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Effects Of Dietary Application Of Two Antagonistic Gut-Isolated *Bacillus* Species On The Immune Response Of *Oreochromis niloticus* To *Aeromonas hydrophila* Infection

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ABSTARCT

Two *Bacillus* species isolates (B_1 and B_2), showed *in-vitro* antagonistic activity against *Aeromonas hydrophila* isolated from the intestinal tract of apparently healthy *Oreochromis niloticus*. Both antagonistic isolates were identified as *Bacillus licheniformis* and *Bacillus cereus/thuringiensis,* respectively by using Biolog's microbial identification system, and they were evaluated to be safe to *O. niloticus* when inoculated intra-peritoneal (I/P). Feeding experiments were carried out *in-vivo* to investigate the effect of both antagonistic isolates (1 X 10^7 CFU/g diet) either alone or combined on fish immune response and resistance to *A. hydrophila* infection. Immunological parameters (phagocytic, lysozyme and serum bactericidal activities) were evaluated as well as a challenge test using pathogenic *A. hydrophila*. Results revealed that, antagonistic Bacillus isolates either alone or combined triggered significant ($P \leq 0.05$) increase in phagocytic, lysozyme and serum bactericidal activities; with the highest values in fish received a mixture of both antagonistic isolates. Survival of *A. hydrophila* challenged fish was highest in fish fed on both antagonistic isolates, followed by *B. cereus/thuringiensis*, and then *B. licheniformis* fed fish.

INTRODUCTION

Nile tilapia, *Oreochromis niloticus*, is considered to be the predominant and most commonly cultured species among tilapias in many countries around the world *(1,2)* including Egypt *(3)*. They are increasingly recognized as the species of choice for intensive aquaculture. However, a major problem associated with intensive fish culture operations increased susceptibility of fish to infectious diseases *(4)*. From which, Aeromonasis has become a serious problem to fish aquaculture all over the world in recent years *(5)*. In particular, outbreaks caused by *Aeromonas hydrophila* infection, a most common problem to fish in Egyptian aquaculture *(6)*, was one of the most common bacterial diseases affecting *Oreochromis niloticus* leading to severe losses on the production. Therefore, till present, aeromonasis can be partially controlled by fish farmers with crude application of antibiotics *(7)*. However, the traditional use of antibiotics has received criticism due to the potential for development of antibiotic resistant bacteria, the presence of antibiotic residues in fish tissue, negative impacts on microbial populations in the aquaculture environment, and suppression of the cultured species' immune system *(8,9)*.

Therefore, there is an increasing need to find safe alternatives to antibiotics *(4)*. An alternative method is the use of antagonistic or

probiotic, bacterial strains to control populations of potential pathogens through competitive exclusion and/or enhancement of fish immunity *(10)*.

Most attempts to propose probiotics for aquaculture have been undertaken by isolating and selecting microbial strains from aquatic environment. These microbes included members from Vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts *(11)*.

Members of the genus Bacillus, one of the dominant probiotic bacteria commonly used in aquaculture, being a normal component of the gut and pond microflora, are able to out-compete other fish pathogenic bacteria for nutrients and space and can exclude them through the production of antibiotics *(11,12)*. Also numerous studies have found that endogenous or exogenous Bacillus strains could be effective in improving growth, immunity and disease resistance in fish *(13)*.

Therefore, the current study was carried out to investigate the effect of two antagonistic *Bacillus* species isolated from the gastrointestinal tract of *Oreochromis niloticus* on the immune response and resistance to *Aeromonas hydrophila* infection.

MATERIAL AND METHODS

Bacterial isolation and identification

Apparently healthy *Oreochromis niloticus (O. niloticus)* with different body weights were collected alive from Abbassa fish farms, Abbassa, Sharkia, Egypt. These fish were used for the isolation of *Bacillus* species from their gastrointestinal tract by a method previously described *(14)*. Identification of the selected *Bacillus* species were carried out by using the Biolog's microbial identification system (Biolog Inc, Hayward, CA, USA) using the Biolog GEN III MicroPlates according to the manufacturer's instructions.

A pathogenic strain of *Aeromonas hydrophila,* previously isolated from naturally diseased *O. niloticus* in the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, was used in the current study.

In-vitro antagonistic activity of isolated *Bacillus* species against *Aeromonas hydrophila*

The interaction between isolated *Bacillus* species and *A. hydrophila* were examined using agar diffusion method and the determination of the inhibition zones were recorded *(15)*.

