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Evaluation of Immunostimulant Activity of *Spirulina platensis* (Arthrospira platensis) and Sage (*Salvia officinalis*) in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Immunostimulants have been found to be attractive and promising agents for disease management in aquaculture. The current study aimed to assess the impacts of feeding with *Spirulina platensis* (SP) and *Salvia officinalis* (SO) for 28 days on the immune status of Nile tilapia (*Oreochromis niloticus*). A total of 180 Nile tilapia (30.6 ± 0.12 g) were divided into 3 groups (triplicates of 20 fish/group). Control group was fed on a basal diet only, while the other two groups were supplemented with SP (10 gm/kg diet) and SO (7.5 gm/kg diet) respectively. Both of SP and SO showed *in vitro* antimicrobial activity against *Pseudomonas aeruginosa*. The immune response of Nile tilapia supplemented with SP and SO revealed significant increases in lysozyme, nitric oxide activities and IgM titer with enhancement of IL-1β and TNF-α genes expression before and after infection with *P. aeruginosa*. Fish challenged with *P. aeruginosa* showed signs of infection with high mortality rate and low relative percentage survival which elevated to be 83% in SP group and 75% in SO group. It could be concluded that dietary supplementation with SP and SO improved immune response and protected Nile tilapia against infection. Moreover, SP showed better results than SO before and after infection.

Keywords: *Spirulina platensis, Salvia officinalis,* Nile tilapia, IL-1β, TNF-α.

Introduction

Over the most recent three decades, aquaculture represents about 40% of the global fish production with a reliable growth of 1% every year. Soon it will be the main source of marine and fresh water sustenance everywhere throughout the world [1-3].

The incidence of microbial pathogens severely affected fish industry [4]. Significant losses have occurred and have subsequently limited aquaculture production in intensive fish culture [5]. Attributable to the wide spread of bacterial resistance to antibiotics, the negative effect on the indigenous microflora of fish and residues of antibiotic which accumulated in fish tissue and environment leading to health hazards in human and animal, the utilization of antibiotics for treatment is not productive and maintainable [6]. Also, the worry of excessive use of antibiotics in aquaculture developed an urgent need to look for an alternative health management strategy, which can be attained by microbial arbitration [7].

The immune-stimulant is defined as a substance which improves the innate or non-specific immune response by cooperating straightforwardly with cells of the system actuating them. It could be nutritional factors, bacterial preparations, polysaccharides, extracts of animal or plant source, cytokines or chemical agents [8]. In aquaculture, a diversity of substances has been suggested to be used as immune-stimulants including synthetic [9], bacterial [10], animal and plant products [11].

Spirulina platensis (SP), is a cyanobacterium that has been utilized for as long as 20 years as a model organism in numerous investigations for the cultivation of algal biomass to obtain protein and chemicals [12]. It has been built up to be a reasonable natural antioxidant and immune-stimulant to humans and animals with less adverse effects and more cost adequacy than synthetic

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products [13]. Early reports demonstrated the pharmacological activities of *S. platensis* which include antioxidant [14] antiviral [15], antibacterial [16], antiplatelet [17], anticardiotoxic [18], hypocholesterolemic [19], antinephrotoxic [20] and anti-hepatoxic effect [21].

Salvia officinalis (SO) or sage, (Lamiaceae family) is local to the Mediterranean area. The principal constituents of sage leaves are tannic acid, oleic acid, ursonic acid, ursolic acid, niacin, nicotinamide, flavones, flavonoid glycosides, cornsole, cornsolic acid, fumaric acid, chlorogenic acid, caffeic acid, and estrogenic substances [22]. The antioxidant [23], immunomodulatory [24], antimicrobial [25] antinociceptive and anti-inflammatory [26], anticancer [27], antidiabetic [28], anti-hyperlipidemic [29], diuretic [30], neuroprotective [31], spasmolytic and hypotensive [32] activities of sage have been recorded.

The current study aimed to demonstrate the immune-stimulant effect of *S. platensis* and *S. officinalis* on Nile tilapia before and after infection with *Pseudomonas aeruginosa.*

Materials and Methods

S. platensis and *S. officinalis*

Pure premium powder of SP was obtained from (HerbaForce, UK). SO leaves were collected from local market, cleaned, shadow dried at 25ºC then ground to powder.

