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Ability of *Lactobacillus plantarum* lipoteichoic acid to inhibit *Vibrio anguillarum*-induced inflammation and apoptosis in silvery pomfret (*Pampus argenteus*) intestinal epithelial cells

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ABSTRACT

Lipoteichoic acid (LTA) is a major constituent of the cell wall of Gram-positive bacteria. The structure and immunomodulation of LTA vary greatly between different species. LTA from *Lactobacillus plantarum* has been shown to exert anti-pathogenic effects. *Vibrio anguillarum* is a major causative agent of vibriosis, one of the most prevalent fish diseases. The purpose of this study was to examine the effects of *L. plantarum* LTA on *V. anguillarum* growth, adhesion, and induced inflammation and apoptosis in intestinal epithelial cells of silvery pomfret (*Pampus argenteus*). Our results showed that *L. plantarum* LTA was unable to inhibit *V. anguillarum* growth; however, it significantly inhibited adhesion of *V. anguillarum*. It also showed significant inhibitory effects on EHEC-induced inflammation and apoptosis by modulating the expression of NF-κB (nuclear factor kappa B), IκB (inhibitor of NF-κB), Bcl2 (B-cell leukemia/lymphoma-2), BAX (Bcl-2-associated X protein), IL-8 (interleukin 8) and TNF-α (tumor necrosis factor-α), and via inhibition of caspase-9 and caspase-3 activation. These data extend our understanding of the beneficial effects of *L. plantarum* LTA, which is related to the inhibition of *V. anguillarum*, and suggest that *L. plantarum* LTA has potential as a new therapeutic agent against *V. anguillarum*-caused vibriosis in fish.

1. Introduction

Probiotics are beneficial microbes that, when administered in adequate amounts, provide health benefits to the host. The health benefits of probiotics include maintaining intestinal epithelial-microbial homeostasis, treating diarrhea-predominant irritable bowel syndrome and helping the organism to assimilate and absorb nutrients from foods and supplements [1]. Recently, probiotics have been successfully employed in promoting growth of fish and in regulating immunological responses. *Lactobacillus plantarum* is a probiotic bacterial species, commonly found in marine environments as well as in intestinal tracts of marine animals. In aquaculture, administration of *L. plantarum* has been shown to promote digestion, increase growth performance, enhance immune response and prevent microflora disturbances [2–4]. However, the mechanisms employed by *L. plantarum* for a healthy intestine need to be better understood to develop more efficient probiotics.

Recent studies demonstrated that components of the cell wall of *L. plantarum*, such as surface-bound proteins, peptidoglycans and lipoteichoic acid (LTA), play important roles in the prevention and treatment of intestinal inflammatory diseases [5–7]. Among these bacterial cell wall components, the mechanisms of beneficial effects exerted by LTA are well known. An increasing body of evidence indicates that *L. plantarum* LTA reduces pro-inflammatory responses, thereby attenuating intestinal inflammation and disease [8]. For example, LTA isolated from *L. plantarum* has been shown to regulate innate and adaptive immunity, protect against inflammation and promote animal intestinal health by blocking TNF-α (tumor necrosis factor-α), IL-8 (interleukin 8) and IL-12 production induced by pathogens and, instead, to increase the

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expression of anti-inflammatory cytokines [9–11]. Therefore, it is reasonable to hypothesize that *L. plantarum* LTA has promising potential as a new therapeutic agent against pathogen-induced intestinal disorders.

Vibrio anguillarum, an important pathogen of cultured fish widely distributed across the world, causes a disease known as vibriosis, a lethal hemorrhagic septicemia affecting marine and estuarine fish species, and resulting in great economic losses [12]. As *L. plantarum* LTA has gained increasing medical importance in preventing pathogen-induced inflammation, the positive effects of *L. plantarum* LTA on the intestinal epithelium in response to *V. anguillarum* were investigated, which could support our attempts to characterize the beneficial properties of this compound further. A study was designed for this purpose, employing fish intestinal epithelial cells (FIECs) isolated from silvery pomfret (*Pampus argenteus*) as an *in vitro* model.

2. Materials and methods

2.1. Bacteria strains and culture conditions

L. plantarum subsp. Plantarum (CICC6257) used in this study was obtained from the China Center of Industrial Culture Collection (CCICC) and cultured in MRS broth at 37 °C. *V. anguillarum* (CCTCCM203069) was purchased from the China Center for Type Culture Collection (CCTCC) and cultured in Bacto marine broth at 28 °C.

