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Cytokine responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells induced with heat-killed probiotics isolated from the Mongolian dairy products

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ABSTRACT

Cytokine responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney (HK) cells to heat-killed lactic acid bacteria probiotics isolated from the Mongolian dairy products were investigated by transcriptomic examination. The HK cells were incubated with two heat-killed bacteria, namely *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) and *L. plantarum* (strain 06CC2) and the responses of 16 cytokine genes at 0 (control), 1, 4, 8, 12, 24 and 48 h post-stimulation were assayed by multiplex RT-PCR analysis (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter, Inc.). The 16 genes included in the assay were pro-inflammatory cytokines (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N), cell-mediated immune regulators (IL-12p35, IL-12p40 and IL-18), antiviral (I-IFN-1 and IFN-γ) and other regulatory (IL-2, IL-7, IL-15, IL-21, IL-10 and TGF-β1) cytokines. Despite the differences in the transcriptional profiles, expression of all the cytokines tested here was significantly elevated by both the probiotic bacterial stimulants compared with the unstimulated control. Therefore, this *in vitro* study has demonstrated the modulation of cytokine defense mechanisms in the HK cells by the two heat-killed probiotics indicating their potentiality as novel immunostimulants to fish. However, strain-dependent varied expression of important cytokines (cell-mediated immune regulators, antiviral and anti-inflammatory cytokines) suggests better efficacy of *L. paracasei* spp. *paracasei* strain as fish immunostimulant. Further *in vivo* studies to elucidate the cytokine regulation networks will validate our present observations. A careful evaluation of ant-inflammatory properties may be undertaken using single strain to affirm the immunostimulatory capability. Moreover, application timings and frequency to assess the longevity of immunostimulant effects and to make the application cost-effective need to be evaluated before any practical use in aquaculture.

1. Introduction

Cytokines, small protein mediators produced by immune cells, regulate and mediate immunity, inflammation and hematopoiesis [1]. Cytokines include interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), transforming growth factor (TGF) and chemokines. To date, several cytokine genes have been discovered employing various molecular techniques. More than 37 different IL genes have thus far been characterized in mammals [2,3]. In teleost, genes encoding 19 ILs, namely IL-1, -2, -4, -6, -7, -8, -10, -11, -12, -13, -15, -16, -17, -18, -19, -20, -21, -22, and -26 [1,4–8] have been identified. In addition to ILs, members of the fish TNF superfamily (TNF-α and -N) [9] and the IFN family (type-I IFN and IFN-γ) [10,11] genes have been isolated from several fish species. Gene encoding regulatory factor of hematopoiesis like TGF-β1 [12] has also been described. The role of various cytokines in fish immunity has been established. Cytokines act through their receptors and have pro-inflammatory, anti-inflammatory and pathogen-killing roles. For

Abbreviations: HK, head kidney; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; LPS, lipopolysaccharide; polyI:C, polyinosinic-polycytidylic acid; RT-PCR, reverse transcription-polymerase chain reaction.

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instances, IL-1 family members (IL-1α and β, IL-18, IL-33), IL-6, IL-17 and TNFs play pro-inflammatory role, IL-2 subfamily members (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21), known as gamma chain cytokines affect the responses of Th cells and the maintenance of T cell memory, IL-10 acts as anti-inflammatory by suppressing the expression of pro-inflammatory cytokines, the heterodimeric IL-12 provides defense against parasites, viruses and intracellular bacteria by stimulating IFN-γ production and IFNs are responsible for defense against virus infection and killing of intracellular pathogens. However, comprehensive analyses of fish cytokines have only recently commenced and less progressed compared to mammalian studies [2]. Since cytokines are important regulators of the immune system, investigating their functions may provide significant basis for the development of vaccines and immunostimulants for fish. Identification of potential indirect immunological markers from the expression profile of cytokine genes and their products involved in immune defense may be an added outcome.

Probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of its ambient environment [13]. Since last few years, use of probiotics as bio-control agents has become a popular technique to improve and maintain a healthy environment as well as farmed animal health. However, the use of probiotics in aquaculture is a newer concept compared to their usage in human, pig, cattle and poultry nutrition. The role of lactic acid bacteria (LAB) as probiotics has been well established. LAB are Gram-positive, non-sporulating and catalase-negative rods or cocci with the ability to ferment various carbohydrates mainly to lactate and acetate [14]. In the last two decades, application of LAB and their metabolic products as potential probiotics has been tested to improve immune status and disease resistance in higher animals [15,16] as well as in fish [17–22]. In fish, elevated expression of cytokine genes such as IL-1β, IL-8, IL-10, TNF-α and TGF-β was caused by killed or live probiotics in several *in vitro* and *in vivo* studies [18–23]. Use of probiotics in elicitation of cell-mediated immune regulators, IL-12 and IL-18 to induce IFN-γ production by natural killer (NK) and Th cells in fish has been scarcely investigated. However, in most of these studies, only a single or few cytokine genes were analyzed. Cytokine system seems complex and involves several genes functioning in a cascading manner. Therefore, simultaneous analysis of more number of cytokines in probiotic treated fish would provide a useful and reliable understanding on the innate immune system and substantiate the use of that probiotic. Recently, a novel technique, multiplex reverse transcription-PCR (RT-PCR) assay (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter, Inc.) to analyze expression of over 15 genes simultaneously has been established for the Japanese pufferfish, *Takifugu rubripes* [24]. This method can detect up to 30 gene expressions within a reaction tube and has been found to economize cost, labor, time, sample number and experiment frequency. Probiotic research, more specifically the use of LAB probiotics in the Japanese pufferfish remains unaccomplished compared with the studies involving other fish species. Lately, 10 LAB strains have been isolated from traditional Mongolian dairy products and their probiotic function was tested in mice [25]. Subsequently, oral administration of these heat-killed LAB strains exhibited immunomodulatory activity in influenza virus (IFV) infected mice, resulting in alleviation of IFV infection by activation of IL-12 and IFN-γ production following enhancement of NK cell activity [16]. These findings strongly indicated the potential immunostimulatory effects of these heat-inactivated LAB strains through their interaction with cytokines and receptors. As the information on IFN-mediated antiviral immunity regulated by IL-12 and IL-18 in response to probiotic treatment is non-existent in fish, we investigated these gene expressions including other cytokines. To evaluate the immunostimulatory function of a novel immune modulating substance, firstly an *in vitro* experiment is conducted using cells isolated from immune-competent organs. Therefore, our study aimed to analyze the expression level of 16 cytokine genes using the multiplex RT-PCR assay in the Japanese pufferfish head kidney (HK) cells induced with two heat-killed LAB probiotics isolated from the Mongolian dairy products. Finally, this experiment evaluated the immunostimulatory effects of these two probiotic strains through elicitation of cytokine gene responses.

