and qPCR assays targeting the genes involved in lipid and sterol metabolism. This contrasts with the major transcriptional changes associated with other intestinal disturbances in salmon, such as dietary induced inflammation (Kortner et al., 2012). Slight but consistent down-regulation of immune genes from several functional groups and pathways might be interpreted as mild immune suppression with cholesterolbeing the most potent. Down-regulation of several digestive proteolytic enzymes was observed, which, nonetheless did not affect growth and feed efficiency. Expression changes of genes involved in lipid metabolism were limited. Intestinal lipid absorption and transepithelial transport, including temporary storage of lipid in cytosolic lipid droplets, are natural metabolic processes undertaken by all healthy animals upon ingestion of a high fat meal. It is possible that the lipid load in the present study did not exceed the threshold that requires compensatory changes of transcriptome. However, the trend towards lower lipid digestibility in the fish fed the LF diet may indicate that the fish capacity for lipid absorption was surpassed, leading towards lipid malabsorption syndrome (LMS) (Hanche-Olsen, 2013; Penn, 2011). Lack of strong gene expression responses could also be related to the general understanding that Atlantic salmon most likely do not encounter such problems in their natural environment and mechanisms for adaptation to high-fat plant diets have not evolved.

4.2. Choline supplements to the LF diet

The most pronounced effects of the choline supplementation were the significant reduction in lipid vacuolation in pyloric intestine and the organo-somatic index for the pyloric intestine (PISI) and mid intestine (MISI) compared to all the other treatments. These observations corresponded to the histological findings showing normal, low degree of enterocyte vacuolation in the proximal intestine region in the LF_Cl fed fish, in contrast to the marked to severe vacuolation in all the other LF treatments, except the LF_PC (see discussion below). These results indicate that dietary choline is a key component for efficient transport of lipid across the intestinal mucosa and that the level in the LF basal diet, in the form of free and bound choline, was not sufficient. These results correspond to our previous study (Hansen et al., 2020) and are further in line with the key role choline plays in lipid transport as part of phosphatidylcholine, an essential, structural component of lipoproteins (Harvey, 2011). Choline metabolism has not been studied in sufficient detail in fish. It is however, likely that choline is metabolized in salmonids as in other monogastric mammals. A major difference may be the route from the enterocytes to the peripheral tissues as a lymphatic system has not been identified in salmon. Also birds lack lymphatic vessels in the mesentery (Whittow, 2000). Without lymphatic vessels, the major route for lipoproteins and their phosphatidylcholine would be via *vena porta*, directly to the liver. Free dietary choline is absorbed by the enterocytes via choline transporters and immediately phosphorylated to phosphocholine and further bound to diacylglycerol to form phosphatidylcholine (Fig. 1) (Li and Vance, 2008).

Table 5
The number of differentially expressed genes (DEG).

Supplemented diets	HF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LH_Cl	LF_CY	LF_ME
Number of DEG	42	25	40	171	75	65	38	57	78

Supplement diet groups compared to the low fish meal control group (LF).

