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Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements

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ABSTRACT

The objective of the present study was to determine the action of β-glucans as feed additives on the gene expression profile of some inflammatory-related cytokines from common carp (*Cyprinus carpio* L.) during the early stages of a non-lethal bacterial infection with *Aeromonas salmonicida*. β-glucan (MacroGard®), was administered daily to carp (6 mg per kg body weight) in the form of supplemented commercial food pellets for 14 days prior to infection. Control and treated fish were then intraperitoneally injected with PBS or 4×10^8 bacteria per fish and were sampled at time 0 and 6 h, 12 h, 1 day, 3 days and 5 days post-injection. Head kidney and gut were collected and the gene expression patterns for *tnfα1*, *tnfα2*, *il1β*, *il6* and *il10* were analyzed by quantitative PCR. Results obtained showed that treatment with β-glucans generally down-regulated the expression of all measured genes when compared to their corresponding controls. After injection, highest changes in the gene expression levels were obtained at 6 h; particularly, in head kidney there was higher up-regulation of *tnfa1* and *tnfa2* in infected fish fed β-glucans in comparison to control feed; however, in gut there was a significant down-regulation of *tnfα1*, *tnfα2*, *il1β* and *il6* in infected fish fed β-glucans. Analysis of carp specific antibodies against *A. salmonicida* 30 days after injection revealed their levels were reduced in the infected β-glucan group. In conclusion, a diet supplemented with β-glucan (MacroGard®) reduced the gene expression levels of some inflammation-related cytokines in common carp. Such a response appears to be dependent of organ studied and therefore the immunostimulant may be preventing an acute and potential dangerous response in gut, whilst enhancing the inflammatory response in head kidney when exposed to *A. salmonicida*.

1. Introduction

In the medical and veterinary fields there is now a recognized philosophy that prevention of infection is a more desirable goal than treating an infected host. Whilst in aquaculture there have been significant advances in the development of vaccines and the application of chemotherapy, the specificity of the protection induced and the possibility of the induction of resistance, respectively, has recently led immunologists to investigate alternative strategies in disease prevention. The use of stimulants of the innate immune system as feed supplements in intensive fish farming is a promising method to control pathogenic outbreaks and several studies, for example Dalmo and Bogwald [1] have speculated that the use of immunomodulators such as β-glucans enhances innate resistance to infection.

β-glucans are glucose polysaccharides that are connected by β-glycosidic bonds or D-glucose molecules which are repetitively linked at a specific position. These complex carbohydrates also have branching glucose side-chains and are found in plants, algae, fungi and some bacteria where they are a major structural component of the cell wall. β-glucan forms derived from yeast mainly comprise D-glucose units with β-1,3-linkages and side-chains of D-glucose at position six. These homopolysaccharides are denominated as β-1,3/1,6-glucans and have been shown to reduce the susceptibility to infection [1,2].

The resistance conferred by β-glucans against pathogens has been reported in several fish species; for instance, Asian catfish (*Clarias batrachus*) [3,4], common carp (*Cyprinus carpio*) [5], catla (*Catla catla*) [6], Nile tilapia (*Oreochromis niloticus*) [7], rohu (*Labeo rohita*) [8] and zebrafish (*Danio rerio*) [9] infected with *Aeromonas hydrophila*, grass carp (*Ctenopharyngodon idella*) infected with grass carp hemorrhage virus (GCHV) [10]; large yellow croaker (*Pseudosciaena crocea*) infected with *Vibrio harveyi* [11]; and rohu infected with *Edwardsiella tarda* [12]. In general, the protective activities induced by β-glucans in fish include an increase in a number of

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immune parameters such as antibody production or response [6,12,13], increased adjuvant effect [5,6], number of leucocytes (particularly neutrophils and/or monocytes) [8,9,13], lysozyme activity [11,14,15], macrophage bactericidal activity [9,13], phagocytic activity [3,8,11,14], serum lytic or bactericidal activity [8,14,15] and superoxide anion production [3,5,10,13,14]. However, there are inconsistencies between these findings, with several studies highlighting the action of β-glucan as controversial. For instance, in comparison with the above studies, diets containing β-glucan administered to channel catfish (*Ictalurus punctatus*) over a 4 weeks period did not significantly affect several immune parameters such as plasma lysozyme, bactericidal and hemolytic complement activities, respiratory burst of phagocytes and the number of lymphocytes found. In addition, changes were not observed in the survival rate in fish infected with *Edwardsiella ictaluri* [16].

