FULL PAPER *Immunology*

Protection against Atypical *Aeromonas salmonicida* Infection in Common Carp, *Cyprinus carpio* L., by Oral Administration of a Mixed Microbial Culture of *Lactobacillus paracasei*, *Pichia membranifaciens* and *Saccharomyces cereviciae*

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ABSTRACT. A microbial culture was prepared by co-cultivation of *Lactobacillus paracasei*, *Pichia membranifaciens* and *Saccharomyces cereviciae* for 48 hr at 30°C in rice bran extract medium, supplemented with dextrose. Oral administration of the resulting non-viable heat-inactivated microbial culture to common carp, *Cyprinus carpio* L., delivered in feed for four weeks, induced effective protection against experimental atypical *Aeromonas salmonicida* infection which causes "ulcer disease". After challenge of the carp by immersion, fish mortality and development of skin lesions such as hemorrhages and ulcers were significantly suppressed in carp treated with mixed microbial culture adsorbed on dry pellets relative to carp treated with medium or without extract. Atypical *A. salmonicida* was re-isolated from ulcerative lesions in parts of dead and surviving fish, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying microbial population changes during the progression of skin lesions. Among interleukin-1β (IL-1β), tumor necrosis factor-α, as well as CXC-α and CXC-β chemokines, gene expression of IL-1β was up regulated in the spleen and head kidney three weeks after administration of the mixed microbial culture. These results clearly show that this mixed microbial culture, delivered in feed, is effective in preventing *A. salmonicida* disease in carp.

KEY WORDS: atypical *Aeromonas salmonicida*, *Lactobacillus paracasei*, *Pichia membranifaciens*, *Saccharomyces cereviciae*, ulcer disease.

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Atypical *Aeromonas salmonicida* is a fish pathogen that causes several diseases in freshwater fish, such as goldfish, *Carassius auratus* L. [19], eel, *Anguilla japonica* [10], and also seawater fish [17, 21]. The most common clinical manifestation of infection is skin ulceration that leads to high mortality. Since 1996, *A. salmonicida* infection, characterized by the formation of ulcers on the body surface and fins ("ulcer disease"), has become common in colored carp, *Cyprinus carpio koi* L., cultivated in Japan [16]. The use of chemotherapeutic agents as an infection control measure is in doubt because of the acquisition of antimicrobial resistance in *A. salmonicida*. Vaccination is believed to be potentially effective against the disease, but no vaccine has yet been developed.

Alternatives to chemotherapy and vaccination in disease control include the enhancement of natural immunity in the host. The administration of immuno-stimulants activates the immune system in a non-specific way, providing resistance against a variety of pathogens [14, 15, 22]. Oral administration of an immuno-stimulator is an efficient means. Also, probiotic strains are administered to fish to prevent infection by pathogens through modulation of their immune response. Probiotics should be applicable to fish farming to reduce the incidence of various infectious diseases [2, 20], since components of microbial cell walls may cause a range of non-specific and specific host immune responses. However, they are best delivered to fish in large numbers of live bacteria. We therefore investigated the effect of oral administration of small numbers of non-viable heat-inactivated microbial culture comprising *Lactobacillus paracasei*, *Pichia membranifaciens* and *Saccharomyces cereviciae* on the protection of carp from infection by *A. salmonicida*. We also measured the expression of several cytokine genes in spleen and head kidney by polymerase chain reaction (PCR) in order to study the mechanisms of anti-*A. salmonicida* activity. Our results clearly show that treatment of fish with small amounts of heat-killed mixed microbial culture is effective in preventing "ulcer disease" in carp.

