The influence of chitosan on immune status and survival rate of *Cyprinus carpio* L. challenged with *Aeromonas hydrophila*

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Abstract

The aim of our study was to evalute the effect of chitosan on non- specific immunity and survival rate of *Cyprinus carpio* challenged with *Aeromonas hydrophila*. A total of 80 common carp (36.4 ± 0.73 g) were devided into four dietary groups: The first group (G1) was served as control fed on chitosn-free diet, the second group (G2) was fed on diet supplemented with 0.75% chitosan, the third group (G3) was fed on diet supplemented with 1.5% chitosan and the fourth group (G4) was fed on diet supplemented with 2% chitosan for 45 days. After 45 day of feeding trail, 6 fish per treatment were sampled for immunity determination (respirotary burst activity, bacteriocidal actvity and lysozyme arctivity). 36 fish in all treatment groups were challenged intramusculary with *Aeromonas hydrophila* on day 45. The results indicated that the fish fed on diet containing 2% chitosan (G4) had the significant increased (*P*<0.05) in respirotary burst activity, bacteriocidal actvity and lysozyme arctivity compared to control group and to other treatment groups, followed by G3 and G2 respectively. Also, G4 showed highest resistance to challenged *A. hydrophila* compared to other groups. Control group (G1) showed the decreased performance in all non sepecific immune parameters and simultaneously increased in mortality rate. Therefore, these results indicate that 2% chitosan as addititive fed could be used as prophylactic in common carp culture to enhance the protection against any possible infection by *A. hydrophila* which is useful for practical fish culture.

Key words: *Cyprinus carpio*, *Aeomonas hydophila*, Chitosan, immunity, prebiotics.

التاثير الغذائي للكيتوسان في الاستجابة المناعية ومعدل البقاء في اسماك الكارب الشائع المصابه بجراثيم الايروموناس هايدروفيلا

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الخلاصة:

اجريت الدراسة لتقييم تأثير اضافة الكيتوسان للعليقه في الاستجابة المناعيه ودراسة معدل البقاء قي اسماك الكارب الشائع بعد اجراء فحص التحدي باستخدام جرثومة الإيروموناس هايدروفيلا. اجريت هذه الدراسة على 80 سمكة من الكارب الشائع (36.4 ± 0.73 غم) قسمت إلى أربع مجموعات غذائيه: المجموعة الأولى (G1) مثلت مجموعة السيطرة وتم تغذيتها على نظام غذائي خالي من الكيتوسان والمجموعة الثانية (G2) مع اضافة 0.75% كيتوسان من وزن العليقة و المجموعة الثالثة (G3) مع اضافة 1.5% كيتوسان من وزن العليقة والمجموعة الرابعة (G4) مع اضافة 2٪ الكيتوسان من وزن العليقة لمدة 45 يوما. بعد 45 يوم تغذية تم أخذ 6 عينات من الأسماك لدراسة بعض معايير الاستجابة المناعية. تم اجراء فحص التحدي بعد يوم 45 في 36 سمكة من كل معاملة عن طريق الحقن في العضلة بجرثومة الإيروموناس هايدروفيلا. أظهرت النتائج أن الأسماك المغذاة على نظام غذائي يحتوي على 2٪ من الكيتوسان (G4) كانت هنالك زيادة معنوية (P<0.05)في معايير الاستجابة المناعية مقارنة بمجموعة السيطره والمجاميع الأخرى، ثم G3 و G2على التوالي كما أظهرت G4 أعلى مقاومة للتحدى ضد جرثومة الايروموناس هايدروفيلا بالمقارنه مع المجموعات ألاخرى بينما أظهرت مجموعة السيطره (G1) انخفاض الأداء في جميع المعايير المناعية وزيادة في معدل الهلاكات في وقت واحد. وبالتالي، فإن هذه النتائج تشير الى ان اضافة نسبة 2٪ كيتوسان من وزن العليقه يمكن أن يعزز الاستجابه المناعيه ضد الاصابات البكتيرية ويؤدي الى انخفاض معدل الهلاكات تحت ظروف الإجهاد وبالتالي يمكن أن يكون مفيدا لتربيةالاسماك.

