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Research Article
Effect of a Phytogenic Feed Additive on the Growth Performance and Susceptibility of *Oreochromis niloticus* to *Aeromonas hydrophila*

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Abstract
Background and Objective: Phytogenic feed additives which include essential oils, can be applied in aquaculture for improving the growth performance and immune defense mechanisms of aquatic animals. This study aimed to investigate the effect of a phytogenic feed additive on the growth performance and susceptibility of *Oreochromis niloticus* to *Aeromonas hydrophila*. **Methodology:** Nile tilapia (0.89 g fish^{-1}) were fed basal diet (29.7% crude protein kg^{-1}, 4425 kcal kg^{-1} gross energy) supplemented with different concentrations (0, 0.5, 1.0 and1.5 g kg^{-1} diet) of the phytogenic feed additive (Silaacid® Encapsulated). Fish (240 all-male) were randomly distributed in triplicate into 4 treatment groups. Treatments were performed in 12 aquaria (20 fish per aquarium). Fish were fed their respective diets twice a day for 8 weeks at 8% of their body weight for the first 2 weeks then 6% for 2 weeks, finally 3% for the last 4 weeks. At the end of feeding trial, fish were challenged with pathogenic *Aeromonas hydrophila* by intraperitoneal injection. **Results:** The optimum growth performance, feed utilization and resistance against *A. hydrophila* were obtained at 0.5 g phytogenic feed additive (Silaacid® Encapsulated) kg^{-1} diet. **Conclusion:** Phytogenic feed additives were promising for enhancing the fish growth performance and protection against *A. hydrophila* infections in Nile tilapia which may improve fish production.

Key words: Phytogenic feed additive, Nile tilapia, growth performance, resistance, *Aeromonas hydrophila*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diversification and intensification of aquaculture operations has become the global trend of commercial aquaculture[1]. Intensification of culture practice is usually coupled with poor managemental conditions because of the overcrowding and overfeeding applied to increase productivity per unit area[2,3]. Aquatic animals reared in these conditions experience high magnitude of stress, leading to decreased performance and subsequently compromised immune defense mechanisms, ultimately leave fish prone to numerous opportunistic pathogens[4,5]. Nevertheless, the stressful conditions associated with the intensive aquaculture practice is likely to continue in many parts of the world with the need to cover the growing global animal protein demand as well as the amplified pressure on aquaculturists to reduce production cost without affecting the cost to the final consumers[6]. Hence, the production of high quality feed that improve growth performance as well as enhance the fish immune competences could be of benefit to intensive aquaculture operations[7,8]. It is well established that the long-term administration of antibiotics in aquafeeds either for treatment or as growth promoters, creates the most favorable environment to enable antibiotic resistance genes to multiply[9]. The treated animals become large reservoirs for the production and spreading of antibiotic-resistant bacteria in their cohabitant environment. Plentiful variety of natural growth promoters, including plant extracts, prebiotics, probiotics and organic acids, has been broadly applied worldwide with reasonable success[10]. Phytogenic feed additives have been addressed on the top list of the natural substances intended for improving the growth performance and immune defense mechanisms of aquatic animals[11,12]. Phytogenic compounds are natural plant-derived bioactive compounds with beneficial effects on growth performance and health of farmed animals[13]. Some phytogenic compounds are recognized to have antiviral, antimicrobial, antifungal, anti-inflammatory and anti-oxidative activities[14]. Traditionally, phytogenic compounds have been used as alternative or complementary medicines to improve human health or cure human diseases[15]. Some scientific publications have tackled the immune stimulatory and antimicrobial effects of plant extracts against microbial infections affecting fish[1]. Phytogenic feed additives have been investigated in channel catfish[16] as well as rainbow trout[17], interestingly, their results demonstrated the improvements in weight gain, Food Conversion Ratio (FCR) and resistance to disease. However, field application of phytogenic derived compounds in aquaculture is still far from achieving the desired objectives.

