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Hany M. R. Abdel-Latif
Department of Poultry and Fish diseases, Faculty of Veterinary Medicine, Alexandria University, Egypt.

Riad H. Khalil
Department of Poultry and Fish diseases, Faculty of Veterinary Medicine, Alexandria University, Egypt.

Correspondence:
Hany M. R. Abdel-Latif
Department of Poultry and Fish diseases, Faculty of Veterinary Medicine, Alexandria University, Edfina, Behera, P.O. Box: 22758, Egypt.

Evaluation of two Phytobiotics, *Spirulina platensis* and *Origanum vulgare* extract on Growth, Serum antioxidant activities and Resistance of Nile tilapia (*Oreochromis niloticus*) to pathogenic *Vibrio alginolyticus*

Hany M. R. Abdel-Latif, Riad H. Khalil

Abstract
Fodder additives using medicinal plants or plants extracts can be considered as a novel trend for control of fish diseases and hoping to achieve the same results as in the use of antibiotics and to overcome the problem of antibiotic resistance. The aim of our research is mainly focused to evaluate two different herbal additives on growth performance, antioxidant and immune potentiating effects. Ropadiar powder plus® (Ropadiar) (Oregano essential oil) and Jade Spirulina® (***Spirulina platensis***) were evaluated separately and used as feed additives for cultured Nile tilapia whereas; a total of six treatments, i.e., negative control group (Fed on basal diet without herbs), three groups fed on Spirulina diets (2.5 %, 5% and 10%) and others fed on Ropadiar diets (5 % and 10%) to investigate their effects on the growth performance, body composition and serum antioxidant activity. After eight weeks of feeding, all fish were experimentally challenged with pathogenic ***Vibrio alginolyticus*** and mortalities were daily recorded for six days post infection. Results showed that fish fed on 10% Ropadiar diet significantly enhanced the growth performance, promoted the deposition of muscle protein and enhanced serum antioxidant activities of treated fish. Moreover, all fish groups fed on diet with 10 % Spirulina and both concentrations of Ropadiar reduced the cumulative mortality percentage (%) following infection, but the lowest mortality rate was observed in the group fed with 10 % Ropadiar diet. It can be concluded that both tested Phytobiotics have an ideal growth promoting and immune enhancing effects for cultured Nile tilapia and they can successfully replace the addition of antibiotics in fish diets.

Keywords: Nile tilapia - Jade Spirulina - Ropadiar powder plus - Growth performance - Antioxidant activity - Disease resistance.

1. Introduction

Phytobiotics can be described generally as a term applied for algae or aromatic plants and essential substances or oils extracted from them, which help to increase the growth performance and have antimicrobial activity to replace the addition of antibiotics in feed of animals. Blue green algae, *Spirulina platensis* (*Sp. platensis*) can be attributed to the group of phytobiotics as it can be used as a food supplement [1] as it is a rich source of nutrients such as vitamins, minerals, carbohydrates, and γ-linolenic acid [2] and a good source of protein [3]; help in the development of potential pharmaceuticals [4] and finally has antioxidant properties [5]. Studies indicated the usefulness of Spirulina for partial or complete replacement of fish meal in the diets of two Indian major carps, Catla (*Catla catla*) and rohu, (*Labeo rohita*) [3] and up to 40% of the fish meal protein in Nile tilapia diets [6]. The dietary Spirulina has immune potentiating effects on the Carp (*Cyprinus carpio* L.) [7] and Nile tilapia, *Oreochromis niloticus* (*O. niloticus*)[8]. Oregano essential oil (OEO) that extracted from *O. heracleoticum* L. plants are characterized by a high phenolic content (Carvacrol and thymol comprising 78.27% of the total oil) and two monoterpene hydrocarbons, γ-terpinene and ρ-cymene (5.54 and 7.35% of the total oil, respectively) [9]. The dietary Oregano oil improved not only the growth performance, muscle protein content and feed utilization but also the survival rates and disease resistance of shrimp [10], Nile tilapia fingerlings [11]; Channel catfish (*Ictalurus punctatus*)[12]; Common carp (*Cyprinus carpio L.*) and farmed gilthead seabream (*Sparus aurata*)[13]. In this study, cultured *O. niloticus* groups were fed on a diet containing different concentrations of Jade Spirulina® and

Ropadiar powder plus® (abbreviated as "Ropadiar") and the growth performance parameters, effects on serum enzymatic activities and resistance against *V. alginolyticus* infection to determine the possibilities to be alternatives for antibiotics addition in fish diets.

