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Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection

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ABSTRACT

The effect of β-glucans as feed additive on the profile of C-reactive protein (CRP) and complement acute phase responses was studied in common carp *Cyprinus carpio* after exposition to a bacterial infection with *Aeromonas salmonicida*. Carp were orally administered with β-glucan (MacroGard®) for 14 days with a daily β-glucan intake of 6 mg per kg body weight. Fish were then intraperitoneally injected with either PBS or 1×10^8 bacteria per fish and sampled at time 0, 6, 12, 24, 48, 72, 96 and 120 h post-injection (p.i.) for serum and head kidney, liver and mid-gut tissues. CRP levels and complement activity were determined in the serum samples whilst the gene expression profiles of CRP and complement related genes (*crp1*, *crp2*, *c1r/s*, *bf/c2*, *c3* and *masp2*) were analysed in the tissues by quantitative PCR. Results obtained showed that oral administration of β-glucan for 14 days significantly increased serum CRP levels up to 2 fold and serum alternative complement activity (ACP) up to 35 fold. The bacterial infection on its own (i.e. not combined with a β-glucan feeding) did have significant effects on complement response whilst CRP was not detectably induced during the carp acute phase reaction. However, the combination of the infection and the β-glucan feeding did show significant effects on both CRP and complement profiles with higher serum CRP levels and serum ACP activity in the β-glucan fed fish than in the control fed fish. In addition, a distinct organ and time dependent expression profile pattern was detected for all the selected genes: a peak of gene expression first occurred in the head kidney tissue (6 h p.i. or 12 h p.i.), then an up-regulation in the liver several hours later (24 h p.i.) and finally up- or down-regulations in the mid-gut at 24 h p.i. and 72 h p.i. In conclusion, the results of this study suggest that MacroGard® stimulated CRP and complement responses to *A. salmonicida* infection in common carp.

1. Introduction

Infectious diseases cause major economic losses in aquaculture and whilst prophylactic measures such as antibiotics and vaccine administration have been generally adopted, antibiotic resistance and vaccination costs have stimulated the need to develop alternative strategies to control pathogenic outbreaks [1]. One of the most promising prevention methods in fish farming is the enhancement of the fish innate immune defences through the administration of immunostimulants such as β-glucans [2].

β-glucans are polysaccharides consisting of a backbone of repetitive D-glucose monomer units linked by β-(1,3) glycosidic bonds with β-(1,6) branching glucose side-chains. These carbohydrates are mostly found in algae, plants, fungi and in some bacteria where they represent a major component of the cell wall. The use of β-glucans as immunostimulants has revealed an array of beneficial properties [2], including enhanced protection against pathogens in several fish species such as: *Aeromonas hydrophila* in common carp *Cyprinus carpio* [3–6], rohu *Labeo rohita* [7], Nile tilapia *Oreochromis niloticus* [8] or zebrafish *Danio rerio* [9]; *Aeromonas salmonicida* in rohu *L. rohita* [10]; *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* [11]; grass carp hemorrhage virus in grass carp *Ctenopharyngodon idella* [12] and *Ichthyophthirius multifiliis* in rainbow trout *Oncorhynchus mykiss* [13]. However, the beneficial effects of

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β-glucans are very dependent on the dose, the duration of the treatment and the route of administration [5]. For instance, if the administration of low doses of β-glucan (0.1% in the diet) to the fish has positive stress reducing effects, high doses (such as 1%) may have adverse effects such as high mortality rate during pathogen infection [14]. Also, a long-term dietary administration of β-glucans resulted in a negative effect on the survival of common carp challenged with *A. hydrophila* [15]. β-Glucans have numerous effects in a number of fish immune parameters such as increases in lysozyme activity [3,10,13,14], respiratory burst activity [3–5,8,12,14], phagocytic activity [7,8,11,14] and granulocyte number [4,5,7,9,10].

In addition, it has been shown that β-glucans can also have an effect on complement activity [6,10]. Complement, which is an essential part of the innate immune system, involves a large range of membrane-bound and soluble proteins which lead to the creation of a membrane attack complex that kills the pathogen. Activation of the complement system is also associated to opsonisation and phagocytosis [16,17]. The mammalian system is certainly the most described and probably the best understood [16]. However, complement component homologues have now been partially identified and described in teleost fish [17], and for some complement components the existence of several protein isoforms have been reported [18]. Recently there has been increased interest in how the complement expression profile is affected by pathogenic bacteria [19,20], viruses [21,22] and parasites [18,23], with or without previous immunostimulation [24–26]. In contrast, the effects of β-glucan on the complement expression profile are still unclear. For example, the expression of three C3 sub-types were differentially up and down-regulated in liver, spleen and head kidney tissues 48 h after a β-glucan stimulation in rainbow trout [24], whilst two sub-types of C5 were up-regulated in hepatopancreas of common carp 48 h after intraperitoneal injection of scleroglucan [27]. Complement activation occurs in fish via three pathways: the classical, the mannose binding lectin and the alternative pathways [18]. Upstream to the classical pathway trigger action, the complex C1q-C1r/s is itself activated by C-reactive protein (CRP) – C1q binding [28]. CRP, which belongs to the pentraxin family, is commonly associated with the acute phase response [29,30] and often considered as a biomarker of infection [31], with serum CRP levels increasing significantly following tissue injury, trauma or infection. For example, serum CRP levels in common carp infected with *A. hydrophila* increased up to 6 fold 40 h post-infection [31], whilst in channel catfish exposed to turpentine a 18-fold increase in serum CRP occurred compared to the control [32] and a 20-fold increase was noted in *rainbow trout* exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) [33]. However, the relationship between β-glucan immunostimulation and CRP profiles in fish has not been ascertained.

We report in the present study the first investigation of the relationship between β-glucan immunostimulation, *A. salmonicida* infection and acute phase protein profiles in common carp (*C. carpio*). The expression profile of two recently described CRP-like genes, *crp1* and *crp2* [34] and several carp complement component genes, *c1r/s*, *bf/c2*, *c3* and *masp2* [23], as markers of the acute phase response have been analysed in head kidney, liver and mid-gut tissues from common carp in response to β-glucan oral administration. Complement component genes have been selected depending on their functional role and their position in the complement pathways i.e. classical for *c1r/s*, classical and alternative for *bf/c2* and lectin for *masp2*. In addition, *c3* has been selected for its central role in the complement cascade [18]. Moreover, CRP levels and alternative complement pathway activity have also been determined from the serum of these fish.

2. Materials and methods

2.1. Experimental animals

The Ukrainian line of common carp [35] was kept at 19 ± 1 °C in re-circulating systems at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz, Poland. 150 fish, one year old with an average weight of 78.4 g, were acclimatised to the re-circulating system for 3 weeks, during which they were fed control feed lacking β-glucan at 3% body weight per day.

2.2. Experimental diet formulation and feeding regimes

Experimental diet was formulated and supplied by Tetra GmbH (Germany) as a standard pellet feed form fortified with 0.1% MacroGard® supplied by Biorigin (Brazil), a feed material which was chosen as a source of β-glucan in this study. The exact food composition has been given by Falco et al. [36]. Following the acclimatisation phase, one group of fish was kept on the control diet (0% MacroGard®) while the other group was fed with the experimental diet (0.1% MacroGard®), both feeding regimes were carried out at 1% fish body weight per day for a two week period. Thus, fish fed with the experimental diet received a daily dose of 10 mg/kg body weight MacroGard® (recommended dose by the commercial supplier, Biorigin), corresponding to a daily intake of 6 mg β-glucan per kg of fish body weight as MacroGard® contains 60% of β-glucan.

2.3. Immunological challenge with A. salmonicida

A. salmonicida subsp. *salmonicida* strain A449 from Polish origin [37] was chosen to ensure the fish survival over the challenge period. This strain was originally isolated and identified from naturally infected carp and its pathogenicity characterized by intraperitoneal injection in carp [38]. Bacteria were grown in lysogeny broth (LB) medium for 18 h at 25 °C, centrifuged at 1600× *g* for 10 min and the bacterial pellet reconstituted in PBS (pH 7.4). Optical density was measured at 540 nm (UV-1601 PC, UV–Visible Spectrophotometer, Shimadzu) and data were aligned with a previously derived McFarland scale to determine the bacterial concentration. Fish which had been fed for two weeks with MacroGard® supplemented food or control food were either injected intraperitoneally with a non-lethal dose of *A. salmonicida* (1×10^8 bacteria in 250 μL PBS per fish) or 250 μl PBS only (for the control fish). No mortality was observed during the feeding and infection periods.