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MATERIALS AND METHODS

Mixed microbial culture: Mixed microbial culture was prepared by cultivating a seed lot of microbial mixture containing *L. paracasei* (strain TLP), *P. membranifaciens* (strain TPM) and *S. cereviciae* (strain TSC) for 48 hr at 30°C in rice bran extract medium, supplemented with 5% dextrose. The seed lot had been stored at –80°C until use. Each microbial strain was identified by biochemical tests, and gene sequence analysis of 16S, 26S and 18S ribosomal RNA fragments respectively, amplified by PCR. The mixed microbial culture contained $5.4 \pm 0.3 \times 10^7$/m*l* of *L. paracasei*, $2.4 \pm 0.1 \times 10^6$/m*l* of *P. membranifaciens* and $4.4 \pm 0.3 \times 10^7$/m*l* of *S. cereviciae*. Number of each bacterial and yeast cells in mixed microbial culture was counted

under microscope using hemocytometer. The culture was inactivated by heating at 121°C for 15 min. The sterility was assessed by cultivation of mixed microbial culture on heart infusion (HI) agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan).

Fish: *Aeromonas salmonicida*-free common carp were donated from Kyorin Fisheries Laboratory, Himeji, Hyogo, Japan). Absence of *A. salmonicida* in carp was affirmed periodically by cultivation of skin and visceral specimen on HI agar. They were reared in 170 *l* plastic aquaria filled with dechlorinated tap water (passing through once at a flow-through rate of 40 *l*/hr), and aerated. The fish were fed a commercial floating dry pellet twice daily. A daily regimen was maintained of 15 hr of light followed by 9 hr dark. Water temperature is shown in Table 1. Total 69 and 16 fish were used in challenge test and cytokine gene expression experiment, respectively. Fish were reared and treated in accordance with the ethical standards of the Osaka Prefecture University Committee on Animal Care and Use.

Administration of mixed microbial culture: Experiments were performed two times individually at different water temperatures: high (permissive temperature for microbial growth; first trial) and low (non-optimum temperature for the growth; second trial) temperature (Table 1). Fish were divided in each group and acclimatized in 20 *l* aquaria (flow-through rate 11 *l*/hr) that were aerated. Fish weighing 30 ± 4 and 32 ± 5 g were used in each experiment. Mixed microbial culture was sprinkled on commercial dry pellets so as to provide final concentrations of 5 to 0.1% of the dry weight, and was adsorbed into the pellets, which were then dried using a blower in a room temperature. The fish were fed pellets containing mixed microbial culture twice daily (total 1% of the fish body weight per day) for 25 and 32 days in the first and second trials, prior to challenge by *A. salmonicida*. Control fish were fed the dry pellet without mixed microbial culture, or the pellet containing 5% culture medium.

In the experiment for cytokine gene expression, fish were administered with 3% mixed microbial culture for 2, 3 and 4 weeks.

Bacterial challenge: The fish were challenged with virulent atypical non-pigment producing *A. salmonicida*, as reported previously [11]. Strain T1031, donated by Niigata Prefectural Inland Water Fisheries Experimental Station, Nagaoka, Niigata, Japan, was cultured in HI broth at 23°C for 5 days with shaking. The fish were immersed in 4.1×10^6 cfu/m*l* of the bacteria for 60 min in the first experiment and 6.7×10^6 cfu/m*l* in the second. Bacterial number was estimated by the calculation of bacterial colonies formed on HI agar for 5 days after inoculation with serial 10-fold dilution of the bacterial culture at 23°C. The water temperatures at challenge were respectively 18 and 13°C. Mixed microbial culture was administered for 32 and 33 consecutive days after the challenge in each experiment. The fish were observed in order to determine survival, and any formation of ulcers and hemorrhagic lesions on the skin. Hemorrhagic lesions and ulcerations were categorized as: no lesion (score 0), small nodule formation or loss of scale (score 1), early stage of hemorrhage (score 2), moderate hemorrhage and ulceration (score 3), severe hemorrhage and ulceration (score 4) and death (score 6). Bacterial isolation was performed by cultivation from hemorrhagic and ulcerative lesions, and from visceral organs of dead fish. This was done also in all surviving fish. *Aeromonas salmonicida* was identified by a slide agglutination test using anti-*A. salmonicida* rabbit serum and biochemical characterization.

