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The Role of Formic Acid as an Antibacterial Feed Additive on Growth Parameters and Health of the Nile Tilapia *(Oreochromas niloticus)*

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ABSTRACT

This study aimed to conclude the impacts of three different dosages of formic acid (an organic acid) on various biological and physiological parameters in the mono-sex Nile tilapia (*Oreochromis niloticus*). The study examined growth performance parameters, hematological and serum biochemical profiles, and the activity of antioxidant enzymes. One hundred and eighty healthy fish, weighing about 30±5g, were stocked in glass tanks (60L/volume) to acclimate. There were 45 fish in each group (G1, G2, G3, and G4). Over eight weeks, groups 2, 3, and 4 were given experimental diets containing formic acid at concentrations of 0.3%, 0.4%, and 0.5%, respectively. Group 1 was used as a control and kept on the basal diet. Groups given diets supplemented with 0.4 and 0.5% formic acid demonstrated a noticeably better feed utilization and weight gain than the 0.3% supplementation group. Overall, during the first 4 weeks, FCR was significantly enhanced compared with the following 4 weeks. The numbers of red blood cells (RBCs), platelets, hemoglobin, white blood cells (WBCs), and serum proteins (total protein, albumin, and globulin) were almost unchanged across all groups. Even liver function and creatinine levels were unchanged. On the other hand, formic acid at the concentrations of 0.4 and 0.5% showed significant improvements in antioxidant enzyme activity (SOD, CAT, and TAC), while the 0.3% group showed a moderate decrease compared to the control. Additionally, when fish was challenged by *Aeromonas hydrophila*, the 0.5% formic acid group had the lowest mortality rate (MR%). The 0.3% group showed better immunological responses, with increased serum bactericidal activity (SBA) and oxidative burst activity (OBA). These results point to formic acid as a potential feed addition for improving growth, boosting immunity, and attaining sustainable aquaculture methods. Dietary formic acid is not recommended to exceed the 4 weeks addition, high period may cause a decrease in FCR.

INTRODUCTION

Among agriculture subsectors, aquaculture is enjoying the most rapid expansion **(FAO, 2010)**. Its principal goal is to promote the development and well-being of aquatic

animals. A new and promising way to improve feed quality and maximize growth performance is by using feed additives **(Cowey & Cho, 1993)**. However, adding antibiotics as growth promoters in aquafeeds has been banned by the EU for the harm these additives cause to the consumers **(Heuer *et al.*, 2009)**. According to **Devasree *et al.* (2014)**, **Reda and Selim (2015)** and **Romano *et al.* (2015)**, there has been an increase in the investigation of alternative solutions, such as acidifiers, probiotics, prebiotics, and plant extracts, which can enhance immunity, inhibit pathogens, and support growth.

There has been an increase in antibiotic-resistant bacteria due to their overuse as a disease-control measure in aquaculture **(Defoirdt *et al.*, 2011)**. To lessen reliance on antibiotics, organic acid salts and essential oils have recently been the focus of researches in disease management in aquaculture **(Koh, 2008)**. The use of antibiotics in aquaculture has been restricted due to the increasing number of resistant bacteria to these drugs and their damage to aquatic ecosystems **(Hassaan *et al.*, 2018)**. Some fish species may be amenable to non-chemical methods such as acidifiers and probiotics that boost their growth performance according to recent research **(Romano *et al.*, 2016)**. Many alternatives were used as replacers such as titanium dioxide nanoparticle **(Sherif *et al.*, 2019)**, *Spirulina platensis* and *Chlorella vulgaris* algae **(Sherif *et al.*, 2020a; Okasha *et al.*, 2024; Tawfeek *et al.*, 2024)**, mitigating hazardous of antibiotics **(Sherif *et al.*, 2021a,b, 2022a, b, 2023a)**. A blend of chitosan-vitamin C and vitamin E nanoparticles can serve as an alternative for the same purpose **(Elnagar *et al.*, 2024)**. Furthermore, organic acids are one of the most affordable and beneficial alternatives for feed additives.

