The Effect of β-glucan on Blood and Mucus of Catfish *Clarias gariepinus* Infected with *Pseudomonas florescence*

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Abstract

The present study was planned to investigate the immunological changes in blood and mucus of catfish *Clarias gariepinus* infected with *Pseudomonas florescence* along with trial for control using (β-glucan). A total number of 240 catfish were collected from Ismailia channel and subdivided to 4 equal groups: the control group, infected group with virulent strain of *P. florescence*, β-glucan group and β-glucan infected group fed on diet supplemented with β-glucan. The blood and mucus samples were taken at 2 days, 3 and 5 weeks for immunological examinations. There were significant decreases in total protein and albumin of the infected group, while β-glucan and β-glucan infected groups revealed significant increases in total protein and globulin compared to the control group. The serum and mucus immunological studies revealed enhanced immune parameters (lysozyme activity and IgM) in all groups with non-significant change in protease level in serum but increased in mucus only compared to the control group. These results concluded that dietary supplementation of β-glucan enhanced skin mucosal and serum humoral defence responses and resistance of catfish against infection *with P. florescence*. The results could be useful for better understanding the role of skin mucus as a key component of the innate immune system, which could be beneficial in control of fish health.

Introduction

In recent years, the production of catfish has suffered massive financial losses due to pathogen spread and break outs. Innate immunity plays a crucial role in increasing resistance to pathogenic organisms and has generated increasing interest in the past few years ***(Magnadottir, 2010).*** Pseudomonas septicemia is one of the most prevalent fish diseases in aquaculture due to its ubiquitous nature in aquaculture environment ***(Fernandez et al, 1990)***.The innate

immune system of fish is divided into physical barriers, cellular and humoral components ***(Uribe et al, 2011)***.Since the majorities of the infectious agents affect or initiate the process of infection on the mucus surfaces, the mucosal immune response plays a crucial role in the course of the infection ***(McNeilly et al, 2008)***. Unfortunately, the complete repertoire of immune factors present in the skin mucus and their precise role on fish immunology and defence is poorly understood ***(Li et al, 2013)***. Nowadays, a wide variety of natural growth promoters (NGPs), including plant extracts, prebiotics, probiotics and organic acids, have been broadly applied worldwide with reasonable success to reduce the risk of diseases and improve fish welfare by enhancement of non-specific defence system ***(Nermeen and Naela, 2014).*** The most promising group of immune-stimulants and also prebiotic are the β-1,3/1,6-glucans, a soluble carbohydrates from the cell walls of yeast *Saccharomyces cerevisiae*, because they have a well-defined chemical structure and mode of action on the immune system, ***(Raa, 2000).*** This study was designed to investigate the immunological changes in both blood and mucus of catfish (*Clarias gariepinus*) infected with *Pseudomonas florescence* with trial for control using (β-glucan) and the role of skin mucus as a key component of the innate immune system.

Materials and methods

Fish:

A total number of (240) apparently healthy African catfish *Clarias gariepinus* were randomly collected alive from Ismailia channel with an average body weight 150 ± 25g. They were transported in its natural water tank to the lab. at Fish Diseases and Management Department, Faculty of Veterinary Medicine, Suez Canal University and kept for 2 weeks under observation for acclimation in glass aquaria and the water was renewed daily. The temperature (25 ± 1°C) was adjusted thermostatically and continuous aeration using electric air pumping compressors.

Preparation of fish diets and feed additives:

Two experimental rations were used. The control ration consisted of the basal commercial without any treatment. The second ration, contained the basal commercial ration, treated with β-1, 3 glucan extracted from *S. cerevisiae* at a concentration of 1g/ 1 kg ration (obtained from Anhui ZhengZheng Biology Technology Co., Ltd.).

Experimental design:

The pre-acclimated catfish *Clarias gariepinus* were divided into 4 groups, each group subdivided into 3 subgroups (replicates, each 20 fish/ aquarium). As shown in table (1) using well identified virulent strain of *Pseudomonas florescence* kindly supplied from Dept. of Fish

Diseases and Management, Faculty of Vet. Medicine, Suez Canal University.

