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Practical aspects of phytobiotic (Veto-Acid®) supplemented to Nile tilapia (*Oreochromis niloticus*) diets and its susceptibility to *Aeromonas hydrophila* challenge

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Abstract : Objective: The present study was carried out to investigate the effect of a phytobiotic feed additive on growth performance, feed utilization, body composition and susceptibility of Nile tilapia (*Oreochromis niloticus*) fingerlings to *Aeromonas hydrophila* challinge. **Methodology:**240 all-male Nile tilapia (0.55g 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 phytobiotic feed additive (Veto-Acid®). Fish were randomly distributed in triplicate into four treatments groups. Treatments were performedin twelve aquaria (20 fish / 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 four weeks. At the end of feeding trial, fish were challenged with pathogenic*Aeromonashydrophila* by intraperitoneal injection. **Results:** The results detected no improvement in growth performance or feed utilization in fish fed tested diets. However the resistance against *A. hydrophila* was obtained at 1.5 g phytogenic feed additive (Veto-Acid®) /kg diet. **Conclusion**: phytobiotic feed additive (Veto-Acid®) are promising for protection against *A. hydrophila* infections in Nile tilapia.
Key word : Phytobiotic, Nile tilapia, Growth Performance Resistance, *Aeromonas hydrophilla*.

Introduction

1. Introduction

The accelerated growth of aquaculture all over the world during the last decades, a combined with gradual alternation from semi intensive to intensive and super intensive in fish farming with climate changes has resulted in fish health problems[1]. In a context of pressure from different community to get healthy food combined with eco-friendly aquaculture products, led to reduce using antibiotics in aquaculture[2]**.** Major targets for improvement in the aquaculture industry include better maintenance of fish health as well as increased fish performance[3]. The main problem for the development and sustainability of the aquaculture industry is the infectious diseases[4] which decreased performance and subsequently compromised immune defense mechanisms, ultimately leave fish prone to numerous opportunistic pathogens[5,6]. These goals have led to develop an alternative feed additive that serve as functional dietary supplements in commercial fish and shrimp feeds. such products include prebiotics, probiotics, immunostimulants[7] and medicinal plants or plant derivatives, or phytogenics or called Phytobiotics, phytochemical or botanicals substances used in animals feed [8].Their use not restricted as alternatives for antibiotic growth promoters but also be extended into areas such as improving growth performance via affecting on feed intake[9,10], gut function[11]; Antioxidant effect [12,13,14] ,

enhance palatability[8]; anti –inflamatory activity [15] and antimicrobial effect [16,17,18] but may also be extended into areas such as disease prevention and immune and stress resistance in aquatic species [19,20,21].

Therefore, The present study, was planned to assess the effect of a phytobiotic feed additive (Veto-Acid®) on the growth performance, feed utilization, body composition and susceptibility of Nile tilapia (*Oreochromis niloticus*) fingerlings to pathogenic strain of *Aeromonas hydrophila.*

2. Materials and Methods

2.1 Fish and culture facilities

Monosex, all male, Nile tilapia fingerlings (0.55 g) were obtained from a commercial tilapia hatchery at Kafr El Sheikh Governorate, Egypt. Fish were randomly distributed in triplicate into four treatments groups in the Fish Nutrition Laboratory, National Research Centre, Egypt. Treatments were performed in twelve glass aquaria (60×30×40 cm^3) at a rate of 20 fish / aquarium. The experimental fish were acclimated to the culture system for 2 weeks. Initially, fifty fish were randomly collected, group weighed and the average initial weights were recorded then frozen at - 20 ^oC for chemical analysis. Water temperature, dissolved oxygen and pH were adjusted around 26.5, 6 mg/L and 7.5 respectively in all treatments and monitored daily.

2.2 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 phytobiotic feed additive (Veto-Acid) containing(Lactic acid(80%) 285gkg^{-1}; Malic acid (99%) 100gkg^{-1}; Citric acid (99%) 200gkg^{-1}; Cinnamaldehyde (Cinnamon extract) 0.75gkg^{-1} and Colloidal silica up to one kg)was 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 followed by 6% for 2 weeks, finally 3% for the last four weeks. The average weight of fish was recorded every 15-day intervals, and the daily rations were readjusted accordingly.

2.3 Chemical analysis of diets and fish

The tested diet and fifteen fish collected from each treatment at the beginning and at the end of feeding experiment were analyzed for moisture content, protein, fat and ash according to the standard methods of AOAC [22].

2.4 Calculations of fish performance

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

Weight gain (WG) = final weight – initial weight.

Specific growth rate (SGR) = 100 (ln W_2 – ln W_1) / T

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.

