Damanhour Journal of Veterinary Sciences 3 (2), (2019) 21-26

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

Efficacy of Dietary Bioorgano Supplementation with Inclusion of QZ Toss™ on Nile Tilapia

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ABSTRACT

The current study was designed to investigate the potential of bioorgano (thymol and carvacrol) with and without the water quality improvement product QZ Toss™ on growth, immune and health performance of Nile tilapia (*Oreochromis niloticus*). Six fish groups were maintained on control diet supplemented with the bioorgano for one month. Both bioorgano dietary levels exhibited significant increase in growth parameters as well as in an example of immune parameters; the white blood cell count; with no negative impacts on both hepatic and renal functions. The histopathological examination revealed better intestinal epithelial status in bioorgano treated groups than other groups, and an increase in intestinal villi length regardless of bioorgano concentration used**.** while the posterior kidney showed activation of melanomacrophages centers (MMCs) which is linked to enhancement of fish immune response. Therefore, we can recommend the dietary inclusion of bioorgano in aqua-feed along with QZ Toss™ application in rearing water as an efficient method to achieve feasible and sustainable fish production.

Keywords: Bioorgano®**;** Q Z Toss™**;** Biochemical**;** Histopathological

1. Introduction

For maximization of the efficacy and profitability of fish production, the global aquaculture is becoming more intensified. That is meant to achieve maximum production of fish from a minimum quantity of water, thus increasing fish yield. However, intensified fish culture is usually associated with poor managemental conditions because of fish overfeeding and overcrowding applied to increase productivity (Pulkkinen et al., 2009). Cultured aquatic animals under such stressful condition are usually prone to be infected with multiple opportunistic pathogens and leading to a reduction in fish immune response and performance. Among the major challenges of this system is rapid water quality deterioration, and higher susceptibility to fish diseases (AboState et al., 2017).

The excessive use of antibiotics for the treatment of fish diseases led to the development of antimicrobial-resistant pathogens, kills the beneficial microbiota in the gastrointestinal tract (GIT), and produces antibiotic residues in the fish body that may be harmful for human consumption (Gupta et al., 2019). To counteract these issues, scientists are exploring new strategies to improve water quality and replace the use of the antibiotics in fish farming. Probiotics, prebiotics and plants extracts have been proposed as candidates for these purposes (Mohammadian et al., 2018; Nandi et al., 2017).

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P ISSN: 2636-3003 EISSN: 2636-2996

Bioorgano is a commercial product containing two plant extracts (90% carvacrol, 10% thymol). Carvacrol and thymol are natural products present in the essential oil of oregano, oil of thyme and other plants.

QZ Toss™ is a commercially available product containing a probiotic bacterial mixture. QZ Toss™ is a special blend of three probiotic microbial cultures: *Bacillus subtilis*, *Bacillus lichenformis*, and *Bacillus megaterium*.

Here, we studied various health, growth, immune and biochemical parameters following the use of Bioorgano and, QZ Toss™. Additionally, we studied whether there is a synergistic effect of combing both Bioorgano and, QZ Toss™ on fish health.

2. Material and methods

2.1. Fish

We used a total number of 180 apparently healthy Nile tilapia *Oreochromus niloticus* (*O. niloticus*) fish with average body weight 50±5 gm in the experimental work. Fish were obtained from a private fish farm in El-Beheira Governorate and transported alive to the experimental facility in aerated plastic tanks.

2.2. Experimental tanks

Throughout the experimental period, fish were kept in 6 prepared concrete tanks (3×4×1 m. each).

Prior to the experimental period, fish were acclimatized for 2 weeks. The tanks were supplied with deep well water according to Innes (1966).

The continuous aeration was maintained in each pond using a 3hp electric air pump. Water temperature was kept naturally at 24±1 ºC.

2.2.1. Fish diets:

Fish were fed floating fish pellets containing 30% crude protein (Aller Aqua Egypt). According to the used fish size, the diet was daily provided at 5% of body weight as described by Eurell et al. (1978). The daily amount of food was offered on two occasions over day (at 9 AM and 1 PM).

2.2.2. Probiotics

1. Bioorgano (90% carvacrol, 10% thymol).
2. QZ Toss™:

As confirmed by the manufacturer (Keeton Industries USA), it's a blend of bacillus species contains 2×10^{12} cfu/kg namely, *Bacillus subtilis* 9×10^{11} cfu, *Bacillus amyloliquefaciens*, 8×10^{11} cfu, and *Bacillus lichinformis*, $3X10^{11}$ cfu/kg.

2.3. Preparation of experimental feed

Biogen® was mixed with the diet in a ratio of 1000 gm/ton feed with sunflower oil, applied and mixed with the feed and then left for drying.

