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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

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ABSTRACT

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.

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.

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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

Analysis System for capillary electrophoresis and fragment size analysis as per the previously described protocol [26]. 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. The relative expression level of each cytokine gene was calculated by normalization to the reference gene, β-actin using GeXP Quant Tool.

2.6. Isolation of HK phagocytic cells

The HK phagocytic cells of the infected pufferfish ($n = 5$) from both treated and control groups were isolated at 4, 8, 12, 24, 72 and 120 h post *V. harveyi* infection according to the method described by Braun et al. [29] with slight modifications. Briefly, the isolated cells were filtered through a nylon mesh (Φ40 μm; Becton, Dickinson and Company, NJ, USA) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen) and a 1% solution of 10,000 g mL^{-1} streptomycin + 10,000 U mL^{-1} penicillin (Invitrogen) and were then centrifuged at 400 × *g* for 20 min at 4 °C. The macrophage-enriched cell interface was re-centrifuged at 500 × *g* for 5 min and was then washed three times with the medium. Viable phagocytic cells including neutrophils (about 10%) and macrophages (about 90%) were counted following trypan blue exclusion method.

2.7. Detection of superoxide anions in phagocytic cells (NBT reduction)

Superoxide anions produced by phagocytic cells were determined using the reduction of nitroblue tetrazolium (NBT; Sigma–Aldrich, St. Louis, MO, USA) assay. The NBT assay was performed at 4, 8, 12, 24, 72 and 120 h post *V. harveyi* infection. Isolated HK phagocytic cells (10^8 cells mL^{-1}) from each sample were seeded at 100 μL in wells of a 96-well plate (Nunc A/S, Roskilde, Denmark) and incubated at 4 °C for 24 h. NBT was dissolved in RPMI 1640 medium (1 mg mL^{-1}) and filtered through a 40 μm mesh (Ministart; Sartorius Stedim Biotech, Goettingen, Germany). Viable phagocytic cells were stimulated with 100 μL NBT solution at 25 °C for 1 h. After removal of the non-adherent cells by washing with PBS, 120 μL of 2 M potassium hydroxide (KOH; Wako, Tokyo, Japan) and 140 μL of dimethylsulfoxide (DMSO; Sigma–Aldrich) were added. The optical density of the solution was then measured at 620 nm using a micro-plate photometer (Multiskan FC, Thermo Scientific).

2.8. Determination of phagocytic activity using flow cytometry (FCM)

The phagocytic activity using FCM was performed at 4, 8, 12, 24, 72 and 120 h post *V. harveyi* infection. Isolated HK phagocytic cells from each sample were seeded at 1 mL in wells of a 24-well plate (Nunc A/S) and incubated at 4 °C for 24 h. After removing the non-adherent cells by washing with PBS, the viable cells (10^7 cells mL^{-1}) were incubated with a suspension of yellow-green fluorescent latex beads (Molecular Probes, Invitrogen, Karlsruhe, Germany) of 1 μm diameter at a ratio of approximately 30 beads per cell. Samples were incubated at 25 °C for 2 h and cells associated fluorescence was evaluated. The samples were analyzed in a Coulter Epics XL flow cytometer (Beckman Coulter, Inc.) with a single air-cooled laser adjusted to 675 nm (FL4) to detect the fluorescence of beads engulfed by phagocytic cells. In this experiment, FCM was calibrated so that the fluorescence of free beads was adjusted to 10^0 FL4-Log fluorescence units. The fluorescence histograms of cell number versus fluorescence intensity were analyzed. Phagocytic activity was accurately evaluated mathematically using the FlowJo software (Tree Star, Inc., San Carlos, CA, USA) to calculate percent cells with fluorescence higher than one or more beads.

2.9. Data analysis

Data on quantified relative expression of a particular gene, NBT reduction and phagocytic activity in the *V. harveyi* infected fish from Lpp-treated and the control groups at each time point were compared using an independent samples *t*-test for equality of means. Statistical analysis was performed using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (S.D.).

3. Results

3.1. Expression of cytokine genes

Based on their functions, 16 cytokines have been categorized into five groups, *viz.* pro-inflammatory, cell-mediated immune inducing, antiviral/intra-cellular pathogen killing, anti-inflammatory and lymphocyte agonist cytokines. A summary of fold change of cytokine gene expression levels in the head kidney and spleen of *V. harveyi* infected Japanese pufferfish after oral administration with the heat-killed *L. paracasei* spp. *paracasei* is presented in Table 1.

3.1.1. Pro-inflammatory cytokines

The pro-inflammatory IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N were expressed at significantly higher level ($P < 0.01$; 0.05) in the HK and spleen of probiotic treated pufferfish during the infection with *V. harveyi* at all the time points (Fig. 1). These pro-inflammatory cytokine responses were acute and gradually reduced from 24 h of infection in both HK and spleen. From fold-change data, higher expression levels were evident in HK compared to spleen tissues (Table 1). Among these cytokines, IL-1β and TNF-α showed the highest expression level (over 16 and 25-fold, respectively) in the infected fish HK at 4 hpi.