Feed conversion ratio (FCR) = dry feed intake (g) / fish live weight gain (g).

Protein efficiency ratio (PER) = 100 (weight gain (g) / protein intake (g)

Protein productive value (PPV) = 100 (protein gain (g) /protein fed (g)).

Energy Retention (ER) = Retained energy in carcass (Kcal)/energy intake (Kcal) ×100.

2.5 Challenge with Aeromonas hydrophila

Virulent *A. hydrophila* strain previously isolated from mortalities affecting *Oreochromis niloticus* [23] were obtained, inoculated into a blood agar plate and incubated at 25°C overnight. Bacterial culture was adjusted to 1 x 10^7 Colony Forming Unit (CFU)/ml 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 x 10^7 CFU/ml live virulent *Aeromonas hydrophila* (*A. hydrophila*) according to Abu-Elala and Raja[24]. 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 fishon the specific Aeromonas isolation agar (Oxoid) and confirmation of retrieved isolates phenotypic and PCR according to Buller[25].

2.6 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) at a 95% confidence limit, using SPSS software, version 16. Duncan's Multiple Range [26] test was used to compare means when F-values from the ANOVA were significant (P<0.05).

3. 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 phytobiotic feed additive (Veto-Acid®) are illustrated in Table (1). The initial weight was nearly similar in all treatment groups with no significant differences (P>0.05). Final fish weight (FW), weight gain (WG), and specific growth rate (SGR) showed numerical difference among treatments supplemented with phytobiotic feed additive in fish diets. but no significant difference (*P*> 0.05) were obtained among the control group fed the basal diet (1.700 g, 1.150 g, 1.483 g) or the other levels 0.5 gkg^{-1} diet; (1.840g, 1.290g, 1.583) 1 g kg^{-1} (1.640 g, 1.090 g, 1.430 g) neither 1.5 gkg^{-1} (1.736g, 1.860g, 1.513 g) were illustrated in Table (1)for FW, WG and SGR respectively. Obviously, phytobiotic feed additive in fish diets did not enhance the growth performance parameters.

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

Treatments gkg^{-1}	Initial Weight (g)	Final Weight (g)	Weight gain (g)	Specific growth rate
0	0.55	1.700±0.08	1.150±0.08	1.483±0.06
0.5	0.55	1.840±0.18	1.290±0.18	1.583±0.13
1.0	0.55	1.640±0.16	1.09±0.16	1.43±0.13
1.5	0.55	1.736±0.10	1.86±0.11	1.513±0.08

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

Treatments gkg^{-1}	Feed intake	FCR	PER	PPV	ER
0	2.316±0.01	2.033±0.12	1.670±0.10	25.613±2.86	18.00±1.26
0.5	2.160±0.14	1.746±0.28	2.023±0.28	31.313±5.46	22.490±3.40
1.0	2.086±0.16	1.970±0.23	1.746±0.18	27.490±3.19	19.960±1.89
1.5	2.256±0.07	1.923±0.26	1.763±0.11	26.56±2.06	19.493±1.64

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) were illustrated in Table (2).

The results clearly demonstrated there were no significant difference (P>0.05) among the different treatments in feed intake (FI) and Feed conversion ratio (FCR) among treatments (control, 0.5, 1 and 1.5 $gkg^{-1)}$. Control group fed the basal diet 3.316and 2.033, 0.5 g kg^{-1}2.160, 1.746,1 g kg^{-1}; 2.086, 1.970and1.5 gkg^{-1}; 2.256 and 1.923, respectively.

Results indicated also no significant differences (p> 0.05) in protein efficiency ratio (PER) among fish fed diets supplemented with the all treatments of phytobiotic feed additive (0.5,1 and 1.5kg^{-1})

(2.023,1.746,1.763) respectively in comparison to the control group without supplementation, 1.670. Similarly, protein productive value (PPV) were not significantly different ($p>0.05$) among fish fed control treatment 25.613.compared with other treatments(31.313,,27.490and 26.560)for 0.5 ,1.0 and 1.5 gkg^{-1}respectivley. Additionally, the lowest Energy retention value ER, 18.00was recorded in fish group fed with control group compared with the other groups (0.5, 1 and 1.5gkg^{-1})22.490,19.960 and19.493respectively although there were numerical differences among treatments. Results of body composition are illustrated 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.

