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Protective effect of *Pseudomonas fluorescens* as a probiotic in controlling fish pathogens

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Abstract: The use of *Pseudomonas fluorscens* isolates as biological control agents against two fish pathogens; *Pseudomonas angulliseptica* and *Streptococcus faecium* in Nile tilapia were investigated in vivo and vitro. *Pseudomonas fluorscens* biovars I, II & III were tested in vitro using the agar diffusion method and showed effectiveness in inhibiting growth of *P. angulliseptica* and *Strep. faecium*. *Pseudomonas fluorscens* biovar II was the most effective with largest inhibition zones against both pathogens. For oral administration, *P. fluorscens* biovars were incorporated into the mixed feed diet. A significant reduction in mortality rate and a significant increase in hematological parameters, total protein, and globulin in fish groups fed *Pseudomonas fluorscens* incorporated diet following challenge by *P. angulliseptica* and *Strep. faecium*. It could be concluded that *P. fluorescens* has a protective effect against different Nile Tilapia pathogens and could be used as a probiotic bacteria and an eco-friendly alternative measure to chemical antimicrobials and further research studies to clarify its protective mechanisms on cellular and molecular levels.

Keywords: Probiotics, Pseudomonas, Fish Diseases, Biological Control, Hematological Parameters

1. Introduction

Aquaculture is an emerging industry as one of the promising enterprises for providing nutritional and food security to humans and supplying the protein demands since there are some critical problems with other resources. However, most of the intensive aquaculture farms have been facing major problems resulted from outbreaks caused by several pathogens, especially bacterial ones which are leading to high mortality and limiting the aquaculture industry expansion. *Pseudomonas anguilliseptica* is an opportunistic pathogen for many cultured fish species in marine and brackish waters worldwide [1] and can cause certain outbreaks under stressful conditions and considered the most significant pathogen among pseudomonas species for cultured fish [2]. *Pseudomonas anguilliseptica* was originally described as the bacterial causative agent of “Sekiten-byo” (red spot disease) in pond-cultured Japanese eel, *Anguilla japonica* [3] and in Finland; it was identified as the etiology of several disease out-breaks in several species of farmed salmonid fish [4]. In Egypt, *P. anguilliseptica* was isolated from naturally infected tilapia [5,6]. Fish streptococcosis is a reemerging disease affecting a variety of wild and cultured fish throughout the world and a limiting factor for aquaculture industry [7]. Streptococcosis have triggered significant economic losses in the aquaculture industry worldwide causing high mortality, reduced growth, and unmarketable appearance. Streptococcus affects a wide range of cultured fish species, including hybrid striped bass, tilapia [8], rabbit fish, *Siganus canaliculatus* [9], rainbow trout, *Oncorhynchus mykiss* [10], red drum, *Sciaenops ocellatus* [11] and European sea bass, *Dicentrarchus labrax* [12]

The increased public awareness of the negative drawbacks caused by over-exposure to synthetic chemicals as well as emerging antimicrobial resistance led to search for alternatives and unique solutions such as organic and synthetic chemical-free food products. To promote organic fish production, it is necessary to develop antibacterial treatments that are based on materials from natural sources. Probiotic bacteria, a live micro-organism that when managed in adequate amounts can confer a health benefit on the host [13], could protect fish and other aquatic animals through different ways such as antagonizing the pathogens for living space, adhesion sites, energy and essential nutrients, producing inhibitory compounds and improving the immune response of the fish [14]. Probiotic bacteria has the ability to adhere to and colonize into the gut and so form a barrier against pathogenic microorganisms and/or to stimulate the host's immune system and this consider the most common concern about the mode of action [15]. In particular, innate immunity has been shown to be affected by feeding fish with probiotic [15]. Non-pathogenic pseudomonas can be used for control of some bacterial pathogens [2] *in vivo* and *in vitro* antibacterial activity against *Aeromonas hydrophila* [16, 17, 18], *Aeromonas salmonicida* [19], *Staphylococcus aureus* and *Aeromonas sobria* [20], Saprolegnia and other fungi [21, 22], Vibrio [23] and *Flavobacterium psychrophilum* [24].

