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Modulation of immune response, physical barrier and related signaling factors in the gills of juvenile grass carp (*Ctenopharyngodon idella*) fed supplemented diet with phospholipids

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ABSTRACT

This study was conducted to investigate the effects of dietary phospholipids (PL) on the gill immune response and physical barrier of juvenile grass carp (*Ctenopharyngodon idella*). A total of 1080 juvenile grass carp with an average initial weight of 9.34 ± 0.03 g were fed six semi-purified diets containing 0.40% (unsupplemented control group), 1.43%, 2.38%, 3.29%, 4.37% and 5.42% PL for 2 months. Compared with the control group, optimal PL supplementation increased ($P < 0.05$): (1) the lysozyme activity, acid phosphatase activity, complement component 3 (C3) content, liver expressed antimicrobial peptide 1 (LEAP-1) and LEAP-2 mRNA expression; (2) the relative mRNA expression of interleukin 10, transforming growth factor β1, inhibitor factor κBα (IκBα) and target of rapamycin (TOR); (3) the activities of anti-superoxide anion (ASA), anti-hydroxyl radical (AHR), copper/zinc superoxide dismutase (SOD1), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), glutathione content and mRNA levels of SOD1, CAT, GPx, GR and NF-E2-related factor 2 (Nrf2) genes; (4) the transcription abundance of occludin, claudin b, claudin c, claudin 12 and zonula occludens 1 genes. At the same time, appropriate PL supplementation decreased ($P < 0.05$): (1) tumor necrosis factor α, interleukin 1β, nuclear factor κB p65 (NF-κB p65), IκB kinase β (IKKβ) and IκB kinase γ (IKKγ) mRNA expression; (2) malondialdehyde (MDA), protein carbonyl (PC) and reactive oxygen species (ROS) content and the relative mRNA expression of Kelch-like-ECH-associated protein 1a (Keap1a) and Keap1b; (3) the transcription abundance of myosin light chain kinase (MLCK) and p38 mitogen-activated protein kinase (p38 MAPK) genes. In conclusion, the positive effect of PL on gill health is associated with the improvement of the immunity, antioxidant status and tight junction barrier of fish gills. Finally, based on ACP activity, C3 content, PC content and ASA activity in the gills, the optimal dietary PL level for juvenile grass carp (9.34–87.50 g) was estimated to be 3.62%, 4.30%, 3.91% and 3.86%, respectively.

1. Introduction

The gills have the largest organ-specific surface interacting with the external milieu and provide an initial barrier to the entry of pathogens in fish [1]. The gill-associated lymphoid tissues play important roles in governing immune repertoire in teleost mucosal immunity [2]. It is reported that gill dysfunction led to the seriously impaired growth performance [3], implying the importance of gill health status. Phospholipids (PL) serve as the integral part of the structure of the biological membranes in fish [4], may participate in maintaining fish gill health; however, to date, no studies have addressed about this topic. Study reported that phospholipids had antioxidant activity by means of its major functional groups

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(choline, ethanolamine and side-chain moieties) [5]. And Jiang et al. [6] found that the important component of PL, inositol, could attenuate oxidative damage in the gills of Jian carp (*Cyprinus carpio* var. Jian). It appears that there may be a close relationship between PL and the health status of fish gills, which needs to be investigated.

The gill health status in fish mainly depends on the gill mucosal immune function [7], which is governed by gill-associated lymphoid tissue that consists of variably sized immune cells, such as lymphocytes, macrophages and granulocytes [8]. The immune cells could secrete humoral components, such as lysozyme (LZ), acid phosphatase (ACP), complement component 3 (C3) and antimicrobial peptides, which play crucial roles in the gill innate immune response of fish [7]. However, no studies have addressed the effects of dietary PL on the humoral components in the gills of fish. The available study showed that dietary PL improved plasma LZ activity in juvenile channel catfish, *Ictalurus punctatus* [9]. These data indicated a possible correlation between PL and the humoral components in fish gills, which is valuable for investigation. Furthermore, inflammation is a key element in the response of the innate immune system and is mediated by cytokines, such as IL-1β, TNF-α and IL-10 [10]. Meanwhile, the production of cytokines could be regulated by the signaling pathways of NF-κB [11] and TOR [12]. To date, no study has investigated the effect of PL on the cytokines through NF-κB and TOR signaling pathways in fish gills. Studies have shown that the important component of PL, choline, could inhibit NF-κB activation in endotoxin stimulated mouse macrophage-like cells [13] and modulate cytokines and TOR expression in fish [14]. These appear that PL may be related to the NF-κB and TOR signaling pathways of fish gills to influence cytokines production. This possibility is worth investigating.

