Dietary lysophosphatidylcholine regulates diacylglycerol, cardiolipin and free fatty acid contents in the fillet of turbot

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Abstract

Lysoosphatidylcholine (LPC) has been widely used as emulsifier in animal feeds to enhance the lipid utilization. However, the effects of LPC on fillet quality has rarely been known. The present study was the first time to investigate the response of fish muscle lipidomics to dietary LPC supplementation. Turbot muscle samples were collected after a 56-day feeding trial where the experimental diet contained 0 or 0.25% LPC. Targeted tandem mass spectrometry was used in the lipidomic analysis. A total of 62 individual lipids (58 up-regulated and 7 down-regulated by LPC) showed significant difference in concentration in response to dietary LPC. Most of these differentially abundant lipids were diacylglycerol, free fatty acid and cardiolipin, and they all were up-regulated by dietary LPC. However, LPC exerted only marginal effects on muscle fatty acid composition and lipid content. The effects of dietary LPC on fillet lipid composition cannot be neglected in fish product evaluation.

1. Introduction

Emulsifier-like feed additives have been widely used in terrestrial animals (Saleh et al., 2020; Wickramasuriya et al., 2020), and also in fish (Bergman, Trushenski & Drawbridge, 2018; Medagoda, Kim, Gunathilaka & Lee, 2021) Compared to other emulsifiers, which has been widely investigated such as bile acids (Jin et al., 2019; Liao et al., 2020), lysophospholipids (LPL) attracted less interest in both terrestrial animals (Saleh et al., 2020; Wickramasuriya et al., 2020), and also in fish (Hosseini, Nourmohammadi, Nazarizadeh & Latshaw, 2016). However, in recent years, increasing attention is being paid on the application of LPL (mostly lysolecithin) in fish feeds. Efficacy of dietary supplementation of LPL has been evaluated in fish species such as hybrid channel catfish (Ictalurus punctatus) (Liu et al., 2010), rainbow trout (Oncorhynchus mykiss) (Taghavizadeh, Shekarabi, Mehrghan & Islami, 2020), Turbot is a worldwide important aquaculture fish species. It has a relatively low lipid content in the muscle, making the muscle lipid susceptible to dietary influence. Dietary LPL is expected to regulate the muscle lipid profile via multiple mechanisms including emulsifying actions in lipid absorption, modifying lipoprotein synthesis, and influencing lipid transport across different tissues. With a feeding trial on turbot followed by a muscle lipidomic analysis, the present study was...
aimed at preliminarily evaluating the effects of dietary LPC supplementation on the lipid profile of turbot muscle. This was the first time to evaluate the LPC effects on fish muscle lipidome. Results of this study could be beneficial to better management of LPL application in fish feeds.

2. Materials and methods

2.1. Experimental diets, feeding trial and sampling

Two experimental diets without or with LPC (0.25% dry matter) were used in the feeding trial (see Supplementary Table S1 for formulation and proximate composition of experimental diets). The LPC product was supplied by Weifang Kenon Biological Technology Co., Ltd. (Weifang, China). The phosphatidylcholine purity of this soy-derived product was 98%, and the available LPC concentration was 5%. The diets were made, packed and stored following the standard procedures in our laboratory. During the preparation of the experimental diets, the LPC was thoroughly mixed into the feeding ingredients with the method of 1:1 gradual dilution (1 LPC:1 feed ingredient). The fatty acid profiles of the experimental diets are presented in Supplementary Table S2.

The feeding trial, with juvenile turbots of an average initial body weight of approx. 8 g, was conducted in Huanghai Aquaculture Co. Ltd. (Haiyang, China). Flow-through deep-well seawater was used in the 56-day feeding trial period. Each diet was randomly assigned to triplicate polyethylene tanks (200 L, 42 × 72 × 72 cm). Each tank was stocked with 30 fish. The experimental fish were hand-fed to apparent satiation two times each day (7:30 and 17:30). During the feeding trial, the water temperature ranged from 16.2 to 16.6 °C; salinity, 27 ~ 29; pH, 7.4 ~ 7.9; and dissolved oxygen, 7.5 ~ 8.1 mg L⁻¹.

At the end of the feeding trial, the average final weight of experimental fish in the control and LPC group was 34.65 g and 38.63 g, respectively. After anesthetized with eugenol (1:10,000), the muscle samples from 6 fish each tank were collected, with samples from 3 fish pooled as one sample. Finally, 2 pooled muscle samples in each tank were used for the lipidomic analysis (6 biological replicates each group). In the lipidomic analysis, the 6 biological replicates for the control group were labeled as rM1-1, rM1-6, rM2-3, rM2-8, rM3-6, and rM3-8, while those for the LPC group were labeled as rM4-2, rM4-3, rM5-3, rM5-6, rM6-3, and rM6-6. An accident happened when sample rM5-3 was processed, and thus this sample was eliminated in the final lipidomic analysis. All sampling protocols, as well as fish rearing practices, were reviewed and approved by the Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute.

