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Morphologic, digestive enzymes and immunological responses of intestine from *Litopenaeus vannamei* after lipopolysaccharide injection

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ABSTRACT

The shrimp intestine barrier serves as the first line of the host defense against pathogen infection. Lipopolysaccharide (LPS) is the cell wall component of gram-negative bacteria, which known as endotoxin and induce the intestine inflammation. In this study, the acute toxicity effects of LPS injection on the morphology, digestive enzymes and immunological responses of intestine from *Litopenaeus vannamei* was investigated. HE stain showed that LPS injection damaged the intestine connective and epithelium tissue. Specifically, a decrease in the activities of digestive enzymes including of amylase, lipase, trypsin and pepsin was observed. Moreover, LPS injection increased the content of oxidative stress parameters ($^{\cdot}O_2^-$ generation capacity, LPO, MDA and PC), and the expression of *HIF-1α* gene. Alternatively, the antibacterial activities (PO and T-NOS), and the expression of the antibacterial genes (*proPO*, *ALF*, *Toll* and *Imd*) and pathogen pattern recognition genes (*LGBP* and *Lec*) increased at first and then decreased. Significant variations in anitioxidant enzyme activity of T-AOC, and the expression of the anitioxidant-related genes (*SOD*, *HSP70* and *Trx*). These results revealed that LPS injection harmed the intestine barrier of *L. vannamei* by damaging intestine mucosal structure, increasing oxidative stress, and suppressing the digestive and immune status.

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is a species important to the global economy (Duan et al., 2017). Shrimp aquaculture can suffer economic losses caused by diseases, for which effective control measures are still lacking (Flegel, 2012). Disease results from a complex interaction of the host, environment, and pathogen, and gram-negative bacteria particularly *Vibrio* species, is a crucial inducing factor (Joshi et al., 2014). The shrimp intestine is an important organ for nutrient uptake and immunity, which continuously exposed to foreign substances especially the pathogen rich aquatic environment, the immunity of the organ serves as the first line of host defense against pathogen infection (Rungrassamee et al., 2016). The shrimp intestine harbors high amount of microbiota including of some opportunistic bacteria, which can increase the risk of pathogen invasion, and produce lipopolysaccharide (LPS) to disorder the immunity of the host (Suo et al., 2017).

LPS is an integral component of the cell wall of Gram-negative bacteria, and play important roles in pathogenesis, drug resistance, and protecting bacteria from harsh environments (Dong et al., 2017). LPS is known as endotoxin that have highly antigenic and cytotoxic (Xian et al., 2009), which can induce intestine inflammation, and is commonly used to build the model of the intestine injury (Yi et al., 2017). Additionally, LPS can induce the immune response through several signaling pathways. For example, LPS increased inflammatory responses via the TLR4-NF-κB signaling pathway, induced autophagy by targeting the AMPK-mTOR pathway in epithelial cells (Yan et al., 2017; Wang et al., 2017).

In shrimp, LPS is used as an potent stimulants of the shrimp immunity, and it can improve the survival of shrimp after *Vibrio* exposure (Rungrassamee et al., 2013). However, a high dose of LPS could kill shrimp in a dose dependent manner (Lorenzon et al., 1999, 2002). LPS could be recognized as non-self molecules by LPS and β-1,3-glucan binding protein (LGBP), activated proPO system, induced oxidative stress, and cause cell apoptosis on shrimp hemocytes (Xian et al., 2013, 2016). However, a clear understanding of the effects of LPS on the intestine barrier of *L. vannamei* is still lacking.

Considering the importance of intestine immune in resisting the pathogen infection, we investigated the effects of LPS injection on the intestine morphology, digestive capacity, oxidative stress responses, and immune indicators in *L. vannamei*. These results will provide new insight on the mechanisms of intestine immune of *L. vannamei* to LPS

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injection.

