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Cytokine responses in the common carp, *Cyprinus carpio* L. treated with baker's yeast extract

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ABSTRACT

Cytokines are important regulators of the immune system and investigation of their functions may prove useful for the development of vaccines and immunostimulants for aquaculture. We therefore investigated the cytokine [interleukin (IL)-1β, IL-10, IL-12 p35 and p40, tumor necrosis factor (TNF)-α, CXC-chemokine and interferon (IFN)-α and γ] responses of the common carp, *Cyprinus carpio* L., upon treatment with a commercial baker's yeast extract (CW-I) that contained nucleotides and β-glucan. Additionally, to confirm that the CW-I-induced cytokines were functional, we also assessed the effect of CW-I administration on superoxide anion production and phagocytic activities of head kidney leucocytes and resistance to *Aeromonas hydrophila* infection in the common carp. Our results demonstrate that baker's yeast extract-treated fish displayed a significant up-regulation of pro-inflammatory cytokine (IL-1β, IL-12 p35 and p40, TNF-α, CXC-chemokine, IFN-γ2) gene expression and a down-regulation of anti-inflammatory cytokine (IL-10) gene expression. Furthermore, significantly increased phagocytic activity and superoxide anion production in kidney cells, and resistance to a bacterial pathogen, were observed in the yeast extract-treated fish compared to non-treated fish. The current study indicates the immunostimulatory effects of a baker's yeast extract rich in nucleotides and β-glucan on the carp immune system and supports its potential use in aquaculture.

1. Introduction

Aquaculture, which produces 52.5 million tonnes (MT) of fish per year with an estimated value of 98 billion US$, is one of the primary food-producing industries (FAO, 2010). With the rapid intensification of aquaculture, infectious disease occurrences that are attributed to bacterial, viral and fungal pathogens have increased. These diseases continue to be one of the most important problems in aquaculture and lead to considerable economic loss (Smith et al., 2003). The control of diseases that occur in intensive aquaculture by the use of chemicals or antibiotics leads to unwanted developments such as bioaccumulation, pollution, antibiotic-resistant pathogens, immunosuppression and high expenditure. The prohibition of treatment of these diseases with antibiotics and chemotherapeutics has necessitated a search for alternative preventive measures that are eco-friendly and are not hazardous to human health. In this regard, since fish depend mostly on innate immune systems rather than on specific immunity, immunostimulants can play a major role in disease protection by enhancing resistance through eliciting innate immunity (Sakai, 1999).

Cytokines, which are protein mediators produced by immune cells, are mainly responsible for host innate defense mechanisms. Cytokines include interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factor (TGF), chemokines and interferons (IFNs) which act through cytokine receptors and have pro-inflammatory, anti-inflammatory and pathogen-killing activities. Since cytokines are important regulators of the immune system, investigation of cytokine functions may provide data that can be used as a basis for the development of vaccines and immunostimulants for aquaculture (Sahoo and Sakai, 2010). Analysis of the expression profile of cytokine genes and assessment of their products involved in immune defense also have the added advantage of identifying potential indirect immunological markers.

Baker's yeast is a particularly important natural bio-product since it contains immunostimulating compounds such as β-glucan, nucleotides, mannan oligosaccharides and chitin and has been proved to influence the immune response of terrestrial animals (Sohn et al., 2000), rodents (Breivik et al., 2005) and aquatic animals such as fish and shellfish (Abdel-Tawwab, 2012; Li et al., 2004; Ortuño et al., 2002; Sahoo and Mukherjee, 2001; Sarlin and Philip, 2011; Selvaraj et al., 2005; Tewary and Patra, 2011). However, most of the studies of the effect of baker's yeast on the immune response in different

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction.