Therefore, this study was designed as initial study to test the capability of non-pathogenic *Pseudomonas fluorescens* Biovars to control and prevent *Pseudomonas anguilliseptica* and *Streptococcus faecium* which are considered important pathogens in many cultured and wild fish species *in vitro* and *in vivo*. And, the main objective of this study was designed to prevent, control or limit the virulence of these pathogens especially with using the beneficial bacteria as a feed additive. Having native probiotic strains is a big advantage toward the successful and effective probiotic application and generally in the aquaculture industry, the concept is prophylaxis is always better than treatment.

2. Materials and Method

2.1. Bacteria and Growth Medium

Non-pathogenic *Pseudomonas fluorescens* Biovars I, II & III were previously isolated from naturally infected tilapia, identified and their safety tested by published work [5, 18]. *Pseudomonas anguilliseptica* was evaluated for its pathogenicity by [5] and *Streptococcus faecium* was obtained from Aquatic Diseases Lab., National Institute of Oceanography & Fisheries (NIOF), Egypt. Bacteria were kept frozen in 15% glycerol, 85% saline solution in aliquots, at - 80 °C for further use.

2.2. In-Vitro Antimicrobial Activity

In *vitro* antimicrobial activity was assessed using the agar diffusion method and the inhibition zone was determined according to [25]. The three *P. fluorescens* biovar I, II and III isolates were inoculated in the center of culture plates, containing tryptic soya agar and incubated at 30 °C for 24 h. Then, a fresh Tryptic Soya Broth (TSB) containing the pathogenic *Pseudomonas anguilliseptica* and another fresh TSB containing *Streptococcus faecium* were spread over the plates, previously inoculated with the tested bacteria. Incubation at 30 °C for 24 h and checked for the appearance of inhibition zones and their sizes were recorded.

2.3. Experimental Fish

A total of 270 healthy Nile tilapia (70 ± 5 g/fish) were obtained from Fish Farm Research Station, NIOF, Egypt. Fish were randomly stocked into nine indoor glass aquaria at a rate of 30 fish / aquarium and acclimatized for two weeks prior to experiments, at 22 ± 1 °C and subjected to 12 h light/ 12 h dark. Fish were fed a commercial tilapia diet contains on protein 26 % and Fat 3 % (Zoocontrol®, Egypt) twice daily at a rate of 2% of fish body weight during acclimation and experiment. To verify that the fish were free of bacterial infection, fish were randomly sampled and their livers and kidneys were aseptically streaked on Tryptic Soya Agar and incubated at 25 ºC for 48 h.

2.4. Water Quality Evaluation

Aquaria water quality parameters were monitored daily. Dissolved oxygen was measured with a dissolved oxygen meter (YSI® Incorporated, OH, USA). Ammonia and nitrites were measured using visocolor kits (Macherey-Nagel®, Germany). Temperature, Oxygen, ammonia and nitrite levels were maintained at 22 ± 1 °C, 6.8 mg/l, 0.1 ± 0.01 and 1 ± 0.5 mg/l, respectively. The aquaria were cleaned daily by siphoning off two thirds of the water and replacing it with fresh water.

2.5. Feed Preparation

Incorporated diets with non-pathogenic *P. fluorescens* were prepared according to the method described by [14]. Bacterial colonies (*P. fluorescens* biovars I, II, III) were grown on TSB, harvested by centrifugation at 1000 RPM for 10 min, washed with saline and re-suspended in saline to 10^{10} cells mL^{-1}. Thereafter, volumes were mixed thoroughly in 100 g of the commercial dry feed contains on protein 26 % and Fat 3 % (Zoocontrol®, Egypt) to achieve a dose equivalent to 10^8 bacterial cells g^{-1} of feed.

