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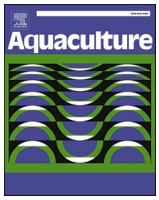

Interaction between dietary saturated fatty acids and cholesterol on mineral and fatty acid availability and postprandial lipids absorption kinetics in Atlantic salmon (*Salmo salar*)

HaoHang Fang [a,b,*], Nini H. Sissener [a], Antony J. Prabhu Philip [c], Grethe Rosenlund [d], Ingunn Stubhaug [d], Øystein Sæle [a,*]

[a] Feed and nutrition, Institute of Marine Research, Bergen, Norway
[b] Department of Biological Sciences (BIO), University of Bergen, Bergen, Norway
[c] Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima), Bergen, Norway
[d] Skretting Aquaculture Innovation (AI), Stavanger, Norway

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ABSTRACT

Atlantic salmon (*Salmo salar*) with an initial weight of approximately 160 g were fed with one of four experimental diets containing low / high levels of saturated fatty acids (SFA, 10 % vs. 24 % of total fatty acid) / cholesterol (CHO, 200 vs. 2400 mg kg^{-1}) in a 2 × 2 design, lasting for 3 months. Plasma samples were collected at 7 postprandial time points (0 h–35.5 h) after 50 days of feeding, and fish were sampled at the end of the trial. Results showed that the increased SFA level in the feed significantly increased the whole-body selenium and iron concentrations. However, whole-body zinc status was neither affected by dietary SFA nor CHO. Additionally, increased SFA level in feed significantly increased the postprandial plasma CHO, low density lipoprotein (LDL) and total protein levels. Also, increased CHO level in feed increased the postprandial plasma astaxanthin, CHO, high density lipoprotein (HDL) and LDL levels. However, analyzed markers related to intestinal triacylglycerol transport were not affected by dietary SFA. Due to dramatic reductions of SFA and CHO in plant-based feed, understanding their nutritional function in *Salmo salar* is important for the aquaculture industry.

1. Introduction

The fast development of aquaculture has forced a change of the feed ingredients from marine- to plant-based. In Norwegian *Salmo salar* feed, fish oil (FO) has been partially replaced by rapeseed oil (RO), accounting for approximately 60 % of the total lipid source in 2020 (Aas et al., 2022a). In addition to containing the valuable eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), FO also contains cholesterol (CHO) and saturated fatty acids (SFA, 20–30 % of total fatty acid) (Sissener, 2018). In comparison, RO does not contain CHO and is lower in SFA (7.5 % of total fatty acids) (Sissener, 2018). Therefore, partly replacing FO with RO results in a huge change in dietary lipid composition, including reductions in dietary SFA and CHO levels. SFA does not only act as an energy supply for β-oxidation but also as a crucial component of cell membranes (Calder, 2015). CHO can regulate membrane fluidity and act as a precursor for many bioactive compounds, such as bile acid, vitamin D, and steroid hormones (Norambuena et al., 2013). SFA are not classified as essential fatty acids because fish can synthesize them de novo (Tocher, 2003), resulting in limited research focusing specifically on SFA in fish. Similarly, CHO is not an essential nutrient for fish, as it can be synthesized from acetate (Kortner et al., 2014). However, feeding fish with plant-oil based feed has been shown to increase cytosolic lipid droplets (CLDs) in the intestine compared to fish-oil based feed (Bell et al., 2001; Olsen et al., 2003; Ruyter et al., 2006). A study on *Salvelinus alpinus* observed that supplementing the diet with 4 % SFA (of feed) reduced the intestinal CLDs, suggesting that dietary SFA might impact intestinal fat transport (Olsen et al., 2000). Intestinal fat transport disorder may induce excessive fat accumulation (termed as steatosis), which may destroy intestinal integrity and thus impede fish health (Olsen et al., 2003; Olsen et al., 2000). Therefore, a better understanding of how dietary lipids impact intestinal fat transport is important for the aquaculture industry.

Additionally, a previous study on *S. salar* reported that partially replacing fish oil with rapeseed oil in feed reduced whole-body zinc

* Corresponding authors at: Feed and nutrition, Institute of Marine Research, Bergen, Norway.
E-mail addresses: fhaohang@126.com, Haohang.Fang@student.uib.no (H. Fang), Oystein.Saele@hi.no (Ø. Sæle).

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(Zn), selenium (Se), and manganese (Mn) contents in fish (Selvam et al., 2022a). Notably, this replacement also reduced the levels of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA, such as EPA and DHA), SFA, and CHO in the feed, making it difficult to isolate the impact of dietary lipids on body mineral content. Zn and other minerals play many important physiological roles in fish (Lall and Kaushik, 2021). Limited research has been conducted on the effect of dietary SFA and CHO on mineral status in fish. However, increased SFA in feed has been shown to elevate liver and plasma Zn levels in rats (Knudsen et al., 1990). Additionally, higher CHO levels in feed have been shown to reduce serum Zn levels in rats (Koo and Ramlet, 1983).

The aim of this feeding trial is to investigate the interaction effect of dietary SFA and CHO in *S. salar*. Data on growth performance and feed utilization are reported in another manuscript (Sissener et al., 2025). Specifically, the specific growth rate, feed intake, and feed conversion ratio of *S. salar* were not significantly impacted by dietary SFA and CHO after 3 months of feeding. Additionally, this study reports on the post-prandial plasma lipid profiles and postprandial intestinal lipid transport in *S. salar* as affected by dietary SFA and CHO. The availability of minerals and fatty acids (FAs) as influenced by dietary nutrients was also analyzed.

The SFA level in Norwegian *S. salar* commercial feed is around 16 % of total fatty acids (Ørnsrud et al., 2020), and the CHO level in traditional marine-based feed is more than 1 g kg^{-1} (Tocher et al., 2008). Although the designed levels of SFA (10 % vs. 24 % of total fatty acid) and CHO (0.2 vs. 2.4 g kg^{-1}) in the present test feed were not representative of practical standards, variations in dietary SFA and CHO were substantial. This approach aimed to provoke potential effects of dietary SFA and CHO on the availability of minerals and lipids, which is beneficial for follow-up work on mineral and lipid nutrition. Additionally, in recent years, the increase in plant-based ingredients in commercial salmon feed has further reduced SFA and CHO levels, making it significant to understand the interaction between these components and mineral/lipid metabolism.

Table 1
Ingredients of the four experimental diets.