The experimental design thus comprised four treatment groups: control fed and PBS injected, control fed and *A. salmonicida* injected, MacroGard® enriched diet and PBS injected, and MacroGard® enriched diet and *A. salmonicida* injected. In addition to 35 fish being utilised per treatment, 10 extra fish were used for time point 0, i.e. just before the exposure to the bacteria. Feeding was stopped after the injections and five fish from each group were sacrificed by exposure to a lethal dose of Propiscin (2 mL/L, 2% etomidate, produced by Inland Fisheries Institute, Poland) [39] at 6, 12, 24, 48, 72, 96 and 120 h post-injection (p.i.) when blood was then taken from the caudal vein. The blood was allowed to clot overnight at 4 °C, then centrifuged (2500× *g* for 10 min at 4 °C) and the serum stored at −80 °C. Head kidney, mid-gut and liver tissues were removed at time 0, 6, 12, 24, 72 and 120 h p.i. and stored in RNAlater (Invitrogen) at −80 °C. Samples (organs and serum) were transported to Keele University, UK, for further analysis.

2.4. Quantification of free-phase carp CRP (cCRP) in serum

Free-phase carp CRP (cCRP) was quantified with a competitive enzyme-linked immunosorbent assay (ELISA) using CRP extracted

and purified from healthy *C. carpio* serum using a two-step affinity chromatography procedure developed by Cartwright et al. [30] and MacCarthy et al. [31]. Briefly, the first step removes the serum high-density lipoprotein complexes containing apolipoprotein A-I which is known to be a major contaminant in the affinity isolation of carp CRP [30] and the second step exploits the calcium dependent binding affinity of CRP for phosphocholine. The free-phase purified cCRP was stored at 4 °C in a calcium buffer (10 mM $CaCl_2$, 50 mM Tris base, 15 mM $NaCl_2$, pH 8) until required. CRP levels in the serum of experimental and control fish were analysed using the competitive ELISA described by MacCarthy et al. [31]. Briefly 0.2 μg of cCRP diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) was bound to individual wells of a high-binding capacity 96-well plate (Corning) and incubated 20 h at 4 °C. The unbound cCRP was removed by a 3 times wash in a PBS-T buffer (PBS with 0.2% Tween-20) and remaining binding sites were blocked using PBS containing 5% of dried milk powder (Marvel) for 30 min at 37 °C. Excess of blocking agent was removed by a 3 times PBS-T wash. Carp serum (diluted 1:10 in PBS) was incubated with 160 μg/μL of rabbit anti-cCRP IgG (produced by Cartwright et al. [30]) at 20 °C for 30 min. In addition, to produce a standard curve, carp serum was replaced with purified free-phase cCRP serially diluted (0–0.5 μg/mL) in PBS. After incubation with either carp serum or standards, 100 μL of the treated rabbit anti-cCRP IgG solution was applied to the cCRP-coated wells and incubated for 1 h at 37 °C. After removal of the excess antibody and cCRP-rabbit IgG complexes by washing with a PBS-T, 100 μL of the secondary antibody, goat anti-rabbit IgG peroxydase conjugate (Sigma, dilution 1:8000 in PBS) was applied to the wells for 1 h at 37 °C. After a final 5 times PBS-T wash step, 100 μL of ophenylenediamine dihydrochloride (Sigma) was prepared according to manufacturer's instructions and added to each well. The plate was incubated in the dark for 1 h at 37 °C after which the enzymatic reaction was stopped with 25 μL of 2.5 M HCl, and the optical density read at 492 nm (Labsystems Multiskan MS plate reader). GraphPad Prism v5 software was used to determine the cCRP concentration of the individual serum samples by comparison between the sample absorbance value and those from the standard curve for which the free-phase cCRP concentration is known.

2.5. Alternative complement pathway activity assay in carp serum

Alternative complement pathway (ACP) activity was assayed according to previously described methods [4,5,40–43] with slight modifications. Sheep red blood cells (SRBC) (sheep blood in Alsevers supplied by TCS Biosciences) were washed with 0.85% saline solution and resuspended in gelatin veronol buffer (GVB) (0.1% gelatin, 250 mM NaCl, 50 mM barbitone, 40 mM sodium barbitone, 10 mM $MgCl_2$, 2 mM $CaCl_2$, all from Sigma) to obtain a standard working concentration of 8×10^6 SRBC per mL. A serial dilution of carp serum (from 0.5% to 7%) was prepared. Briefly, for each serum dilution, a positive control which reflects 100% haemolysis (X μL of serum, 100 μL distilled water and 100 μL SRBC) and a sample test (X μL of serum, 100 μL 10 mM EGTA-GVB and 100 μL SRBC) were incubated 1 h at 20 °C. The samples were then centrifuged at 400× *g* for 5 min at 4 °C and kept at 4 °C to stop the lytic reaction. 200 μL of supernatant was transferred to a 96-well plate (Corning) and the haemoglobin release optical density (OD) was read at 410 nm (Labsystems Multiskan MS plate reader). For each serum dilution, the rate of haemolysis is calculated by dividing the OD value for the sample test by the OD value for the positive control. GraphPad Prism v5 software was used to build a graph by plotting the log(dilution) against the log(rate of haemolysis). The ACH50 (in ACH50 units/mL), which correspond to the serum dilution giving 50% of SRBC haemolysis, was determined.

2.6. Gene expression by real time PCR

2.6.1. RNA isolation and cDNA synthesis

RNA was extracted and purified from mid-gut, head kidney and liver tissues using the RNeasy Mini Kit (Qiagen, UK) following manufacturer's instructions and concentration determined by Nanodrop 1000 (Thermo Scientific, UK). The RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) and stored at −80 °C prior to cDNA synthesis according to manufacturer's instructions (Invitrogen). Briefly, 0.5 μg RNA was added to random hexamers, 10 mM dNTPs, 25 mM $MgCl_2$, 10× PCR Buffer II, DEPC-treated water and the M-MuLV. Reactions were carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and cDNA samples were stored at −20 °C.

2.6.2. Real time RT-PCR

Real time RT-PCR assays were performed using the ABI PRISM® 7000 Sequence Detector System (Applied Biosystems). A quantity of cDNA corresponding to 5 ng of RNA was added to 900 nM of each primer (Table 1) and 1× Power SYBR Green PCR Master Mix (Applied Biosystem) in a final volume adjusted to 20 μL with DEPC-treated water. The thermal cycling conditions comprised 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each run a melting curve was created and checked to ensure only a single product had been amplified. The results were analysed using the $2^{-\Delta\Delta Ct}$ method [44] and normalized against expression of the 40S ribosomal protein S11 reference gene [45]. For each time point, all treatment groups were compared to the control group in order to reduce the variation due to injection stress.

Table 1
Primers used in this study. Specific primers for targeted genes were supplied by Eurofins MWG Operon, London, UK.

Gene	Gene function	Primer type	Sequence 5′–3′	GenBank accession no.	Reference
40S	housekeeping gene	Forward	CCGTGGGTGACATCGTTACA	AB012087	[44]
		Reverse	TCAGGACATTGAACCTCACTGTCT		
crp1	CRP response	Forward	AGCAATGCAACATTTTTCCGTC	JQ010977	[34]
		Reverse	ACTTGCGTCAAAGCCACCCAC		
crp2	CRP response	Forward	GATGCTGCAGCATTTTTCAGTC	JQ010978	[34]
		Reverse	CTCCGCATCAAAGTTGCTCAAAT		
c1rs	classical complement pathway	Forward	CAAGCCCATCTTGGCTCCTGG	AB042609	[23]
		Reverse	GTCCAGATCAAGCGGGGACGT		
bf/c2	classical and alternative complement pathways	Forward	CGGTCATGGGAAAAAGCATTGAGA	AB047361	[23]
		Reverse	GATATCTTTAGCATTTGTCGCAG		
c3	central component of the complement system; marker of the three pathways	Forward	GGTTATCAAGGGGAGTTGAGCTAT	AB016215	[23]
		Reverse	TGCTGCTTTGGGTGGATGGGT		
masp2	MB-Lectine complement pathway	Forward	CAAGCTGTCCAAGGTGATTG	AB234294	[23]
		Reverse	AGCAGTGAGGACCCAGTTGT		

2.7. Statistical analysis

All data (cCRP serum levels, serum alternative complement pathway activity and gene expression profiles) are given as means ± standard error of the mean (SEM) of five independent individuals. Statistical analysis was carried out using GraphPad Prism v5 and PASW Statistics 18 software. Data were tested for normality and homoscedasticity prior to further analysis. A two-way analysis of variance (ANOVA) and post-hoc Bonferroni's multiple comparisons test have been performed on serum cCRP levels and complement activity to determine significant differences between the different treatment groups. A student *t*-test was performed for the pre-injection analysis. In order to investigate whether or not serum cCRP levels and serum complement ACP activity were correlated, Pearson correlation statistical analyses were performed. Additional post-hoc Pearson correlation statistical analyses were performed to investigate whether or not variations observed in serum levels or activities were correlated to specific related gene up- or down-regulations. Gene expression data were normalised using a log-transformation prior to a two-way ANOVA and subsequent Bonferroni post-hoc test analysis. Significance was defined as $p \leq 0.05$.

