Effects of Lactobacillus delbrueckii on Immune and Antioxidant Parameters in Skin Mucus and Antimicrobial Peptide Gene Expression in Skin of Yellow River Carp (Cyprinus carpio) Postprint

Authors: Zeng Qinghui, Liu Min, Yao Yuhong, Ding Xiangfei, Shi Lisha, Li Xiaofan, Zhang Chunnuan

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Abstract

This experiment primarily investigated the effects of Lactobacillus delbrueckii on immune and antioxidant indices in skin mucus and antimicrobial peptide gene expression in the skin of Yellow River carp (Cyprinus carpio Huanghe var.). A total of 450 Yellow River carp with an initial weight of $(15.0\$\pm0.5)gwererandomlydividedinto5groups, with3replicatespergroupand30fishperreplicate.Theexperime$ CFU/g, respectively. After 8 weeks of feeding, skin mucus and skin samples were collected to determine immune and antioxidant indices in skin mucus and the relative expression levels of antimicrobial peptide genes [liver-expressed antimicrobial peptide 1 (Leap-1) and liver-expressed antimicrobial peptide 2 (Leap-2)] in the skin. The results showed: The 1$×10^{6}$ and 1$×10^{7}$ CFU/g Lactobacillus delbrueckii groups exhibited significantly higher activities of lysozyme (LYZ), alkaline phosphatase (AKP), superoxide dismutase (SOD) and contents of complement 3 (C3) and complement 4 (C4) in skin mucus compared with the control group (P<0.05), while their skin mucus malondialdehyde (MDA) content was significantly lower than that of the control group (P<0.05). However, the above indices in the 1$×10^{5}$ and 1$×10^{8}$ CFU/g Lactobacillus delbrueckii groups showed no significant differences from the control group (P>0.05). No significant differences were observed in acid phosphatase (ACP) activity in skin mucus among all groups (P>0.05). The 1$×10^{6}$ CFU/g Lactobacillus delbrueckii group showed significantly higher activities of catalase (CAT) and glutathione peroxidase (GPx) in skin mucus compared with the control group (P<0.05), and the $1\$\times10^{\{5\},1\times10}\{6\},and1\times10^{7}\$$ CFU/g Lactobacillus delbrueckii groups exhibited significantly higher total antioxidant capacity (T-AOC) in skin mucus than the control group (P<0.05). The relative expression levels of

antimicrobial peptide genes Leap-1 and Leap-2 in the skin of all experimental groups were significantly higher than those of the control group (P<0.05), with the highest values observed in the 1$×10^{7}$ CFU/g Lactobacillus delbrueckii group. The results indicate that dietary supplementation with Lactobacillus delbrueckii at 1$×10^{6}-1×10^{7}$ CFU/g can enhance the immune function and antioxidant capacity of the skin in Yellow River carp, and upregulate the expression of antimicrobial peptide genes.

Full Text

Effects of *Lactobacillus delbrueckii* on Immune and Antioxidant Indexes in Skin Mucus and Antimicrobial Peptide Gene Expression in Skin of Yellow River Carp (*Cyprinus carpio* Huanghe var.)

ZENG Qinghui, LIU Min, YAO Yuhong, DING Xiangfei, SHI Lisha, LI Xiaofan, ZHANG Chunnuan*