It has been suggested that the immunostimulant activity of β-glucans could be mediated by the modulation of the pro-inflammatory cytokines and chemokines gene expression profile. For example, Selvaraj et al. [13] reported that β-glucan injection induced the gene expression of interleukin (IL)1β in carp head kidney macrophages. Also, in rainbow trout (*Oncorhynchus mykiss*), *il1β* and *il6* transcript levels increased in liver, head kidney and spleen after β-glucan injection [17]. *In vitro* studies showed that treatment with β-glucan of head kidney leukocytes from trout up-regulated *il1β*, *il6*, *il10* and tumor necrosis factor α (TNFα) and decreased transforming growth factor β (TGFβ) transcript levels [18]. Similar results were obtained for *il1β* and *il6* expression levels when treating trout head kidney macrophages [17].

Most of these studies have involved injection of this immunostimulant or direct application to isolated cells. Oral administration is potentially the most effective practice for mass immune-stimulation with a reduction of stress in fish kept in aquaculture. In this paper we report for the first time how cytokines involved in the inflammatory response and antibody levels in carp (*C. carpio*) respond to a diet supplemented with *Saccharomyces cerevisiae* derived β-1,3/1,6-glucan (MacroGard®, Biorigin) and exposure to a non-lethal dose of the gram negative bacteria *Aeromonas salmonicida*, causative agent of the disease furunculosis which produces severe losses in aquaculture [19].

2. Materials and methods

2.1. Experimental animals

Common carp from Ukrainian line were grown in re-circulating systems at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Poland. Fish, 12 months of age (average weight: 78.4 g) at the time of infection, were maintained at 18 °C. Prior to experiment fish were acclimatized to the re-circulating system and fed with control feeding at 3% body weight per day for a period of 14 days.

2.2. Diet formulation and feeding regime

Feed was prepared and provided by Tetra GmbH in a standard pellet feed (Table 1) fortified with 0.1% MacroGard® supplied by Biorigin. Fish received either standard or β-glucan supplemented feed at 1% of their body weight per day, for 14 days prior to injection. Thus, β-glucan intake was 6 mg per kg of fish body weight per day.

2.3. Infection with Aeromonas salmonicida

A. salmonicida subsp. salmonicida strain A449 from Polish origin was initially isolated and identified from naturally infected carp, followed by pathogenecity tests conducted by intraperitoneal injection in carp [20]. Bacteria were grown in lysogeny broth (LB) medium for 18 h at 25 °C. The culture was centrifuged at 1600 × *g* for 10 min and the pellet reconstituted in PBS (pH 7.4). Optical density was measured (UV-1601 PC, UV–Visible Spectrophotometer, Shimadzu) and aligned with a previously derived McFarland scale. Fish were intraperitoneally injected with either 250 μl of bacteria (4×10^8/ml, a sub-lethal dose) or 250 μl PBS only (control fish). No mortality was observed during the feeding and infection periods.

Table 1
Diet formulation.

Component	%
Fish protein concentrate	45.00
Wheat starch[a]	41.00
Cellulose	2.57
Soybean oil	4.50
Fish oil	4.50
Ethoxyquin	0.02
Vitamin-premix	0.25
Stabilized vitamin C	0.11
Mineral-premix	2.06

[a] β-glucan diet is fortified with 0.1% MacroGard® (this percentage is taken out from wheat starch).

