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Three differently generated salmon protein hydrolysates reveal opposite effects on hepatic lipid metabolism in mice fed a high-fat diet

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ABSTRACT

This study investigates the effects of salmon peptide fractions, generated using different enzymatic hydrolyzation methods, on hepatic lipid metabolism. Four groups of mice were fed a high-fat diet with 20% casein (control group) or 15% casein and 5% of peptide fractions (treatment groups E1, E2 and E4) for 6 weeks. Weight gain was reduced in mice fed E1 and E4-diets compared to control, despite a similar feed intake. Reduced plasma and liver triacylglycerol levels in E1 and E4-mice were linked to reduced fatty acid synthase (FAS) activity and hepatic expression of lipogenic genes. By contrast, plasma and liver lipids increased in the E2 group, concomitant with increased hepatic FAS activity and Δ9 desaturase gene expression. Shotgun lipidomics showed that MUFAs were significantly reduced in the E1 and E4 groups, whereas PUFAs were increased, and the opposite was observed in the E2 group. In conclusion, bioactive peptides with distinctive properties could potentially be isolated from salmon hydrolysates.

1. Introduction

The role of dietary protein is to provide the body with essential amino acids for protein synthesis and energy. Beyond this nutritional role, ingested proteins have a wide range of biological functions affecting protein, glucose and lipid metabolism, transportation, immune function, blood pressure and hormonal functions (Chou, Affolter, & Kussmann, 2012). It has been increasingly clear that the dietary source of protein can affect cellular energy metabolism, and that hydrolyzed peptides can have potent and specific bioactive potential (Erdmann, Cheung, & Schroder, 2008). Health benefits from fish consumption have been attributed to the *n*-3 polyunsaturated fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, recent studies have drawn attention towards proteins from marine sources, which are considered valuable bioactive components as their amino acid composition and protein profile differ from terrestrial sources (Kim, Ngo, & Vo, 2012; Larsen, Eilertsen, & Elvevoll, 2011). According to Kelleher, 7 million tons of fish byproducts were discarded as processing waste in 2005 (Kelleher, 2005), which constituted 50% of the total catch being used for human consumption (Rustad, 2003). Fish byproducts can be hydrolyzed enzymatically, using various techniques, liberating potentially bioactive peptides incorporated in the parental molecule, according to molecule size, stability in water, refined protein and protein mix. Rodent studies on fish protein hydrolysates have shown that marine proteins exhibit cholesterol-lowering (Shukla et al., 2006), antihypertensive (Je, Park, Kwon, & Kim, 2004; Kim & Mendis, 2006), immunomodulating and antioxidant effects (Ahn, Cho, & Je, 2015), in addition to reparative properties in the intestine (Fitzgerald et al., 2005), and increased insulin sensitivity (Pilon et al., 2011). How fish protein hydrolysate may affect lipid metabolism in liver is less clear. The liver is the major site of lipid metabolism and both fatty acid oxidation and liponeogenesis are carried out here. These processes are dependent on rate-limiting enzymes, e.g., carnitine palmitoyltransferase (CPT)-1 and 2, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). Several desaturases are involved in *de novo* fatty acid synthesis. The Δ9 desaturase, encoded by the gene *Scd1*, generates monounsaturated fatty acids. Along with elongases, the Δ5 and Δ6 fatty acid desaturases, encoded by the genes *Fads1* and *2*, respectively, are important in the biosynthesis of essential polyunsaturated fatty acids (PUFAs). Regulation of these enzymes will influence fatty acid composition

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and thus important cellular functions, including cell signaling (Nakamura & Nara, 2004).

In this study, we investigated the effect of three different salmon peptide fractions (designated E1, E2, and E4), generated using different enzymatic hydrolyzation and microfiltration methods, on hepatic lipid metabolism in male C57BL/6J mice. We show that different peptide compositions, generated from the same species of fish, varied in their beneficial effects on body weight and lipid metabolism.

2. Materials and methods

2.1. Animals and diets

The animal experiments were carried out with ethical permission obtained from the Norway State Board for Biological Experiments and followed the Norwegian Research Councils ethical guidelines. Nine to ten weeks old male C57BL/6J mice were housed, 3 per cage, at constant temperature (22 ± 2 °C) and humidity (55 ± 5%), and exposed to a 12 h light–dark cycle with unrestricted access to food and tap water. After 1 week of acclimatization to these conditions, they were divided into 4 groups and fed either a high-fat (HF) diet containing 24% (w/w) fat (21.3% lard and 2.7% soy oil) and 20% casein (control group, $n = 9$), or the HF diet supplemented with protein hydrolysate E1, E2 or E4 from salmon byproducts (a generous gift from Marine Bioproducts, Storebø, Norway) (15% casein and 5% peptide, group E1, E2 and E4, $n = 6$) *ad libitum* for 6 weeks. The different salmon peptide fractions were produced as follows: for fractions E1 and E2, fish material (spine) was treated enzymatically with alkaline protease and a neutral protease and the resulting protein hydrolysate was subjected to a second enzymatic treatment. Fraction E1 was treated with an Acid Protease A, while fraction E2 was treated with the proteolytic enzyme Umamizyme from *Aspergillus oryzae*. The final hydrolysate was then filtered, using micro- and ultra-filtration, and the size distribution of the peptides analyzed. For both fractions, more than 50% of the final preparation consisted of peptides in the range 200–1200 Da and approximately 25% of the preparation consisted of peptides below 200 Da. Salmon backbones, including heads, were hydrolyzed with proteolytic Alcalase 2.4 L (Novozymes, Denmark) and subjected to micro- and ultra-filtration and constituted peptide fraction E4. Nearly 60% of the final preparation consisted of peptides in the range below 1200 Da.

Amino acid composition of the control and peptide diets is given in Supplementary Table 1. Diets were packed airtight and stored at −20 °C until used to prevent lipid oxidation. Mice were housed in groups of three per cage at a constant temperature of 22 ± 2 °C and a dark/light cycle of 12/12 h. Body weights of the mice were measured approximately every seventh day and food intake was measured 3 times during the study. At sacrifice, animals were fasted overnight, anesthetized with 2% isoflurane (Schering-Plough, Kent, UK) and blood was collected by heart puncture. The blood was centrifuged, EDTA–plasma separated and frozen prior to further analysis. Livers were collected and immediately frozen in liquid nitrogen and stored at −80 °C prior to further analysis.