2. Materials and methods

2.1. Fish and culture conditions

A total number of 120 apparently healthy *O. niloticus*, with average body weight of (50 ± 5 g / fish) were obtained from Barseek fish farm at Behera Governorate and transported a live to the laboratory of the department of poultry and fish diseases, Faculty of veterinary medicine, Alexandria University in large plastic bags containing water enriched by oxygen (2/3). All fish were placed in aquaria and left acclimated for 2 weeks prior to the experiments. After that, the fish were divided into six aquaria (20 fish per each). Experiments were conducted in prepared glass aquaria (90 x 50 x 35 Cm), supplied with chlorine free tap water [14].

The continuous aeration was maintained in each aquarium using an electric air pumping compressor. Settled fish wastes were cleaned daily by siphoned with three quarters of the aquarium's water, which was replaced by aerated water from the water storage tank. Water temperature was kept at 22 ± 1 °C and pH 8.5 throughout whole experiments.

2.2. Tested phytobiotics

2.2.1. Jade Spirulina algae®: It is a high-quality spray-dried form of the blue green algae, Sp. platensis. This is the commercial product of Salt Creek, Inc. 3528 West 500 South Salt Lake City, UT 84104 USA.

2.2.2. Ropadiar Powder plus®: Which is composed of Wheat fiber, ethereal oil (Origanum oil) that is extracted from Origanum heracleoticum L. This is the commercial product of Ropapharm International, Ronde Tocht 48 1507 CK Zaandam - Netherlands.

Both concentrations of Ropadiar Powder plus® (5 % and 10 %) were used in this study.

2.3. Experimental design and diets

Six diets were formulated to be isocaloric (2.98 kcal / kg diet) and isonitrogenous (25 % crude protein). All ingredients were finely ground, mixed in a Hobart mixer and pelleted through a 2.4 mm diameter diet in a Hobart meat grinder. The pellets were air-dried at room temperature, broken into small pieces and stored in a freezer until used. Ingredients and proximate composition of the experimental diets without adding any of the tested phytobiotics were presented in Table (1).

Table 1: Formulation (%) of the basal diet without addition of any medicines and herbs.

Ingredients	%
Fish meal (72%)	27
Soybean meal (44%)	23
Ground yellow corn	35
Wheat bran	10
Binders *	2
Mineral premix (a)	1.5
Vitamin premix (b)	1.5

* Binders: Sodium carboxy methyl cellulose (high viscosity) for increasing the pelleting ability [15]

(a) Provided the following vitamins (mg kg^{-1} diet unless otherwise stated): vitamin A, 4000 IU; vitamin D3, 2000 IU; vitamin K, 10; vitamin E, 50; thiamine, 10; riboflavin, 12; pyridoxine, 10; pantothenic acid, 32; nicotinic acid, 80; folic acid, 2; biotin, 0.2; vitamin B12, 0.01; L-ascorbyl-2-polyphosphate (25% vitamin C activity), 60.

(b) Provided the following minerals (mg kg^{-1} diet): zinc (as $ZnSO_4 \cdot 7H_2O$), 150; iron (as $FeSO_4 \cdot 7H_2O$), 40; manganese (as $MnSO_4 \cdot H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as KI), 5; cobalt (as $CoCl_2 \cdot 6H_2O$), 0.05; selenium (as Na_2SeO_3), 0.0.

The addition of the various substances in each group was in amounts as following:-

Group 1 (Con):- Control group without medicines and herbs;

Group 2 (Sp. 2.5 %):- 2.5 % Jade Spirulina® diet;

Group 3 (Sp. 5 %):- 5% Jade Spirulina® diet;

Group 4 (Sp. 10 %):- 10 % Jade Spirulina® diet;

Group 5 (Ropadiar 5%):- 5% Ropadiar Powder plus® diet and

Group 6 (Ropadiar 10%):- 10% Ropadiar Powder plus® diet.