Therefore, the present study, was planned to assess the effect of a phytogenic feed additive (Silaacid® Encapsulated) on the growth performance, feed utilization, body composition and survival of Nile tilapia, *Oreochromis niloticus,* fingerlings challenged with pathogenic *Aeromonas hydrophila*. It will strengthen the benefits of the field application of phytogenic feed additives in fish farming operations as alternative to antibiotics to comate microbial infections and to improve growth performance of cultured aquatic animals.

MATERIALS AND METHODS

Fish and culture facilities: Monosex, all male, Nile tilapia fingerlings (0.89 g) were obtained from a commercial tilapia hatchery at Kafr El Sheikh Governorate, Egypt. Fish were randomly distributed in triplicate into 4 treatments groups in the Laboratory of Fish Nutrition, National Research Centre, Egypt. Treatments were performed in 12 glass aquaria ($60\times30\times40$ cm^3) at a rate of 20 fish per aquarium. The experimental fish were acclimated to the culture system for 2 weeks. Initially, 50 fish were randomly collected, group weighed and the average initial weights were recorded then frozen at -20°C for chemical analysis. Water temperature, dissolved oxygen and pH were adjusted around 26.5°C, 6 and 7.5 mg L^{-1}, respectively in all treatments and monitored daily.

Test diets and feeding regime: A basal diet was formulated to contain (29.73% crude protein, 6.67% ether extract, 3.53% crude fibers, 3.94% ash and 4425 kcal kg^{-1} gross energy. A commercial phytogenic feed additive (Silaacid® Encapsulated) containing oregano oils (Carvarcol 75 g kg^{-1}, thymol 25 g kg^{-1}); acidifier; 100% fumaric acid (250 g kg^{-1}); 68% sodium formate (250 g kg^{-1}); 78% calcium propionate (90 g kg^{-1}); potassium sorbate (10 g kg^{-1}) encapsulated with palm oil (200 g kg^{-1}) and soya oil (100 g kg^{-1}), were added to the basal diet to represent the levels of 0.0 (control), 0.5, 1.0 and 1. 5 g kg^{-1} diet. Fish were fed their respective diets twice a day (at 8 am and 13 pm) for 8 weeks at 8% of their body weight for the first 2 weeks then 6% for 2 weeks, finally 3% for the last 4 weeks. The average weight of fish was recorded every 15 day intervals and the daily rations were readjusted accordingly.

Chemical analysis of diets and fish: The tested diet and 15 fish collected from each treatment at the beginning and at the end of feeding experiment were analyzed for moisture

Table 1: Growth performance parameters of Nile tilapia fed the tested diets

Treatments (g kg^{-1})	Initial weight (g)	Final weight (g)	Weight gain (g)	Specific growth rate
0 (Control)	0.90	2.36±0.07^{b*}	1.46±0.01^{b}	1.61±0.08^{b}
0.5	0.90	2.48±0.06^{a}	1.58±0.03^{a}	1.70±0.02^{a}
1.0	0.88	2.34±0.07^{b}	1.45±0.01^{b}	1.61±0.07^{b}
1.5	0.89	2.11±0.04^{c}	1.23±0.03^{c}	1.44±0.03^{c}

*Values in the same column with different superscripts are significantly different at p<0.05, values are given as Meant±SE

Table 2: Feed utilization parameters of Nile tilapia fed tested diets

Treatments (g kg^{-1} diet)	Feed intake (g)	FCR	PER	PPV	ER
0	3.22±0.07^{ab*}	2.21±0.08^{ab}	1.52±0.05^{ab}	26.62±1.98^{b}	19.18±1.47^{b}
0.5	3.29±0.06^{a}	2.08±0.08^{b}	1.62±0.06^{a}	27.06±0.57^{a}	19.76±0.56^{a}
1.0	3.27±0.07^{a}	2.24±0.07^{ab}	1.50±0.05^{ab}	25.98±1.10^{b}	18.95±0.68^{b}
1.5	3.02±0.04^{b}	2.46±0.10^{a}	1.37±0.06^{b}	22.58±1.75^{c}	16.33±1.09^{c}

*Values in the same column with different superscripts are significantly different at p<0.05, FCR: Feed conversion ratio, PER: Protein efficiency ratio, PPV: Protein productive value, ER: Energy Retention, values are given as Meant±SE

content, protein, fat and ash according to the standard methods of AOAC[18].