2. Materials and methods

2.1. Shrimp and culture conditions

Healthy juvenile *L. vannamei*, averaging weight 5.4 ± 0.3 g, were randomly collected from a local hatchery and reared in a semi-intensive culture pond in Shenzhen, China. Shrimp were cultured in filtered aerated seawater (salinity 30‰, pH 8.2, dissolved oxygen 6.0 ± 0.5 mg/L) at 30 °C for one week before starting the experiment, and fed daily with a ratio of 5% of body weight using formulated pellet feed (Haida Feed, Jieyang, China).

2.2. LPS injection and sampling

LPS (from *Escherichia coli* 055:B5) was obtained from Sigmae-Aldrich (USA), and dissolved with the sterile 0.9% saline solution to the concentration of 2 mg/mL. After acclimation, the shrimp were examined health status. Briefly, the pathogenic bacteria of shrimp such as *Vibrio* spp. was detected using the plate count method with *Vibrio* spp. chromogenic medium per the method of Zhang et al. (2016), and the shrimp with no signs of infection. Then, the experimental shrimp were divided into two groups: the control group, and the LPS injection group, with three replicate tanks per group. Shrimp were housed at 50 shrimp per tank and the tanks contained 500 L of filtered, aerated seawater. For the LPS injection group, the shrimp was injected individually with LPS solution prepared above at the second abdominal segment of each shrimp, resulting in 8 μg/g per shrimp (wet weight), per the method of Xian et al. (2016). For the control group, the shrimp was injected individually with the equal amount of sterile 0.9% saline solution at the same body position as the LPS injection group. The whole intestine of three shrimp from each replicate tank were collected individually at 0, 6, 12, 24, 48, and 72 h after injection, and snap frozen in liquid nitrogen for biochemical assays and gene expression analysis.

2.3. Histological analysis

Intestine of nine shrimp from each group were randomly sampled at 72 h post-injection, respectively, and stored in Davidson's solution for 24 h. After rinsed with flow water 8 h, the tissues were dehydrated in series of ethanol (70%, 80%, 90% and 100%), transparented with xylene, embedded in paraffin and cut in a microtome (Leica, RM2016, Germany) at 4 μm thickness. After hematoxylin and eosin (HE), stained sections were observed and photographed under the light microscopy (Olympus, Japan).

2.4. Biochemical analysis

Intestine of nine shrimp from each group were homogenized by adding sterile 0.9% saline solution to prepare 10% (*w*:*v*) homogenates, then centrifuged at 3500 rpm for 10 min at 4 °C. After removing precipitates, supernatants were immediately used for biochemical parameters analyze with a microplate reader (Bio-Rad, USA). Total protein concentration in tissue homogenates was measured by Coomassie brilliant blue protein assay kit (Jiancheng, Ltd., Nanjing, China). Assays were all run in three replicate samples.

Digestive enzymes such as amylase (AMS), lipase (Lip), trypsin (Tryp) and pepsin (Pep) activity were determined with commercial test kits respectively (Jiancheng, Ltd., Nanjing, China). AMS activity was detected spectrophotometrically based on starch as substrate at 660 nm, and one unit of which was defined as the amount of enzyme per mg tissue protein every 30 min hydrolyzes 10 mg starch at 37 °C. Lip activity was detected spectrophotometrically at 420 nm, and one unit of which was defined as the amount of enzyme per g tissue protein every minute catalyzes 1 mmol substrate at 37 °C. Tryp activity was detected spectrophotometrically at 253 nm, and one unit of which was defined as the amount of enzyme per mg tissue protein every minute make the absorbance change 0.003 at 37 °C under the pH 8.0 assay condition. Pep activity was detected spectrophotometrically at 660 nm, and one unit of which was defined as the amounts of enzyme per mg tissue protein every minute generate 1 mg amino acid by hydrolyzing protein at 37 °C.