College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, China

*Corresponding author: ZHANG Chunnuan, Lecturer, E-mail: zhangchunnuan12@163.com

Abstract

This study investigated the effects of *Lactobacillus delbrueckii* on immune and antioxidant indexes in skin mucus and antimicrobial peptide gene expression in skin of Yellow River carp (*Cyprinus carpio* Huanghe var.). A total of 450 Yellow River carp with initial body weight of $(15.0\pm0.5)gwererandomlydividedintofivegroupswiththreereplicatespergroupand30fishperreplicate.Theexp$ CFU/g *L. delbrueckii* for 8 weeks. At the end of the feeding trial, skin mucus and skin samples were collected to determine immune and antioxidant indexes in skin mucus and the relative expression levels of antimicrobial peptide genes [liver-expressed antimicrobial peptide-1 (Leap-1) and liver-expressed antimicrobial peptide-2 (Leap-2)] in skin. The results showed that the activities of lysozyme (LYZ), alkaline phosphatase (AKP), superoxide dismutase (SOD) and the contents of complement 3 (C3) and complement 4 (C4) in skin mucus of the 1$×10^{6}$ and 1$×10^{7}$ CFU/g groups were significantly higher than those of the control group (P<0.05), while the malondialdehyde (MDA) content was significantly lower (P<0.05). No significant differences were observed in these parameters between the 1$×10^{5}$ or 1$×10^{8}$ CFU/g groups and the control group (P>0.05). Acid phosphatase (ACP) activity in skin mucus did not differ significantly among all groups (P>0.05). The activities of catalase (CAT) and glutathione peroxidase (GPx) in skin mucus of the 1$×10^{6}$ CFU/g group were significantly higher than those of the control group (P<0.05), and the total antioxidant capacity (T-AOC) in skin

mucus of the 1$×10^{5}, 1×10{6}, and1×10^{7}$ CFU/g groups was significantly higher than that of the control group (P<0.05). The relative expression levels of antimicrobial peptide genes Leap-1 and Leap-2 in skin were significantly up-regulated in all experimental groups compared with the control group (P<0.05), with the highest values observed in the 1$×10^{7}$ CFU/g group. These results demonstrate that dietary supplementation with 1$×10^{6}$ to 1$×10^{7}$ CFU/g *L. delbrueckii* can enhance immune function, antioxidant capacity, and antimicrobial peptide gene expression in Yellow River carp.

Keywords: *Lactobacillus delbrueckii*; *Cyprinus carpio* Huanghe var.; skin; immune; antioxidant

Introduction

Fish are relatively primitive vertebrates with immune systems far less developed than those of mammals and birds. The fish immune system can be divided into external and internal components, with skin being a crucial part of the external immune system. Fish skin contains numerous mucous cells, macrophages, and various lymphocytes along with other active substances that form a protective barrier against pathogenic microorganisms on the body surface, serving as an effective defense line against infection [1]. The composition of fish skin mucus is complex; studies have revealed that it contains not only immunoglobulins [2] but also various enzymes and immune factors, including lysozyme, cathepsin, esterase, metalloproteinase, lectin, interferon, calmodulin, complement, antimicrobial peptides, histones, and ribosomal proteins [3]. In recent years, the molecular mechanisms of mucosal immune responses in fish skin have become a hot topic in fish immunology research. Investigating the role of immune-related genes in fish skin can help elucidate the molecular regulatory mechanisms of skin immune responses and provide scientific references for disease prevention and treatment in fish.

Lactic acid bacteria can enhance animal immunity, improve gastrointestinal microbiota balance, and exhibit antioxidant, anti-tumor, anti-hypertensive, and cholesterol-lowering effects [4]. As an immunostimulant, lactic acid bacteria can enhance macrophage activity, promote the development of immune organs, improve phagocytic capacity, and induce interferon production, cell division, antibody generation, and cellular immunity through continuous low-level stimulation, thereby elevating both humoral and cellular immune levels [5]. However, reports on the effects of *Lactobacillus delbrueckii* on skin immunity and antioxidant function in fish remain limited. Therefore, this study used Yellow River carp as a model to investigate the effects of dietary supplementation with different levels of *L. delbrueckii* on immune and antioxidant indexes in skin, providing a theoretical basis for the application of *L. delbrueckii* in enhancing fish immunity.

1.1 Experimental Materials

Lactobacillus delbrueckii was purchased from Fluka Company in Switzerland with a viable count of 1.0$×10^{10}$ CFU/g. Yellow River carp were obtained from a fish farm in Luoyang City with an initial body weight of (15.0$±$0.5) g. The fish were acclimated for 2 weeks before the experiment and fed commercial carp feed (purchased from Luoyang Hefeng Feed Company) containing 33.6% crude protein and 6.62% crude fat three times daily during acclimation.