2. Materials and methods

2.1. LAB strains

Two LAB strains, namely *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) (Lpp) and *L. plantarum* (strain 06CC2) (Lp) isolated and identified previously from the Mongolian fermented camel milk (Tarag) and cow milk cheese (Aaruul), respectively, were cultured in Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h as described [25]. They were 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 LAB were washed again with PBS and lyophilized. The lyophilized Lpp and Lp powders containing 1.1×10^{11} and 2.4×10^{11} cells g^{-1}, respectively, were suspended in PBS for treatment of fish HK cells.

2.2. Experimental fish

Japanese pufferfish, *T. rubripes* (body weight, 205 ± 8 g) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan. Fish were first acclimatized in an aerated seawater tank at 20 °C and fed a commercial diet (Sango, Higashimaru Co. Ltd., Kagoshima, Japan) at 1% body weight per day for two weeks under a natural photoperiod prior to their use in the study. The health status of experimental fish was checked by examining presence of any abnormal lesions or parasites on body surfaces and internal organs of few randomly sampled animals and culturing smears from slimes, head kidney, spleen and blood from the same fish on Marine Agar Broth 2216E (Difco, Detroit, Michigan, USA) for existence of any bacterial pathogens. The results showed presence of no pathogenic bacteria in fish. Fish handling and experimental procedure were conducted in accordance with the guidelines for the care and use of laboratory animals of the University of Miyazaki.

2.3. Isolation of HK cells

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. Pufferfish HK cells were isolated following the method described elsewhere [26]. Briefly, HK tissue was aseptically excised from freshly euthanized pufferfish ($n = 5$) and gently pushed through a 100-μm nylon mesh (John Staniar, Whitefield, Manchester, UK) 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). After washing with the above medium and depletion of erythrocytes, the cells were then pushed again through a 40-μm nylon mesh cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Finally, the number of prepared cells was adjusted to 1×10^7 cells mL^{-1}.

2.4. In vitro stimulation of HK cells and isolation of total RNA

In order to determine if LAB strains can elicit different transcriptional profiles of the cytokine genes, we used heat-inactivated form of two strains isolated from Mongolian dairy products as prepared in Section 2.1. Two milliliters of the HK cell suspension in RPMI 1640 medium (Invitrogen) supplemented with 5% FBS and 1% streptomycin/penicillin (Invitrogen) (see Section 2.3) was placed in each well of a 24-well plate (Nunc A/S, Roskilde, Denmark), stimulated with the heat-killed bacteria (Lpp and Lp) at 20 μg mL^{-1} and incubated for 1, 4, 8, 12, 24, and 48 h at 25 °C. Cells collected at 0 h without stimulation served as a control for the experiment. Each treatment (stimulation) and control had three replicates. Sampling of incubated cells was conducted at the time points mentioned above and the cells were stored at −80 °C prior to RNA extraction. Total RNA was extracted from the stimulated HK cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. To avoid the presence of 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).

2.5. GeXPS primer design, multiplex RT-PCR and capillary electrophoresis

Primer design (16-cytokine plex) and multiplex analysis were conducted using the multiple assay panel reported previously [24]. RT to cDNA was performed using reverse primer set mix (Supplementary Table 1), reverse transcriptase, RNase inhibitor, Kan^r RNA and 1× RT Master Mix Buffer supplied in the GenomeLab GeXP Start Kit (Beckman Coulter) according to manufacturers' protocols. RT reaction mixtures were incubated at 48 °C for 1 min, followed by 42 °C for 60 min and then 95 °C for 5 min. Subsequent to RT, PCR was carried out with each reaction containing 9.3 μL RT reaction mixture, 0.02 μM forward primer set mix, 5 mM $MgCl_2$, 3.5 U Thermo Start *Taq* DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA, USA), and 1× PCR Master Mix Buffer (GenomeLab GeXP Start Kit; Beckman Coulter) containing 10 mM HCl, 50 mM KCl, 0.3 mM of each dNTP, 0.02 μM Kan^r gene PCR forward primer, 1 μM universal reverse primer, and 1 μM D4-labeled universal forward primer. The cycling conditions for PCR were as follows: one cycle of 95 °C for 10 min, 45 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min. The PCR products from multiplex RT-PCR were then prepared for capillary electrophoresis by adding 1 μL of each reaction to its corresponding well in a 96-well electrophoresis micro-plate containing 38.5 μL of sample loading solution and 0.5 μL of DNA size standard-400 (GenomeLab GeXP Start Kit; Beckman Coulter). The samples were mixed well by pipetting up and down. The micro-plate containing samples mix was then covered with aluminum foil lid and centrifuged at 2000 rpm for 1 min to consolidate the liquid to the bottom of the wells. Finally, each well was overlaid with one drop of mineral oil (GenomeLab GeXP Start Kit; Beckman Coulter). The samples were then placed in a GeXP Genetic Analysis System for capillary electrophoresis and fragment size analysis.

2.6. Multiplex data analysis

The GeXP Genetic Analysis system matched each PCR product based on size by capillary gel electrophoresis (CEQ8000 Automated Sequencer; Beckman Coulter) with the appropriate gene and measured their dye signal strength in arbitrary units of optical fluorescence, defined as the fluorescent signal minus background. Next, the data was normalized to the external synthetic reference control transcript, kanamycin using GeXP profiler (eXpress Analysis) software, with the area-under-the-curve set to 1. This step minimizes inter-capillary variation. The relative expression level of each cytokine gene was calculated by normalization to the reference gene, β-actin using GeXP Quant Tool. Differences between the quantified expressions of a particular gene in stimulated HK cells at different time ponits and in untreated control cells were evaluated with one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for their comparison. Statistical analysis was performed using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL). All data are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Optimization and preparation of the multiplex GeXP 16-cytokine plex

Prior to preparation of a multiplex primer mix, quality of each gene-specific primer pair was tested in a singlet RT-PCR reaction (single-plex). This determined that a single peak of the expected size was generated, with no nonspecific fragments produced. A multiplex primer mix of selected cytokine gene-specific primers (Supplementary Table 1) was prepared for analysis. As different gene targets including the housekeeping reference genes (β-actin and GAPDH) yielded low or high signal peaks in the multiplexed GeXP assay, the concentrations of gene-specific reverse primers were increased or decreased to improve detection in multiplex reactions. Finally, the 16-cytokine primer plex was prepared with the selected primer concentrations of reverse primers giving optimal signal detection (Supplementary Table 1).