Antibiotic resistance genes may be transferred from farm animals to humans, according to research of **Olson and Dinerstein (2005)**, **Poirel *et al.* (2012)** and **Watts *et al.* (2017**), which is a major worry for public health. Despite organic acids' common use as acidifiers in animal feeds on land, studies on fish have shown that these compounds can boost growth, increase nutrient intake, and strengthen resistance to disease owing to their potent antibacterial characteristics **(NRC, 2011)**. This study aimed to see how dietary formic acid affected the Nile tilapia growth performance, feed consumption, biological indices, and disease resistance.

MATERIALS AND METHODS

1. Fish collection and rearing conditions

180 disease-free Nile tilapia *(Oreochromis niloticus)* specimens, mono-sex (all males) were obtained from a private aquaculture farm in Tolompat 7 village, Kafrelsheikh Governorate. Each fish weighed 30±5g. The fish were placed in the aquarium at the Animal Health Research Institute (AHRI). Glass aquariums with dimensions 60×40×30cm were filled with sixty liters of dechlorinated tap water as soon as the fish arrived. Fish were fed a regular diet for 15 days during the acclimatization

period. The water in each aquarium had a temperature value of 25 ± 1°C and a dissolved oxygen concentration of 6.9 ± 0.5mg/ L. The aquarium was refilled with about 30% of its original volume on daily-basis following a partial drain to keep the water quality at its best **(Sherif *et al.*, 2020b; Eldessouki *et al.*, 2023)**.

2. Experimental design and diet preparation

The fish were divided into four equal groups, with 45 individuals named G1, G2, G3, and G4. The group that did not receive any supplement was G1 that represented the control. Food diets containing 0.3, 0.4, and 0.5% formic acid were given to the G2, G3, and G4 experimental groups, respectively (Table 1). All experimentals diets were designed according to the dietary requirements stated by the **NRC (2011)**, and the feeding trial lasted for 8 weeks. A 31.8% crude protein, 6.2% crude fat, and 4% crude fiber composition made up the basal diet. Experimental diets composition are illustrated in Table (2).

The diets ingredients used in the experiments were grinded and sieved using a grain grinding machine (FFC-45, JIMO, China) with a 0.05mm sieve. The ground materials were combined with the right amount of water and formic acid to get a good consistency for making pellets. The 1.5mm diameter pellets were extruded using an experimental extruder (Model SYSLG30-IV), air dried for 24h at ambient temperature, and thereafter sealed in plastic bags and kept at -20°C until needed. The diets were administered to the fish twice a day at 8:00 a.m. and 3:00 p.m., at a rate of 3% of their body weight (b.w.). Fish weight were monitored every two weeks, and the feeding amounts were changed accordingly.

Table 1. Experimental design and group distribution

Group	Treatment	No. of fish	Period of experiment
G (1)	Control (basal diet)	45	8 weeks
G (2)	formic acid 0.3 % of the diet	45	8 weeks
G (3)	formic acid 0.4 % of the diet	45	8 weeks
G (4)	formic acid 0.5% of the diet	45	8 weeks

3. Growth performance

The number and weight of fish were tracked every two weeks to record weight gain (WG). Among the measurments that were analyzed were weight gain (WG), daily weight gain (DWG), and feed conversion ratio (FCR). Growth parameters were calculated following these equations **(Castell & Tiews 1980; Tacon 1987)**:

- weight gain (WG) = Final body weight (g) − Initial body weight (g)
- Daily weight gain (DWG) $= \frac{\text{TG}}{\text{time (days)}}$
- Feed conversion ratio (FCR) $= \frac{\text{Feed intake (FI)}}{\text{TG}}$