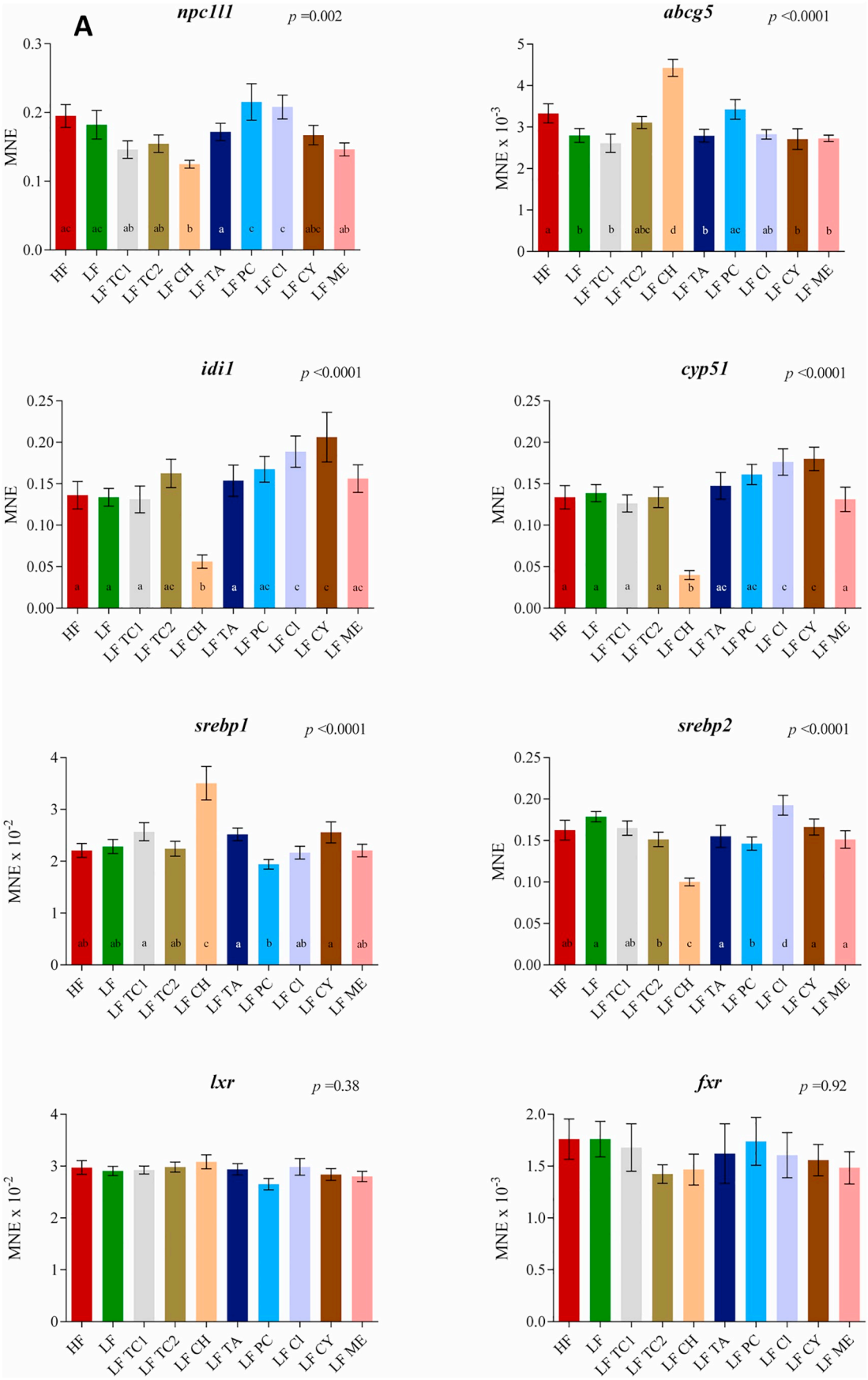


Fig. 7. A–C Gene expression profiling of pyloric caeca samples by qPCR. Values are expressed as mean normalized expression (MNE), with their standard errors represented by bars (n = 8 fish per group). Different levels denote significant differences between diet groups (p < .05). For full genes names see S1 Table.