2.2. Lipid and fatty acid analysis

Liver lipids were extracted according to Bligh and Dyer (1959), solvents were evaporated under nitrogen and samples re-dissolved in isopropanol before analysis. Lipids from liver extracts or plasma were then measured enzymatically on a Hitachi 917 system (Roche Diagnostics, Mannheim, Germany), using kits for analyzing total TAG (GPO–PAP kit, Roche Diagnostics), cholesterol (CHOD–PAP kit, Roche Diagnostics), and total phospholipids (Diagnostic Systems GmbH, Holzheim, Germany). Fatty acid composition was analyzed in extracted liver lipid, using gas chromatography, as described previously by Grimstad et al. (2012).

2.3. Lipidomic analysis

Liver samples were stored at −80 °C prior to analysis and lipidomics analysis was performed on 50–100 mg of liver tissue from each mouse from two groups, E1 and E2, in addition to the control group. The tissue samples were pulverized with a CP02 CryoPrep Dry Pulverization System (Covaris), and resuspended in ice-cold methanol containing 0.1% butyl-hydroxytoluene (BHT) at a concentration of 100 mg/mL.

For lipidomics analysis, lipids were extracted from liver homogenates, using a modified Folch lipid extraction procedure (Ekroos, Chernushevich, Simons, & Shevchenko, 2002). Samples were spiked with known amounts of deuterium-labeled or heptadecanoyl-based synthetic internal standards, serving for quantification of the endogenous lipid species, as previously described (Bergan et al., 2013). The samples were stored at −80 °C prior to mass spectrometry analysis.

Molecular glycerophospholipids, glycerolipids, cholesteryl esters sphingomyelins and triacylglycerols (TAGs) were analyzed by shotgun analysis on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, ABSCIEX) equipped with a robotic nanoflow ion source (NanoMate HD, Advion Biosciences) (Stahlman et al., 2009). In shotgun lipidomics a 5 μL volume was infused at a concentration of 0.5 μg liver/μL. For TAG analysis, the concentration was diluted to 0.05 μg/μL. The analyses were performed in both positive and negative ion modes, using multiple precursor ion scanning (MPIS) and neutral loss (NL)-based methods (Ekroos et al., 2002, 2003). Sphingolipids were analyzed by reverse phase ultra-high pressure liquid chromatography (Rheos Allegro UHPLC, Flux Instruments AG), using an Acquity BEH C18, 2.1 × 50 mm column with a particle size of 1.7 μm (Waters, Milford, Massachusetts, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, ABSCIEX). A 25 min gradient, using 10 mM ammonium acetate in water with 0.1% formic acid (mobile phase A) and 10 mM ammonium acetate in acetonitrile:2-propanol (4:3, v/v), containing 0.1% formic acid (mobile phase B), was used. The column temperature was set to 60 °C and the flow rate to 500 μL/min. 10 μL samples were injected. Sphingolipids were monitored, using multiple reactions monitoring (MRM) as described by Merrill, Sullards, Allegood, Kelly, and Wang (2005).

The MS data files were processed, using Lipid Profiler™ and MultiQuant™ software for producing a list of lipid names and peak areas. The individual lipids measured can be found in Supplementary Table 2. Masses and counts of detected peaks were converted into a list of corresponding lipid names. Lipids were normalized to their respective internal standard (Bergan et al., 2013) and tissue weight to retrieve their concentrations. Data were analyzed, using the software Tableau Desktop 7.0 and the percentage differences between the groups (E1 vs. controls and E2 vs. controls) were estimated in pairwise comparisons, using a Hodges–Lehmann estimator, and the significances were calculated using Wilcoxon rank-sum t-test.

2.4. Hepatic enzyme activities

Liver samples were homogenized and a post-nuclear fraction was prepared as previously described (Berge, Flatmark, & Osmundsen, 1984). The activity of CPT-1 was measured in the presence and absence of malonyl-CoA, as previously described (Vik et al., 2014). The assay conditions for CPT-2 were identical to CPT-1, apart from some changes in the reaction mix; BSA and KCN were

exchanged with 0.01% Triton X-100, and 35 μg of total protein were used for the assays. Acyl-CoA oxidase (ACOX)-1 activity was measured, using 20 μg protein, by a coupled assay, described by Small, Burdett, and Connock (1985), with some modifications (Madsen et al., 1999). Glycerol-3-phosphate acyltransferase (GPAT) activity was measured, as described by Skorve et al. (1993).

2.5. RNA isolation, cDNA synthesis, and real-time PCR

Total RNA from liver tissue was purified, using the MagMax total RNA isolation system (Applied Biosystems, Carlsbad, CA, USA) after tissue homogenization. The quantity of the RNA was measured spectrophotometrically, using a NanoDrop 1000 (NanoDrop Products, Wilmington, DE, USA) and the quality of the RNA was analyzed, using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). Quality limit for further use of RNA was set to a R/Q value of $\geqslant 7$ (out of 10). cDNA was synthesized with 500 ng of RNA per reaction, using High Capacity RNA to cDNA Mastermix (Applied Biosystems). Genes of interest were analyzed in individual samples from liver, using SYBR Green gene expression assays, and using primers for acetyl-CoA carboxylase alpha (*Acaca*, 5′acctgtacaagcagtgtgggct, 3′cacatggcctggcttggaggg), acyl-CoA thioesterase (*Acot1*, 5′ctggcgcatgcaggatc, 3′ggcacttttcttg-gatagctcc), fatty acid desaturase 1 (*Fads1*, 5′ggctcccgggtcatcag, 3′acccttgttgatgtggaatgc), (*Fads2*, 5′ggacataaagagcctgcatgtg, 3′gg gcaggtatttcagcttcttc), (*Fas*, 5′ggcatcattgggcactcctt, 3′gctgcaag-cacagcctctct), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*, 5′ccggcaacaacaagatctgtg, 3′atgtacaggatggcgatgca), fatty acid binding protein, liver (*L-fabp*, 5′ccatgactggggaaaaagtc, 3′gc-ctttgaaagttgtcaccat), stearoyl-CoA desaturase (*Scd1*, 5′acgggctccg gaaccgaagt, 3′ctggagatctcttggagcatgtggg). SYBR Green primers were used at concentrations ranging from 100 to 200 nM and run with the Power SYBR Green Master Mix (Applied Biosystems) in an ABI Prism 7500 sequence detection system. Gene expression was estimated, using the average threshold (Ct) value, in triplicate calculated, using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001), using hypoxanthine phosphoribosyltransferase 1 (*Hprt1*, 5′ggtgaaaaggac-ctctcgaagtg, 3′atagctaagggcatatccaacaac) as reference gene and one individual sample in the high-fat group as calibrator.