3.1.2. Cell-mediated immune inducing cytokines

Transcript levels of cell-mediated immune inducing cytokines, IL-12p35, IL-12p40 and IL-18 were significantly higher ($P < 0.01$; 0.05) in the HK and spleen of *V. harveyi* infected pufferfish at all the time points (Fig. 2). Although, higher expression level of these three cytokines was observed in the HK tissues, higher fold-change (3.7–8.9) of IL-12p40 was recorded in spleen tissues of the infected fish (Table 1).

3.1.3. Antiviral/intra-cellular pathogen killing cytokines

Significantly higher expression level ($P < 0.01$; 0.05) of I-IFN-1 and IFN-γ was detected in the HK at all the time points except 72 hpi during *V. harveyi* infection in the treated fish compared to control ones (Fig. 3). Spleen of the treated fish had higher transcript level of both the cytokines at all through the time course excluding 120 hpi for I-IFN-1. Overall, higher fold-change of both the cytokines was noticed in HK than in spleen during the infection of treated fish (Table 1).

3.1.4. Anti-inflammatory cytokine

IL-10 gene expression was higher (2.74-fold relative to the control) in the HK of infected fish at 8 hpi only (Table 1). However, an enhanced mRNA level of IL-10 ($P < 0.01$; 0.05) was recorded in spleen of probiotic treated fish compared to untreated fish during the infection at 4 and 120 hpi (Fig. 3).

Table 1
Fold change of mean expression level ($n = 5$) of cytokine genes relative to that of control fish (=1) in the head kidney and spleen of *Vibrio harveyi* infected Japanese pufferfish after orally administered with the heat-killed *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22).

Gene	Head kidney						Spleen					
	Hours postinfection											
	4	8	12	24	72	120	4	8	12	24	72	120
A. Pro-inflammatory cytokines												
1. IL-1β	16.61**	10.18**	9.12**	5.71**	3.02**	2.85**	3.31**	2.80**	3.41**	1.76**	1.84**	1.38*
2. IL-6	9.00**	2.14**	4.13*	4.06**	3.06**	2.94*	2.24**	2.86**	3.00**	2.21**	1.73**	1.62**
3. IL-17A/F-3	8.27**	5.90**	4.92**	3.28**	1.08	2.21**	4.51**	6.36**	7.95**	8.08**	5.19**	3.73*
4. TNF-α	25.45**	17.50**	16.91**	4.16**	3.88**	2.62**	2.63**	6.51**	4.91**	4.67**	2.39**	1.35*
5. TNF-N	3.04**	2.58**	2.61**	2.39**	2.05**	1.95**	2.08**	2.72**	2.76**	2.34**	2.00**	1.24*
B. Cell-mediated immune inducing cytokines												
1. IL-12p35	9.18**	2.00**	5.86**	2.32**	2.60**	8.00**	2.61**	2.35**	1.74**	2.99**	1.50**	2.10**
2. IL-12p40	2.41**	2.26**	2.69**	2.51**	2.12**	4.53**	3.70**	4.06**	6.95**	5.63**	8.90**	3.89*
3. IL-18	8.19**	7.01**	10.51**	4.39**	7.84**	12.33**	6.11**	3.43**	5.47**	2.13**	3.00**	8.52**
C. Antiviral/intra-cellular pathogen killing cytokines												
1. I-IFN-1	4.30**	2.10**	3.59**	2.28**	1.07	7.88**	3.72**	1.88**	1.63*	2.71**	1.75**	1.42
2. IFN-γ	6.20**	2.92**	3.88**	3.10**	1.21	4.71**	4.19**	3.36**	2.27**	2.62**	2.59**	8.76**
D. Anti-inflammatory cytokine												
1. IL-10	1.00	2.74**	1.43	1.00	0.43	1.52	3.70**	0.55	1.83	0.45	0.65	2.03*
E. Lymphocyte agonists												
1. IL-2	1.35	6.10**	3.94**	1.12	3.13**	5.62**	5.00**	2.04**	1.77*	7.15**	4.00**	1.21*
2. IL-7	6.66**	8.32**	10.56**	6.87**	4.74**	3.11**	18.24**	13.30**	7.48**	6.26**	4.02**	3.98**
3. IL-15	1.00	0.89	1.00	0.78	0.38	0.50	3.61**	1.10	1.37	1.43	1.00	0.76
4. IL-21	4.56**	1.13	1.02	2.20**	1.04	2.43**	1.96**	1.43*	1.06	1.18	1.50**	1.44*
5. TGF-β1	58.71**	63.14**	40.64**	39.92**	25.75**	0.89	1.39**	1.04	1.16	1.40**	1.50**	1.02

Asterisk indicates significantly higher fold change of a particular gene at each time point (*$P < 0.05$; **$P < 0.01$).

3.1.5. Lymphocyte agonists

In the present study, a significant increase ($P < 0.01$; 0.05) in expression of IL-2, IL-7, IL-21 and TGF-β1 genes was observed in *V. harveyi* infected Japanese pufferfish following administration with Lpp at almost all the time points except in case of IL-21 (Fig. 4). However, a variation in tissue expression between HK and spleen was observed for these cytokines. Lpp treatment did not cause higher expression of IL-15 in the HK, whereas a significant 3.61-fold increased transcript level ($P < 0.01$) was observed in spleen of the treated fish compared with control ones at 4 hpi only (Table 1).