2.2. Quantitative analysis of muscle lipidomics

Lipids were extracted from approximately 20 mg tissues according to Lam et al. (2016) with modifications. Briefly, tissue samples were homogenized in 900 μL of chloroform:methanol 1:2 (v/v) with 10 % deionized water on a bead ruptor (OMNI, USA). The homogenate was then incubated at 1500 rpm for 1 h at 4 °C. At the end of the incubation, 350 μL of deionized water and 250 μL of chloroform were added to induce phase separation. The samples were then centrifuged and the lower organic phase containing lipids was extracted into a clean tube. Lipid extraction was repeated once by adding 500 μL chloroform to the remaining tissues in aqueous phase, and the lipid extracts were pooled into a single tube and dried in the SpeedVac under organic mode. Samples were stored at −80 °C until further analysis.

The targeted lipidomic analysis was conducted in collaboration with LipidALL Technologies Company Limited (Changzhou, Jiangsu Province, China). The analysis used an extensive, targeted library tailored for animal tissue and cells that confers sufficient lipid coverage to render global lipid pathway analysis. All quantification experiments were conducted using internal standard calibration. Briefly, polar lipids were analyzed using an Agilent 1290 UPLC system coupled with a triple quadrupole/ion trap mass spectrometer (6500 Plus Qtrap; SCIEX) as described previously (Song et al., 2020; Lam et al., 2021). Separation of individual lipid classes of polar lipids by normal phase (NP)-HPLC was conducted using internal standard calibration. Briefly, tissue samples were homogenized using an Agilent 1290 UPLC system coupled with a triple quadrupole/ion trap mass spectrometer (6500 Plus Qtrap; SCIEX) as described previously (Song et al., 2020; Lam et al., 2021). Separation of individual lipid classes of polar lipids by normal phase (NP)-HPLC was conducted using a Phenomenex Luna 3 μm column (i.d. 4.6 × 100 mm) under the following conditions: mobile phase A (chloroform:methanol:ammonium hydroxide = 89.5:10.0:0.5) and mobile phase B (chloroform:methanol:ammonium hydroxide:water = 55:39:0.5:5.5). MRM transitions were set up for comparative analysis of various polar lipids.

Individual lipid species were quantified by referencing to spiked internal standards of the same lipid class including d₈-PC32:0(16:0/16:0), d₈-PE33:1(15:0/18:1), d₈-PS, d₈-PA33:1(15:0/18:1), d₈-PG33:1(15:0/18:1), d₈-PI33:1(15:0/18:1), Cer d18:1/15:0-d₈, d₈-SM d18:1/18:1, and C₂₂-GluCer, which were obtained from Avanti Polar lipids (Alabaster, Alabama, USA). Glycerol lipids including diacylglycerols (DAG) and triacylglycerols (TAG) were quantified using a modified method of reverse phase HPLC/HRM. Separation of neutral lipids were achieved on a Phenomenex Kinetex-C18 2.6 μm column (i.d. 4.6 × 100 mm) using an isocratic mobile phase containing chloroform:methanol:0.1 M ammonium acetate 100:100:4 (v/v/v) at a flow rate of 170
µL for 17 min. Levels of short-, medium-, and long-chain TAGs were calculated by referencing to spiked internal standards of TAG(14:0)-d5, TAG(16:0)-d5 and TAG(18:0)-d5, respectively, obtained from CDN isotopes (Pointe-Claire, Quebec, Canada). DAGs were quantified using d5-DAG17:0/17:0 and d5-DAG18:1/18:1 as internal standards (Avanti Polar Lipids). Free cholesterol and cholesteryl esters were analysed as described previously with d6-cholesterol and d6-C18:0 cholesteryl ester (CE) (CDN isotopes) as internal standards (Shui et al., 2011). Free fatty acids were quantitated using d31-16:0 (Sigma-Aldrich) and d8-20:4 (Cayman Chemicals) as internal standards, while d3-16:0-acylcarnitine (Cayman Chemicals) were used for quantification of acyl-carnitines.

Lipid levels were expressed in µmol/g muscle sample. However, in the results presentation below, most of the result were expressed as relative concentration between the LPC group and the control group (Fold_LPC/control). Mann-Whitney-U test was used in the non-parametric test of difference between the LPC group and the control group. The difference was considered as significant when P < 0.05.