Polymerase chain reaction: RNA was isolated and cDNA was synthesized as follows. Total RNA was isolated from 20 mg of the head kidney and spleen samples using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. The genomic DNA removing column enabled us to perform cDNA synthesis without DNase I treatment. Final elution was carried out by adding 30 μl of RNase free water, to maximize the concentration of sample RNA. Immediately after RNA isolation, cDNA was synthesized with an ExScript RT reagent Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. In summary, 10 μl of reaction mixture containing 2 μl of 5× ExScript buffer, 0.5 mM dNTP mixture, 50 units of ExScript RTase, 10 units of RNase inhibitor, 50 pmol random 6-mers and 1 μl of total RNA sample was incubated for 15 min at 42°C, followed by heating at 95°C for 2 min for RTase inactivation. The samples were stored at –20°C prior to further use.

Table 1. Experimental design

	Experiment 1	Experiment 2
Materials administered	3%, 0.5%, 0.1% extract, and none	5% extract, 5% medium, and none
Duration	25 days	32 days
Fish number in each group	10 or 9	10
Body weight at the start of the experiment	30 ± 4 g	32 ± 5 g
Feeding	1% of the body weight/day	1% of the body weight/day
Water temperature at start of feeding	15°C	18°C
at challenge	18°C	13°C
end of the experiment	20°C	10°C
Challenge dose	4.1×10^6/m*l* for 60 min	6.7×10^6/m*l* for 60 min
Observation period	32 days	33 days

Table 2. Oligonucleotide primers used in real time PCR

Gene/Accession number	Forward primer	Reverse primer
IL-1β/AJ245635	AAG GAG GCC AGT GGC TCT GT	CCT GAA GAA GAG GAG GCT GTC A
TNFα/AJ311800	GCT GTC TGC TTC ACG CTC AA	CCT TGG AAG TGA CAT TTG CTT TT
CXCa/AJ421443	CTG GGA TTC CTG ACC ATT GGT	GTT GGC TCT CTG TTT CAA TGC A
CXCb/AB082985	GGG CAG GTG TTT TTG TGT TGA	AAG AGC GAC TTG CGG GTA TG
40S RNA/AB012087	CCG TGG GTG ACA TCG TTA CA	TCA GGA CAT TGA ACC TCA CTG TCT

Real-time quantitative PCR was performed using StepOnePlus with Power SYBR Green PCR Master mix according to the method described by Chadzinska *et al.* [5].

Real-time quantitative PCR (RT-qPCR) was performed using StepOnePlus with a Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, U.S.A.) according to the procedure described by Chadzinska *et al*. [5]. The 1 μl of cDNA sample and primer sets shown in Table 2 were added to Master mix at a concentration of 5 μM, and the final volume was brought to 20 μl by adding deionized water. The reaction mixtures were processed via the following cycling program: incubation for 10 min at 95°C for enzyme activation; 40 cycles of 15 sec at 94°C for denaturation; 30 sec at 60°C for annealing; and 30 sec at 72°C for elongation. A melting curve was acquired by performing continuous fluorescence acquisition, starting at 60°C with a rate of 1°C/5 sec, up to 90°C to check the amplification specificity. In all cases the amplifications were specific and no amplification was detected in the non-reverse transcription negative control. The acquired fluorescence data were analyzed using StepOne Software version 2.1 (Applied Biosystems, Osaka, Japan), to determine the mRNA expression ratio (*R*) relative to house keeping gene 40S mRNA expression.

Statistical analysis: Data were analyzed by χ^2 test to determine differences in survival rate between groups. Statistical significance in cytokine gene expression between two groups was analyzed by Student's *t*-test.

