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Cytokine mediated immune responses in the Japanese pufferfish (*Takifugu rubripes*) administered with heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22) isolated from the Mongolian dairy product

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ABSTRACT

The important role played by cytokines in host innate immunity and the interaction of subsets of immune and inflammatory cells through cytokines offer avenues for immune interventions. We investigated 16 cytokine gene responses in the Japanese pufferfish, *Takifugu rubripes* orally treated with a heat-killed lactic acid bacterium (LAB), *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) (Lpp) isolated from a Mongolian dairy product at 1 mg g^{-1} body weight d^{-1} for 3 days. Additionally, we assessed superoxide anion production (SAP) and phagocytic activity (PA) of head kidney cells and resistance to *Vibrio harveyi* infection in treated fish. Significant up-regulation of pro-inflammatory (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N), cell-mediated immunity inducing (IL-12p35, IL-12p40 and IL-18), antiviral/intra-cellular pathogen killing (I-IFN-1 and IFN-γ), anti-inflammatory (IL-10) and peripheral T cell expansion and survival controlling (IL-2, IL-7, IL-15, IL-21 and TGF-β1) cytokines was observed in the treated fish. Furthermore, significantly increased SAP, PA and pathogen resistance were observed in the treated fish compared to untreated fish. Our results indicate the enhancement of cytokine mediated immunity in *T. rubripes* by the use of the heat-killed Lpp as a potential immunostimulant and would be of great use in immunomodulation trials with the possibility to monitor positive immune response.

1. Introduction

Cytokines, the low molecular weight protein mediators are often glycosylated and produced by activated immune cells in response to invasion by parasitic, bacterial or viral pathogenic components [1]. They act through their corresponding receptors in an autocrine or paracrine fashion with pro-inflammatory, anti-inflammatory, cell-mediated immune inducing, lymphocyte agonistic and pathogen-killing properties. Cytokines originate from macrophages, lymphocytes, granulocytes, dendritic cells (DCs), mast cells, and epithelial cells and include interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factor (TGF), interferons (IFNs) and chemokines [2]. They play a major role in host innate immunity and are indispensable for recruitment and activation of macrophage, neutrophil, and lymphocyte to the infection sites for pathogen elimination [3]. Aquaculture with 63.6 million ton production and 8.8% annual growth rate [4] is the only option available to meet up increasing fish demand due to population explosion, as the contribution from marine capture is almost static. Increasing infectious disease occurrences with rapid intensification and expansion of aquaculture have also led to considerable economic loss [5]. Treatment of diseases using chemotherapeutics and antibiotics at farm level is either infeasible or prohibited and it is necessary to search for alternative preventive measures that are ecofriendly and unhazardous to human health. Since innate immunity as a first line of defense, plays a major role in rendering protection against pathogens in fish, the use of bio-products as immunostimulants would provide substantial resistance through its elicitation [6]. Therefore, evaluation of cytokine response can be a valid method to assess efficacy of a novel immunostimulant.

During the last two decades, the probiotic role of lactic acid bacteria (LAB) and their metabolic products has been evaluated to improve immune status and disease resistance in higher animals [7–9] as well as in fish [10–13]. Several *in vitro* and *in vivo* studies demonstrated elevated expression of cytokine genes such as IL-1β, IL-8, IL-10, TNF-α and TGF-β caused by live or killed probiotics in fish [11,12,14,15]. However, information on IFN-mediated antiviral immunity and cell-mediated

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immune inducing cytokines, IL-12 and IL-18 to stimulate IFN-γ production by natural killer (NK) cell and Th1 cells in response to probiotic treatment in fish is scantily available. Most of the previous studies targeted a single or few cytokine gene analyses. Since cytokine system is complex and involves several genes functioning in a cascading manner, simultaneous analysis of different cytokine family members in fish would elucidate a useful and more reliable understanding on the innate as well as cell-mediated immune systems. Probiotic research, more specifically the use of LAB probiotics as immune enhancers has not been tested in the Japanese pufferfish (*Takifugu rubripes*) *in vivo* compared with extensive studies involving other fish species [10–13]. Traditionally, Mongolian nomadic people have been consuming several dairy products processed from milk using natural tools and ingredients. These dairy products are prepared using different types of milk from domestic animals such as cows, sheep, goats, yaks, horses and camels. Recently, 10 LAB strains have been isolated from these traditional dairy products and oral administration of these heat-killed LAB strains exhibited immunomodulatory activity in influenza virus (IFV) infected mice [9]. Moreover, in an *in vitro* study, modulation of cytokine defense mechanisms in the pufferfish head kidney (HK) cells by two heat-killed probiotics suggested better efficacy of *Lactobacillus paracasei* spp. *paracasei* strain as immunostimulant [16]. Based on these findings, we hypothesized that IFN and other cytokine mediated immunity may be positively influenced by this heat-inactivated LAB strain in fish. Therefore, to validate this hypothesis, our *in vivo* study aimed at examining the expression of 16 functionally different cytokine genes in the Japanese pufferfish orally administered with this heat-killed probiotic strain. Additionally, to confirm the functionality of cytokines induced by this immunostimulant, we also assessed superoxide anion production, phagocytic activity using flow cytometry and burden of infected *Vibrio harveyi* in the orally administered pufferfish.