Blood sampling:

The blood samples were collected from the caudal blood vessels of fish and placed in a clean centrifuge tube, then centrifuged at 3000 r.p.m for 5 minutes for estimation of serum biochemical and immunological parameters.

Mucus sampling:

Fish were anesthetized prior to sampling with MS222 (Tricaine methanes ulphonate,) from (Argent Chemical Laboratories, USA) with a dose of 100mg/l + 50 mg Sod bicarbonate. Skin mucus samples were collected from fish specimens using the method of ***Guardiola et al (2014)***. Skin mucus was collected by gentle scraping the dorso-lateral surface of catfish specimens using a cell scraper with enough care to avoid contamination with blood and urino-genital and intestinal excretions. The sample then homogenized with 1 volumes of Tris-buffered saline (TBS, 50 mM Trise HCl, pH 8.0, 150 mM Sod chloride). The homogenate was vigorously shaken and centrifuged in cool centrifuge at 14.000 rpm for 15 min at 4°C. and the collected supernatant was stored frozen until use.

Serum and mucus immunological assay:

A-Lysozyme activity:

Serum and mucus lysozyme activities were determined by the turbidometric assay as described by ***Esteban et al (2001)***. Twenty five ul serum or mucus was added on 175 µl (0.75 mg/ml *Micrococcus lysodeikticus*) together with the assay buffer in flat-bottomed 96-well plates. The reduction in absorbance at 450 nm was measured from 0 to 15 min. at 25° C using an ELISA reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min and the units of lysozyme activity were calculated using the hen egg white lysozyme standard curve.

B- Protease activity:

Protease levels in serum and mucus of skin were analyzed using the enzyme-linked immunosorbent assay (ELISA) according to ***Tian et al (2004).*** The optical density (OD) was measured at 540 nm using Tekan micorplate reader. The optical-value was directly related to the amount of protease.

C- Total immunoglobulin M levels:

Total IgM levels in serum and mucus of body surface were analyzed using the enzyme-linked immunosorbent assay (ELISA) according to ***Lee (2013).*** The optical density (OD) was measured at 560 nm using Tekan micorplate reader. The optical-value was directly related to the amount of total IgM.

Table 1: *Experimental design:*

Group	Diet	Infection
Control	Basal diet	Not infected
Infected	Basal diet	infected i.p. with 1 ml ($1x10^7$ CFU/fish) of the virulent strain of *P. florescence*.
β-glucan	Basal diet containing 1g β-1,3 glucan/kg diet	Not infected
β-glucan infected	Basal diet containing 1g β-1,3 glucan/kg diet	infected i.p. with 1 ml ($1x10^7$ CFU/fish) of the virulent strain of *P. florescence* after 5 weeks of treatment with β-1,3 glucan

Results and Discussion

The present work revealed significant decreases in total protein and albumin with hyperglobulinemia at the 3rd and 5th weeks in the infected group compared to the control group (table 2). The hypoproteinemia may be due to the decrease in protein synthesis by the liver due to the hepatopathy occurs in the infected fish ***(Coles, 1986)***. ***Rehulka (2002)*** attributed the results to the loss of albumin from the skin lesions or increase its catabolism in acute inflammation caused by the infection. The results agreed with ***Wafaa (2007) and Ghada (2011).***

The present study showed significant increases in serum total protein and globulin levels in β-glucan supplemented diet groups with reduction of A/G ratio compared to the control one. These results agreed with ***Amnah (2012)***. Also, ***El-Komy and Shehab El-Din (2014)*** who obtained the same result in *O. niloticus* fed probiotics supplemented diets and they attributed the results to the immuno-modulatory effect of yeast on the liver cells activating the anabolic capacity to produce blood proteins particularly globulin indicating a positive effect on the integrity of hepatocytes.

As shown in table (3), Pseudomonas infected group revealed a significant increase in serum lysozyme level compared to the control one which could be attributed to the stimulation of immune system as a part of body defense mechanism against the infection**.** Serum lysozyme activity presents a first line of defence mechanism together with lytic factors by acting on the peptidoglycan of bacterial cell walls leading to breakdown of bacteria ***(Wang et al, 2010)***.