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

Treatments	DM	Crude Protein	Ether Extract	Ash
Initial	16.88	52.27	19.91	16.02
0 gkg^{-1}	22.613±1.26	58.350±0.14	28.26±0.48	10.650±0.35
0.5 gkg^{-1}	22.786±0.71	58.600±0.92	29.840±0.79	9.923±0.32
1.0 gkg^{-1}	23.41±0.70	57.100±1.47	29.223±1.06	10.473±0.28
1.5 gkg^{-1}	22.416±0.81	57.766±0.48	30.600±0.92	10.033±0.27

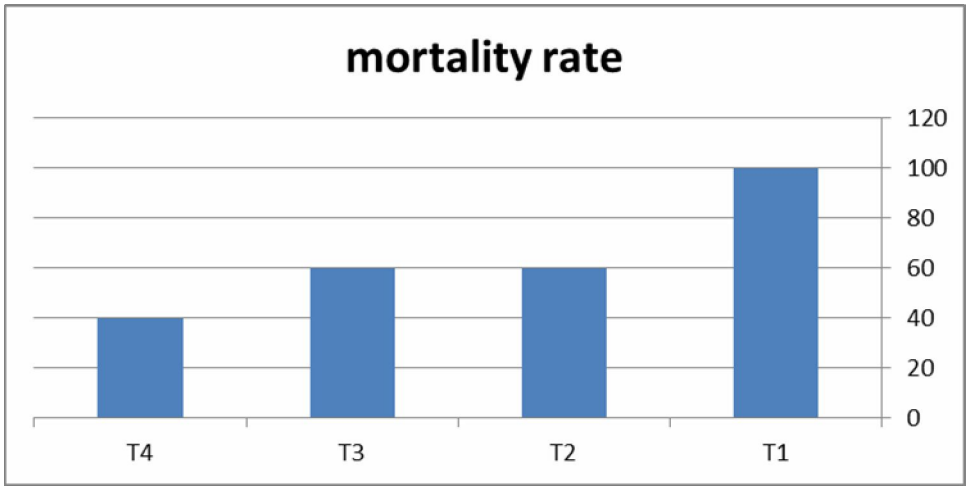

Fig (1) : Mortality rate of *O. niloticus* experimentally infected with *A. hydrophila*

2.6 Challenge with *Aeromonas hydrophila*

Fish survivability after IP injection with *A. hydrophila* increased in all groups fed with diets supplemented with the commercial phytobiotic compound. The lowest mortality (40 %) was noticed in fish fed on 1.5 supplementation followed by 1.0 (60 %) the same trend was observed at 0.5supplementation (60 %). On the other hand, the highest cumulative mortality (100 %) was noticed in the control group fed with the basal diet without phytobiotic 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 spleen, liver and kidneys were commonly detected. *A. hydrophila* were re-isolated from all succumbed fish. All fish injected with saline showed no mortalities Figure (1)

4. Discussion

Dietary supplementation of the basal diet fed to *O. niloticus* with the phytobiotic feed additive (Veto-Acid®) not significantly improved the growth performance or feed utilization efficiency neither chemical body composition of *O. niloticus*. In 0.5;1.0 or 1.5gkg^{-1}compared with control treatment without any supplementation . The results detected negative effect on growth performance concomitantly with increasing the supplementation of the phytobiotic feed additive .These results in agreement with the result of Peterson *et al* [27] showed that products from essential oils or phytochemicals did not improve growth performance in Channel

cat fish (*Ictalurus punctatus*). In the same trend with our study, some previous studies[28] concerned with diet supplementation with herbal extracts was reflected negatively on the growth performance of rainbow trout fish as well as their resistance to infectious agents which in concordance with the extracted results in the current study indicating that excessive levels of phytobiotic compounds may have negative impacts on the growth performance of fish.

The results of this study are contrary to other studies found that the effect of Cinnamaldehde or 3-phenl-2- propenal, a natural flavoring substance,occurs in the leaves of Cinnamon trees (found in Veto-Acid). this, Aromatic compound has been demonstrated to stimulate induce the secretion of the digestive enzymes (amylase production) which results in appetite stimulation and increased food consumption and efficiencies [29]. Moreover, phytobioticfeed additives improve the flavor, palatability of feed, stimulate digestive secretions as well as enhance enzyme activity[11,30]. In another study, addition of phytobiotic feed additives containing active natural compounds 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 [31]. Our Results indicated that SGR and FCR values of Nile tilapia were not affected significantly by the supplementation of the Cinnamaldehyde feed additive. In contrary with the result observed by [29].

Concerning the presence of organic acid in(Veto-Acid®) and In agreement with our study, Petkam *et al* [32]; Liber*et al*[33]; Zhou *et al*[34] and Da Saliva *et al*[35] 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.