The first four experimental diets used; diet + 0 % algae, diet + 2.5 % Spirulina, diet + 5 % Spirulina and diet + 10% Spirulina respectively according to [8]. The feeding trial lasted eight (8) weeks and the diet was daily provided at a fixed feeding ratio of 3% of body weight of fish [16]. The quantity of feed related to fish weight was adjusted through weekly weighing at early morning before feeding. The daily amount of food was offered as two equal meals / day on two occasions over the day (at 9 am and 12 pm).

2.4. Sample collection and analytical methods

2.4.1. Growth performance parameters

After four (4) weeks from the start of the experiment, Fish in each group were weekly weighted and the growth performance parameters were calculated [17] as follows:-

Weight gain (WG %) = 100 × (final bodyweight − initial bodyweight) / initial body weight.

Specific growth ratio (SGR) = 100 × (final weight / initial weight) / days of the experiment

Feed conversion ratio (FCR) = feed consumed (g, dry weight) / weight gain (g)

Protein efficiency ratio (PER) = weight gain (g) / protein intake (g)

2.4.2. Proximate analysis of the muscle composition of treated fish: At the end of the feeding trial, dorsal musculature of fish were sampled, sealed in plastic bags and stored frozen (-20 °C) until analysis for muscle composition [18]. Crude protein (N × 6.25) was determined by the Kjeldahl method after acid digestion using an Auto Kjeldahl System (1030-Auto-analyzer, Tecator, Hoganos, Sweden); Crude lipid was determined by the ether-extraction method using a Soxtec System HT (Soxtec System HT6, Tecator, Sweden); Moisture was determined by oven drying at 105 °C until a constant weight was achieved and finally ash content was measured after placing the samples in a muffle furnace at 550 °C for 24 hours.

2.4.3. Antioxidant activity measurements

After four (4) weeks from the start of the experiment, Citrated blood samples (6 fish / group) were collected weekly from the caudal vessels after the end of feeding trial. Individual fish were sampled only to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. Samples were centrifuged at 3000 rpm for 30 minutes and plasma was separated and stored at −20 °C for future analysis. Kits used depend on the Quantitative Colorimetric Determination methods (Bioassay Systems; Solutions for Research and Drug discovery); Plasma Superoxide Dismutase (SOD) activity was determined using EnzyChrom™ Kit (ESOD-100) [19]; Plasma Catalase (CAT) activity was determined using EnzyChrom™ kit (ECAT-100) [20] and Plasma Glutathione Peroxidase (GPX) activity was determined using EnzyChrom™ Kit (EGPX-100) [21].

2.4.4. Challenge test with *V. alginolyticus*

At the end of the feeding trial, a challenge test was performed on each group with *V. alginolyticus* that was isolated in previous work [22]. Bacteria were inoculated into 10 ml of liquid Trypticase soy broth (TSB, Sigma) and were grown overnight at 28 °C. Cultures were centrifuged at 1000 rpm for 10 minutes. Supernatant was removed and the pelleted bacteria were washed twice in sterile phosphate buffered saline (PBS) solution. The concentration of bacteria was adjusted to McFarland standard no. 2 (5×10^6 CFUs ml $^{-1}$) by the optical density of suspension. About 0.1 ml of suspended bacteria was injected intraperitoneally of fish [23]. Mortality was recorded for 6 days following infection to estimate the cumulative mortality percentage (%).

2.4.5. Statistical analysis

The statistical analysis was made using Analysis of Variance (ANOVA) for detection the differences among different treatments used in this study, also when significant differences occurred, the group means were further compared with (Chi^2) test according to [24].

3. Results

3.1. Growth performance parameters

Data on the growth performance of *O. niloticus*, including weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) are shown in Table (2). Weight gain (WG) of fish fed on the 10% Ropadiar diet was significantly higher than other groups; Specific growth rate (SGR) of fish fed on the 5% and 10% Ropadiar diets was significantly higher than other groups and similarly FCR value ($P < 0.01$). Lowest FCR and highest PER values were observed for fish fed on the 10% Ropadiar diet compared to fish fed on the other five diets ($P < 0.01$).