The result of our study showed significant increase of serum lysozyme activity in β-glucan supplemented groups compared to the control group**.** Lysozyme is constitutively expressed, synthesized and secreted by neutrophils, monocytes and macrophages; the greatest concentration of lysozyme is directly proportional to the leukocytic count. As the supplementation of β-glucan in fish diet increased the leukocytic count, the lysozyme concentration and activity were increased ***(Gado et al, 2014).***

Moreover, the result revealed a high lysozyme activity in mucus of the infected group compared to the control group (table 4)**.** Change in cutaneous mucus lysozyme activity are common once a fish becomes infected with a pathogen, depending on the level of infection, the lysozyme enzyme may act as a first encounter defense to bacteria by existing externally on the fish, such as in the skin mucus and gills ***(Rodriguis, 2008). Fast et al (2002)*** found that infested rainbow and Atlantic salmon with sea lice showed significant increase in mucus lysozyme activity.

The present study revealed high lysozyme activity in mucus of β-glucan supplemented groups compared to the control group. ***Guardiola et al (2014)*** demonstrated that gilthead seabream skin mucus contains higher level of lysozyme than serum. Therefore, the lysozyme activity in skin mucus showed no significant correlation with other immune substances, which suggested that, the lysozyme is constitutively, secreted in the skin mucus of these fish species ***(Jung et al, 2012)***. ***Sheikhzadeh et al (2012b)*** found that dietary supplementation of prepiotic Hilyses (fermented *S. cerevisiae*) significantly increased lysozyme level in the skin mucus of rainbow trout**.**

Protesases in the skin mucus may play a protective role against pathogens by both direct, cleaving their proteins thus damaging the pathogens ***(Subramanian et al, 2008),*** and indirect, by hampering their colonization and invasion due to modifications in the consistency of mucus surfaces and/or increasing the sloughing of these mucus layers, leading to pathogen removal from the body surface ***(Chen et al, 2008).***

In the present study, the level of protease was significantly increased in mucus of β-glucan supplemented groups compared to the control group. Increased epidermal protease activity was shown following the administration of some immunostimulants such as Ergosan and Hilyses (fermented *Saccharomyces cerevisiae*) in rainbow trout ***(Sheikhzadeh et al, 2012a and b).***

The present study showed significant increase in mucus protease level of the infected group and decreased at the end of the

experiment compared to the control group**.** Many pathogens like Pseudomonas produce virulence factors (extracellular products) including proteases. Pathogens use proteases to facilitate entry into host organisms or for extracellular digestion ***(Rodrguis, 2008)***. This result agreed with, ***Ross et al (2000)*** who observed highly significant increase of protease level in mucus of Atlantic salmon infested with salmon lice**.**

Immunoglobulins, principally immunoglobulin M (IgM), are major components of the teleost humoral immune system, and is found in blood and other fluids including mucus ***(Cuesta et al, 2004).*** Regarding the infected group, there were significant increases in both serum and mucus IgM of the infected group compared to the control group. It is found that, Feeding *Aeromonas hydrophila* ghosts to carp resulted in higher IgM titers in the intestine and serum; it is interesting that in this study gut mucus IgM titers were twice as high as those found in serum ***(Tu et al, 2010). Reyes-Becerril et al (2015)*** observed increased serum IgM level at 24 or 48 hr in Pacific red snapper fish experimentally exposed to *Aeromonas veronii*. ***Ghada (2011)*** recorded significant increase in serum IgM in *O. niloticus* infected with *A. hydrophila*.

The total serum immunoglobulin IgM result showed significant increase value in β-glucan groups compared to the control group**.** β-glucan, because of their large molecular weight, they cannot penetrate the cell membrane and therefore they must interact with cell-surface receptors; it has been shown that β-glucans are recognized by several receptors found on neutrophils, macrophages, and dendritic cells ***(Tanioka et al, 2013)***. The interaction of β-glucans with specific receptors on macrophages and dendritic cells can induce the production of several cytokines, which in turn responsible for enhanced immunoglobulin and antibody levels in the body by stimulating the lymphocyte proliferation (both B and T cells), leading to elevation of immunoglobulin level in both *in vitro* and *in vivo* conditions ***(Tanioka et al, 2013*** *and* ***Rufchaie and Hoseinifar (2014)***.