3. Results

3.1. Effect of 14 days of feeding with the MacroGard® supplemented diet on CRP and complement profiles

The basal serum CRP level (Fig. 1) was significantly higher ($t = 3.562$, $p = 0.0074$) in the MacroGard® fed group (38 μg/mL) compared to the control fed group (18 μg/mL). The serum alternative complement pathway basal activity (Fig. 1) was also significantly higher ($t = 3.115$, $p = 0.0143$) in the MacroGard® fed fish (1752.1 ACH50 units/mL) compared to the control fed fish (50.3 ACH50 units/mL).

The expression of CRP and complement genes (Fig. 1) changed significantly in the mid-gut and the head kidney tissues ($F = 4.84$, $p = 0.0012$ and $F = 5.32$, $p = 0.0006$ respectively) but not in the liver ($F = 0.35$, $p = 0.8775$). There was a significant down-regulation of *crp2* in both mid-gut and head kidney tissues (reduced to 0.22 fold, $p < 0.001$ and to 0.36 fold, $p < 0.001$ respectively) as well as a significant up-regulation of *c1rs* in the same mid-gut and head kidney tissues (1.66 fold increase, $p < 0.05$ and 2.25 fold increase, $p < 0.05$ respectively).

3.2. CRP and complement profiles after injection with *A. salmonicida*

3.2.1. Serum CRP levels

Whilst serum CRP levels (Fig. 2) were significantly different between the treatments over the whole challenge period ($F = 9.89$, $p < 0.0001$), time of treatment had no significant effect ($F = 1.93$, $p = 0.0819$) on the levels of this acute phase protein. In the MacroGard® fed/*A. salmonicida* injected group, serum CRP levels were significantly higher during the first few hours p.i. compared to the control group (i.e. 48.8 μg/mL and 28.6 μg/mL respectively at 6 h p.i., $p < 0.05$) as well as at 12 h p.i. compared to the control group (50.3 μg/mL and 21.4 μg/mL respectively, $p < 0.05$) and the control fed/*A. salmonicida* injected group (50.3 μg/mL and 18.2 μg/mL respectively, $p < 0.01$). The levels then decreased and were similar to those for the control group until the end of the challenge but significantly different from those obtained in the control fed/*A. salmonicida* injected group (36.5 μg/mL and 9.2 μg/mL respectively, $p < 0.05$). In contrast, CRP levels for the control fed/*A. salmonicida* injected group were lower to those obtained for the control group (8 μg/mL less in average), except at 6 h p.i. (28.6 μg/mL and 18.6 μg/mL for the control). Finally the cCRP levels for the MacroGard® fed/PBS injected group were always higher than in the control group with a significant increase in CRP levels being noted at 48 h p.i. (40 μg/mL, $p < 0.05$). It is interesting to note that the CRP levels for the groups fed with the MacroGard® enriched diet were higher than in the control group during the whole challenge period.

3.2.2. Serum alternative complement pathway activity

Serum alternative complement pathway (ACP) activity (Fig. 3) was significantly different between the treatments over the whole challenge period ($F = 17.4$, $p < 0.0001$) although the time effect was not significant ($F = 1.46$, $p = 0.197$). In the MacroGard® fed/*A. salmonicida* injected group, ACP activity decreased from 6 h p.i. to 48 h p.i. i.e. from 2150 to 400 ACH50 units/mL, and then increased significantly to reach a peak at 96 h p.i. (4800 ACH50 units/mL, $p < 0.01$). In contrast, ACP activity for the control fed/*A. salmonicida* injected group was very low until 48 h p.i. (90 ACH50 units/mL) and then started to slowly increase until the end of the challenge (up to 1320 ACH50 units/mL at 120 h p.i.). Finally the ACP activity for the MacroGard® fed/PBS injected group increased from 12 h p.i. to reach significant levels above 3000 ACH50 units/mL until the end of the challenge (4140 ACH50 units/mL, $p < 0.01$ at 48 h p.i.; 3100 ACH50 units/mL, $p < 0.05$ at 72 h p.i. and 3520 ACH50units/mL, $p < 0.01$ at 96 h p.i.). It is interesting to note that the ACP activity for the groups fed with the MacroGard® supplemented diet was higher than in the control group during the whole challenge period. Pearson correlation statistical analyses performed on serum cCRP levels and serum ACP activity revealed that CRP levels and complement activity were not correlated in *Aeromonas* infected fish nor in PBS injected fish nor in control fed fish but were significantly positively correlated in β-glucan fed fish ($r = 0.2406$, $p = 0.0448$), indicating increasing complement ACP activity when CRP levels increase in those fish.

3.2.3. CRP and complement gene expression profiles

In the head kidney tissue (Fig. 4), with the exception of *crp2*, the expression of all genes analysed was significantly affected by the treatments utilised ($F = 3.76–49.64$, $p \leq 0.0139$), whilst the time effect was significant for all the genes ($F = 3.84–100.14$, $p \leq 0.0066$) with the exception of *c3* ($F = 1.87$, $p = 0.1230$). Post-hoc analysis revealed a pattern of significant gene expression up-regulations occurring at 6 h p.i. in all of the analysed genes except for *c1rs*. The greatest significant up-regulation was detected in the MacroGard® fed/*A. salmonicida* injected group for *crp1* (5.6 fold, $p < 0.001$), *crp2* (2 fold, $p < 0.01$) and *masp2* (8.3 fold, $p < 0.001$) and in the control fed/*A. salmonicida* injected group for *bf/c2* (2.7 fold, $p < 0.01$) although significant up-regulations were also detected for this group for *crp1* (3.4 fold, $p < 0.001$) and *masp2* (4.6 fold, $p < 0.01$). Time of infection also appeared to affect the expression of all the genes analysed. After 5 days post-infection (120 h p.i.), *masp2* was significantly up-regulated in all groups compared to the control (3.6–5.4 fold, $p \leq 0.05$) whereas *crp2* was down-regulated (0.43–0.66 fold), *c3* up-regulated (1.4–1.9 fold) and *crp1* up-regulated in the *A. salmonicida* infected groups (1.7 fold, $p < 0.05$). In addition, between 6 h p.i. and 120 h p.i., several effects on the gene expression were detected. For example, in all the groups *crp2* was down-regulated at 24 h p.i. (0.4–0.66 fold) and *c1* up-regulated at 72 h p.i. (1.8–3.5 fold) whereas *bf/c2* was down-regulated at 72 h p.i. (0.34–0.58 fold). In the *A. salmonicida* infected groups, *c3* was up-regulated at 12 h p.i. (1.9–3.1 fold).