Table 2. Feed ingredients physical and chemical analyses of experimental diet

Physical analysis	%	Chemical analysis	%
Soy meal (44%)	39.5	Moisture	9.9
Fish meal	8.5	Dry matter	90.1
Yellow corn	35.1	Crude protein	31.8
Corn gluten	9	Ether extract	6.2
Wheat bran	2	Ash	5.5
Soy oil	5	Crude fiber	4
Vitamins & minerals mixture	0.3	Nitrogen free extract	42.6
Salt	0.25	Calcium	0.76
Carboxymethylcellulose	0.2	Phosphorus	0.59
DL. methionine	0.15	Lysine, methionine	1.69, 0.75
	Digestible energy (kcal/kg)		3437.3

Note: Vitamin premix contains vitamin A 12000000 IU, vitamin K3 2g, vitamin C 250g, vitamin E 10g, vitamin D3 2200000 IU, vitamin B1 1g, vitamin B2 5g, vitamin B6 1.5g, vitamin B12 0.01g, Biotin 0.050g, Niacin 30g, Folic acid 1g and Pantothenic acid 10g and carrier to 1000g. 3Mineral premix contains Copper 4g, Manganese 60g, Zinc 50g, iron 80g, Iodine 1g, Cobalt 0.1g, and Selenium 0.1g with calcium carbonate (CaCO3) carrier to 1000g.

4. Hematological parameters

Blood samples were collected from five fish at the end of the trial from each group. Caudal vein blood is divided in two halves, one of them mixed 10% EDTA as an anticoagulant. The serum that had not been hemolyzed was separated from the blood by centrifugation at 3000rpm for 10 minutes after coagulation at 4°C. Biochemical analysis required serum to be kept at -20°C. **Reitman and Frankel (1957)** approach was used to detect serum aspartate and alanine aminotransferases (AST and ALT, respectively). Total protein concentrations were assessed using the method of **Henry (1964)**, and serum

albumin concentrations were assessed by using the method of **Wotton and Freeman (1982)**. Via implementing the method of **Coles (1974)**, globulin concentrations were determined by subtracting total protein from albumin.

5. Antioxidant enzyme activity and non-specific immune parameters

The oxidative status of the fish was assessed by measuring superoxide dismutase (SOD), lipid peroxidase (LPO), and total antioxidant capacity (TAC). The method of **Lartillot *et al.* (1988)** was followed to measure CAT activity spectrophotometrically at 240nm, with the result shown as μmol H_2O_2 decomposed per mg protein per minute. Hepatic tissues were tested for LPO and TAC levels following the feeding trial, and SOD activity was ascertained using the method outlined by **Paya *et al.* (1992)**. A phosphate-buffered saline (pH 7.4) and all the other reagents and buffers were purchased from the Biodiagnostic® Chemical Company.

To evaluate characteristics of immunological response, blood samples were taken from five randomly chosen fish from each group at the end of the eigh-week feeding trial.

- **Serum bactericidal activity (SBA)**

For serum bactericidal activity (SBA%), the method described by **Kajita *et al.* (1990)** was followed. A bacterial culture of *A. hydrophila* (2×10^8 CFU/ml) and *O. niloticus* (2×100μl) serum were mixed and left to incubate at 25°C for one hour. Substituting sterile PBS for serum allowed us to create a blank control. Placing the mixture on blood agar followed incubation. It was then diluted in PBS at a 1:10 ratio. The plates were incubated at 27°C for 24 hours to assess the antibacterial activity, and the number of live bacterial colonies was counted.

- **Neutrophil glass-adhesion assay**

Following the protocol laid out by **Anderson *et al.* (1992)**, the nitroblue tetrazolium (NBT) test was used to measure neutrophil oxidative burst activity (OBA). The neutrophils were allowed to attach to the glass surface by incubating a drop of heparinized blood onto a cover slip right after collection for 30 minutes at 25°C in a humid atmosphere. Fifty microliters of a 0.2% NBT solution (Fluka Buchs, Co., Switzerland) were added to microscope slides after the coverslips were delicately rinsed with PBS (pH 7.4). After 30 minutes of incubation, neutrophils were counted using a light microscope after staining them in dark blue.