The total mucus immunoglobulin IgM results showed significantly higher values in β-glucan groups compared to the control. It was found that, dietary supplementation of fish with *C. butyricum* at higher dose treatment caused secretion of IgM in the blood sera and the skin mucus in comparison with the control ***(Song et al, 2006).*** Delivery of probiotic bacteria results in greater numbers of IgM+ B cells in the gut lamina propria both in juveniles and developing larvae ***(Abelli et al, 2009).***

It could be concluded that dietary supplementation of β-glucan

enhanced skin mucus and serum humoral defence responses in catfish *Clarias gariepinus*.

Table2: Proteingram (mean value ± SE) in different experimental groups.

Groups	Time	Total protein gm/dl	Albumin gm/dl	Globulin gm/dl	A/G ratio
Control	**2 days**	4.46 ± 0.06^{a}	2.63 ± 0.09^{a}	1.83 ± 0.10^{a}	1.43 ± 0.12^{a}
	3 weeks	4.57 ± 0.16^{b}	2.59 ± 0.08^{a}	1.98 ± 0.08^{b}	1.30 ± 0.14^{a}
	5 weeks	4.42 ± 0.05^{b}	2.63 ± 0.09^{a}	1.79 ± 0.06^{c}	1.46± 0.04^{a}
Infected	**2 days**	4.13 ± 0.05^{b}	2.21 ± 0.09^{b}	1.92 ± 0.09^{a}	1.15 ± 0.18^{a}
	3 weeks	4.25 ± 0.10^{c}	1.91 ± 0.05^{c}	2.34 ± 0.12^{a}	0.81 ± 0.05^{b}
	5 weeks	4.01 ± 0.06^{c}	1.99 ± 0.07^{b}	2.02 ± 0.06^{b}	0.98± 0.03^{b}
β-glucan	**2 days**	4.52 ± 0.11^{a}	2.68 ± 0.05^{a}	1.84 ± 0.13^{a}	1.45 ± 0.16^{a}
	3weeks	5.01 ± 0.09^{a}	2.62 ± 0.09^{a}	2.39 ± 0.06^{a}	1.09 ± 0.12^{a}
	5 weeks	5.15 ± 0.12^{a}	2.62 ± 0.10^{a}	2.53 ± 0.12^{a}	1.03 ± 0.06^{b}
β-glucan infected	**2 days**	4.37 ± 0.04^{a}	2.56± 0.07^{a}	1.81 ± 0.03^{a}	1.41 ± 0.13^{a}
	3 weeks	5.12 ± 0.16^{a}	2.60± 0.05^{a}	2.52 ± 0.07^{a}	1.03 ± 0.16^{a}
	5 weeks	4.95 ± 0.19^{a}	2.56± 0.11^{a}	2.39 ± 0.09^{a}	1.07 ± 0.07^{b}

Superscript with different letters in the same colum at the same week are significant at ($p < 0.05$)

Table 3: *Some serum immunological parameters (mean value ± SE) in different experimental groups*

Group	Time	Lysozyme µg/ml	Protease µg/ml	ALP U/ml
Control	2 days	9.79 ± 0.23^{b}	25.93 ± 0.25^{a}	65.19 ± 0.27^{b}
	3 weeks	8.77 ± 0.28^{c}	27.49 ± 0.45^{a}	66.62 ± 0.20^{c}
	5 weeks	10.05 ± 0.16^{d}	24.96 ± 0.15^{a}	64.18 ± 0.28^{d}
Infected	2 days	10.99 ± 0.16^{a}	26.15 ± 0.18^{a}	67.34 ± 0.32^{a}
	3 weeks	16.62 ± 0.18^{a}	26.72 ± 0.32^{a}	80.44 ± 0.31^{a}
	5 weeks	14.63 ± 0.24^{c}	24.67 ± 0.45^{a}	69.57 ± 0.29^{c}
β-glucan	2 days	9.92 ± 0. 24^{b}	26.52 ± 0.39^{a}	65.56± 0.23^{b}
	3 weeks	12.15 ± 0.23^{b}	27.37 ± 0.41^{a}	68.43 ± 0.35^{b}
	5 weeks	17.43 ± 0.21^{b}	25.34± 0.30^{a}	73.15 ± 0.23^{b}
β-glucan infected	2 days	10.05 ± 0.17^{b}	26.11± 0.33^{a}	64.89 ± 0.16^{b}
	3 weeks	12.75 ± 0.39^{b}	27.22 ± 0.37^{a}	67.89 ± 0.20^{b}
	5 weeks	20.96 ± 0.26^{a}	25.35± 0.26^{a}	75.04 ± 0.24^{a}