3.2. NBT reduction activity of phagocytes

The production of superoxides assessed by NBT reduction, was significantly higher ($P < 0.001$; 0.05) in *V. harveyi* infected pufferfish treated with Lpp than in control fish at all the time points (Fig. 5). Over the time course, there was a conspicuous decreasing trend in optical density (O.D.) level.

3.3. Phagocytic activity

The phagocytic activity of head kidney leukocytes from *V. harveyi* infected pufferfish treated with Lpp showed a significant increase ($P < 0.01$; 0.05) compared with that of the control fish at all the time points (Fig. 6; Supplementary fig. 1). However, on the whole, there was a decreasing trend noticed in phagocytic activity with the lowest value at 8 hpi.

4. Discussion

To date, the beneficial effects of probiotic administration against *V. harveyi* infection in the Japanese pufferfish have not been demonstrated, however in a previous *in vitro* study, the same heat-killed *L. paracasei* spp. *paracasei* was found to modulate cytokine responses in the HK cells [26]. In this study, the up-regulated 16 cytokine genes in the HK and spleen of *V. harveyi* infected fish following administration with the heat-killed probiotic strain indicated the immunostimulatory effects of this probiotic bacterium.

4.1. Cytokine gene expressions

4.1.1. Pro-inflammatory cytokines

As primary sites of infection and inflammation, the kidney and spleen may exhibit up-regulation of the genes encoding pro-inflammatory cytokines, IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N during a bacterial infection. Similarly, in our study, these pro-inflammatory cytokines were up-regulated in the HK and spleen of *V. harveyi* infected fish treated with the heat-killed *L. paracasei* spp. *paracasei*. Here, an early and significantly elevated expression of IL-1β was observed in the HK and spleen at 4 hpi, and the expression sustained until 120 hpi at a decreasing level. Increased levels of IL-1β transcript have previously been reported in rainbow trout (*Oncorhynchus mykiss*) infected with bacterial pathogens, notably *Aeromonas salmonicida* [23] and *Yersinia ruckeri* [30]. IL-1β produced by macrophages, monocytes and lymphocytes, is a key molecule in acute response to microbial invasion and tissue injury [31] and it acts through binding to its receptor (IL-1R), leading to a series of signal transduction events and activation of the classical nuclear factor (NF)-κB pathway and downstream release of other cytokines [32]. Therefore, this acute and sustained IL-1β expression would have mediated the inflammatory response and tissue damage [33] and also influenced the subsequent cytokine release cascade [11]. During the cytokine release cascade, IL-6 originates from endothelial cells, fibroblasts, monocytes, and macrophages in response to other cytokine stimuli (IL-1, IL-17, and TNF-α) following bacterial infection to perform a key role in cellular and tissue homeostasis through activation of target genes involved in growth, differentiation, survival, apoptosis, and proliferation [34]. Increased acute-phase and sustained expression of IL-6 in this study may be related to the involvement of humoral as well as cellular defense in response to *V. harveyi* infection by the activation of various target cells, such as hepatocytes, leukocytes, T and B cells and hemopoietic cells. The pro-inflammatory Th17 cytokine, IL-17A/F-3 gene was