3.2. Expression of cytokine genes in stimulated HK cells

Table 1 displays the summary of the transcriptional responses of HK cells stimulated with the two heat-killed probiotic bacteria isolated from Mongolian dairy products.

3.2.1. Pro-inflammatory cytokines

IL-1β was constitutively expressed in the HK cells of pufferfish and its transcript levels were significantly enhanced by both the heat-killed bacteria (Fig. 1A). Cells stimulated with *L. plantarum* (Lp) showed significantly higher expression level ($P < 0.05$) of IL-1β (over 10-fold) gene all through the time points. However, compared to the control there was a significant increase ($P < 0.05$) in the expression level of IL-1β in cells induced with *L. paracasei* spp. *paracasei* (Lpp) at 1, 4, 8, and 12 h post-stimulation (hps). The pro-inflammatory IL-6 was higher expressed in HK cells stimulated with Lpp only at 4 hps, while significant increased transcript level ($P < 0.05$) of this gene was observed in Lp stimulation at all the time points with over 100-fold higher expression at 12 and 24 hps compared to the control (Fig. 1B). Another pro-inflammatory cytokine, IL-17A/F-3 exhibited significantly increased expression ($P < 0.05$) in HK cells stimulated with heat-killed Lpp at all the time points except 48 hps (Fig. 1C). However, stimulation of cells with Lp did not cause higher expression of this gene. Similar pattern of significantly higher expression ($P < 0.05$) of TNF-α and TNF-N was exhibited by HK cells stimulated with Lp at all the time points. However, Lpp stimulation also caused significantly increased ($P < 0.05$) TNF-α expression at all the time points excluding 8 hps compared to that of control (Fig. 1D). Although the transcript level of TNF-N was low in cells treated with Lpp compared to Lp stimulated cells, it was below detection level in unstimulated control cells at 0 h (Fig. 1E).

Table 1
Summary of the transcriptional responses of HK cells stimulated with heat-killed probiotic bacteria isolated from Mongolian diary products.

Genes	*Lactobacillus paracasei* spp. *paracasei* (06TCa22)						*Lactobacillus plantarum* (06CC2)					
	Hours post-stimulation						Hours post-stimulation					
	1	4	8	12	24	48	1	4	8	12	24	48
A. Pro-inflammatory cytokines												
1. IL-1β	↑↑↑	↑↑↑	↑↑↑	↑↑↑	–	–	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
2. IL-6	–	↑↑↑	–	–	–	–	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑
3. IL-17A/F-3	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	–	–	–	–	–	–	–
4. TNF-α	↑↑↑	↑↑	–	↑↑↑	↑↑	↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
5. TNF-N	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑↑
B. Cell-mediated immune regulators												
1. IL-12p35	–	–	–	–	↑↑↑	–	–	–	–	–	–	–
2. IL-12p40	↑↑↑	↑↑↑	–	–	–	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
3. IL-18	↑↑	↑↑	–	–	–	–	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑
C. Antiviral cytokines												
1. I-IFN-1	↑↑	↑↑	–	–	–	–	↑↑	–	–	–	–	↑↑
2. IFN-γ	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	–	–	–	–	–	–	–
D. Other regulatory cytokines												
1. IL-2	↑↑	–	–	–	–	–	↑↑	–	–	↑↑	↑↑	↑↑
2. IL-7	↑↑	–	–	–	–	↑↑	–	–	–	↑↑	↑↑	↑↑↑
3. IL-15	–	–	–	–	–	–	–	↑↑	↑↑	↑↑	–	↑↑
4. IL-21	↑	–	–	–	–	–	↑↑	–	–	–	↑↑	↑↑
5. IL-10	↑↑↑	↑↑↑	–	–	–	–	–	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
6. TGF-β1	↑↑	–	–	–	–	–	–	↑↑	–	–	–	–

↑ = Significantly higher compared to control (0 h) with expression level between 0 and 1.
↑↑ = Significantly higher compared to control (0 h) with expression level between 1 and 5.
↑↑↑ = Significantly higher compared to control (0 h) with expression level more than 5.
– = Not significantly different from control (0 h).

3.2.2. Cell-mediated immune regulators

Significantly increased expression (almost 50-fold) of IL-12p35 was detected in HK cells incubated with Lpp only at 24 hps ($P < 0.05$). However, higher expression compared to the control was observed at all the time points in stimulation with both the heat-killed bacteria (Fig. 2A). IL-12p40 transcript level was consistently enhanced by Lp stimulation at all the time points, but not by Lpp treatment which induced significantly higher expression ($P < 0.05$) at 1, 4 and 48 hps (Fig. 2B). IL-18 gene was constitutively expressed in the HK cells of pufferfish and its transcript levels were significantly enhanced by both the heat-killed bacteria (Fig. 2C). Cells stimulated with *L. plantarum* (Lp) showed significantly higher expression level ($P < 0.05$) of IL-18 (over 3-fold) gene all through the time points. However, compared to the control there was a significant increase in the expression level ($P < 0.05$) of IL-18 in cells induced with *L. paracasei* spp. *paracasei* (Lpp) at 1, and 4 hps.

3.2.3. Antiviral cytokines

Significantly higher expression level ($P < 0.05$) of I-IFN-1 was detected in cells incubated with Lpp at 1, and 4 hps and with Lp at 1, and 48 hps (Fig. 3A). However, significantly lower ($P > 0.05$) transcript level of this gene was observed at 8, 12, 24, and 48 hps in Lpp stimulation and at 4, 8, and 12 hps in Lp treatment compared to that of control. IFN-γ expression was significantly increased ($P < 0.05$) by heat-killed Lpp stimulation at all the time points excluding 48 hps (Fig. 3B). Similar expression profile compared with control was observed in HK cells treated with Lp at all the time points.