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fish species have focused on analysis of the effects of whole yeast cells, or of specific components that were isolated from whole yeast cells, such as nucleotides or β-glucan. There is little information regarding the potential effect of a baker's yeast extract that contains both nucleotides and β-glucan on fish immune responses. In particular, there is limited information regarding the expression of cytokine genes in fish treated with a yeast extract. One exception is a report by Reyes-Becerril et al. (2008), which demonstrated down-regulated expression of IL-1β and TNF-α genes in the gilthead seabream, *Sparus aurata*, when it was fed with live yeast, *Debaryomyces hansenii*. We assessed the efficacy of a baker's yeast extract, in which nucleotides and β-glucan are major components, in eliciting cytokine functions in the common carp, *Cyprinus carpio*. This fish species was selected because it is one of the most highly cultivated fish and contributes to around 5.6% of the global aquaculture production (FAO, 2010). We investigated the effect of feeding common carp with a commercial baker's yeast extract (CW-I) on the expression of cytokine genes such as IL-1β, TNF-α, IL-12p35, IL-12p40, CXC-chemokine, IL-10, Type-1IFN-α, IFN-γ1 and IFN-γ2 in the carp head kidney leucocytes. Additionally, to confirm that the cytokines induced by CW-I were functional, we also assessed superoxide anion production (NBT assay), phagocytic activities and resistance to *Aeromonas hydrophila* infection in the CW-I fed common carp.

2. Materials and methods

2.1. Fish

Common carp, *C. carpio* L. (mean weight, 100 g), were obtained from Mera Fisheries Farm (Miyazaki, Japan). The fish were firstly acclimatized in an aerated freshwater tank at 20 °C and fed with a commercial diet (Hikari Staple, KYORIN Co. Ltd., Hyogo, Japan) containing 35% crude protein at 1% body weight daily in 3 equal meals for 2 weeks under a natural photoperiod prior to their use in the study.

2.2. Preparation and administration of baker's yeast extract

In this study, we used the baker's yeast extract CW-I (TableMark Co., Ltd., Tokyo, Japan). This yeast extract was produced as follows. Following cultivation of the baker's yeast (*Saccharomyces cerevisiae*), the yeast was washed and the yeast-fungus was separated. Next, a hot-water extract of the separated yeast-fungus was filtered and condensed into an extract using a concentration can. A suitable concentration of the condensed extract was turned into a powder using a spray dryer. The resulting product had the form of fine granules. Nucleotides and β-glucan were major components of this CW-I, representing 10% and 7%, respectively, of the total CW-I (Table 1). Fish were divided into two groups, viz. treatment and control group (n = 60) and maintained in freshwater flow-through system at 22 ± 2 °C under a natural photoperiod. CW-I granules were orally administrated at 5 mg fish^{-1} once a day for 3 days to all fish of the treatment group and the fish of the control group were fed a commercial diet as mentioned at 1% body weight once daily for the same period. The fish in both the groups were force-fed using a disposable feeding needle (1.2Φ × 75 mm; Fuchigami, Kyoto, Japan). Sampling was performed at 1, 3, 5, 7 and 10 days post administration period. The experiment and fish maintenance were conducted in accordance with the guidelines for the care and use of laboratory animals at the University of Miyazaki.

Table 1
Composition of baker's yeast extract (CW-I) used in the study.

Component	%
Nucleotides[a]	10.44
β-glucan	7.03
Total amino acids	48.40
Total fiber	17.01
Minerals	5.05
Total vitamins	1.07
Organic acids	0.85
Others	10.15

[a] Nucleotides (10.44%) composed of disodium cytidine-5′-monophosphate (CMP) — 2.10%, disodium uridine-5′-monophosphate (UMP) — 2.49%, disodium inosine-5′-monophosphate (IMP) — 2.98%, disodium guanidine-5′-monophosphate (GMP) — 2.78% and disodium adenosine-5′-monophosphate (AMP) — 0.09%.

2.3. Serum preparation

Blood was drawn from a peripheral vein of the fish using a syringe (Terumo, Japan) and was incubated for 1 h at room temperature. Subsequently, the sample was incubated on ice for 1 h, and centrifuged at 4 °C, 400 × *g* for 10 min. The obtained serum was stored at −20 °C until further use.