Ingrediens, %	**LSLC**	**HSLC**	**LSHC**	**HSHC**
Wheat	6.9	6.9	6.7	6.7
Fish meal	5.0	5.0	5.0	5.0
Wheat gluten	16.6	16.6	16.6	16.6
Pea protein concentrate	7.2	7.2	7.2	7.2
Soyprotein concentrate	30.0	30.0	30.0	30.0
Sunflower meal	2.0	2.0	2.0	2.0
Faba bean dehulled	2.0	2.0	2.0	2.0
Guar meal	2.0	2.0	2.0	2.0
Rapeseed oil	16.49	9.39	16.49	9.39
Incromega E1050	1.55	0.0	1.55	0.0
Veramaris oil	1.19	3.07	1.19	3.07
Palm oil	0.0	8.71	0.0	8.71
Camelina Oil	4.10	2.15	4.10	2.15
Water change	0.023	0.023	0.023	0.023
Astaxanthin	0.05	0.05	0.05	0.05
Ytrium premix	0.1	0.1	0.1	0.1
Vitamin and mineral mix	4.751	4.751	4.751	4.751
Cholesterol	0.0	0.0	0.22	0.22

Wheat: Lantmännen Ek För; fish meal: Pelagia AS; wheat gluten: Tereos Starch and Sweet BE; Pea protein concentrate: Pelagia AS; Soyprotein concentrate: European Commodity Company S.a.; Sunflower meal: Bunge Novenyolajipari Zrt; Faba bean dehulled: SAS Soufflet Negoce; guar meal: Meghraj international; rapeseed oil: ADM International Sarl (NL dep); Incromega E1050: Croda Europa limited; Veramaris oil: Veramaris V.O.F; palm oil: AAK AS; camelina oil: European commodity company Russia; cholesterol: Carbogen AMCIS.

2. Materials and methods

2.1. Experimental diet

As presented in Table 1, four experimental diets were formulated with low / high levels of SFA / CHO (2 × 2) (Produced by Skretting Aquaculture Innovation (AI) Feed Technology Plant, Stavanger, Norway) and were named as follows (SFA, % of total fatty acid / CHO, mg kg^{-1}): LSLC, 10 %/200; HSLC, 24 %/200; LSHC, 10 %/2400; HSHC, 24 %/2400. The differences between high SFA diets and low SFA diets were different levels of rapeseed oil, camelina oil, incromega E1050 (Croda Europa limited), veramaris oil (Veramaris V.O·F), and palm oil. This oil blend was used in order to reach the lowest levels of SFA in the feeds. Specifically, compared to low SFA diets, high SFA diets had increased SFA (mainly 16:0) and reduced 18:1n-9, 18:3n-3 and 18:2n-6, while keeping n-3 LC-PUFA at a constant level. In addition, the CHO level in low CHO diets only came from the dietary ingredients, and additional CHO (2.2 g kg^{-1}) was provided to achieve high CHO diets. Yttrium oxide was added to the feed as an indigestible marker. Dietary nutrient compositions, minerals and FA profiles were analyzed and presented in Table 2.

2.2. Fish feeding and management

Feeing trial was conducted at Skretting AI Lerang Research Station (Strand, Norway). Atlantic salmon (neomales, Stofnfiskur, Benchmark Genetics Limited, Iceland, hatched July 2020) with an initial weight of approximately 160 g were distributed to 12 tanks (1 m diameter, 450 L, 90 fish/tank) and fed with one of four experimental diets in triplicate (Fig. 1). Before the feeding trial, fish were fed a commercial diet (Produced by Skretting Norway, Stavanger, Norway) for acclimation (2 weeks). The fish were reared in running sea water at 11.97 ± 0.04 °C, 106 ± 1 % O_2 saturation, and exposed to 24-h light. During the feeding period, fish were fed for 2 h, 3 times per day, with collection of uneaten feed after each meal. Diets were distributed in slight excess using automatic feeders (Hølland Teknologi AS). Standard husbandry procedures at the research station were applied.

2.3. Sampling

After 50 days of feeding treatment, postprandial samples (plasma and mid-intestine, for absorption kinetics) and feces samples (for apparent digestibility coefficient (ADC)) of Atlantic salmon were collected, as described below. Further, the fish trial continued for another 40 days (a total 3 months of the feeding trial), where fish were sampled for whole-body mineral analysis. A 50-day feeding treatment was used to investigate absorption kinetics and apparent digestibility coefficient, following a previous report that utilized an 8-week feeding treatment (Prabhu et al., 2014). Additionally, short feeding durations might obscure dietary effects on whole-body minerals, as suggested by our recent study (Fang, 2024). Therefore, extending the feeding trial to 3 months ensures that the dietary effects are not masked by an insufficient feeding duration.

Before sampling, Atlantic salmon were starved for 48 h to establish a pre-prandial status, termed 0 h (Fig. 1). Next, fish were fed the experimental diets for 1 h, and the postprandial time was calculated after this feeding. Fish were sampled at postprandial time points: 0 h, 1.5 h, 3.5 h, 7.5 h, 11.5 h, 23.5 h, 35.5 h. At each time point, 5 individuals per tank were euthanized with an overdose of anaesthetic (Tricaine Pharmaq, 0.2 g/L). Further, blood was collected from the caudal vein by heparin-treated vacutainer and centrifuged for plasma separation. Furthermore, the mid-intestinal filling degree was assessed through visual inspection by the same person, with a score ranging from 0 to 3 indicating the degree of mid-intestinal filling, from empty to fully filled (Supplementary table 1). Also, the mid-intestine (just behind the pyloric caeca) was sampled, stored in *RNAlater* solution overnight. Plasma and mid-

Table 2
Nutritional analysis of different experimental diets.

	LSLC	HSLC	LSHC	HSHC
Analyzed composition **(wet matter)**				
Protein, %	45.2	46.1	45.9	45.9
Fat, %	26.8	27.7	27.1	27.5
Moisture, %	7.3	6.7	6.7	7.3
Ash, %	4.6	4.7	4.4	4.4
CHO, mg kg^{-1}	509	234	2307	2384
Analyzed mineral **(wet matter, mg kg^{-1})**				
Zn	202.1	211.9	203.5	199.1
Se	0.47	0.50	0.47	0.45
Mn	50.2	54.4	51.1	50.5
Fe	187.6	193.9	195.4	213.7
Cu	11.5	11.4	12.0	11.2
Y	65.8	66.3	68.1	65.9
Analyzed fatty acid composition **of** *selected fatty acids (mg/g)*				
Σ SFA	25.65	60.58	25.13	60.61
C10:0	0.02	0.03	0.02	0.03
C12:0	0.05	0.27	0.04	0.25
C14:0	0.85	1.84	0.71	1.76
C15:0	0.15	0.22	0.14	0.21
C16:0	16.62	49.16	16.35	49.18
C18:0	5.16	6.9	5.1	6.99
C20:0	1.65	1.31	1.64	1.34
C22:0	0.77	0.54	0.74	0.56
C24:0	0.38	0.31	0.39	0.29
Σ MUFA	125.68	108.08	121.8	110.25
C16:1n-7	0.87	0.67	0.75	0.67
C18:1n-5	0.05	0.03	0.04	0.04
C18:1n-7	5.98	4.06	5.79	4.13
C18:1n-9	107.33	97.08	104.54	99.04
Σ C18:1	113.36	101.17	110.37	103.21
C20:1n-7	0.22	0.12	0.22	0.11
C20:1n-9	7.96	4.41	7.65	4.53
C20:1n-11	0.13	0.07	0.11	0.07
Σ C20:1	8.31	4.6	7.98	4.71
C22:1n-9	1.66	0.85	1.57	0.88
C22:1n-11	0.94	0.51	0.59	0.49
Σ C22:1	2.6	1.36	2.16	1.37
C24:1n-9	0.54	0.28	0.54	0.29
Σ PUFA	97.61	78.89	95.2	80.18
Σ n-6	51.2	42.71	50.3	43.57
C18:2n-6	48.97	40.72	48.14	41.57
C20:2n-6	0.75	0.38	0.73	0.4
C20:4n-6	0.69	0.74	0.67	0.73
C22:5n-6	0.79	0.87	0.76	0.87
Σ n-3	46.29	36.1	44.81	36.53
C18:3n-3	26.49	15.14	25.89	15.57
C18:4n-3	0.35	0.27	0.27	0.28
C20:3n-3	0.39	0.25	0.44	0.2
C20:4n-3	0.25	0.28	0.22	0.28
C20:5n-3 (EPA)	5.03	6	4.73	6.01
C21:5n-3	0.12	0.05	0.1	0.04
C22:5n-3	0.73	0.9	0.69	0.9
C22:6n-3 (DHA)	12.93	13.21	12.47	13.25
n-6/n-3	1.11	1.18	1.12	1.19
EPA + DHA	17.96	19.21	17.2	19.26
C16:2n-4	0.06	0.03	0.04	0.03
C16:4n-1	0.06	0.05	0.05	0.05
Σ other FA	0.12	0.08	0.09	0.08
Σ FA	248.94	247.55	242.13	251.04
Σ SFA (% of total fatty acids)	10.3 %	24 %	10.4 %	23.7 %

