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Exogenous phospholipids supplementation improves growth and modulates immune response and physical barrier referring to NF-κB, TOR, MLCK and Nrf2 signaling factors in the intestine of juvenile grass carp (*Ctenopharyngodon idella*)

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A B S T R A C T

This study was conducted to investigate the effects of dietary phospholipids (PL) on the growth performance, intestinal enzyme activity and immune response and intestinal 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. Results indicated that 3.29% PL increased lysozyme (LZ) and acid phosphatase (ACP) activities and complement component 3 (C3) content ($P < 0.05$), up-regulated the mRNA relative expression levels of interleukin 10, transforming growth factor β1 (TGF-β1), inhibitor protein κBα (IκBα), target of rapamycin (TOR) and casein kinase 2 (CK2) ($P < 0.05$), and down-regulated tumor necrosis factor α (TNF-α), interleukin 1β, nuclear factor κB p65 (NF-κB p65), IκB kinase β (IKKβ) and IκB kinase γ (IKKγ) mRNA relative expression levels ($P < 0.05$) in the intestine, suggesting that optimum PL could improve fish intestinal immunity. In addition, 3.29% PL increased the activities of anti-superoxide anion (ASA), anti-hydroxyl radical, copper/zinc superoxide dismutase (SOD1), glutathione peroxidase (GPx) and glutathione reductase (GR), the content of glutathione ($P < 0.05$), and the mRNA relative expression levels of occludin, zonula occludens 1 (ZO-1), claudin 3, claudin 12, claudin b, claudin c, SOD1, GPx, GR and NF-E2-related factor 2 (Nrf2) and decreased malondialdehyde (MDA), protein carbonyl (PC) and ROS content ($P < 0.05$), the mRNA relative expression levels of Kelch-like-ECH-associated protein 1a (Keap1a), myosin light chain kinase (MLCK) and p38 mitogen-activated protein kinase (p38 MAPK) in the intestine, indicating that the optimum PL could improve fish intestinal physical barrier. Finally, based on the PWG, C3 content in the DI, ACP activity in the DI, intestinal PC content and intestinal ASA activity, the optimal dietary PL levels for juvenile grass carp (9.34–87.50 g) were estimated to be 3.46%, 3.79%, 3.93%, 3.72%, and 4.12%, respectively.

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1. Introduction

Intestine plays an important role in the physiology of fish by performing the dual function of maintaining immune homeostasis and digesting/absorbing nutrients [1]. Due to the durative exposure to its environment, the intestinal mucosa is constantly contacted with a wide variety of antigens, including potential pathogens and

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food antigens present in the lumen [2], highlighting the importance of intestinal health status. Previous studies in our laboratory found that exogenous nutrients, such as valine [3] and tryptophan [4], had beneficial effects on fish intestinal health status. Phospholipids (PL) serve as the integral part of the biological membranes structure in fish [5], may participate in maintaining fish intestinal health; however, to date, no studies have addressed in this topic. The available study in piglets showed that dietary PL could attenuate intestinal histologic lesions that caused by the malnutrition process [6]. It appears that PL could affect the intestinal health status of fish, which warrants investigation.

In fish, the intestinal health status is strongly associated with its immunity [7], which mainly depends on their immune response [8]. The humoral components, such as lysozyme (LZ), acid phosphatase (ACP) and complement component 3 (C3) [9] and cytokines, such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) [10] play important role in orchestrating the immune response in fish intestine. Furthermore, the production of cytokines could be involved in signaling pathway of nuclear factor κB (NF-κB) [11]. In grass carp, NF-κB inhibition reduced the release of TNF-α in the head kidney leukocytes [12]. However, to date, studies to investigate the effects of dietary PL on the humoral components and NF-κB in regulating production of cytokines in animal have not been carried out. In't Veld et al. [13] reported that PL could affect leucine transport activity in *Lactococcus lactis*. Study from our laboratory has shown that leucine could increase intestinal LZ and ACP activities and C3 levels in grass carp [14]. In addition, Baskaran et al. [15] found that PL could promote the intestinal absorption of β-carotene in mice. Study reported that β-carotene inhibited the expression and production of TNF-α and IL-1β in RAW264.7 cells [16]. And study found that PL could significantly improve inhibitor protein κB (IκB) gene expression in rainbow trout fry [17]. To our knowledge, IκB has been identified as an NF-κB p65-binding protein, which prevents NF-κB p65 translocation to the nucleus, thereby decrease the production of cytokines [11]. These observations indicated a possible correlation between dietary PL and the humoral components and cytokine production and related signaling molecule involved in the NF-κB signaling pathway of the intestine in fish, which is valuable for investigation.