In contrast to the head kidney tissue, only the gene expression of *crp2* and *bf/c2* ($F = 3.66$ and $F = 12.49$ respectively, $p \leq 0.0157$) was significantly influenced by the different treatments in the liver tissue (Fig. 5). However, time significantly affected all the genes

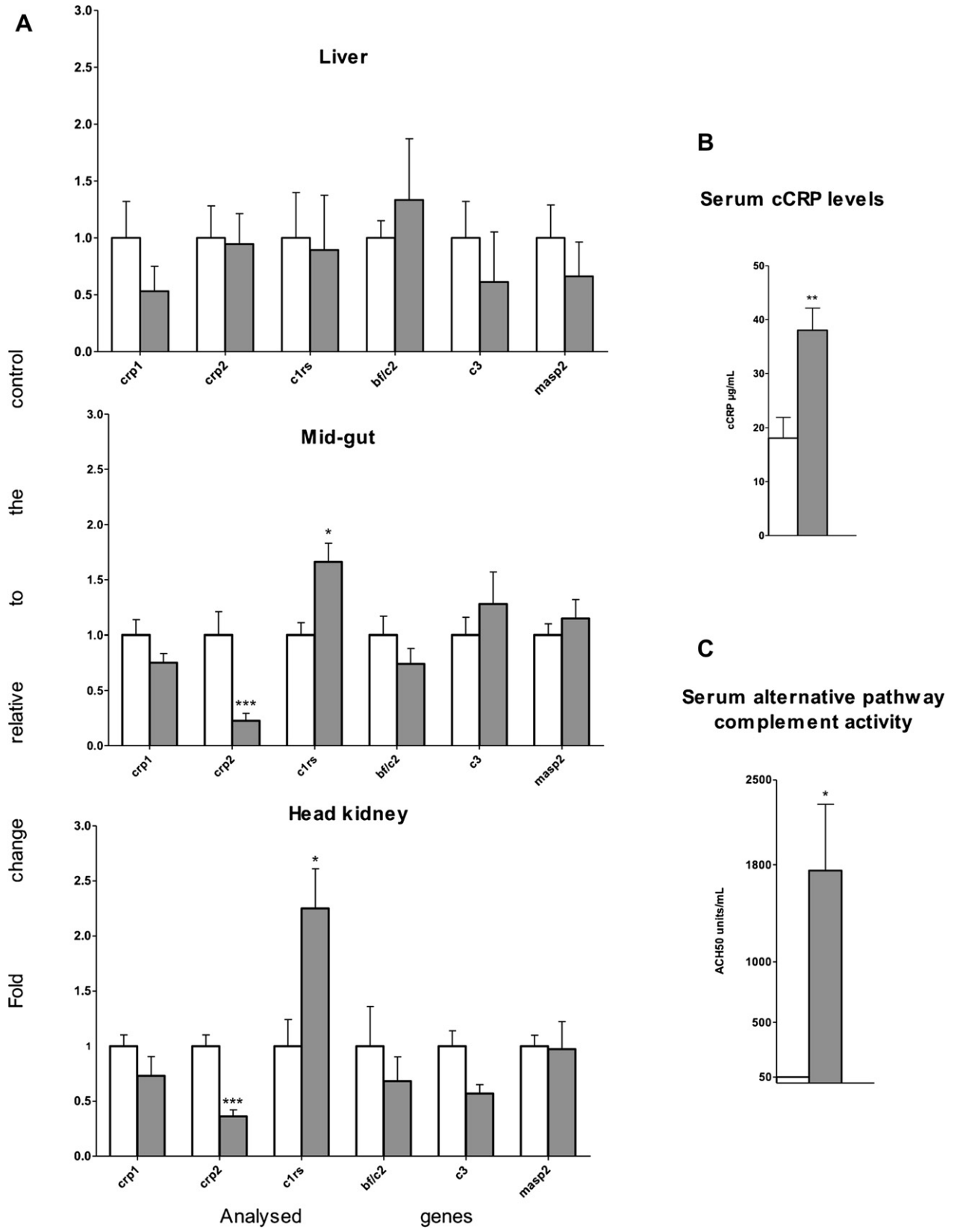

Fig. 1. Effect of the MacroGard® supplemented diet on carp CRP and complement profiles after 14 days of feeding.
A: *crp1*, *crp2*, *c1rs*, *bf/c2*, *c3* and *masp2* gene transcript levels in liver, mid-gut and head kidney analysed by RT-PCR. Expression levels of MacroGard® supplement fed fish samples (grey bars) are shown as x-fold change compared to the control fed fish samples (white bars).
B: Serum cCRP levels of the control fed fish (white bars) and the MacroGard® supplemented fed fish (grey bars).
C: Serum alternative complement pathway activity of the control fed fish (white bars) and the MacroGard® supplemented fed fish (grey bars).
All the graphs show mean ± SEM, $n = 5$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

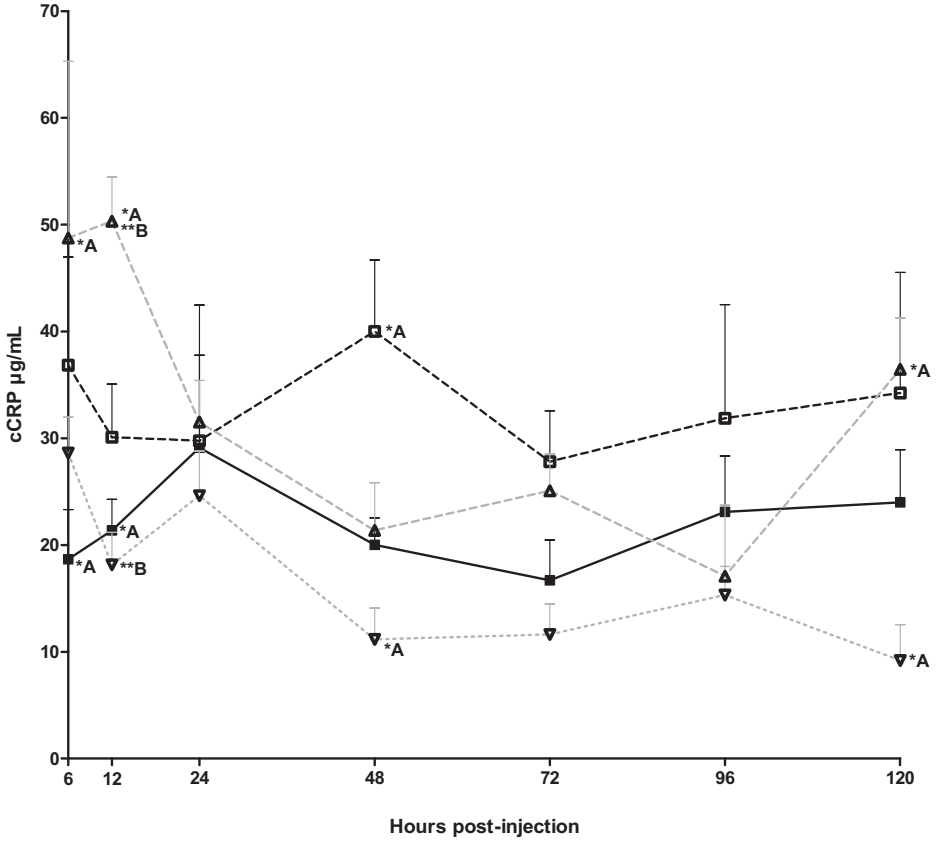

Fig. 2. Serum carp CRP levels during an immunological challenge with *Aeromonas salmonicida*. Carp were previously fed for 14 days with either control feed or MacroGard® supplemented diet. Fish were then injected with *A. salmonicida* (1×10^8 bacteria in 250 µL PBS per fish) or injected with PBS only (250 µL).
-■- = control feed and PBS injection, □ = MacroGard® feed and PBS injection, ∇ = control feed and *A. salmonicida* injection, Δ = MacroGard® feed and *A. salmonicida* injection. Mean ± SEM, $n = 5$. *A and **B represent Bonferroni's post-hoc two-way ANOVA $p \leq 0.05$ (*) and $p \leq 0.01$ (**) where A and B label the groups significantly different.

analysed ($F = 3.2–14.59$, $p \leq 0.0171$) with the exception of *crp1* and *c1rs*. In contrast to the pattern detected at 6 h p.i. in the head kidney tissue, a post-hoc analysis revealed a pattern of significant gene expression up-regulations occurring at 24 h p.i. in the liver tissue: four of the analysed genes (*crp2*, *bf/c2*, *c3* and *masp2*) were up-regulated in the *A. salmonicida* infected groups whereas *crp1* was significantly down-regulated and no effect was detected in the expression of *c1rs*. As in the head kidney tissue, the greatest significant up-regulations were detected in the *A. salmonicida* infected groups: in the control fed/*A. salmonicida* injected group for *crp2* (12.4 fold, $p < 0.001$), *bf/c2* (12.7 fold, $p < 0.001$), *c3* (4.3 fold, $p < 0.01$) and *masp2* (4.5 fold, $p < 0.01$) and in the MacroGard® fed/*A. salmonicida* injected group for *bf/c2* (8.1 fold, $p < 0.001$) and *masp2* (5 fold, $p < 0.001$). Up-regulation of *bf/c2* was also observed in the infected groups at 12 h p.i. (7.1 and 7.9 fold compared to the control, $p \leq 0.01$).