2.4. Analysis of cytokine gene expression

Total RNA was isolated from the head kidney of the carp (n = 5) at 1, 3, 5, 7 and 10 days post treatment using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. Poly (A) RNA was then purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). To avoid presence of DNA, RNA samples were treated with recombinant DNase (RNase-free) as per the manufacturer's protocol (Takara Bio Inc., Japan). This recombinant DNase is an endonuclease that catalyzes degradation of both single- and double-stranded DNA randomly. cDNA synthesis was performed using ReverTra Ace (Toyobo, Japan). The PCR reactions were performed according to the following protocol: 1 μL of cDNA was mixed with 5 μL (5 μM) dNTPs (10 μM of each dNTP), 0.5 μL *Taq* polymerase (5 units μL^{-1}), 5 μL of each gene-specific primer (IL-1β, IL-12p35, IL-12p40, TNF-α, CXC-chemokine, IL-10, Type-IIFN-α, IFN-γ1, IFN-γ2 and β-actin as internal control) and 28.5 μL of nuclease-free water. Primers and annealing conditions are presented in Table 2. PCR products were electrophoresed on a 1.5% agarose gel to detect specific bands.

To reach an optimum semi-quantitative approach for analyzing gene expression, cytokine genes and β-actin gene were amplified using a series of cycle numbers (20–35) under the above mentioned conditions. After determining the optimal cycle number, specific PCR was conducted thrice. The relative level of transcripts was quantified for each gene by densitometry, which was performed by measuring the photo-stimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Japan) (Tanekhy et al., 2010; Tassakka et al., 2006). The ratio of cytokine gene/β-actin product was subsequently calculated for each gene of interest and was used to assess the difference in expression levels between the CW-I administered group at different time intervals and control.

2.5. Isolation of phagocytic cells

The head kidney phagocytic cells of carp (n = 5) were isolated according to the method described by Braun et al. (1982) with slight modifications. Briefly, the isolated cells were filtered through a nylon mesh (Φ40 μm) with RPMI1640 medium (Nissui, Japan) containing 1% streptomycin/penicillin (Gibco, USA), and 5% carp serum (CS) and were then centrifuged at 400 × *g* for 40 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 using trypan blue exclusion.

Table 2
Gene specific primers, annealing temperature and their corresponding product sizes of carp β-actin and cytokine genes used for PCR analysis in the study.

Gene	Primer sequence (5′→3′)	Annealing temperature (°C)	Accession number
β-actin Fw[a]	ACCTCATGAAGATCCTGACC	60	M24113
β-actin Rv[b]	TGCTAATCCACATCTGGTGG		
IL-1β Fw	GGAGAATGTGATGGAAGAGC	58	AJ245635
IL-1β Rv	GTAGAGGTTGCTGTTGGA		
TNF-1,2α Fw	AGTCAGCTTCTTGAAGAAGGA	62	α1 — AJ311800
TNF-1,2α Rv	TGGAAAGACACCTGGCTGTA		α2 — AJ311801
TNF-1,2,3α Fw	AGAACAATCAGGAAGGTGGAA	63	α3 —
TNF-1,2,3α Rv	TGGAAAGACACCTGGCTGTA		AB112424
IL-12p35 Fw	TGCTTCTCTGTCTCTGTGATGGA	60	AJ580354
IL-12p35 Rv	CACAGCTGCAGTCGTTCTTGA		
IL-12p40 Fw	GAGCGCATCAACCTGACCAT	60	AJ621425
IL-12p40 Rv	AGGATCGTGGATAGTGACCTCTAC		
CXC-chemokine Fw	GTGTGAACATGGTTCCTCCA	61	AB082985
CXC-chemokine Rv	GGATTGAAGCATTTCTGCTCT		
IL-10 Fw	TGATGACATGGAACCATTACTGG	60	AB110780
IL-10 Rv	CACCTTTTTCCTTCATCTTTTCA		
Type1-IFNα Fw	GGATCCTGCAGATGGCTCGGCAGAT	58	AB376666
Type1-IFNα Rv	ACTAGTTTAAACTCTTTTTTTAGCATTGTTT		
IFN-γ1 Fw	GTCGCTGCTGCTTGATA GAA	60	AM261214
IFN-γ1 Rv	CTGAAGCTCCCTCCATACTT		
IFN-γ2 Fw	GAGGAACCTGAGCAGAATCT	60	AM168523
IFN-γ2 Rv	CCTTGATCGCCCATAGTGTT		

[a] Fw = forward.
[b] Rv = reverse.