2.6. Statistics

All values are presented as means ± standard deviation (SD). One-way ANOVA was used for analysis of differences between groups, followed by Dunnett's multiple comparison test between all groups vs. control when data followed Gaussian distribution and the Kruskal–Wallis test, with Dunn's multiple comparison test, was used when data were not normally distributed. Significance was set to ($P < 0.05$). All statistics were calculated using GraphPad Prism 6 for Mac OS X with the exception of the lipidomics data where software Tableau Desktop 7.0 was used.

3. Results

3.1. Body weight was influenced by dietary intake of salmon peptide fractions

C57BL/6J mice, fed high-fat diets with 15% casein and 5% (w/w) of salmon peptide fractions E1 or E4, demonstrated lower body weight gain throughout the 6 weeks feeding period (Fig. 1A), as well as a significantly lower total weight gain than did control mice fed 20% (w/w) casein as protein source (Fig. 1B). By contrast, mice fed peptide E2 had a body weight curve and total weight gain more similar to the control group. Feed intake was measured 3 times during the experiment, and the average feed intakes were similar in control, E2 and E4-fed mice (Fig. 1C), while it was higher in peptide E1-fed mice, despite the low weight gain in this group. A small, but significant reduction was observed in the liver index (% liver weight/body weight) of E4-fed mice compared to controls (Fig. 1D).

3.2. The salmon peptide fractions differentially affected plasma lipids but not bile acid levels

Plasma lipids were measured in samples from fasted animals. While mice fed peptides E1 and E4 demonstrated significantly lower plasma TAG levels than did the control, the TAG level in mice fed peptide E2 was elevated (Fig. 2A). The peptide E2 group also demonstrated elevated levels of total cholesterol, phospholipids and HDL-cholesterol compared to the control (Fig. 2B–D). No change was observed in LDL-cholesterol or the HDL/LDL ratio in any of the feeding groups (Fig. 2E and F), but non-esterified fatty acids showed a tendency to increase in the E4 group (Fig. 2G, control vs. E4, Mann–Whitney, P-value = 0.044). There was a small increase in plasma bile acids in the E4 group, when measured in plasma samples pooled from 2 to 4 animals (Fig. 2H). However, statistical significance could not be analyzed (n = 2–3).

3.3. The effect of salmon peptide fractions on hepatic enzymes

As the studied salmon peptide fractions affected weight gain and lipid levels differently in C57BL/6J mice, we analyzed the activity of hepatic enzymes involved in lipid catabolism and synthesis. CPT-1 is the rate-limiting enzyme in fatty acid import into mitochondria for β-oxidation. In mice fed E1, no change was observed in the activity of CPT-1 (Fig. 3A), or its % inhibition by malonyl-CoA (Fig. 3B). CPT-2 activity was also unchanged, further supporting no influence on mitochondrial β-oxidation in this group (Fig. 3C). The activity of GPAT, involved in the synthesis of TAG, was unchanged (data not shown). However, *de novo* lipogenesis could be affected, as FAS activity tended to be reduced in E1-fed mice compared to control-mice (Fig. 3D). In E4 mice, a potential concomitant increase in β-oxidation and reduction in lipogenesis was observed; CPT-1 and -2 activities demonstrated small, but significantly higher levels than the control, suggesting more CPT-mediated mitochondrial import of fatty acids in these mice. Also, FAS activity was significantly lower in E4-fed mice than in control mice. By contrast, E2 mice demonstrated a significantly higher FAS activity than did control mice, suggesting increased *de novo* lipogenesis in this group, while CPT-1 and -2 activity were unchanged.

We further analyzed hepatic expression of genes involved in fatty acid metabolism. The liver fatty acid binding protein (*L-fabp*) was not affected by the diets (Fig. 4A). *Acot1*, which regulates the cytosolic levels of acyl-CoA, CoASH and free fatty acids, was significantly increased in the E2 and E4 groups compared to control mice (Fig. 4B). In agreement with the enzyme activity data, *Fas* gene expression was significantly reduced in the E1 and E4 groups compared to control (Fig. 4C). The expression of another important gene in fatty acid synthesis, *Acaca*, was significantly decreased in the E1 group compared to the control (Fig. 4D). The Δ9 desaturase (*Scd1*) mRNA level tended to be increased in livers from E2-fed mice, supporting an increase in *de novo* lipogenesis in this group (Fig. 4E). There was also indication of a differential effect on Δ5 and Δ6 desaturation of fatty acids, as *Fads1* was significantly lower expressed in the E4 group vs. the control group (Fig. 4F) and *Fads1* and *Fads2* tended to be lower in the E1 group compared to the control (Fig. 4G). HMG-CoA reductase (*Hmgcr*), the rate-limiting enzyme in cholesterol synthesis, was significantly reduced in the E1 and E4 groups compared to the control (Fig. 4H).