Zn, Zinc; Mn, manganese; Fe, iron; Cu, copper; Se, selenium; Y, Yttrium; CHO: Cholesterol; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

intestine samples were stored in liquid nitrogen until analysis. One week after, feces were collected by stripping all remaining fish. Before stripping the fish to obtain the feces, the fish had been starved for 12 h. One pooled sample from all remaining fish per tank was gently mixed and stored at −20 °C until further analysis.

2.4. Chemical analysis

After homogenization, moisture, crude protein and ash in feeds were analyzed according to the AOAC official methods (Thiex, 2009). Briefly, moisture was analyzed by drying at 105 °C until constant weight; crude protein was analyzed by the Kjeldahl method (N x 6.25); ash was analyzed by complete combustion (550 °C) in a muffle furnace; fat level was analyzed after acid hydrolysis and extraction with diethyl ether.

Minerals (including Zn, Se, Mn, Fe, copper (Cu), yttrium (Y)) in diets, feces and whole fish were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Silva et al., 2019). Before analysis, samples were freeze dried and homogenized. Next, 0.2 g sample, 0.5 ml deionized water and 2 ml concentrated HNO_3 were added to a test tube and then digested in a Milestone UltraWave Microwave Digestion System (Milestone Inc., USA). Further, digested samples were diluted to 25 ml with deionized water. Furthermore, the eluate was introduced into the nebulizer tube of the ICP-MS (Thermo Scientific, USA), and minerals were analyzed in the kinetic energy discrimination (KED) reaction mode. To correct for instrumental drift during the analysis, a solution of germanium and rhodium was added online. The ICP-MS was tuned before analysis using a 1 ppb tuning solution B (Thermo Fisher, in 2 % HNO_3 and 0.5 % HCl). Data collection and processing were carried out using Qtegra software (Thermo Scientific). An external calibration curve ranging from 10 to 500 ng ml^{-1} was utilized to quantify the minerals.

FA compositions in diets and feces were analyzed according to a previous report (Hundal et al., 2021a, 2021b). Briefly, total lipid was isolated from the sample by chloroform-methanol (v:v, 2:1), with a 20 times weight of the sample (chloroform-methanol (g) = sample (g) × 20). Further, methylated 19:0 (internal standard) was added to the isolated lipid. Additionally, FA methyl esters were obtained using boron trifluoride (12 % BF3 in methanol) after saponification (2 % NaOH in methanol). Besides, FA methyl esters were isolated from solution by hexane. Furthermore, individual methyl esters were analyzed by Scion GC (Bruker SCION 436-GC), following the instructions of the manufacturer. Results were analyzed by the software of Chromeleon® version 6.8.

Plasma lipids, including triacylglycerol (TAG, Horiba Medical, A11A01640), CHO (Horiba Medical, A11A01634), high density lipoprotein (HDL, Horiba Medical, A11A01636), low density lipoprotein (LDL, Horiba Medical, A11A01638) and total protein (Horiba Medical, A11A01669), were analyzed by Pentra C400 (HORIBA; Montpellier, France). Plasma astaxanthin was analyzed by HPLC, with detection at 470 nm, and then quantified using an external calibration curve (Ørnsrud et al., 2004).

2.5. Intestinal gene expression

Intestinal samples from three postprandial time points, including 1.5 h, 11.5 h and 23.5 h, were selected for mRNA expression. Intestinal RNA was isolated by the Maxwell® HT simplyRNA Kit (Promega, USA) and the Biomek® 4000 automated liquid handler (Beckman Coulter, USA. Next, RNA integrity and quantity were measured by Bioanalyzer (Agilent 2100) and spectrophotometer (NanoDrop ND-1000), respectively. Next, cDNA was synthesized using the inverse transcription kit (N808–0234; Applied Biosystems, Foster City, Ca, USA). Further, candidate genes were quantified by real-time PCR (qPCR) in the qPCR instrument (Bio-Rad, US), with SYBR GREEN PCR Master Mix (Roche-Norge, Norway). qPCR program as follows: 10 min preincubation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a melting curve. Candidate genes were normalized after the stable calculation of the housekeeping genes (*β-actin* and *ef-1α*) by the software CFX Maestro (Bio-Rad, US). Candidate genes for qPCR are listed in Table 3. The housekeeping genes chosen for this study were selected due to their stable expression (Olsvik et al., 2005). Additionally, these two genes are commonly used in Atlantic salmon studies, as demonstrated in (Hundal et al., 2021b; Radhakrishnan et al., 2023; Selvam et al., 2022a).

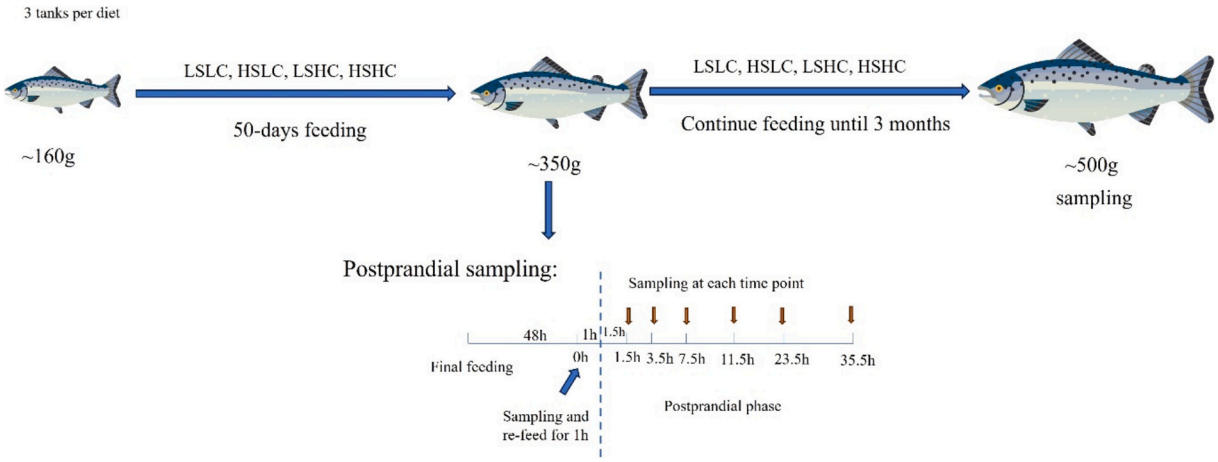

Fig. 1. Schematic diagram of feeding management and sampling in this manuscript.