2.6. Feeding Experiment

To study the effect of *P. fluorescens* against *P. anguilliseptica* and *Streptococcus faecium* infection *in vivo,* the following feeding experiment was conducted. Fish were fed with incorporated diet with non-pathogenic *P. fluorescens* biovars for 7 days then injected intra-peritoneal with pathogenic *P. anguilliseptica* at dose 3 x 10^7 CFU/ml/ fish as its LD50 2-4 x 10^7 CFU/ml/ fish and *Streptococcus faecium* at dose 3 x 10^8 CFU/ml/fish which has LD50 1.5 – 4 x 10^8 CFU/ml/fish. Infected and non-infected control groups were used as positive and negative experimental control groups. Fish continued to be fed with the non-pathogenic *P. fluorescens* incorporated diet for another 7 days. Behavioral alterations, feeding response, and mortality for all experimental groups were examined and recorded daily. Dead fish were removed daily and subjected to bacteriological examination for presence of pathogens. Groups and dose of inoculation were represented and tabulated (Table 1).

2.7. Hematological Analysis

At the end of the experiment, 10 fish from each aquarium were anaesthetized and blood samples were collected from the caudal vein into heparinized tubes. Blood samples were used for determination of RBCs, Hb, PCV, WBCs and differential leukocytic count according to [26]. And, Serum samples were obtained by blood centrifugation at 3000 RPM for 15 minutes for estimation of the total protein content calorimetrically according to method described by [27] and albumin content by a colorimetric method at wave length 550 nm according to [28].

***Table 1.** Feeding experiment design to evaluate in vivo antimicrobial efficiency of isolated non-pathogenic Pseudomonas fluorescens.*

Groups		**Inoculating organisms**			**Inoculating bacteria (pathogenic)**	
Group No.	**Fish No.**	**Species of bacteria**	**Dose**	**Route**	**Species of bacteria**	**Dose**
1st group	30	*P. fluorescens* biovar I	10^8 cells /g	Feeding	*P. anguilliseptica*	0.1ml (3 x 10^7 CFU)
	30	*P. fluorescens* biovar II	10^8 cells /g	Feeding	*P. anguilliseptica*	0.1ml (3 x 10^7 CFU)
	30	*P. fluorescens* biovar III	10^8 cells /g	Feeding	*P. anguilliseptica*	0.1ml (3 x 10^7 CFU)
	30	Control infected	Normal diet	Feeding	*P. anguilliseptica*	0.1ml (3 x 10^7 CFU)
2nd group	30	*P. fluorescens* biovar I	10^8 cells /g	Feeding	*Strep. faecium*	0.1ml (3 x 10^8 CFU)
	30	*P. fluorescens* biovar II	10^8 cells /g	Feeding	*Strep. faecium*	0.1ml (3 x 10^8 CFU)
	30	*P. fluorescens* biovar III	10^8 cells /g	Feeding	*Strep. faecium*	0.1ml (3 x 10^8 CFU)
	30	Control infected	Normal diet	Feeding	*Strep. faecium*	0.1ml (3 x 10^8 CFU)
3rd group	30	Control non infected	Normal diet	Feeding	Saline 0.9%	0.1 ml

2.8. Statistical Analysis

Analysis of variance (One way ANOVA) and Duncan's multiple range test (DMRT) were carried out to determine differences between treatments at probability level $P > 0.01$ according to [29]. All the statistical analysis was done by using the software program SPSS (version 22).

3. Results and Discussion

The antibacterial effect of probiotic bacteria may be due to either individual or joint production of antibiotics, bacteriocins, siderophores [30], lysozymes and proteases and alteration of pH by organic acid production [31]. In present study, in *vitro* evaluation showed that non-pathogenic *Pseudomonas fluorescens* biovar I, II and III had an antagonist effect against *Pseudomonas angulliseptica* and *Streptococcus faecium* in fish. In *vitro* assay, *P. fluorescens* biovars had antibacterial activity against both bacterial pathogens *Pseudomonas angulliseptica* and *Streptococcus faecium.* And *P. fluorescens biovar II* resulted in a larger inhibition zone (6 & 5 mm) than biovar I (3 & 3 mm) and III (4 & 3 mm) against *P. angulliseptica* and *Streptococcus faecium* respectively. Previous studies have the same results against different pathogenic organisms such as *P. fluorescens* had an antimicrobial effect in vitro on microbiological plates in the laboratory against *Aeromonas hydrophila* and *Aeromonas salmonicida* [16-18, 32]. *Pseudomonas fluorescens*'s antimicrobial effect *in vitro* may be attributed to the production of several antibiotic-like substances as bacteriocins and a phenazine antibiotic [33], non-nitrogen-containing compound [34] and siderophores production which mediated competition for iron [20, 35, 36].