1.2 Experimental Diets

A commercial feed served as the basal diet, which used fish meal, soybean meal, rapeseed meal, and cottonseed meal as protein sources, and soybean oil and fish oil as lipid sources. The composition and nutrient levels are shown in Table 1 . Four experimental diets were prepared by supplementing the basal diet with $1\times10^{5}, 1\times10^{6}, 1\times10^{7}, and 1\times10^{8}$ CFU/g *L. delbrueckii*, respectively. All feed ingredients were ground to pass through a 60-mesh sieve and mixed stepwise. The *L. delbrueckii* was dissolved in an appropriate amount of water, thoroughly mixed with the feed ingredients, and processed into 2 mm diameter pellets using a meat grinder. The pellets were dried at 40 °C for 15 hours to achieve moisture content below 10% and stored at -4 °C until use.

Table 1 Composition and nutrient levels of the basal diets (air-dry basis)

Ingredients	Content
Fish meal	
Soybean meal	
Rapeseed meal	
Cottonseed meal	
Wheat middling	
Wheat bran	
$Ca(H_2PO_4)_2$	
Premix	
Soybean oil	
Bentonite	
NaCl	
Total	

Nutrient levels
Crude protein CP
Crude fat EE
Crude ash Ash

Supplied per kg of premix: $CuSO_4 \cdot 5H_2O$ 2.0 g, $FeSO_4 \cdot 7H_2O$ 25 g, $ZnSO_4 \cdot 7H_2O$ 22 g, $MnSO_4 \cdot 4H_2O$ 7 g, Na_2SeO_3 0.04 g, KI 0.026 g, $COCl_2 \cdot 6H_2O$ 0.1 g, VA 900,000 IU, VD 200,000 IU, VE 4,500 mg, VK_3 220 mg, VB_1 320

mg, VB_2 1,090 mg, VB_5 2,000 mg, VB_6 500 mg, VB_{12} 1.6 mg, VC 10,000 mg, choline chloride 60,000 mg, pantothenate 1,000 mg, folic acid 165 mg.

1.3 Feeding Management

After 2 weeks of acclimation, 450 healthy Yellow River carp of uniform size were randomly divided into five groups with three replicates per group and 30 fish per replicate. The control group was fed the basal diet, while the experimental groups were fed diets supplemented with $1\$\times10^{\{5\},1\times10}\{6\},1\times10^{\{7\},and1\times10}\{8\}\$$ CFU/g *L. delbrueckii*, respectively, for 8 weeks. Fish were fed three times daily at 07:00, 12:00, and 17:00 at a feeding rate of 4%–5% of body weight, adjusted according to actual feed intake. During the experimental period, water temperature was maintained at $(25.0\$\pm0.2)°C, dissolvedoxygenat(6.0\pm0.2)mg/L, pHat7.2\pm\$0.2$, and total ammonia nitrogen below 0.2 mg/mL.

1.4 Sample Collection and Analysis

1.4.1 Sample Collection At the end of the feeding trial, fish were fasted for 24 hours and anesthetized with MS-222 (100 mg/L). Skin mucus was gently scraped with a clean glass slide, and 5 mL was collected and stored at -20 °C for subsequent analysis. Skin tissue was then excised, frozen in liquid nitrogen for 1 hour, and stored at -80 °C until analysis.

1.4.2 Determination of Immune and Antioxidant Indexes in Skin Mucus The activities of lysozyme (LYZ), acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and the contents of complement 3 (C3), complement 4 (C4), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) in skin mucus were determined using assay kits from Nanjing Jiancheng Bioengineering Institute following the manufacturer' s instructions.

1.4.3 Determination of Antimicrobial Peptide Gene Expression in Skin Approximately 0.1 g of skin tissue was used for total RNA extraction according to the Trizol protocol. RNA concentration and quality were measured, with OD_{260}/OD_{280} ratios of 1.8–2.0 indicating good quality. RNA samples were diluted to the same concentration for cDNA synthesis. cDNA was synthesized using a kit from Dalian Bao Biological Company with the following program: 42 °C for 40 min, 90 °C for 2 min, and storage at 4 °C. The cDNA was then diluted 10-fold for real-time quantitative PCR.

Primers were designed using Primer5 software and synthesized by Shanghai Yingjie Company (Table 2). Real-time quantitative PCR was performed using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa) under the following conditions: 94 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 55.9 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. β-actin served

as the internal reference gene, and the relative expression levels of target genes Leap-1 and Leap-2 were calculated using the $2^{-}\Delta\Delta$Ct method.