2.4. Experiment

Table 1: Body weight in fish groups. Average initial fish body weight in all groups was about 50 g per fish

	Body weight (g)
Control	786±17
QZ Toss™	708±55
Bioorgano 250 gm/ton + QZ Toss™	814±20*
Bioorgano 250 gm/ton	798±25
Bioorgano 500 gm/ton + QZ Toss™	856±22*
Bioorgano 500 gm/ton	780±30

* significant at $p \leq 0.05$

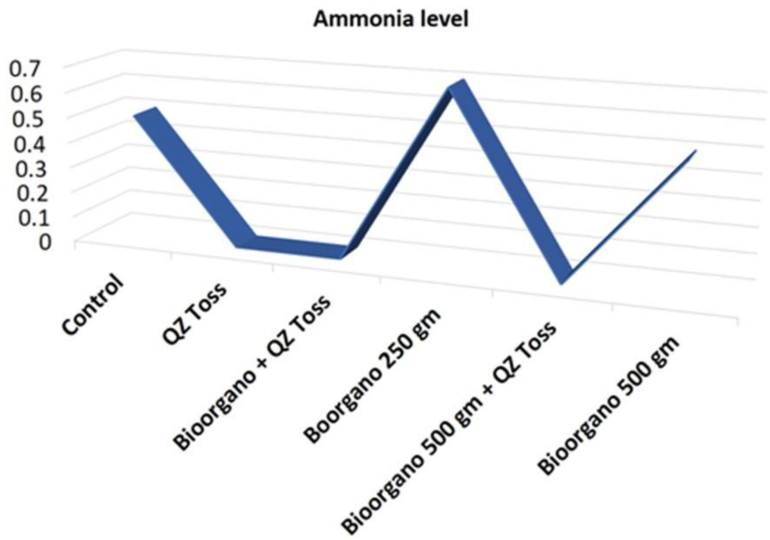

Figure (1): Water levels of ammonia in fish groups.

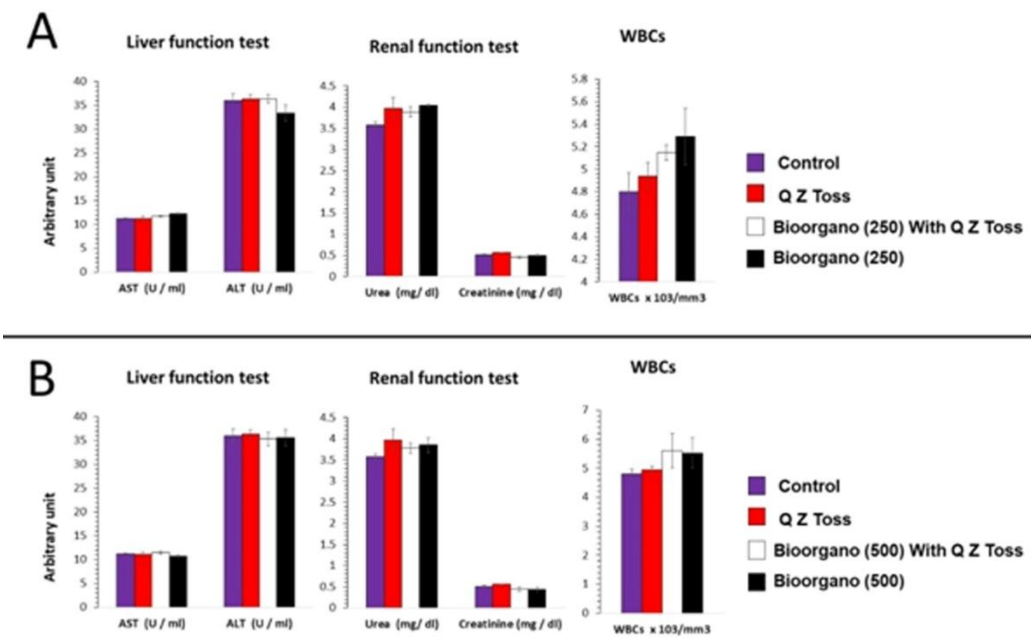

Figure (2): Biochemical analysis of Nile Tilapia after treatment with QZ Toss™ in water and bioorgano at 250 mg/kg (A) and bioorgano at 500 mg/kg of food (B). Statistical differences (P < 0.05) are denoted by asterisks. Data are expressed as the mean ± SE (n = 3).