Safety of the isolated antagonistic *Bacillus species* (B_1 and B_2)

The two *Bacillus* species isolates (B_1 and B_2) that showed inhibitory activity against pathogenic *A. hydrophila in-vitro*, were evaluated for its safety to *O. niloticus.* A bacterial suspension of each of the two antagonistic *Bacillus* species (B_1 and B_2) was prepared and adjusted to 10^9 CFU/ml using McFarland standard tubes *(6)*. A total number of 90 apparently healthy *O. niloticus* with an average body weight of 45±5.0g were obtained from a private fish hatchery. They were kept in well prepared glass aquaria (each of 80cm X 40cm X 30cm, filled with dechlorinated tap water and supplied by an air pump) for 2 weeks under observation for acclimation to the new environment. These fish were divided into 3 equal groups in three replicates (10 $fish^{-1}$). Fish of the first and second groups were injected intra-peritoneal (I/P) with 1.0 ml of the prepared bacterial suspension of first and second antagonistic isolates (B_1 and B_2), respectively, while fish of the third group were kept as a control and injected I/P with 1.0 ml of sterile physiological saline (0.85% NaCl). Fish of all groups were kept under daily observation for 15 days post inoculation at water temperature of 26±2°C, PH (8±0.5), salinity (0.5±0.1 ‰), Dissolved oxygen (6.5±0.5 mg/L), Nitrite (less than 0.05mg/L),

Ammonia (less than 0.02mg/L) and water hardness of 190±10 mg/L as $CaCO_3$.

Feeding experiments

Feeding experiments were carried out to investigate the effect of dietary supplementation of the two antagonistic *Bacillus* species (B_1 and B_2) either alone or combined on *O. niloticus* immune response and resistance to *A. hydrophila* infection.

Diets used for fish

Basal Diet

A pelleted ration (D_1) contained 30% crude protein obtained from the Fish Researches Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt, were used as a control diet (without any Bacillus supplementation) in the feeding experiments.

Experimental Diets

Three *Bacillus* species supplemented diets (D_2, D_3 and D_4) were prepared. D_2 was a basal diet in which first antagonistic *Bacillus* species (B_1) (1 X 10^7 CFU/g) was incorporated, D_3 was a basal diet in which second antagonistic *Bacillus* species (B_2) (1 X 10^7 CFU/g) was incorporated, while D_4 was a basal diet in which a mix of first *Bacillus* species (B_1) (0.5 X 10^7 CFU/g) and second *Bacillus* species (B_2) (0.5 X 10^7 CFU/g) were incorporated *(6)*. These prepared diets were kept in a refrigerator at 4°C and were used in the feeding experiments. These diets prepared every two weeks.

Experimental design

A total number of 120 *O. niloticus* with an average body weight of 25±5.0g were obtained from a private fish hatchery. They were kept in well prepared glass aquaria (each of 80cm X 40cm X 30cm, filled with dechlorinated tap water and supplied by an air pump) for 2 weeks under observation for acclimation to the new environment. These fish were divided into 4 equal groups (T_1, T_2, T_3 and T_4), 30 fish for each in three replicates (10 fish^{-1}). The first group (T_1) kept as a control and fed on the basal diet (D_1), while groups T_2, T_3 and T_4 were fed on Bacillus supplemented diets (D_2, D_3 and D_4), respectively (**Table 1**). Fish of all groups were fed twice a day at a rate of 3% of the biomass for 30 days. The water of the aquaria was changed daily. At the end of feeding period (30 days), some immunological parameters were evaluated as well as a challenge test using *A. hydrophila* strain was carried out.

Table 1. Design of feeding experiments

Group *n = 30	Diet	Incorporated *Bacillus* species	
		B_1 (CFU/g)	B_2 (CFU/g)
T_1 (control)	D_1	-	-
T_2	D_2	1 X 10^7	-
T_3	D_3	-	1 X 10^7
T_4	D_4	0.5 X 10^7	0.5 X 10^7

*n = number of fish per group.

Immunological aspects

At the end of the feeding period, heparinized blood samples were collected from the caudal vessels of fish of each group using sterile syringes rinsed with heparin and used for leukocytes separation *(16)* for determination of the phagocytic activity *(17)*. While, another blood samples were withdrawn into Eppendorf tubes and centrifuged at 3000

rpm for 15 minutes for serum separation which used for determination of lysozyme activity *(18)* and serum bactericidal activity against *A. hydrophila (19)*.

Challenge test

At the end of the feeding period, a challenge test using a pathogenic *A. hydrophila* strain was done. Ten fish from each group were challenged by I/P injection with 0.5 ml of *A. hydrophila* fresh culture suspension containing 10^8 bacterial cells ml^{-1}, while another 10 fish were I/P injected with 0.5 ml of sterile physiological saline (0.85% NaCl). The challenged fish were kept under daily observation for up to 15 days. The mortalities were recorded and the relative level of protection (RLP) among the challenged fish was determined *(20)* according to the following equation:

RLP = 1- (percentage of treated mortality ÷ percentage of control mortality) X 100

Statistical analysis

Statistical analysis was performed using the SPSS Statistics program, where the analyses of variance (ANOVA) and Duncan`s Multiple Range Test *(21)* were used to determine differences between treatments (levels of significance are expressed as $P \leq 0.05$).