Table 3
Primers of candidate genes for qPCR analysis.

Gene	Primer sequence (5′– 3′)	Accession no. [a]	Efficiency (%)	Production (bps)	Tm (°C)
β-actin	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	BG933897	105.4	91	57.56 61.14
ef- 1α	F: TGCCCCTCCAGGATGTCTAC R: CACGGCCCACAGGTACTG	AF321836	87.2	57	60.69 60.05
npc1l1	F: CCCGTCATGAGCCAGGATAC R: GCATGGGGCAGACCTTTTTG	XM_014171081.2	111.0	162	59.97 60.04
cd36	F: ACCCCCAGCAGTCACATTATT R: GTATGTAGGTCCCAGCAGCA	XM_014153607.2	104.2	131	59.36 59.17
fatp4	F: TCTGGAACACATGACAAGCC R: GCGAACAAGTTGTGTCCTTCC	XM_014125609	105.5	170	58.10 60.00
fabp2	F: GGATTATGCCTCGACTGCCA R: GCCACTCTGGGGAATTGCTA	BT048647	106.0	149	59.89 59.74
dgat1	F: CGGTAACGGAATGGTGCGT R: CAGCCTCTGACATCAATTGCCT	XM_014124329.1	108.9	148	60.74 60.94
mtp	F: TGATCATTGTAAAATGTGTGCCTTT R: ACAGCTAGCAAGTTAGCCTC	XM_014195517.2	115.5	124	58.24 57.32
apob	F: TGGGCTTGACTGGCAAGATT R: TCCCTCATCTTGGCGTTTCT	X81856	105.0	101	59.89 59.02
cpt1	F: TAAGAGGCCGTGGACCAATC R: ATTGCGCTGAGCACATTGGA	XM_045708590.1	107.5	195	59.46 60.96
mgat2	F: CGAGTGCAAGCTCTGCAAGG R: GAGGTCGGGCAAGATGAAGT	XM_014205344	102.5	75	61.91 59.75
apoa4	F: TGAAGGTGTTGGTGGTGCTT R: TGTTGCCTTGGCGACATAGT	XM_014201582	102.8	131	60.03 59.96

[a] : GenBank (http://www.ncbi.nlm.nih.gov/); *ef- 1α*: elongation factor 1 alpha; *npc1l1*: niemann-Pick C1-Like 1; *cd36*: cluster of differentiation 36; *fatp4*: fatty acid transporter protein 4; *fabp2*: fatty acid-binding protein 2; *dgat1*: diglyceride acyltransferase 1; *mtp*: microsomal triglyceride transfer protein; *apob*: apolipoprotein B; *cpt1*: carnitine O-palmitoyltransferase 1; *mgat2*: monoacylglycerol O-acyltransferase 2; *apoa4*: apolipoprotein A-IV.

Except for *β-actin* and *ef- 1α* (Olsvik et al., 2010), other primers were newly designed, through NCBI online primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?). One step qPCR (QIAGEN one step qPCR kit) was used to evaluate the primer specificity.

Candidate genes for qPCR including two house-keeping genes (*β-actin* and *ef- 1α*) and ten genes related to lipid transport and lipid metabolism (niemann-Pick C1-Like 1 (*npc1l1*), cluster of differentiation 36 (*cd36*), fatty acid transporter protein 4 (*fatp4*), fatty acid-binding protein 2 (*fabp2*), diglyceride acyltransferase 1 (*dgat1*), microsomal triglyceride transfer protein (*mtp*), apolipoprotein B (*apob*), carnitine O-palmitoyltransferase 1 (*cpt1*), monoacylglycerol O-acyltransferase 2 (*mgat2*), apolipoprotein A-IV (*apoa4*)).

2.6. Statistical analysis

The Shapiro–wilk test and Levene's test were used to test the normal distribution and variance of the data. Results from postprandial plasma parameters (except for plasma astaxanthin) and postprandial intestinal mRNA expression (5 samples from each tank, total 15 samples for each diet), were analyzed by three-way nested ANOVA (random factor: tank), with Time, SFA and CHO as independent variables. Postprandial plasma astaxanthin results (1 sample from each tank, total 3 samples for each diet) were analyzed by three-way ANOVA, with time, SFA and CHO as independent variables. Other results, including ADC of minerals, whole-body minerals and ADC of FAs were analyzed by two-way ANOVA, with SFA and CHO as independent variables. The significant results from the plasma curve between "0 h" and the "selected time point" were analyzed by multiple comparisons with Dunnett's test. If the p value was <0.05, results between groups were considered statistically significant. Whenever the interaction effect was significant, one-way ANOVA followed by Tukey's multiple comparison analysis was performed. All results are presented as mean ± standard deviation (SD). GraphPad Prism 8 (Insightful Science, U.S.) and R (R Development Core Team, 2011) were used for statistical analysis.

3. Results

3.1. Apparent Digestibility Coefficient (ADC) of minerals and whole-body minerals

S. salar fed diets high in SFA had significantly higher whole-body Se and Fe contents compared to fish fed low SFA diets ($p < 0.05$) (Table 4). Additionally, the high CHO diet treatment significantly increased whole-body Se content compared to the low CHO diet treatment ($p < 0.05$). However, the whole-body levels of Zn, Mn, and Cu were neither affected by the individual nor interactive effects of dietary SFA and CHO ($p > 0.05$). Also, the ADC of all selected minerals (Zn, Se, Mn, Fe and Cu) in *S. salar* were not influenced by dietary treatments (SFA, CHO and SFA × CHO) ($p > 0.05$).

3.2. The ADC of fatty acids

The sequence of ADC values for three FA classes in four experimental groups is: Σ PUFA > Σ Monounsaturated fatty acids (MUFA) > Σ SFA (Table 5). Compared to low SFA diet treatments, high SFA diet treatments significantly reduced the ADC of total FA, as well as three FA classes (Σ SFA, Σ MUFA and Σ PUFA) ($p < 0.05$). Nevertheless, the digestibility of all FAs was very high in all four diet groups (ranging from 94 %–100 %).