2.4. Experimental design

The experimental design comprised four treatment groups i.e. control feeding injected with PBS, β-glucan feeding injected with PBS, control feeding injected with *A. salmonicida* and β-glucan feeding injected with *A. salmonicida*, each comprising 33 fish. Also, 10 extra fish were used for time point 0, i.e. just before the injection. Five fish from each group were sacrificed by exposure to 2 mL L^{-1} Propiscin (2% etomidate, produced by Inland Fisheries Institute, Poland) [21]. A section of the midgut and head kidney tissues were removed at time 0, 6 h, 12 h, 1 day, 3 days and 5 days post-injection. Samples were stored in RNA later (Invitrogen) at −80 °C and transported to Keele University, UK, for molecular analysis. The remaining fish (8 fish per group) were used at 30 days post-injection to detect the presence of specific antibodies against *A. salmonicida* as described below.

2.5. RNA isolation and cDNA synthesis

"RNeasy" kit (Quiagen) was used to isolate and purify gut and head kidney total RNA following manufacturer's instructions. Isolated RNAs were re-suspended in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) and stored at −80 °C. Template RNA (0.5 μg) was used to obtain cDNA using random hexamer (Applied Biosystems) and the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen) according to manufacturer's instructions in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems).

2.6. RT-qPCR

RT-qPCR assays were performed using the ABI PRISM® 7000 Sequence Detector System (Applied Biosystems) in a final volume of 20 μl, containing 900 nM of each primer, an amount of cDNA corresponding to 5 ng of RNA and 1× Power SYBR Green PCR Master Mix (Applied Biosystems). All primers used (Eurofins MWG Operon, London, UK) are listed in Table 2. Primers for amplification of the *il6* gene were designed using the Primer Express® software v3.0 (Applied Biosystems). Thermal cycling conditions followed the

Table 2
Oligonucleotide primers used in this study.

Gene	Primer	Sequence (5′–3′)	GenBank accession no.	Reference
40s	40S.FW	CCGTGGGTGACATCGTTACA	AB012087	[39]
	40S.RV	TCAGGACATTGAACCTCACTGTCT		
tnfα1	TNFα1.FW	GAGCTTCACGAGGACTAATAGACAGT	AJ311800	[40]
	TNFα1.RV	CTGCGGTAAGGGCAGCAATC		
tnfα2	TNFα2.FW	CGGCACGAGGAGAAACCGAGC	AJ311801	[40]
	TNFα2.RV	CATCGTTGTGTCTGTTAGTAAGTTC		
il1β	IL1β.FW	AAGGAGGCCAGTGGCTCTGT	AJ245635	[39]
	IL1β.RV	CCTGAAGAAGAGGAGGCTGTCA		
il6 Family	IL6fam.FW	GCAGCGCATCTTGAGTGTTTAC	AY102632	Primer express v3.0
	IL6fam.RV	CTGCTGCTCCATCACTGTCTTC		
il10	IL10.FW	CGCCAGCATAAAGAACTCGT	AB110780	[41]
	IL10.RV	TGCCAAATACTGCTCGATGT		

standard default protocol of the instrument. A melting curve for each PCR was conducted to ensure that only a single product had been amplified. Gene expression results were analyzed using the $2^{-\Delta\Delta Ct}$ method [22] and data for all treatment groups were compared to the control group at each time point (absolute values of the control group at each time point are shown in supplemented data Fig. S1 to confirm gene expression stabilization). The data obtained for each gene were normalized against those obtained for the expression of the 40S ribosomal protein S11 reference gene which did not change significantly at each time point. Data are represented as the mean fold changes ± standard deviation for five independent individuals, each one performed in duplicate.

2.7. IgM antibody response

The presence of specific antibodies against *A. salmonicida* in common carp sera was determined 30 days post-injection through an enzyme-linked immunosorbant assay (ELISA). The protocol was adapted from Cochet et al. [23] using whole bacteria, cultured as above. Between each stage of the assay, plates were washed three times with 0.05% Tween20 (Sigma) in 10% PBS. Briefly, bacteria were diluted in bicarbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$/L, pH9.6) and used to coat microtitre plates, 3×10^7 bacteria per well. Plates were incubated overnight at 4 °C and then wells were blocked with 4% skimmed milk powder in PBS. Serum from infected and non-infected carp was added at 1:30, 1:60 and 1:120 in dilution buffer (2% skimmed milk powder in PBS) and incubated at 25 °C for 80 min. The presence of aeromonas-specific carp antibodies were detected using WCI12 (1:500 in dilution buffer) [24], followed by goat-anti-mouse IgG conjugated with horse-radish peroxidase (1:2000 in dilution buffer) (Bio-Rad). Both antibodies were incubated on the plate at 25 °C for 60 min. The substrate, 3, 3′, 5, 5′ tetramethyl benzidine (TMB; Sigma), was then added and allowed to develop for 10 min before being stopped using 1 M H_2SO_4. Assay absorbance values were then measured at 450 nm (Tecan Sunrise Automatic Plate Reader, Austria). All samples were run in triplicate, alongside positive and negative sera as well as a dilution buffer control and reagent blanks.