Calculations of fish performance: The growth performance and feed utilization efficiency were calculated as following:

$$\text{Weight Gain (WG)} = \text{Final weight-Initial weight} \quad (1)$$

$$\text{Specific Growth Rate (SGR)} = \frac{\ln W_2 - \ln W_1}{T} \times 100 \quad (2)$$

where, W_1 and W_2 are the initial and final weight, respectively, ln represent Natural logarithm and T is the number of days in the feeding period.

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Dry feed intake (g)}}{\text{Fish live weight gain (g)}} \quad (3)$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}} \times 100 \quad (4)$$

$$\text{Protein Productive Value (PPV)} = \frac{\text{Protein gain (g)}}{\text{Protein fed (g)}} \times 100 \quad (5)$$

$$\text{Energy Retention (ER)} = \frac{\text{Retained energy in carcass (kcal)}}{\text{Energy intake (kcal)}} \times 100 \quad (6)$$

Challenge with *Aeromonas hydrophila*: Virulent *A. hydrophila* strain previously isolated from mortalities affecting *Oreochromis niloticus*[19] were obtained, inoculated onto a blood agar plate and incubated at 25°C overnight. Bacterial culture was adjusted to 1×10^7 CFU mL^{-1} in Phosphate Buffer Saline (PBS). At the end of feeding experiment, 20 fish were randomly collected from each experimental group then divided into 2 groups each of 10 fish. The first group was i.p. (intraperitoneally) injected with 0.2 mL PBS containing 1×10^7 CFU mL^{-1} live virulent *Aeromonas hydrophila* (*A. hydrophila*) according to Elala *et al.*[20]. The second group was i.p. injected with 0.2 mL of saline solution as a control. All fish were kept under observation for 10 days to record the daily mortalities and abnormal clinical signs. Mortalities were only considered with the re-isolation of the injected bacterial isolate from dead fish on the specific *Aeromonas* isolation agar (Oxoid) and confirmation of retrieved isolates phenotypic and PCR according to Buller[21].

Statistical analysis: All data were subjected to one-way analysis of variance (ANOVA) at a 95% confidence limit, using SPSS software, version 16[22]. Duncan's Multiple Range[23] test was used to compare means when F-values from the ANOVA were significant (p<0.05).

RESULTS

Growth performance of Nile tilapia: Average values of initial weight, final body weight, weight gain and specific growth rate of Nile tilapia fingerlings fed different levels of commercial phytogenic feed additive (Silaacid® Encapsulated) are show, in Table 1. The initial weight was nearly similar in all treatment groups with no significant differences (p>0.05). The optimum growth performance was obtained significantly (p<0.05) at 0.5 g kg^{-1} diet; (2.48, 1.58 and 1.70 g); followed by 1 g kg^{-1} (2.34, 1.45 and 1.61 g) with no significant difference with control group (2.36, 1.46, 1.61) for final Fish Weight (FW), Weight Gain (WG) and Specific Growth Rate (SGR), respectively. Interestingly, 1.5 g kg^{-1} phytogenic feed additive in fish diets did not enhance the growth performance parameters and produced the lowest fish growth performance parameters; (2.11, 1.23 and 1.44 g) for the same parameters respectively.

Feed utilization: Feed and protein utilization parameters expressed as Feed Intake (FI), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), Protein Productive Value (PPV) and Energy Retention (ER) are given in Table 2.

Table 3: Body composition on dry matter basis of Nile tilapia fed tested diets

Treatments	DM	Crude protein	Ether extract	Ash
Initial	16.88	52.27	19.91	16.02
0 g kg^{-1}	24.41±0.65	57.95±0.55	28.96±0.52	9.73±0.08
0.5 g kg^{-1}	24.28±0.85	57.27±0.90	29.38±1.16	10.16±0.39
1.0 g kg^{-1}	24.63±0.28	57.23±0.68	29.23±0.20	9.73±0.44
1.5 g kg^{-1}	23.36±0.68	56.73±1.05	28.20±0.16	10.58±0.36

Values are given as Meant±ES, DM: Dry matter

Table 4: Mortality patterns of *O. niloticus* experimentally infected with *A. hydrophila*