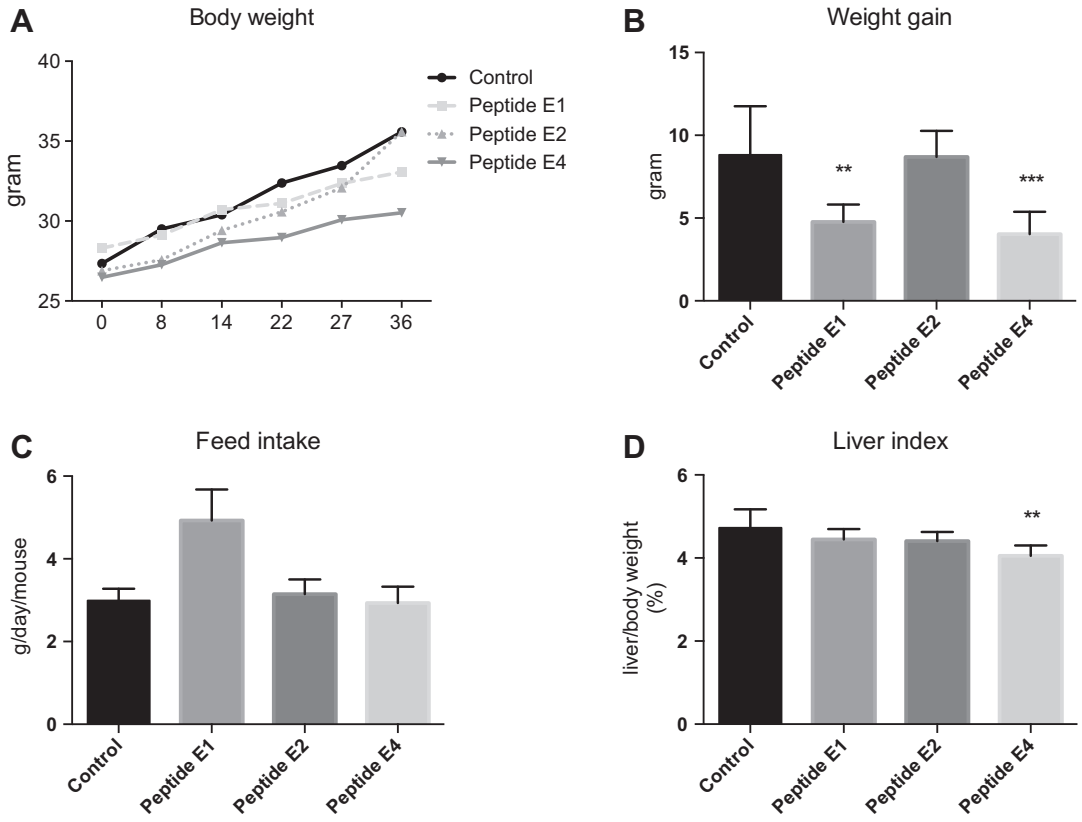

Fig. 1. (A) Body weight curve, (B) weight gain, (C) feed intake, and (D) hepatic index in mice fed a high-fat casein diet (control, $n = 9$) or a diet where 5% of the protein source was replaced with 5% of three different salmon protein hydrolysates E1, E2 or E4 ($n = 6$). Data are shown as means with SD and dissimilar letters indicate significantly different values ($P < 0.05$). Statistical significance could not be calculated in (C) since data from 3 mice were pooled.

3.4. A differential effect of salmon peptide fractions on hepatic fatty acid composition

To determine if the differently processed peptide fractions could influence fatty acid composition, total fatty acids in liver were analyzed. Saturated fatty acids (SFAs) were unaffected in all peptide diet groups compared to the control. Largely similar effects on monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) composition were observed in the E1 and E4 groups: While MUFAs tended to be reduced in the E1 group, and were significantly reduced in the E4 group (Table 1), *n*-3 PUFAs were significantly increased in the E4 group, and *n*-6 PUFAs increased in the E1 group compared to the control (Table 1). The opposite effect was observed in the liver of E2 mice, where MUFAs increased strongly, while *n*-3 and *n*-6 PUFAs were significantly reduced compared to control mice. In line with these results, the Δ9 desaturase index, calculated by the ratio C16:1*n*-7/C16:0 or the ratio C18:1*n*-9/C18:0, was increased in the E2 group compared to the control. The Δ5 desaturase (*n*-6) index, calculated by the ratio C20:4*n*-6/C20:3*n*-6, increased in the E1 and E4 group, while the Δ6 desaturase (*n*-3) index, calculated by the ratio C20:5*n*-3/C20:4*n*-3, was significantly increased only in E4 (Table 1). In addition, the Δ5 desaturase (*n*-3) index was significantly increased in the E4 group compared to the control, while no change was seen in the Δ6 desaturase (*n*-6) index (data not shown).

3.5. Comparison of the effect of salmon peptide fractions on hepatic lipids

In the enzymatic measurements of lipids from liver extracts, we found that TAG tended to be reduced by peptides E1 and E4 (E1 vs. control, student's *t*-test, *P*-value = 0.042) (Supplementary Table 3). Surprisingly, cholesterol showed a small but significant increase in E4 vs. control, while phospholipids were increased by both E1 and E4. However, the total hepatic lipid levels (TAG, cholesterol and PL) were unchanged in E1 and E4 compared to the control. In the livers of E2 mice, both TAG and total hepatic lipids were significantly increased (Supplementary Table 3), in line with plasma lipid results.

Since the E1 and E2 salmon peptide fractions were generated from the same raw-material, but showed opposite effects on hepatic lipid metabolism, liver samples from mice fed these diets were further compared to controls in three separate analytical runs in shotgun, sphingolipid and TAG lipidomics. Fig. 5A shows a summary of the effects of these two salmon protein hydrolysate fractions at the level of the major lipid classes. In line with the enzymatic measurement, the relative difference (%) of TAG was significantly elevated in the E2 group vs. control, and tended to decrease in the E1 group vs. control. Striking differences were also seen with the ceramides (Cer) and glycerophosphoethanolamines (PE), which increased significantly in E1 vs. control, diacylglycerols (DAG), which increased in E2 vs. control, and glycosyl/galactosylceramide (Glc/GalCer), which were specifically reduced in E1 vs. control. The difference in TAG in the E1 group compared to the control was seen as a reduction in all species of TAG, whereas the E2-diet primarily increased the TAG species containing short, saturated or monounsaturated fatty acids compared to the control (Fig. 5B). The peptide fractions had a prominent different effect on hepatic ceramide levels. The relative differences of all d18:1 ceramides were increased with the E1 diet compared to the control, while largely the opposite effect was observed in the E2 group, apart from a statistically significant increase in Cer (d18:1/18:0) (Fig. 5C).