RESULTS

Fish mortality after A. salmonicida challenge and development of skin lesions: In the first experiment, at higher water temperature (18°C at challenge), the protective effect of mixed microbial culture was observed in a significant reduction of fish mortality (Fig. 1A and Table 3-1). Of the non-treated carp in the control group, following challenge with *A. salmonicida*, seven carp died at 32 days after the challenge. In contrast, 100% and 90% of fish treated with 3% (4.9×10^5 cfu of *L. paracasei*, 2.2×10^4 cfu of *P. membranifaciens* and 4.0×10^5 cfu of *S. cereviciae* per day) and 0.5 % (8.1×10^4, 3.6×10^3 and 6.6×10^4 cfu per day, respectively) of mixed microbial culture survived at 32 days after challenge ($P<0.01$ compared to control fish by χ^2 test). Delivery of mixed microbial culture-containing food at 0.1% had no obvious effect on the survival of fish (60% of fish died at 32 days after the challenge). Small nodules first appeared on various sites on the body surface, which developed into localized hemorrhages and loss of scales. The lesions progressed to broad and severe hemorrhages, resulting in severe ulcer formation. Gross lesions observed in the control fish group were much more severe than those in the fish groups administered 3% or 0.5% mixed microbial culture (Fig. 1B and Table 3-1). The mean gross lesion score ± SE (Fig. 1B) was calculated according to the score points shown in Table 3. Atypical *A. salmonicida* was re-isolated from hemorrhagic and ulcerative lesions of both dead and surviving fish of the control group (60%), whereas no bacteria were isolated from fish treated with 3% mixed microbial culture.

Figure 1C and 1D, and Table 3–2 also show that the administration of 5% mixed microbial culture to carp (8.1×10^5 cfu of *L. paracasei*, 3.6×10^4 cfu of *P. membranifaciens* and 6.6×10^5 cfu of *S. cereviciae* per day) induced effective protection against experimental *A. salmonicida* infection in the second experiment (performed at low water temperature; 13°C at challenge). Of the control group (5 % medium or without mixed microbial culture), following challenge with *A. salmonicida*, the majority of carp had severe skin lesions such as prominent hemorrhages and ulcers (Fig. 1D and Table 3-2); 1 and 2 carp died in each control group, respectively (Fig. 1C and Table 3-2). The control fish showed skin hemorrhages at 6 days after challenge, and skin ulcers developed 9 days after challenge. In contrast, all fish survived that had been treated with 5% of mixed microbial culture, and development of skin lesion was significantly reduced (restricted and mild hemorrhages in 3 fish) or was completely suppressed (7 fish). In this test *A. salmonicida* was isolated from control fish administered 5% rice bran extract medium (40%) or from untreated fish (30%). No *A. salmonicida* was isolated from fish treated with 5% mixed microbial culture.

Cytokine gene expression in spleen and head kidney of fish administered with mixed microbial culture: Expression of the cytokine genes IL-1β, TNF-α, CXC-α and CXC-β was measured in the spleen and head kidney of carp administered with 3% mixed microbial culture at 2, 3 and 4 weeks and also untreated control fish. Higher levels of IL-1β were observed in the spleen, namely 19.1 times higher three weeks after the administration of mixed microbial culture, than in the untreated control (0 week) (Figure 2A; $P<0.05$ analyzed by Student's *t*-test). No significant regulation of TNF-α, CXC-α and CXC-β was observed in the spleen during the experimental period. There were no significant changes in cytokine and chemokine expression in the head