Treatments	No of mortality day^{-1}										
(g kg^{-1})	1	2	3	4	5	6	7	8	9	10	Total %
0.0	-	-	-	4	3	3	-	-	-	-	100
0.5	-	-	-	-	1	1	-	-	-	-	20
1.0	-	-	-	-	2	1	1	-	-	-	40
1.5	-	-	-	-	1	2	2	-	-	-	50

The results clearly demonstrated the enhancing effect of phytogenic feed additive on the feed utilization. There were significant difference ($p<0.05$) among the different treatments in FI and FCR. The highest FI and best FCR values were observed in the group fed on 0.5 g kg^{-1} phytogenic feed additive; 3.29, 2.08 followed by 1 g kg^{-1}; 3.27, 2.24 with no significant differences with the control group fed the basal diet 3.22 and 2.21, respectively. The lowest values were noticed in the group fed 1.5 g kg^{-1}; 3.02 and 2.46, respectively.

Results indicated a significant differences ($p< 0.05$) in PER between fish fed diet supplemented with the 0.5 g kg^{-1} phytogenic feed additive (1.62) and that fed with 1.0 g kg^{-1}; control group (1.50 and 1.52), respectively. On the other hand, fish fed diets supplemented with 1.5 g kg^{-1} Silaacid® Encapsulated exhibited the lowest PER; 1.37. Similarly, PPV were significantly different between fish fed with 1.5 g kg^{-1} Silaacid® Encapsulated and the other groups (Control, 0.5 and 1 g kg^{-1} diet). The highest PPV values were recorded in fish fed with 0.5 g kg^{-1} Silaacid® Encapsulated, 27.06 and the lowest, 22.58 were noticed in fish fed with 1.5 g kg^{-1}. Additionally, the highest energy retention value, 19.76 was recorded in fish group fed with 0.5 g kg^{-1}concentration while the lowest, 16.33 was noticed with feeding phytogenic feed additive at 1.5 g kg^{-1}. Results of body composition are given in Table 3. No significant difference ($p>0.05$) was noticed between all treatments in Dry Matter (DM), Crude Protein (CP), Ether Extract (EE) or ash content.

Challenge with *Aeromonas hydrophila*: Fish survivability after i.p. injection with *A. hydrophila* increased in all groups fed with diets supplemented with the commercial phytogenic compound. The lowest cumulative mortality (20%) was noticed in fish fed on 0.5 g kg^{-1} concentration followed by 1.0 g kg^{-1} (40%) then 1.5 g kg^{-1} (50%). On the other hand, the highest cumulative mortality (100%) was noticed in the control group fed with the basal diet without phytogenic supplementation. Majority of fish died without exhibiting any clinical signs. Others showed petechial hemorrhages widely distributed on different parts of the external body surfaces. Internally, congestion of liver, spleen and kidneys were commonly detected. *Aeromonas hydrophila* were re-isolated from all succumbed fish. All fish injected with saline showed no mortalities except in the group fed on the basal diet without phytogenic compound that noticed 1% mortality but *A. hydrophila* was not detected on bacteriological examination of this sample (Table 4).

DISCUSSION

Dietary supplementation of the basal diet fed to *O. niloticus* with the phytogenic feed additive (Silaacid® Encapsulated) significantly improved the growth performance and feed utilization efficiency of *O. niloticus* at 0.5 g kg^{-1} diet compared to other concentrations. Interestingly, increasing the level of phytogenic feed additive did not accompanied with continued improvement of growth performance and feed utilization efficiency. Fish fed diet supplemented with 1.5 g kg^{-1} showed the lowest values of growth parameters. The improvement of growth performance concomitantly with the supplementation of the phytogenic feed additive may be attributed to the effect of its beneficial ingredients including; oregano oils such as carvacrol and thymol, acidifiers; organic acids, fumaric acid and organic acids salts; sodium formate, calcium propionate and potassium sorbate. The effect of oregano oil on fish productive performance might be relevant to enhancement of digestibility, as well as absorption of nutrients[24]. Supplementation of fish diet with essential oils also increase the availability of nutrients and lead to a higher protein synthesis, which in turn could explain the better