Introduction:

Despite the global aquaculture has been expanding with the fast development in the past 16 years [1]. However unmanged fish culture paractices and adverse environmental conditions affect the fish health leading to economic losses in fish production. Fish diseases are the major problems causing heavy loss to fish farmers thoughout the world [2]. Among the different types of disease causing agents, bacterial pathogens are the most important and responsible for severe mortalities in a wide range of fishes at different stages of growth [3]. *Aeromonas hydrophila* has been isolated from a wide range of freshwater fish species in Asia. It causes several signs of ill health including tail and skin rot, fatal haemorrhagic septicemias and in

human; it is responsible for soft-tissue wound infection and diarrheic disease [4].

Vaccination is an important prophylactic measure that could be used to prevent diseases, even though disease caused by bacteria like *A. hydrophila* has been not contolled by vaccination due to hetrogenicity [5]. Traditional diseases control strategies employ antibiotics and chemical disinfectants. However, these substances are not recommended due to the emergence of bacterial resistance [6]. From a scientific point of view, the use of immunostimulants has been suggested to become an alternative way for the prevention and control of various diseases in aquaculture.

[7, 8, 9, 10]. Moreover, recently many researchers have been documented that probiotics can provide significant protection against pathogens of shrimp/fish by suppressing the pathogens, enhancing the immunity or improving water quality [7, 8].

Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics [10, 11], which are classified as complex low molecular weight oligosaccharides and generally cannot be digested by the fish but are metabolized by promoting healthy bacteria within the fish gut. Correspondingly, the beneficial effects of some oligosaccharides have also been demonstrated on fish [9].

Among the novel families of biological macromolecules, whose relevance is becoming increasingly evident, are chitin and its main derivative, chitosan. Potential and usual applications of chitin, chitosan and their derivatives are estimated to be more than 200 [12]. In fact, some studies regarding chitosan have already been conducted in broiler chickens [13], pigs [14] or fishes [15, 16], and beneficial effects on growth performance, or blood profiles have been reported [16]. As for common carp, there is little information about the efficiency of any prebiotics including chitosan oligosaccharides. Thus, the aim of the present study was to assess the influence of chitosan on non-specific immune response and on relative percentage survival of *C. carpio* challenged by *A. hydrophila.*

Materials and Methods

Diet preparation

Forty five days feeding trial was initiated with four dietary treatments including: wheat flour 45%, soybean meal 32%, yellow corn 11%, animal protein 10%, premix 1% and dicalcium phosphate 1%. Control diet as it was not supplemented with chitosan, whereas Chitosan diets comprised the same ingredients of control diet but these were supplemented with Chitosan at concentration of 0.75%, 1.5% and 2%. All the ingredients were properly were ground separately in an electric grinder and thoroughly mixed and water was added in sufficient quantity. Pellets (2.0×4.0 mm) were prepared using a dry power press. The dried pellet diets were packed in bags and stored at -20 °C.

Experimental design

C. carpio L., (weighing 30 g, n = 150) were obtained from a local fish farm (Babyle Fish Farm, Iraq) and transported to the aquarium facilities. After 2 weeks acclimation and on-growing, 80 fish (average weight (36.4 ± 0.73 g) were randomly distributed into 12×80 L fiberglass tanks (10 fish tank^{-1}). Each treatment was conducted in duplicate (two tanks treatment^{-1}). Fish within different treatment groups were fed three times daily at a rate of 3% of average body mass for 60 days according to their respective treatment as follows: group 1 (G1), fed control diet (no added chitosan); group 2 (G2), fed bassanal diet supplemented with 0.75% chitosan; group 3 (G3), fish fed diet 2% chitosan. Each group was placed in a fully prepared aquarium containing dechlorinated tap water, the average of water temperature was 22± 1.5 ^{o}C, dissolved oxygen was in the range 7-8.6 and the pH was in the range 7.1- 8.19 using YSI D.O. meter Model 55 and pen-type HANNA.Throughout experiments/trials, fish were reweighed every two week and within this period feed input was adjusted daily based on weight gain.

Challenge Study

Fish fed diets supplemented with and without chitosan were challenged intramuscularly with *A. hydrophila* on day 60. A virulant strain of *A. hydrophila* were recieved in Tryptose Soy Broth (TSB) from Al-Kendi company. Sub culture were made on Tryptose Soy Agar (TSA) at 25 ^{o}C. The day seven. LD50 was determined by intramuscular injection of 48 fish with graded doses of *A. hydrophila* (10^6, 10^7, 10^8, 10^9 and 10^{10} CFU ml^{-1}) at 25°C, and the result showed that the LD50 on day 7 was 108 CFU/fish. Fish were injected intramusculary (im) with 0.2 ml of *A. hydrophila* at a concentration 1.2×10^7 CFU ml^{-1} in phosphate buffer saline (PBS) as a medium. After injection all the groups were observed for their response against bacterial strain.