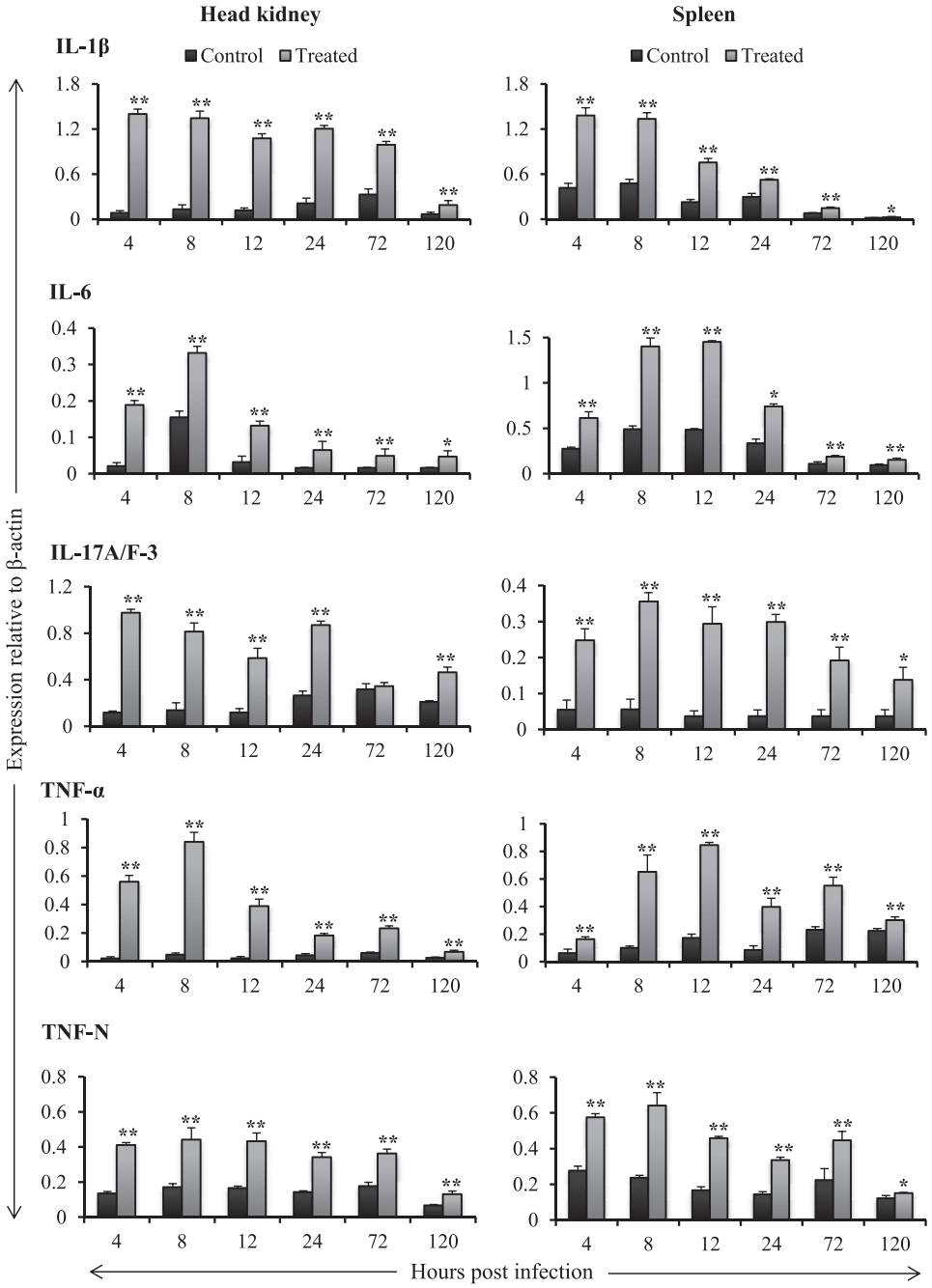

Fig. 1. Relative expression of pro-inflammatory cytokine genes at different time points in the head kidney and spleen of *Vibrio harveyi* infected Japanese pufferfish after oral administration with the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (06TCa22) at 1 mg g^{-1} body weight d^{-1} for 3 days. Asterisk indicates significantly higher expression in the treated fish compared with control group at each time point ($^{*}P < 0.05$; $^{**}P < 0.01$). Values are mean ± S.D. of 5 fish.

up-regulated in the HK and spleen of the infected pufferfish following Lpp administration, which is in agreement with our previous *in vitro* study, where higher expression was observed in the HK cells at 1–24 h poststimulation [26]. This elevated expression of IL-17A/F-3 might have occurred due to the exposure of naïve T cells to the already elevated IL-1β, IL-6 and TGF-β1 during Th17 differentiation [35]. TNFs, produced mainly by macrophages/monocytes and T lymphocytes have a variety of functions in the regulation of inflammation and cellular immune responses including the stimulation of respiratory burst activity and phagocytosis [36]. In this experiment, expression of TNF-α and the fish-specific ligand TNF-N genes was enhanced in the infected fish following treatment with Lpp. Similar to our observation of increased expression of TNF-α gene, feeding LAB probiotics, such as *Lactobacillus plantarum* [20] and *Lactobacillus rhamnosus* [24] caused elevated expression in rainbow trout. In our recent *in vitro* experiment, elevated expression of the pro-inflammatory cytokine genes was observed in the pufferfish HK cells stimulated with the heat-killed form of Lpp and *L. plantarum* (06CC2) isolated from the Mongolian dairy products [26]. Our results coupled with