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

Superoxide anions produced by phagocytic cells were determined using the reduction of nitroblue tetrazolium (NBT; Dojindo, Japan) assay. The NBT assay was performed on days 1, 3 and 5 after oral CW-I administration. NBT was dissolved in RPMI1640 medium (1 mg mL^{-1}) and filtered through a mesh. The phagocytic cell stimulant PMA (Phorbol 12-Myristate 13-Acetate; Wako, Japan) was then added to the NBT solution to a final concentration of 20 ng mL^{-1}. Viable phagocytic cells (1×10^7 cells mL^{-1}) were stimulated with a 50% NBT-PMA solution at 20 °C for 1 h. To fix and adhere the cells, methanol (2 M) was then added. After removal of the methanol, the cells were air-dried and 120 μL of 2 M potassium hydrate (KOH; Wako, Japan) and 140 μL of dimethylsulfoxide (DMSO; Wako, Japan) were added. The optical density of the solution was then measured at 620 nm using a microplate reader.

2.7. Determination of phagocytic activity

The number of kidney cells was counted using a hemocytometer (Funakoshi, Japan) and cell density was adjusted to 10^7 cells mL^{-1} in RPMI1640 culture medium containing 5% CS. Cells were allowed to adhere to glass cover slips (22 mm × 22 mm) for 1 h and non-adherent cells were removed by washing with fresh medium. An aliquot (0.1 mL) of latex particles (0.85 μm; 10^9 particles mL^{-1}; Difco, USA) was suspended in 1 mL of RPMI1640 medium containing 5% CS and this mixture was added to the cells on the cover slips and incubated for 1.5 h at 25 °C. The cover slips were then picked up with a forceps and washed with the medium for 1 min. The cells were fixed with methyl alcohol, air-dried and stained with Giemsa. The number of adhered cells was about 5×10^5 cells per cover slip and 300 cells were counted microscopically. Cells that had phagocytized particles were determined by analysis using phase contrast microscopy, and were counted. Phagocytic activity (PA) was determined by using the following formula:

$$PA(\%) = \frac{\text{Number of phagocytizing cells}}{\text{Number of total cells}} \times 100.$$

The phagocytosis assay was performed on days 1, 3 and 5 after CW-I administration. Five individual fish were analyzed at each sampling time. The phagocytic index (PI) was also determined by counting the number of phagocytizing cells in each group

2.8. A. hydrophila infection test

A preliminary pathogenicity test of *A. hydrophila* (strain MU9901) for carp was conducted to ascertain the bacterial injection dose. The artificial infection was done by an intraperitoneal (i.p.) injection of 0.1 mL of four different bacterial suspensions containing 1×10^{10}, 1×10^9, 1×10^8 and 1×10^7 cfu (colony forming units) mL^{-1} into the carp (n = 20). PBS (0.1 mL) was injected into a control group. In the high dose (1×10^{10} cfu mL^{-1}) group, all carps died 1 day after challenge. However, half of the carps died at a dose of 1×10^9 cfu mL^{-1} after 7 days of injection and there was 20% mortality in 1×10^8 or 1×10^7 cfu mL^{-1} injected group at 7 days post injection. In the control group no mortality occurred. Therefore, we decided to use 1×10^9 cfu mL^{-1} for this study. However, finally artificial infection of *A. hydrophila* to the baker's yeast extract treated and control fish groups (n = 30) was conducted with i.p. injection of 0.1 mL bacterial suspension (3.4×10^9 cfu mL^{-1}) which was marginally higher than the previously ascertained dose. The artificial infection of the fish was conducted on day 1 after the CW-I administration period. At 2, 6, 12 and 24 h post-injection, blood, spleen and head kidney (n = 5) from treated and control group fish were removed and homogenized. The serially diluted homogenate was then plated onto LB agar plates. These plates were incubated at 37 °C for 60 h, and then examined for bacterial colony growth. The number of colonies formed on each plate was multiplied by the reciprocal value of dilution to determine the cfu per unit sample volume.

