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# Dietary pantothenic acid depressed the gill immune and physical barrier function via NF-κB, TOR, Nrf2, p38MAPK and MLCK signaling pathways in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

This study explored the effects of pantothenic acid (PA) on the immune and physical barrier function, and relative mRNA levels of signaling molecules in the gill of grass carp *(Ctenopharyngodon idella)*. The results indicated that compared with optimal PA supplementation, PA deficiency (1.31 mg/kg diet) decreased gill interleukin 10, transforming growth factor β1, inhibitor of κBα (IκBα), eIF4E-binding protein 2, Claudin b and ZO-1 mRNA levels; anti-superoxide anion activity, and activities and mRNA levels of copper/zinc superoxide dismutase, manganese superoxide dismutase, glutathione peroxidase, glutathione reductase and NF-E2-related factor ($P < 0.05$). Additionally, PA deficiency and excess (75.08 mg/kg diet) decreased gill complement 3 and glutathione contents, lysozyme and acid phosphatase, anti-hydroxy radical, catalase and glutathione S-transferases activities, and liver-expression antimicrobial peptide 2, hepcidin, Claudin 3, Claudin c and Occludin mRNA levels ($P < 0.05$). Conversely, PA deficiency increased gill reactive oxygen species and protein carbonyl contents, and interferon γ2, interleukin 8, nuclear factor kappa B P65, Claudin 15a, Kelch-like ECH-associating protein 1a and Kelch-like ECH-associating protein 1b mRNA levels ($P < 0.05$). Moreover, PA deficiency and excess increased gill malondialdehyde content, and tumor necrosis factor α, interleukin 1β, IκB kinase α, IκB kinase β, IκB kinase γ, target of rapamycin and ribosomal S6 protein kinase1 p38 mitogen-activated protein kinases and myosin light-chain kinase mRNA levels ($P < 0.05$). In conclusion, PA deficiency decreased immune and physical barrier function, and regulated relative mRNA levels of signaling molecules in fish gill. Based on the quadratic regression analysis of gill lysozyme activity, the optimal PA levels in grass carp (253.44–745.25 g) were estimated to be 36.97 mg/kg diet.



## 1. Introduction

The fish gill represents an immune-competent organ for it is characterized by large mucosal surfaces [1], and gill health status is utmost importance for fish survival [2]. Uribe et al. [3] found that the fish gill health status is largely dependent upon its immune function and physical barrier function. Up to now, limited study has shown that nutrients could enhance gill health status of fish. Study from our lab showed that arginine could protect the gill against copper-induce damage, thereby maintaining gill health status of grass carp *(Ctenopharyngodon idella)* [4]. As we know, pantothenic acid (PA), one essential water-soluble vitamin, is a component of coenzyme A (CoA), acyl CoA and acyl carrier protein, and the coenzyme form of PA is involved in acyl group transfer reactions, tricarboxylic acid cycle and acetylation of choline [5]. Study from

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our lab reported that PA deficiency could cause growth retardation in juvenile Jian carp *(Cyprinus carpio var. Jian)* [6]. In fish, the growth is closely related to the health status of gill [2]. In addition, study in fish showed that PA deficiency could significantly decrease the concentrations of free PA in the gill [7]. These data suggest that PA may be related to the gill health of fish, further studies are warranted to address these important questions.

To our knowledge, the fish gill health status is largely dependent upon its immune function [3]. The immune function of gill has been found to be correlated with antibacterial compounds like lysozyme (LA), acid phosphatase (ACP), complement and antimicrobial peptides in fish [8,9], and cytokines such as interleukin 1β (IL-1β), interleukin 8 (IL-8) and interferon-γ (IFN-γ) [10]. The transcription levels of cytokines could be mediated by nuclear factor kappa B (NF-κB) and target of rapamycin (TOR) signaling pathways in fish [11]. In human umbilical vein endothelial cells, NF-κB inhibition could decrease IL-8 gene expression [12]. In mice bone marrow neutrophils, inhibition of TOR caused a decrease in expression of tumor necrosis factor α (TNF-α) [13]. However, studies have not addressed the effects of PA on the gill immune function and its possible mechanisms involved in NF-κB and TOR signaling pathways in fish. In mice, PA deficiency could decrease the level of insulin in serum [14]. Study has implied that insulin could inhibit NF-κB activity in mononuclear cells of humans [15]. In addition, in rats, PA deficiency could decrease progesterone level in plasma [16]. Study in mice showed that progesterone could suppress TOR gene expression [17]. These appear that PA may have effects on the fish gill immune and its possible mechanisms in fish, which is valuable for investigation.