2.8. Statistical analysis

Data were statistically analyzed by using two way analysis of variance (ANOVA) (repeated-measure analysis for IgM antibody response) and Bonferroni's multiple comparisons to determine significant differences between the different treatments and their respective control groups. GraphPad Prism v4 software was used for creating the graphs and statistical analysis.

3. Results

3.1. Effect of β-glucan supplemented diets on the expression of selected inflammatory genes

The expression of selected inflammatory genes (Fig. 1) in carp fed control and β-glucan supplemented diets changed significantly in gut ($F = 29.25$; $P < 0.0001$) but not in head kidney tissues ($F = 1.93$; $P = 0.095$) after 14 days. All genes analyzed were down-regulated in gut as were *tnfα2* and *il6fam* in head kidney when fed with β-glucan supplemented diets, although Bonferroni's pairwise comparison indicated that only *tnfα2* in gut ($P < 0.001$) and head kidney ($P < 0.05$) and *il10* in gut ($P < 0.05$) were significantly down-regulated. The expression levels for *tnfα2* in gut and

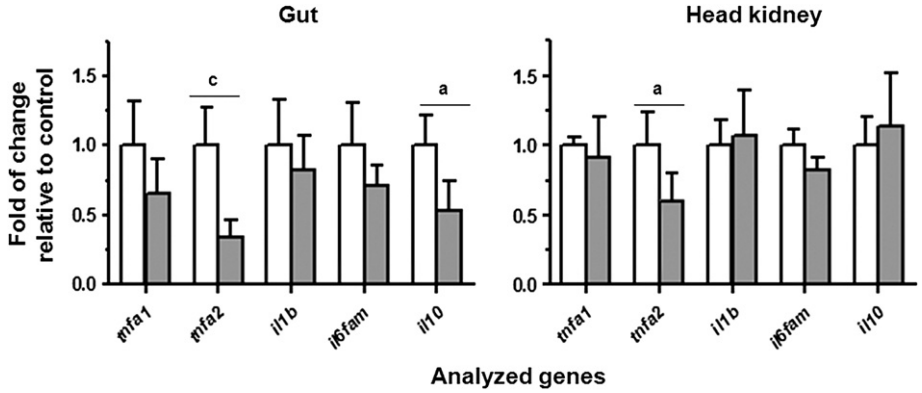
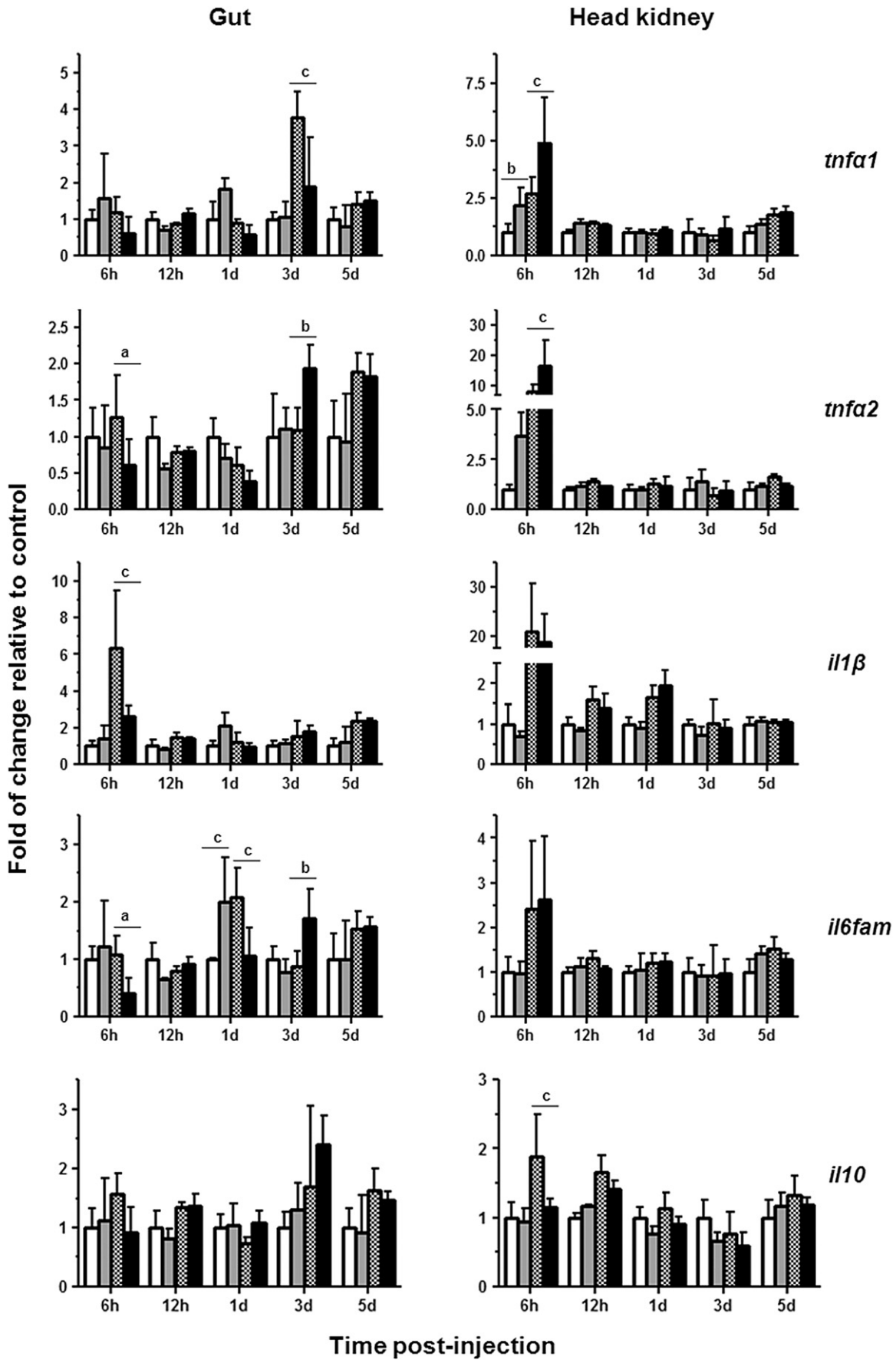


Fig. 1. Effect of the β-glucan supplemented diet on the transcript levels of selected inflammatory-related genes in gut and head kidney of carp after 14 days of experimental feeding. *tnfα1*, *tnfα2*, *il1β*, *il6fam* and *il10* genes transcript levels were analyzed by RT-qPCR. Expression levels of β-glucan supplemented feeding samples (grey bars) are reported as fold of change compared to the control feeding samples (white bars). Significant differences when comparing β-glucan supplemented diet groups with their associated control diet groups are represented by a ($P < 0.05$) and c ($P < 0.001$). Mean ± SD; $n = 5$.


Gut
Head kidney
tnfα1
tnfα2
il1β
il6fam
il10
Fold of change relative to control
Time post-injection
6h
12h
1d
3d
5d

head kidney were reduced to 0.34- and 0.61-fold, respectively, and the expression level for *il10* in gut was 0.53-fold all in comparison to their corresponding controls.