RESULTS AND DISCUSSION

The use of antagonistic probiotic bacteria is widely expected to become an alternative method for the prevention and control of bacterial diseases in fish *(22)*. There are numerous reports of *Bacillus* species being isolated from fish and shellfish which are often antagonistic against other microorganisms, including pathogenic bacteria *(6,11,12,14,23)*. In the current study, the *in-vitro* antagonistic test revealed that, two isolates (B_1 and B_2) out of 97 *Bacillus* species isolates showed antibacterial activity against *Aeromonas hydrophila* with clear inhibition zones of 20 mm and 16 mm diameter respectively. The antibacterial effect is generally due to any of the following factors, either singly or in combination: production of antibiotics, bacteriocins, siderophores, lysozymes or proteases, and alteration of pH values by the organic acids produced *(24)*. Antagonism may be also due to competition for nutrients that favour the growth of antagonistic bacteria, or the expression of their inhibitory effects *(11)*. *Bacillus* species are known to produce a large number of antimicrobials *(25)*. These include bacteriocins and bacteriocin-like inhibitory substances (BLIS) (e.g., Subtilin and Coagulin) as well as antibiotics *(26)*.

In this study, the two antagonistic *Bacillus* species (B_1 and B_2) could be identified according to the Biolog's microbial identification system as *Bacillus licheniformis* and *Bacillus cereus/thuringiensis* respectively. Whatever, the *Bacillus species* with probiotic activity is not restricted to a single species of this genera, but includes many species such as *B. subtilis, B. cereus, B. coagulans, B. clausii, B. megaterium, B. licheniformis (27,28), B. pumilus, B. firmus (6), B. brevis (2), B. endophyticus, B. tequilensis (29)* and *B. thuringiensis (30)*.

Both antagonistic *Bacillus* species isolates were evaluated to be harmless and safe to *O. niloticus* following I/P inoculation with 1.0 X 10^9 CFU/fish, where neither abnormal signs nor mortalities were observed or recorded on the inoculated fish with either Bacillus bacteria or sterile saline solution during the experiment period. Also the postmortem examination of the inoculated fish did not show any abnormal lesions indicating the safety of *B. licheniformis* and *B. cereus/thuringiensis* to *O. niloticus* under the existing conditions, and therefore were considered safe to be used in the fish diets. These findings agreed with *Aly et al. (6,31)* who proved the safety of a number of antagonistic *Bacillus* species to the same fish species.

In the current study, significant ($P \leq 0.05$) increases of phagocytic percent (P%) and phagocytic index (PI) were recorded in *O. niloticus* fed diets contained *B. licheniformis* and/or *B. cereus/thuringiensis* **(Table 2).** These findings may be due to feeding fish with *Bacillus* species could stimulate phagocytosis activity of phagocytes. Similar finding were previously recorded *(32-34)*.

Serum lysozyme activities of *O. niloticus* increased significantly ($P \leq 0.05$) after feeding on *B. licheniformis* and/or *B. cereus/thuringiensis* containing diets **(Table 2).** This may be attributed to the immunostimulatory effect of ingested *Bacillus* species on fish as previously reported *(31,34)* .

Significant ($P \leq 0.05$) increases in serum bactericidal activity (SBA) against *A. hydrophila* were recorded in *O. niloticus* fed on diets contained *B. licheniformis* and/or *B. cereus/thuringiensis* **(Table 2).** The increase of SBA activities can possibly be due to a higher concentration of lysozymes *(30)* and other various humoral factors which involved in innate and/or adaptive immunities and are elevated in the serum to protect the fish effectively from infection *(35)*.

Mechanisms through which the probiotic bacteria exert their immunostimulant effects have not been cleared in fish. However, ingested Bacillus spores delivered to the small intestine in large numbers can interact with the gut-associated lymphoid tissue (GALT) and priming the stimulation the immune system *(26)*. Also, probiotics may interact with the immune cells such as mononuclear phagocytic cells (monocytes, macrophages) and polymorphonuclear leucocytes (neutrophils) and NK cells to enhance innate immune responses *(36)*.