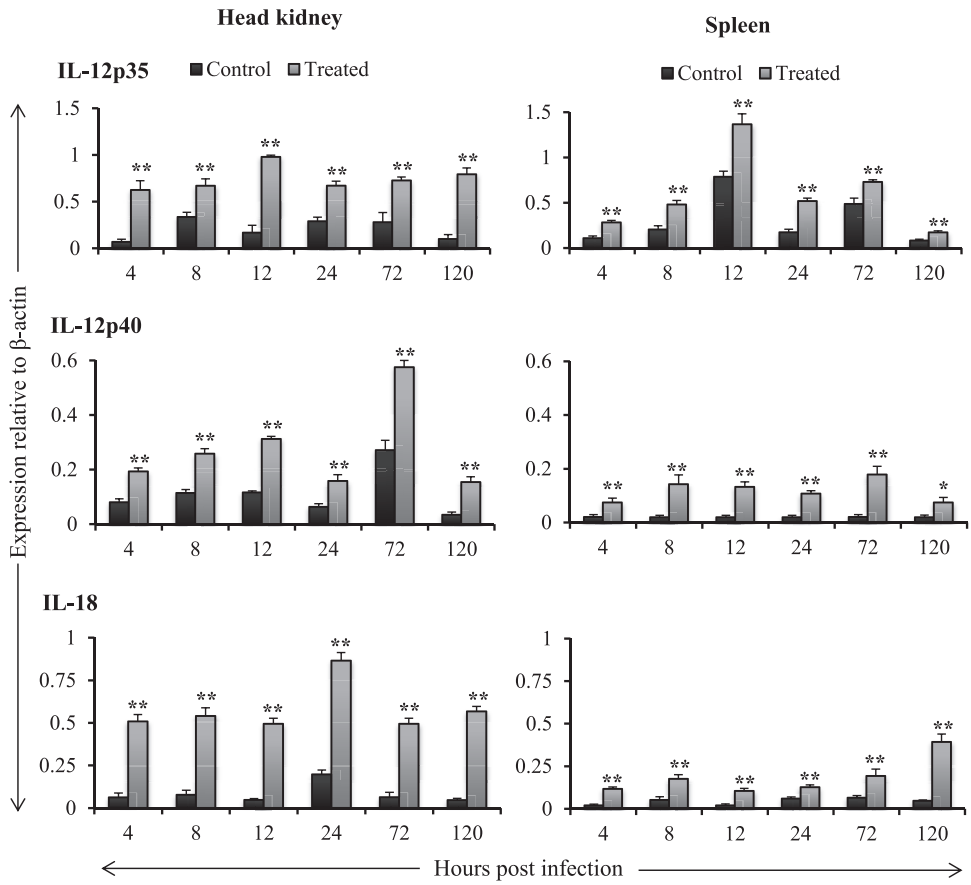

Fig. 2. Relative expression of cell-mediated immune inducing cytokine genes at different time points in the head kidney and spleen of *Vibrio harveyi* infected Japanese pufferfish after oral administration with the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (06TCa22) at 1 mg g^{-1} body weight d^{-1} for 3 days. Asterisk indicates significantly higher expression in the treated fish compared with control group at each time point ($^{*}P < 0.05$; $^{**}P < 0.01$). Values are mean ± S.D. of 5 fish.

these reports, suggest the acute and synergistic induction of pro-inflammatory cytokines to enhance immune responses to *V. harveyi* infection in the treated Japanese pufferfish.

4.1.2. Cell-mediated immune inducing cytokines

IL-12 is a heterodimeric cytokine composed of two covalently linked peptide chains termed as IL-12p35 (or IL-12α) and IL-12p40 (or IL-12β) and offers defense against parasites, viruses and intracellular bacteria by stimulating the production of IFN-γ by Th1 cells and NK cells [37]. In the present study, enhanced expression of IL-12p35 and IL-12p40 genes in the HK and spleen of the heat-killed probiotic-treated fish compared with untreated control during *V. harveyi* infection may indicate co-expression of these subunits in the same cell to generate the bioactive IL-12 [38]. Previously, heat-killed *L. plantarum* and Lpp isolated from Mongolian dairy products stimulated production of IL-12 in bronchoalveolar lavage fluid of mice [17] and expression of both the subunits of IL-12 in HK cells of the Japanese pufferfish [26], respectively. Therefore, these results including our present findings indicate the immunostimulatory property of Lpp in induction of IL-12 for the development and maintenance of Th1 cells and enhancing immunity against bacterial pathogens in the Japanese pufferfish. The cell-mediating pleiotropic cytokine, IL-18 is produced by monocytes/macrophages and like IL-12, it participates in clearance of intracellular pathogens through the induction of IFN-γ, and of viruses by activation of cytotoxic T cells [39]. Compared to the previous *in vitro* study where the heat-killed Lpp induced higher expression of this gene in the HK cells at 1 and 4 h poststimulation only [26], a sustained and longer duration expression was observed in the HK and spleen of infected fish treated with Lpp. IL-18 was initially identified as an inducer of IFN-γ production, however it alone induces only small amount of IFN-γ, while in combination with IL-12 it causes high levels of IFN-γ production by T cells [40]. Therefore, in the current study, the heat-killed Lpp apparently stimulated pro-IFN-γ monokines, IL-12 and IL-18 which in turn may induce considerable amount of IFN-γ production during *V. harveyi* infection [39].

4.1.3. Antiviral/intra-cellular pathogen killing cytokines

There are three sub-families of IFNs, namely type I, II and III. Among these, type I and III IFNs trigger specific signaling pathways for activation of innate immune defenses against viral infection, while type II IFN, also known as IFN-γ mainly participates in cell mediated and adaptive immunity [41]. We observed constitutively up-regulated expression of type I-IFN-1 and IFN-γ genes in the Lpp administered pufferfish HK and spleen during infection with *V. harveyi*. Therefore, this probiotic treatment would possibly trigger protection against virus infection by the activated type I-IFN-1 [11] and may also increase intracellular pathogen killing ability of leukocytes by the stimulated IFN-γ [26]. This higher level of IFN-γ expression may be a consequence of combined effects of already activated IL-12 and IL-18. Similarly, in the previous *in vitro* study, IFN-γ gene was up-regulated in the pufferfish HK cells by Lpp stimulation [26]. Increased expression of IFN-γ has also been observed in the proximal intestine of rainbow trout infected with *A. salmonicida* [23]. The LAB probiotic, *L. rhamnosus* feeding