Table 2 Primer information

Genes	Primer sequence (5´-3´)	GenBank accession No.
Leap-1	F: CAGTACATCGTCTCT-GTCGTCR: CAGTAGGCGCCATGAG-GTTT	KC551971.1
Leap-2	F: ACAGTACATCGTCTCT-GTCGTCR: CGAGAAGAGCC-CGTTTGTGA	XM_{019099090}.1
β-actin	F: TTTG-GCGCTTGACTCAGGATR: AGGC-CATAAGGGAAGGGACA	M24113.1

1.5 Statistical Analysis

Raw data were initially processed using Excel 2010 and then subjected to one-way ANOVA using SPSS 17.0 software. When significant differences were detected among groups, Duncan' s multiple range test was used for mean comparison. Data are expressed as mean ± standard error, with P<0.05 considered statistically significant.

Results

2.1 Effects of *L. delbrueckii* on Immune Indexes in Skin Mucus

As shown in Table 3 , all experimental groups exhibited varying degrees of elevation in immune indexes compared with the control group. Specifically, the activities of LYZ and AKP and the contents of C3 and C4 in skin mucus of the 1$×10^{6}$ and 1$×10^{7}$ CFU/g groups were significantly higher than those of the control group (P<0.05), while no significant differences were observed in these parameters between the 1$×10^{5}$ or 1$×10^{8}$ CFU/g groups and the control group (P>0.05). Acid phosphatase (ACP) activity in skin mucus did not differ significantly among all groups (P>0.05).

Table 3 Effects of *Lactobacillus delbrueckii* on skin mucus immune indexes of Yellow River carp

L. delbrueckii addition (CFU/g)	LYZ (U/mL)	C3 (mg/mL)	C4 (mg/mL)	ACP (U/mL)	AKP (U/mL)
0 (Control)	65.79$±3.37	75	10	23.09	

Values in the same column with different letter superscripts were significantly different (P<0.05). The same as below.

2.2 Effects of *L. delbrueckii* on Antioxidant Indexes in Skin Mucus

As shown in Table 4 , SOD activity in skin mucus of the 1$×10^{6}$ and 1$×10^{7}$ CFU/g groups was significantly higher than that of the control group (P<0.05), with no significant differences among other groups (P>0.05). CAT and GPx activities in skin mucus of the 1$×10^{6}$ CFU/g group were significantly higher than those of the control group (P<0.05), while other experimental groups did not differ significantly from the control (P>0.05). Total antioxidant capacity (T-AOC) in skin mucus of the $1\$\times10^{\{5\},1\times10}\{6\},and1\times10\hat{}\{7\}\$$ CFU/g groups was significantly higher than that of the control group (P<0.05), with no significant differences in other groups (P>0.05). Malondialdehyde (MDA) content in skin mucus of the 1$×10^{6}$ and 1$×10^{7}$ CFU/g groups was significantly lower than that of the control group (P<0.05), while other groups showed no significant differences (P>0.05).

Table 4 Effects of *Lactobacillus delbrueckii* on skin mucus antioxidant indexes of Yellow River carp

L. delbrueckii addition (CFU/g)	SOD (U/mL)	CAT (U/mL)	GPx (U/mL)	T-AOC (U/mL)	MDA (nmol/mL)
0 (Control)	22.55$±0.52				

2.3 Effects of *L. delbrueckii* on Gene Expression in Skin

As shown in Figure 1 [Figure 1: see original paper], the relative expression levels of antimicrobial peptide genes Leap-1 and Leap-2 in skin were significantly higher in all experimental groups compared with the control group (P<0.05), reaching the highest values in the 1$×10^{7}$ CFU/g group.