3.2.4. Other regulatory cytokines

Incubation with heat-killed Lpp caused a significant increase ($P < 0.05$) in IL-2 expression only at 1 hps, whereas significantly higher expression ($P < 0.05$) of IL-2 gene was observed at 1, 12, 24 and 48 hps in HK cells stimulated with Lp. In case of IL-7 gene, higher expression compared to control was exhibited at 1, and 48 hps and 12, 24, and 48 hps in cells stimulated with Lpp and Lp, respectively. However, IL-7 transcript in Lp treated cells was a whopping 25 times higher at 48 hps compared with that of control cells. Cells incubated with heat-killed Lpp showed a significant decrease ($P > 0.05$) in IL-15 expression at all the time points, while elevated transcript level of this gene was detected in HK cells treated with Lp at 4, 8, 12 and 48 hps. Similar to IL-2, the expression level of IL-21 was significantly enhanced ($P < 0.05$) by Lpp treatment only at 1 hps. However, at 1, 24 and 48 hps, its expression was elevated by Lp. Almost similar expression pattern related to IL-6 was recorded for the anti-inflammatory IL-10 gene with the enhanced expression level at 1 and 4 hps by Lpp treatment and at all the time points except 1 hps by Lp stimulation. TGF-β1 was significantly expressed ($P < 0.05$) in HK cells exposed to heat-killed Lpp and Lp at 1 and 4 hps, respectively. However, significantly lower expression level ($P > 0.05$) of this gene was observed at 8, 12, 24 and 48 hps in both Lpp and Lp stimulated cells compared to that of control cells.

4. Discussion

In this study, we have demonstrated that bacteria isolated from the Mongolian dairy products can regulate expression of 16 cytokine genes in HK cells of the Japanese pufferfish using the GeXPS multiplex RT-PCR assay. The targeted genes were from different cytokine categories, namely IL-1 subfamily, IL-2 subfamily, pro-inflammatory IL-6 and IL-17A/F-3, ant-inflammatory IL-10, heterodimeric IL-12, TNF superfamily, IFN family and TGF. The up-regulated cytokine genes in the HK cells following stimulation with two heat-killed probiotic strains indicated the immunostimulatory effects of these bacteria. Previously, oral administration of the same heat-inactivated strains exhibited immunomodulatory activity through elicitation of intestinal immunity in mice [16]. Here, the increased transcription of cytokine genes could provide an early protective immunity in the pufferfish during infection with pathogens.

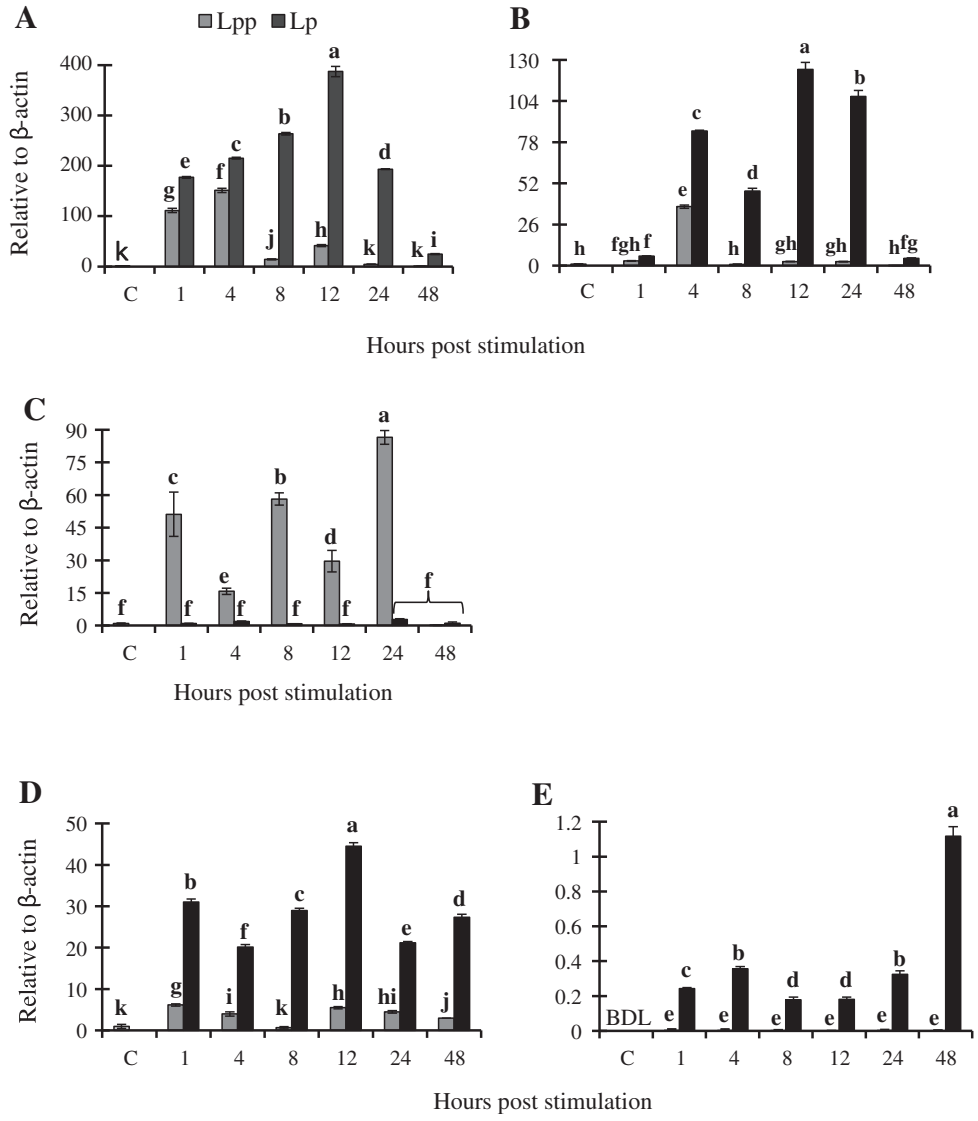

Fig. 1. Relative expression profiles (mean ± SD; $n = 3$) of pro-inflammatory cytokine genes (A. IL-1β; B. IL-6; C. IL-17A/F-3; D. TNF-α; E. TNF-N) in HK cells stimulated with two heat-killed probiotic bacteria, *Lactobacillus paracasei* spp. *paracasei* 06TCa22 (Lpp) and *L. plantarum* 06CC2 (Lp). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells at various time points and in unstimulated cells at 0 h (C). BDL; below detection level.

