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Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.)

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Abstract

Altered lipid metabolism has been shown in fish fed plant protein sources. The present study aimed to gain further insights into how intestinal and hepatic lipid absorption and metabolism are modulated by plant meal (PM) and soya-saponin (SA) inclusion in salmon feed. Post-smolt Atlantic salmon were fed for 10 weeks one of four diets based on fishmeal or PM, with or without 10 g/kg SA. PM inclusion resulted in decreased growth performance, excessive lipid droplet accumulation in the pyloric caeca and liver, and reduced plasma cholesterol levels. Intestinal and hepatic gene expression profiling revealed an up-regulation of the expression of genes involved in lipid absorption and lipoprotein (LP) synthesis (apo, fatty acid transporters, microsomal TAG transfer protein, acyl-CoA cholesterol acyltransferase, choline kinase and choline-phosphate cytidylyltransferase A), cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase) and associated transcription factors (sterol regulatory element-binding protein 2 and PPARγ). SA inclusion resulted in reduced body pools of cholesterol and bile salts. The hepatic gene expression of the rate-limiting enzyme in bile acid biosynthesis (cytochrome P450 7A1 (*cyp7a1*)) as well as the transcription factor liver X receptor and the bile acid transporter *abcb11* (ATP-binding cassette B11) was down-regulated by SA inclusion. A significant interaction was observed between PM inclusion and SA inclusion for plasma cholesterol levels. In conclusion, gene expression profiling suggested that the capacity for LP assembly and cholesterol synthesis was up-regulated by PM exposure, probably as a compensatory mechanism for excessive lipid droplet accumulation and reduced plasma cholesterol levels. SA inclusion had hypocholesterolaemic effects on Atlantic salmon, accompanied by decreased bile salt metabolism.

Key words: Lipoproteins: Sterols: Plant meal: Soya-saponins: Quantitative real-time PCR

For the majority of intensively reared carnivorous finfish species, such as salmonids, fishmeal (FM) is traditionally used as the protein source. While global aquaculture production has continued to grow, FM usage has remained static[1]. The limited supply of FM has led to increased interest in using plant proteins as alternatives to marine fishery-derived proteins. Nevertheless, inclusion of plant protein sources in aquafeeds is limited since a number of disadvantages have been recognised. Besides the negative effect on growth performance and intestinal function[2–5], plant protein inclusion in fish feeds has been reported to be associated with decreased lipid digestibility, reduced bile salt levels and hypocholesterolaemia, suggesting an altered regulation of lipid metabolic pathways[6–13]. The increased economical and quantitative importance of dietary plant protein sources in commercial fish feeds demands a better understanding of lipid metabolic processes such as absorption, transport and synthesis to improve feed utilisation and production cost optimisation.

The major lipid constituents of both natural and commercial salmonid diets are generally TAG with smaller amounts of phospholipids and cholesterol esters[14]. In monogastrics, TAG are hydrolysed in the intestinal lumen into NEFA (FA) and monoacylglycerols. Intracellularly, these hydrolysis products are carried by specific binding proteins to the endoplasmic reticulum, where they are resynthesised into TAG and incorporated into lipoproteins (LP). The formation of LP is

Abbreviations: *abcg5*, ATP-binding cassette G5; *cd36*, cluster of differentiation 36; *fabp2*, fatty acid-binding protein 2; *fatp*, fatty acid transport protein; FA, fatty acids; FM, fishmeal; *lxr*, liver X receptor; LP, lipoproteins; MTP, microsomal TAG transfer protein; PC, phosphatidylcholine; PM, plant meal; *srebp2*, sterol regulatory element-binding protein 2; SA, soya-saponin.

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mediated by the microsomal TAG transfer protein (MTP), which shuttles resynthesised TAG onto a newly formed apoB molecule. This primordial LP particle undergoes further lipidation with the addition of TAG and cholesterol esters by MTP, and phospholipids, apoAIV and, at a later stage, apoAI are added to the surface of the LP particle. This LP particle is then secreted by exocytosis via the basolateral membrane into the lymph[15,16]. It has been suggested that the mechanism of lipid absorption and LP formation in fish intestine does not differ fundamentally from that in monogastric mammals[17,18]. However, information is still lacking regarding the routes of lipid transport from the intestine. A lymphatic system has not been identified in fish yet.

Saponins are heat-stable glycosides present in legumes, such as soyabean, pea and lupin[19,20]. Saponins, with their membrane-permeabilising activity[21], are considered to be the factors responsible for the development of the distal intestine enteritis induced by soyabean meal in Atlantic salmon[22,23]. Based on their detergent and surfactant properties[21], dietary saponins probably disturb fat emulsification and micelle formation, but information necessary to understand their significance in lipid metabolism in fish is scarce. In general, saponins exhibit hypocholesterolaemic effects in a variety of animals[24–28], but these effects may be dependent on interactions with other plant components[29–31]. It has also been noted that a saponin-induced reduction of serum cholesterol levels occurs only when an otherwise hypocholesterolaemic diet has been fed[32]. It appears as if interactions between saponins and other components in plant meal are necessary for the effects on lipid metabolism, but information on these interactions is scarce.

The present study is a part of a larger experiment investigating the dose response of soya-saponin (SA) inclusion in plant meal- and FM-based diets at levels of 0, 2, 4, 6 and 10 g/kg. This part of the study aimed to understand how lipid absorption and metabolism are modulated by variation in protein source, saponin inclusion (10 g/kg), and the possible interaction between them in salmon feed. Intestinal and hepatic transcript levels of specific apo (*apoAI*, *apoAIV* and *apoB*), key enzymes (*mtp*, acyl-CoA cholesterol acyltransferase (*acat*), monoacylglycerol acyltransferase (*mgat*), choline kinase (*chk*) and choline-phosphate cytidylyltransferase A (*pcyt1a*)) and FA transporters (cluster of differentiation 36 (*cd36*), fatty acid transport protein (*fatp*) and fatty acid-binding protein 2 (*fabp2*)) were quantified using quantitative real-time PCR. We also quantified the hepatic expression of key genes involved in cholesterol and bile salt metabolism as described previously[33]. Total bile salt levels in the plasma and intestinal contents as well as plasma cholesterol levels were measured. Histomorphological alterations in the pyloric caeca and liver after dietary treatments were evaluated. Our hypothesis was that plant meal and SA inclusion in salmon feed would cause alterations in the expression levels of genes involved in lipid and sterol metabolism and that the differential expression would be reflected in alterations in blood plasma sterol levels and tissue lipid accumulation.

Materials and methods

Feed ingredients and diet formulation

SA (95 % concentrate) was obtained from Organic Technologies. FM, lupin meal and wheat gluten meal were used as primary protein sources. Fish oil and wheat meal were used as lipid and carbohydrate sources, respectively. For the present experiment, two basal diets were formulated: a FM-based diet (FM diet) with 587 g/kg FM and a plant meal-based diet (PM diet) with 60 % substitution of FM (235 g/kg FM, 200 g/kg lupin meal and 190 g/kg wheat gluten meal). The diets were formulated to be isoenergetic (24 MJ/kg gross energy). The fish investigated in the present study were fed one of the basal diets or diets produced from the basal diets supplemented with 10 g SA/kg. This saponin level is supposed to correspond to the level present in a diet in which a saponin-rich soyabean meal provides all the dietary protein. All diets were supplemented with standard vitamins and minerals to meet the requirements[34]. The diets were produced by Nofima AS, with a pellet size of 5 mm. Detailed diet formulations are given in Table 1.