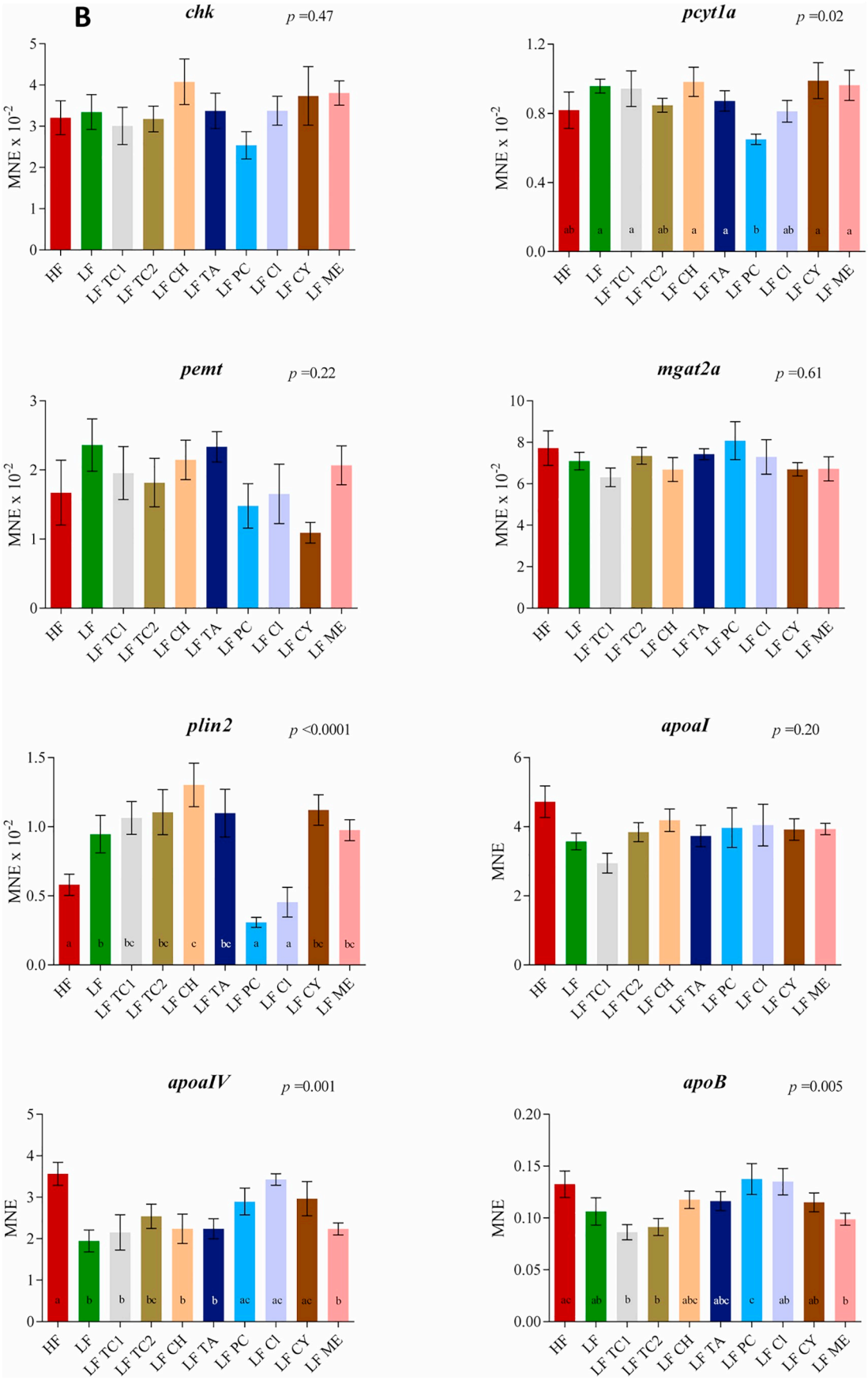


Fig. 7. (*continued*)