Superscript with different letters in the same colum at the same week are significant at ($p < 0.05$)

Table 4: *Some mucus immunological parameters (mean value ± SE) in different experimental groups*

Group	Time	Lysozyme µg/ml	Protease µg/ml	ALP U/ml
Control	**2 days**	11.77 ± 0.20^{b}	27.04 ± 0.12^{b}	70.24 ± 0.34^{b}
	3 weeks	11.94 ± 0.24^{c}	28.21 ± 0.47^{c}	71.30 ± 0.43^{c}
	5 weeks	12.82 ± 0.22^{c}	27.66 ± 0.17^{c}	69.41 ± 0.32^{d}
Infected	**2 days**	13.24 ± 0.23^{a}	29.68 ± 0.25^{a}	74.21 ± 0.28^{a}
	3 weeks	18.77 ± 0.17^{a}	34.15 ± 0.19^{a}	89.03 ± 0.41^{a}
	5 weeks	15.71 ± 0.23^{b}	25.40 ± 0.23^{d}	75.30 ± 0.29^{c}
β-glucan	**2 days**	11.89 ± 0.19^{b}	27.41± 0.18^{b}	71.20 ± 0.65^{b}
	3 weeks	17.60 ± 0.24^{b}	31.93± 0.17^{b}	78.10 ± 0.45^{b}
	5 weeks	23.91 ± 0.51^{a}	37.84± 0.24^{b}	90.57 ± 0.25^{b}
β-glucan infected	**2 days**	11.46 ± 0.25^{b}	26.85± 0.39^{b}	70.65 ± 0.31^{b}
	3 weeks	18.10 ± 0.26^{b}	31.41± 0.37^{b}	79.13 ± 0.59^{b}
	5 weeks	24.31 ± 0.26^{a}	39.35± 0.21^{a}	92.54 ± 0.30^{a}

Superscript with different letters in the same colum at the same week are significant at ($p < 0.05$)

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تأثير البيتاجلوكان على دم ومخاط أسماك القط الأفريقي المصابة بمرض التسمم الدموي السيدوموناس

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تم عمل هذه الدراسة بغرض دراسة التقصي عن الإصابة بمرض التسمم الدموي السيدوموناسي على دم ومخاط أسماك القط الأفريقي من الناحية المناعية والى دراسة تأثير الوقاية باستخدام البيتاجلوكان كمحفز مناعي طبيعي. وقد أستخدم في هذه الدراسة ٢٤٠ سمكة وتم تقسيمها إلى أربع مجموعات (المجموعة الضابطة ، المجموعة المصابة، مجموعة البتاجلوكان ومجموعة البيتاجلوكان المصابة). و قد تم تجميع عينات الدم والمخاط من المجموعات المختلفة فى اليوم الثاني والاسبوع الثالث والاسبوع الخامس. أسفرت النتائج عن وجود نقص في البروتين الكلي والزلال في الأسماك المصابة وإلى وجود زيادة في مستوى البروتين الكلي والجلوبيولين مع تقدم التجربة في أسماك كل من مجموعتى البيتاجلوكان والبيتاجلوكان المصابة بالمقارنة بالمجموعة الضابطة. أسفرت النتائج المناعية للسيرم والمخاط الى وجود زيادة معنوية في مستويات كل من إنزيم الليزوزيم ، والاميونوجلوبين ام في كل المجموعات أما انزيم البروتييز فقد أظهر زيادة معنوية فقط في مخاط كل المجموعات بالمقارنة بالمجموعة الضابطة. كما أوضحت الدراسة إلى أهمية استخدام البيتاجلوكان كإضافة لعليقه أسماك القط لدورها في تحسين الحالة المناعية ودورها في مقاومة هذا المرض وإلى ضرورة الاهتمام بدراسة دور المناعة المخاطية في مجال أمراض الأسماك.