In vitro screening of antimicrobial activity

The *in vitro* antibacterial activity of SP and SO against *P. aeruginosa* was assessed by the method of agar well diffusion [33]. Four ml 0.15 M NaCl of fresh *Pseudomonas aeruginosa* suspension with 10^6 CFU ml^{-1} for bacteria at OD 620 nm, based on MacFerland's scale were mixed well with 100 ml melted warm nutrient agar. Wells of 9 mm diameter were cut in the agar with sterilized cork borer and filled with 100 µl of SP or SO with a final concentration of 8 mg/ml for SP and 10 mg/ml for SO. To allow the antimicrobial substance to diffuse through the inoculated medium, plates were kept in a refrigerator for 6 hours. After incubation at 30 ± 2°C for 48 hours, the clearance zones were recorded in mm.

Fish

Nile tilapia (*Oreochromis niloticus*) (180 fish) with average body weight of 30.6 ± 0.12 g were got from Fish Research Unit of Faculty of Veterinary Medicine, Zagazig University, Egypt. For acclimation, the fish were kept in glass aquaria (80×40×30cm) filled with dechlorinated tap water and fed on basal diet 15 days before beginning of the experiment. Water dissolved oxygen, salinity, temperature, pH, carbonate hardness, ammonia –N, nitrite-N and nitrate-N were monitored once per week as indicated by APHA [34].

Diets and experimental design

The commercial ingredients used for formulation of fish basal diet and its chemical analysis were showed in Table 1. The fish were arbitrarily partitioned into 3 equal experimental groups (triplicates of 20 fish/ each group). Control group was nourished on a basal diet only, while the other two groups were supplemented with diets contained SP (10 gm/kg diet) [35] and SO (7.5 gm/kg diet) [36] respectively. The diets were supplied at the rate of 2 % of the fish biomass twice daily for 28 days and adjusted every two weeks according to fish weight.

Table 1: Basal diet composition and its chemical analysis

Ingredients	Percentage of diet
Fish meal	25
Soy bean meal	22
Ground yellow corn	26.5
Meat meal	20
Fish oil	5
Vitamin and mineral mixture (premix)	1.5
Calculated chemical analysis	
Crude protein	39.9
Crude lipid	10.89
Crude fiber	3.68
Moisture	10.58
Ash	9.11

Sampling

Blood was gathered from (five fish/group) from the caudal veins at the 2nd and the 4th weeks of the experiment to be centrifuged at 3000 rpm/ 15 min to get clear serum which was kept at -20 ºC till be used.

Head kidney tissues were collected from 5 fish/group at the 2nd and the 4th weeks of the experiment to be used for detection of gene expression.

Non-specific and specific immune assays

Serum lysozyme activity was detected using the method of Ellis [37], serum nitric oxide (NO) was determined according to Rajaraman [38]. The level of immunoglobulin (IgM) was measured in serum according to Siwicki and Anderson [39].

The gene expression of IL-1β and TNF-α

Extraction of Total RNA from the head kidney tissue was applied utilizing RNeasy Mini Kit, (QIAGEN, Germany) following the manufacturer's instructions. The yields and purity of Total RNA were detected by using Spectrophotometer 260 and 280 nm absorbance (Thermo Scientific, USA). RevertAid Reverse Transcriptase kit (Thermo Fisher) and oligo-dT were utilized for cDNA synthesis. Initial heat denaturation (65°C for 5 min) was performed for 1 µg of total RNA, then 20 µl of the reactions were incubated at 42°C/ 1 h and then at 85°C/15 min. After that, cDNA was added to a SYBR Green qPCR Master Mix (QIAGEN, Germany) containing 30 pg/ml of primer pairs specific for target genes, [40,41] (Table 2) designed by the Primer 3 program. Initial denaturation was done at 95°C/ 1 min, then 40 cycles were utilized for denaturation at 95°C/ 15 s, annealing at 57°C/ 20s and extension at 72°C/ 45 s. The extent of all amplicons was affirmed on 2% agarose gel electrophoresis stained with SYBR Safe DNA gel stain (Invitrogen). The reference gene utilized was EF-1α [42]. The calculation of the levels of gene expression levels was performed according to Schmittgen and Livak [43].