In addition, fish intestinal health also depends on the intestinal physical barrier, which is associated with enterocyte structure integrity and the tight junction (TJ) complex (occludin, zonula occludens 1 (ZO-1) and claudins) [7]. Beutheu et al. [18] reported that the up-regulation of occludin and ZO-1 expression contributed to limit barrier disruption during anticancer chemotherapy in Caco-2 cells. Furthermore, the myosin light chain kinase (MLCK) and mitogen-activated protein kinases, such as p38 MAPK have emerged as key regulators of the tight junctions in terrestrial animals [19]. However, no study has addressed the effect of dietary PL on the tight junction protein in fish. As we know, PL metabolite, phosphate, is basal component of nicotinamide adenine dinucleotide phosphate (NADPH) [20]. Shi et al. [21] found that NADPH is required as a crucial cofactor during the biosynthesis of isoleucine in *Corynebacterium glutamicum*. Studies have shown that isoleucine could regulate tight junction proteins and p38 MAPK mRNA expression in the intestine of Jian carp [22]. These data indicated PL might affect the tight junction proteins expression, however, these warrant investigation. Moreover, the oxidative damage customarily leads to the destruction of enterocyte structure integrity in fish [23]. Fish combat oxidative damage with antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [24]. To our knowledge, the enzyme activity is partly related to enzyme gene transcription [25]. Study reported that the gene transcription of antioxidant enzymes were regulated by the NF-E2-related factor 2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) pathway in aquatic organisms [26]. However, no study has been conducted to investigate the effect of PL on the antioxidant enzymes and signal molecules Nrf2 and Keap1 in fish intestine. Inositol as the basic component of inositol phospholipids, is the important component of PL [27]. Previous study from our laboratory showed that inositol could regulate the gene relative expression levels of Nrf2 and Keap1 and increase SOD and GPx activities in the intestine of juvenile Jian carp [28]. These observations indicated a possible correlation between dietary PL and the gene expression of antioxidant enzymes and related signaling molecule involved in the Nrf2/Keap1 signaling pathway in the fish intestine, which is valuable for investigation.

The grass carp (*Ctenopharyngodon idella*), one of the commercial herbivorous species, is widely distributed in many countries [29]. The optimal levels of dietary PL for growth have been estimated for several carnivorous and omnivorous fish (ayu, *Plecoglossus altivelis*: 3%; rainbow trout, *Oncorhynchus mykiss*: 4%; Atlantic salmon, *Salmo salar*: 6%) [5]. However, the nutrient requirement for fish are various a lot among different feeding habits [5] and physiological indices [9]. Up to now, no study has been conducted to investigate the optimal levels of dietary PL for herbivorous fish. Thus, one purpose of the present study was to evaluate the optimal dietary PL levels for juvenile grass carp, especially basing on immunity and antioxidant status indices.

From the foregoing statement, the objective of this study was to explore the effects of dietary PL on fish growth, intestinal function and immune response and intestinal physical barrier as well as the related signaling molecule of juvenile grass carp (Fig. 1). The resultant findings could provide partial theoretical evidence for the potential molecular mechanisms behind the effects of dietary PL on the intestinal health status. The optimal levels of dietary PL for the immunity response, antioxidant status and growth of juvenile grass carp were also evaluated, which may be used in formulating commercial feeds for the intensive culture of grass carp.

2. Materials and methods

2.1. Experimental diets

The diets formulation and composition are 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

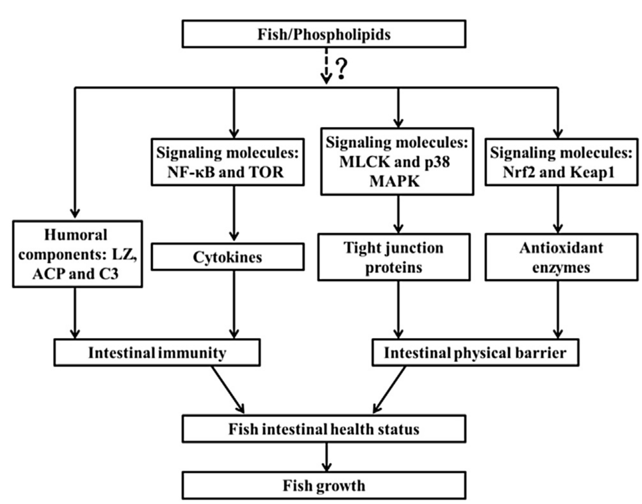

Fig. 1. The potential action pathway of phospholipids-regulated intestinal health in fish.

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