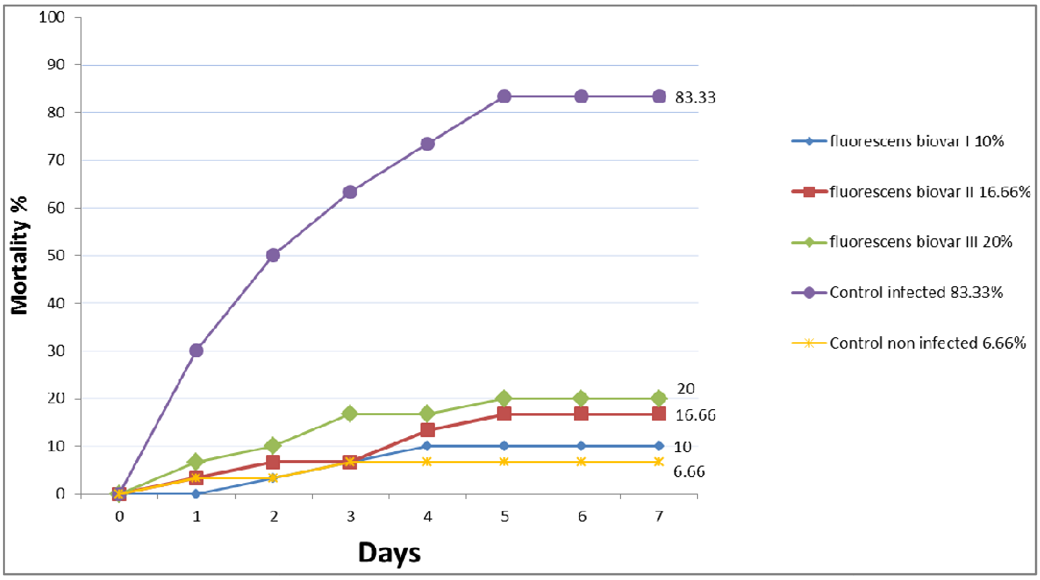

***Figure 1A.** Cumulative mortality rate of O. niloticus fed a diet containing 10^8 cells /g P. fluorescens biovar I, II & III for 7 successive days and then challenged I/P with 0.1 ml (3 x 10^7 cells) of P. anguilliseptica*