4.1. Pro-inflammatory cytokines

The pro-inflammatory IL-1β is a key mediator in response to microbial invasion and tissue injury and can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines that can activate macrophages, NK cells and lymphocytes [27]. A distinguishable up-regulation of IL-1β gene (over 100-fold increase) was observed in both the probiotics-treated HK cells at the immediate phase of incubation (1 and 4 hps) compared to control cells. The involvement of this cytokine at very early stage of immune response was confirmed in Atlantic cod (*Gadus morhua*) HK leukocytes incubated with live and heat-inactivated intestinal bacteria [28]. Therefore, the acute IL-1β expression observed in the present study would have many downstream effects including release of other cytokines [29]. IL-6, another pro-inflammatory cytokine released during the cytokine cascade following bacterial infection, plays a key role in cellular and tissue homeostasis through activation of target genes involved in growth, differentiation, survival, apoptosis, and proliferation [30]. Increased expression of IL-6 in HK cells at 4 hps with Lpp treatment and at all the time points with Lp stimulation in this study indicated the immunostimulating functions of these two probiotic strains. The pro-inflammatory Th17 cytokine, IL-17A/F-3 gene was up-regulated following Lpp stimulation, which is in agreement with the observation of Korenaga et al. [31], who reported higher expression in the pufferfish HK cells induced with LPS at 4 and 12 hps. TNFs are pro-inflammatory cytokines involved in inflammation, apoptosis, cell proliferation and stimulation of various aspects of the immune system [32]. In our experiment, expression of TNF-α and the fish-specific ligand TNF-N genes was enhanced following stimulation with both Lpp and Lp. Similarly, an increase in TNF-α gene expression was reported in the HK of rainbow trout (*Oncorhynchus mykiss*) fed the probiotics, *Canrobacterium maltaromaticum*, and *C. divergens* [23], *L. plantarum* [20] and *L. rhamnosus* [19]. Expression of TNF-N was enhanced in pufferfish HK cells stimulated with either polyI:C or imiquimod [24]. In higher animals, IL-1β expression was induced by *L. acidophilus*, *L. reuteri* and *L. salivarius* in spleen and cecal tonsil cells of chicken [33] and by *L. acidophilus* and *L. plantarum* in human peripheral blood mononuclear cells, PBMC [34]. *L. acidophilus* caused higher production of IL-6 and TNF-α in

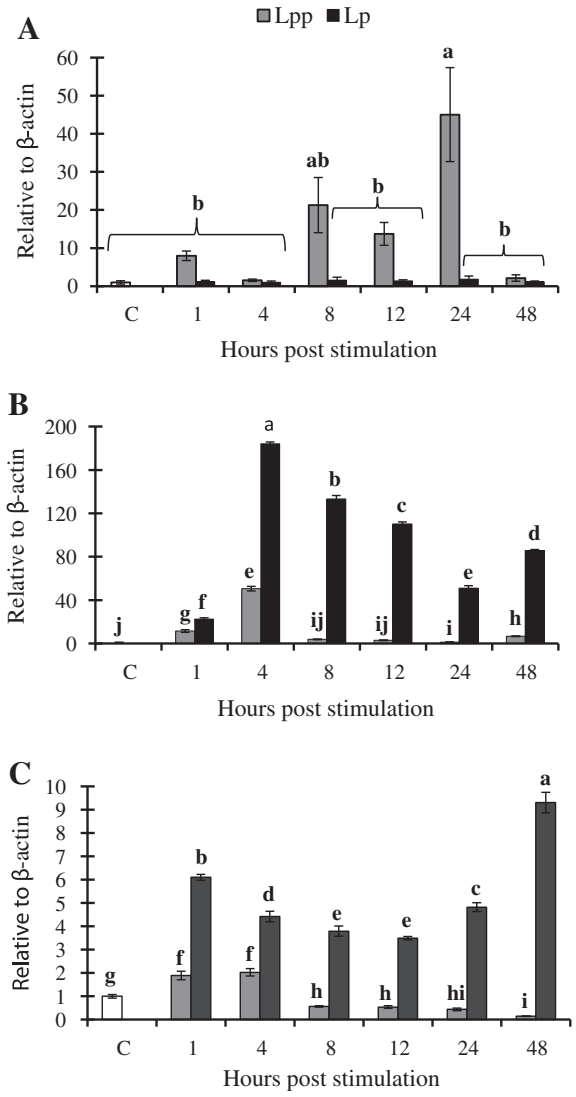

Fig. 2. Relative expression profiles (mean ± SD; $n = 3$) of cell-mediated immune factor genes (A. IL-12p35; B. IL-12p40; C. IL-18) in HK cells stimulated with two heat-killed probiotic bacteria, *L. paracasei* spp. *paracasei* 06TCa22 (Lpp) and *L. plantarum* 06CC2 (Lp). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells at various time points and in unstimulated cells at 0 h (C).

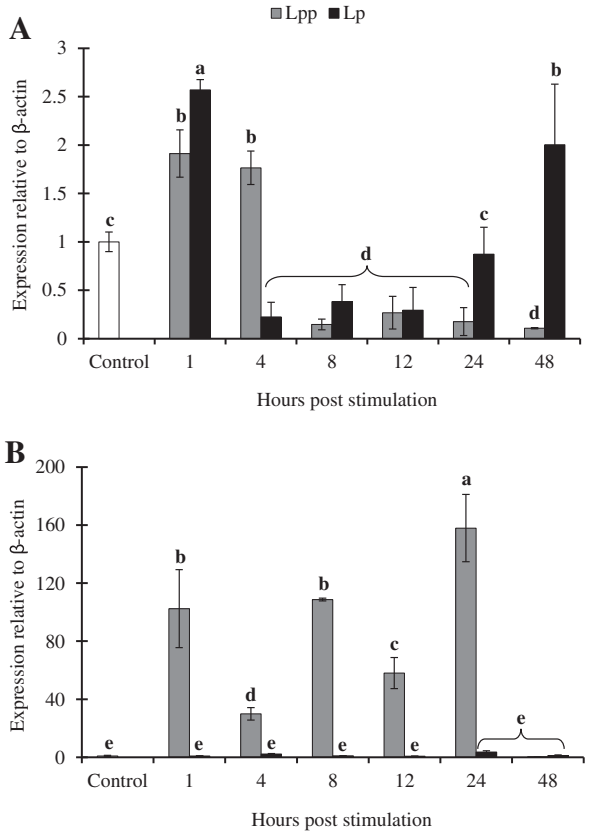

Fig. 3. Relative expression profiles (mean ± SD; $n = 3$) of antiviral cytokine genes (A. I-IFN-1; B. IFN-γ) in HK cells stimulated with two heat-killed probiotic bacteria, *L. paracasei* spp. *paracasei* 06TCa22 (Lpp) and *L. plantarum* 06CC2 (Lp). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells at various time points and in unstimulated cells at 0 h (control).

murine macrophages [35]. In human PBMC macrophages, IL-17 release was elevated by LAB strains both in live and heat-killed forms [36]. Our results coupled with these reports, suggest the capability of LAB probiotics in elicitation of pro-inflammatory cytokine responses.