As in the head kidney tissue, in the mid-gut tissue (Fig. 6) the gene expression with the exception of *crp2 was* affected by the treatments utilised ($F = 6.13–10.07$, $p \leq 0.0008$), whilst the time effect was significant for all the genes ($F = 5.56–28.78$, $p \leq 0.0005$). Unlike the clear patterns observed in the head kidney and the liver tissues (i.e. up-regulations occurring at 6 h p.i. and 24 h p.i. respectively), post-hoc analysis revealed significant up- and down-regulations of the targeted genes during the whole challenge period and not only in the *A. salmonicida* infected groups. At 6 h p.i., *crp2* and *bf/c2* were down-regulated in all the groups (0.23–0.5 fold) while significant up-regulations of *crp1* and *c1rs* have been detected in the *A. salmonicida* injected groups (1.8–8.4 fold). At 12 h p.i., two genes were up-regulated in the *A. salmonicida* infected groups: *crp1* in the MacroGard® fed/*A. salmonicida* injected group(1.6 fold, $p < 0.05$) and *crp2* in the control fed/*A. salmonicida* injected group (2.1 fold, $p < 0.01$). At 24 h p.i., down-regulations of *crp1* and *masp2* were detected in both *A. salmonicida* infected groups ($p \leq 0.05$) while up-regulations of *c1rs* and *bf/c2* were observed in all the groups. At 72 h p.i., four of the six analysed genes (*crp1*, *c1rs*, *bf/c2* and *c3*) were up-regulated in all the groups and finally at 120 h p.i significant up-regulations of *crp1*, *c3* and *masp2* and down-regulation of *crp2* were detected in both infected groups ($p \leq 0.05$).

4. Discussion

The present study reports and highlights for the first time the complexity of the interaction between the immunostimulant β-glucan and the acute phase proteins profiles during an infection in common carp. It has been shown that feeding carp with a 0.1% MacroGard® enriched diet for 14 days significantly affects the acute phase proteins profiles (both gene expression and serum activity or levels). In addition, it was shown that the CRP and complement profiles were also significantly affected during an *A. salmonicida* challenge. The effects of both β-glucan feeding and pathogen injection were found to be dependent on organ, feeding regime and time period.

4.1. Effect of 14 days β-glucan feeding

Oral administration of the immunostimulant was preferred to injection or bath methods because of its mass immune stimulation potential and because it does not cause stress to the animals. Oral administration of 0.1% MacroGard® enriched diet for 14 days

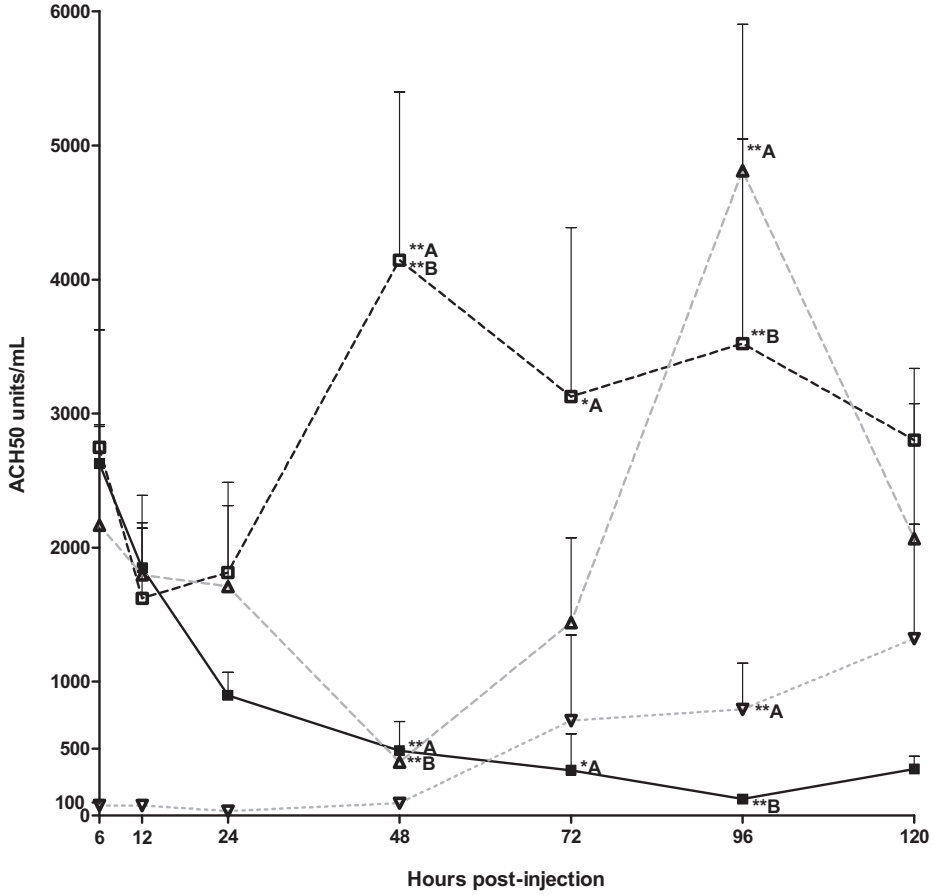

Fig. 3. Alternative complement pathway activity during an immunological challenge with *Aeromonas salmonicida*. Carp were previously fed for 14 days with either control feed or MacroGard® supplemented diet. Fish were then injected with *A. salmonicida* (1×10^8 bacteria in 250 μL PBS per fish) or injected with PBS only (250 μL).
-■- = control feed and PBS injection, □ = MacroGard® feed and PBS injection, ∇ = control feed and *A. salmonicida* injection, Δ = MacroGard® feed and *A. salmonicida* injection. Mean ± SEM, $n = 5$. *A, **A and **B represent Bonferroni's post-hoc two-way ANOVA $p \leq 0.05$ (*) and $p \leq 0.01$ (**) where A and B label the groups significantly different.

significantly increased serum CRP levels up to 2 fold and serum alternative complement activity up to 35 fold. Whilst CRP related genes (*crp1* and *crp2*) were down-regulated, complement related genes expression was not generally affected, except for a significant up-regulation of *c1rs* in the mid-gut and head kidney tissues. Despite the fact that CRP is mainly synthesised in the liver [17], the two CRP-like genes analysed in this study revealed different expression profiles either in liver, mid-gut or head kidney tissues. This supports the previous observations by Falco et al. [34], suggesting a difference in the biological activity of the related isoforms.

Although the literature on the effects of β-glucan on the acute phase protein profiles is inconsistent, the down-regulation observed for CRP related genes is in agreement with other studies conducted with immunostimulants. For instance, Liu et al. [46] have shown that the expression of CRP in rainbow trout hepatocytes, head kidney macrophages and spleen lymphocytes significantly decreased after the injection of turpentine oil as an acute phase immune response inducer. The apparent contradiction between serum CRP levels and CRP related genes expression might be explained by the fact that the levels of free-phase CRP could have reached an optimal circulating concentration and therefore the CRP related genes were down-regulated to bring a rapid return to homeostasis [47]. Another reason is that there are more than two CRP related genes in common carp genome as the recent identification of seven CRP-like genes in zebrafish [34] might suggest and therefore the expression of those related genes might not have been recorded. The up-regulation of *c1rs* might then reflect the need of the C1q-C1rs production in the mid-gut and in the head kidney tissues to initiate the classical pathway by binding to the circulating CRP [28]. Regarding the serum alternative complement activity, although no effects of oral administration of β-glucan was detected in rainbow trout [48], large yellow croaker *Pseudosciaena crocea* [49] or common carp [4,5], our results are in accordance with the findings of several studies performed in sea bass *Dicentrarchus labrax* [50] and in *L. rohita* [10]. The apparent contradiction between the high serum alternative complement activity and the absence of a significant effect on its related gene expression (*bf/c2* and *c3*) might be explained by the fact that the primers utilised for this study did not pick up the appropriate gene isoforms responsible for the Bf/C2 and C3 protein expression [23], highlighting the polymorphic complexity of the common carp complement system [17,18]. In addition, due to post-transcriptional and traductional regulation mechanisms, mRNA level of a given gene may not systematically reflect protein level and activity. Results of this study thus highlight the importance of characterising the most active isoform of individual proteins.

With a significant increase in the level of circulating CRP and in the activity of the alternative complement in the serum, a 14 day β-glucan oral administration period induced an immune response and enhance some immune parameters in carp. The fact that some related genes were either down-regulated or non-affected could perhaps indicate that the effect of the immune stimulation was enough to induce a proper immune response and a subsequent β-glucan feeding might have detrimental effects [2,14,51].