In fish, in addition to the immune function, the physical barrier function is also the main foundation of the gill heath [3]. The fish gill physical barrier is mainly composed of epithelial cells and their intercellular tight junctions (TJ) [18]. The TJ is composed of TJ proteins, which includes cytosolic proteins like zonula occludens 1 (ZO-1) and transmembrane proteins like Occludins and Claudins in fish gill epithelium [19]. The transcript abundance of TJ proteins could be regulated by p38 mitogen-activated protein kinases (p38MAPK) in fish [20]. In rats, inhibition of p38MAPK could increase occludin expression in blood-retinal barrier [21]. However, no study has addressed the effects of PA on TJ proteins and its possible mechanisms related to signaling molecule p38MAPK in fish gill. In rats, PA deficiency could decrease the corticosterone level in plasma [16]. Study has shown that corticosterone could induce p38MAPK phosphorylation in rats [22]. These data indicate that PA may have effects on TJ proteins, which may be partly via influencing p38MAPK in fish gill. However, the topic is worthy of investigation. Additionally, the integrity of epithelial cells is also plays a relatively larger role in the gill physical barrier of fish [18]. It was reported that the fish gill epithelial cells are vulnerable to oxidative damage caused by exceeding reactive oxygen species (ROS) [4]. The ROS removal in large part relies on the non-enzymatic compounds (glutathione (GSH)) and enzymatic antioxidants compounds (copper/zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx), and catalase (CAT)) in fish [23]. Work in fish revealed that the elevation of CuZnSOD and GPx activities may be partly due to increase their mRNA levels [11]. The expression of antioxidant enzyme genes is typically regulated by signaling molecule NF-E2-related factor (Nrf2) in fish [24]. However, no study has addressed the effects of PA on regulating antioxidant enzyme activities by modulating their gene transcriptions related to Nrf2 signaling pathway in fish gill. In humans, it was reported that methylcrotonyl-coenzyme A (one coenzyme of PA) carboxylase catalyzes an essential step in leucine metabolism [25]. Study in our lab showed that leucine could increase Nrf2 mRNA level in fish intestine [24]. These findings lead to the idea that PA may regulate antioxidant enzyme activities via modulating their gene transcriptions, which may be related to the Nrf2 signaling pathway in the gill of fish. However, the topic needs to be investigated.

This study is in line with our previous investigation, which was a larger research study on how dietary PA deficiency and excess decreased growth performance and intestinal health in fish [26]. In fish, it was reported that the growth is also closely related to the health of gill [2]. Hence, the aim of the present study was for the first time to investigate the effects of dietary PA on the gill immune and physical barrier function of fish. Additionally, mRNA levels of cytokines, antioxidant enzymes, TJ proteins and signal molecules (NF-κB P65, TOR, Nrf2 and p38MAPK) were measured to provide a potential way for PA mediating the gill immune and physical barrier function of fish. Meanwhile, the dietary PA requirement according to gill immune indicator was also evaluated, which may provide a reference for formulating feed of grass carp.

## 2. Materials and methods

### 2.1. Experimental design and diets

As shown in Table 1, formulation of the basal diet was the same as our previous study [26]. 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 dietary protein sources. Fish oil (CIA. Pesquera Camanchaca S.A., Santiago, Chile) and soybean oil (Kerry Oils & Grains Industrial Co., Ltd., Sichuan, China) were used as dietary lipid sources. Six experimental diets were supplemented with calcium-D-pantothenate (Sigma, St. Louis, MO, USA) at concentrations of 0 (un-supplemented control), 15.00, 30.00, 45.00, 60.00 and 75.00 mg/kg diet, and the corn starch amount was reduced to compensate, according to the method of Wen et al. [6]. The final PA concentrations of the six experimental diets were 1.31

**Table 1**
Composition and nutrients content of basal diet.

| Ingredients | g/kg | Nutrients content | g/kg |
|---|---|---|---|
| Fish meal | 37.50 | Crude protein[d] | 293.20 |
| Casein | 248.10 | Crude lipid[d] | 45.40 |
| Gelatin | 75.00 | n-3[e] | 10.00 |
| DL-Met (99%) | 1.40 | n-6[e] | 10.00 |
| α-Starch | 240.00 | Available phosphorus[e] | 6.00 |
| Corn-starch | 231.20 | | |
| Fish oil | 25.00 | | |
| Soybean oil | 18.90 | | |
| Cellulose | 50.00 | | |
| $Ca(H_2PO_4)_2$ | 22.40 | | |
| Vitamin premix[a] | 10.00 | | |
| Mineral premix[b] | 20.00 | | |
| Pantothenicic acid premix[c] | 15.00 | | |
| Choline chloride (60%)[a] | 5.00 | | |
| Ethoxyquin (30%)[1] | 0.50 | | |

[a] Vitamin premix (g/kg premix): retinyl acetate (500 000 IU/g), 2.40; cholecalciferol (500 000 IU/g), 0.40; D,L-a-tocopherol acetate (50%), 12.54; menadione (23%), 0.79; thiamine nitrate (98%), 0.04; ribofiavin (80%), 0.55; pyridoxine hydrochloride (98%), 0.59; cyanocobalamin (1%), 0.81; niacin (99%), 2.17; D-biotin (2%), 4.91; mesoinositol (99%),19.19; folic acid (96%), 0.40; ascorhyl acetate (93%), 7.16. All ingredients were diluted with corn starch to 1 kg.

[b] Mineral premix (g/kg premix): $FeSO_4 \cdot H_2O$, 22.900; $CuSO_4 \cdot 5H_2O$, 0.020; $ZnSO_4 \cdot H_2O$, 0.630; $MnSO_4 \cdot H_2O$, 1.650; KI, 0.070; $NaSeO_3$, 0.004; $MgSO_4 \cdot H_2O$, 56.200. All ingredients were diluted with corn starch to 1 kg.

[c] Pantothenic acid premix: premix was added to obtain graded levels of calcium-D-pantothenate (Sigma, St. Louis, MO, USA) and the amount of corn starch was reduced to compensate.

[d] Crude protein and crude lipid contents were measured value.

[e] Available phosphorus, n-3 and n-6 contents were calculated according to NRC (2011).