4.2. Cell-mediated immune regulators

IL-12, a regulator of cell-mediated immune responses, provides defense against parasites, viruses and intracellular bacteria by stimulating the production of IFN-γ by Th1 cells and NK cells [37]. IL-12 is active in its heterodimeric form, which is composed of two covalently linked peptide chains: a 35-kDa chain termed IL-12p35 (or IL-12α) and a 40-kDa chain termed IL-12p40 (or IL-12β) [38]. In the present study, the expression of IL-12p35 and IL-12p40 genes at higher levels in heat-killed probiotics-stimulated HK cells compared with untreated control cells indicates co-expression of these subunits in the same cell to generate the bioactive IL-12 [39]. Previously, heat-killed *Lactococcus lactis* subsp. *lactis* and *L. plantarum* isolated from Mongolian dairy products were found to induce large quantity production of IL-12 in spleen cells [15] and bronchoalveolar lavage fluid [16] of mice, respectively. Therefore, these observations along with our results of elevated expression suggest that IL-12 may also play a pivotal role in inducing antibacterial and antiviral immune responses in the Japanese pufferfish. IL-18, a pleiotrophic cytokine produced by monocytes/macrophages, like IL-12 plays a major role in cell-mediated immune responses and it is also important for the clearance of intracellular pathogens through the induction of IFN-γ, and of viruses by activation of cytotoxic T cells [40]. A slight but significant increase in expression of the IL-18 gene was observed at 1–4 hps with Lpp. In contrast, stimulation with Lp induced consistently higher expression of this gene at all the time points. Similarly, *L. rhamnosus* HN001 was shown to induce IL-12 and IL-18 following direct *in vitro* co-culture with mice spleen macrophages [41]. Therefore, it is indicated that the two heat-killed probiotic strains used in this study are capable of directly stimulating pro-IFN-γ monokines, IL-12 and IL-18 which induce high level IFN-γ production [40].

4.3. Antiviral cytokines

IFNs induce an antiviral state of cells and contribute to the defense against virus infection in vertebrates. Three IFN subfamilies include type I, II and III. The type I (mainly IFN-α and IFN-β) and III IFNs have a major role in the first line of defense against viruses, whereas type II IFN is identical to IFN-γ, which plays a major role in innate, as well as in adaptive cell-mediated immune responses for

removal intracellular pathogens and tumor control [29]. We found that type I-IFN-1 gene expression was induced by both the heat-killed probiotic bacteria, whereas IFN-γ gene expression was up-regulated only by Lpp. Therefore, these immunostimulant treatments may provide protection against virus infection and also might have elevated the IFN-γ mediated intracellular pathogen-killing ability of leukocytes in the Japanese pufferfish. Higher level of IFN-γ induction may be the consequence of combined effects of activated IL-12 and IL-18. Consistent to our results, up-regulated expression of IFN gene expression was noticed in the HK and spleen of rainbow trout fed the probiotic, *L. rhamnosus* for 30 days [19]. In human, *L. acidophilus* [42] and *L. plantarum* [34] stimulated higher IFN-γ production in PBMCs, and *L. salivarius* led to a sustained increment in production of IFN-γ in macrophage-like cell lines [43]. However, Lp in our study could not mount IFN-γ induction, which is contrary to the ability of IFN-γ stimulation by the same strain in IFV infected mice [16]. This observed larger variation in IFN-γ modulation was strictly strain dependent and could be of practical importance for the screening of new probiotic strains. Furthermore, the up-regulation of IFN-γ gene in our study may be induced by the elevated IL-15, as a positive feedback loop exists between these two cytokines [44].

4.4. Other regulatory cytokines

The IL-2 subfamily members, known as gamma chain (γC or CD132) cytokines, include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 in mammals and except IL-19, all of these molecules have been discovered in fish [38]. IL-2, IL-4, IL-9 and IL-21 are released from Th cells that affect their responses, whilst IL-7 and IL-15 are particularly involved in the maintenance of T cell memory [45]. In the present study, a significant increase in expression of the IL-2 gene was observed following stimulation with Lpp at 1 hps and Lp at 1, 12, 24 and 48 hps. IL-15 gene was significantly up-regulated by the treatment of Lp only at 4, 8, 12 and 48 hps. Expression of the IL-21 gene was enhanced by the stimulation with Lpp at 1 hps and Lp at 1, 24 and 48 hps. Expression of these genes, in our study, was consistent till 48 hps mainly in Lp stimulation. Therefore, the results indicate that induction of these gene expression in HK cells persists for longer duration compared to the previous reports of expression at 1, 3 and 6 [46] or 4 hps [47]. However, higher expression of IL-2 was observed at 72 hps in pufferfish T cell enriched cultures isolated from peripheral blood leukocytes (PBL) [48]. The present information will be useful in predicting T and B cells survival and proliferation in fish after immune-stimulation. In the current study, increased expression of IL-7 was observed at 1 hps in stimulation with Lpp and at 12, 24 and 48 hps with Lp. This late and longer duration induction of IL-7 may contribute to the development of non-regulatory T cells and survival and function of naive and memory T cells [49]. The anti-inflammatory IL-10 is multi-functional with cytokine synthesis inhibitory and immunosuppressive functions [50]. Expression of the gene encoding IL-10 was higher (over 15-fold relative to the control) in HK cells stimulated with Lpp but not with Lp. Similarly, an increase in IL-10 gene expression was reported in the HK of rainbow trout fed the probiotic, *L. plantarum* [20]. In contrast to our observation, dietary administration of LAB probiotic, *L. delbrueckii* ssp. *delbrueckii* to European sea bass (*Dicentrarchus labrax*) larvae induced a decrease in IL-10 gene expression [21]. Similarly, IL-10 was significantly up-regulated in colitis mouse challenged with *L. plantarum* [51]. It is therefore possible that the up-regulated function of IL-10 might have controlled the regulation of the inflammatory response induced by the stimulated pro-inflammatory cytokines, thereby minimizing damage to the host due to an excessive response [52]. Furthermore, in this study, the induction of this cytokine was found to be strain dependent. TGF-β1 is a potent regulatory cytokine with a variety of cellular functions, such as regulation of cell proliferation, differentiation, migration, and apoptosis under both physiological and pathological conditions [53]. Although we observed enhanced expression of the TGF-β1 gene in HK cells at 1 hps with Lpp and 4 hps with Lp, the expression level decreased compared to the control at other post-stimulation time points. In the line with our findings, higher expression of TGF-β1was observed in the HK and spleen of rainbow trout fed the probiotics, *L. rhamnosus* for 30 days [19] and *L. rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* for 45 days [18]. Therefore, our results suggest that TGF-β1 induction may be acute but inconsistent and the enhanced activation of TGF-β1 in conjugation with the stimulated IL-10 in the pufferfish HK cells could possibly have had a suppressive effect on T cell proliferation [54]. In the present study, we recorded differential effects of the two LAB strains especially on the induction of IL-2 subfamily and anti-inflammatory (IL-10 and TGF-β1) cytokines and the strain Lp induced them more consistently compared to Lpp. These strain-dependent varied cytokine responses may be related to functional and constituent differences of these two strains and these differences are caused by genetic, nutritional and environmental factors and more importantly by the origin of the strains, as Lpp is from fermented camel milk and Lp is from cow milk cheese.