6. Statistical analysis

Investigations were conducted applying the analysis of variance (ANOVA) for statistical purposes. The SPSS software (version 22) was used for all computations and statistical evaluations.

RESULTS

Table (3) shows that when the dietary percentage of formic acid increased, the fish's performance was significantly and linearly improved. G4 had the greatest feed intake (FI) and weight gain (WG) at 140 and 50.26g, respectively. G3 and G2 were closely behind, with 129.2 and 121.4g; 50.42 and 43.1g, respectively. These numbers were significantly higher ($P< 0.05$) than the control group (G1), which measured 101.9 and 39.33g, respectively. This pattern was accompanied by a notable decline in feed conversion ratio (FCR), which indicated an enhanced feed efficiency in the groups that were supplemented with formic acid.

Table 3. Fish growth performance

Items	IW	FW	FI	WG	DWG	FCR
G1	30.33C ±0.84	69.74C ±0.67	101.9D ±1.4	39.33C ±0.9	0.7C ±0.02	2.59B ±0.05
G2	33.4AB ±0.64	76.55B ±1.42	121.4C ±2.4	43.1B ±0.82	0.77B ±0.02	2.82A ±0.01
G3	32.8B ±0.29	83.2A ±0.3	129.2B ±0.7	50.42A ±0.6	0.9A ±0.01	2.56B ±0.04
G4	35.5A ±0.9	85.8A ±0.6	140A ±0.74	50.26A ±1.44	0.9A ±0.03	2.79A ±0.1

Note: G; Group, IW; initial weight, FW; final weight, FI; feed intake, WG; weight, DWG; daily weight gain, FCR; food conversion rate. Group (G) G1, G2, G3, and G4: fish-fed diets supplemented with formic acid 0, 0.3, and 0.4 % of fish feed.

The dietary formic acid led to a decrease in feed conversion ratio (FCR) during the first four weeks of the feeding trial ($P< 0.05$), showing high feed utilization, then groups G2, G3, and G4 had FCR values of 6.63, 6.1, and 6.6 when administered formic acid for an extended period of 8 weeks, which were significantly higher compared to the control group (2.62) (Table 4).

Table 4. Weekly changes food conversion ratio (FCR)

Items	Week2	Week4	Week6	Week8
G1	3.4Aa ±0.7	2.04Ab ±0.11	2.8Bab ±0.09	2.62Bab ±0.15
G2	2.2Bd ±0.08	1.78Bc ±0.03	2.69Bb ±0.08	6Aa ±0.14
G3	1.56Bc ±0.01	1.7Bbc ±0.08	2.63Bb ±0.08	6.1Aa ±0.6
G4	1.45Bc ±0.12	1.62Bc ±0.03	4.3Ab ±0.25	6.63Aa ±0.34

Different capital letters in the same column mean significant differences between groups, while small letters in the same row mean substantial differences between periods at $P< 0.05$.

Table 5. Blood parameters, serum protein, and liver enzymes

Items	RBCs	Hg	PCV	WBCs	TP	ALB	GLO	ALT	AST	Creatinine
G1	3.57A ±0.19	10.7A ±0.6	34.7A ±3.12	39.6A ±2.3	5.9A ±0.35	3.08A ±0.05	2.82A ±0.36	18.5A ±0.3	23.8B ±1.01	0.76A ±0.03
G2	3.33A ±0.12	10A ±0.36	32.4A ±1.17	43.9A ±1.14	5.5A ±0.17	2.82AB ±0.03	2.68A ±0.17	18.3A ±0.98	20.9C ±0.3	0.66AB ±0.02
G3	2.71B ±0.13	8.12B ±0.4	26.3B ±1.27	37.1AB ±1.63	4.73B ±0.12	2.92B ±0.05	1.82B ±0.13	18.33A ±0.4	25.5AB ±0.7	0.58B ±0.02
G4	2.4B ±0.12	7.2B ±0.35	23.33B ±1.12	38.2B ±3.1	4.37B ±0.17	2.75C ±0.04	1.61B ±0.16	17.43A ±0.8	27.2A ±0.83	0.6B ±0.06

Notes: Different capital letters in the same column mean significant differences between groups at $P< 0.05$.