3.2. Inflammatory-related cytokine gene expression after injection with A. salmonicida

The expression of *tnfα1* (Fig. 2) changed significantly after injection of the bacteria between times and treatments in both gut and head kidney tissues ($F = 8.67$; $P < 0.0001$ and $F = 7.82$; $P < 0.0001$, respectively). Bonferroni's pairwise comparison indicated there was a significant down-regulation at 3 days post-injection in gut from infected fish fed β-glucan supplements in comparison to those fish fed control diet ($P < 0.001$), and a significant up-regulation at 6 h post-injection in head kidney from either PBS injected and infected fish fed with β-glucan supplemented feed compared to their corresponding control groups ($P < 0.01$; $P < 0.001$, respectively). In this latter case, a 4.91 ± 1.95-fold increase in the expression of this gene was found for infected fish fed with β-glucan supplemented diet compared to 2.68 ± 0.75-fold increase for infected fish with control feed.

The expression of *tnfα2* (Fig. 2) also changed significantly after injection between times and treatments in both gut and head kidney tissues ($F = 5.14$; $P < 0.0001$ and $F = 12.02$; $P < 0.0001$, respectively). However, in comparison to *tnfα1* gene expression there were significant down- and up-regulation at 6 h ($P < 0.05$) and 3 days ($P < 0.01$) post-injection respectively in gut tissue from infected fish fed with β-glucan supplemented diet. Like *tnfα1*, *tnfα2* was significantly up-regulated in head kidney at 6 h post-injection ($P < 0.001$) in injected fish fed with β-glucan supplements (16.55 ± 8.29-fold increase) compared to corresponding control group (7.98 ± 2.25-fold increase).

The expression of *il1β* (Fig. 2) also changed significantly after injection between times and treatments in both gut and head kidney tissues ($F = 7.13$; $P < 0.0001$ and $F = 17.55$; $P < 0.0001$, respectively). Significant decrease was however only found in *il1β* transcript levels at 6 h post-injection in the gut tissue from infected fish fed with β-glucan supplemented diet in comparison to control feeding ($P < 0.001$).

The expression of *il6* (Fig. 2) changed significantly after injection between times and treatments in both gut and head kidney tissues ($F = 5.41$ and $P < 0.0001$ and $F = 2.63$; $P = 0.0051$, respectively). Several significant differences in gene expression in the gut were observed whilst none were noted in head kidney. In the gut tissue, *il6* was significantly down-regulated (over 2-fold) at 6 h ($P < 0.05$) and 1 day ($P < 0.001$) post-injection in infected fish fed with β-glucan supplemented diets and significantly up-regulated at 3 days.

The expression of the regulatory cytokine *il10* (Fig. 2) also changed significantly after injection between times and treatments in both gut and head kidney tissues ($F = 2.3$; $P = 0.014$ and $F = 4.17$; $P < 0.0001$, respectively). Significant decrease in gene expression was however only found at 6 h post-injection in the head kidney tissue from infected fish fed with β-glucan supplemented feed in comparison to control feed ($P < 0.001$).

3.3. Specific IgM response to A. salmonicida

The amount of specific antibodies to *A. salmonicida* differed significantly between feeding treatments ($F = 40.45$; $P < 0.0001$), although no specific antibodies were detected in the PBS injected groups either fed control or β-glucan supplemented diets. However, specific antibodies against bacteria were detected in those groups injected with the pathogen. Feeding fish with a β-glucan supplemented diet also reduced significantly ($P < 0.001$, $P < 0.001$ and $P < 0.05$ for titres 1/30, 1/60 and 1/120, respectively) the amount of specific antibodies present in comparison to the infected group with control feeding. In these infected fish fed normal diet the antibody titre was 4-fold higher than in infected fish fed with β-glucan supplemented diet (Fig. 3). In order to confirm this effect a second smaller-scale experiment and similar results were obtained (data not shown).

4. Discussion

In the present study it has been shown that feeding a β-glucan supplemented diet to carp affects the inflammatory response and health status of the fish although the changes noted are dependent on organ analyzed.