5. Conclusion

Our findings provide a comparative understanding on the immunomodulatory functions of two probiotic strains isolated from the Mongolian dairy products by examining their role in transcriptional responses of 16 cytokines in the HK cells of the Japanese pufferfish. Despite the differences in the transcriptional profiles, increased or induced expression of the cytokine genes proves the potentiality of these two probiotics as novel immunostimulants to fish. However, strain-dependent varied expression of important cytokines (cell-mediated immune regulators, antiviral and anti-inflammatory cytokines) suggests better efficacy of *L. paracasei* spp. *paracasei* 06TCa22 strain as fish immunostimulant. Further *in vivo* studies to elucidate the cytokine regulation networks will validate our present observations. A careful evaluation of ant-inflammatory properties may be undertaken using single strain to affirm the immunostimulatory capability. Moreover, application timings and frequency to assess the longevity of immunostimulant effects and to make the application cost-effective need to be evaluated before any practical use in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.fsi.2013.01.024.

References

[1] Savan R, Sakai M. Genomics of fish cytokines. Comp Biochem Physiol D Genomics Proteomics 2006;1:89–101.
[2] Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. J Allergy Clin Immunol 2011;127:701–21.
[3] Chen Q, Carroll HP, Gadina M. The newest interleukins: recent additions to the ever-growing cytokine family. Vitam Horm 2006;74:207–28.
[4] Corripio-Miyar Y, Bird S, Tsamopoulos K, Secombes CJ. Cloning and expression analysis of two pro-inflammatory cytokines, IL-1 beta and IL-8, in haddock (*Melanogrammus aeglefinus*). Mol Immunol 2007;44:1361–73.
[5] Igawa D, Sakai M, Savan R. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Mol Immunol 2006;43:999–1009.
[6] Kono T, Bird S, Sonoda K, Savan R, Secombes CJ, Sakai M. Characterization and expression analysis of an interleukin-7 homologue in the Japanese pufferfish, *Takifugu rubripes*. FEBS J 2008;275:1213–26.
[7] Ohtani M, Hayashi N, Hashimoto K, Nakanishi T, Dijkstra JM. Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. Immunogenetics 2008;60:383–97.
[8] Wen Y, Shao JZ, Xiang LX, Fang W. Cloning, characterization and expression analysis of two *Tetraodon nigroviridis* interleukin-16 isoform genes. Comp Biochem Physiol B, Biochem Mol Biol 2006;144:159–66.
[9] Savan R, Kono T, Igawa D, Sakai M. A novel tumor necrosis factor (TNF) gene present in tandem with theTNF-alpha gene on the same chromosome in teleosts. Immunogenetics 2005;57:140–50.
[10] Zou J, Yoshiura Y, Dijkstra JM, Sakai M, Ototake M, Secombes C. Identification of an interferon gamma homologue in Fugu, *Takifugu rubripes*. Fish Shellfish Immunol 2004;17:403–9.
[11] Zou J, Tafalla C, Truckle J, Secombes CJ. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. J Immunol 2007;179:3859–71.
[12] Yang M, Zhou H. Grass carp transforming growth factor-β1 (TGF-β1): molecular cloning, tissue distribution and immunobiological activity in teleost peripheral blood lymphocytes. Mol Immunol 2008;45:1792–8.
[13] Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biocontrol agents in aquaculture. Microbiol Mol Biol Rev 2000;64:655–71.
[14] Vijayabaskar P, Somasundaram ST. Isolation of bacteriocin producing lactic acid bacteria from fish gut and probiotic activity against common fresh water fish pathogen *Aeromonas hydrophila*. Biotechnol 2008;7:124–8.
[15] Kimura M, Danno K, Yasui H. Immunomodulatory function and probiotic properties of lactic acid bacteria isolated from Mongolian fermented milk. Biosci Microflora 2006;25:147–55.
[16] Takeda S, Takeshita M, Kikuchi Y, Dashnyam B, Kawahara S, Yoshida H, et al. Efficacy of oral administration of heat-killed probiotics from Mongolian dairy products against influenza infection in mice: alleviation of influenza infection by its immunomodulatory activity through intestinal immunity. Int Immunopharmacol 2011;11:1976–83.
[17] Balcázar JL, de Blas I, Ruiz-Zarzuela I, Vendrell D, Gironés O, Muzquiz JL. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). FEMS Immunol Med Microbiol 2007;51:185–93.
[18] Panigrahi A, Kiron V, Satoh S, Hirono I, Kobayashi T, Sugita H, et al. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev Comp Immunol 2007;31:372–82.
[19] Panigrahi A, Kiron V, Satoh S. Real-time quantification of immune gene expression in rainbow trout fed different forms of probiotic bacteria *Lactobacillus rhamnosus*. Aqua Res 2011;42:906–17.
[20] Pérez-Sánchez T, Balcázar JL, Merrifield DL, Carnevali O, Gioacchini G, de Blas I, et al. Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection. Fish Shellfish Immunol 2011;31:196–201.
[21] Picchietti S, Fausto AM, Randelli E, Carnevali O, Taddei AR, Buonocore F, et al. Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.). Fish Shellfish Immunol 2009;26:368–76.
[22] Pirarat N, Pinpimai K, Endo M, Katagir T, Ponpornpisit A, Chansue N, et al. Modulation of intestinal morphology and immunity in nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. Res Vet Sci 2011;91:e92–7.
[23] Kim DH, Austin B. Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics. Vet Immunol Immunopathol 2006;114:297–304.