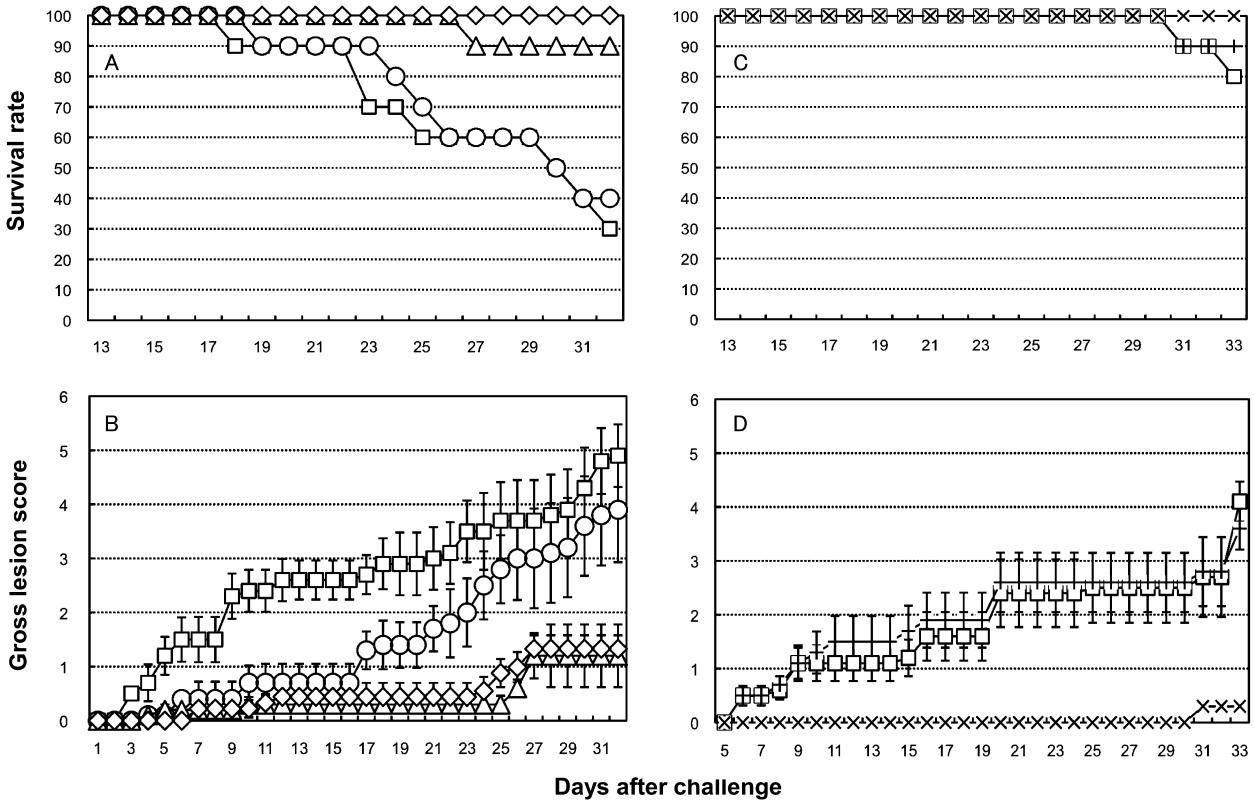

Fig. 1. Mortality and mean gross lesions in carp after challenge with the T1031 strain of atypical *Aeromonas salmonicida*. In the first experiment, carp were fed dry pellet total 1% of body weight per day containing 3% (rhombus), 0.5% (triangle), or 0.1% (circle) heat-inactivated mixed microbial culture. The microbial culture was administered for 25 days orally prior to challenge. Control fish were administered feed containing no microbial culture (square). The water temperature was 18°C at challenge. Figure 1A shows the survival rate 32 days after the challenge. The mean gross lesion score ± SE (Fig. 1B) was calculated according to the score points shown in Table 3. In the second experiment, carp received pellet containing 5% (X) mixed microbial culture for 32 days (total 1% of body weight per day) and challenged at a water temperature 13°C. Control fish were administered feed containing 5% rice bran medium (cross) or without any medium (square). Figure 1C shows the survival rate 33 days after the challenge. The mean gross lesion score (Fig. 1D) is also shown.

Table 3. Survival and formation of gross lesions of carp after the challenge with *Aeromonas salmonicida*

Experiment	Treatment	Gross lesions*						Bacterial re-isolation
		None	+**	++	+++	++++	Dead	
1	Mixed microbial culture (3%)	1***	4	4	0	0	0	0%
	(0.5%)	3	6	0	0	0	1	10%
	(0.1%)	2	1	1	0	0	6	40%
	Untreated	0	1	0	2	0	7	60%
2	Mixed bacterial culture (5%)	7	3	0	0	0	0	0%
	Medium control (5%)	0	0	2	2	5	1	40%
	Untreated	0	0	0	3	5	2	30%

*Thirty-two and thirty-three days after challenge in experiments 1 and 2, respectively. ** Hemorrhagic lesions and ulcerations were categorized as: no lesion (none; score 0), small nodule formation or loss of scale (+; 1), early stage of hemorrhage (++; 2), moderate hemorrhage and ulceration (+++; 3), severe hemorrhage and ulceration (++++; 4) and death (dead; 6). ***Number of fish.

kidney compared to those in control fish (data not shown).