Oxidative stress parameters such as reactive oxygen species (ROS) production ($^{\cdot}O_2^-$ generation capacity), lipid peroxidation (LPO), malondialdehyde (MDA), and protein carbonyl (PC) content were determined with commercial test kits respectively (Jiancheng, Ltd., Nanjing, China). $^{\cdot}O_2^-$ generation capacity was measured spectrophotometrically using xanthine/xanthine oxidase as the $^{\cdot}O_2^-$ generator and the product were read spectrophotometrically at 550 nm. LPO and MDA content was measured spectrophotometrically with thiobarbituric acid (TBA) method and the product were read spectrophotometrically at 586 nm and 532 nm respectively. PC content was measured spectrophotometrically at 370 nm, and expressed as nmol/mgprot.

Immune biochemical parameters such as total antioxidant capacity (T-AOC), phenoloxidase (PO) and total nitric oxide synthase (T-NOS) activity, and nitric oxide (NO) content were determined with

Table 1
Primer sequences used in this study.

Primer name	Sequence (5′–3′)	GenBank accession number
proPO-F	CAATGACCAGCAGCGTCTTC	AY723296
proPO-R	CACGGAAGGAGGCGTATCAT	
ALF-F	GGTGTTCCTGGTGGCACTCT	GQ227486
ALF-R	AGCTCCGTCTCCTCGTTCCT	
Toll-F	TGGTCCTCAGCCTTGGAGAT	DQ923424
Toll-R	CTCCATCACTGGCGCACTTA	
Imd-F	GCGAGATCGAGGAACGAGAC	FJ592176
Imd-R	CGCGTGTGGTCAGCTATCAT	
LGBP-F	GGCAACCAGTACGGAGGAAC	KF911077
LGBP-R	AATCATCGGCGAAGGAGTCT	
Lec-F	GCAGCAACCTGATAATGCACA	DQ871245
Lec-R	TCGGATGGTGGCTTCACATA	
HSP70-F	CAACGATTCTCAGCGTCAGG	AY645906
HSP70-R	ACCTTCTTGTCGAGGCCGTA	
Trx-F	TTCCTGAAGGTGGATGTGGA	EU499301
Trx-R	AGTTGGCACCAGACAAGCTG	
SOD-F	CCGTGCAGATTACGTGAAGG	DQ005531
SOD-R	GTCGCCACGAGAAGTCAATG	
HIF-1α-F	GGAGTCTTTGAGAGAGAG	FJ807918
HIF-1α-R	GCCTCCTTCCGTGATCTTC	
β-actin-F	GCCCTGTTCCAGCCCTCATT	AF300705
β-actin-R	ACGGATGTCCACGTCGCACT	

proPO, prophenoloxidase; *ALF*, anti-lipopolysaccharide factor AV-K; *Imd*, immune deficiency; *LGBP*, lipopolysaccharide and β-1,3-glucan binding protein; *Lec*, C-type lectin; *HSP70*, heat shock protein 70; *Trx*, thioredoxin 1; *SOD*, cytosolic Mn superoxide dismutase; *HIF-1α*, hypoxia inducible factor 1 alpha.

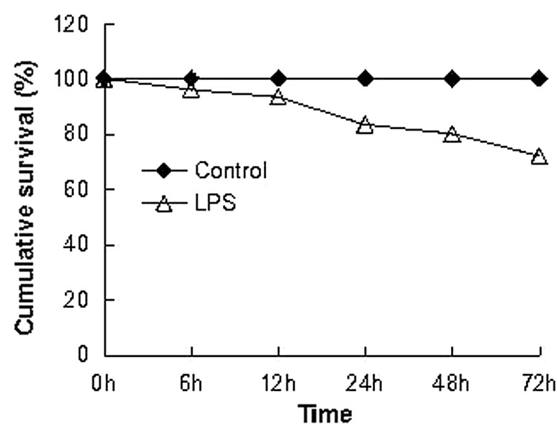

Fig. 1. Cumulative survival of *L. vannamei* at different time intervals after LPS injection. Vertical bars represented the mean ± SE (*N* = 3).

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