Blood and Serum Collection from Fish

At the end of the exposure period (i.e. pre and post challenge), three fish per tank (n=6) were netted randomly and quickly anaesthetised in a buffered solution of clove powder (eugenol; 25-50 mg L^{-1} water for 10 min). Fresh blood samples were immediately obtained from the caudal vein for determination of respirotary burst activity. Prior to serum collection, another six fish from each group were anaesthetized and blood was collected in 5 ml test tube without anticoagulant and allowed to clot for 2 h and then centrifuged at 2500 rpm for 15 min; collected supernatant was subjected to a further 1 min spinning at 2500 rpm. The serum samples were stored at – 20 ^{o}C until they were needed for analysis of serum lysozyme activity and baceriocidal activity .

Determination of immunological parameters

Serum Lysozyme Activity

Lysozyme level in serum of six fish in each treatment group was detected by using turbidimetric assay according to the method described by [17]. Briefly, test serum (0.1 ml) was added to 1.9 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was achieved at 25 °C and the absorbance was measured at 530 nm after 0.5 and 5 min using spectrophotometer. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1}.

Respiratory Burst Activity

This assay was carried out using the reduction of nitroblue tetrazolium (NBT) to formazon as a measure of superoxide anion (O_2^-) production. According to method described by [18]. Briefly, the blood samples were collected by piercing the caudal peduncle in a test tube containing 2.5% EDTA as anticoagulant. Fifty μl of blood was placed into the wells of U bottom microtitre plates (three replicate wells were used sample^{-1}) and incubated at 22 °C for 1 h to facilitate adhesion of cells. Afterwards, step, the supernatant was removed and the adhered wells were washed three times in PBS. After washing, 50 μl of 0.2% NBT+1 μl ml^{-1} of phorbol myristate acetate (PMA) were added and resulting solution was incubated for a further h at 22 °C. The cells were then fixed with 100% methanol for 2–3 min and again washed (3x) with 70% methanol. Then, the plates were air dried. Sixty μl 2 M potassium

hydroxide and 70 μl dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The optical density (OD) of the turquoise blue solution was then read in a plate reader at 540 nm.

Serum Bactericidal Activity

Serum bactericidal activity was determined according to [19]. Briefly, serum samples from each group were combined to three numbers. Pooled sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVB+2 pH 7.5, containing 0.5 mM ml^{-1} Mg2+ and 0.15 mM/ml Ca2+). The bacteria *A. hydrophila* were suspended in the same buffer to make a concentration of 1×105 CFU ml^{-1}. The diluted sera and bacteria were mixed at 1:1, incubated for 90 min at 25 °C and then shaken. The numbers of viable bacteria was then calculated by counting the colonies from the resultant incubated mixture on TSA plates in duplicate (two plates per sample) after 24 h incubation. The bactericidal activity of test serum was expressed as percentage of colony forming units in test group compared to the control group.

Relative Percentage Survival (RPS)

Average mortality (%) data was used for calculating Relative Percentage Survival (RPS) as follows:

RPS (%)= 1- [(Mortality (%) in treated group) ÷ (Mortality (%) in control group)] ×100

Statistical analysis

Data were analyzed by one way ANOVA using SPSS V. 16 Software. When the differences were significant at $P<0.05$ level, multiple range test was utilized to compare the mean values among the treatments due to the main effects.

Results and Discussion

Serum Lysozyme Activity

Serum lysozyme activity (U ml^{-1}) of *C. carpio* after 45 feeding trail on Chitosan and after challenge with *A. hydrophila* is shown in Figure 1. The results exhibited the highest lysozyme activity (77 U ml^{-1}) in fish group fed 2% Chitosan for 45 days. There was significant difference between G4 and control group ($P<0.05$). Also, G4 showed significantly different compared to G2 and G3 ($P<0.05$). After challenge with *A. hydrophila* (60 days) 2% Chitosan group revealed significantly different in comparison to control group and also significant difference than G2 and G3 ($P<0.05$).

