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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).

Table 1
Expression of cytokine genes relative to β-actin gene at 24 h post treatment in the head kidney of the Japanese pufferfish administered with three doses of the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22).

Cytokine gene	Treatment/dose of LAB			
	Control	0.5 mg g^{-1} BW	1 mg g^{-1} BW	2 mg g^{-1} BW
IL-1β	0.197 ± 0.046^{c}	0.560 ± 0.020^{b}	1.074 ± 0.070^{a}	1.123 ± 0.189^{a}
IL-6	0.224 ± 0.044^{c}	0.613 ± .090^{b}	3.286 ± 0.408^{a}	3.230 ± 0.145^{a}
IL-17A/F-3	0.181 ± 0.074^{c}	0.762 ± 0.180^{b}	3.247 ± 0.293^{a}	3.265 ± 0.388^{a}
TNF-α	0.283 ± 0.045^{b}	0.792 ± 0.115^{b}	2.992 ± 0.322^{a}	2.799 ± 0.339^{a}
TNF-N	0.458 ± 0.088^{d}	1.261 ± 0.120^{c}	2.114 ± 0.069^{b}	2.443 ± 0.217^{a}
IL-12p35	0.809 ± 0.037^{c}	0.876 ± 0.062^{c}	3.902 ± 0.433^{a}	3.147 ± 0.329^{b}
IL-12p40	0.673 ± 0.102^{c}	1.495 ± 0.101^{b}	2.710 ± 0.384^{a}	2.790 ± 0.143^{a}
IL-18	0.430 ± 0.085^{b}	0.531 ± 0.051^{b}	1.807 ± 0.282^{a}	1.971 ± 0.082^{a}
I-IFN-1	0.500 ± 0.166^{b}	0.848 ± 0.300^{b}	3.398 ± 0.360^{a}	3.449 ± 0.369^{a}
IFN-γ	0.250 ± 0.107^{b}	0.647 ± 0.141^{b}	2.773 ± 0.275^{a}	3.139 ± 0.334^{a}
IL-10	0.691 ± 0.227^{c}	1.964 ± 0.451^{b}	2.430 ± 0.310^{ab}	2.598 ± 0.146^{a}
IL-2	0.337 ± 0.044^{d}	0.871 ± 0.113^{c}	1.888 ± 0.131^{b}	2.590 ± 0.349^{a}
IL-7	0.438 ± 0.067^{b}	0.487 ± 0.055^{b}	1.835 ± 0.204^{a}	1.835 ± 0.165^{a}
IL-15	0.391 ± 0.069^{c}	0.498 ± 0.073^{c}	1.721 ± 0.248^{b}	2.445 ± 0.219^{a}
IL-21	0.414 ± 0.041^{c}	0.365 ± 0.073^{c}	2.117 ± 0.200^{b}	3.776 ± 0.241^{a}
TGF-β1	0.334 ± 0.063^{d}	0.750 ± 0.058^{c}	1.588 ± 0.146^{b}	3.820 ± 0.328^{a}

Means with different superscripts in a row differ significantly ($P < 0.05$); values are mean ± S.D. of five fish; BW, body weight.

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

HK tissues from randomly sampled (n = 5) treated and control fish were isolated at 4, 8, 12, 24, 72 and 120 h post treatment; total RNA extraction and multiplex RT-PCR assay were performed as per the protocols described in Section 2.3.2.

2.4.2. Isolation of HK phagocytic cells

The HK phagocytic cells of the pufferfish (n = 5) were isolated at 4, 8, 12, 24, 72 and 120 h post oral Lpp administration according to the method described by Braun et al. [19] with slight modification. 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.

2.4.3. 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) assay. The NBT assay was performed at 4, 8, 12, 24, 72 and 120 h post oral administration. Isolated HK phagocytic cells (1×10^8 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 RPMI1640 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).

2.4.4. 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 oral administration. 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 (1×10^7 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 cell 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, the flow cytometer was calibrated/optimized such that the fluorescence of free or no beads was adjusted to 10^0 FL4-Log fluorescence units (negative gate setting). The resulting fluorescence histograms plotting 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 the percentage of cells with fluorescent intensity equivalent to at least one bead.