Experimental animals and design

The feeding trial was carried out at the Nofima Marine research station at Sunndalsøra (Norway) and in compliance with laws regulating experimentation with live animals in Norway, as overseen by the Norwegian Animal Research Authority (Forsøksdyrutvalget). The experiment was carried out using a 2 × 2 factorial design with four dietary treatments. Furthermore, two factors, PM inclusion and SA inclusion, were

Table 1. Formulation of the experimental diets

Ingredients (g/kg)	FM	FM-SA	PM	PM-SA
FM*	587	587	235	235
Lupin meal†			200	200
Wheat gluten‡			190	190
Wheat meal	156	156	67	67
Soya-saponin§		10		10
Wheat starch	10		10	
Fish oil‖	221	221	242	242
Vitamin mix¶	20	20	20	20
Mineral mix¶	5	5	5	5
$Ca(H_2PO_4).2H_2O$			10	10
Carophyll pink	0·4	0·4	0·4	0·4
Yttrium oxide**	0·1	0·1	0·1	0·1
Lys HCl			17	17
DL-Met			3·6	3·6
Chemical composition (%)				
Crude protein	42·3	42·3	44·1	44·1
Crude lipid	29·3	29·3	30·3	30·3
Starch	10·3	10·3	7·5	7·5
Energy (MJ gross energy/kg)	23·9	23·9	24·2	24·2

FM, fish meal; FM-SA, fish meal with soya-saponin; PM, plant meal; PM-SA, plant meal with soya-saponin.
* Fishmeal 122/11, LTQ fishmeal, produced by Welcon AS.
† Supplied by Holtermann AS.
‡ Wheat gluten 159/10, Amytex 100, produced by Syral.
§ 95 % Concentrate, supplied by Organic Technologies.
‖ Fishoil O5/10, NorSalmOil, produced by Norsildmel.
¶ Supplemented to meet the requirements.
** Marker for the evaluation of nutrient digestibility.

Table 2. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes* used for real-time PCR

Gene symbols	5′–3′ primer sequence		Amplicon size (bp)	Annealing temperature (°C)	Primer efficiency	GenBank accession no.
	Forward	Reverse				
mgat2a	acgctacaggcttcaggaaa	ggaatcagacctgccatcat	116	60	2·05	NM_001140718
$apoB_{liver}$†	ttgcagagacctttaagttcattca	tgtgcagtggttgccttgac	120	60	1·94	X81856
$apoB_{intestine}$‡	ccctgagatggtgtccgtat	gcgtcgacttccatagcttc	131	63	1·80	CB504205
apoAI	ctggtcctcgcactaaccat	tggacctctgtgcagtcaac	144	60	1·96	NM_001123663
apoAIV	caggaccagtctcagcaaca	gttgacttcctgtgccacct	131	60	1·90	BT048822
mtp	aacgtgacagtggacatgga	ggaccgtggtgatgaagtct	89	60	2·00	CA042356
acat	tgctggagtttgacctgttg	gctgcgatggtagagagtcc	139	60	1·98	GE793368
chk	ctcaagtttgcccgtctgat	cacaggggaatgagtggagt	88	60	1·94	DY706802
pcyt1a	cgggtctatgcagatggaat	gctcgtcctcgttcatcact	166	60	2·09	BT045986
cd36	caagtcagcgacaaaccaga	aggagacatggcgatgtagg	91	60	1·93	AY606034
fatp	aggagagaacgtctccacca	cgcatcacagtcaaatgtcc	159	60	1·88	CA373015
fabp2a1§	ggtgctgaaaactaccagagcca	ggatttgaacgtagctcttcttgg	152	60	2·00	EU880417
fabp2b§	tgccttcccctcattctcta	ggtgatacggtcttcatccaa	82	60	2·00	EU880419
pparα§	gcttcatcaccagggagttt	tcactgtcatccagctccag	113	60	1·99	NM_0011235960
pparγ§	tgctgcaggctgagtttatg	caggggaaagtgtctgtggt	107	58	1·96	NM_0011235946
hmgcr‖	ccttcagccatgaactggat	tcctgtccacaggcaatgta	224	60	1·88	NM_001173919
srebp2‖	tcgcggcctcctgatgatt	agggctaggtgactgttctgg	147	60	1·91	HM561861
cyp7a1¶	tcctcaacaccctggagaac	cagcatggtcttagccaggt	125	63	2·03	BT059202
abcg5¶	agactgcctcgtccaacact	ccattttcgtgaacgtgtacc	157	60	1·94	CU073172
*abcb11***	ccgaccagggcaaagtgatt	cagaatgggctcctgggatac	101	60	1·92	NM_001124656
fxr¶	ttcaacatctcaactcatca	tagcaggtcctcattgat	102	60	2·01	NM_001173830
lxr‖	gccgccgctatctgaaatctg	caatccggcaaccaatctgtagg	210	60	1·88	FJ470290

mgat2a, monoacylglycerol acyltransferase 2-A; *mtp*, microsomal TAG transfer protein; *acat*, acyl-CoA cholesterol acyltransferase; *chk*, choline kinase; *pcyt1a*, choline-phosphate cytidylyltransferase 1A; *cd36*, cluster of differentiation 36; *fatp*, fatty acid transport protein; *fabp2a1*/*fabp2b*, fatty acid-binding protein 2 isoforms; *pparα*/*pparγ*, PPAR isoforms; *hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *srebp2*, sterol regulatory element-binding protein 2; *cyp7a1*, cytochrome P450 7A1; *abcg5*, ATP-binding cassette G5; *abcb11*, ATP-binding cassette B11; *fxr*, farnesoid X receptor; *lxr*, liver X receptor.

* Readers can refer to a previous study[35] for reference gene primer details.

† Primers obtained from Torstensen *et al.*[77]. High expression was found in the liver and very low expression in the pyloric caeca.

‡ Primers designed in 5′ end of apoB transcript. High expression was found in the pyloric caeca.

§ Primers obtained from Venold *et al.*[78].

‖ Primers obtained from Minghetti *et al.*[79].

¶ Primers obtained from Kortner *et al.*[33].

** Primers obtained from Zaja *et al.*[80].

tested separately and in combination. Post-smolt Atlantic salmon (*Salmo salar* L.) of the Sunndalsøra strain with an initial weight of 442 (SEM 4) g were allocated to eight 1 m^3 fibreglass tanks, twenty-two fish per tank, with 250 litres of seawater flowing at a rate of 20 litres/min. Fish present in duplicate tanks were fed one of the four experimental diets. During the feeding trial, water temperature was decreased from 12 to 9°C. The oxygen content and salinity of the outlet water were monitored. Salinity ranged between 32 and 33 g/l. Fish were continuously fed by automatic disc feeders. A 24 h lighting regimen was employed during the experimental period.