3.3. Postprandial plasma lipid profiles

After 50 days of feeding the dietary treatments, the changes in postprandial plasma parameters of *S. salar* were analyzed (Fig. 2, all results presented in supplementary table 2). Firstly, high SFA diet treatments significantly increased postprandial plasma CHO ($p < 0.01$), LDL ($p < 0.05$) and total protein ($p < 0.05$) levels compared to low SFA diet treatments. Besides, compared to low CHO diet treatments, high CHO diet treatments significantly increased the postprandial plasma CHO ($p < 0.01$), HDL ($p < 0.01$) and LDL ($p < 0.01$) levels and tended to increase postprandial plasma astaxanthin ($p = 0.06$) and total protein ($p = 0.08$) levels. Secondly, plasma TAG ($p = 0.05$) and astaxanthin ($p < 0.01$) levels were affected by postprandial time. Compared to the pre-prandial status (0 h), significantly higher plasma TAG and astaxanthin levels were observed postprandially 3.5 h–23.5 h and 1.5 h– 35.5 h, respectively ($p < 0.05$). Thirdly, Time x SFA significantly impacted the postprandial plasma total protein ($p < 0.05$) levels. Increased SFA in feed significantly increased plasma total protein at 3.5 h, 7.5 h and 11.5 h postprandially.

Table 4
The apparent digestibility coefficient (ADC) of minerals and whole-body minerals of *Salmo salar* as affected by different dietary treatments.

	LSLC	HSLC	LSHC	HSHC	p value		
					S × C effect	SFA effect	CHO effect
ADC (%, After 50-days feeding)							
Zn	26.4 ± 2.9	29.6 ± 1.0	26.7 ± 3.9	25.2 ± 1.8	0.17	0.22	0.61
Se	59.3 ± 3.2	63.7 ± 1.7	60.8 ± 2.6	59.0 ± 4.1	0.11	0.39	0.46
Mn	4.3 ± 7.8	9.2 ± 0.3	6.4 ± 6.3	3.0 ± 4.2	0.22	0.53	0.82
Fe	0.0 ± 14.1	−3.7 ± 2.2	−4.2 ± 6.3	6.6 ± 1.8	0.15	0.53	0.46
Cu	11.3 ± 4.8	9.4 ± 4.3	13.0 ± 4.9	7.3 ± 3.6	0.48	0.95	0.17
Whole-body minerals (mg kg^{-1}, After 3-months feeding)							
Zn	28.3 ± 2.6	28.8 ± 1.0	27.2 ± 1.7	31.8 ± 4.6	0.27	0.18	0.61
Se	0.17 ± 0.00	0.18 ± 0.00	0.16 ± 0.01	0.18 ± 0.01	0.19	**0.01**	**0.02**
Mn	2.0 ± 0.5	1.8 ± 0.1	1.5 ± 0.3	1.9 ± 0.3	0.17	0.56	0.49
Fe	8.8 ± 0.2	9.9 ± 0.3	8.6 ± 0.3	9.2 ± 0.5	0.39	**0.01**	0.08
Cu	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	0.81	0.10	0.24

ADC (%) = (1- (selected nutrient in Feces (%) * yttrium in Feed (%)) / (selected nutrient in Feed (%)* Yttrium in Feces(%))) *100; p values were performed by two-way ANOVA among four experimental groups; values = mean ± SD ($n = 3$); CHO: cholesterol; SFA: saturated fatty acid; C × S: CHO × SFA; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Se, selenium.

3.4. Postprandial intestinal mRNA expression

As presented in Fig. 3 (all results presented in supplementary table 3), intestinal mRNA expression of *apoa4, apob* and *mtp* were not affected by dietary SFA ($p > 0.05$). Nevertheless, increased SFA in feed significantly increased the intestinal *mgat2* expression at 1.5 h, 11.5 h and 23.5 h postprandially ($p < 0.01$). Additionally, the intestinal *npc1l1* expression level was significantly affected by the interaction of SFA and CHO ($p < 0.01$), and the HSLC diet treatments significantly upregulated this gene compared to other dietary treatments at 23.5 h postprandial. None of the analyzed genes were affected by dietary CHO ($p > 0.05$).

4. Discussion

4.1. Postprandial lipid kinetics

After dietary FAs are absorbed into the intestinal epithelial cells, they are primarily re-esterified into TAG, and either incorporated into TAG-rich lipoproteins (such as chylomicron) or stored as cytosolic lipid droplets (CLDs) (Iqbal and Hussain, 2009; Selvam et al., 2022b). Phosphatidylcholine (PC) is essential for the synthesis of TAG-rich lipoproteins (Olsen et al., 2003; Sæle et al., 2018), and its synthesis requires the participation of SFA (Van Greevenbroek et al., 1995). Additionally, a previous study on *Salvelinus alpinus* found that fish fed a diet containing 20 % linseed oil (high in 18:3n − 3) led to excessive CLD accumulation in their intestines, whereas fish fed a diet containing 16 % linseed oil and 4 % SFA (16:0) showed a reduction in this negative effect (Olsen et al., 2000). Olsen and colleagues speculated that insufficient SFA in the diet might impair PC synthesis, thereby hindering TAG-rich lipoprotein formation and depressing TAG transport. Additionally, feeding zebrafish a diet deficient in PC upregulated the mRNA expression level related to intestinal apolipoprotein synthesis (*apoa4*) (Sæle et al., 2018). On the contrary, the present study observed that intestinal mRNA expressions related to apolipoprotein synthesis (*apoa4* and *apob*) and TAG-rich lipoprotein assembly (*mtp*) were not affected by dietary SFA. Additionally, postprandial plasma TAG level was not affected by dietary SFA. These results suggested that dietary levels of SFA of 10 %–24 % of total fatty acid did not impact TAG-rich lipoprotein formation and intestinal TAG transport. Similarly, a recent study using RTgutGC cells reported that intestinal mRNA expression related to apolipoprotein synthesis was not affected by 16:0 (SFA) treatment compared to cells given monosaturated FA or polysaturated FA (Selvam et al., 2022b). Another probable mechanism in the previous study (Olsen et al., 2000) is that SFA are less dependent on TAG-rich lipoprotein for transportation than PUFA, alternatively being transported by an albumin complex. In the present study, increased dietary SFA significantly increased the plasma total protein level at postprandial 3.5 h, 7.5 h and 11.5 h, suggesting a possible increase of the SFA-albumin complex in plasma during these periods (van der Vusse, 2009). An in vitro study reported that fish intestinal cells can directly transfer partly free fatty acids (FFA) from the apical region to the basolateral chamber (and into circulation) (Selvam et al., 2022b). They observed higher transfer rates of 16:0 (SFA) compared to 20:4n-6 (PUFA). Similarly, in an earlier study on carp, after feeding fish with radiolabeled palmitic acid and -tripalmitin diets,

Table 5
The apparent digestibility coefficient (ADC) of selected fatty acids of *Salmo salar* as affected by different dietary treatments.