growth performance[25]. Moreover, phytogenic feed additives improve the flavor, palatability of feed, stimulate digestive secretions as well as enhance enzyme activity[26]. Similarly, addition of phytogenic feed additives containing oregano oils to channel catfish feed , *Ictalurus punctatus*, have been found to promote their growth performance, increase antioxidant activity, enhance muscle protein sedimentation as well as improve disease resistance of fish to invading pathogens[16]. Diet supplementation with thymol and carvacrol also improved the growth performance of rainbow trout, *Oncorhynchus mykiss*[27]. Results indicated that SGR and FCR values of Nile tilapia were positively affected by the supplementation of phytogenic feed additive. Similar results were observed by Hong *et al.*[28]. On the other hand, previous study of Sonmez *et al.*[29] concerned with diet supplementation with herbal extracts containing oregano oils like mint oil was reflected negatively on the growth performance of rainbow trout fish as well as their resistance to infectious agents which is in contrast with this study. That were noticed in fish groups fed with 1.5 g kg^{-1} (Silaacid® Encapsulated) indicating that excessive levels of phytogenic compounds behind the optimum level may have negative impacts on the growth performance of fish.

The growth promoting effects of the phytogenic feed additive (Silaacid® Encapsulated) can also be attributed to the presence of acidifiers including; organic acid and their salts (fumaric acid, sodium formate, calcium propionate and potassium sorbate). Dietary acidifiers have been found to improve the growth performance and the nutrient availabilities in various aquatic species[20] Acidifiers reduce the pH of stomach and the upper gut, which in turn stimulates the pepsin activity, enhance protein digestibility, nitrogen retention and mineral absorption[30]. Additionally, lowering the gut pH has beneficial effects on lactic acid bacteria that able to grow at a relatively low pH[31]. These indigenous probiotic bacteria colonize the intestinal surface and form a barrier, serving as the first defence to restrict attachment of fish pathogenic bacteria to the gut mucosa[32,33].

Inclusion of citric acid/formic acid in fish diets has been found to enhance the bioavailability of minerals, including phosphorus, magnesium, calcium and iron in rainbow trout; *Oncorhynchus mykiss*, sea bream; *Pagrus major* and Indian carp *Labeo rohita*[34]. Organic acids were absorbed through the intestinal epithelia by passive diffusion, providing energy for renewing the intestinal epithelia and maintaining the gut health[35]. Oral administration of potassium diformate significantly improves the feed intake, the feed conversion ratio, the live weight gain and the protein efficiency ratio of various tilapia species[36]. In contrast, contradictory results also have been reported in some studies of Petkam *et al.*[37] and Zhou *et al.*[33] who reported no significant improvement in the growth performance of tilapia fed on organic acids/salt blend or formic acid salts, respectively, at various dietary levels (0, 9 and 12 g kg^{-1} diet). The encapsulation of oregano oils by plant palm oils and soya oils are supposed to potentiate the effect of active principal contained in the phytogenic feed additive (Silaacid® Encapsulated) as it protect the volatile components in oregano oils and make it more stabilized, accordingly, remain active throughout the fish gastrointestinal tract. Additionally, coating prevents the damaging effect of gastrointestinal tract secretions on the organic acids until reaching the hind gut as well as deterioration by temperature during pelleting the diet.

Fish survivability following challenge with *A. hydrophila* increased concomitantly with the supplementation of diet with the phytogenic feed additive (Silaacid® Encapsulated). Phytogenic compound in diet at 0.5 g kg^{-1} showed the highest protective efficiency (20% mortality) against *A. hydrophila* in comparison to other concentrations. Increasing levels of phytogenic compound in the diet did not enhance the resistance of fish against bacterial challenge indicating that 0.5 g kg^{-1} supplementation in the diet is the optimum level. Additionally, fish fed only on the basal diet demonstrated 100% mortality. Earlier studies by Yang *et al.*[10] and Peterson *et al.*[11] have demonstrated that plant-based additives containing essential oils could improve the immune competence and resistance against invading pathogens. Essential Oils (EO) like thymol and carvacrol have been reported to improve survival against *A. hydrophila* infection in channel cat fish[16]. Similarly survivability of channel catfish challenged with *E. ictaluri* has been enhanced with the supplementation of commercial phytogenic feed additive containing essential oils; carvacrol, thymol, anethol and limonene[38]. Volpatti *et al.*[39] have also demonstrated that diet supplementation with the essential oil carvacrol strongly reduced the mortality rate of *Vibrio anguillarum* infection in *Dicentrarchus labrax* fish compared to control fish fed on the basal diet only.