Sampling

Sampling was conducted following 10 weeks of feeding the experimental diets. Randomly selected fish were anaesthetised with tricaine methanesulphonate (MS222; Argent Chemical Laboratories) and subsequently killed by a sharp blow to the head. Only the fish that had digesta throughout the intestinal tract were sampled to ensure intestinal exposure to the diets. The intestines were cleared of all fatty tissue and intestinal content before the collection of tissue samples. Pyloric caecal and liver tissues were sampled from four fish from each tank for mRNA extraction, placed in RNAlater (Ambion) at 4°C for 24 h and then stored at −20°C. Blood (four fish per tank) was collected in heparinised vacutainers for plasma preparation. Intestinal contents (four fish per tank) from the pyloric intestine, mid-intestine and distal intestine were frozen in liquid N_2 and then stored at −80°C. Pyloric caecal and liver tissues (six fish per tank) were sampled for histological evaluation, placed in 4% phosphate-buffered formaldehyde solution for 24 h and subsequently stored in 70% ethanol until further processing.

Quantitative real-time PCR

RNA purification and quality control, DNase treatment, complementary DNA synthesis, primer optimisation and quantitative PCR assays were carried out as described in detail elsewhere[35]. Quantitative PCR primers were obtained from the literature or designed using Primer3 (http://frodo.wi.mit.edu/primer3). The details of the primers are given in Table 2. Elongation factor 1α (*ef1a*), β-actin (*actb*), RNA polymerase II (*rnapolII*), hypoxanthine phosphoribosyltransferase 1 (*hprt1*), ribosomal protein S20 (*rps20*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were evaluated for use as reference genes by ranking the expression levels according to their stability, as described previously[35]. For pyloric caecal and liver samples, *rps20* was used as the normalisation factor. The mean normalised expression of the target genes was calculated from raw quantification cycle (Cq) values[36].

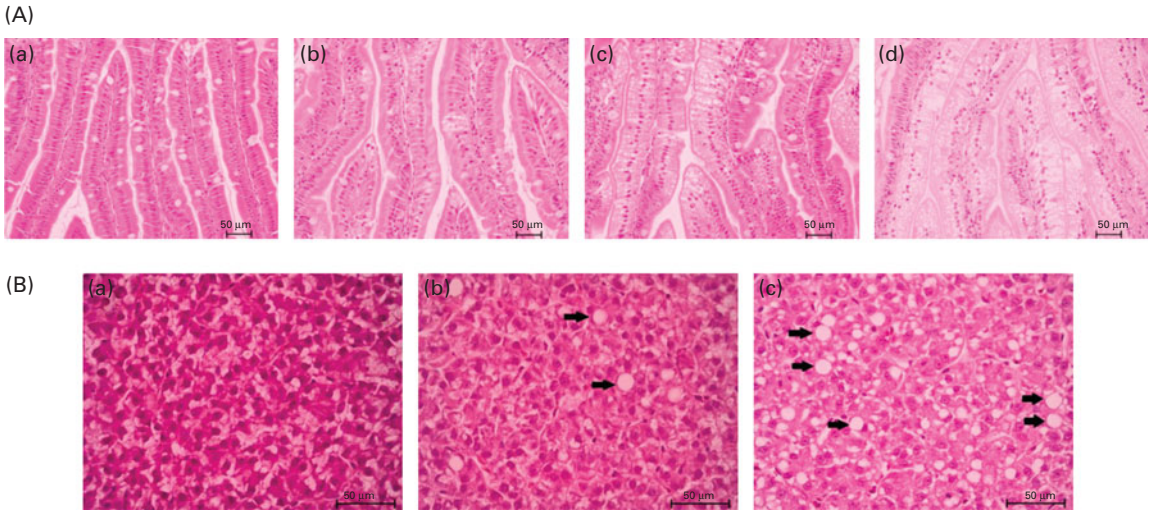

Fig. 1. Severity of steatosis of the pyloric (A) caeca and (B) liver of fish fed the experimental diets, representative for (a) absent (normal), (b) slight, (c) moderate and (d) marked. →, Large vacuoles of TAG fat. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Histology

From each treatment group, twelve fish were used for histological evaluation. Pyloric caecal and liver samples were dehydrated in ethanol, equilibrated in xylene, embedded in paraffin and sectioned (5 μm) according to standard histological techniques. Tissues were stained with haematoxylin and eosin. Blinded histological examination was carried out using a light microscope by paying particular attention to (presumed) lipid accumulation (steatosis) within enterocytes and hepatocytes. Steatosis was assessed using a categorical scoring system with grades of absent, slight, moderate and marked (Fig. 1).

Cholesterol and bile salt analyses

Plasma cholesterol and bile salt concentrations, as well as bile salt concentrations in the intestinal contents, were determined as described previously[12]. All analyses were carried out on Advia® 1800 (Siemens Healthcare Diagnostics) at the Central Laboratory of the Norwegian School of Veterinary Science, Oslo, Norway.

Statistical analyses

Statistical analyses were carried out using JMP 10 (SAS Institute, Inc.). PM inclusion and SA inclusion were evaluated as class variables in a two-way ANOVA with interaction. For the histological evaluation of intestinal and liver samples, results were compared using a χ^2 test. The level of significance was set at $P<0{\cdot}05$.

Results

Growth performance

Growth performance was significantly affected by PM inclusion, but not by SA inclusion or the interaction between them (Fig. 2(A); Table 3). Feeding the PM-based diets resulted in significantly decreased final weight and specific growth rate (SGR) compared with feeding the FM diets by 24·8 and 20·7 %, respectively.

Histology

Lipid droplet accumulation in pyloric caecal enterocytes was significantly affected by PM inclusion (Table 4). Feeding the PM diets resulted in increased lipid droplet accumulation in enterocytes compared with feeding the FM diets. Similarly, the frequency of hepatic steatosis was significantly affected by PM inclusion (Table 4). Whereas none of the sampled fish fed the FM diets exhibited steatosis in the liver, several fish fed the PM diets exhibited slight-to-moderate hepatic steatosis. No significant interaction between PM inclusion and SA inclusion was observed.

Cholesterol and bile salt levels

Blood plasma cholesterol levels were significantly affected by both PM inclusion and SA inclusion (Fig. 2(B); Table 3). PM and/or SA supplementation significantly decreased blood plasma cholesterol levels. Moreover, a significant interaction between PM inclusion and SA inclusion was observed for this variable. The magnitude of the hypocholesterolaemic effects of SA on fish fed the PM-based diets was not as large as that of the effects on fish fed the FM diets. Plasma bile salt levels of fish were significantly decreased by SA inclusion. No effect of PM inclusion and no interaction between the saponin and dietary treatments were observed (Fig. 2(B); Table 3).

Digesta bile salt levels in the intestinal sections were significantly reduced by SA inclusion but not by PM inclusion (Fig. 2(C); Table 3).