2.9. Statistical analysis

Differences between the quantified relative expressions of a particular gene in treated fish at different time intervals and in control fish were evaluated with one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for their comparison. Data on NBT reduction and phagocytic assays of the yeast extract-treated and the control groups at each time point were compared using Student's *t*-test. 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. Analysis of cytokine gene expression

The expression levels of cytokine genes in the head kidney of CW-I treated carp over 10 days post-treatment, as analyzed by PCR, were significantly different than those in the control fish. The expression of IL-1β, TNF-α, IL-12p35, IL-12p40 and IFN-γ2 genes was significantly increased ($P<0.05$) on day 1 post-treatment compared with that in the control fish (Fig. 1A and B). CXC-chemokine gene expression was increased compared to control ($P<0.05$) on days 1, 5, and 7 post-treatment (Fig. 1A). However, a reduction in IL-10 gene expression was apparent in the CW-I treated fish compared with the control group (Fig. 1B). Expression of Type-I IFN-α and IFN-γ1 genes was not detected in this study.

3.2. NBT reduction activity of leucocytes

The production of superoxide, as assessed by NBT reduction, was significantly higher ($P<0.01$) in fish treated with CW-I than in control fish on day 3 post-treatment. However, on day 1 post-treatment, the production of superoxide by leucocytes was lower in CW-I treated fish than in control fish (Fig. 2).

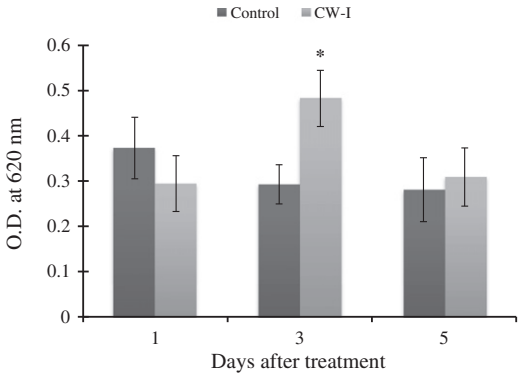

Fig. 2. The production of superoxide anion in the phagocytic cells of CW-I treated or non-treated (control) common carp. Superoxide anion production was measured as optical density (O.D.) at 620 nm in the nitroblue tetrazolium (NBT) assay. Values are mean ± S.D. in 5 fish. *$P<0.01$.

3.3. Phagocytic activity

The phagocytic activity of head kidney leucocytes from carp treated with CW-I showed a significant difference compared with that of the control fish ($P<0.01$; 0.05). Thus, treated fish showed higher phagocytic indices at all sampling times than those of the control. The phagocytic activity of CW-I-treated carp compared to control was highest on day 3 post-treatment (Fig. 3). The phagocytic index of head kidney leucocytes was significantly higher ($P<0.01$) in the CW-I treated fish at 3 days post-treatment compared to that of the control.

3.4. Resistance to A. hydrophila

An increase in viable pathogen cell counts in the blood, spleen and head kidney was initially observed in both the CW-I treated and control groups. However, the viable pathogen cell counts were decreased in the CW-I treated group from 6 h post-injection compared with the counts in the control group and it showed a decreasing trend till 24 h post-injection with lower values in the baker's yeast extract treated group (Fig. 4). There was no mortality recorded during the study period as it was for a short duration (24 h).