Table 2: Weight gain (WG), Specific growth rate (SGR), Feed conversion ratio (FCR) and Protein efficiency ratio (PER) of *O. niloticus* fed different diets with feed additives.

	Cont.	2.5% Sp.	5% Sp.	10% Sp.	5% Ropadiar	10% Ropadiar
WG	0.10 ± 0.01 ^{A}	0.13 ± 0.01 ^{E}	0.14 ± 0.01 ^{E}	0.27 ± 0.01 ^{D}	0.58 ± 0.05 ^{C}	0.67 ± 0.01 ^{B}
SGR	0.04 ± 0.001 ^{E}	0.10 ± 0.01 ^{D}	0.12 ± 0.01 ^{D}	0.16 ± 0.01 ^{C}	0.25 ± 0.02 ^{B}	0.31 ± 0.01 ^{A}
FCR	10.33 ± 1.55 ^{A}	7.17 ± 1.17 ^{B}	6.83 ± 1.61 ^{C}	3.60 ± 0.13 ^{D}	1.70 ± 0.01 ^{E}	1.67 ± 0.11 ^{E}
PER	0.48 ± 0.14 ^{E}	0.70 ± 0.11 ^{D}	0.73 ± 0.12 ^{D}	1.39 ± 0.11 ^{C}	2.93 ± 0.12 ^{B}	3.00 ± 0.11 ^{A}

Capital liters: Indicated that means within the same row of different litters are significantly different at ($P < 0.01$).

3.2. Moisture, crude protein, crude lipid and ash of dorsal muscle samples:

In terms of muscle composition, no significant differences among treatments for moisture, ash and lipid content, but the muscle protein content was affected by dietary Ropadiar and Spirulina diets Table (3). The protein content in muscle of fish fed (5 % and 10 %) Ropadiar and 10 Spirulina diets were the highest ($P < 0.01$).

Table 3: Moisture (%), crude protein (%), crude lipid (%) and ash (%) of dorsal muscle of *O. niloticus* treated groups

	Cont.	2.5% Sp.	5% Sp.	10% Sp.	5% Ropadiar	10% Ropadiar
Moisture	72.13 ± 7.12 ^{A}	71.03 ± 7.13 ^{A}	72.31 ± 7.14 ^{A}	72.46 ± 7.44 ^{A}	72.03 ± 7.23 ^{A}	71.90 ± 7.20 ^{A}
Protein	25.20 ± 5.12 ^{B}	25.66 ± 5.44 ^{B}	26.43 ± 5.48 ^{AB}	26.61 ± 6.45 ^{AB}	27.11 ± 7.11 ^{A}	28.32 ± 7.18 ^{A}
Lipid	8.37 ± 1.82 ^{A}	8.71 ± 1.87 ^{A}	8.48 ± 1.48 ^{A}	9.53 ± 1.45 ^{A}	8.88 ± 1.48 ^{A}	9.46 ± 1.49 ^{A}
Ash	1.66 ± 0.55 ^{B}	1.87 ± 0.77 ^{AB}	1.81 ± 0.76 ^{AB}	2.10 ± 0.44 ^{A}	1.72 ± 0.73 ^{B}	1.78 ± 0.77 ^{B}

Capital liters: Indicated that means within the same row of different litters are significantly different at ($P < 0.01$).

3.3. Antioxidant activity measurements
Antioxidant activity was measured by Superoxide dismutase (SOD) Table (4); Glutathione peroxidase (GSH-PX) Table (5) and Catalase (CAT) activity Table (6). All data were increased weekly with the increase the period of feeding of both Spirulina and Ropadiar diets in comparison with control diet. Furthermore, among all treated groups, SOD, GSH-PX and CAT activities in plasma of fish fed with the 10% Ropadiar diet were significantly higher than fish from the other four treatments (P < 0.01).

Table 4: Effects of *Sp. platensis* and Ropadiar on Superoxide dismutase (SOD) enzyme activity (U/L) in serum of cultured *O. niloticus* among different weeks.