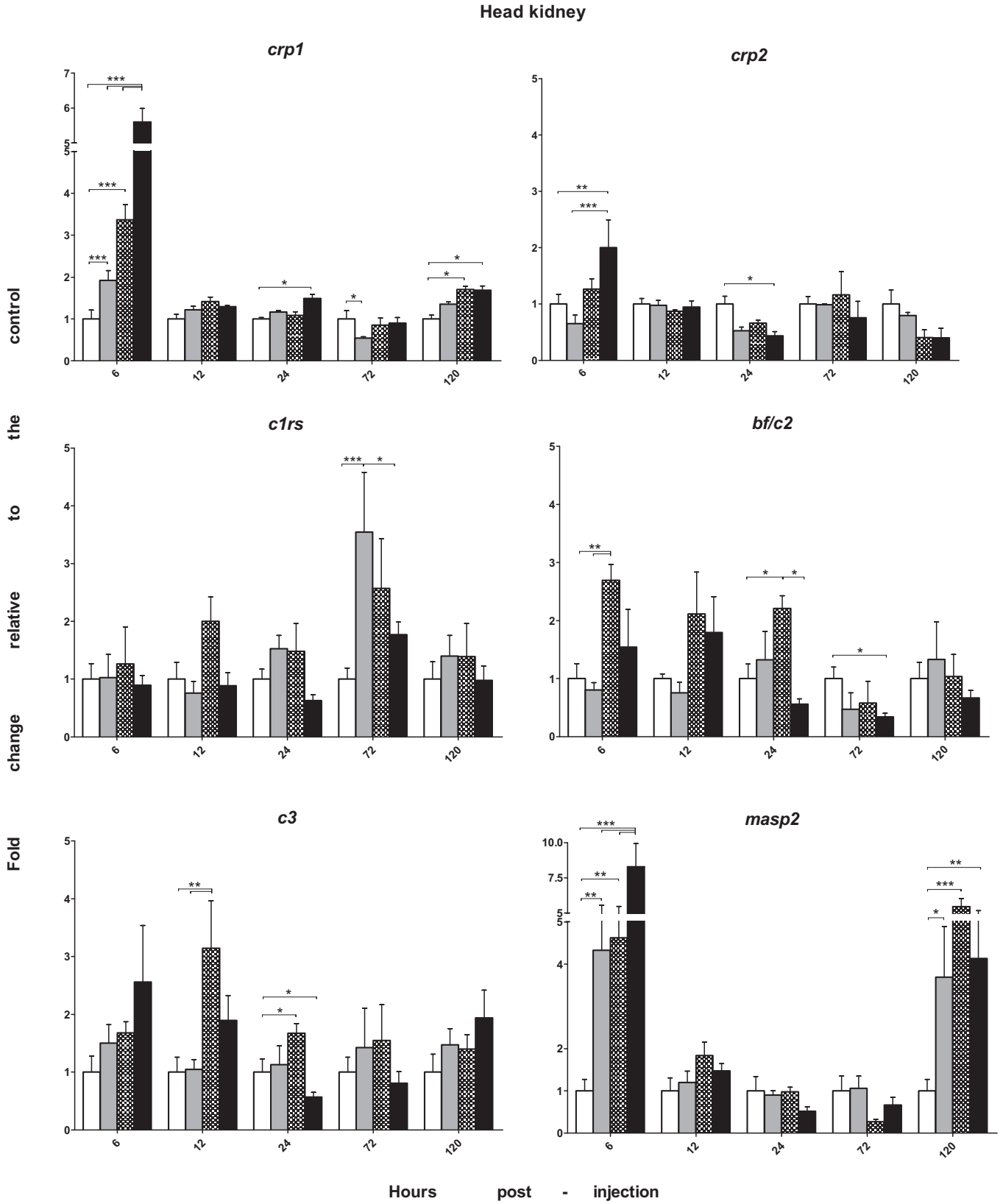

Fig. 4. *crp1*, *crp2*, *c1rs*, *bf/c2*, *c3* and *masp2* gene transcript profiles in the head kidney of carp during an immunological challenge with *A. salmonicida*. Expression levels of the different treatment groups are shown as x-fold change compared to the control fed fish samples (white bars) at each time point.
□ = control feed and PBS injection (control group), ▒ = MacroGard® feed and PBS injection, ▓ = control feed and *A. salmonicida* injection, ■ = MacroGard® feed and *A. salmonicida* injection.
Significant differences are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. All the graphs show mean ± SEM, $n = 5$.

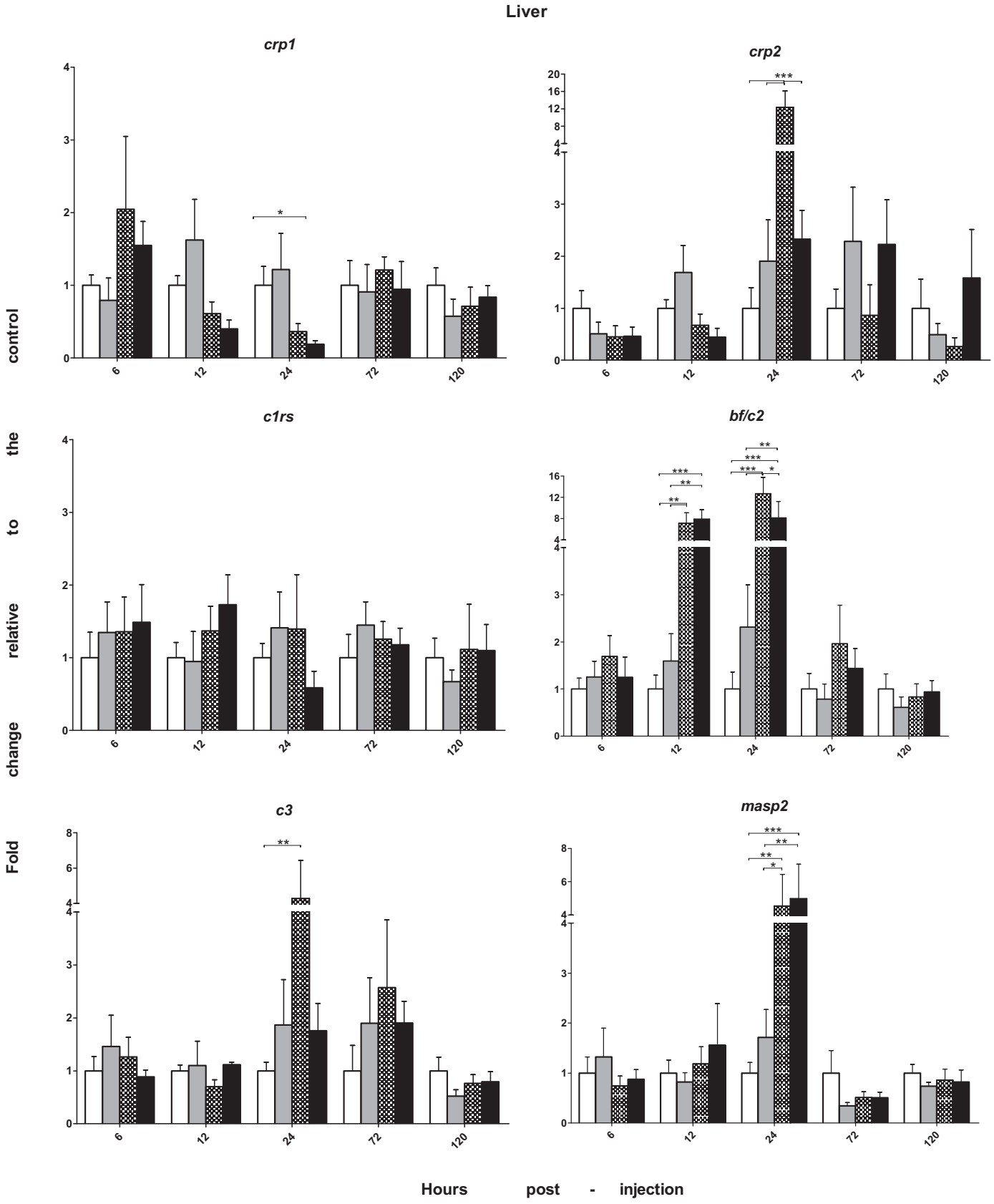

Fig. 5. *crp1*, *crp2*, *c1rs*, *bf/c2*, *c3* and *masp2* gene transcript profiles in the liver of carp during an immunological challenge with *A. salmonicida*. Expression levels of the different treatment groups are shown as x-fold change compared to the control fed fish samples (white bars) at each time point.
□ = control feed and PBS injection (control group), ▒ = MacroGard® feed and PBS injection, ▓ = control feed and *A. salmonicida* injection, ■ = MacroGard® feed and *A. salmonicida* injection.
Significant differences are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. All the graphs show mean ± SEM, $n = 5$.

