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Elevated cytokine responses to *Vibrio harveyi* infection in the Japanese pufferfish (*Takifugu rubripes*) treated with *Lactobacillus paracasei* spp. *paracasei* (06TCa22) isolated from the Mongolian dairy product

G. Biswas [a], H. Korenaga [a], R. Nagamine [b], S. Kawahara [c], S. Takeda [d], Y. Kikuchi [d], B. Dashnyam [e], T. Yoshida [c], T. Kono [b,]**, M. Sakai [c,]*

[a] *Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan*
[b] *Interdisciplinary Research Organization, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan*
[c] *Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan*
[d] *Minami Nihon Rakuno Kyodo Co. Ltd., Miyakonojo 885-0017, Japan*
[e] *Mongolian Biotechnology Association, Ulaanbaatar, Mongolia*

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A B S T R A C T

With the aim of evaluating the effect of a Mongolian dairy product derived *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) (Lpp) on the cytokine-mediated immune responses to *Vibrio harveyi* infection, we examined 16 cytokine expressions in the Japanese pufferfish, *Takifugu rubripes*. Fish were orally treated with the heat-killed Lpp at 1 mg g^{-1} body weight d^{-1} for 3 days. At 24 h posttreatment, fish were infected by an intramuscular injection of 0.1 mL *V. harveyi* bacterial suspension (10^8 cfu mL^{-1}). Additionally, superoxide anion production (SAP) and phagocytic activity (PA) of head kidney cells were assessed during 120 h postinfection period. Significant up-regulation of pro-inflammatory (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N), cell-mediated immune inducing (IL-12p35, IL-12p40 and IL-18), anti-viral/intra-cellular pathogen killing (I-IFN-1 and IFN-γ), anti-inflammatory (IL-10) and lymphocyte agonistic (IL-2, IL-7, IL-15, IL-21 and TGF-β1) cytokines was observed in the treated fish compared to control ones during the pathogen infection. Furthermore, significantly increased SAP and PA ($P < 0.01$; 0.05) were recorded in the treated fish compared to untreated fish. These results suggest the beneficial role of Lpp in enhancement of cytokine-mediated immunity in the Japanese pufferfish against *V. harveyi* infection and application of this product as a potential fish immunostimulant.

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1. Introduction

Vibriosis caused by several *Vibrio* spp., such as *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum*, and *Vibrio parahaemolyticus*, is an important disease to the aquaculture animals. Among these bacteria, *V. harveyi* is a severe pathogen with a wide host range covering both vertebrates and invertebrates [1,2]. *V. harveyi* is a Gram-negative, luminous bacterium omnipresent in marine environment. In addition to crustaceans, this bacterium is also known to cause disease outbreaks in several teleost species, notably brill *Colistium guntheri*, Japanese flounder *Paralichthys olivaceus*, seabass *Lates calcarifer*, sea bream *Sparus aurata*, Senegalese sole *Solea senegalensis*, seven-band grouper *Epinephelus septemfasciatus*, and turbot *Colistium nudipinnis* [3–8]. Recently, high mortality (about 40%) of the river puffer, *Takifugu obscures* with clinical signs of a single large and deep ulcer on the lateral or ventral skin and hemorrhage at the base of the pectoral, pelvic and anal fins was reported to be etiolated by *V. harveyi* in a public aquarium in Korea [9]. Later, the Japanese pufferfish, *Takifugu rubripes* displaying nodular lesions in the branchial chamber as well as on the inner surface of operculum had a moderate mortality caused by this bacterium in a mariculture farm in Western Japan [10]. Therefore, this bacterium has also been found to be a virulent pathogen to pufferfish.

In aquaculture, control of bacterial disease outbreaks has been depending mainly on antibiotics, which are often ineffective and lead to antibiotic-resistance, immunosuppression, bioaccumulation and high expenditure [11]. Moreover, prohibition on the use of

Abbreviations: HK, head kidney; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; RT-PCR, reverse transcription-polymerase chain reaction; LAB, lactic acid bacteria; Th, T helper.

* Corresponding author. Tel./fax: +81 985 587219.
** Corresponding author. Tel./fax: +81 985 587866.
E-mail addresses: tkono@cc.miyazaki-u.ac.jp (T. Kono), m.sakai@cc.miyazaki-u.ac.jp (M. Sakai).