	LSLC	HSLC	LSHC	HSHC	p value		
					S × C effect	SFA effect	CHO effect
ADC (%, After 50-days feeding)							
Σ SFA	98.0 ± 0.3	94.9 ± 0.1	98.5 ± 0.2	94.6 ± 1.0	0.25	**0.00**	0.79
16:0	98.4 ± 0.2	95.1 ± 0.1	98.7 ± 0.3	94.8 ± 1.0	0.33	**0.00**	0.99
18:0	97.5 ± 0.4	93.5 ± 0.2	98.0 ± 0.4	93.2 ± 1.1	0.22	**0.00**	0.86
Σ MUFA	99.7 ± 0.1	98.9 ± 0.1	99.8 ± 0.0	98.6 ± 0.6	0.45	**0.00**	0.45
18:1n-9	99.8 ± 0.1	98.9 ± 0.1	99.8 ± 0.0	98.7 ± 0.6	0.52	**0.00**	0.52
Σ PUFA	99.7 ± 0.0	99.4 ± 0.0	99.7 ± 0.0	99.2 ± 0.3	0.24	**0.01**	0.24
Σ n-3	99.7 ± 0.0	99.6 ± 0.0	99.8 ± 0.0	99.4 ± 0.2	0.15	**0.00**	0.15
18:3n-3 (ALA)	99.8 ± 0.0	99.5 ± 0.0	99.9 ± 0.0	99.4 ± 0.3	0.26	**0.00**	0.44
20:5n-3 (EPA)	99.8 ± 0.0	99.7 ± 0.0	99.8 ± 0.0	99.6 ± 0.2	0.08	**0.03**	0.22
22:6n-3 (DHA)	99.6 ± 0.0	99.6 ± 0.0	99.6 ± 0.1	99.3 ± 0.2	**0.04**	0.17	0.31
Σ EPA + DHA	99.6 ± 0.0	99.7 ± 0.0	99.7 ± 0.0	99.4 ± 0.2	**0.03**	0.15	0.15
Σ n-6	99.6 ± 0.0	99.3 ± 0.1	99.6 ± 0.1	99.0 ± 0.3	0.22	**0.00**	0.48
18:2n-6 (LA)	99.6 ± 0.0	99.2 ± 0.1	99.7 ± 0.1	99.0 ± 0.4	0.40	**0.00**	0.40
20:4n-6 (ARA)	99.3 ± 0.1	99.3 ± 0.0	99.3 ± 0.3	99.0 ± 0.2	0.33	0.33	0.33
TFA	99.6 ± 0.1	98.1 ± 0.1	99.6 ± 0.0	97.8 ± 0.6	0.31	**0.00**	0.63

ADC (%) = (1- (selected nutrient in Feces (%) * yttrium in Feed(%)) / (selected nutrient in Feed (%) * Yttrium in Feces (%))) *100; p values were performed by two-way ANOVA among four experimental groups; If the interaction effect was significant, one-way ANOVA followed by Tukey's multiple comparison analysis was performed; values = mean ± SD (n = 3); CHO: cholesterol; SFA: saturated fatty acid; C × S: CHO × SFA; ALA: alpha-linolenic acid; LA: linoleic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; TFA: total fatty acid.

significant levels of labeled SFA (as FFA-albumin complexes) and TAG (as lipoproteins) were observed in plasma, suggesting the crucial role of FFAs in the fat transport system of fish (Kayama and Iijima, 1976). However, the intestinal fat transport mechanism in fish remains incompletely understood. Overall, dietary SFA did not impact mRNA expression associated with intestinal lipoprotein formation and TAG transport. Besides, the effects of dietary SFA on intestinal CLDs and fish health require further investigation.

In the present study, plasma TAG content at 3.5 h postprandial was significantly higher than that pre-prandially (0 h). This increase is primarily due to the secretion of ultra-low lipoproteins (ULDL, similar to chylomicron in mammals) from the intestine, although hepatic VLDL secretions might also contribute to a small extent (Cohn et al., 1988). In a human study, it has been shown that an early release of postprandial chylomicron may come from the previous meal (Fielding et al., 1996). In the present study, fish were fasted for 48 h before the postprandial trial to ensure their gastrointestinal tracts were empty at pre-prandial status. This fasting period eliminated the influence of residual chyme on the postprandial parameters. In addition, the TAG absorption peak appeared at 11.5 h (12 °C) postprandially, compared to 24 h in *Oncorhynchus mykiss* at 10 °C (Eliason et al., 2010), 24 h in *Dicentrarchus labrax* L. at 15 °C (Santulli et al., 1988) and 6 h in *Sparus aurata* at 24 °C (Castro et al., 2016). These different results could be attributed to different gastrointestinal systems between species, and different environmental temperatures. Gastric fish, such as *S. salar*, *Oncorhynchus mykiss* and *Dicentrarchus labrax L.*, regulate the retention time of chyme in their stomach (Hoar et al., 1983). In addition, increased water temperature is correlated with increasing digestive enzyme activities and intestinal nutrient transport efficiency in fish (Volkoff and Rønnestad, 2020).

In the present study, the increased CHO level in feed tended to increase the postprandial plasma astaxanthin level. The promotive effect of CHO on astaxanthin utilization was reported in previous *S. salar* studies (Chimsung et al., 2014; Chimsung et al., 2013; Sissener et al., 2017a, 2017b). The possible mechanism is that increased dietary CHO increases the bile acid synthesis (Horton et al., 1995), thereby enhancing intestinal astaxanthin absorption (Olsen et al., 2005). Further, increased CHO level in feed could increase the plasma astaxanthin transport by increasing lipoprotein level, such as HDL (Dietschy et al., 1993). Additionally, we observed a significant increase in plasma astaxanthin content at 3.5 h postprandial (compared to pre-prandial levels). It is known that astaxanthin is transported by incorporation into TAG-rich lipoprotein, such as ULDL and VLDL (Guo et al., 2024). In the present study, ULDL entered the circulatory system at 3.5 h postprandially. Therefore, the elevation of plasma astaxanthin at 3.5 h postprandial could be attributed to intestinal ULDL secretion. Moreover, we observed a significantly higher plasma astaxanthin level at 1.5 h postprandial compared to pre-prandial levels. Since the plasma TAG level did not increase significantly at 1.5 h postprandial, the elevated astaxanthin content at this time point is unlikely to come from the meal. One possible explanation might be that feeding triggers tissues HDL synthesis and releases into circular system, as the plasma CHO level significantly increased, and plasma HDL showed an apparent increase (though not significant) at 1.5 h postprandial. However, knowledge about HDL metabolism during the postprandial state in fish remains unclear.

In the present study, postprandial plasma CHO levels were correlated with dietary CHO levels. Additionally, plasma HDL and LDL levels, two lipoproteins responsible for transporting CHO between the liver and peripheral tissues (Lie et al., 1993), were significantly increased by higher dietary CHO. These results are consistent with previous findings in *S. salar*: feeding fish marine-based diets supplemented with CHO for five months significantly increased their plasma total CHO and correspondingly HDL and LDL levels (Farrell et al., 1986). Furthermore, the present study observed that postprandial plasma CHO and LDL levels were correlated with dietary SFA. The mechanism by which SFA increases plasma CHO levels remains unclear. As described in mammals (Grundy and Denke, 1990), this effect might be associated with increased SFA in the feed suppressing vascular LDL clearance. In contrast, Torstensen et al. (2000) reported that *S. salar* fed diets containing a range of 12.5 % to 45.3 % SFA of total FA for 23 months did not impact their plasma CHO level. These different results might be attributed to different feeding durations, with the longer feeding period in the previous study (Torstensen et al., 2000) allowing fish to better adjust to the diets.