2.4.5. V. harveyi infection and assessment of its burden in fish

The bacterium *V. harveyi* (strain HTPV-0710) [20] was used for artificial infection of the fish at 24 h after the Lpp administration period. Artificial infection was conducted by intramuscular (i.m.) injection to the Lpp treated and control pufferfish groups (n = 35) with 0.1 mL of the bacterial suspension (1×10^8 cfu mL^{-1}). At 4, 8, 12, 24, 72 and 120 h post-injection, blood, spleen and HK (n = 5) were removed and homogenized. The serially diluted homogenate was then plated onto Marine Agar (Difco, Detroit, MI, USA) plates. These plates were incubated at 25 °C for 72 h, and then examined for bacterial colony growth. The number of colonies grown on each plate was multiplied by the reciprocal value of dilution to determine the cfu per unit sample volume. Identity of the recovered bacterium was confirmed by PCR amplification and sequencing of 16S rDNA using 2 primer sets, F1 (5′-TGGAAACGATGGC TAATACCGCA-3′)-R2 (5′-ATCTGAGTGTCAGTATCTGTCCA-3′) and F2 (5′-TGGACAGATACTGACACTCAGAT-3′)-R1 (5′-CAGTCATGAACCACAAA GTGG-3′) designed based on Mohi et al. [20].

2.5. Data analysis

Difference between the quantified relative expressions of a particular gene in treated fish and control fish of the dose finding experiment was evaluated with one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for their comparison. Data on quantified relative expression of a particular gene, NBT reduction, phagocytic activity and infected bacterial burden in the 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 expressed as mean ± standard deviation (S.D.).

3. Results and discussion

3.1. Expression of cytokine genes

Transcriptional responses of 16 cytokines in the HK of the Japanese pufferfish administered with the heat-killed *L. paracasei* spp. *paracasei* and control fish are presented is Fig. 1. For easier and better understanding, a summary of fold change of expression level of cytokine genes in the head kidney of the treated fish relative to that of control fish (=1) at different time points is represented in Supplementary Table 2. Based on their functions, 16 cytokines have been categorized into five groups, *viz*. pro-inflammatory, cell-mediated immunity inducing, antiviral/intra-cellular pathogen killing, anti-inflammatory and lymphocyte agonist cytokines.

3.1.1. Pro-inflammatory cytokines

The pro-inflammatory IL-1β, IL-6, and IL-17A/F-3, and TNF-α and TNF-N (TNF-Novel) were constitutively expressed in the HK of immunostimulant treated pufferfish. The transcript levels of these cytokines were significantly enhanced ($P < 0.01$) by the heat-killed bacterium administration at all the time points compared with the