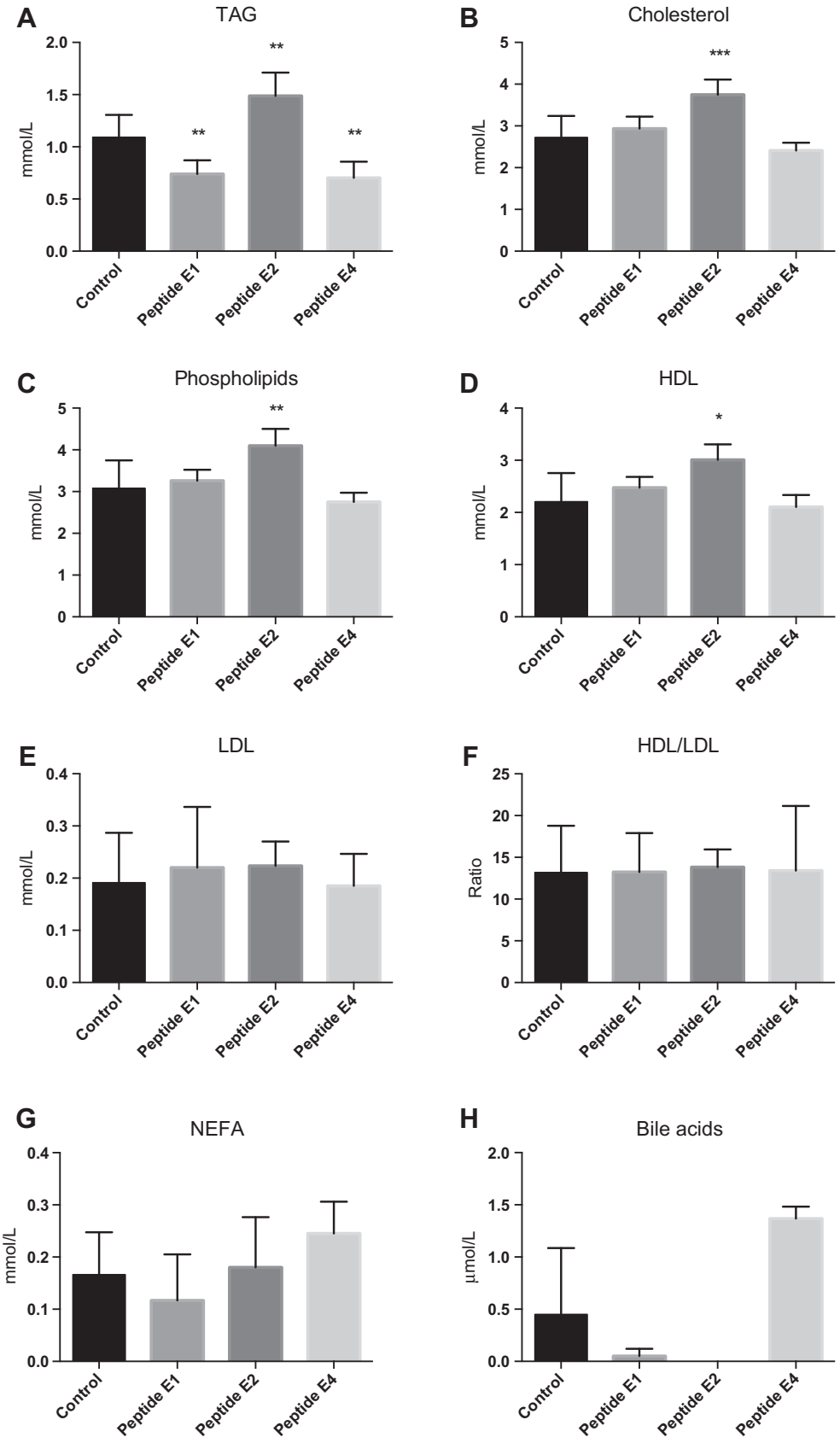

Fig. 2. Plasma lipids in mice fed a high-fat casein diet (control, $n = 8$) or a diet where 5% of the protein source was replaced with 5% of three different salmon protein hydrolysates, E1, E2, and E4, respectively ($n = 6$). (A) Triacylglycerol (TAG), (B) cholesterol, (C) phospholipids, (D) high-density lipoprotein (HDL), (E) low-density lipoprotein (LDL), (F) HDL/LDL ratio, and (G) non-esterified fatty acids (NEFA). Bile acids in pooled plasma samples from 2 to 4 mice ($n = 2–3$) are shown in (G). Data are shown as means with SD and values significantly different from control were determined by one-way ANOVA (A–C and F) or Kruskal–Wallis test (D, E and G), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistics could not be performed in (H).