Gene expression profiling

As shown in Fig. 3 and Table 3, PM inclusion had significant effects on pyloric caecal expression of key genes involved in

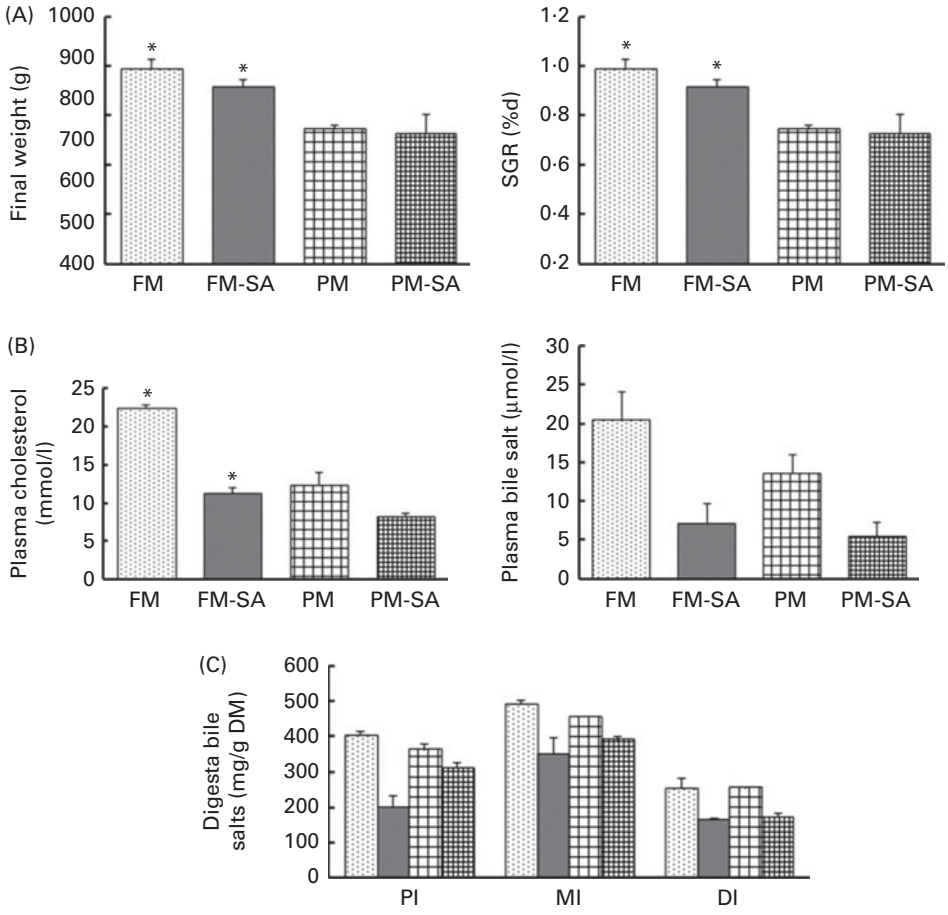

Fig. 2. Growth performance (A), concentrations of blood plasma cholesterol and bile salts (B) and concentrations of bile salts in digesta (C) of fish fed the experimental diets: fish meal (FM, ⊡); fish meal with soya-saponin (FM-SA; ▩); plant meal (PM; ▦); plant meal with soya-saponin (PM-SA; ▦). Values are means, with standard errors represented by vertical bars. * Mean values were significantly different from those for plant meal inclusion ($P<0·05$; two-way ANOVA). For plasma cholesterol, plasma bile salts and digesta bile salts, there was a significant effect for soya-saponin inclusion ($P<0·05$; two-way ANOVA). For plasma cholesterol, there was a significant interaction between plant meal and soya-saponin inclusion ($P<0·05$; two-way ANOVA). SGR, specific growth rate; PI, pyloric intestine; MI, mid-intestine; DI, distal intestine.

lipid metabolism. Generally, PM inclusion induced the up-regulation of the expression of genes involved in LP assembly (*apoB*, *apoAI*, *apoAIV*, *mtp*, *acat*, *chk* and *pcyt1a*) and FA transport (*cd36*, *fatp* and *fabp2a1*). SA inclusion up-regulated the expression of genes encoding FA transporters (*cd36*, *fatp* and *fabp2b*) as well as the associated transcription factor *pparγ*. Neither PM inclusion nor SA inclusion changed the expression of *mgat2a*.

In the liver (Fig. 4; Table 3), similar to the results observed in the pyloric caeca, PM inclusion resulted in the up-regulation of the expression of genes involved in LP synthesis (*apoB*, *apoAI* and *mtp*) and fatty acid transport (*fatp*). PM inclusion had significant effects on the expression of genes involved in cholesterol metabolism. PM inclusion resulted in the up-regulation of the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) as well as the associated transcription factors (sterol regulatory element-binding protein 2 (*srebp2*) and *pparγ*). SA inclusion had significant effects on the expression of genes involved in bile acid metabolism. Feeding SA-supplemented diets resulted in 11·5- and 8·3-fold down-regulation of the expression of cytochrome P450 7A1 (*cyp7a1*) compared with feeding the FM and PM diets, respectively. In parallel, SA supplementation resulted in reduced expression levels of liver X receptor (*lxr*), bile acid transporter (ATP-binding cassette B11 (*abcb11*)) and cholesterol transporter (ATP-binding cassette G5 (*abcg5*)).

Discussion

Effects of plant meal inclusion

In the present study, dietary inclusion of PM reduced growth performance, induced lipid droplet accumulation in the pyloric caeca and liver, and decreased plasma cholesterol levels in Atlantic salmon. The reduction of growth performance by PM inclusion is in accordance with several previous studies in Atlantic salmon[2,6,37].

The present study indicates that the capacity for intestinal LP assembly was up-regulated in the PM diet-fed fish, probably as a result of excessive lipid droplet accumulation. Interestingly, the expression of *mgat2a*, which is responsible for TAG re-esterification, remained unaffected by PM inclusion. In accordance with this observation, a number of studies have

Table 3. Two-way ANOVA of the data for growth, cholesterol and bile salt levels, and gene expression of fish fed the experimental diets

	P		
Parameters	PM	SA	PM×SA
Final weight (g)	0·004	0·279	0·378
Specific growth rate (%/d)	0·007	0·353	0·547
Plasma cholesterol (mmol/l)	0·002	0·001	0·018
Plasma bile salt (μmol/l)	0·172	0·014	0·375
Digesta bile salt (mg/g DM)			
Pyloric intestine	0·164	0·003	0·020
Mid-intestine	0·941	0·011	0·169
Distal intestine	0·760	0·007	0·880
Gene expression in the pyloric caeca			
mgat2a	0·710	0·176	0·829
apoB	0·025	0·730	0·955
apoAI	<0·001	0·843	0·052
apoAIV	<0·001	0·824	0·318
mtp	0·001	0·214	0·686
acat	0·014	0·624	0·885
chk	<0·001	0·708	0·887
pcyt1a	<0·001	0·239	0·892
cd36	0·001	0·002	0·389
fatp	0·002	0·022	0·269
fabp2a1	0·026	0·272	0·631
fabp2b	0·297	<0·001	0·188
pparα	0·243	0·160	0·740
pparγ	0·476	0·003	0·298
Gene expression in the liver			
hmgcr	0·005	0·412	0·940
srebp2	0·008	0·104	0·675
cyp7a1	0·160	<0·001	0·201
abcg5	0·556	0·006	0·478
abcb11	0·149	0·035	0·246
lxr	0·104	<0·001	0·127
fxr	0·088	0·625	0·182
pparα	0·308	0·189	0·458
pparγ	<0·001	0·489	0·853
apoB	0·038	0·437	0·262
apoAI	0·001	0·201	0·682
mtp	0·007	0·218	0·875
acat	0·852	0·113	0·459
fatp	0·001	0·591	0·475
cd36	0·247	0·277	0·967