2.2. Purification of LTA from L. plantarum

Highly purified LTA was isolated from *L. plantarum* by *n*-butanol according to a previously described method [13]. The purity of LTA was determined by measuring protein and endotoxin contents using the conventional silver staining after polyacrylamide gel electrophoresis and *Limulus amebocyte lysate* (LAL), respectively. DNA and RNA contaminations were assessed through measuring UV absorptions at 260 and 280 nm, respectively. The integrity of the LTA structure was confirmed through nuclear magnetic resonance (NMR) spectrometry and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

2.3. Isolation and culture of primary FIECs

Apparently healthy silvery pomfret were used for this study. FIECs were prepared and cultured, as previous described [14]. The intestinal tissues were dissected and washed three times with phosphate buffered saline (PBS) containing gentamicin, penicillin and streptomycin, and then treated with 0.1% sodium hypochlorite for 1 min in an ice bath. The tissues were washed with PBS and incubated for 1 min in 75% ethanol. The tissue preparations were cut into 0.1 × 0.1 mm pieces and incubated with 0.25% trypsin solution and continuously stirred at 28 °C for 30 min. The released cells were cultured in DMEM media supplemented with 10% fetal calf serum at 28 °C in a humidified atmosphere of 5% CO_2 for about 48 h.

2.4. Antimicrobial activity

The inhibitory effect of LTA on *V. anguillarum* was carried out by agar plate diffusion test, as previously described [15]. Two wells per dish were created using a 14-mm-diameter gel punch. A total volume of 450 μl (3 × 150 μl) from LTA or MRS broth control (30 μg/ml) was added to the respective well. To accelerate the diffusion, the dishes were incubated at 50 °C after each addition of 150 μl. From the stationary growth phase of *V. anguillarum*, 500 μl of 1×10^5 CFU/ml was added to 5 ml MRS broth (45 °C) containing 0.8% agar. The agar was rapidly dispersed and poured into the dishes, which were then incubated overnight before assessment of the diameters of the inhibition zones.

2.5. Adhesion inhibition assay

An adhesion inhibition assay was performed according to a previously described method [15]. Three different procedures were used in order to differentiate exclusion, competition or displacement of *V. anguillarum* by LTA. *V. anguillarum* was collected and re-suspended in DMEM media for 10^8 CFU/ml. For exclusion tests, intestinal cell monolayers were cultured, washed three times with PBS solution and incubated with LTA for 30 min. Then, *V. anguillarum* was added and incubated for another 30 min. For the competition test, LTA, *V. anguillarum* and intestinal cells were mixed and incubated for 1 h. For displacement test, *V. anguillarum* and the intestinal cells were incubated together for 30 min. After removal of non-adherent *V. anguillarum*, LTA was added and incubated for a further 30 min. After incubation, all epithelial cells were washed three times with PBS solution, fixed in PBS containing 4% (w/v) paraformaldehyde and observed microscopically following Gram staining. For each well, 50 cells with *V. anguillarum* were inspected to assess the number of *V. anguillarum* attached to cells. Each assay was conducted at least three times.

2.6. Stimulation of cells

The FIECs were allowed to attach and grow in 6-well tissue culture plates (Costar) for 48 h. Before the stimulation assays, the bacteria were collected and re-suspended in antibiotic-free DMEM media at a density of 1×10^8 CFU/ml. Then, FIECs were co-incubated with DMEM media, LTA, *V. anguillarum*, and the mixture of LTA and *V. anguillarum* in 5% CO_2 at 28°C for 2 h. After incubation, the culture media and cells were collected for ELISA analysis, western blot analysis, caspase activity assays, and assessment of apoptotic and necrotic cells [16].

2.7. Assessment of apoptotic and necrotic cells

Apoptosis and necrosis of FIECs were assessed with an Annexin V-FITC apoptosis detection kit (BD, USA). The cells were stained with Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) for analysis by flow cytometry (FCM). FITC and PI fluorescence were measured at 530 nm and 585 nm emission, respectively. A positioning of quadrants on Annexin V/PI dot plots was performed. Live cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+) and necrotic cells (Annexin V^-/PI^+) were distinguished. Therefore, the total apoptotic proportion included the percentage of cells with fluorescence Annexin V^+/PI^- and Annexin V^+/PI^+ [15].

2.8. Western blot analysis

The expression of NF-κB (nuclear factor kappa B; p65), IκB, BAX (Bcl-2-associated X protein) and Bcl2 (B-cell leukemia/lymphoma-2) were analyzed by western blot according to a previous method [16]. The total cellular and nuclear proteins were extracted. The nuclear extracts were used to determine NF-κB protein levels and the cytoplasmic extracts were used to determine IκB, BAX and Bcl2 protein levels. The total protein content was determined using the BCA protein assay. Fifty micrograms of total protein from each sample were subjected to SDS-PAGE. Then, the proteins were electroblotted onto Hybond-CExtra nitrocellulose membrane (Amersham, USA). After blocking at room temperature with 5%