2.3. Analysis of proximate composition and fatty acid composition in the muscle

The proximate composition analysis of fish muscle was performed according to the standard methods of Association of Official Analytical Chemists (AOAC) (2000). Samples were oven-dried at 105 °C to a constant weight for moisture analysis. Crude protein was assayed by measuring nitrogen (N × 6.25) using the Kjeldahl method. However, the lipid concentration in the muscle was analyzed with the chloroform–methanol method. The fatty acid composition in the muscle was analyzed with gas chromatograph (GC-2010 Pro, Shimadzu, Japan). Samples were firstly freeze-dried, and fatty acids in the samples were esterified with KOH-methanol (1 mol/L) and with HCL-methanol (2 mol/L), on 72 °C water bath. Fatty acid methyl esters were extracted with hexane and then separated via gas chromatography equipped with a fused silica capillary column (SHRT-2560, 100 m × 0.25 mm × 0.20 µm, Shimadzu, Japan). The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C min⁻¹, and then from 200 °C to 250 °C at a rate of 2 °C min⁻¹. Both the injector and detector temperatures were 250 °C. Results were expressed as the percentage of each fatty acid with respect to total fatty acids (TFA).

The data were arcsine transformed firstly, and then subjected to one-way analysis of variance (ANOVA) in SPSS 16.0 for Windows. Levene test was performed to test the homogeneity of variance. Significant differences between the means were detected by Tukey’s multiple range test. The level of significance was chosen at P < 0.05. The results were presented as means ± standard errors of means (SEM).

3. Results and discussion

After management of the raw data, a total of 615 lipids were successfully identified and subsequently quantified (Supplementary Fig. S1). The biggest lipid class quantified was TAG (208 individual lipids), followed by CL (62 individual lipids), while there were 10–30
individual lipids quantified in most other lipid classes. However, only 1 individual lipid was quantified for S1P, Sph, and Cho. The Principal Component Analysis (PCA) showed that the samples of the LPC group and control group generally clustered separately (Supplementary Fig. S2 and S3). However, there was a small distance between samples rM4-3 and rM6-3 of the LPC group and sample rM2-8 of the control group.

In total, 62 individual lipids showed significant difference in concentration between the LPC group and the control group (58 up-
regulated and 7 down-regulated by LPC) (Table 1, Fig. 1). Most of these lipids were distributed in DAG, FFA, CL, acylcarnitine, and PA (Fig. 1). When the total concentration of a specific lipid class was evaluated between the two experimental groups (Fig. 4), although the total CL concentration was not significantly different between the two groups (P = 0.3290, Fold\_LPC/control = 0.9581). As a key phospholipid of the mitochondria, CL is an anionic phospholipid mainly located in the inner mitochondrial membrane, where it helps regulate bioenergetics, membrane structure, and apoptosis (Scherer & Schmitz, 2010; Pennington et al., 2019). PA and PG are precursors of the biosynthesis of CL (Houthoofter & Vaz, 2008). PA and LPG can be formed to PG and then further converted to CL. In other ways, acyl groups can be exchanged between multiple phospholipid species, including LPL and CL (Malhotta et al., 2009; Moncada et al., 2017). The remodeling of the mitochondrial CL requires a phospholipid:lysophospholipid transacylase, tafazzin. The transacylation reaction alters the molecular species composition and, as a result, the physical properties of lipids. In vivo, the most important substrate of tafazzin is the mitochondria-specific lipid cardiolipin (Schiame & Xu, 2020), and remodeled CL contains predominantly unsaturated fatty acids (Ye et al., 2016). The transacylations mediated by tafazzin showed the highest rate for the phosphatidylcholine-cardiolipin transacylation. Transacylation activities were about 10-fold higher for linoleoyl groups than for oleoyl groups, and they were negligible for arachidonoyl groups (Xu et al., 2006). In the present study, significant inter-group difference was observed in CL\_7\_16(16:1), CL\_7\_6(16:1), CL\_8\_6(16:1), CL\_8\_7(16:1), CL\_9(16:1), CL\_10\_6(16:2), CL\_10\_7(18:2), CL\_10\_7(18:2), CL\_11\_8(18:2), CL\_12\_8(18:2), CL\_12\_8(20:3), CL\_12\_8(20:3), CL\_16\_10(20:3), and CL\_13\_20(20:3) (Table 1, Fig. 4), but was indeed not observed in CL with oleoyl or arachidonoyl groups. Other studies suggested that tafazzin itself lacks acyl specificity, but the acyl specificity is driven by the packing properties of certain lipid domains (Schiame et al., 2012). Nevertheless, it has to be noted that all these findings mentioned above were from mammalian studies. Relevant studies on fish are warranted.