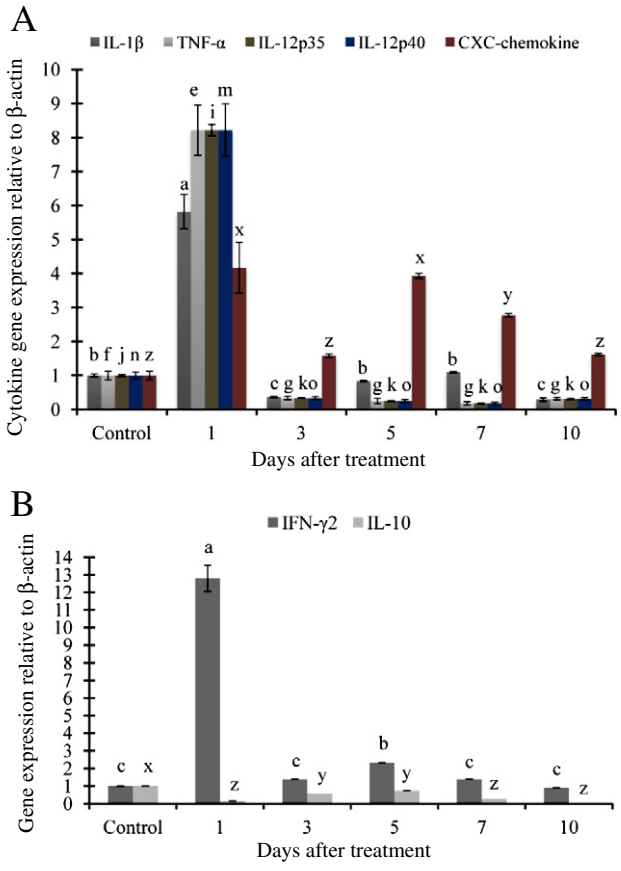

Fig. 1. A. Densitometric quantification of PCR-analysis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-12 and CXC-chemokine) gene expression relative to the β-actin gene transcript in the head kidney of control and CW-I treated carp. Different superscript letters for each gene indicate a significant difference ($P<0.05$) in expression levels in treated fish at various time intervals and in control fish. B. Densitometric quantification of PCR-analysis of IFN-γ2 and IL-10 gene expression relative to the β-actin gene transcript in the head kidney of control and CW-I-treated carp. Different superscript letters for each gene indicate a significant difference ($P<0.05$) in expression levels in treated fish at various time intervals and in control fish.

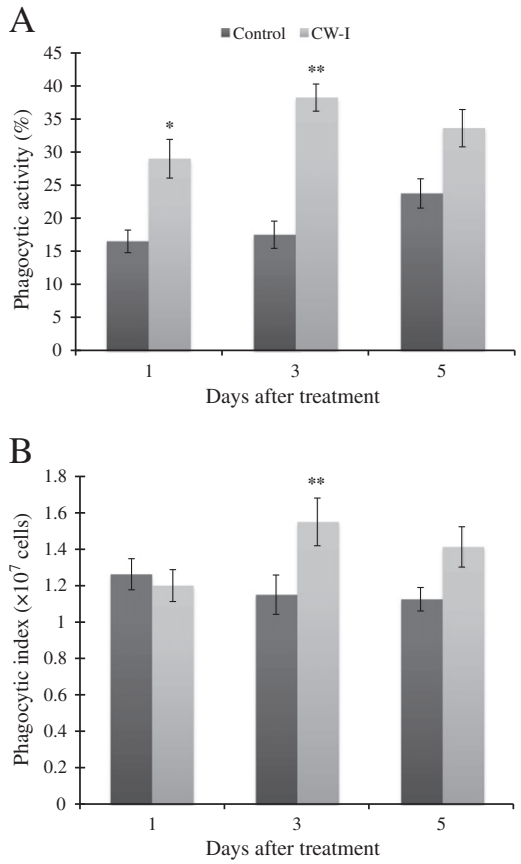

Fig. 3. Phagocytic activity and phagocytic index of leucocytes of carp in CW-I treated group and non-treated group (control). Phagocytic activity indicates the percentage of cells that phagocytosed latex beads. The phagocytic index reflects the number of phagocytic cells. Values are mean ± S.D. in 5 fish. **$P<0.01$; *$P<0.05$.

4. Discussion

In the present study, dietary baker's yeast extract administration resulted in an increase in the cytokine responses of carp head kidney leucocytes, as assessed by increased cytokine gene expression. 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, natural killer (NK) cells and lymphocytes (Low et al., 2003). TNF is a pro-inflammatory cytokine involved in inflammation, apoptosis, cell proliferation and the stimulation of various aspects of the immune system (Savan and Sakai, 2004). Significant up-regulation of IL-1β and TNF-α gene expression was observed in the yeast extract-treated common carp compared to control fish. Similar up-regulation of IL-1β and TNF-α gene expression was observed in turbot treated with nucleotides (Low et al., 2003) and in common carp fed with Spirulina (Watanuki et al., 2006) or with human IFN-α (Watanuki et al., 2009). However, down-regulation of IL-1β and TNF-α gene expression was recorded in the gilthead seabream (*S. aurata*) fed with the live yeast (*D. hansenii*) (Reyes-Becerril et al., 2008). IL-1 secretion results in a cascade of effects including IL-2 release from stimulated T cells and NK cell activity. Therefore, the increased IL-1β expression that was observed in the present study would have many downstream effects on the immune system of the fish. Increased IL-1β production may also result in increased phagocytic activity (Low et al., 2003).