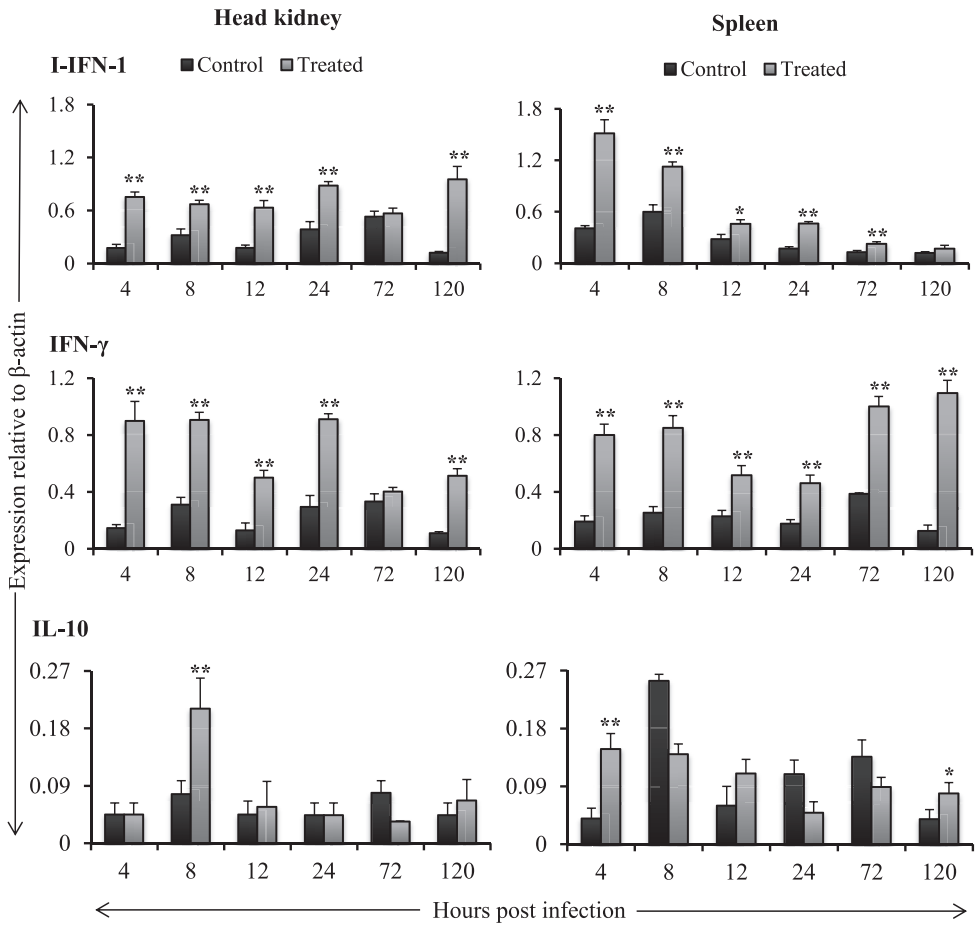

Fig. 3. Relative expression of antiviral (I-IFN-1)/intra-cellular pathogen killing (IFN-γ) and anti-inflammatory (IL-10) cytokine genes at different time points in the head kidney and spleen of *Vibrio harveyi* infected Japanese pufferfish after oral administration with the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (06TCa22) at 1 mg g^{-1} body weight d^{-1} for 3 days. Asterisk indicates significantly higher expression in the treated fish compared with control group at each time point (*$P < 0.05$; **$P < 0.01$). Values are mean ± S.D. of 5 fish.

induced IFN gene expression in the HK and spleen of rainbow trout with no mention on the IFN type [24]. However, our findings clearly demonstrated enhancement of both the antiviral (type I-IFN-1) and intracellular pathogen removal (IFN-γ) defenses in the infected fish.

4.1.4. Anti-inflammatory cytokine

It is generally believed that the process of inflammation is regulated through the balanced functioning of pro- and anti-inflammatory cytokines [30]. The anti-inflammatory cytokine, IL-10 produced mainly by monocytes, T cells (mainly Tr1 cells), B cells, NK cells, macrophages and DCs [42] regulates the inflammatory process by inhibiting over expression of many pro-inflammatory cytokines, chemokines and chemokine receptors [43]. IL-10 gene expression was increased only at 8 hpi in the HK and 4 and 120 hpi in the spleen of the Lpp administered pufferfish during the postinfection period. Similarly, an increase in IL-10 gene expression after *Lactococcus garvieae* infection was reported in the HK of rainbow trout fed the probiotic, *L. plantarum* [20]. Furthermore, several probiotic strains induced higher level of IL-10 production to provide defense against diseases in mice [44]. The inconsistent IL-10 expression observed in the bacterial pathogen infected fish might have allowed the occurrence of pro-inflammatory responses which were consistent and necessary in the host to get rid of the infection.

4.1.5. Lymphocyte agonists

The gamma chain (γC or CD132) cytokines, IL-2, IL-4, IL-9 and IL-21 act mainly as growth and proliferation factors for progenitors and mature immune cells and also have roles in lineage-specific cell differentiation [40]. In the present study, a significant increase in expression of IL-2, IL-7, IL-15 and IL-21 genes was observed in the infected Japanese pufferfish following administration with Lpp at almost all the time points except in case of IL-15 and IL-21. Similar longer duration expression of IL-2 gene was observed in Lpp-treated pufferfish HK cells, whereas IL-7 and IL-21 were up-regulated at 1 h poststimulation only [26]. Therefore, this longer duration induction of IL-7 in the HK and spleen and short time expression of IL-15 in the spleen only may contribute to the development of non-regulatory T cells and survival and function of naïve and memory T cells [45]. Another potent regulatory cytokine TGF-β1 was up-regulated in the infected pufferfish HK more consistently than in spleen. In agreement with our results, rainbow trout fed the probiotics, *L. rhamnosus* [24] and *L. rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* [19] had elevated expression of TGF-β1 in the HK and spleen. On the contrary, rainbow trout infected with *A. salmonicida* displayed down-regulated expression of TGF-β in the distal intestine [23]. Therefore, the present consistent TGF-β1 induction along with the stimulated IL-10 in the pufferfish would possibly have provided a suppressive effect on T cell proliferation [46].