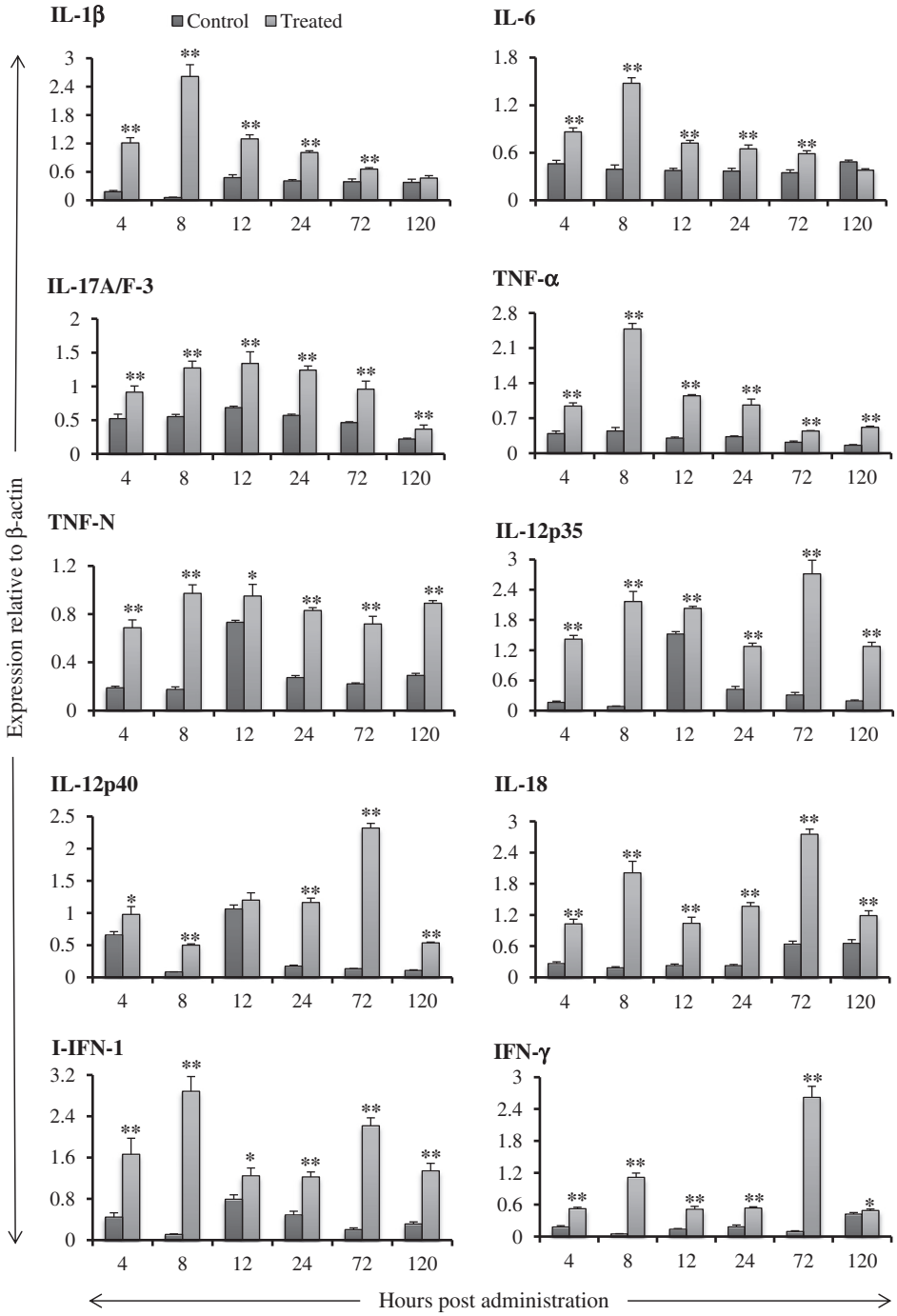

Fig. 1. Relative expression of cytokine genes at different time points in the head kidney of the Japanese pufferfish administered with the heat-killed LAB, *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) at 1 mg g^{-1} body weight d^{-1} for 3 days. Asterisk indicates significantly higher 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 five fish.