IL-12 is a pro-inflammatory cytokine that provides defense against parasites, viruses and intracellular bacteria by stimulating the production of IFN-γ by NK cells and T cells (Øvergård et al., 2012). 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) (Kobayashi et al., 1989). In the present study, the expression of IL-12p35 and IL-12p40 genes at higher levels in yeast extract-administered fish compared with non-treated fish indicates that these IL-12 chains are co-expressed in the same cell and generate bioactive IL-12 (Gubler et al., 1991). To date, IL-12p35 and IL-12p40 subunits have been characterized in common carp (Forlenza et al., 2008; Huising et al., 2006), European sea bass (Nascimento et al., 2007) and pufferfish (Yoshiura et al., 2003), and more recently, IL-12p35 (IL-12β) has been identified in the Atlantic halibut (Øvergård et al., 2012).

Human chemokines induce the migration of leucocytes towards infection or injury sites (Dixon et al., 1998) by acting as chemoattractants that cause an influx of nuetrophils, monocytes, T cells and basophils into these sites. In the present study, we observed that a high level of CXC-chemokine expression was found mainly in fish treated with yeast extract. The homology of the gene expressed may be similar to CXCL10, which has been reported in the same species by Savan et al. (2003a). In other fish studies, it was shown that trout CXCL8 expression is increased in head kidney macrophages following exposure to LPS and recombinant human TNF-α (Sangrador-Vegas et al., 2002). CXC-chemokine gene expression was also observed in common carp treated with cytidine-phosphateguanosine (CpG) dinucleotides (Tassakka and Sakai, 2004; Tassakka et al., 2006) and zebrafish to which β-glucan had been administered (Rodríguez et al., 2009).

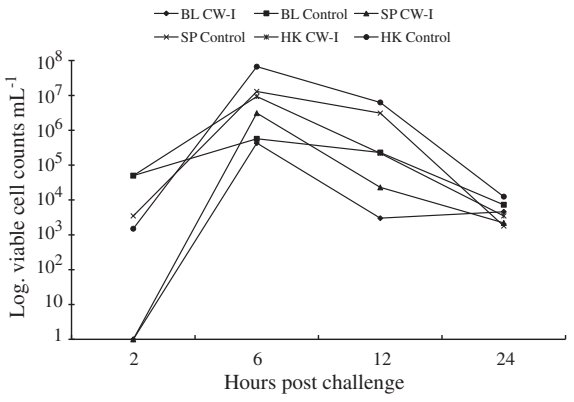

Fig. 4. Distribution of *Aeromonas hydrophilla* in the blood, spleen and head kidney of CW-I-treated and control carp (n = 5) challenged with 3.4×10^9 cfu bacteria mL^{-1}. BL = blood; SP = spleen; HK = head kidney.

Dietary baker's yeast extract down-regulated the expression of the IL-10 gene. IL-10 is a multifunctional cytokine with cytokine synthesis inhibitory and immunosuppressive functions (Savan et al., 2003b). In contrast to our observation, an increase in IL-10 gene expression was reported in sea bass treated with LPS (Buonocore et al., 2007) and in common carp fed with human IFN-α (Watanuki et al., 2009). However, Spirulina-treatment of common carp induces a decrease in IL-10 gene expression similar to the decrease that we observed (Watanuki et al., 2006). It is therefore possible that down-regulation of the function of the anti-inflammatory cytokine IL-10 might have favored the enhanced expression of pro-inflammatory cytokines in the yeast extract-treated fish.

IFNs are important cytokines that induce an antiviral state of cells and contribute to the defense against virus infection in vertebrates. There are three sub-families of IFNs, types I, II and III identified in vertebrates. Type I (mainly IFN-α and IFN-β) and type III IFNs have a major role in the first line of defense against viruses. However, type II IFN is identical to IFN-γ which plays a major role in innate, as well as in adaptive cell-mediated immune responses in which it mainly removes intracellular pathogens, and IFN-γ also plays a role in tumor control. This cytokine stimulates macrophage-mediated phagocytosis as well as macrophage production of pro-inflammatory cytokines and antimicrobial oxygen radicals (Schoenborn and Wilson, 2007). We found that IFN-γ2 gene expression was induced by yeast extract administration to common carp, whereas induction of Type I IFN (IFN-α) expression was not detected. No expression of type I IFN indicates that the baker's yeast extract treatment may not provide protection against virus infection. In contrast, the baker's yeast extract might have elevated the IFN-γ2 mediated intracellular pathogen killing ability of leucocytes in common carp. The existence of two IFN-γ genes (IFN-γ1 and 2) in common carp has already been described based on in vitro experiments (Stolte et al., 2008). Therefore, the present study reconfirmed the existence and expression of the IFN-γ2 gene in common carp.