After 14 days of feeding a β-glucan supplemented diet, there was a down-regulation of expression of the selected genes. Decrease of the gene expression was more pronounced in gut, although only *tnfα2* and *il10* were significantly down-regulated in comparison to control tissues from fish that were fed control diet. *tnfα2* was the only gene of those analyzed that was significantly down-regulated in head kidney. Since β-glucan was orally administrated, it is reasonable expectation that its effect should initially be located in gut tissue. Although the literature on the effects of β-glucan on cytokine expression is inconsistent, the observed down-regulation of inflammatory-related genes noted is in agreement with other studies performed in fish and mammals. For example, Djordjevic et al. [25] showed that the expression of several immune-genes (including genes related with the inflammatory process) were reduced in spleen from rainbow trout after feeding with experimental diets containing lentinan and in rat, orally administration of β-glucan reduced some parameters associated with sepsis-induced oxidative organ injury, including serum TNFα levels [26,27]. Our studies therefore confirm the differential response of organs to the administration of β-glucan. Such a response, which was manifested in the profile of gene regulation, may relate to the duration and administration route of the immunostimulant or reflect the different residential or migratory cellular composition in the organs examined. Indeed, the induction of leukocyte migration and/or alternative polarization of macrophages triggered by the β-glucan content of the diets been previously reported by Chadzinska et al. [28] when injecting zymosan intraperitoneally to carp.

Previous studies have revealed that the expression profile of selected immune-genes associated with inflammation is affected by infection with pathogens such as *A. salmonicida*. Expression profiles evaluated after infection with the bacteria in several tissues for different fish species such as Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), rainbow trout (*O. mykiss*) and turbot (*Scophthalmus maximus*), showed significant changes in a variety of components of the immune system. This included the up-regulation of relevant genes involved in the inflammatory response, such as *tnfα* and *il1β* and interferon-related genes [29–36]. In our studies, the pattern of the gene expression involved in the inflammatory response observed to β-glucan administration

Fig. 2. Time course of the expression of the inflammatory-related cytokines genes *tnfα1*, *tnfα2*, *il1β*, *il6* and *il10* in gut and head kidney of carp after intraperitoneal injection with *A. salmonicida* fed control or β-glucan supplemented diet. Expression was determined by RT-qPCR and normalized to 40S ribosomal protein S11 gene expression. The gene expression data for all treatment groups were compared to the control group (control diet, non-infected) at each time point. Data are represented as the mean fold changes ± SD ($n = 5$). Significant differences when comparing β-glucan supplemented diet groups with their associated control diet groups are represented by a ($P < 0.05$), b ($P < 0.01$) and c ($P < 0.001$). Groups: □, control diet, non-infected; ▒, β-glucan supplemented diet, non-infected; ▓, control diet, infected; ■, β-glucan supplemented diet, infected.

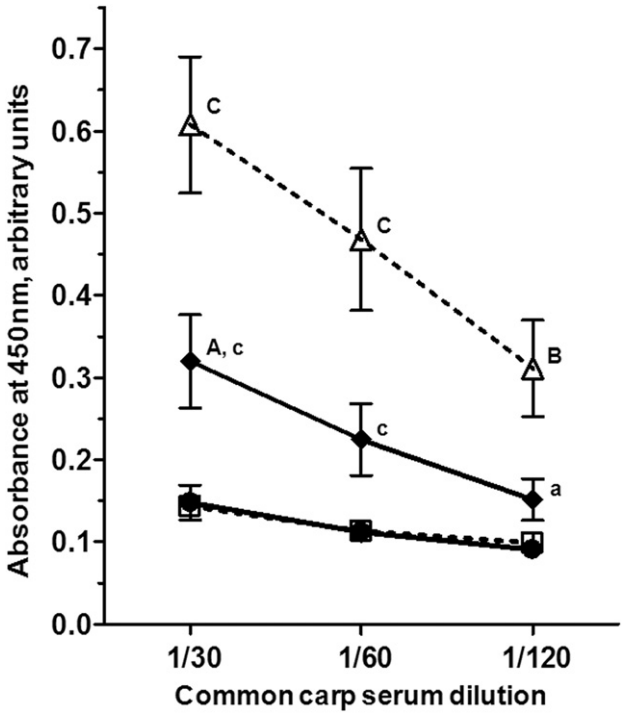


Fig. 3. Specific antibodies in sera from common carp fed with or without β-glucan supplemented diets and injected with *A. salmonicida*. Data are represented as average values ± standard error of the mean (SEM) ($n = 8$). Significant differences are represented by: A, a ($P < 0.05$); B, b ($P < 0.01$) and C, c ($P < 0.001$), where A, B and C represent significant differences within the same feeding group and a, b and c within the same time point. --□--, control diet, non-infected; —●—, β-glucan supplemented diet, non-infected; --△--, control diet, infected; —◆—, β-glucan supplemented diet, infected.