PM, plant meal; SA, soya-saponin; PM × SA, interaction between plant meal and soya-saponins; *mgat2a*, monoacylglycerol acyltransferase 2-A; *mtp*, microsomal TAG transfer protein; *acat*, acyl-CoA cholesterol acyltransferase; *chk*, choline kinase; *pcyt1a*, choline-phosphate cytidylyltransferase 1A; *cd36*, cluster of differentiation 36; *fatp*, fatty acid transport protein; *fabp2a1*/*fabp2b*, fatty acid-binding protein 2 isoforms; *pparα*/*pparγ*, PPAR isoforms; *hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *srebp2*, sterol regulatory element-binding protein 2; *cyp7a1*, cytochrome P450 7A1; *abcg5*, ATP-binding cassette G5; *abcb11*, ATP-binding cassette B11; *lxr*, liver X receptor; *fxr*, farnesoid X receptor.

demonstrated that the accumulation of large lipid droplets (mainly consisting of TAG) in the intestine of fish is not due to the increased TAG re-esterification but rather due to lipid export rate reduction[38–42]. Therefore, the excessive lipid droplet accumulation observed in the present study, most probably, was a result of reduced lipid export from the intestinal mucosa to the circulatory system. The reason for this is not clear; however, deficient amounts of one or more of the major constituents of the polar LP surface, such as phosphatidylcholine (PC), apo and cholesterol, are probably responsible for this. In support of this, it has been shown that dietary supplementation with PC prevents intestinal steatosis in common carp larvae[38].

In mammalian systems, molecular regulation of lipid absorption and transport from the intestine has been studied extensively[15,16,43,44]. The present study reveals novel information on the regulation of lipid absorption and transport in a piscine model, which is assumed to be generally similar to that in mammals[18,45,46]. A summary of the proposed regulation is shown in Fig. 5. Dietary lipids are exported from the intestine in the form of large, neutral lipid-rich LP. LP production in the enterocytes involves a series of biosynthetic processes that results in the formation of a large hydrophobic core surrounded by a thin coat of phospholipids (mainly PC), cholesterol and apo[43]. Clearly, phospholipids, cholesterol and apo are required for LP assembly. The positive effects of intestinal PC on LP formation have been demonstrated in mammals[47–49]. In fish, the ability of the enterocytes to export lipids may be limited by the absence of dietary PC[41,42]. As in mammals, *de novo* PC synthesis in fish occurs primarily via the cytidine diphosphate (CDP)-choline pathway with choline kinase producing phosphocholine recognised as the committing enzyme, followed by phosphocholine cytidylyltransferase producing CDP-choline, recognised as the rate-limiting and rate-regulatory enzyme[45]. In the present study, the expression of both *chk* and *pycta1* was up-regulated in the PM diet-fed fish, indicating that the capacity for PC synthesis from choline was increased. PM inclusion also induced the up-regulation of the expression of genes encoding the *apoB, apoAI* and *apoAIV*, and an increased capacity for cholesterol esterification was indicated by the increased *acat* levels. In mammals, the increased mRNA expression of *mtp* in the intestine has been considered to be a response to excessive lipid droplet accumulation[16]. The present study is in line with these studies, showing an up-regulation of the expression of *mtp* in the pyloric caeca when steatosis was observed. In parallel to the overall transcriptional induction of LP synthesis, the expression of genes involved in FA transport was also induced, showing an up-regulation of the FA transporters *cd36* and *fatp*, as well as *fabp2a1*, which is responsible for FA transport from the cytoplasm to the endoplasmic reticulum[48]. In FA transporter knockout animals, lipids tend to accumulate in the proximal small intestine primarily because of the decreased transport of FA targeted for transport to the circulatory system through their use in the assembly of LP[50]. However, it is likely that an efficient FA uptake from the intestinal lumen requires both spontaneous diffusion and protein-mediated transfer, and the relative importance of these two mechanisms is probably highly dependent on the microenvironment and tissue phenotype[44]. Altogether the present results suggest that PM inclusion up-regulated the capacity for LP synthesis and FA transport. The correlation between induced gene expression and increased lipid droplet accumulation suggests an induction of regulatory mechanisms to compensate for the reduced lipid export rate.

In line with our observations in the pyloric caeca, PM treatment resulted in hepatic fatty change and increased levels of genes (*apoB*, *apoAI* and *mtp*) encoding key proteins involved in LP synthesis (mainly VLDL). These results suggest that similar regulatory mechanisms may exist in both the intestine and liver of fish fed the PM diets to compensate for the reduced

Table 4. Severity of steatosis of the pyloric caeca and liver of fish fed the experimental diets*

					Two-factor χ^2 test†		
	FM	FM-SA	PM	PM-SA	PM	SA	PM × SA
Intestinal steatosis						1·000	1·000
Absent (normal)	10	9	0	0	<0·001		
Slight	2	3	1	2			
Moderate	0	0	8	7			
Marked	0	0	3	3			
Hepatic steatosis						1·000	1·000
Absent (normal)	12	12	8	4	<0·001		
Slight	0	0	4	6			
Moderate	0	0	0	2			

FM, fish meal; FM-SA, fish meal with soya-saponin; PM, plant meal; PM-SA, plant meal with soya-saponin; PM × SA, interaction between plant meal inclusion and soya-saponin inclusion.
* The numbers represent the number of fish. In each treatment group, twelve fish were used for histological evaluation.
† The statistical analyses pertain to all the data for each tissue.

lipid export rate. However, the increased lipid droplet accumulation in the liver of fish fed the PM may also result from an increase in lipolysis from adipose tissue[51–53].

Gene expression profiling also indicated that the capacity for cholesterol synthesis was up-regulated in the PM diet-fed fish. This is in accordance with several previous studies[13,33,54–56] and could reflect a general need for *de novo* cholesterol synthesis in PM-fed fish, probably as a direct result of reduced cholesterol body pools. The proposed regulation of hepatic cholesterol and bile acid synthesis is shown in Fig. 5. In mammals, the committed step in hepatic cholesterol biosynthesis is the synthesis of mevalonate by Hmgcr, and *hmgcr* mRNA transcription is controlled by Srebp[57,58]. At low sterol levels, Srebp are cleaved and translocated to the nucleus and these up-regulate the expression of genes involved in sterol metabolism[58]. The involvement of Srebp2 in the up-regulation of