Lysozyme is an important hydrolytic enzyme that breaks cell wall of bacteria by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell wall. It is known to attack both Gram negative and positive bacteria. The increased level of lysozyme and bactericidal activity indicated increased level of protection against bacterial infection [20]. This result are in agreement with [21] who reported significantly increased in lysozyme activity in chitosan supplemented groups in Asian seabass *Lates calcarifer* on day 60 compared to control group. The result of the present investigation indicated that Chitosan incorporated in diet in order to stimulate immune response and protection against infection of *A. hydrophila.*
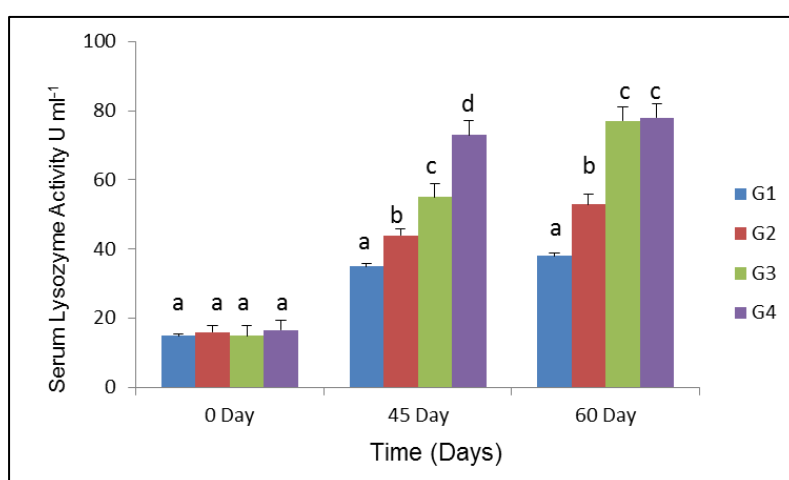

Figure1: Serum lysozyme activity (U ml^{-1}) in *C. carpio* following feeding trial on different concentration of chitosan for 0, 45 and 60 days. Values are mean ± S.E. Different letters indicated significant different between groups ($P<0.05$); n =6.

Respiratory burst activity

Respiratory burst activity (NBT reduction) in *C. carpio* neutrophils indicated significant increased (P<0.05) in all Chitosan groups compared to control group after 45 days feeding of Chitosan (Figure 2). There was significant difference (P <0.05) between G2, G3 and G4. After challenge (i.e 60 days) with *A. hydrophila*, respiratory burst activity increased significantly in all Chitosan groups in comparison to control group. G4 showed the highest level of NBT reduction which was significantly different than other groups (G2 and G3).

Similar to this, [22] had reported increase in respiratory burst activity in rainbow trout treated with chitosan. Enhanced respiratory burst activity was observed till 45th day in low- and medium dose chitosan-fed groups and 30th day in high dose fed group. [21]) also reported enhanced respiratory burst activity up to 60 days in Asian seabass fed 1% chitosan in diet. However, [23] reported significantly

stimulated neutrophil respiratory burst activity even after 12 weeks of feeding chitosan-coated diet to *Paralichthys olivaceus*. Previous studies were performed using dietary chitosan as an immunostimulant to protect brook trout against *Aeromonas salmonicida* [24], augmenting the respiratory burst and phagocytic activities in the gilthead sea bream [25,26]. As the derivatives of chitosan, chitosan oligosaccharides (COS) have better biocompatibility and solubility [27]. For these reasons, the biological activities of COS are of increasing interest in recent research. This study also confirmed that the dietary chitosan had beneficial effects on the some non-specific immune response of *C. carpio*. Similar results have been reported in other animals including pigs [14], broilers [13] or fishes [15, 28]. The mechanism by which COS enhance innate immune responses is still unknown to date. [29] showed significantly elevated immune responses in comparison to controls and individual application.
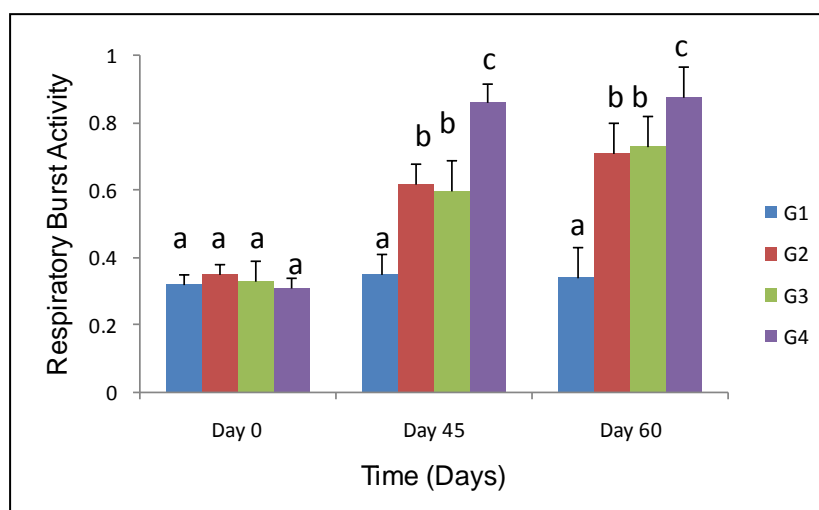
Figure 2: Respiratory burst activity (NBT reduction) in *C. carpio* neutrophils following feeding trial on chitosan for 0, 45 and 60 days. Values are mean ± S.E. Different letters indicated significant different between groups (P<0.05); n =6.