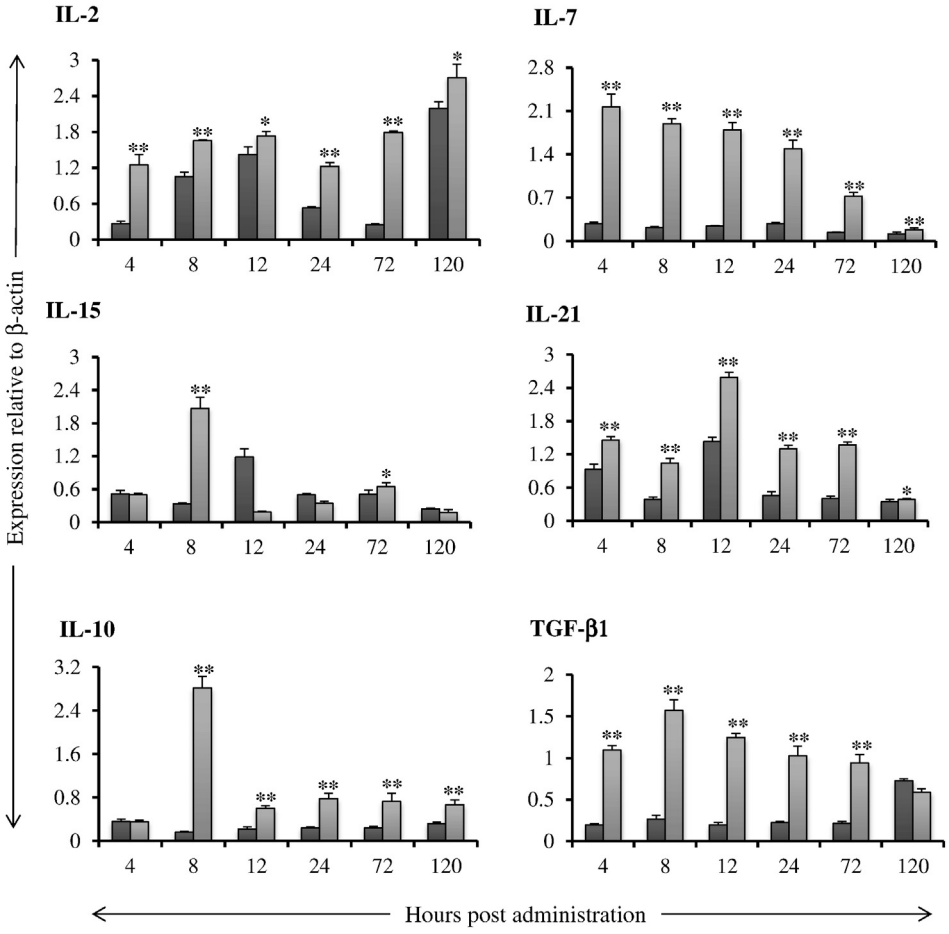

Fig.1 (*continued*).

untreated fish (Fig. 1; Supplementary Table 2). IL-1β originates from macrophages, monocytes and lymphocytes and plays a pivotal role in acute immune response to microbial invasion and tissue injury by activating lymphocytes or inducing release of other cytokines [21]. A distinguishable up-regulation of IL-1β gene (1.24 to 45.07-fold increase) was observed in the HK of the probiotic-treated fish compared to the control group at all the time points except 120 h post administration (hpa) (Supplementary Table 2). Therefore, this acute IL-1β expression would have influenced the downstream cytokine release cascade [22] and activated humoral immune response by elevating T-cell dependent antibody production [23]. However, the lower expression level of IL-1β in the present study compared to the previous *in vitro* experiment [16] may be attributed to the direct exposure of HK cells to Lpp causing higher stimulation in the latter. As a component of the cytokine cascade, IL-6 is released by 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 [24]. Increased acute-phase expression of IL-6 (1.69 to 3.77-fold; Supplementary Table 2) in this study may be related to the involvement of humoral as well as cellular defense by the activation of various target cells, such as hepatocytes, leukocytes, T and B cells and hemopoietic cells. IL-17A, produced mainly by T cells acts as pro-inflammatory in a synergistic mode with other cytokines and plays a central role in inflammatory process [25]. It has also a potential role in the recruitment and activation of neutrophils. The up-regulated expression of IL-17A/F-3 following Lpp administration to pufferfish is in agreement with the observation of Korenaga et al. [26], who reported higher expression in the pufferfish HK cells induced with LPS at 4 and 12 h. This higher level expression of IL-17A/F-3 might have occurred due to the involvement of regulatory T cells which control IL-17 expressing cells in fish [27]. TNFs take part in inflammation, autoimmunity, apoptosis, cell proliferation and differentiation, and stimulation of the immune system [28]. Similar to our observation of enhanced expression of TNF-α gene, rainbow trout (*Oncorhynchus mykiss*) fed different probiotics, such as *Canrobacterium maltaromaticum*, and *Canrobacterium divergens* [29], *Lactobacillus plantarum* [12] and *Lactobacillus rhamnosus* [30] displayed increased expression.

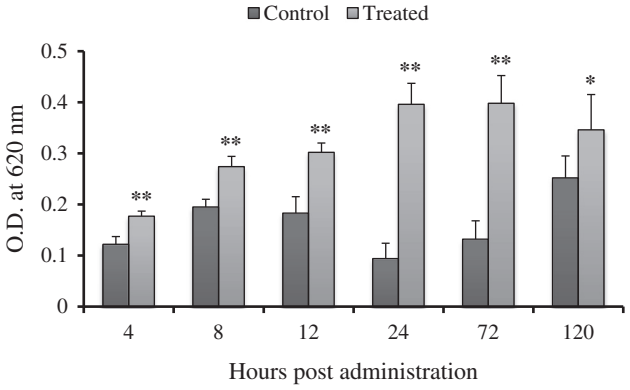

Fig. 2. Production of superoxide anion in the phagocytic cells of the heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22) treated or untreated (control) Japanese pufferfish. Superoxide anion production was measured as optical density (O.D.) at 620 nm in the nitroblue tetrazolium (NBT) assay. Values are mean ± S.D. in 5 fish. $^{*}P < 0.05$; $^{**}P < 0.001$.