As expected, the LPC concentration in the muscle was significantly affected by dietary LPC (Supplementary Fig. S5). However, although the LPC product used in the present study was soy-derived, which meant that it lacks long chain-polyunsaturated fatty acids (LC-PUFA), concentrations of LPC\_20:5, LPC\_22:6, and LPC\_22:5 were still increased by dietary LPC supplementation. This indicates the possible wide existence of transacylation reaction between PL and LPC. Concentration of certain PL was also significantly regulated by dietary LPC. The esterification of LPL mediated by acyl-CoA-dependent lysophospholipid acyltransferase contributes to PL synthesis and remodeling (Renaier et al., 2015).

Besides, concentration of several acylcarnitines in the muscle were significantly up-regulated by dietary LPC (Supplementary Fig. S6). Acylcarnitines transport fatty acids into mitochondria and are essential for β-oxidation and energy metabolism (Jarrell et al., 2020). Although the crude lipid content in the muscle was only slightly decreased by the dietary LPC in the present study (Supplementary Table S4), other recent fish studies with species, such as channel catfish (Liu et al., 2020) and rainbow trout (Taghavizadeh et al., 2020; Adhami et al., 2021), showed that dietary LPC supplementation reduced the cholesterol and lipid contents in whole body and liver. Up-regulation of acylcarnitines may contribute to the lipid-reducing effects of dietary LPC on farmed fish.

In conclusion, dietary LPC supplementation significantly regulated the lipid profile in farmed turbot muscle. Most of these differentially abundant lipids were DAG, FFA and CL, and they all were up-regulated by dietary LPC. Concentrations of certain lipid species in PA, LPC and acylcarnitine were also significantly regulated by dietary LPC. However, only marginal effects of dietary LPC supplementation was observed in total fatty acid composition and lipid content of muscle. The full change

| Table 2 | Fatty acid composition in the muscle (% total fatty acid) |
|---------|----------------------------------------------------------|
| Fatty acid | Control | LPC |
| C14:0 | 2.06 ± 0.17 | 1.99 ± 0.05 |
| C16:0 | 18.2 ± 0.60 | 18.3 ± 0.29 |
| C16:1-7 | 2.16 ± 0.16 | 2.06 ± 0.06 |
| C18:0 | 6.09 ± 0.06 | 5.77 ± 0.08 |
| C18:1-9t | 0.19 ± 0.03 | 0.22 ± 0.02 |
| C18:2-9c | 18.4 ± 0.38 | 17.7 ± 0.32 |
| C18:2-6t | 0.13 ± 0.01 | 0.14 ± 0.01 |
| C18:2-6c | 25.6 ± 0.99 | 25.2 ± 0.39 |
| C20:0 | 0.29 ± 0.02 | 0.26 ± 0.01 |
| C18:3-6 | 0.10 ± 0.05 | 0.17 ± 0.00 |
| C20:1-9 | 1.51 ± 0.03 | 1.42 ± 0.02 |
| C18:3-6c | 2.14 ± 0.17 | 2.09 ± 0.07 |
| C20:2-6 | 1.22 ± 0.05 | 1.16 ± 0.02 |
| C22:0 | 0.12 ± 0.00 | 0.09 ± 0.00 |
| C22:1-9 | 0.26 ± 0.02 | 0.21 ± 0.02 |
| C20:3-6 | 0.22 ± 0.01 | 0.23 ± 0.00 |
| C20:4-6 | 1.19 ± 0.08 | 1.23 ± 0.03 |
| C22:2-6 | 0.08 ± 0.01 | 0.07 ± 0.01 |
| C20:5-6 | 4.25 ± 0.17 | 4.35 ± 0.13 |
| C24:1-9 | 0.17 ± 0.01 | 0.13 ± 0.00 |
| C22:6-6 | 15.2 ± 0.81 | 16.5 ± 0.68 |

Data in the same row not sharing a same superscript letter were significantly different (P < 0.05).
Fig. 4. Heatmap of identified cardiolipin (CL). The average concentration of a lipid metabolite was standardized to be 0. Higher concentration than the average was labeled as orange, and lower concentration was labeled as purple. The color value as indicated in the right color bar means fold of standard deviation distant to the average concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
scenario of fish muscle lipid profile in response to dietary LPC supplementation, as well as the involved mechanisms, remain not well-known. Further research in this area will be beneficial to better management of LPC supplementation in fish feeds.

CRediT authorship contribution statement

Houguo Xu: Conceptualization, Software, Writing – original draft. Xing Luo: Formal analysis. Yuliang Wei: Data curation, Methodology. Mengqing Liang: Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foch.2022.100293.

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