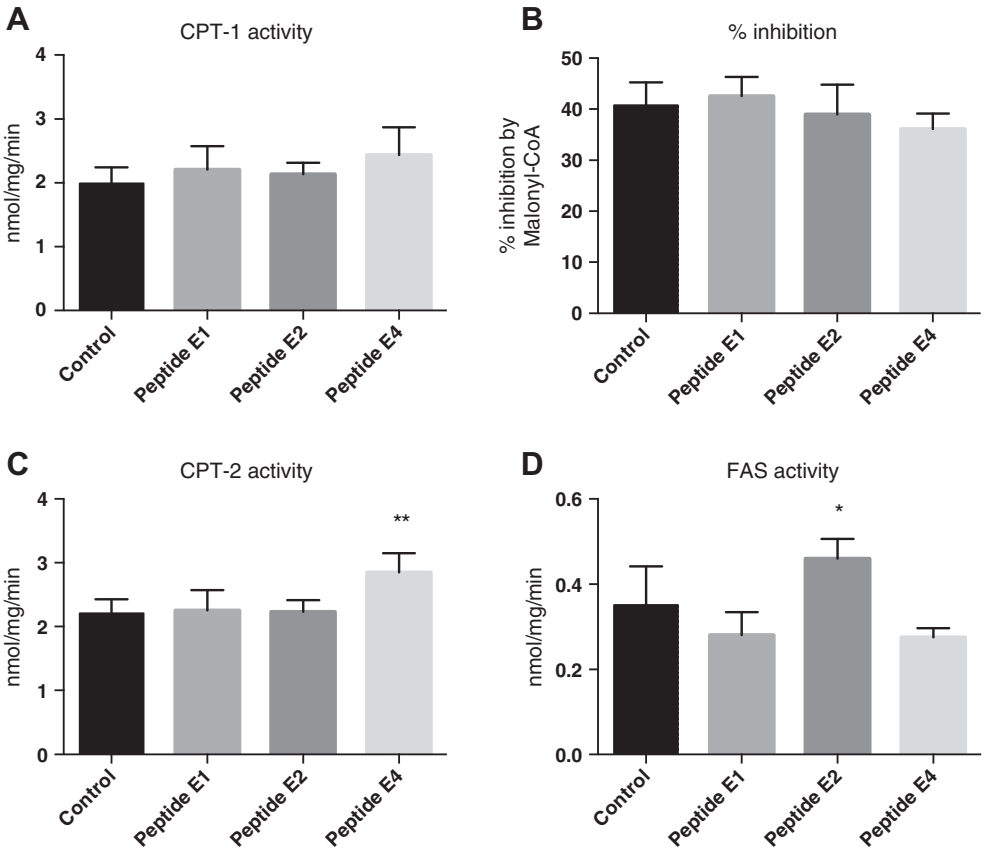

Fig. 3. Hepatic enzyme activities in mice fed a high-fat casein diet (control) or a diet where 5% of the protein source was replaced with 5% of three different salmon protein hydrolysates, E1, E2, and E4, respectively. (A) Carnitine palmitoyltransferase-1 (CPT-1) activity, (B) % inhibition of CPT-1 activity by malonyl-CoA, (C) carnitine palmitoyltransferase-2 (CPT-2) activity and (D) fatty acid synthase (FAS) activity. Data are shown as means with SD ($n = 6$) and values significantly different from control were determined by one-way ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$).

4. Discussion

Based on recent studies, fish proteins and peptides have diverse bioactive properties (Kim & Mendis, 2006). The purpose of our study was to evaluate the effects of three different fractions of salmon protein hydrolysate on body weight development and plasma lipids, as well as hepatic lipid metabolism, as the liver is the main organ maintaining lipid homeostasis. The novel findings of our study were the divergent effects on body weight, plasma and hepatic TAG levels, hepatic lipogenesis and β-oxidation, and hepatic fatty acid composition by peptide fractions generated using different enzymatic methods.

The groups fed peptide fractions E1 and E4 exhibited a lower weight gain throughout the study, despite the same or higher feed intake than control. Furthermore, both E1 and E4-fed groups had reduced plasma TAG levels and a tendency to reduced liver TAG. In E1 mice, this was linked to decreased expression of the *Fas* and *Acaca* genes, as well as a tendency to decrease FAS activity. The reduction in these rate-limiting steps in fatty acid synthesis indicates a repression of *de novo* lipogenesis by the E1 peptide. Similar to E1-fed mice, key enzymes in lipogenesis were significantly reduced by E4, and in addition, an increased CPT-1 and -2 activity suggested that increased fatty acid oxidation could have contributed to the lower body weight and plasma TAG observed in this group.

The generation of the E2 peptide fraction differed from that of the E1 peptide fraction in the secondary enzymatic treatment, and opposite effects were observed in these treatment groups. E2-fed mice had a body weight gain curve similar to that of control-fed mice, and importantly, displayed increased hepatic TAG, as well as plasma TAG, cholesterol, and phospholipid levels. FAS activity was significantly increased by E2, and thus the differences in plasma and liver TAG could partly be explained by an opposite regulation of fatty acid synthesis in mice fed the E2 vs. the E1 and E4 peptide fractions. In support of this, the increased Δ9 index, and the tendency to increase in hepatic gene expression of *Scd1*, the enzyme performing the crucial Δ9 unsaturation step during fatty acid synthesis, indicates stimulated fatty acid synthesis in E2-fed mice. The GPAT activity was not altered in the intervention groups; thus TAG synthesis was probably not affected by the diets.

A number of studies, using high doses of fish protein or protein hydrolysate from Atlantic salmon (15–20% of diet), have demonstrated reduced plasma cholesterol levels in Wistar rats (Hosomi et al., 2011; Liaset et al., 2009). This has been linked to increased plasma bile acid levels, and cholesterol clearance through the bile. However, in a number of comparable studies in rabbits and mice, no influence on plasma cholesterol levels has been shown (Bergeron & Jacques, 1989; Bjørndal et al., 2013). In line with this, no plasma cholesterol-reducing effects or increases in plasma bile acids were observed in the treatment groups compared to control, despite a reduction in the gene expression of *Hmgcr*, involved in cholesterol biosynthesis, in the E1 and E4 groups. This could either be due to the animal model or the lower dose of salmon protein hydrolysate used compared to previous studies.

Different sources of protein are assumed to differ in digestibility, thus contributing unequally to energy supply; other proteins or peptides are believed to suppress appetite (Nishi, Hara, Asano,