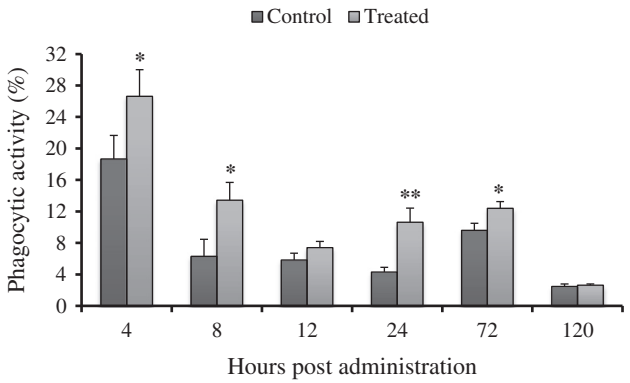

Fig. 3. Phagocytic activity of head kidney cells of the Japanese pufferfish in the heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22) treated group and untreated group (control). Phagocytic activity indicates the percentage of cells that phagocytosed yellow–green fluorescent latex beads detected using flow cytometry. Values are mean ± S.D. in 5 fish. $^{*}P < 0.05$; $^{**}P < 0.01$.

The result of expression reconfirmed the existence of TNF-N gene in the Japanese pufferfish. TNF-N, a TNF-β like gene was discovered for the first time, apart from mammals, from the Japanese pufferfish and zebrafish and is present in tandem with TNF-α gene on the same chromosome [31]. Previously, an *in vitro* experiment demonstrated elevated expression of these pro-inflammatory cytokine genes in the pufferfish HK cells stimulated with the heat-killed form of Lpp and *L. plantarum* (strain 06CC2) isolated from the Mongolian dairy products [16]. Our results coupled with these reports, suggest the acute and synergistic induction of pro-inflammatory cytokines to enhance immunity in the treated Japanese pufferfish.

3.1.2. Cell-mediated immunity 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 renders defense against parasites, viruses and intracellular bacteria by stimulating the production of IFN-γ by Th1 cells and NK cells [32]. In the present study, enhanced expression of IL-12p35 and IL-12p40 genes was observed in the heat-killed probiotic-treated fish compared with untreated control (Fig. 1; Supplementary Table 2). Therefore, this simultaneous expression of the two subunits might have caused to yield the functional IL-12p70 heterodimer as seen in carp [33]. Previously, heat-killed *L. plantarum* and Lpp isolated from Mongolian dairy products stimulated the production of IL-12 in bronchoalveolar lavage fluid of mice [9] and expression of both the subunits of IL-12 in HK cells of the Japanese pufferfish [16], respectively. Another cell mediating pleiotropic cytokine, IL-18 is produced by monocytes/macrophages and similar to IL-12, it plays important role in the clearance of intracellular pathogens through the induction of IFN-γ, and of viruses by the activation of cytotoxic T cells [34]. A significant 1.81 to 10.88-fold increase ($P < 0.01$) in the expression of the IL-18 gene was observed in the treated Japanese pufferfish compared to control fish at all the time points (Supplementary Table 2). However, in the previous *in vitro* study, same heat-killed Lpp induced higher expression of this gene in the HK cells at 1 and 4 h post stimulation only [16]. Therefore, in our study, the distinct stimulation of pro-IFN-γ monokines, IL-12 and IL-18 by the heat-killed Lpp induced considerable increase in IFN-γ expression.

3.1.3. Antiviral/intra-cellular pathogen killing cytokines

IFNs provide defense against virus infection in vertebrates. Among the three sub-families of IFNs, Type I and III IFNs trigger specific signaling pathways for the activation of innate immune defenses against viral infection and type II IFN (=IFN-γ) primarily promotes cell mediated and adaptive immunity [35]. We observed significantly up-regulated expression ($P < 0.01$; 0.05) of type I-IFN-1 and IFN-γ genes in Lpp administered pufferfish (Fig. 1; Supplementary Table 2). Therefore, this immunostimulant treatment may provide protection against virus infection by activating type I-IFN-1 [22] and also might have elevated the IFN-γ mediated intracellular pathogen killing ability of leukocytes in the Japanese pufferfish [16]. As a consequence of combined effects of activated IL-12 and IL-18, IFN-γ expression in the HK was induced at higher level. Similarly, in the previous *in vitro* study strain-dependent IFN-γ expression was noticed in the pufferfish HK cells with elevated expression caused by Lpp [16]. In another experiment, the LAB probiotic, *L. rhamnosus* feeding induced IFN gene expression in the HK and spleen of rainbow trout [30] without specifying the IFN class. On the contrary, our results clearly indicated elevation of both the antiviral (type I-IFN-1) and intracellular pathogen removal (IFN-γ) defenses in the treated fish.