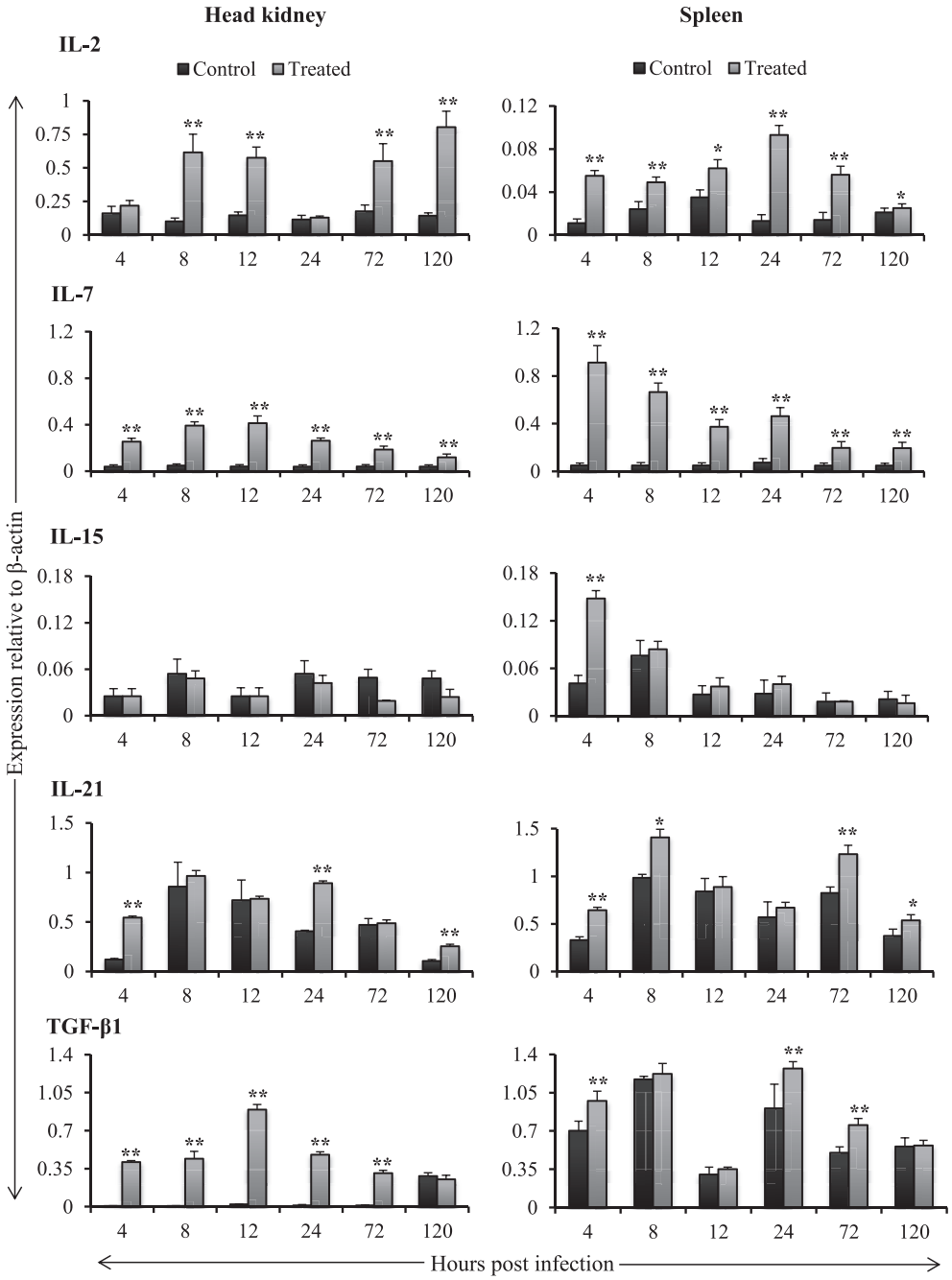

Fig. 4. Relative expression of lymphocyte agonistic cytokine genes at different time points in the head kidney and spleen of *Vibrio harveyi* infected Japanese pufferfish after oral administration with the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (06TCa22) at 1 mg g^{-1} body weight d^{-1} for 3 days. Asterisk indicates significantly higher expression in the treated fish compared with control group at each time point (*$P < 0.05$; **$P < 0.01$). Values are mean ± S.D. of 5 fish.