Table 2. Effect of dietary supplementation of antagonistic *Bacillus* species on some immunological parameters in *Oreochromis niloticus*

Group	Diet	Supplemented *Bacillus* species (1.0×10^7 CFU/g diet)	Phagocytic activity		Lysozyme activity (µg/ml)	Serum bactericidal activity (..X 10^4 cfu/ml)
			P%	PI		
Control (T_1)	D_1	--	$44.66^{B} \pm 1.76$	$1.77^{C} \pm 0.043$	$155.00^{C} \pm 0.00$	$806.67^{A} \pm 52.06$
T_2	D_2	*B. licheniformis*	$72.66^{A} \pm 2.90$	$2.88^{B} \pm 0.003$	$192.67^{B} \pm 7.33$	$493.33^{B} \pm 35.27$
T_3	D_3	*B. cereus/thuringiensis*	$78.66^{A} \pm 2.90$	$2.90^{B} \pm 0.020$	$207.33^{AB} \pm 14.66$	$340.00^{C} \pm 34.64$
T_4	D_4	*B. licheniformis* + *B.cereus/thuringiensis*	$80.66^{A} \pm 3.52$	$3.85^{A} \pm 0.073$	$229.33^{A} \pm 7.33$	$353.33^{C} \pm 17.63$

Means carrying different superscript in the same column are significantly different ($P \leq 0.05$).

High levels of Protection against *A. hydrophila* challenge were recorded in *O. niloticus* previously fed on *B. licheniformis* and/or *B. cereus/thuringiensis* **(Table 3).** Similar observations were previously recorded *(6,30,31)*. The effectiveness of protection against infection is often attributed to the enhanced immune response *(4)*.

The current study cleared that feeding of *O. niloticus* on a mixture of the two antagonistic Bacillus isolates triggered the highest and best effect for both immune response and disease resistance. This may be

attributed to some of proposed mechanisms include greater survival, growth, viability or adhesion to mucosal surfaces of one species in the presence of another species, the production of different enzymes or other proteins, the creation of a probiotic niche and additive/ synergistic effects of strain specific properties *(33)*.

It could be concluded that some endogenous antagonistic *Bacillus* species could be selected and used not only to suppress the growth of opportunistic bacterial pathogens (e.g. *Aeromonas hydrophila*) but also to enhance the immune defense of fish and thereby improving their resistance against infections, as a way to minimize the massive use of antibiotics. However further advanced studies were necessary, before recommending its application in aquaculture.

Table 3. Effect of dietary supplementation of antagonistic *Bacillus* species on *Aeromonas hydrophila* challenged *Oreochromis niloticus*

Group	Diet	Supplemented *Bacillus* species (1.0×10^7 CFU/g diet)	Mortality (%)	RLP (%)
Control (T_1)	D_1	--	80.00	0.00
T_2	D_2	*B. licheniformis*	40.00	50.00
T_3	D_3	*B. cereus/thuringiensis*	30.00	62.50
T_4	D_4	*B. licheniformis* + *B.cereus/thuringiensis*	20.00	75.00

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الملخص العربى

تأثير استخدام نوعين من بكتريا الباسيلس المعزولة من أمعاء الأسماك على الاستجابة المناعية للبلطي النيلي والعدوى بالايروموناس هيدروفيلا

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أوضحت الدراسة أنه تم التوصل الى عزلتين من بكتريا الباسيلس المثبطة لنمو الايروموناس هيدروفيلا معملياً من القناة الهضمية لأسماك البلطي النيلي. وباستخدام نظام البيولوج لتعريف الميكروبات تم تعريف تلك العزلتين على ان احداهما باسيلس ليشنيفورميس والأخرى باسيلس سيريس/ثورينجينسيس. وبحقن تلك العزلتين داخل التجويف البريتوني لأسماك البلطي النيلي وجد أنهما امنين وليس لهما اي تاثير ضار. ثم تم دراسة تأثير اضافة تلك العزلتين (باسيلس ليشنيفورميس & باسيلس سيريس/ثورينجينسيس) بجرعة 10^7 خلية بكتيرية/ جرام عليقة (كلا على حدى أو خليط منهما) في تغذية أسماك البلطي النيلي على الاستجابة المناعية والمقاومة للعدوى بالايروموناس هيدروفيلا. تم تقييم بعض الأنشطة المناعية (قدرة الخلايا المناعية علي الالتهام ، نشاط الليزوزيم & قدرة مصل الدم على قتل البكتريا) كما تم اجراء اختبار تحدي باستخدام الايروموناس هيدروفيلا الممرضة. وقد أظهرت النتائج ان عزلتي بكتريا الباسيلس المثبطة سواء كلا على حدى أو خليط منهما أدى الى زيادة معنوية في قدرة الخلايا المناعية علي الالتهام و نشاط الليزوزيم و قدرة مصل الدم على قتل البكتريا وكانت اعلى قيم في الأسماك التي تغذت خليط من العزلتين. كما اظهر اختبار التحدي أعلى نسبة اعاشة في الأسماك التي تغذت على العزلتين معا تليها التي تغذت على باسيلس سيريس/ثورينجينسيس فقط ثم التي تغذت على باسيلس ليشنيفورميس فقط.