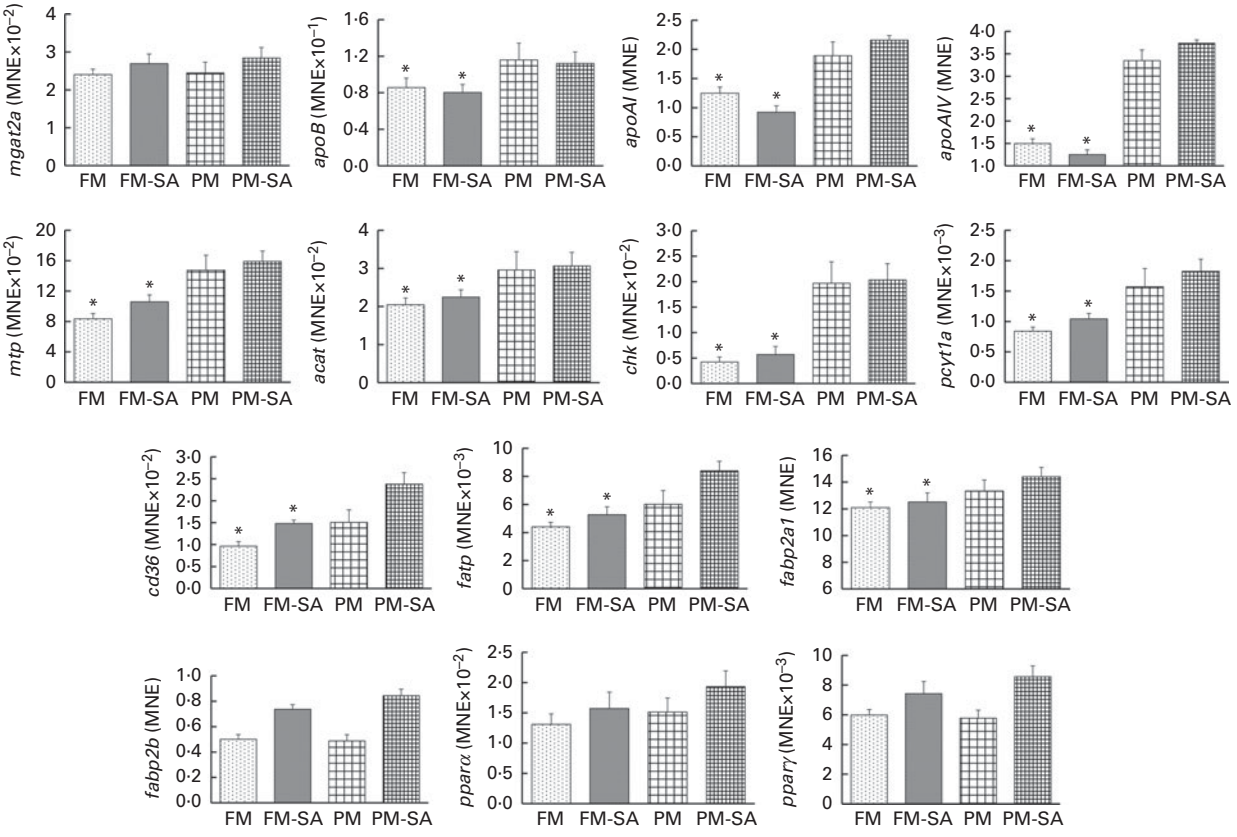

Fig. 3. Pyloric caecal gene expression of monoacylglycerol acyltransferase 2-A (*mgat2a*), *apoB*, *apoAI*, *apoAIV*, microsomal TAG transfer protein (*mtp*), acyl-CoA cholesterol acyltransferase (*acat*), choline kinase (*chk*), choline-phosphate cytidylyltransferase 1A (*pcyt1a*), cluster of differentiation 36 (*cd36*), fatty acid transport protein (*fatp*), fatty acid-binding protein 2 isoforms (*fabp2a1/fabp2b*) and PPAR isoforms (*pparα/pparγ*). Values are means, with standard errors represented by vertical bars. * Mean values were significantly different from those for plant meal inclusion ($P<0·05$; two-way ANOVA). For *cd36*, *fatp*, *fabp2b* and *pparγ*, there was a significant effect for soya-saponin inclusion ($P<0·05$; two-way ANOVA). FM, Fish meal (□); FM-SA, fish meal with soya-saponin (▨); PM, plant meal (▦); PM-SA, plant meal with soya-saponin (▦); MNE, mean normalised expression.