Figure 1 Effects of *Lactobacillus delbrueckii* on relative expression levels of Leap-1 (A) and Leap-2 (B) genes in skin of Yellow River carp

Discussion

3.1 Effects of *L. delbrueckii* on Immune Indexes in Skin Mucus

Fish skin contains numerous mucous cells, macrophages, and antibody-secreting cells that, together with other active substances, form an effective defense line against pathogenic microorganisms [6]. The present results showed that dietary supplementation with different levels of *L. delbrueckii* increased the activities of LYZ and ACP and the contents of C3 and C4 in skin mucus of Yellow River carp to varying degrees, demonstrating enhanced immune function. Lysozyme is an important immune factor primarily secreted by immune cells, and fish skin mucus contains various enzymes and immune factors that play crucial roles in immune responses. As a probiotic, lactic acid bacteria can promote the proliferation of beneficial bacteria while inhibiting harmful bacteria. The cell walls of beneficial bacteria such as lactic acid bacteria and bifidobacteria contain lipoteichoic acid (LTA) and peptidoglycan-teichoic acid complexes with strong immunoadjuvant activity that can enhance fish immunity [7]. Pan et al. [8] reported that lactic acid bacteria can improve immune recognition in aquatic animals, induce T and B lymphocytes and macrophages to produce cytokines, and activate the systemic immune system through lymphocyte recirculation, thereby enhancing both non-specific and specific immune functions. Previous studies have reported that probiotics can increase LYZ activity in fish blood [9-10], and complement components in skin play important roles in skin immune responses [11]. Specifically, C3 and C4 are crucial for resisting pathogen invasion, with C3 being a key factor in all complement activation pathways [12-13]. While studies on *L. delbrueckii* increasing C3 and C4 contents have been reported in Yellow River carp blood, no such studies have been conducted on skin. The immune-enhancing effects of C3 and C4 may be related to their activation of key genes at critical sites in immune pathways, though the specific molecular mechanisms require further investigation.

3.2 Effects of *L. delbrueckii* on Antioxidant Indexes in Skin Mucus

SOD and CAT are primary antioxidant enzymes; SOD converts superoxide radicals ($O_2^{-}\cdot$) into oxygen (O_2) and hydrogen peroxide (H_2O_2), while CAT decomposes H_2O_2 into O_2 and water (H_2O) [14] to eliminate excess free radicals. Total antioxidant capacity (T-AOC) is a comprehensive indicator reflecting overall antioxidant function. The present results showed that SOD, CAT, GPx activities and T-AOC in skin mucus of Yellow River carp increased initially and then decreased with increasing *L. delbrueckii* supplementation, with the highest values observed in the 1$\times$10^{6}$ CFU/g group. The antioxidant function of *L. delbrueckii* may be related to its beneficial bacterial protective effects. Early studies demonstrated that metabolites of lactic acid bacteria possess antioxidant functions [15-17], and lactic acid bacteria may also promote antioxidant capacity by regulating intestinal microbial balance. Previous research reported that bacteriocins from lactic acid bacteria improved antioxidant function in broilers [16]. These findings indicate that dietary supplementation with appropriate lev-

els of *L. delbrueckii* can enhance the antioxidant capacity of Yellow River carp to a certain extent.

3.3 Effects of *L. delbrueckii* on Antimicrobial Peptide Gene Expression in Skin

Antimicrobial peptide genes are widely present in fish and constitute an important component of the innate immune system, playing crucial roles in defending against pathogenic microorganisms [18]. In this study, dietary supplementation with *L. delbrueckii* significantly up-regulated the relative expression levels of antimicrobial peptide genes Leap-1 and Leap-2 in skin compared with the control group, indicating that *L. delbrueckii* enhanced the immunity and disease resistance of Yellow River carp. This effect may be attributed to the increased activity of immune enzymes in skin mucus, which play important roles in immune responses. Additionally, immunoglobulins and cytokines can serve as key regulators of immunity, and short-chain fatty acids produced by lactic acid bacteria can promote the proliferation of beneficial bacteria such as lactic acid bacteria in the intestine while inhibiting harmful bacteria, which is closely related to immune regulation. Similar studies on lactic acid bacteria enhancing fish immunity have been reported [19-20], which also concluded that excessively high concentrations of *L. delbrueckii* did not produce optimal effects, suggesting that appropriate dosage is critical for its efficacy.

Conclusion

Dietary supplementation with appropriate levels of *Lactobacillus delbrueckii* can enhance skin immune function and antioxidant capacity and up-regulate antimicrobial peptide gene expression in Yellow River carp. Under the conditions of this study, the optimal dietary supplementation level of *L. delbrueckii* for Yellow River carp was 1$×10^{6}$ to 1$×10^{7}$ CFU/g.

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Note: Figure translations are in progress. See original paper for figures.