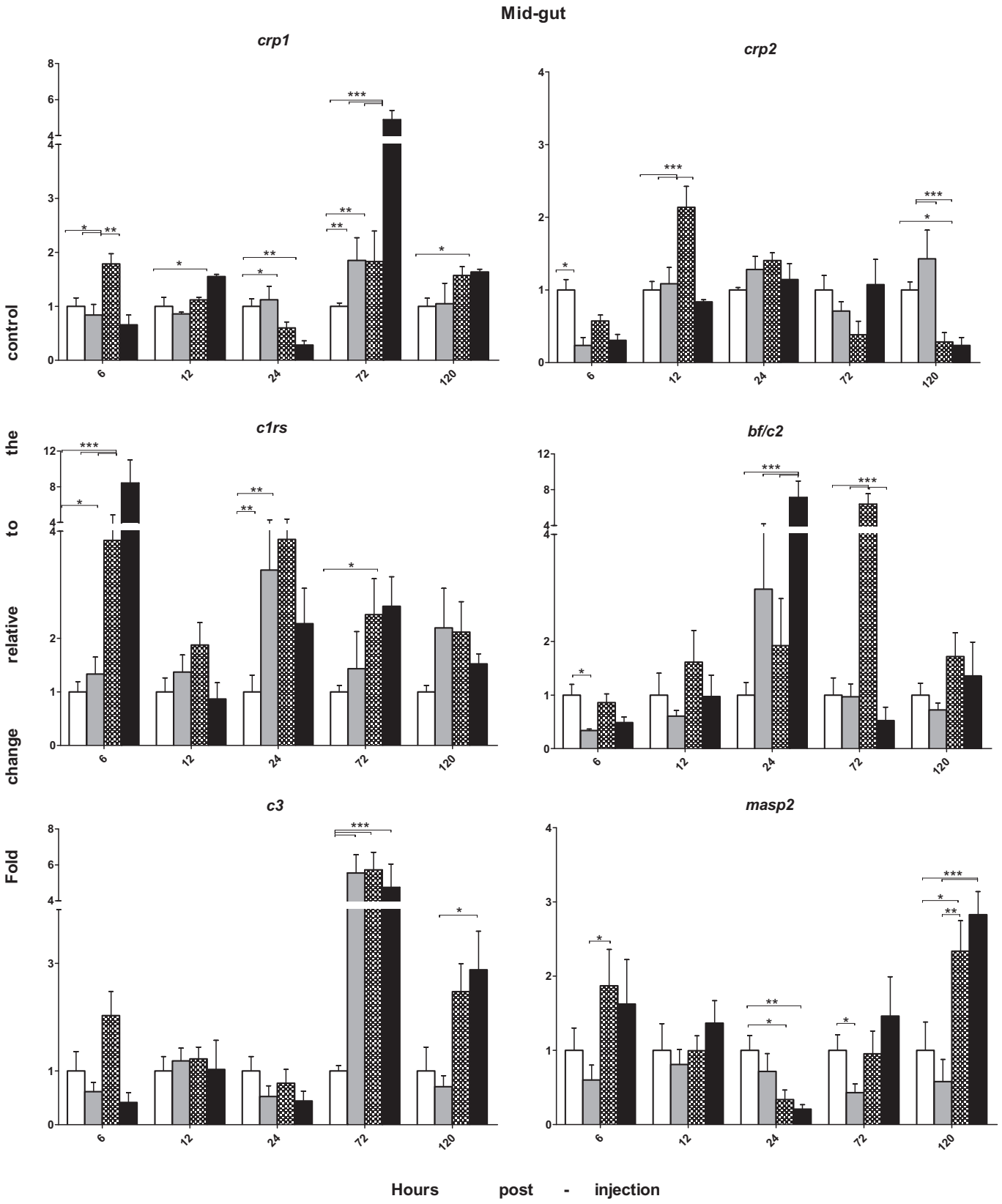

Fig. 6. *crp1*, *crp2*, *c1rs*, *bf/c2*, *c3* and *masp2* gene transcript profiles in the mid-gut section of carp during an immunological challenge with *A. salmonicida*. Expression levels of the different treatment groups are shown as x-fold change compared to the control fed fish samples (white bars) at each time point.
□ = control feed and PBS injection (control group), ▒ = MacroGard® feed and PBS injection, ▓ = control feed and *A. salmonicida* injection, ■ = MacroGard® feed and *A. salmonicida* injection.
Significant differences are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. All the graphs show mean ± SEM, $n = 5$.

Indeed, when the oral administration of the β-glucan immunostimulant was stopped after 14 days, the carp immune response was still stimulated as serum CRP levels and ACP activity were still found to be at very high levels, supported by the positive correlation observed between these two immune parameters in β-glucan fed fish. Serum CRP levels in the group fed with MacroGard® and injected with PBS were 1.5–2 fold higher than in the control group during the whole duration of the challenge and the CRP related genes were found to be up-regulated with several important up-regulation for *crp1* in the kidney at 6 h p.i., in the liver at 12 h p.i. and in the gut at 72 h p.i. Regarding complement, the ACP in the MacroGard® fed PBS injected group was found to be up to 4 times more active than in the control group during the whole duration of the challenge. This very high level of complement activity was in correlation with the related genes expression profiles as the expression of *bf/c2* and *c3* were overall up-regulated. A Pearson correlation statistical analysis revealed a significant positive correlation between ACP activity and *bf/c2* gene expression in the liver ($r = 0.4836$, $p = 0.0359$), which suggest that the very high level of serum complement activity could be due to a specific *bf/c2* up-regulation in the liver of those fish. Several peaks of up-regulations were also detected for *c1rs* and *masp2*, suggesting that the other complement pathways were stimulated as well.

In conclusion, a 14 day period of β-glucan oral administration was sufficient enough to induce and enhance an immune response in carp and maintain the immune parameters at elevated levels for up to 5 days after feeding had stopped. These findings are in agreement with other studies performed on common carp [4–6]. β-Glucan stimulating effects were initially expected to be detected in the mid-gut as the β-glucan was incorporated into food. However early effects were also detected in the head kidney and liver tissues, suggesting that the β-glucan response was systemic but also organ and time dependent.

4.2. CRP and complement profiles during an Aeromonas salmonicida infection

CRP and complement profiles have shown significant variations in fish fed with control diet during a challenge with *A. salmonicida*, the aetiological agent of the atypical form of furunculosis in salmonids and other freshwater fish [52,53].