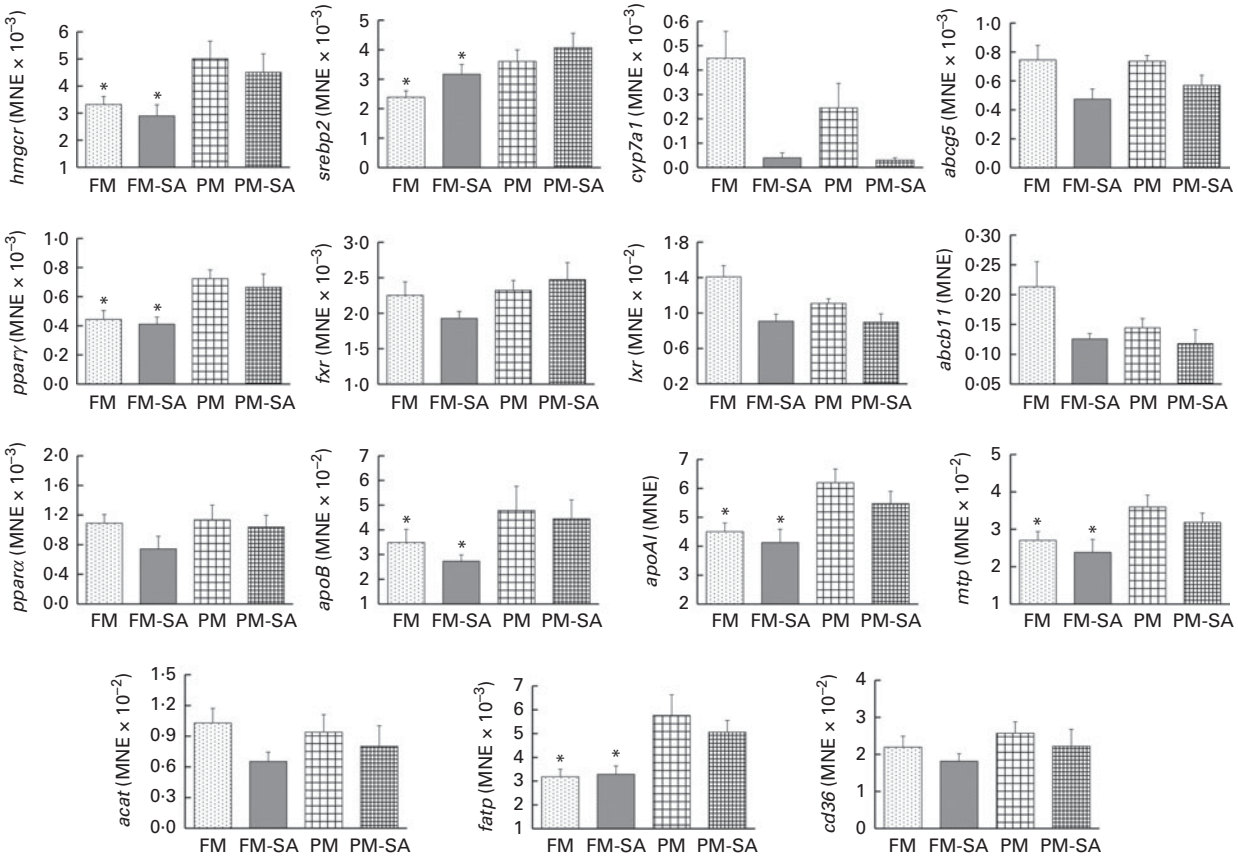

Fig. 4. Hepatic gene expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*), sterol regulatory element-binding protein 2 (*srebp2*), cytochrome P450 7A1 (*cyp7a1*), ATP-binding cassette G5 and B11 (*abcg5* and *abcb11*), liver X receptor (*lxr*), farnesoid X receptor (*fxr*), PPAR isoforms (*pparα/pparγ*), *apoB*, *apoAI*, microsomal TAG transfer protein (*mtp*), acyl-CoA cholesterol acyltransferase (*acat*), fatty acid transport protein (*fatp*) and cluster of differentiation 36 (*cd36*). Values are means, with standard errors represented by vertical bars. * Mean values were significantly different from those for plant meal inclusion ($P<0·05$; two-way ANOVA). For *cyp7a1*, *abcg5*, *lxr* and *abcb11*, there was a significant effect for soya-saponin inclusion ($P<0·05$; two-way ANOVA). FM, fish meal (□); FM-SA, fish meal with soya-saponin (▨); PM, plant meal (▦); PM-SA, plant meal with soya-saponin (▦); MNE, mean normalised expression.

cholesterol biosynthetic pathways has been shown in salmon fed plant products[33,54,57,59]. The present study is in line with these previous studies, showing increased mRNA levels of *srebp2*, as well as *hmgcr*, in fish fed the PM diets.

In addition to Srebp, Ppar are also important regulators of lipid and sterol metabolism and may interact and crosstalk with Srebp, Lxr and Fxr[60]. In an earlier study also conducted in salmon, *pparγ* showed a stronger response to the diet supplemented with PM when compared with *pparα*[33]. The present study is in line with this report, as the hepatic expression of *pparα* remained unaffected by PM inclusion, whereas that of *pparγ* was up-regulated. The observed induction of the expression of *pparγ* could be a result of alterations in hepatic fat homeostasis, as some studies have demonstrated an enhanced expression of *pparγ* in animal models of steatotic liver[61–63]. Recent mammalian studies have also shown that Pparγ regulates the expression of many genes involved in cholesterol transport and metabolism[64–67].

Effects of soya-saponin

In the present study, SA inclusion significantly decreased plasma cholesterol levels. A number of studies have shown that saponin from different sources lowers plasma cholesterol levels in various animals (reviewed in Francis *et al.*[21]). These reports suggest that dietary saponins may be, at least partly, responsible for the hypocholesterolaemic effects of dietary PM in fish studies[33,68]. Moreover, the present results showed a reduction in bile salt concentrations after SA exposure both in the intestinal lumen and in blood plasma. Many factors may possibly play a role in causing the decreased body pools of cholesterol and bile salt observed with dietary SA inclusion. For example, most saponins can form complexes with cholesterol and bile salts, thereby increasing their faecal excretion[69,70]. Additionally, SA has been shown to be an important factor responsible for the enteritis induced by dietary soyabean meal[22,23,71,72]. The saponin-induced intestinal inflammation seems to impair enterocyte maturation and may, therefore, limit bile salt reabsorption from the gut. Furthermore, the results of the present study indicated that the capacity for hepatic biosynthesis and secretion of bile salts was down-regulated by saponin exposure, which may aggravate the depletion of bile salts.

The classical pathway of primary bile acid synthesis mediated by the rate-limiting enzyme Cyp7a1 is thought to be the major contributor to bile salt synthesis in humans[60]. The basal expression of *cyp7a1* is primarily controlled by one or more specific transcription factors, wherein positive and negative regulation is mediated by Lxr and Fxr, respectively[73]. In the