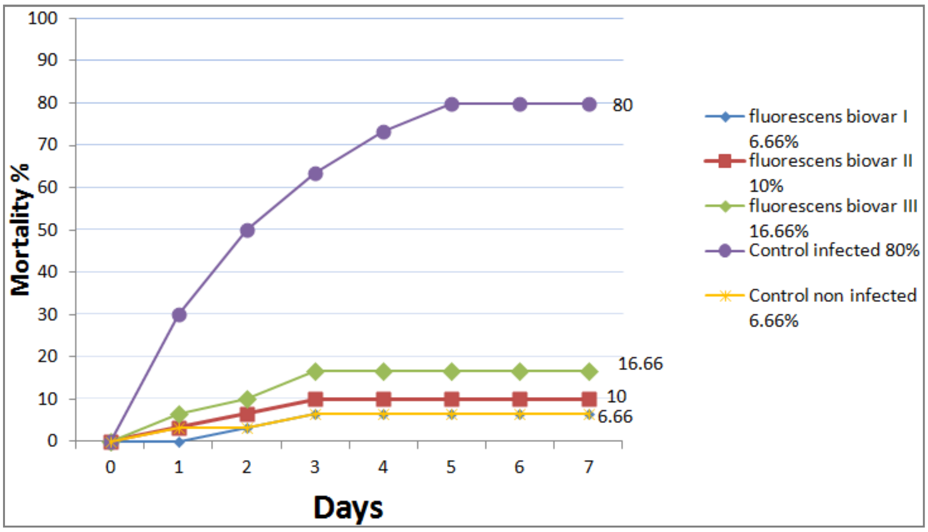

Figure 1B. *Cumulative mortality rate of O. niloticus fed a diet containing 10^8 cells /g P. fluorescens biovar I, II & III for 7 successive days and then challenged I/P with 0.1 ml (3 x 10^8 cells) of Strept. faecium*

Table 2. *Hematological parameters of fish groups challenged by P. angulliseptica. Means with the same letter within a column are not significantly different (P < 0.01).*

Groups	Hb g/100ml	PCV %	RBCs 106/mm3	WBCs 103/mm3	Lymphocytes 103/mm3	Monocytes 103/mm3	Protein g/100ml	Albumin g/100ml	Globulin g/100ml
Control	4.3± 0.066^c	15.2 ± 0.13^c	1.31 ± 0.014^c	58.2 ± 0.97^c	47.33 ± 0.57^d	5.61 ± 0.036^d	2.75 ± 0.13^c	0.75 ± 0.04^a	2.06 ± 0.15^d
P. fluorescens biovar I	5.21 ± 0.18^b	21.95 ± 0.3^b	1.47 ± 0.035^b	69.65 ± 0.59^b	52.66 ± 0.68^c	10.25 ± 0.29^c	3.01 ± 0.085^b	0.68 ± 0.021^b	2.62 ± 0.60^c
P. fluorescens biovar II	5.42 ±0.16^a	22.33 ± 0.21^a	1.55 ± 0.17^a	71.33 ± 0.89^a	53.04 ± 0.73^b	11.66 ± 0.35^a	3.55 ± 0.36^a	0.56 ± 0.07^c	3.21 ± 0.022^a
P. fluorescens biovar III	5.12 ±0.13^b	23.25 ± 0.42^a	1.54 ± 0.13^a	71.88 ± 0.61^a	55.11 ± 0.56^a	10.88 ± 0.45^b	3.12 ± 0.045^b	0.54 ± 0.017^d	3.01 ± 0.046^b

Table 3. *Hematological and physiological parameters of fish groups challenged by Strep. faecium. Means with the same letter within a column are not significantly different (P < 0.01).*

Groups	Hb g/100ml	PCV %	RBCs 10^6/mm^3	WBCs 10^3/mm^3	Lymphocytes 10^3/mm^3	Monocytes 10^3/mm^3	Protein g/100ml	Albumin g/100ml	Globulin g/100ml
Control	4.3 ± 0.066^c	15.2 ± 0.13^d	1.31± 0.014^d	58.2 ± 0.97^d	47.33 ± 0.57^d	5.61± 0.036^c	2.75 ± 0.13^d	0.75 ± 0.04^d	2.06 ± 0.15^c
P. fluorescens biovar I	5.01 ± 0.13^b	20.66±0.01^c	1.41± 0.029^c	68.88± 0.46^c	51.94 ± 0.83^c	10.07± 0.35^b	3.21 ± 0.04^c	0.65 ± 0.045^a	2.83 ± 0.70^b
P. fluorescens biovar II	5.11 ± 0.11^a	21.48± 0.33^b	1.53 ± 0.14^a	71.02± 0.93^a	53.13 ± 0.37^b	10.96± 0.65^a	3.42 ± 0.24^b	0.58 ± 0.01^c	3.02 ± 0.075^a
P. fluorescens biovar III	5.14 ± 0.10^a	22.25± 0.40^a	1.50 ± 0.16^b	70.12± 0.43^b	54.01 ± 0.86^a	10.89± 0.63^a	3.67± 0.052^a	0.60 ± 0.072^b	3.0 ± 0.044^a