In contrast, contradictory results also have been reported in some studies; showed that dietary acidifiers have been found to improve the growth performance and the nutrient availabilities in various aquatic species [24, 36]. Acidifiers reduce the pH of stomach and the upper gut, which in turn stimulates the pepsin activity, enhancing protein digestibility, nitrogen retention and mineral absorption[37,38]. Additionally, Lowering the gut pH has beneficial effects on lactic acid bacteria that able to grow at a relatively low pH[34]. These indigenous probiotic bacteria colonize the intestinal surface and form a barrier, serving as the first defense to restrict attachment of fish pathogenic bacteria to the gut mucosa [35]. In the same trend, 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 *Labeorohita* [39]. Baruah *et al*[40] found that citric acid of microbial phytase have a synergistic effect on Indian carp (*Labeorohita*). Organic acids are absorbed through the intestinal epithelia by passive diffusion, providing energy for renewing the intestinal epithelia and maintaining the gut health[41]. 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[32]; shrimp[42].

Fish survivability following challenge with *A.hydrophila* increased concomitantly with the supplementation of diet with the phytobiotic feed additive (Veto-Acid®). Phytobiotic compound in diet at the concentration of 1.5 showed the highest protective efficiency (40% mortality) against *A. hydrophila* in comparison to other concentrations. Increasing levels of phytogenic compound in the diet enhance the resistance of fish against bacterial challenge indicating that $1.5kg^{-1}$ supplementation is the optimum level. Additionally, fish fed only on the basal diet demonstrated (100 % mortality). Earlier studies have demonstrated that plant-based additives containing Cinnamaldehyde could improve the immune competence and resistance against invading pathogens[43]. Cinnamaldehydehas been reported to worked effectivelyagainst all Chryseobacterium and Myroides spp. in Tilapia[43]. 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 [44].

Phytobiotic feed additive containing Cinnamaldehyde could play a decidedly active role as powerful antibacterial agents[43]. Cinnamaldehyde acts by inhibiting the proton motive force, respiratory chain, electron transfer and substrate oxidation, resulting in uncoupling of oxidative phosphorylation, inhibition of active transport, loose of pool metabolites, and disruption of synthesis of DNA, RNA, proteins, lipids and polysaccharides[45]. This is in agreement with Chang *et al* [46] and Ooi *et al* [47] they found that cinnamaldehde had excellent antibacterial activity against diverse gram negative and gram positive bacteria. The antagonistic and inhibitory effects of phytogenic, phytobiotic compounds containing essential oils, cinnamaldehyde and turmeric have been noticed against variety of fish bacterial pathogens including; *Vibrio harveyi*, *A. hydrophila* and *A. salmonicida* [21,48].

Phytobiotic compounds containing cinnamaldehyde also increase resistance against microbial infections via enhancing the immune competence of fish [43]. Diverse mechanisms through which cinnamaldehyde could enhance fish immunity. It's used at sub-inhibitory levels is not only a potent inhibitor of autoinducer-2-based quorum sensing(QS), but also impacted in *Vitro* the production of multiple virulence factors and biofilm formation, and reduced in vivo the mortality of Artemia shrimp exposed to vibrio harveyiBB120 [49]. According to Brackman *et al*.[49], cinnamaldehyde and cinnamaldehyde derivatives are potentially useful antipathogenic lead compounds for treatment of vibriosis. Additionally, fish fed on diets supplemented with cinnamaldehyde have higher serum lyzozyme levels[21]. Lyzozymes is critical for protection against fish pathogens as it directly activates the polymorphonuclear leucocytes and macrophages or it promotes phagocytosis as an opsonic of freshwater and marine fish[50].

On the other hand, the Organic acids ingredients included in the commercial (Veto-Acid) phytobiotic feed additive are also supposed to potentiate the resistance of tilapia against *A. hydrophila* challenge [51,52]. Weak organic acids can easily penetrate the bacterial plasma membrane and thus acidify the cell's interior eventually killing the bacterium[53]. Studies also Rmli *et al* [54] found that the use of potassium diformate at 0.2% is an efficient tool to control vibrio anguillarum in tropical tilapia culture.

In the same context, Ng *et al* [55] suggest that cumulative mortality of fish fed no organic acids was higher compared with fish fed organic acid supplemented diets at 16 days post challenge with *Streptococcus agalactiae* .which is in an agreement with our results.

5.Conclusion

Supplementation of aqua feeds with phytobiotic feed additive (Veto-Acid®) containing cinnamaldehyde and organic acids did not improve the growth performance or feed utilization in Nile tilapia, *O. niloticus,* however, it's promising for protection against *A. hydrophila* infections when obtained at 1.5 gkg^{-1}diet and increase their resistance against invading pathogens.

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