The hematological and biochemical parameters shows that the Nile tilapia were affected by nutritional supplementation with formic acid (Table 5). At the end of the feeding trial, a substantial decline ($P< 0.05$) in RBCs, PCV, Hb, and WBC values was detected in groups G3 and G4, which had 0.4 and 0.5% formic acid, respectively, when compared with the control group (G1). Instead, there were no discernible changes in these hematological markers between the control and the G2 group (0.3% formic acid). G3 and G4 had considerably lower levels of total protein (TP), albumin (ALB), and globulin

(GLO) than G1 did ($P< 0.05$). However, in G2 (0.3% formic acid), these metabolic indicators had no discernible alterations compared to the control group.

Analyses of liver enzyme and creatinine levels are presented in Table (5). ALT activity was stable throughout all experimental groups, with no significant differences observed compared to the control group (G1). While comparing G1 to fish fed diets containing 0.4% (G3) and 0.5% (G4) formic acid, AST activity was significantly higher in the latter two groups ($P< 0.05$). In contrast, AST levels in G2 (containing 0.3% formic acid) were lower ($P< 0.05$) compared to the control group. Creatinine levels were significantly lower in fish of groups G3 and G4 than G1 ($P< 0.05$). Similarly, there was no observed change in creatinine levels between the control and G2 groups.

Table 6. Antioxidants and immune parameters of the experimental Nile tilapia

Item	SOD	CAT	LPO	TAC	OBA	SBA
G1	0.44D ±0.02	5.4B ±0.15	2.26C ±0.06	0.6AB ±0.05	13.7B ±0.88	39.7A ±4.8
G2	0.66C ±0.03	4.4C ±0.23	2.29C ±0.15	0.78A ±0.07	17.7A ±0.3	39.7A ±2.73
G3	0.91B ±0.03	6.77A ±0.1	3.9B ±0.4	0.65AB ±0.16	13.3B ±0.9	30.3AB ±0.9
G4	1.25A ±0.029	7.22A ±0.07	4.97A ±0.2	0.39B ±0.05	9C ±0.6	24.3B ±0.88

Notes: Different capital letters in the same column mean significant differences between groups at $P< 0.05$. CAT; catalase, SOD; superoxide dismutase, LPO; lipid peroxidase, TAC; total antioxidant capacity, OBA; oxidative burst activity, SBA; serum bactericidal activity.

All groups supplemented with formic acid (G2, G3, and G4) had significantly increased SOD levels ($P< 0.05$) (Table 6), but was less in G2. While, G3 and G4 had significantly higher CAT activity than the control group, whereas group G2 had a observed lower activity level. G3 and G4, which received 0.4% and 0.5% formic acid, respectively, showed a notable increase in LPO levels, although G2 did not differ with the control group. In contrast to the control group, there were no statistically significant changes in TAC levels among the experimental groups.

Based on Table (6), when it comes to the immunological status, G4 (fed 0.5% formic acid) had significantly lower levels of SBA and OBA ($P< 0.05$). In contrast to the control group, fish of G2 had significantly improved OBA levels, indicating the boost of the immunological status, meanwhile G3 showed no difference.