[24] Kono T, Takayama H, Nagamine R, Korenaga H, Sakai M. Establishment of a multiplex RT-PCR assay for the rapid detection of fish cytokines. Vet Immunol Immunopathol 2013;151:90–101.
[25] Takeda S, Yamasaki K, Takeshita M, Kikuchi Y, Tsend-Ayush C, Dashnyam B, et al. The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. Animal Sci J 2011;82:571–9.
[26] Kono T, Hamsuna A, Korenaga H, Lizasa T, Nagamine R, Ida T, et al. The role of neuromedin U during inflammatory response in the common carp. Fish Shellfish Immunol 2012;32:151–60.
[27] Low C, Wadsworth S, Burrells C, Secombes CJ. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture 2003;221:23–40.
[28] Lazado CC, Caipang CMA, Gallage S, Brinchmann MF, Kiron V. Expression profiles of genes associated with immune response and oxidative stress in Atlantic cod, *Gadus morhua* head kidney leukocytes modulated by live and heat-inactivated intestinal bacteria. Comp Biochem Physiol Part B 2010;155:249–55.
[29] Biswas G, Korenaga H, Takayama H, Kono T, Shimokawa H, Sakai M. Cytokine responses in the common carp, *Cyprinus carpio* L. treated with baker's yeast extract. Aquaculture 2012;356–357:169–75.
[30] Fischer P, Hilfiker-Kleiner D. Survival pathways in hypertrophy and heart failure: the gp130-STAT axis. Basic Res Cardiol 2007;102:393–411.
[31] Korenaga H, Kono T, Sakai M. Isolation of seven IL-17 family genes from the Japanese pufferfish *Takifugu rubripes*. Fish Shellfish Immunol 2010;28:809–18.
[32] Savan R, Sakai M. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L): genomic and expression analysis. Fish Shellfish Immunol 2004;17:87–94.
[33] Brisbin JT, Gong J, Parvizi P, Sharif S. Effects of Lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin Vaccine Immunol 2010;17:1337–43.
[34] Vissers YM, Snel J, Zuurendonk PF, Smit BA, Wichers HJ, Savelkoul HFJ. Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells. FEMS Immunol Med Microbiol 2010;59:60–70.
[35] Morita H, He F, Fuse T, Ouwehand AC, Hashimoto H, Hosoda M, et al. Cytokine production by the murine macrophage cell line J774.1 after exposure to Lactobacilli. Biosci Biotechnol Biochem 2002;66:1963–6.
[36] Donkor ON, Ravikumar M, Proudfoot O, Day SL, Apostolopoulos V, Paukovics G, et al. Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure. Clin Exp Immunol 2012;167:282–95.
[37] Øvergård A, Nepstad I, Nerland AH, Patel S. Characterization and expression analysis of the Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines: IL-1β, IL-6, IL-11, IL-12β and IFNγ. Mol Biol Rep 2012;39:2201–13.
[38] Secombes CJ, Wang T, Bird S. The interleukins of fish. Dev Comp Immunol 2011;35:1336–45.
[39] Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc Natl Acad Sci U S A 1991;88:4143–7.
[40] Arend WP, Palmer G, Gabay C. IL-1, IL-18, and IL-33 families of cytokines. Immunol Rev 2008;223:20–38.
[41] Cross ML, Mortensen RK, Kudsk J, Gill HS. Dietary intake of *Lactobacillus rhamnosus* HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice. Med Microbiol Immunol 2002;191:49–53.
[42] Gackowska L, Michalkiewicz J, Krotkiewski M, Helmin-Basa A, Kubiszewska I, Dzierzanowska D. Combined effect of different lactic acid bacteria strains on the mode of cytokines pattern expression in human peripheral blood mononuclear cells. J Physiol Pharmacol 2006;57(Suppl. 9):13–21.
[43] Drago L, Nicola L, Iemoli E, Banfi G, De Vecchi E. Strain-dependent release of cytokines modulated by *Lactobacillus salivarius* human isolates in an *in vitro* model. BMC Res Notes 2010;3:44.
[44] Wang T, Holland J, Bols N, Secombes CJ. Molecular and functional characterization of interleukin-15 in rainbow trout *Oncorhynchus mykiss*: a potent inducer of interferon-gamma expression in spleen leucocytes. J Immunol 2007;179:1475–88.
[45] Osborne LC, Abraham N. Regulation of memory T cells by gamma c cytokines. Cytokine 2010;50:105–13.
[46] Bei JX, Suetake H, Araki K, Kikuchi K, Yoshiura Y, Lin HR, et al. Two interleukin (IL)-15 homologues in fish from two distinct origins. Mol Immunol 2006;43:860–9.
[47] Bird S, Zou J, Kono T, Sakai M, Dijkstra H, Secombes CJ. Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, *Fugu rubripes*, following their discovery by synteny. Immunogenetics 2005;56:909–23.
[48] Sugamata R, Suetake H, Kikuchi K, Suzuki Y. Teleost B7 expressed on monocytes regulates T cell responses. J Immunol 2009;182:6799–806.
[49] Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol 2000;1:426–32.
[50] Savan R, Igawa D, Sakai M. Cloning, characterization and expression analysis of interleukin-10 from the common carp *Cyprinus carpio* L. Eur J Biochem 2003;270:4647–54.
[51] Duary RK, Bhausaheb MA, Batish VK, Grover S. Anti-inflammatory and immunomodulatory efficacy of indigenous probiotic *Lactobacillus plantarum* Lp91 in colitis mouse model. Mol Biol Rep 2012;39:4765–75.
[52] Raida MK, Buchmann K. Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. Fish Shellfish Immunol 2008;25:533–41.
[53] Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99–146.
[54] Tang H, Guo Z, Zhang M, Wang J, Chen G, Cao X. Endothelial stroma programmes hematopoietic stem cells to differentiate into regulatory dendritic cells through IL-10. Blood 2006;108:1189–97.