In addition to mucosal immunity, the physical barrier function is also critical in maintaining gill health status [15]. Structural integrity of the gills is the foundation of its physical barrier function in fish, and fish have developed an antioxidant defense system to protect the structural integrity of the gills [16]. Antioxidant defense systems in fish include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and non-enzymatic compounds, such as glutathione (GSH) [17]. Moreover, the expression of antioxidant enzyme is mainly regulated by nuclear factor-E2-related factor 2 (Nrf2) in zebrafish [18]. However, there is no information about the effect of PL on antioxidant enzymes and Nrf2 in the gills of fish. The available study showed that dietary PL could increase SOD and GPx activities in the liver of blunt snout bream fingerlings [19]. The above data indicates that PL may affect the antioxidant capacity of fish gills. Moreover, the tight junction complex is also an important part of the physical barrier for the fish gills [20]. The tight junction (TJ) complex was mainly made up of both integral TJ proteins and TJ plaque proteins [21]: the integral TJ proteins, such as occludin and claudins, bridge the apical intercellular space and form a regulated permeability barrier; the TJ plaque proteins, such as zonula occludens 1 (ZO-1), serve as links between the integral TJ proteins and the actin cytoskeleton and as adapters for the recruitment of cytosolic molecules implicated in cell signaling. The myosin light chain kinase (MLCK) has emerged as key regulators of the tight junctions in terrestrial animals [22]. However, to date, studies to investigate the effect of dietary PL on TJ proteins and MLCK in fish gills have not been carried out. In terrestrial animal, dietary PL could inhibit intestinal cholesterol absorption [23]. Zhu et al. [24] reported that high cholesterol level could increase MLCK expression and activity in the aortas of rabbits. These data indicated PL might affect the tight junction via influencing the signaling molecules of MLCK in fish gills, however, these warrant investigation.

This study is a part of a larger study aimed at determining the effects of PL on fish growth using the same growth trial as the previous study [25]. The objective of this study was to further investigate the effects of PL on the immunity and the physical barrier function of fish gills, which could be used to preliminarily determine the PL-dependent mechanism of improving disease resistance. The optimum dietary PL levels for gill health related parameters in juvenile grass carp was also evaluated.

2. Materials and methods

2.1. Experimental diets

The diet formulation and composition is shown in Table 1. Fish meal (Pesquera Lota Protein Ltd., Lota, Chile), casein (Hulunbeier Sanyuan Milk Co., Ltd., Inner Mongolia, China) and gelatin (Rousselot Gelatin Co., Ltd., Guangdong, China) were used as the protein sources. Linseed oil (Hunan Yama biotechnology Co., Ltd., Hunan, China), safflower oil (Shanghai Yuan Tian Edible Agricultural Products Ltd., Shanghai, China), coconut oil (Lvyuan natural flavor oil refinery, Jiangxi, China) and soybean lecithin (Shaanxi Huicheng biotechnology Co., Ltd., Shaanxi, China) were used as lipid sources. Six experimental diets were obtained by supplementing the control diet with soybean lecithin at concentrations of 0.00% (un-supplemented control), 1.00%, 2.00%, 3.00%, 4.00% and 5.00% diet, while adjusting coconut oil to maintain the diets iso-lipidic, the method according to Niu et al. [26]. The analyzed PL levels were 0.40% (un-supplemented control), 1.43%, 2.38%, 3.29%, 4.37% and 5.42% of the diets according to the method described by Juaneda and Rocquelin [27] and Li et al. [28]. After being prepared completely, the pellets were stored at −20 °C until feeding according to Zhao et al. [29].

2.2. Feeding trial

All protocols were approved by the Institutional Animal Care

Table 1
Diet formulation and composition.[a]

Ingredient	% (diet)
Fishmeal	3.00
Casein	28.00
Gelatin	7.50
DL-Met (99%)	0.14
Corn starch	11.55
Alpha-starch	24.00
Cellulose	5.00
Vitamin premix[b]	1.00
Mineral premix[c]	2.00
$Ca(H_2PO_4)_2$ (22%)	2.26
Choline chloride (60%)	0.50
Ethoxyquin (30%)	0.05
Oil premix[d]	5.00
Soybean lecithin premix[e]	10.00

[a] Crude protein and total lipids were measured to be 30.16% and 9.06% respectively.
[b] Per kilogram of vitamin premix: retinyl acetate (500 000 IU g^{-1}), 2.40 g; cholecalciferol (500 000 IU g^{-1}), 0.40 g; D,L-a-tocopherol acetate (50%), 12.55 g; menadione (23%), 0.80 g; thiamine nitrate (98%), 0.05 g; riboflavin (80%), 0.55 g; pyridoxine hydrochloride (98%), 0.59 g; cyanocobalamin (1%), 0.83 g; niacin (99%), 2.24 g; D-biotin (2%), 4.91 g; mesoinositol (99%), 19.39 g; folic acid (96%), 0.40 g; ascorhyl acetate (93%), 7.16 g. All ingredients were diluted with corn starch to 1 kg.
[c] Per kilogram of mineral premix: $MgSO_4 \cdot H_2O$, 60.530 g; $FeSO_4 \cdot H_2O$, 23.110 g; $CuSO_4 \cdot 5H_2O$, 0.010 g; $ZnSO_4 \cdot H_2O$, 0.620 g; $MnSO_4 \cdot H_2O$, 1.640 g; KI, 0.070 g; $NaSeO_3$, 0.005 g. All ingredients were diluted with corn starch to 1 kg.
[d] Per kilogram of oil premix: linseed oil 185.80 g, safflower oil 237.60 g, corn starch 576.60 g.
[e] Soybean lecithin premix: premix was added to obtain graded level of phospholipids.