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large number of antibiotics and chemotherapeutics for treatment of diseases has offered an opportunity to search for alternative preventive measures that are eco-friendly and unhazardous to human health. In this regard, since fish depend mostly on innate immune systems rather than on specific immunity, use of bio-products as broad-spectrum immune enhancers can play a major role in disease protection by elevating resistance through elicitation of innate immunity [12]. Over the years application of probiotic microbes to prevent or control pathogenic microorganisms has been increasing [13,14]. Probiotic lactic acid bacteria (LAB) and their products have been proved to enhance immune status and disease resistance in higher animals [15–17] as well as in fish [18–21]. Probiotic research, more specifically the use of LAB probiotics as immune enhancers has not been accomplished in the Japanese pufferfish compared to extensive studies involving other fish species.

Cytokines produced by macrophages, lymphocytes, granulocytes, dendritic cells (DCs), mast cells, and epithelial cells, include interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factor (TGF), interferons (IFNs) and chemokines [22]. Cytokines play an important role in the immune system by binding to specific receptors at the cell membrane, setting off a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus [23]. They elevate the host defense in response to invasion by parasitic, bacterial or viral pathogenic components. Live or killed probiotics can influence cytokine-mediated immunity in fish [19,20,24,25]. Recently, 10 LAB strains have been isolated from the Mongolian traditional dairy products and oral administration of these heat-killed LAB strains exhibited immunomodulatory activity in influenza virus (IFV) infected mice [17]. Moreover, in an *in vitro* study, modulation of cytokine defense mechanisms in the Japanese pufferfish head kidney (HK) cells by two heat-killed probiotics suggested better efficacy of *Lactobacillus paracasei* spp. *paracasei* (06TCa22) as immunostimulant [26]. Therefore, we hypothesized that cytokine-mediated immunity may be induced in response to *V. harveyi* infection by this heat-inactivated LAB strain in fish. Since cytokine system is complex and involves several genes functioning in a cascading manner, simultaneous analysis of different cytokine family members in fish would provide a comparative understanding on the innate immune system. In this context, our study aimed at examining expression of 16 different cytokine genes functionally related to pro-inflammatory, cell-mediated immune inducing, antiviral/intra-cellular pathogen killing, anti-inflammatory and lymphocyte agonist in *V. harveyi* infected Japanese pufferfish after oral administration with the heat-killed *L. paracasei* spp. *paracasei*. Additionally, to confirm the functionality of cytokines induced by this probiotic, we also assessed superoxide anion production and phagocytic activity using flow cytometry in response to *V. harveyi* infection in the treated pufferfish.

2. Materials and methods

2.1. Experimental fish

Japanese pufferfish, *T. rubripes* (body weight, 50.6 ± 2.4 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 elsewhere [26]. All animal experiments were conducted according to the relevant national and international guidelines, 'Act on Welfare and Management of Animals' (Ministry of Environment, Japan). Ethics approval from the local IACUC was not sought since this law does not mandate protection of fish.

2.2. Preparation of LAB

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 [27]. 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.

2.3. Oral administration of the heat-killed LAB

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 1 mg g^{-1} body weight (=0.5 mL) once a day for 3 days to all fish of the treatment group and the fish of the control group were intubated with the same volume of SDW using a disposable feeding needle (1.2Φ × 75 mm; Fuchigami, Kyoto, Japan).

2.4. V. harveyi infection

V. harveyi HTPV-0710 is a pathogenic strain isolated previously from the diseased Japanese pufferfish [10]. The bacterium was grown in Marine Agar Broth 2216E (Difco, Detroit, Michigan, USA) for 16 h at 25 °C in a shaking incubator at 150 rpm. Artificial infection was conducted by intramuscular (i.m.) injection to the pufferfish of treatment and control groups ($n = 35$) with 0.1 mL of the bacterial suspension (10^8 cfu mL^{-1}) at 24 h after the Lpp administration period.

2.5. Tissue isolation, RNA extraction and multiplex RT-PCR assay

HK and spleen tissues from randomly sampled ($n = 5$) infected fish of treated and control groups were isolated at 4, 8, 12, 24, 72 and 120 h postinfection (hpi). For tissue sampling, 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. HK and spleen tissues were 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 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 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 [26,28]. RT and PCR were performed using 16 cytokine primers (Supplementary Table 1). The PCR products from multiplex RT-PCR were prepared and run in the GeXP Genetic

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