4.2. Minerals and fatty acids availability

Based on several studies (Aas et al., 2022b; Aas et al., 2019; Selvam et al., 2022a; Shearer et al., 1994), the whole-body Zn content in commercially farmed *S. salar* (ready to slaughter, around 5 kg) has decreased from 55 mg kg^{-1} in 1991 to 35 mg kg^{-1} in 2016. This reduction is associated with the increased use of high fat and plant-based feeds. Plant-based feeds have lower Zn availability and content compared to marine-based feeds (Prabhu et al., 2019). Additionally, in 2016, the EU reduced the maximum allowable Zn content in salmonid

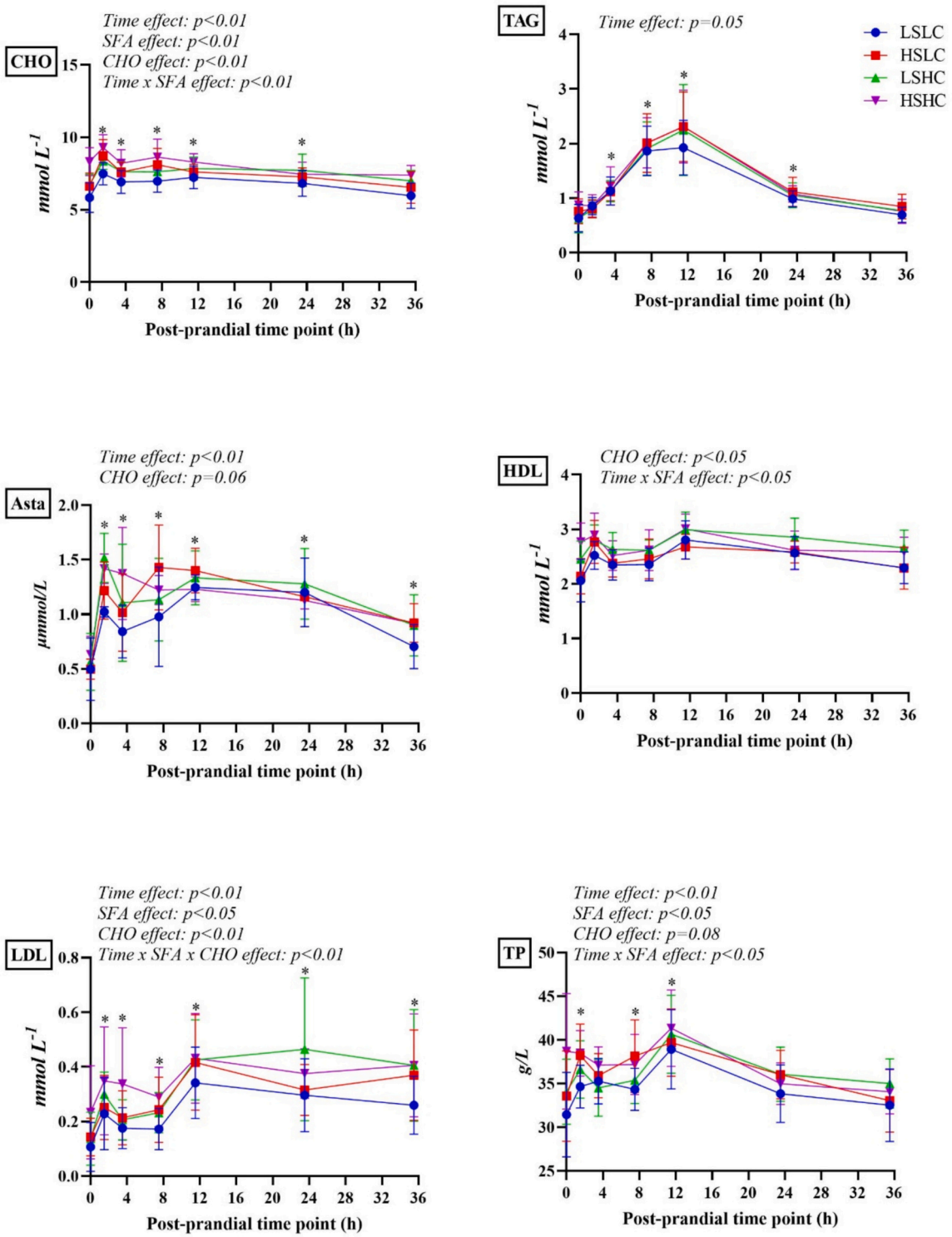

Fig. 2. Postprandial plasma lipids curves of *Salmo salar* fed with different diets for 50-days; *: Significant results compared to pre-prandial results (0 h); CHO: cholesterol; SFA: saturated fatty acid; C × S: CHO × SFA; TAG: triacylglycerol; Asta: astaxanthin; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TP: total protein; Values are mean ± SD; n (CHO, TAG, HDL, LDL, TP) =15; n (Asta) =3.

feed from 200 mg kg^{-1} to 180 mg kg^{-1} (European Commission, 2016). Further, it has been demonstrated that increased fat level in feed reduced whole-body Zn content in *S. salar* (Selvam et al., 2022a, Fang, 2024). Besides, the above studies also reported that replacing part of the dietary RO with FO increased whole-body Zn content. However, increasing FO in the feed increases n-3 LC-PUFA, SFA and CHO. This makes it difficult to determine whether the changes in body Zn status are due to n-3 LC-PUFA, SFA or CHO. In the present study, the effects of dietary SFA and CHO were singled out and whole-body Zn was not affected, indicating that dietary SFA and CHO did not impact whole-body Zn status. In the case of Se, partly replacing plant- with marine-based ingredients in feed increased whole-body Se content (Selvam et al., 2022a) and tissues Se (Betancor et al., 2016) contents in *S. salar*. Selvam et al. attributed this effect to the dietary n-3 LC-PUFA. However,