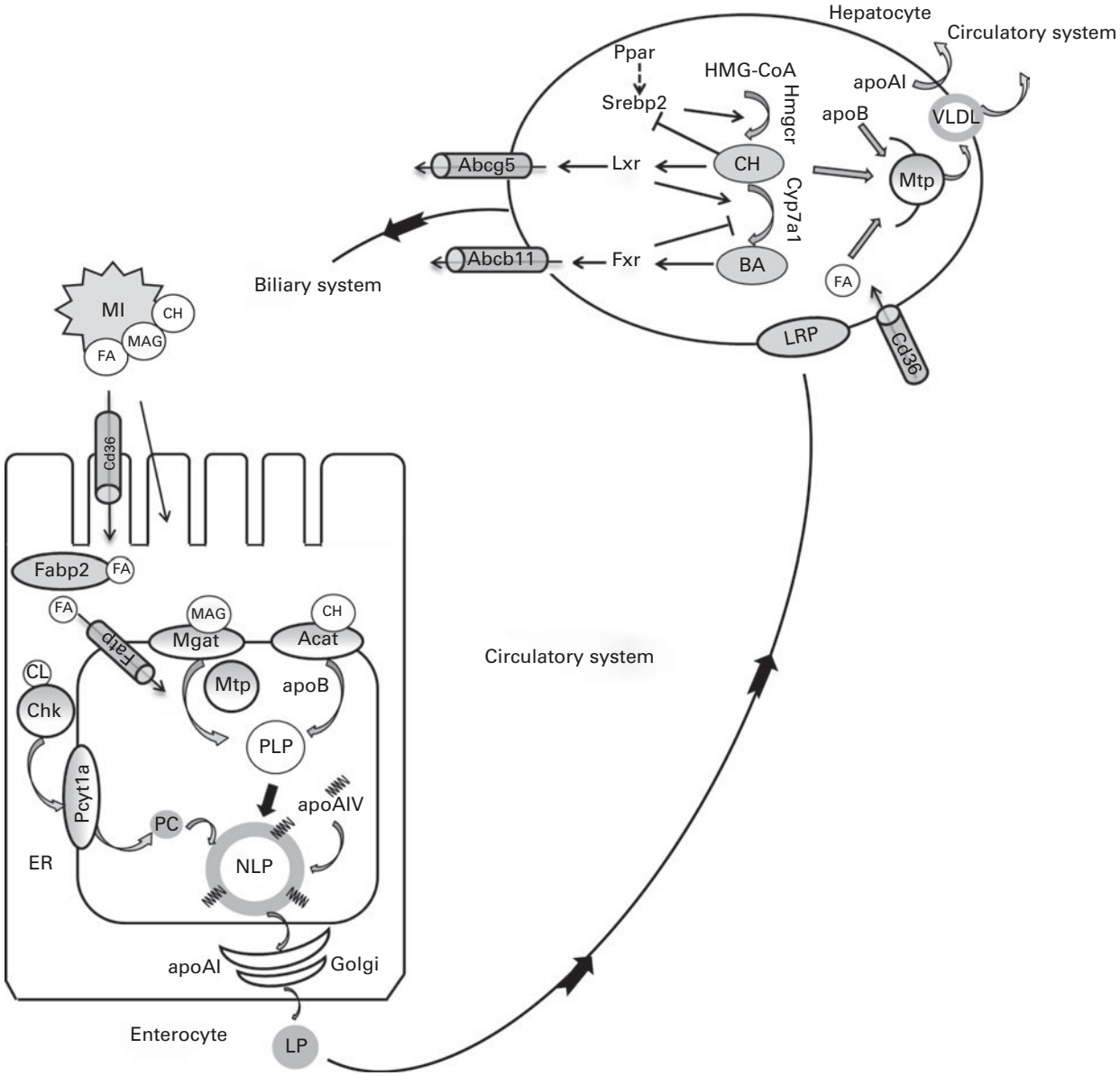

Fig. 5. Proposed molecular regulation of lipoprotein (LP) assembly, and cholesterol (CH) and bile acid (BA) metabolism based on data from the present study and previous studies in fish and mammalian species. In the intestinal lumen, products of lipid hydrolysis are solubilised in micelles (MI) and presented to the apical membrane of enterocytes. Fatty acids (FA) are transported from the intestinal lumen into the enterocytes by protein transporters including Cd36 (cluster of differentiation 36) and fatty acid transport protein (Fatp) or by protein-independent diffusion. Within the cytoplasm, FA are shuttled by fatty acid-binding protein 2 (Fabp2). Acyl-CoA cholesterol acyltransferase (Acat) and monoacylglycerolacyltransferase (Mgat) are located in the endoplasmic reticulum (ER) membrane, where they facilitate the esterification of CH and monoacylglycerols (MAG), respectively. These esterified products are assembled into proximal lipoproteins (PLP) with apoB in a microsomal TAG transfer protein (Mtp)-dependent manner. The PLP is merged with apoAIV to form a nascent lipoprotein (NLP) by core expansion. Choline kinase (Chk) and choline-phosphate cytidylyltransferase (Pcyt1a) are the committed and rate-limiting enzymes in the synthesis of phosphatidylcholine (PC) from choline (CL). PC is used in the formation of the polar lipid coat of NLP. Within the Golgi lumen, apoAI is attached to NLP to form a mature LP. The LP is released from the enterocyte to enter the circulatory system. LP are transported to the liver, in fish supposedly via the portal veins, and recognised by LP receptor-related proteins (LRP). In hepatocytes, the intestinal LP are disassembled and new LP, i.e. VLDL, are formed. apoB and Mtp participate in the formation of VLDL. apoAI is also secreted by the liver. CH and primary BA are the major constituents of bile that are synthesised in the liver and released into the biliary duct via the specific membrane transporters ATP-binding cassette G5 and B11 (Abcg5 and Abcb11), respectively. Both CH and BA are stored in the gall bladder and released into the intestine upon ingestion of feed. In the hepatocytes, sterol regulatory element-binding protein 2 (Srebp2) up-regulates the expression of genes involved in CH synthesis (3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgcr*)). Liver X receptor (Lxr) is activated by oxysterols, and it up-regulates the expression of genes involved in CH catabolism (cytochrome P4507A1 (*cyp7a1*)) and transport (*abcg5*). Farnesoid X receptor (Fxr) is activated by BA and controls intracellular BA levels by the regulation of Cyp7a1. PPAR isoforms (Ppar) are associated with the regulation of CH and BA metabolism, possibly by interaction with Srebp2. →, Activation; —, inhibition; ----, possible interaction.

present study, *lxr* mRNA levels were reduced after SA supplementation, but *fxr* levels remained unaffected. As an important transcription factor in sterol metabolism in mammals and, mostly probably, fish[74], *lxr* transcripts have been cloned and characterised in salmonids[75]. The hepatic expression of *lxr* has been shown to be higher in salmon fed fish oil than in those fed vegetable oil, which could be related to either dietary cholesterol/phytosterol contents or FA composition[75]. The results of the present study showed that saponins could be another factor inducing the down-regulation of the expression of *lxr* in fish fed plant-derived alternatives. Consistent with the *lxr* expression profile, the expression of *cyp7a1* was markedly down-regulated by SA exposure. These results suggest that the capacity for hepatic bile acid biosynthesis was down-regulated in fish fed SA.

In the liver, the ATP-binding cassette Abcg5 (which forms a heterodimer with Abcg8) is regulated by Lxr activation and mediates cholesterol transport from the liver into the bile[76]. In the present study, the expression of both *abcg5* and *lxr* was down-regulated by SA exposure. This result is in line with the previous observation that *abcg5* and *lxr* displayed similar gene expression profiles in the liver of salmon fed soyabean meal[33]. The reason for the down-regulation of the expression of *abcg5* is not clear; however, it is probably a result of alterations in hepatic sterol metabolism induced by SA. Similarly, the hepatic expression of the bile salt transporter *abcb11* was down-regulated by SA. Since the bile salt concentration is about 100- to 1000-fold higher in the bile than in the hepatocytes, canalicular bile salt transport represents the rate-limiting step in bile formation[60], and Abcb11 is mainly responsible for bile salt transport across the canalicular membrane[76]. These results indicate that the capacity for bile salt transport from the liver to the biliary duct was decreased in fish fed SA.

In contrast to PM inclusion, SA inclusion did not affect lipid droplet accumulation or expression levels of genes involved in LP assembly. However, the expression of the FA transporters *cd36*, *fatp* and *fabp2b* was up-regulated in the pyloric caeca, indicative of an enhanced capacity for intestinal FA absorption. SA inclusion decreased plasma and digesta bile salt levels and hepatic capacity for bile salt synthesis and transport. Given the key role of bile salts in fat emulsification and micelle formation, it is likely that the decreased body pools of bile salts affected intestinal lipid absorption. Thus, the enhanced capacity for FA transport could be a response to the decreased body pool of bile salts in fish fed saponins.

Interaction

Studies on interactions between different ingredients would be useful to avoid ingredient combinations causing negative effects in fish when using PM. However, in the present study, the hypocholesterolaemic effects of SA in the PM group were not as obvious as those in the FM group. Hepatic gene expression profiles indicated that the capacity for cholesterol biosynthesis was up-regulated in fish by PM exposure. Hardly any information exists regarding the interactions between SA and ingredients in lupin meal, and the mechanism behind the weakened hypocholesterolaemic effects of SA supplementation in the PM diets is not clear. However, the present results indicated that SA supplementation alone could have hypocholesterolaemic effects on Atlantic salmon.

Conclusion

PM inclusion in salmon feed resulted in decreased growth performance, excessive lipid droplet accumulation in the pyloric caeca and liver and reduced plasma cholesterol levels. Gene expression profiling suggested that the capacity for LP assembly and cholesterol synthesis was up-regulated by PM inclusion, probably occurring as a result of impaired lipid transport and cholesterol metabolism. SA inclusion had hypocholesterolaemic effects on Atlantic salmon, accompanied by decreased bile salt levels. Gene expression profiles indicated that the capacity for hepatic biosynthesis and secretion of bile salts was down-regulated by saponin exposure.

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The authors' contributions were as follows: M. G. performed the gene expression and histopathology work and wrote the manuscript; T. M. K. guided the gene expression work and revised the manuscript; M. P. and Å. K. designed and conducted the feeding trial and reviewed the manuscript. A. K. H. participated in the planning and conductance of the molecular work.

The authors declare that there are no conflicts of interest.

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