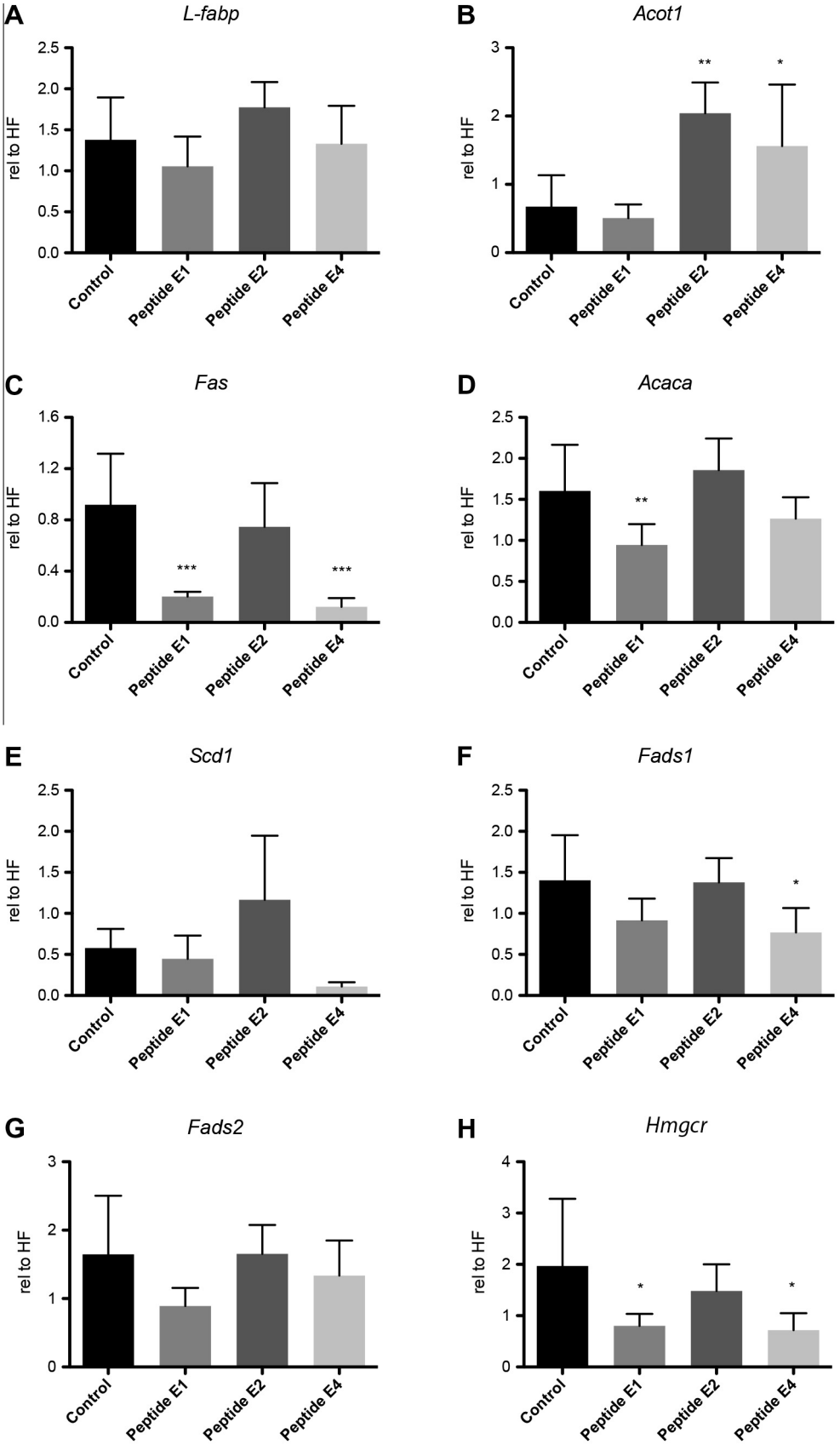

Fig. 4. Hepatic gene expressions in mice fed a high-fat casein diet (control) or a diet where 5% of the protein source was replaced with 5% of three different salmon protein hydrolysates, E1, E2, and E4, respectively. (A) Fatty acid binding protein, liver (*L-fabp*), (B) acyl-coenzyme A thioesterase 1 (*Acot1*), (C) fatty acid synthase (*Fas*), (D) acetyl-coenzyme A carboxylase alpha (*Acaca*), (E) stearyl-coenzyme A desaturase (*Scd1*), (F) fatty acid desaturase 1 (*Fads1*), (G) fatty acid desaturase 2 (*Fads2*), and (H) 3-hydroxy-3-methylglutaryl CoA reductase (*Hmgcr*). Data are shown as means with SD ($n = 6$) and dissimilar letters indicate significantly different values.

Table 1
Hepatic fatty acid composition mice fed a casein control diet or diets with 5% of three different salmon protein hydrolysates for 4 weeks[a].

Fatty acids	Control	E1	E2	E4
SFAs	34.8 ± 1.4	36.3 ± 0.9	34.2 ± 0.7	35.3 ± 1.3
MUFAs	23.8 ± 6.4	17.8 ± 1.8	31.1 ± 4.1**	18.1 ± 4.4
n-3 PUFAs	9.8 ± 1.4	10.7 ± 0.9	7.7 ± 1.1***	14.1 ± 1.4***
n-6 PUFAs	31.4 ± 3.8	34.9 ± 0.7	26.8 ± 2.5***	32.4 ± 1.8
[b]Δ9-desat. (C16:0)	0.06 ± 0.03	0.05 ± 0.01	0.10 ± 0.02**	0.05 ± 0.02
[b]Δ9-desat. (C18:0)	2.1 ± 1.3	1.2 ± 0.1	3.1 ± 0.9**	1.3 ± 0.5
[c]Δ5-desat.	9.5 ± 2.1	13.5 ± 1.2*	9.3 ± 1.3	17.6 ± 4.6***
[d]Δ6-desat.	0.08 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.03*

Data are shown as means ± SD (n = 6–9).
Values significantly different from control were determined by one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).
Abbreviations: SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids, PUFAs, polyunsaturated fatty acids.
[a] wt%.
[b] Delta9 desaturase index (C16:1*n*-7/C16:0 and C18:1*n*-9/C18:0).
[c] Delta5 desaturase index (C20:4*n*-6/C20:3*n*-6).
[d] Delta6 desaturase index (C20:5*n*-3/C20:4*n*-3).

& Tomita, 2003), while some are suggested to increase satiety. In addition, a previous study showed that salmon protein uniquely protects against body weight gain, independently of feed intake (Pilon et al., 2011). In support of this, feed intake in the four groups was unable to explain the divergent effect on body weight. Thus, in the present study, the distinct peptide or amino acid composition of each salmon protein hydrolysate-fraction may explain the variation in body weight gain between the treatment groups (Supplementary Table 1). The degree of hydrolysis of corn gluten meal has been reported to affect bioavailability and weight gain in rats (Jin et al., 2014); however, in our study, the three different hydrolysates had similar compositions with regard to peptide sequence size, indicating similar degrees of hydrolysis.