DISCUSSION

Recent studies have shown that the growth of lactic acid bacteria can be improved by the use of yeast [1]. Co-culture with *S. cervisiae* improved *Lactobacillus* performance. Yeasts actively synthesize substances such as vitamins, amino acids and purines, and breakdown complex carbohydrates, which is essential for the growth of *Lactobacillus*.

Lactobacillus excretes lactic acid which leads to a lowering of the pH, which promotes yeast growth. Multistrain microbial products are expected to show enhanced biological effects when administered *in vivo*, whereas such properties are strain-specific. The three efficacious microbes are compatible in co-culture in the present study. Therefore, the three microbes can be combined for multi-strain immunostimulant with enhanced *in vivo* efficacy.

This study shows that oral administration of non-viable heat-inactivated mixed microbial culture successfully protected carp against experimental *A. salmonicida* infection. A small number of bacteria or a small amount of their products were effective in providing protection, since we administered diets containing 5 to 0.5% of the microbial culture that were fed as 1% of the body weight of carp per day. In the experiment conducted at high water temperature, fish treated with 3 or 0.5% mixed microbial culture had a significantly increased survival rate and mild skin lesions, whereas control fish developed extensive hemorrhages resulting in severe ulcer formation and death (Fig. 1A and 1B, and Table 3–1). The protective effect was also observed in the second experiment; although a small number of control fish died after the challenge due to the low water temperature in which the growth of *A. salmonicida* is not optimal, untreated fish and fish administered rice bran media developed severe hemorrhagic lesions and ulcerations compared to fish treated with 5% mixed microbial culture (Fig. 1C and 1D, and Table 3–2). Atypical *A. salmonicida* was re-isolated from hemorrhagic and ulcerative lesions of dead and surviving fish in the control groups in the two tests, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying bacterial population changes during the growth of skin lesions. No *A. salmonicida*, *A. hydrophila* or *Flavobacterium* was isolated from fish treated with mixed microbial culture. Both experiments indicate that heat-inactivated microbial components, or microbial products contained in mixed microbial culture, are involved in the suppression of lesion formation and the reduced mortality. Since skin is an essential protective barrier for fish, and acts as a first line of defense against ulcer disease, inhibition of bacterial attachment to the skin and suppression of bacterial growth may be important in protection against *A. salmonicida* infection.

The mechanism by which the non-pathogenic microorganisms used in the present study protect against ulcer disease remains unclear. Lactic acid bacteria such as *Lactobacillus* and *Carnobacterium* isolated from salmonids produce compounds that inhibit the growth of Gram-positive and Gram-negative fish pathogens [2, 20]. The inhibitory effects of lactic acid bacteria are due to the production of anti-bacterial low molecular weight substances and bacteriocins [20]. In these experiments, rainbow trout administered with these bacteria in their feed showed reduced mortality after challenge [2]. But the protective effect found in the present study was not due to direct antimicrobial or antibiotic activity, since mixed microbial culture did not inhibit the growth of *A. salmonicida in vitro* (data not shown).

It is well known that administration of probiotic bacteria such as *Lactobacillus*, *Lactococcus* or *Bacillus* in the diet can cause beneficial physiological effects by modulating functions of the immune system and inducing protection against fish infectious diseases [3, 4, 18]. In these systems, however a large number of live probiotic bacteria should be administered to fish; 10^6 cfu/g fish weight [3] or 10^9 to 10^{12} cfu/g [18] of bacteria should be fed at 1% biomass to induce protection. A notable feature of the mixed microbial culture used in the present study is that a small number of heat-inactivated bacteria and a small amount of culture products induce a significant protective effect in carp. For example, 4.9×10^5 cfu of *L. paracasei*, 2.2×10^4 cfu of *P. membranifaciens* and 4.0×10^5 cfu of *S. cereviciae* administered to fish weighing 30 g per day induced protection (3% group in Exp. 1). It is likely that enhancement of innate protective responses would confer protection. For example, we found that the mixed microbial culture activated mice spleen lymphocytes in a dose-dependent manner *in vitro* via a reaction similar to that of Concanavalin A or lipopolysaccharide (LPS) (data not shown). Microbial cell wall components, such as peptidoglycan of *L. paracasei* or glucan of *P. membranifaciens* and *S. cereviciae*, may cause a range of non-specific host immune responses. Microbial metabolites may also be involved in the anti-infectious response. These immunostimulants facilitate the function of phagocytic cells and the induction of cytokines to activate other immunocompetent cells; also phagocytic cells could themselves be stimulated to increase their phagocytic and bactericidal activities.