The fish feeding experiment exhibited lower mortality rate in fish groups fed a *P. fluorescens* biovars incorporated diet than infected control groups. The fish groups fed P. *fluorescens* biovar I, II & III and challenged by *P. angulliseptica* (Figure 1A)*,* resulted in 10, 16.66 and 20% cumulative mortality respectively, and these results could be attributed to many theories for instance pseudomonas produces antibiotic metabolites, which are inhibitory for both gram-negative and gram-positive bacteria [37] and another research study reported that pseudomonas strain of fish origin inhibited a number of pseudomonas strains involved in meat spoilage [38]. Correspondingly, aquatic pseudomonads are often antagonistic against other microorganisms, including fish-pathogenic bacteria and fish-pathogenic fungi, and the antibacterial ability of pseudomonas species may protect host fish against pathogenic bacteria [31]. In 2006, the authors concluded that pseudomonas species PS 102 could be employed as a potential biological control agent in shrimp and prawn aquaculture systems for management and control of bacterial infections [39]. The fish groups fed with *P. fluorescens* biovar I, II & III integrated diet and challenged by *Strep. faecium* (Figure, 1B) exhibited mortality rates of 6.66, 10 and 16.66%, respectively, while the control infected group did not resist *Strep. faecium* while the control infected group had a 83.33% and 6.66 % in control non-infected group mortality rate. These results agreed with that marine pseudomonas species associated with soft coral have antibacterial activity against *Streptococcus equi* [40] and this might be attributed to some of pseudomonas bioactive substances are antimicrobial properties [41]. Additionally, pseudomonas gave an excellent antibacterial activity and inhibited growth of clinical isolates as well as indigenous marine bacteria, but in other hand did not give activity against Streptococcus [42]. *Pseudomonas fluorescens* antimicrobial effect may be resulted from antibacterial substances produced by pseudomonas which have diverse mechanisms of action; some affect the bacterial cell membrane causing bacterial cell lysis, whereas the others act as acetyl-CoA carboxylase and nitrous oxide synthesis inhibitors [41]. Another probability, It also may stimulate the appetite and improve nutrition through vitamins production, detoxification of injurious compounds in the diet, and breakdown of indigestible components [14]. As companion, siderophore production is also a possible action used by Pseudomonas to counteract and constrain pathogens involved in plant diseases [43], in fish, rainbow trout [24] and it was concluded by that pathogens with potential low iron uptake should be more influenced with siderophore assembly of the probiotic used against them.

Hematological and physiological analysis showed that groups fed on a *Pseudomonas fluorescens* biovars incorporated diet had a significant increase in RBCs count, PCV%, Hb content, WBCs, lymphocytes and monocytes levels in comparison to the control group (Table 2&3). This was attributed to those non-pathogenic bacteria which have probiotic effects, could stimulate hemopiotic response resulting in raising hematological parameters [14, 18, 44]. The total protein and globulin (Table 2&3) were higher than the control group in all groups treated with *Pseudomonas fluorescens* and same results were reported by [17, 18]. The significant increase in total protein and globulin and decrease in albumin level accredited to the modulator effect of *Pseudomonas fluorescens* on liver cells resulting in activation of the anabolic capacity of hepatocytes to produce blood proteins, especially globulin [15].

Drug resistance development in bacteria and the accumulation of chemicals in the environment have led to strict regulations that limit the use of antibiotics and chemotherapy in aquaculture industry [45, 46] in many countries to avoid emerging new bacterial resistant strains. This study showed a small picture about the potential use of non-pathogenic bacteria as a progressive new solution for controlling fish pathogens and for organic fish culture which could potentially be used as alternatives to chemotherapy. Afterwards, it can be concluded that probiotics could be a new eco-friendly alternative measure to control fish diseases for sustainable aquaculture. Further studies will focus on manipulation of commensal microbiota to produce organic products to compete with fish pathogens and identify the protective mechanisms of non-pathogenic pseudomonas strains on cellular and molecular levels.

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