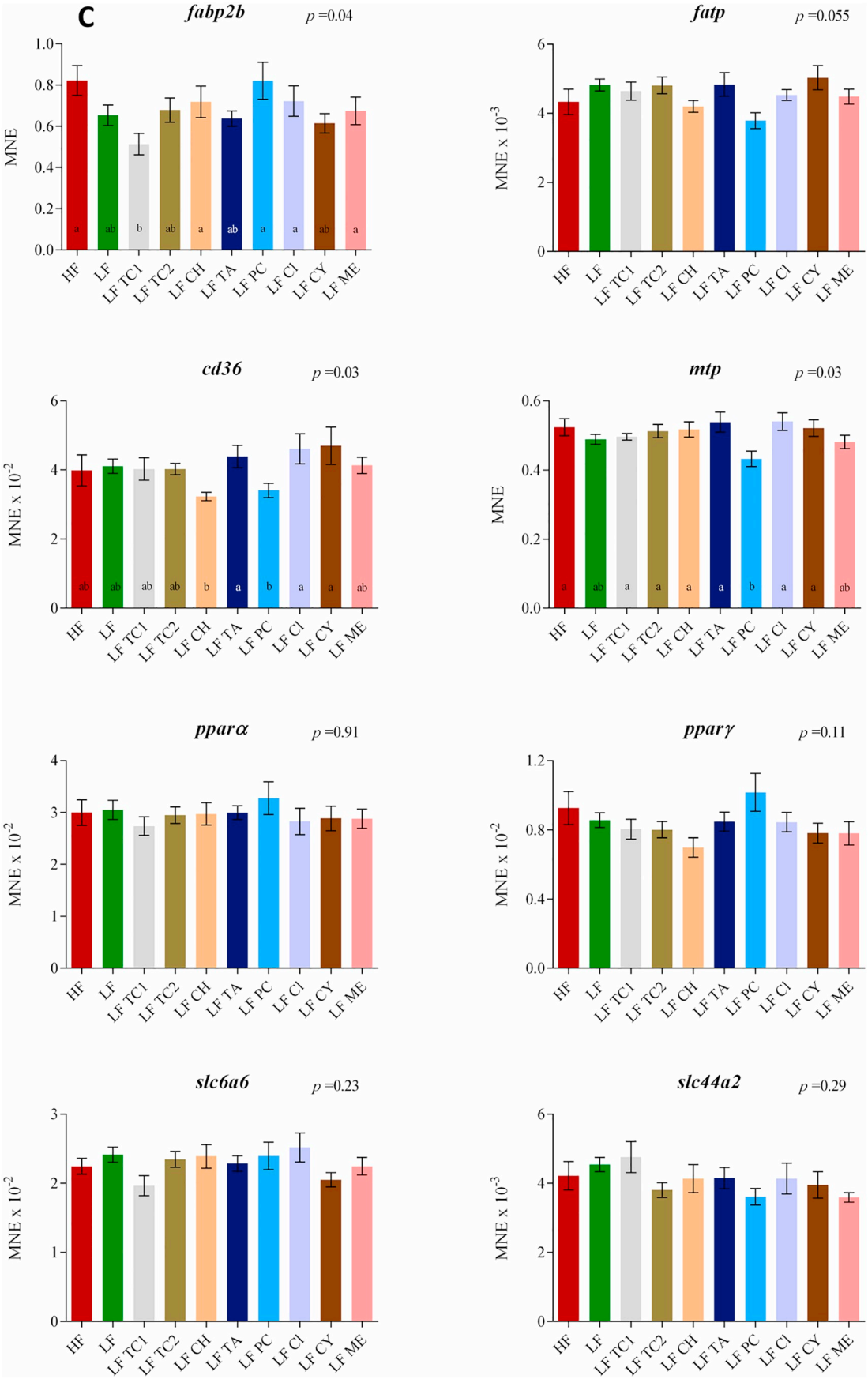


Fig. 7. (*continued*)

Phosphatidylcholine entering the intestine, with food or in bile, is hydrolyzed by phospholipases to lysophosphatidylcholine before uptake into the enterocyte and re-esterification to phosphatidylcholine. Another supply of phosphatidylcholine is endogenous synthesis in the liver from serine, activated diglycerides, ethanolamine and methyl groups from methionine as described in Fig. 1. Endogenous synthesis of phosphatidylcholine seems to be sufficient for many animals at most life stages. A requirement is established also for several fish species, but only at the early life stages (NRC, 2011), and not yet for Atlantic salmon. An early study of effects of dietary supplementation of phospholipid and choline in Atlantic salmon weighing from 1.0 to 7.5 g indicated, based on growth rate, that the smallest fish required a dietary supply, but not the larger (Poston, 1990). Since these conclusions, research on phospholipid and choline metabolism in Atlantic salmon has been conducted only with very young salmon. The most recent studies, also investigating very young fish, have for the first time addressed lipoprotein metabolism in the intestine of the Atlantic salmon (Jalili et al., 2019; Jin et al., 2018a; Jin et al., 2018b). These studies seem to confirm that phospholipid metabolism is immature in the young Atlantic salmon and that an exogenous supply may be necessary.

The results of the molecular studies in the present study are in accordance with our previous work (Hansen et al., 2020) and confirm the role of choline in lipoprotein assembly and lipid transport. Choline supplementation, in both of our studies, seemed to cause a down-regulation of phosphatidylcholine synthesis by decreasing *pcyt1a*. It also seemed to promote intracellular lipid transport by inducing *apoaIV* expression and reducing intracellular lipid storage as indicated by the reduced expression of *plin2*. However, the overall transcriptome response to the choline supplementation was low, as pointed out above. A previous study conducted on first feeding salmon fry has also documented relatively stable transcriptome profiles after dietary phospholipid supplementation (De Santis et al., 2015).