Phytogenic feed additive containing EOs could play a decidedly active role as powerful antibacterial agents. The EOs increase the permeability of bacterial cell membranes resulting in cell contents leakage and eventually killing the cell. The leakage usually occurs through cell wall degradation, cytoplasmic membrane damage, cytoplasm coagulation and membrane proteins destruction[10]. The antagonistic and inhibitory effects of phytogenic compounds containing essential oils have been noticed against variety of fish bacterial pathogens including; *Vibrio harveyi*, *A. hydrophila*

and *A. salmonicida*[40,41]. Fish feed additives containing the oregano essential oil have strong antibacterial activities against *Lactococcus garvieae* in rainbow trout, *Oncorhynchus mykiss* since the supplementation of diet with different concentrations of oregano oil (0.125, 1.5 and 2.5 mL kg^{-1}) significantly reduced fish mortality. The 3.0 mL kg^{-1} diet showed no mortality after challenged with *L. garvieae*[42].

Phytogenic compounds containing essential oils also increase resistance against microbial infections via enhancing the immune competence of fish[42]. Diverse mechanisms through which essential oils could enhance fish immunity. Their ability to increase lectins production together with the capacity to activate complement are important pathways[43]. Lectins recognize, agglutinate and opsonize microbial pathogens hence these substances are strongly involved in the improvement of fish immune defence mechanisms to microbial infections[44,38]. These oils also enhance fish hematological parameters including lymphocytes[27]. Additionally, fish fed on diets supplemented with oregano essential oils have higher serum lyzozyme levels. Lyzozymes is critical for protection against fish pathogens as it directly activates the polymorpho nuclear leucocytes and macrophages or it promotes phagocytosis as an opsonin of freshwater and marine fish[39,42].

On the other hand, the organic acids ingredients included in the commercial (Silaacid® Encapsulated) phytogenic feed additive are also supposed to potentiate the resistance of tilapia against *A. hydrophila* challenge. Theses acids are more effective against Gram negative bacteria than EOs[45,46]. Weak organic acids can easily penetrate the bacterial plasma membrane and thus acidify the cell's interior eventually killing the bacterium[47]. Studies also reported synergistic effects of some EOs and organic acids[48]. Zhou *et al.*[49] reported that EO in combination with organic acids work better against Gram negative bacteria than individual EOs or organic acids. Phenols in EOs damage the bacterial cell membrane increasing its permeability consequently, enhance the susceptibility of bacterial pathogens to the acidic environment[50]. This study highlighted the beneficial effects of phytogenic feed additives containing mixture of oregano oils (carvarcol, thymol) and acidifiers (organic acids and their salts) on the growth performance and resistance of *Oreochromis niloticus* to *A. hydrophila* pathogen. This study potentiated the necessity of the field applications of phytogenic feed additives in fish farming as alternatives to antibiotics to combat microbial infections hence protecting human health from their hazardous outcomes.

CONCLUSION

Supplementation of aqua feeds with phytogenic feed additives containing oregano oils and organic acids have promoting effects on the growth performance of Nile tilapia (*O. niloticus*) and increase their resistance against invading pathogens.

SIGNIFICANCE STATEMENTS

- Results indicated that using commercial phytogenic feed additive (Silaacid® Encapsulated) at 0.5 g kg^{-1} diet for Nile tilapia (*Oreochromius niloticus*) has positive effect on growth performance, feed utilization parameters with no significant effect on chemical body composition
- Results also indicated that the highest protective efficiency against *Aeromonas hydrophila* found at 0.5 g kg^{-1} diet (Silaacid® Encapsulated) compared with other levels (1 and 1.5 g kg^{-1})

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