Dietary proteins may play a role in mechanisms affecting fatty acid composition (Bjørndal et al., 2013; Leveille, Tillotson, & Sauberlich, 1963). The amino acid composition of the diet has been shown to influence lipogenesis and desaturation, as tyrosine down-regulates hepatic Δ6 activity (Peluffo, Nervi, Gonzalez, & Brenner, 1984), and arginine and leucine can regulate the gene expression of *Scd1* and *Fas* in muscle and adipose tissue (Madeira et al., 2014). Here, the amino acid composition did not differ to a large extent since they were all dominated by amino acids from casein. However, tyrosine and leucine were higher in the E2 diet compared to the control, E1 and E4, while arginine was reduced in the E1 and E4 diets compared to the control and E2. In line with this, the E1 and E4 groups displayed similar patterns in fatty acid composition, including an increase in the fatty acid ratios 18:3*n*-6/18:2*n*-6 and 20:4*n*-6/20:3*n*-6 compared to the control. These ratios are commonly used as an index for the activities of Δ6- and Δ5 desaturase, respectively. As also reported by others (Sjogren et al., 2008), the Δ5 and Δ6 desaturation indices are influenced by a number of pathways, and failed to reflect the expression of *Fads1* and *Fads2*. In contrast, *Scd1* expression correlated with the Δ9 desaturase index in liver. Thus, concomitant with increased *Scd1* expression in E2-mice, an increase in monounsaturated fatty acids and the Δ9 desaturase index was observed. Interestingly, while hepatic MUFA increased, *n*-3 PUFA and *n*-6 PUFAs decreased in the E2 group. The main product of Δ9 desaturase, oleoyl-CoA (C18:1), is mainly used to generate TAG (Mauvoisin & Mounier, 2011), and this was reflected in the predominant increase in TAG species containing short MUFAs. Studies investigating the effect of MUFA supplementation on plasma lipid levels in humans are inconclusive; however, in rats, a high MUFA amount in a diet containing 40% energy from fat was shown to increase plasma total cholesterol, TAG and phospholipid levels as compared to a low dietary MUFA amount (Chang & Huang, 1998). The current study suggests that an increased hepatic expression of *Scd1*, and a corresponding increase in MUFAs incorporated into TAG could lead to increased VLDL release.

The E1 and E4 diets resulted in increased hepatic PUFA levels, indicating increased biosynthesis of PUFAs at the expense of MUFAs. Dietary and endogenous fatty acids, in particular PUFAs, are involved in metabolic regulation of lipid and glucose metabolism. Thus, the differential peptide-effects on lipid levels could have been reinforced by their opposite regulation of fatty acids important in cell signaling.

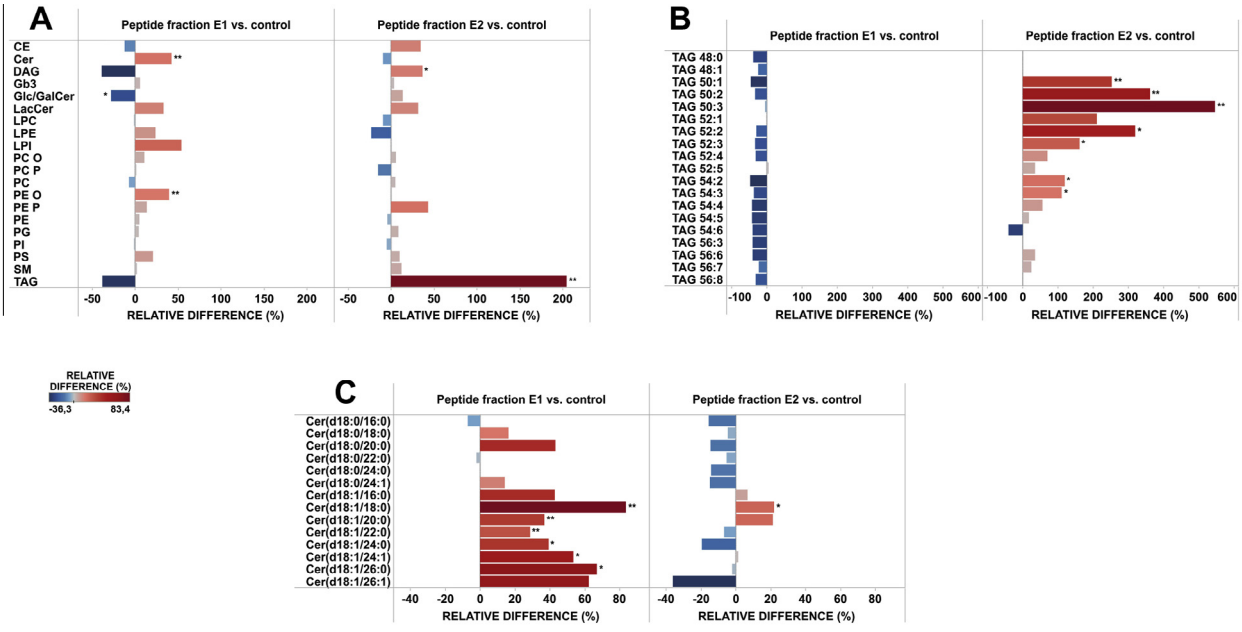

Fig. 5. Summary of lipid classes (A), TAG species (B), and ceramide species (C) in liver, presented as relative molar percentage differences between treatment groups and control, in mice fed a high-fat casein diet (control) or the replacement with 5% of the protein source with 5% of peptide fraction E1 or E2 (n = 6). Significance is indicated as *P < 0.05 and **P < 0.01.

Assessment of liver lipids, using lipidomics, also revealed a significantly elevated level of ceramides in the E1 group. These waxy lipid molecules can induce both inflammation and insulin resistance (Summers, 2006). Increased ceramide levels in adipose tissue have been linked to higher levels of liver fat (Kolak et al., 2012); however, in our study, TAG decreased while ceramides increased in the liver of E1-fed mice. A reduction in diacylglycerols (DAG) in the E1 group could be of significance in view of its physiological importance in activating protein kinase C (PKC). PKC phosphorylates the hydroxyl-groups of serine and threonine amino acid residues on several important target proteins and modulates physiological processes in all cells. In our study, DAG and TAG displayed a simultaneous decrease in the E1 group and increase in the E2 group, relative to control. The significant increase of DAG in the peptide E2 group, combined with increased gene expression of *Scd1*, but not in ceramide levels, is similar to findings in the liver of patients with non-alcoholic fatty liver disease (Kotronen et al., 2009). In further studies, it will be interesting to evaluate the effect of the E1 and E4 fish protein hydrolysate fractions on animal models, on NAFLD, as well as obesity and insulin resistance.

5. Conclusions

The three different peptide fractions from salmon protein hydrolysate show diverse effects on weight gain, plasma and liver lipids and lipid synthesis, suggesting that protein products that are enzymatically hydrolyzed exhibit distinct and, in some cases, opposite effects. Thus, prior to their potential use as dietary supplements, it is of importance to analyze protein hydrolysates thoroughly to exclude possible negative metabolic effects. Predigestion of proteins gives the opportunity to optimize nutritional value and bioavailability of the peptides, thus obtaining a more specific product regarding its use, either as a balanced food supplement, food aid or simply healthy food.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.011.

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