	Cont.	2.5 % Sp.	5% Sp.	10% Sp.	5% Ropadiar	10% Ropadiar
1st wk	6.01 ± 1.01 Ac	6.58 ± 1.03 Cc	18.75 ± 3.75 Cb	19.65 ± 1.57 Cb	20.18 ± 2.19 Cb	42.29 ± 3.28 Ca
2nd wk	6.02 ± 1.02 Af	9.820 ± 1.55 Be	32.23 ± 3.45 Bc	15.35 ± 3.75 Dd	37.32 ± 3.17 Bb	45.57 ± 4.25 Ba
3rd wk	6.06 ± 1.02 Ae	12.45 ± 2.55 Ad	39.79 ± 4.56 Ab	23.29 ± 4.55 Bc	38.40 ± 3.19 Bb	85.60 ± 5.55 Aa
4th wk	6.86 ± 1.03 Ad	13.57 ± 2.67 Ac	40.24 ± 4.57 Ab	42.54 ± 4.59 Ab	42.30 ± 4.19 Ab	86.70 ± 5.77 Aa

Capital liters: Indicated that means within the same column of different litters are significantly different at (P < 0.01).
Small litters: Indicated that means within the same raw of different litters are significantly different at (P < 0.01).

Table 5: Effects of *Sp. platensis* and Ropadiar on Glutathione peroxidase (GSH-PX) enzyme (µg/dl) activity in serum of cultured *O. niloticus* among different weeks.

	Cont.	2.5% Sp.	5% Sp.	10% Sp.	5% Ropadiar	10% Ropadiar
1st wk	0.32 ± 0.03 Cc	0.21 ± 0.02 Dd	0.18 ± 0.01 Dd	0.55 ± 0.01 Cb	0.60 ± 0.03 Cb	1.44 ± 0.04 Ba
2nd wk	0.32 ± 0.03 Cd	0.50 ± 0.03 Cc	0.39 ± 0.03 Cd	0.58 ± 0.03 Cc	1.08 ± 0.02 Bb	1.58 ± 0.05 Ba
3rd wk	0.37 ± 0.04 Be	0.73 ± 0.04 Bc	0.63 ± 0.03 Bd	0.75 ± 0.04 Bc	1.16 ± 0.03 Bb	1.86 ± 0.05 Ba
4th wk	0.43 ± 0.04 Ad	0.81 ± 0.04 Ac	0.86 ± 0.04 Ac	0.88 ± 0.04 Ac	1.93 ± 0.03 Ab	2.09 ± 0.03 Aa

Capital liters: Indicated that means within the same column of different litters are significantly different at (P < 0.01).
Small litters: Indicated that means within the same raw of different litters are significantly different at (P < 0.01).

Table 6: Effects of *Sp. platensis* and Ropadiar on Catalase (CAT) enzyme (µg/dl) activities in serum of cultured *O. niloticus* among different weeks.

	Cont.	2.5% Sp.	5% Sp.	10% Sp.	5% Ropadiar	10% Ropadiar
1st wk	11.32 ± 1.33 Dd	16.48 ± 1.48 Dc	16.95 ± 1.95 Dc	16.12 ± 1.62 Cc	19.50 ± 1.95 Db	29.45 ± 2.30 Ca
2nd wk	15.20 ± 1.55 Cd	19.67 ± 1.66 Cc	18.87 ± 1.88 Cc	19.87 ± 1.89 Bc	22.15 ± 2.24 Cb	33.25 ± 3.34 Ba
3rd wk	20.83 ± 2.50 Ad	21.23 ± 2.44 Bc	22.83 ± 2.88 Bc	21.13 ± 2.22 Ac	24.12 ± 2.25 Bb	30.83 ± 3.30 Ca
4th wk	18.52 ± 1.52 Bd	22.30 ± 2.22 Ac	23.68 ± 2.68 Ac	22.23 ± 2.23 Ac	27.30 ± 2.29 Ab	35.10 ± 3.35 Aa

Capital liters: Indicated that means within the same column of different litters are significantly different at (P < 0.01).
Small litters: Indicated that means within the same raw of different litters are significantly different at (P < 0.01).

3.4. Challenge test with *V. alginolyticus*
After 8 weeks of feeding, fish were challenged with *V. alginolyticus* and cumulative mortality % was recorded for 6 days Figure (1). All treated groups as 2.5% Sp. diet, 5% Sp. diet, 10% Sp. diet, 5% Ropadiar diet and the 10% Ropadiar diet showed reduced mortalities compared to the Cont. diet by 25%, 45%, 55%, 60% and 70% respectively.