Table 2: Oligonucleotide primers and probes used in SYBR Green real time PCR

Gene	Primer sequence (5'-3')	Reference
IL-1B	GCTGGAGAGTGCTGTGGAAGAACATATAG CCTGGAGCATCATGGCGTG	Castro *et al*. [40]
TNF alpha	CCAGAAGCACTAAAGGCGAAGA CCTTGGCTTTGCTGCTGATC	Standen *et al*. [41] Gröner *et al*. [42]
EF-1α	CCTTCAACGCTCAGGTCATC TGTGGGCAGTGTGGCAATC	

Challenge test

After the 28 day feeding experiment, 30 fish from each treatment were challenged intraperiotneally with 0.2 ml of 1x 10^7 cfu of *P. aeruginosa* (previously isolated at the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University under project No.5589 from moribund fish and confirmed to be pathogenic). The challenged fish were observed for 14 days to determine the clinical signs, post-mortem findings and the mortality rates. The moribund fish were used for bacterial re-isolation. Recorded mortalities were utilized to estimate the relative percentage survival (RPS) as following:

RPS = 100 - [(treatment mortality/ control mortality) x 100] [44].

Three days after infection, blood samples were collected to get serum which used for estimation of the same immunity indices and head kidney tissues were obtained from 3 fish in each treatment to be used for estimation of the genes expression of IL-1β and TNF-α.

Statistical analysis

The statistical analysis was applied by using the SPSS (21) package program. Duncan's Multiple Range Test was utilized to detect the statistical differences between groups. Values were expressed as means ± standard error. Differences with $p<0.05$ were considered significant.

Results

In vitro antimicrobial activity

The inhibition zones of SP and SO against *Pseudomonas aeruginosa* were 11 and 10 mm respectively.

Non-specific and specific immune assays

The effects of supplementation with SP and SO on the non-specific and specific immune parameters of Nile tilapia are shown in Table, 3. At the end of the second and the fourth week of feeding trials, the lysozyme activities, nitric oxide assay and the titer of IgM showed a significant increase in SP and SO groups when compared with the control group. That increase noted after 2 and 4 weeks of supplementation was significant in SP group when correlated with SO group.

Supplementation of Nile tilapia infected with *P. aeruginosa* with SP and SO resulted in a significant increase in the lysozyme activities, nitric oxide assay and the titer of IgM when contrasted with the control infected group. That increase was significant in SP group than SO group, (Table, 3).

Table 3: Effect of supplementation with *Spirulina platensis* (10 gm/kg diet) and *Salvia officinalis* (7.5 gm/kg diet) for 28 days on lysozymes, nitric oxide activities and IgM level in Nile tilapia before and after infection with *pseudomonas aeruginosa*.

Time	Parameters / Groups	Lysozymes	Nitric oxide	lgM
2nd week	control	0.243± 0.001^c	43.400±.510^c	0.166±0.008^c
	sp	1.073±0.013^a	59.600±.510^a	0.972±0.001^a
	so	0.617±0.001^b	55.00±.707^b	0.588±0.001^b
4th week	control	0.327±0.007^c	46.60 ±0.510^c	0.254±0.001^c
	sp	1.693±0.001^a	66.60±0.510^a	1.552±0.001^a
	so	1.213±0.001^b	61.40± 0.510^b	1.176±0.001^b
Post infection	infected control	0.969± 0.001^c	56.60 ± 0.510^c	0.859± 0.001^c
	sp	2.569± 0.001^a	73.60± 0.510^a	2.440± 0.001^a
	so	2.153± 0.001^b	68.60± 0.510^b	2.023± 0.001^b

Values are represented as Mean ± SE (n=5/group). Means within the same column carrying different superscripts are significant at ($p<0.05$).

The IL-1β and TNF-α genes expression

As demonstrated in table, 4, the expression of IL-1β and TNF-α genes was enhanced in groups received SP and SO when compared with control group at the end of the second and the fourth week of feeding. That effect was significant in SP group than SO group.

Groups of SP and SO showed a significant increase in IL-1β and TNF-α genes expression when compared with control infected group. That increase was significant in SP group than SO group, (Table, 4).

Table 4: Effect of supplementation with *Spirulina platensis* (10 gm/kg diet) and *Salvia officinalis* (7.5 gm/kg diet) for 28 days on IL-1β and TNF-α genes expression in Nile tilapia before and after infection with *pseudomonas aeruginosa*.