Serum Bactericidal Activity

Serum bactericidal activity of the control group and Chitosan supplemented groups is presented in Figure 3. Serum bactericidal activity after 45 days dietary feeding of chitosan was significantly higher (P<0.05) in Chitosan groups compared to control group. After challenge with *A. hydrophila* 2% Chitosan group (G4) registered the highest bactericidal activity (82%) which was significant difference than control group and also significantly different in compassion to G2 and G3. This result indicating T that 2% Chitosan had effective against the infection of *A. hydrophila*. This could be attributed and well correlated with reduced mortality (%). Chitosan plays a vital role in the stimulation of bactericidal activity and phagocytic cells that stems particularly from its stimulation of the generation of reactive oxygen species like O_2^- by the affected cells [3]. Therefore, Chitosan improved to be an effective immunostimulat preventing the organization of bacterial infection in common carp.
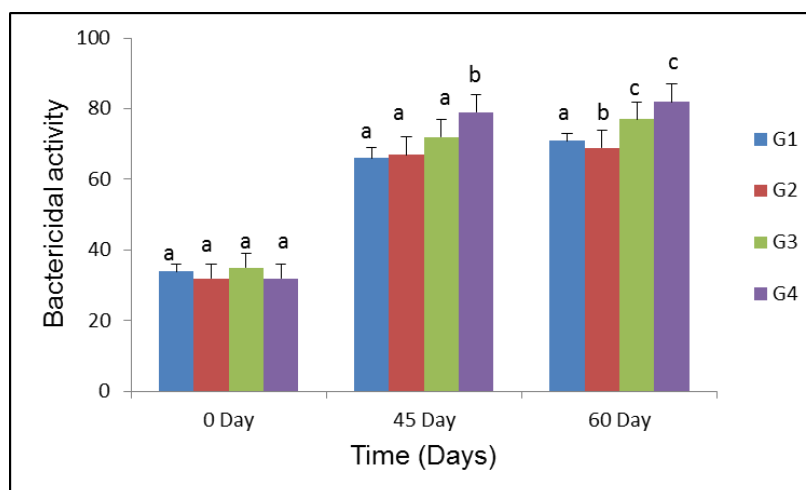
Figure 3: Bacteriocidal activity in *C. carpio* following feeding trial on chitosan for 0, 45 and 60 days. Values are mean ± S.E. Different letters indicated significant different between groups (P<0.05); n =6

Relative Percentage Survival (RPS)

Mortality and survival percentage and RPS of *C. carpio* feeding different concentration of Chitosan and in the control group after challenge by *A. hydrophila* are presented in Table 1. Mortality (%) was highest (40%) in control group. The higher RPS was observed in group feeding 2% Chitosan (100%) followed by G3 and G2 (75% and 50% respectively). This result can be explained on the basis of stimulation of the innate immunity of carp by dietary Chitosan. Similar result also registered by [21, 31].

Treatment	Mortality (%)	Survival (%)	RPS (%)
G1	40%	60%	....
G2	20%	80%	50%
G3	10%	90%	75%
G4	0%	100%	100%

Table 1. Mortality percentage, survival percentage and RPS of *C. carpio* feeding different

   concentration of Chitosan and in the control group after challenge by *A. hydrophila*

G1= control group; G2= Fish group fed 0.75% Chitosan; G3= Fish group fed 1.5% Chitosan; G4= Fish group fed 2% Chitosan

In conclusion, the dietary administration of 2% chitosan revealed increased protection against the infection with *A. hydrophila* and higher survival rate which indicate increased non-specific-immune responses and disease resistance of common carp. These informations will be important for fish aquaculture. More studies are necessary to determine the molecular and cellular mechanisms of the effect of Chitosan.

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