was also affected by injection with *A. salmonicida*. In general, highest changes in the gene expression levels were obtained at 6 h post-injection, predominantly in head kidney, although some significant changes at 1 or 3 days were also detected in the gut. In particular, both *tnfα1* and *tnfα2* transcripts increased significantly at 6 h post-injection in head kidney for both infected and non-infected fish fed with β-glucan supplements in comparison to their corresponding normal feeding controls. In head kidney at the same time point, high increases of *il1β* expression were restricted to infected groups showing a clear response against the presence of bacteria, although differences between diets were not significant. However, expression levels of these genes together with *il1β* and *il6fam* decreased at the same time point in gut for infected fish fed with β-glucan supplements, which is interesting since IL-1, IL-6, and TNFα are considered important inducers of the acute phase response [37].

Regarding the regulatory cytokine IL10, its mRNA levels remained unchanged in the gut during the whole time course studied, while a significant reduction was observed in the head kidney at 6 hours in infected fish fed with β-glucan supplements. This result together with those obtained from the pro-inflammatory cytokines (mainly *tnfα1* and *tnfα2*) suggests that β-glucan containing diets enhanced the inflammatory response in head kidney and reduced it in gut. The β-glucans may therefore be preventing an acute and potential dangerous response in gut, and enhancing the inflammatory response in head kidney. In addition, this anti-inflammatory state found in gut in response to β-glucan treatment may contribute to an enhanced transport of nutrients and metabolic processes which may explain weight gain reported by several authors after feeding fish with β-glucan enhanced diets [11,14,38]. It is interesting that the responses noted, particularly in the head kidney, are transient and were reduced after 6 h. This may reflect that in order to ensure fish survival our study incorporated a relatively low virulent form of *A. salmonicida*. Analysis of the antibody response at thirty days after injection did show the presence of specific antibodies against *A. salmonicida* in both infected groups, however the group fed with control feeding had significantly higher titres than fish fed with a β-glucan supplemented diet. This fact could be partially explained by the decrease of the inflammatory response observed in both head kidney and gut samples after the feeding period and in gut tissue after injection. Also, the significant up-regulation of the pro-inflammatory cytokines observed in head kidney after injection in the fish fed with β-glucan supplemented diet may have triggered other defense mechanisms, such as the activation of the cellular response or a more potent acute phase response, which could have cleared the bacteria more efficiently. It is of interest to note that several other investigations have revealed that β-glucans can affect antibody titres in fish although this interaction may be dependent on delivery route. For example, despite several studies that have reported the adjuvant properties of β-glucans in fish [5,6,13], enhancement of specific antibody levels were only significant when β-glucans were injected intraperitoneally, with no increases seen during oral administration. However, high rates of protection were found in both cases. Sahoo et al. [12] described the adjuvant properties of β-glucan when they were orally administered to Indian major carp (*L. rohita*), although an increase in specific antibody levels and protection against *E. tarda* were only obtained in immunocompromised fish, not healthy fish.

In conclusion, we have shown that β-glucan supplemented diet administered to common carp decreased the transcript levels of several pro-inflammatory cytokines in gut and head kidney tissues. The infection with *A. salmonicida* did not modify this tendency in gut, but levels of tnfα1, tnfα2, il1β and il6fam became significantly higher in fish fed β-glucan supplemented diet at 6 h post-infection. Such differential effects may reflect the complex interactions between the bacterium and the immunostimulant's relationship with the inflammatory response of the host. Analysis of the antibody response however revealed at one month after injection antibody levels against *A. salmonicida* were 4-fold lower in this group. Further research on the molecular aspects of the different β-glucan forms, dose, duration, administration and experimental models (fish-pathogen) are needed to better understand the immunological activities of this immunostimulant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.02.028.

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