Time	Parameters / Groups	Il-1β	TNF-α
2nd week	control	1.808±0.055^{c}	2.114±0.055^{c}
	sp	5.496±0.052^{a}	5.806±0.052^{a}
	so	3.618±0.055^{b}	3.928±0.055^{b}
4th week	control	1.908±0.055^{c}	2.198±0.051^{c}
	sp	6.442±0.053^{a}	6.742±0.053^{a}
	so	4.698±0.055^{b}	5.008±0.055^{b}
Post infection	infected control	.788± 0.009^{c}	1.0880±0.009^{c}
	sp	5.662±0.053^{a}	5.7720±0.167^{a}
	so	4.494±0.050^{b}	4.8040±0.050^{b}

Values are represented as Mean ± SE (n=5/group). Means within the same column carrying different superscripts are significant at ($p < 0.05$).

Clinical and post-mortem findings

Fish challenged with *P. aeruginosa* showed petechial hemorrhages and ulceration on body surface, especially at the base of fins, tail and fins rot, congested gills and abdominal distention. Internally, abdominal dropsy with congestion of internal organs was observed. Mortality rates were 17% SP group and 25% in SO group compared with 75% for the control group. The relative percentage survival (RPS) was 83% and 75% in SP and SO groups respectively.

Discussion

Spirulina is viewed as a standout amongst the most generally utilized microalgal species in aquaculture because of its high constituents of protein, essential amino acids, antioxidant pigments, essential fatty acids, carotenoids, vitamins and minerals [45].

The antimicrobial activity of SP may be attributed to its constituents from antimicrobially active lipids and fatty acids [46]. It was suggested that lipids kill microorganisms by disruption of their cell membrane [47]. The antimicrobial activity of SP observed in the current study against *P. aeruginosa* was in agreement with early observations recorded by Sudha *et al*. [48] and Pradhan *et al*. [49].

Salvia officinalis is cultivated in numerous countries for its dried leaves which are used as raw material in medicine, food-industry and perfumery [50]. It has been found that the essential oil and leaves extracts of SO have a wide range of antimicrobial effects which may be due to its particular chemical constituents [51]. The present observations revealed that the leaves of SO prevented *P. aeruginosa.* Similar findings previously reported [52].

In comparison to mammals, fish for the most part depend on innate immunity [53]. As needs be, awesome great consideration has been centered on the utilization of dietary bioactive materials to fortify innate immunity.

In fish, lysozyme is an essential antimicrobial effector that fills in as an opsonin in complement system and phagocytes initiation [54,55]. It is recognized in the blood, mucus and organs of different fish and assumes a critical bactericidal part in the non - specific defense against pathogens primarily through lytic effects on their cell membrane. By splitting glycosidic bonds between N-acetylmuramic acid and Nacetylglucosamine, it hydrolyzes the peptidoglycan layers of bacterial cell membranes [56].

Nitric oxide (NO) is bactericidal reactive oxygen that is delivered fundamentally by macrophages after incitement with an assortment of operators, for example, microbial segments and cytokines [57, 58].

In the current study, it has been observed that Nile tilapia fed on diets supplemented with SP and SO showed a significant enhancement in the lysozyme activity and a significant increase in the level of NO. The immune-modulatory activity of SP especially, associated with non-specific immune system has been suggested [59, 60]. That effect was attributed to its content of C-phycocyanin which increased the phagocytic and the natural killer activities [61]. Our results were similar to those early observed that SP enhanced the immune response in channel catfish [59] and Nile tilapia [62].

Water soluble polysaccharide as galactose, glucose, mannose, xylose, and fructose are active SO compounds that have been found to possess immune modulatory activity [63], including anti-inflammatory, anti-cancer, anti-ulcer, macrophage phagocytic stimulation and induction of cytokine as well as complement activating potency [64]. In concurrence with our findings, previous reports suggested that supplementation with SO augmented immunity in rainbow trout [65].

Antibodies (immunoglobulins) are glycoproteins that expressed in the membrane of the B lymphocyte (BCR) or free in body fluids, secreted by plasma cells (B lymphocytes activated by antigen connection) [66]. The predominant immunoglobulin in teleosts is a tetramer of the IgM class and contains eight antigenic combining sites [67]. Some teleosts have a monomer of IgM in their serum, although the factors leading to its expression are still unknown [68].

Our findings revealed that addition of SP and SO in the diet of Nile tilapia elevated the level of serum IgM. Those findings are in the same direction with those noted after SP [69] and SO [70] supplementation.

As a member of interleukin-1 (IL-1) cytokine family, Interleukin-1β (IL-1β), is a prototypical pro-inflammatory cytokine and key mediator of the body's reaction to microbial infection, immunological response and tissue damage [71].