4.2. Superoxide anion production (NBT reduction)

In vertebrates, phagocytic process is followed by the production of reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which are highly microbicidal [47]. The NBT assay used in this study indicates the ability of phagocytes to kill pathogens by producing oxygen radicals. NBT test showed significantly increased values in *V. harveyi* infected fish orally administered with the heat-killed Lpp when compared to infected control fish received no Lpp treatment. A similar increase in superoxide anion production was reported in olive flounder, *P.olivaceus* fed diet containing probiotics and probiotics + herbal mixture during infection with *Uronema marinum* and *Streptococcus parauberis*, respectively [47,48]. Therefore, it is anticipated that the increased superoxide anion production observed in this experiment may result in increased pathogen killing ability of the phagocytes.

4.3. Phagocytic activity

Phagocytosis initiates an early activation of the inflammatory response before antibody production, and is mediated by phagocytic cells such as neutrophils, monocytes and macrophages in fish [18]. In agreement with the increased oxygen radical-producing

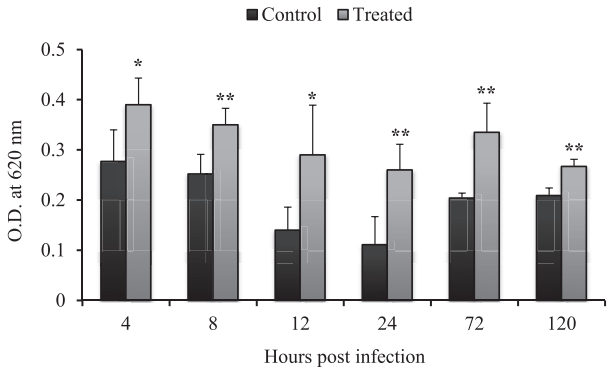

Fig. 5. Production of superoxide anion in the phagocytic cells of *Vibrio harveyi* infected Japanese pufferfish which were orally treated with *Lactobacillus paracasei* spp. *paracasei* (06TCa22) (treated) or untreated (control). Superoxide anion production was measured as optical density (O.D.) at 620 nm in the nitroblue tetrazolium (NBT) assay. Values are mean ± S.D. in 5 fish. $^{*}P < 0.05$; $^{**}P < 0.001$.

activity observed in the infected fish following administration with the Lpp, phagocytic activity was also enhanced with the highest value at 4 hpi. Our results of induction of phagocyte stimulation by probiotic treatment during pathogen infection are consistent with the previous reports of enhanced phagocytic activity in *A. salmonicida* spp. *salmonicida* infected rainbow trout fed with LAB probiotics [18] and *S. parauberis* infected olive flounder fed probiotics and herbal mixture [48]. Our results indicated that the modulation of phagocytic activity is an immediate and key effect induced by the heat-killed Lpp on the immune system of pufferfish during bacterial infection.

We used intubation (force-feeding) method of probiotic administration rather than incorporating in diet to make sure that the results obtained were due to the probiotic effect only without much variation among individual fish for the feeding activity. However, we anticipate that similar results would be obtained in practical feeding trials and need to be further investigated. The 0.1 mL dose of *V. harveyi* (10^8 cfu mL^{-1}) used for the infection did not cause any fish mortality. We continued to maintain the fish until 10 days postinfection and observed no mortalities. Therefore, the dose of bacterium was not sufficient to cause mortality in this bigger size (~50 g) pufferfish group compared to 0.2 mL of 10^8 cfu $fish^{-1}$ bacterial suspension causing 20% mortality in 4–6 days in smaller size group (10 g) previously [10].

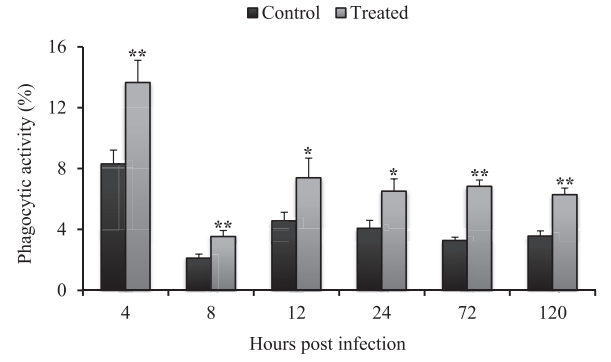

Fig. 6. Phagocytic activity of head kidney cells of the Japanese pufferfish in the heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22) treated group and untreated group (control) during *Vibrio harveyi* infection period. Phagocytic activity indicates the percentage of cells that phagocytosed yellow-green fluorescent latex beads detected using flow cytometry. Asterisk indicates significantly higher value in the treated fish compared with control group at each time point ($^{*}P < 0.05$; $^{**}P < 0.01$). Values are mean ± S.D. in 3 fish.

5. Conclusion

The present findings of increased or induced expression of 16 cytokine genes in the head kidney and spleen, enhanced phagocytic activity and superoxide anion production during *V. harveyi* infection in the Japanese pufferfish following the treatment with a Mongolian dairy product derived heat-killed *L. paracasei* spp. *paracasei* (06TCa22) indicate the immunostimulatory effects of this heat-killed LAB probiotic. Our results suggest the use of this product as a potential fish immunostimulant. Elucidation of the cytokine signaling networks and their various roles in immune responses in fish treated with this probiotic using different treatment modes including feeding trial may be the subject of further research before any practical application in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.06.004.

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