In this study, the fish were administered with baker's yeast extract once a day for 3 days and gene expression level was studied till 10 days post treatment period. Significant up-regulation of most of the cytokine genes was noticed on day 1 post-treatment compared with the other later time points. This may be because of cytokines, as innate immune components for the first line of host immunity in fish are activated rapidly and released in quick succession under stimulation (Tanekhy et al., 2010). At later stages, immunostimulatory effects of baker's yeast might have dwindled down and were responsible for down-regulation of cytokine gene expression.

The NBT assay used in this study indicates the ability of phagocytes to kill pathogens by producing oxygen radicals. This ability of fish macrophages confers protection against diseases. Increased respiratory burst activity can be correlated with increased oxygen radical production and increased killing activity (Sharp and Secombes, 1993). In the present study oxygen radical production in common carp appeared to be positively influenced by yeast extract administration. A similar increase in superoxide anion production was reported in hybrid striped bass (Li and Gatlin, 2003) and in the gilthead seabream (*S. aurata*) (Ortuño et al., 2002) when fed with the brewer's yeast *S. cerevisiae*, in gilthead seabream when fed with the live yeast

D. hansenii (Reyes-Becerril et al., 2008) and in the rohu (*Labeo rohita*) when fed with baker's yeast (Tewary and Patra, 2011) or with a yeast extract (Andrews et al., 2011). In agreement with the increased oxygen radical-producing activity that we observed following dietary administration of the yeast extract, this extract also activated other functions of carp leucocytes, including phagocytosis, and resulted in an increase in the phagocytic index. Higher levels of activation of phagocytic cells were recorded in the leucocytes of treated fish at 1, 3 and 5 days post-treatment compared with control fish. However, significantly increased phagocytic activity and a higher phagocytic index were noticed at 3 days after yeast extract treatment. Our results of induction of phagocyte stimulation by a yeast extract are consistent with previous reports on the effects of treatment of the same fish with human IFN-α (Watanuki et al., 2009), Spirulina (Watanuki et al., 2006) and yeast RNA (Sakai et al., 2001). It is pertinent to assess the resistance conferred to fish treated with the yeast extract. Dietary yeast or yeast extract supplementation can enhance resistance to bacterial pathogens such as *A. hydrophila* in several fish species such as the common carp (*C. carpio*) (Gopalakannan and Arul, 2010), the leopard grouper (*Mycteroperca rosacea*) (Reyes-Becerril et al., 2011), the Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab, 2012; Abdel-Tawwab et al., 2008) and the rohu (*L. rohita*) (Andrews et al., 2011). *A. hydrophila*, which we used for disease resistance studies of yeast extract-treated common carp, is a major bacterial fish pathogen. We compared the resistance levels of the yeast extract-treated and non-treated fish by determining the fluctuation in the number of bacterial cells in their blood and organs after an artificial bacterial challenge. Bacterial loads were lower in the blood, spleen and kidney of carp treated with the yeast extract than in the control group. These data show that administration of dietary baker's yeast extract to the fish in our study enhanced the fish resistance to this bacterial pathogen. The increased fish resistance to *A. hydrophila* infection, together with the increased fish macrophage oxygen radical production and phagocytic activities that we observed in the treated fish in our study, correlate with the observed enhanced regulation of cytokine expression in the treated fish.

The present findings of increased or induced expression of cytokine genes in the head kidney, enhanced phagocytic activity, superoxide anion production and resistance to a bacterial pathogen that resulted from treatment of the common carp with a baker's yeast extract indicate the immunostimulatory effects of this yeast extract that contains both nucleotides and β-glucan. Our results support the use of this extract as a potential fish immunostimulant. Further research is required to elucidate the cytokine signaling networks and their various roles in immune responses in fish treated with the baker's yeast extract using different doses and treatment modes.

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