4.3. Phosphatidylcholine supplements to the LF diet

The effects of supplementation with phosphatidylcholine, were very similar to those caused by supplementation with choline regarding organ indices, lipid vacuolation of the PI and intestinal gene expression. In line with the fact that choline is an integrated component of phosphatidylcholine and both diets contained a choline level just below 3000 mg/kg, this was as expected. Based on the present study, with a TGC of ~4 and dietary lipid level of ~27%, a choline level of 3000 mg/kg is sufficient to secure efficient lipid transport across the intestinal mucosa for post-smolt Atlantic salmon. Phospahtidylcholine supplementation significantly increased the lipid digestibility by 2.4 point per cent, compared to the unsupplemented diet. Choline supplementation elevated the lipid digestibility less than for phosphatidylcholine with 1.1%. The explanation for greater effect of phosphatidylcholine was most likely related to the role of phosphatidylcholine, as one of several phospholipids, in emulsification of lipid in the stomach and intestine and its role in micelle formation following hydrolysis (Bauer et al., 2005). Based on these considerations, it appears likely that supplementation of a choline/phosphatidylcholine deficient diet with phosphatidylcholine would more efficiently rectify the effects of the deficiency on lipid digestibility. The changes in lipid digestibility by phosphatidylcholine would be interesting to follow up in further investigations.

4.4. Methionine supplements to the LF diet

The lack of effects of methionine supplementation suggests that the methyl groups of methionine are not available for the synthesis of phosphatidylcholine from phosphatidylserine, via the pathway known to function in the liver of some other animals, and as shown in Fig. 1. This is in line with the results of Rumsey et al. (Rumsey, 1991) who investigated choline requirement in the young rainbow trout. No replacement value for choline was observed for methionine supplementation. This implies that, Atlantic salmon in salt water, need a dietary supply of choline or phosphatidylcholine. According to nutritional principles, choline will be defined as the essential nutrient, as phosphatidylcholine can be synthesized if sufficient choline is present.

4.5. Taurocholate, taurine and cysteine supplements to the LF diet

Taurocholate, at both inclusion levels, affected only biomarkers related to bile salt metabolism, and the responses were as expected. The only exception was an elevation in protein digestibility observed for the low taurocholate level (LF_TC2), i.e. compared to LF. This might be due to the stabilizing effect of bile salts on intestinal proteases, such as trypsin and chymotrypsin making them more resistant to degradation. Higher stability may secure higher efficiency of protein hydrolysis (Maldonado-Valderrama et al., 2011; Gass et al., 2007). The highest level of taurocholate supplementation (LF_TC1) did not further increase protein digestibility. The explanation might be that the maximum effect was obtained with the lower dose. No indications of either beneficial or detrimental alterations associated with diet-induced enteritis were observed upon inclusion of taurocholate in the diet, in contrast to the detrimental effects of higher levels shown in our previous study (Kortner et al., 2014).

Supplementation with taurine, the conjugate, in taurocholate, did not affect any of the observed biomarkers. The exception was an elevated protein digestibility similar to the effects of the LF_TC2 diet. The plasma level of bile salts in fish fed the LF_TA diets were high, and similar to the levels of the LF_TC fed fish. It may be suggested that the positive effect on protein digestibility, as for the LF_TC fed fish, was related to higher bile salt concentration in the chyme.

In line with the lack of responses of taurine supplementation, responses to supplementation with cysteine, a precursor of taurine, were also insignificant.

4.6. Cholesterol supplements to the LF diet

In general, the cholesterol supplementation produced expected effects on sterol and bile salt metabolism and confirms the findings in our previous study (Kortner et al., 2016; Kortner et al., 2014). Sterol uptake was suppressed as well as de novo cholesterol biosynthesis and, hence, induction of sterol efflux from the intestinal mucosa. The magnitude of response in gene expression was lower in the present study as compared to our earlier work. Lower cholesterol dose used in the present study (0.2% vs. 1.5%) is the likely explanation for this difference.

5. Conclusions

Choline is an essential nutrient for Atlantic salmon in seawater, particularly important in lipid transport across the intestinal mucosa. Phosphatidylcholine is a good source of choline. Neither supplementation with methionine, cysteine, taurine nor taurocholate diminished the symptoms of choline deficiency. High plant diets for Atlantic salmon must include a choline or phosphatidylcholine source.

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Declaration of Competing Interest

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participated in study design, but had no role in data collection, analysis and interpretation, decision to publish or preparation of the manuscript.

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.

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