Serum CRP levels for the control fed *A. salmonicida* injected group were lower to those obtained for the control group and remained stable during the whole duration of the challenge. In addition, when comparing control fed *A. salmonicida* infected fish to MacroGard® fed *A.salmonicida* infected fish, results show that the MacroGard® feeding significantly affected serum CRP levels but that CRP was not detectably induced during the carp acute phase reaction to *A. salmonicida* infection. These findings are in agreement with other studies performed on Atlantic cod *Gadus morhua* [54] and rainbow trout [55] where pentraxins, and especially CRP, did not exhibit typical acute phase protein profiles. Such decreasing serum CRP levels, as in our present study, have been found in Atlantic salmon *Salmo salar* [47]. However, this could be explained by the fact that CRP could potentially bind to bacteria through its affinity for *A. salmonicida* capsular polysaccharide CPS [56] and therefore was not detected by ELISA. Our study represents the first investigation on the expression of CRP related genes during an *A. salmonicida* challenge. Thus, the small peak of serum free-phase circulating CRP at 6 h p.i. could be related to the up-regulation of *crp1* at the same time in the head kidney, the liver and the mid-gut tissues but Pearson correlation statistical analyses did not reveal significant correlations. Following the same reasoning, the other peak in serum levels observed at 24 h p.i. might be related to the down-regulation (0.4 fold) of *crp1* compensated by an up-regulation (12.4 fold) of *crp2* in the liver and in the mid-gut tissue at the same time. Finally, the relative stability of the CRP serum levels after 48 h p.i. may be explained by the absence of significant down- or up-regulations of both *crp1* and *crp2*. In contrast, the ACP activity for the control fed *A. salmonicida* injected group was stable until 48 h p.i. and then started to increase significantly until the end of the challenge. Interestingly, serum ACP activity was very low in the control fed *A. salmonicida* injected group compared to the control fed PBS injected group during the first 24 h post-injection, which may result from the interaction of the complement cascade on the bacterial surface in infected fish. In the literature, the alternative complement pathway activity has been found to be very high in fish serum compared to mammalian serum [41] suggesting that this pathway has a greater role than the classical or the lectin pathways in fish immune response. However, conflicting results have been reported in the correlation between *A. salmonicida* infection and ACP activity. Indeed, several studies carried out on Atlantic salmon [57] or on channel catfish [58] concluded that the ACP was not detectably induced during the immune acute phase reaction to *A. salmonicida*. Nevertheless, our results are in accordance with other studies performed in Atlantic salmon [59,60] and in rainbow trout [61] which have shown that the ACP activity significantly increased during an *A. salmonicida* challenge. Moreover, our study, which represents the first to investigate complement related gene expression during an infection with *A. salmonicida*, has revealed that even if complement precursors are mainly synthesised in the liver, a distinct pattern was found in all the complement related gene expression profiles (*c1rs*, *bf/c2*, *c3* and *masp2*) across the three selected immune organs. A peak of gene expression first occurred in the head kidney tissue (6 h p.i. or 12 h p.i.), then an up-regulation was detected in the liver several hours later (24 h p.i.) and finally both up- or down-regulations appeared in the mid-gut later on 24 h p.i. and 72 h p.i. These organ and time dependent expression profiles are in agreement with the findings from other *in vivo* infection challenges performed in common carp with *Trypanoplasma borreli* [23] and with *Ichtyophthirius multifiliis* [18]. The observed late increase in ACP activity (after 48 h p.i.) could be related to the ACP-related genes *bf/c2* and *c3* expression profiles. Indeed, up-regulations of *bf/c2* and *c3* appeared mainly after 24 h p.i. in all the tissues indicating that the complement cascade has been initiated supported by Pearson correlation analyses which revealed positive correlations between serum ACP and head kidney *bf/c2* and *c3* gene expression profiles at 24 h p.i. ($r = 0.73$, $p = 0.0158$ and $r = 0.63$, $p = 0.0132$ respectively). The constant high level of ACP-related gene expression in either head kidney, liver or mid-gut tissues after 24 h p.i. might indicate that the ACP was stimulated, which is in accordance with ACP serum activity results.

Although variations were detected in CRP related gene expression profiles, no significant effects of the *A. salmonicida* infection were detected on CRP serum levels. This could be explained by the non-virulent bacteria strain, which was chosen to ensure the survival of the fish over the challenge period. The bacteria were perhaps either not pathogenic enough to induce a typical serum CRP acute phase response that can, for example, be obtained in common carp with *A. hydrophila* [31], or the CRP was bound to bacteria and was not detected by the ELISA utilised.

4.3. β-Glucan feeding effects on innate acute phase proteins during an Aeromonas salmonicida challenge

β-Glucan immunostimulation had significant effects on CRP and complement profiles during the *A. salmonicida* challenge. Previous studies on these fish have also revealed a specific IgM response to the *A. salmonicida* challenge [36] and confirmed the presence of specific antibodies against *A. salmonicida* in the infected groups.

Other related studies also revealed that apoptosis was induced in the pronephric cells during the infection and that the β-glucan oral administration was significantly affecting the apoptosis-related gene expression [51].

In fish fed with MacroGard® and challenged with *A. salmonicida* a peak in serum CRP levels was observed at 12 h p.i. which decreased until the levels of circulating CRP were similar to those found for the control group and a final increase was detected at 96 h p.i. to 120 h p.i. Although the peak in CRP levels at 6–12 h p.i. and probably the one at 120 h p.i. could be due to the *Aeromonas* infection, the overall higher free-phase CRP circulating levels may also result from the β-glucan feeding effect described previously as no significant differences were observed between the two groups fed with β-glucan. However, regarding the related gene expression, the profiles obtained for *crp1* and *crp2* may be due to the added effects of both β-glucan feeding and *Aeromonas* infection. Indeed, up- or down-regulation patterns of the genes for the fish fed with MacroGard® and challenged with *A. salmonicida* were similar to those obtained for the two other treatment groups, for example: crp1 up-regulation in the head kidney at 6 h p.i., *crp1* down-regulation at 24 h p.i. in the gut, *crp1* up-regulation at 72 h p.i. in the gut, *crp2* down-regulation at 24 h p.i. in the kidney or *crp2* down-regulation at 120 h p.i. in the gut. In addition, when comparing MacroGard® fed groups, *crp1* and *crp2* genes were significantly more up-regulated in the head kidney at 6h p.i. in the *A.salmonicida* infected fish than in the PBS injected fish, suggesting an effect of the infection on the CRP related gene expression profiles at an early stage of the infection. Although the literature on CRP serum levels and CRP related gene expression profiles during an infection in fish is inconsistent, several studies performed on CRP response in *Limulus* hepatopancreas infected with *Pseudomonas aeruginosa* have shown acute up-regulations by about 60 fold [62]. It would be therefore interesting to investigate CRP related genes expression profiles during immunological challenges with more virulent strains of *A. salmonicida* or other pathogenic strains such as *A.hydrophila* which elicit typical CRP acute phase responses [31]. In contrast to serum CRP circulating levels, ACP activity in fish fed with MacroGard® and challenged with *A. salmonicida* could possibly be due to the effect of both β-glucan feeding and *Aeromonas* infection. Indeed, ACP activity was found to be very similar to that observed in the control group until 48 h p.i., then the activity increased similar to that noted in the infected and normal fed fish group. The β-glucan feeding effect might be related to the large fold increase in ACP activity after 48 h p.i. and to the then very high activity levels detected at this time point (over 2000 ACH50 units/mL after 72 h p.i.). However, it is interesting to note that ACP activity was significantly lower in the MacroGard® fed/*A. salmonicida* infected group than in the MacroGard® fed/PBS injected group at 48h p.i., suggesting that the complement activity was reduced in the infected fish at this specific stage of the infection. Nevertheless, several other studies performed on common carp have also shown ACP activation with intraperitoneal injections of β-glucan [6]. This significant increase in ACP activity has also been observed in other fish species such as in rainbow trout challenged with *A. salmonicida* and orally administered with probiotic strains [63] or in European sea bass *D. labrax* challenged with *Vibrio alginolyticus* and orally administered with mannan oligosaccharides [64]. Complement related genes were overall up-regulated in the head kidney, liver and mid-gut tissues with several peaks of up-regulation occurring in the first few hours post-infection in the head kidney (6 h-12 h p.i.), then in the liver (12 h–24 h–72 h p.i.) and finally in the gut (72 h–120 h p.i.). It is possible therefore that complement operates through its three existing pathways (classical, lectin and alternative), not only through the alternative pathway. The complement related gene expression profiles in fish fed with MacroGard® and challenged with *A. salmonicida* were believed to be due to the added effects of both β-glucan feeding and *Aeromonas* infection. Indeed, up- or down-regulation patterns were often in correlation with patterns observed for either one or both of the other treatment groups, for example: *c1* up-regulations in the mid-gut; *bf/c2* up-regulations in the liver; *c3* up-regulations in the mid-gut or *masp2* up-regulations in the kidney. The effect of either β-glucan or infection or both was dependent on organ, time and targeted gene. This differential regulation of complement component genes has also been found in the rainbow trout after a β-glucan immunostimulation [24] and in common carp after intraperitoneal injection of scleroglucan [27].

4.4. General conclusion of the study

The present study was one of the first to investigate the complexity of the relationship between β-glucan immunostimulation and infection in common carp. Their effects on the carp acute phase protein profiles were numerous, sometimes additive effects, and were dependent on organ, time and targeted protein or gene.

An immune response was induced after 14 days of β-glucan oral administration at the recommended dose by the supplier (Biorigin). Although the *A. salmonicida* infection did not have significant effects on CRP serum levels on its own, the combination of infection and β-glucan feeding did show significant effects on both CRP and complement profiles, suggesting that MacroGard® stimulated CRP and complement responses to *A. salmonicida* infection in common carp.

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