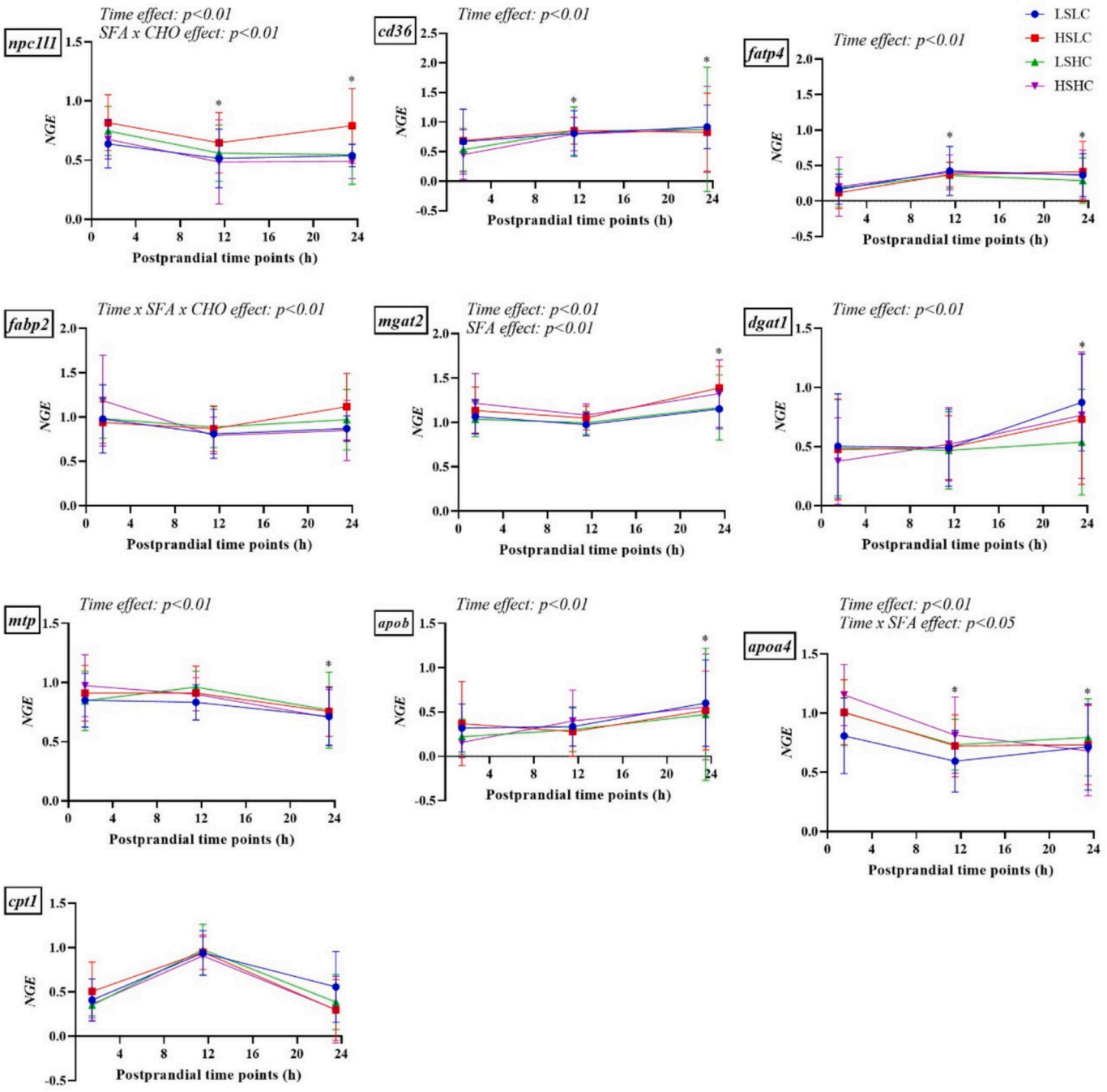

Fig. 3. Postprandial intestinal gene expression related to lipid uptake and transport in *Salmo salar* as affected by dietary saturated fatty acid (SFA) and cholesterol (CHO); Intestinal CHO uptake: NPC1L1; Intestinal FA uptake: CD36; Intracellular FA transportation: FATP4, FABP2; TAG re-esterification: MGAT2, DGAT1; Lipoprotein formation: MTP, APOB, ApoA4; FA β-oxidation: CPT1; Values are mean ± SD ($n = 15$); NGE: normalized gene expression; *: Significant results compared to results at 1.5 h. (After 50-days feeding).

in the present study, increased dietary SFA leads to increased whole-body Se, suggesting that the beneficial effect of marine-based ingredients on body Se status could also be attributed to other FAs, including SFA. Additionally, increased CHO in feed significantly increased whole-body Se content in the present study. However, this effect cannot be conclusively attributed to the dietary CHO levels due to the small differences in Se content between the experimental feeds. Except for Se, dietary SFA had a similar effect on Fe in the present study. Generally, after being reduced to the ferrous state (Fe^{2+}), Fe is taken up and transported by iron transfer proteins (Bury et al., 2003). In addition, in vitro studies have shown that Fe can form a complex with FAs and cross cell membranes, and SFA are more efficient at transferring Fe than MUFA and PUFA (Simpson et al., 1988; Yao et al., 2005). Also, increased SFA in feed increased Fe status in mammals (Kapsokefalou and Miller, 1993; Ortega et al., 2001; Olsen and Ringø, 1998). Further, replacing dietary RO with FO increased hepatic Fe content in *S. salar* (Selvam et al., 2022a), which is partly attributed to increased dietary SFA level.

In the present study, the sequence of FAs digestibility was: Σ PUFA > Σ MUFA > Σ SFA, which is in accordance with other salmonids studies (Caballero et al., 2002; Karalazos et al., 2011; Olsen and Ringø, 1998). This result is correlated with the preference of bile salt activated lipase (BAL) for FA (Iijima et al., 1998; Sæle et al., 2018). BAL has high

efficiency in hydrolyzing PUFA esters in many marine fish (Gjellesvik et al., 1992; Iijima et al., 1998; Patton et al., 1977; Patton et al., 1975). Also, this order is associated with the FAs absorptive preference of intestinal epitheliums: PUFA > MUFA > SFA (Xu et al., 2020). Increased dietary SFA significantly reduced the digestibility of Σ SFA, Σ MUFA and Σ PUFA, which is similar to previous salmonid reports (Caballero et al., 2002; Karalazos et al., 2011; Menoyo et al., 2003; Sissener et al., 2017a, 2017b). Menoyo et al. (2003) suggested that increased saturation and chain length in feed reduced the ADC of FAs in *S. salar*. Although dietary SFA impacted digestibility of individual FA significantly, ADC values in all groups were high and the difference between groups were extremely minor.

5. Conclusion

Overall, in *Salmo salar*, increased SFA level in feed significantly increased the whole-body Se and Fe status. However, dietary SFA and CHO did not impact the whole-body Zn status. Further, increased SFA level in feeds significantly increased postprandial plasma CHO, LDL and total protein levels, whereas increased CHO level in feed significantly increased postprandial plasma CHO, HDL, LDL levels and tended to increase postprandial plasma astaxanthin and total protein levels. However, dietary SFA did not impact analyzed markers related to intestinal triacylglycerol transport.

Author contribution

All authors participated in the manuscript writing and approved the final version. Project manager: Nini; Experiment design and plan: Nini, Øystein, Antony, Grethe, Ingunn; dietary feed formulation and production, fish trial coordinator: Ingunn; Sample collection: Nini, Øystein, Antony; Sample and result analysis: HaoHang; Main author of manuscript writing: HaoHang; All author contributes to manuscript revision.

CRediT authorship contribution statement

HaoHang Fang: Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Nini H. Sissener:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Antony J. Prabhu Philip:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Grethe Rosenlund:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ingunn Stubhaug:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Øystein Sæle:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

No competing interests.

Data availability statement

Experiment data is available from the corresponding author based on reasonable requests.

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Appendix A. Supplementary data

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