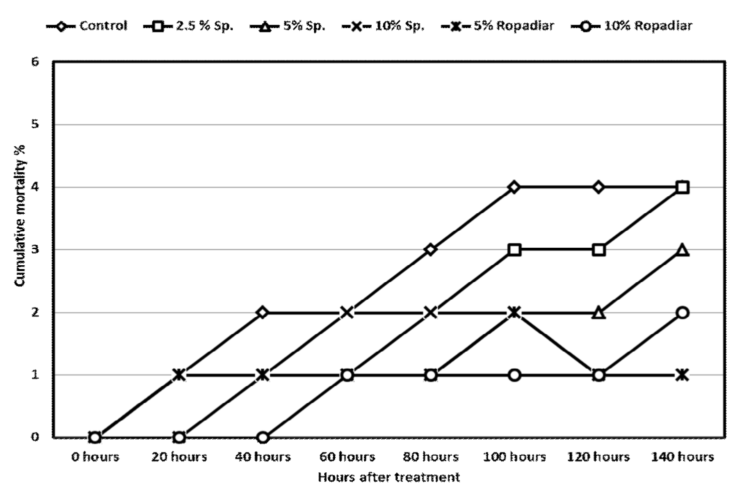

Fig 1: Cumulative mortality (%) of fish in control group and in groups fed diets containing different concentrations of Spirulina (2.5% Sp., 5% Sp. & 10% Sp.) and Ropadiar (5% Ropadiar and 10% Ropadiar) throughout the 140 hours post infection.

4. Discussion

Public awareness had currently focused on the wide spread of fish diseases and the importance of immunostimulants in their prevention in order to prevent their possible transmission to the human beings and to decrease their economic losses from fish [25]. The results of growth performance parameters were similar to that of Orego-stim® (a product containing natural OEO) that improve WG, FCR and PER of channel catfish [12] and this may be attributed to its distinctive aromatic flavor makes it a strong appetizer. Also, results of 10% Spirulina diet improved SGR and WG of for carp fry using six different species of carp: *Catla catla, Labeo rohita, Cirrhinus mrigala, Hypophthalmichthys molitrix* (Silver carp), *Ctenopharyngodon idella* (grass carp) and *Cyprinus carpio* (Common carp) [26], hybrid catfish, *Clarias macrocephalus x Clarias gariepinus* [27] and *O. niloticus* [8]. Other results indicated that Spirulina diet improved the growth rates for striped jack, *Pseudocaranx dentex* at 5% supplementation in the feed [28]. These results may be attributed to that the dietary inclusion of Spirulina leads to improve the protein digestibility [29]; activated protein synthesis and significantly increased collagen content of intramuscular connective tissue [30] and this indicated that both Spirulina and Ropadiar diets improved the protein retention in musculature. The serum antioxidant activities were enhanced in Spirulina diets due to the presence of phycobiliproteins such as C-phycocyanin (CP) and allophycocyanin [5] while in Ropadiar diets due to both Carvacrol and thymol have excellent antioxidant properties [31]. Both Spirulina and Ropadiar diets reduced the mortality of experimentally infected *O. niloticus* and these results may be attributed to the inhibition of growth of different microorganisms by Carvacrol and thymol [32] through disintegration of the membrane of the bacteria, leading to the release of membrane-associated material from the cells to the external medium. Moreover, terpenoids and phenylpropanoids can penetrate the membrane of the bacteria and reach the inner parts of the cell because of their lipophilicity [33] and also their aromaticity [34] which is responsible for their antibacterial activity. While the results of Jade Spirulina® in immune enhancing properties may be due to the fact that antibodies produced through the feeding [35].

5. Conclusions

Finally, it can be concluded that the usage of either Jade Spirulina® or Ropadiar powder plus® can beneficially enhance growth performance parameters, muscle protein composition, serum antioxidant activity and immune status of *O. niloticus*; and both concentrations of Ropadiar powder plus® (5% and 10%) in fish diet achieved the best results in raising the immune response of O. niloticus towards bacterial infections and this can be considered as a good candidate to be alternative the use of antibiotics in feed.

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