3.1.4. Anti-inflammatory cytokine

The anti-inflammatory cytokine, IL-10 is produced mainly by monocytes, T cells (mainly Tr1 cells), B cells, NK cells, macrophages and DCs [36]. It inhibits the expression of many pro-inflammatory cytokines, chemokines and chemokine receptors [37]. IL-10 gene expression was higher (2.08 to 17.73-fold relative to the control) in the Lpp administered pufferfish at 8–120 hpa (Supplementary Table 2). Similarly, an increase in IL-10 gene expression was reported in the HK of rainbow trout fed the probiotic, *L. plantarum* [12]. The up-regulated IL-10 might have controlled the inflammatory response of the stimulated pro-inflammatory cytokines, thereby minimizing damage to the host due to an excessive response [38].

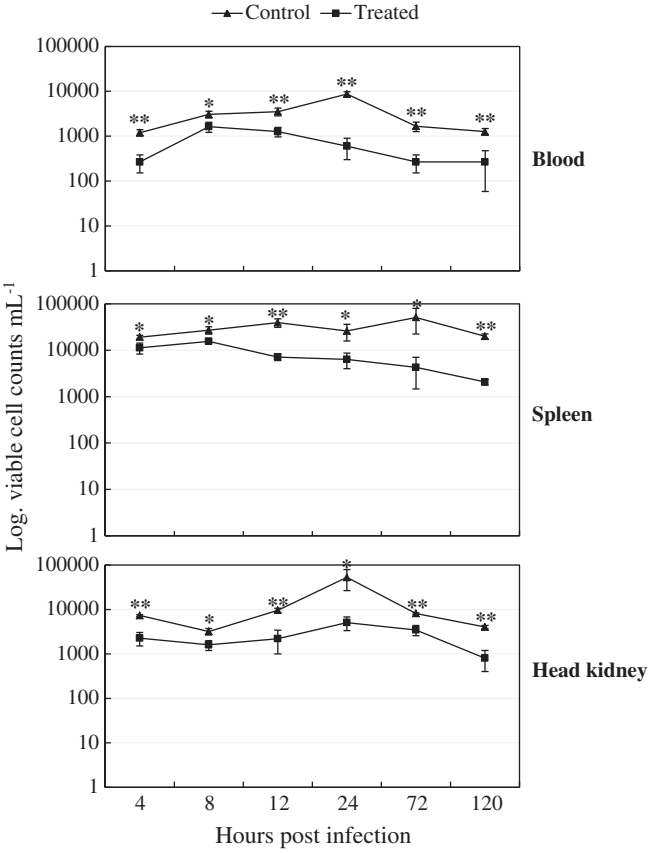

Fig. 4. Burden (mean ± S.D.) of *Vibrio harveyi* in the blood, spleen and head kidney of the heat-killed *Lactobacillus paracasei* spp. *paracasei* (06TCa22)-administered and control Japanese pufferfish (n = 5) infected with 1×10^8 cfu bacteria mL^{-1}. Asterisk indicates significantly higher bacterial cell counts in the control group compared with treated fish at each time point ($^{*}P < 0.05$; $^{**}P < 0.01$).

3.1.5. Lymphocyte agonists

The gamma chain (γC or CD132) cytokines, IL-2, IL-4, IL-9 and IL-21 are released from Th cells that affect their development, maintenance and responses, whilst IL-7 and IL-15 are particularly involved in the maintenance of T cell memory [39]. In general, they 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 ($P < 0.01$; 0.05) in the expression of IL-2, IL-7, IL-15 and IL-21 genes was observed in the Japanese pufferfish following administration with Lpp at almost all the time points except in the case of IL-15 (Fig. 1; Supplementary Table 2). Similar longer duration expression of IL-2 gene was observed in Lpp treated pufferfish HK cells *in vitro*, whereas IL-7 and IL-21 were up-regulated at 1 h post stimulation only [16]. Therefore, in the present study, this longer duration induction of IL-7 and IL-15 may contribute to the development of non-regulatory T cells and survival and function of naïve and memory T cells [41]. Another potent regulatory cytokine TGF-β1 was up-regulated in the treated pufferfish HK at all the time points except at 120 hpa (Fig. 1; Supplementary Table 2). Similar to our findings, rainbow trout fed the probiotics, *L. rhamnosus* for 30 days [30] and *L. rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* for 45 days [11] had higher expression of TGF-β1 in the HK and spleen. Therefore, the present consistent TGF-β1 induction in the pufferfish would possibly have provided a suppressive effect on lymphocyte proliferation [42].