TNF-α, a pro-inflammatory cytokine, is one of the early immune genes expressed at the beginning of infection in fish and has a key part in controlling inflammation. In fish, TNF-α shows overlapping functions with IL-1β. Numerous fish TNF-α s have been generated in bacteria as monomers, dimers and trimers and can enact macrophages/phagocytes and improve their killing activity against microorganisms [72].

Our findings showed a significant increase in the expression of IL-1β and TNF-α of Nile tilapia fed on SP and SO. Early studies recorded similar observations on SP [60, 69] and SO [73].

Concerning the effect of supplementation with SP and SO in Nile tilapia after infection with *p. aeruginosa*, the current data revealed that addition of SP and SO elicited a significant elevation in lysozyme activity, NO level and IgM titer. In addition, the expression of pro-inflammatory cytokine (IL-1β and TNF-α) was up-regulated. Those findings indicated the ability of those supplementations to protect Nile tilapia against infection with *P. aeruginosa*. Our findings are consistent with those early observed by Abu-Elala *et al*. [74]; Mahmoud *et al*., [75] on SP and Velickovic *et al*. [52] on SO.

Fish challenged with *P. aeruginosa* showed petechial hemorrhages and ulceration on body surface, especially at the base of fins, tail and fins rot, congested gills and abdominal distention. Internally, abdominal dropsy with congestion of internal organs was observed. These lesions could be attributed to the different types of toxins produced by *P. aeruginosa* [76]. Likewise, Eissa *et al*. [77]

noted irregular hemorrhages on body surface, cloudiness of eyes, scales detachment and congested gills, In addition to, sanguineous fluids in the abdominal cavity as signs of pseudomoniasis in *Oreochromis niloticus*.

Mortality rates were 17% in SP group and 25% in SO group compared with 75% for control group. The relative percentage survival (RPS) was 83% in SP group and 75% in SO group. The decline in mortality rate in groups supplemented with SP and SO may indicate their activity against *pseudomonas* infection.

Conclusion

Based on the findings of present study, it could be concluded that both of SP and SO has an immunostimulant effect and are recommended to be used as feed additives in Nile tilapia.

Conflict of interest

The authors declare that they have no conflict of interest.

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الملخص العربى

تقييم التأثير المحفز للمناعه للسبيرولينا بلاتنسيس والمريميه فى البلطى النيلى

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في الآونة الأخيرة، وجدت محفزات المناعة انها عاملا جذابا وواعدا للتحكم فى أمراض الأحياء المائية. هدفت الدراسة الحالية إلى تقييم آثار التغذية بالسبيرولينا بلاتنسيس و المريميه لمدة ٢٨ يوما على الاستجابة المناعية للبلطي النيلي (اوريكرومس نيلوتيكس). تم تقسيم عدد ١٨٠ سمكة بلطي نيلي (٣٠,٦ ± ٠,١٢ جم) إلى ٣ مجموعات (ثلاث نسخ من ٢٠ سمكة / مجموعة). تم تغذية المجموعة الضابطة على العليقة الأساسية فقط، بينما تغذت المجموعتان الأخريتان على السبيرولينا بلاتنسيس (١٠ جم / كجم عليقة) والمريميه (٧,٥ جم / كجم عليقة) على التوالي. اظهرت الاستجابة المناعية للبلطي النيلي المغذى بالسبيرولينا بلاتنسيس والمريميه زيادة معنوية في مستويات الليزوزيم و اكسيد النيتريك ومعدل الاجسام المناعيه (IgM) مع زيادة تعبير الجينات IL-1β و TNF-α قبل وبعد العدوى بالسيدوموناس إيريجينوسا. وقد أظهرت كل من السبيرولينا بلاتنسيس والمريميه النشاط المضاد للميكروبات ضد سيدوموناس أيريجينوسا في المختبر. وأظهرت الأسماك المعدية بالسيدوموناس أيريجينوسا علامات العدوى مع ارتفاع معدل النفوق وانخفاض نسبة الاعاشة التي ارتفعت إلى ٨٣٪ في مجموعة السبيرولينا و ٧٥٪ في مجموعة المريميه. يمكن أن نستخلص أن المكملات الغذائية (السبيرولينا بلاتنسيس والمريميه) تعزز الأستجابة المناعية وتحمى البلطي النيلي ضد العدوى.