Macrophages are stimulated by microbial antigens such as LPS, peptidoglycan and other microbial triggers (innate macrophage activation) [8, 12]. Activated macrophages produce pro- and/or anti-inflammatory cytokines. Innate activation of carp macrophages is implied by activation of IL-1β, TNF-α and CXC chemokines genes [9]. *In vivo* and *in vitro* stimulation of carp head kidney phagocytes with LPS or peptidoglycan successfully induced IL-1β gene activation [7, 12, 13]. In the present study, significant up-regulation of the IL-1β gene was observed in the spleen relative to untreated controls three weeks after the administration of mixed microbial culture (Fig. 2A). This implicates the mixed microbial culture in the induction of this mediator of immune response. IL-1β is a multifunctional cytokine that plays a crucial role in the initiation of immune responses, and affects gene expression during inflammation through the promotion of other cytokines and chemokines [6]. We therefore believe that carp administered mixed microbial culture are in an immunologically elevated state ready to counter any possible pathogens.

Further studies are required to determine how innate immune responses are induced in carp following treatment with mixed microbial culture. Normal carp constitutively express IL-1β mRNA, predominantly in the immune organs, spleen and head kidney [7]. The distribution and localization of IL-1β producing cells suggests that they are

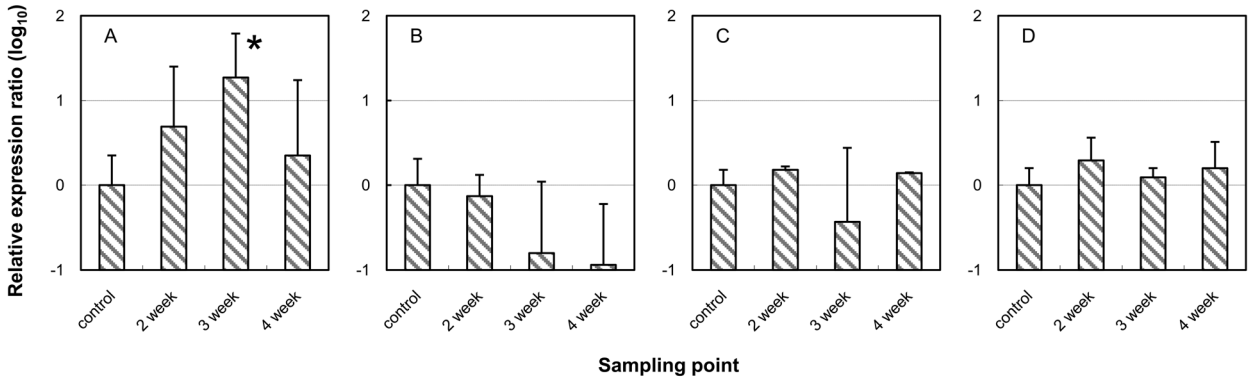

Fig. 2. Real time PCR quantitative expression of carp cytokines after oral administration of mixed microbial culture. A to D show amplification of IL-1β, TNF-α, CXC-α and CXC-β in spleen relative to house keeping gene 40S mRNA expression. Bars represent mean value ± SD of four fish per time point. *Indicates significantly elevated expression (P<0.05).

macrophages [7]. Upregulation of IL-1β induction by our mixed microbial culture may stimulate the production of other cytokines that contribute to the activation of immunocompetent cells so as to inhibit bacterial infections. The effects of mixed microbial cultures in aquaculture are not limited to improving the health of fish by improving physical attributes of fish such as their intestinal environment, but can also control of infectious diseases.

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