Our results indicated increased expression of majority of 16 cytokines with low or insignificant up-regulation at some time points for a few cytokines (IL-10 and IL-15). On the whole, there was a decreasing trend observed in expression level of most of the cytokines over time course. This may be because of cytokines, as components for the first line of host immunity in fish are activated rapidly and released in a quick succession under stimulation [43]. At later stages, immunostimulatory effects of the heat-killed Lpp might have dwindled down and were responsible for decrease in cytokine gene expression.

3.2. Superoxide anion production (NBT reduction)

The production of superoxides assessed by NBT reduction, was significantly higher ($P < 0.001$; 0.05) in the pufferfish treated with Lpp than in control fish at all the time points (Fig. 2). Superoxide anions are produced by phagocytes to attack and kill invasive pathogens. This ability of fish macrophages is used to evaluate the host protection level against diseases. In the present study, oxygen radical production in the Japanese pufferfish appeared to be positively influenced by the oral administration of heat-killed LAB. Similarly, an increase in respiratory burst activity was reported in *Epinephelus coioides* fed diet containing *L. plantarum* 7–40 [13] and *Labeo rohita* fed *L. plantarum* VSG3 supplemented diet [44]. Therefore, increased superoxide anion production can be correlated with the increased pathogen killing ability of the Japanese pufferfish phagocytes.

3.3. Phagocytic activity

The phagocytic activity of head kidney leukocytes from pufferfish treated with Lpp showed a significant difference compared with that of the control fish ($P < 0.01$; 0.05). Thus, treated fish showed higher phagocytic activity than those of the control at 4, 8, 24 and 72 hpa (Fig. 3) with a decreasing trend. 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 as also demonstrated in rainbow trout fed aerobic heterotrophic intestinal bacteria [45].

3.4. Burden of V. harveyi in fish

It is quite pertinent to assess the elevated pathogen removal ability conferred to the pufferfish administered with the heat-killed LAB. Viable *V. harveyi* cell count in the blood, spleen and HK was less in the treated pufferfish compared to control group at all the time points ($P < 0.01$, 0.05; Fig. 4). In our study, a distinct *V. harveyi* removal activity has been induced in pufferfish by the oral administration of the heat-killed Lpp. *V. harveyi* is a luminous bacterium known to cause moderate mortality in the Japanese pufferfish displaying nodular lesions in the branchial chamber as well as on the inner surface of operculum [20]. However, bacterial dose used in our infection study, did not cause fish mortality. Similar to our observation, feeding of LAB probiotics to different fish species reduced bacterial pathogen loads [44,46]. The increased *V. harveyi* removal level, together with the increased fish superoxide anion production and phagocytic activity recorded in the treated fish in our study correlate with the observed enhanced regulation of cytokine expression.

In our experiment, the fish were intubated rather than given the strain in the feed to make it definite that the results obtained were due to the effects of immunostimulant. However, we speculate that similar results would be expected in feeding the microbial products with diet to fish and need to be further investigated.

4. Conclusion

This study provides a comparative understanding on the cytokine mediated immunity in the Japanese pufferfish orally treated with a Mongolian dairy product derived heat-killed *L. paracasei* spp. *paracasei* (strain 06TCa22) at 1 mg g^{-1} body weight d^{-1} by examining transcriptional responses of 16 cytokine genes, superoxide anion production, phagocytic activity and pathogen load. The up-regulation of different cytokine families suggests the efficacy of the heat-killed *L. paracasei* spp. *paracasei* (strain 06TCa22) as a potential fish immunostimulant. Furthermore, elucidation of various cytokine regulation pathways in this immunostimulant treated fish need to be fully disclosed for clearer understanding on host innate and cell-mediated immune defense mechanisms.

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