DISCUSSION

This study found that feed efficiency and growth performance were improved when formic acid was added to the diets of the Nile tilapia. **das Neves *et al.* (2021)** found that dietary acidifiers, can boost feed consumption, growth rates, and disease resistance in aquaculture. This support from previous studies, with the rainbow trout *(Oncorhynchus mykiss)* as an example: after 40 days of supplementation with citric acid, sorbic acid, thymol, and vanillin, there were no discernible changes in their growth parameters. However, FCR and SGR were enhanced with extended supplementation for 82 days **(Pelusio *et al.,* 2020)**. The gigantic groupers fed a meal containing 1% butyric acid did not outgrow the control group. According to **Yong *et al.* (2020)**, various factors, including species, acid type, dosage, and feeding time, influence the outcomes. Additional research on the Nile tilapia has demonstrated that a mixture of medium- and short-chain acids called Bacti-nil® Aqua can enhance growth, immunity, gut microbiota, and disease resistance. For 21 days, young tilapia fed supplemented- diets with 0.5% Bacti-nil® Aqua showed reduced FCR values, increased lysozyme, and bactericidal activity compared to fish fed on a basal diet, according to a study by **da Silva *et al.* (2023)**.

It is worth mentioning that there was no change in hematological indicators in the tilapia given 0.3% formic acid (G2) compared to the control group **(NRC, 2011)**. There was no change in hematological parameters in tilapia-fed acidifier diets, according to the findings of **Lim *et al.* (2010)**, which are in line with this finding. Due to significantly decreased levels of RBCs, Hb, and WBCs in fish groups G3 (0.4% formic acid) and G4 (0.5% formic acid), the immunosuppressive effects may have been more noticeable at higher dosages. **Renuka *et al.* (2014)** and **Hassaan *et al.* (2015)** found that organic acid supplementation improves hematological indices in the juvenile beluga *(Huso huso),* the Nile tilapia, and Catla catla. However, other studies have shown that this response varies by species, acid type, and dosage.

G3 and G4 had significantly reduced levels of immunological, biochemical markers compared to the control group. Similarly, for the total protein (TP), albumin (ALB), and globulin (GLO) **(Tahmasebi-Kohyani *et al.,* 2011)**, G2 did not show any significant differences. More investigations are required on the effects of organic acids concerning these parameters.

Two of the most commonly used liver enzymes for evaluating liver health are ALT and AST **(Lemaire *et al.,* 1991)**. While ALT levels did not differ significantly between groups in this study, AST levels had the superiorty in G4 compared with the control group. Although G2 showed no notable changes, G3 and G4 had significantly lower serum creatinine levels (a marker of kidney function) than the control group, suggesting that the formic acid did not negatively impact renal health. In accordance,

Soltan *et al.* (2017) and **Hassaan *et al.* (2018)** claimed that organic acid blends enhanced fish's protein metabolism and liver function.

A key role in protecting cells from oxidative stress is played by antioxidant enzymes, including SOD and CAT **(Tabrez & Ahmad, 2009; Sherif *et al.*, 2022c, 2023b, 2024)**. While CAT activity increased most dramatically in G3 and G4, SOD activity was substantially higher across the board. That SOD and CAT activity are functionally connected, and the increases in one of them is typically accompanied by an increase in the other **(Halliwell, 1994)**. The body may activate its antioxidant enzymes more effectively to counteract the oxidative stress that high concentrations of formic acid induce.

Organic acids can modulate the immune responses and have antimicrobial properties. The bactericidal serum, lysozyme, and nitric oxide activities were improved in tilapia fed a mixture of short-chain organic acids (propionic and formic acids) compared to those fed a basal diet **(Reda *et al.*, 2016)**. The challenge test results showed a decreased serum bactericidal activity (SBA) and increased levels of formic acid (G3 and G4); these were indicative of immunosuppressive effects, in contrast. According to previous research, sodium butyrate and other organic acids have been found to strengthen the immune system of tilapia **(Dawood *et al.*, 2020)**. Adding formic acid to the diet of the Nile tilapia improves their growth performance, immunity, and antioxidant responses; however, an overabundance of this beneficial substance can have the opposite effect. Our findings emphasize the necessity of dosage adjustment for maximizing formic acid efficacy while minimizing risk to fish health.

CONCLUSION

The results showed that the Nile tilapia's feed efficiency and growth performance were significantly enhanced when formic acid was added to their diets at the first 4 weeks. Immunological responses were boosted during the 8- week trial. Results show that formic acid may be a useful feed supplement to increase aquaculture systems' sustainability and output.

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