2. Materials and methods

2.1. LAB strain and its preparation

A LAB strain, *L. paracasei* spp. *paracasei* (strain 06TCa22) (Lpp) isolated and identified previously from the Mongolian fermented camel milk (Tarag) was cultured in Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h [17]. The bacterium was harvested by centrifugation at 10,000 ×*g* for 5 min, washed twice with phosphate-buffered saline (PBS) and boiled for 1 h. Then, the boiled bacterium was washed again with PBS and lyophilized. The lyophilized Lpp powder containing 1.1×10^{11} cells g^{-1} was suspended in sterilized distilled water (SDW) for oral administration to fish.

2.2. Experimental fish

Japanese pufferfish, *T. rubripes* (body weight, 50.4 ± 2.2 g) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan. Fish were first acclimatized in an aerated seawater tank at 22 ± 2 °C and fed a commercial diet (Sango, Higashimaru Co. Ltd., Kagoshima, Japan) at 1% body weight daily for two weeks under a natural photoperiod prior to their use in the study. The health status of experimental fish was checked following the method described earlier [16]. All animal experiments were conducted according to the relevant national and international guidelines, 'Act on Welfare and Management of Animals' (Ministry of the Environment, Japan). Ethics approval from the local IACUC was not sought since this law does not mandate protection of fish.

2.3. Immunostimulant dose finding experiment

2.3.1. Oral administration of immunostimulant

Lyophilized Lpp powder was suspended in SDW at a concentration of 20, 10 and 5% (w/v). Three groups of fish (n = 5) were orally administered with 0.5 mL of the suspensions to receive the Lpp doses at 0.5, 1 and 2 mg g^{-1} body weight (BW) once a day for 3 days and the fish of a control group (n = 5) received same volume of SDW. The fish in all the groups were intubated using a disposable feeding needle (1.2Φ × 75 mm; Fuchigami, Kyoto, Japan).

2.3.2. RNA extraction from HK tissue and multiplex RT-PCR assay

At 24 h post treatment, individual fish was scooped out of holding tank and anesthetized with 2-phenoxyethanol (0.05%, Sigma–Aldrich, St. Louis, MO, USA) in a bucket containing aerated seawater before being sacrificed for tissue collection. HK tissue was aseptically excised from freshly euthanized pufferfish (n = 5) from all the groups and submerged immediately in RNAlater solution (Ambion, Austin, TX, USA) for overnight and finally stored at −80 °C until use. Total RNA was extracted from the stored HK tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. To avoid the presence of genomic DNA, RNA samples were treated with recombinant DNase I (RNase-free) as per the manufacturer's protocol (Takara Bio Inc., Shiga, Japan). Quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA).

In this study, a multiplex reverse-transcription-polymerase chain reaction (RT-PCR) assay (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter, Inc., Brea, CA, USA) was used to analyze the expression of 16 cytokine genes simultaneously from a single reaction tube. Primer design (16-cytokine plex) and multiplex analysis were conducted using the multiplex assay panel established previously [16,18]. RT and PCR were performed using 16 cytokine universal primers (Supplementary Table 1). The PCR products from multiplex RT-PCR were prepared and run in the GeXP Genetic Analysis System for capillary electrophoresis and fragment size analysis as per the described protocol [16]. The data were normalized to the external synthetic reference control transcript, kanamycin resistance gene using GeXP profiler (eXpress Analysis) software, with the area-under-the-curve set to 1. Although we added primers of two housekeeping reference genes, β-actin and GAPDH in the multiplex RT-PCR assay (Supplementary Table 1), more consistent, uniform and unaffected expression levels of β-actin were obtained in all the samples than that of GAPDH. Therefore, the relative expression level of each cytokine gene was calculated by normalization to β-actin using GeXP Quant Tool.

2.3.3. Determination of dose based on cytokine gene expression

We examined 16 cytokine gene expressions in HK tissue of pufferfish treated with three doses of heat-killed Lpp at 24 h post administration and found significantly higher expression level ($P < 0.05$) of most of the genes at the doses of 1 and 2 mg g^{-1} compared with 0.5 mg g^{-1} BW and control (Table 1). However, the expression levels were not different at the two higher doses ($P > 0.05$). Therefore, we selected the 1 mg g^{-1} BW dose to evaluate the cytokine response in the next experiment.

2.4. Experiment to evaluate cytokine responses by the immunostimulant

Fish were divided into two groups, *viz*. treatment and control group (n = 70) and maintained in seawater flow-through system. Lpp powder suspended in SDW (10% w/v) was orally administered at the previously determined dose of 1 mg g^{-1} BW (=0.5 mL) once a day for 3 days to all fish of the treatment group and the fish of the control group were fed the same volume of SDW using the disposable feeding needle (Fuchigami).