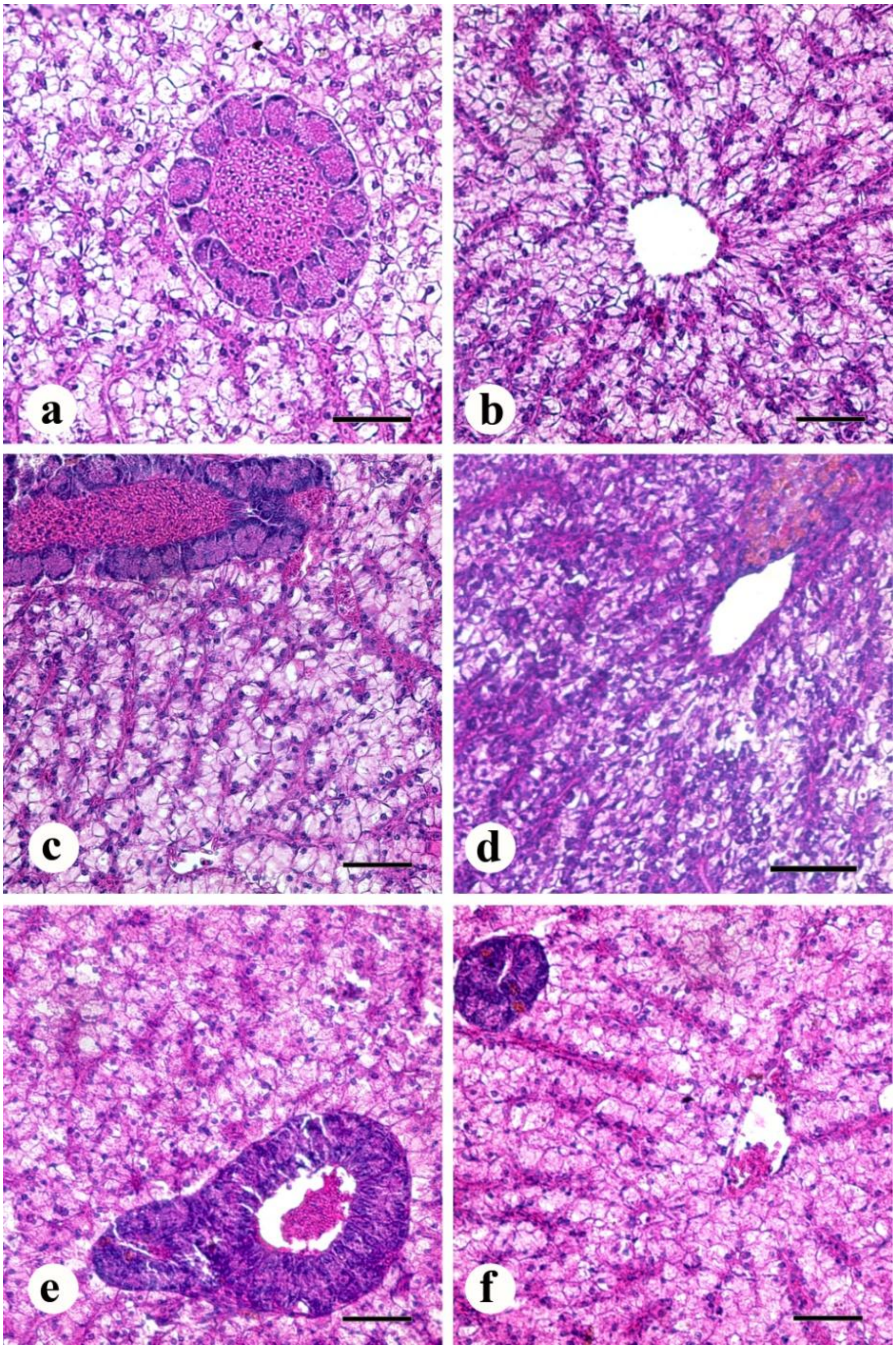

Figure (3): Histomorphology of Nile Tilapia hepatopancreas (a) Control group showing congestion in main blood vessels and sinusoidal spaces with diffuse vacuolar degeneration in hepatocytes. (b) QZ Toss™ only group showing diffuse vacuolar degeneration in hepatocytes with mild congestion in sinusoidal spaces. (c) Bioorgano 250 gm/ton + QZ Toss™ group showing congestion in main blood vessels and sinusoidal spaces with mild vacuolar degeneration in hepatocytes. (d) Bioorgano 250 gm/ton only group showing congestion in main blood vessels and sinusoidal spaces with mild vacuolar degeneration in hepatocytes. (e) Bioorgano 500 gm/ton + QZ Toss™ group showing mild congestion in main blood vessels and sinusoidal spaces with scarce vacuolar degeneration in hepatocytes. (f) Bioorgano 500 gm/ton only group showing mild congestion in sinusoidal spaces with scarce vacuolar degeneration in hepatocytes. Hematoxylin & Eosin stain. Scale bar = 50 μm.

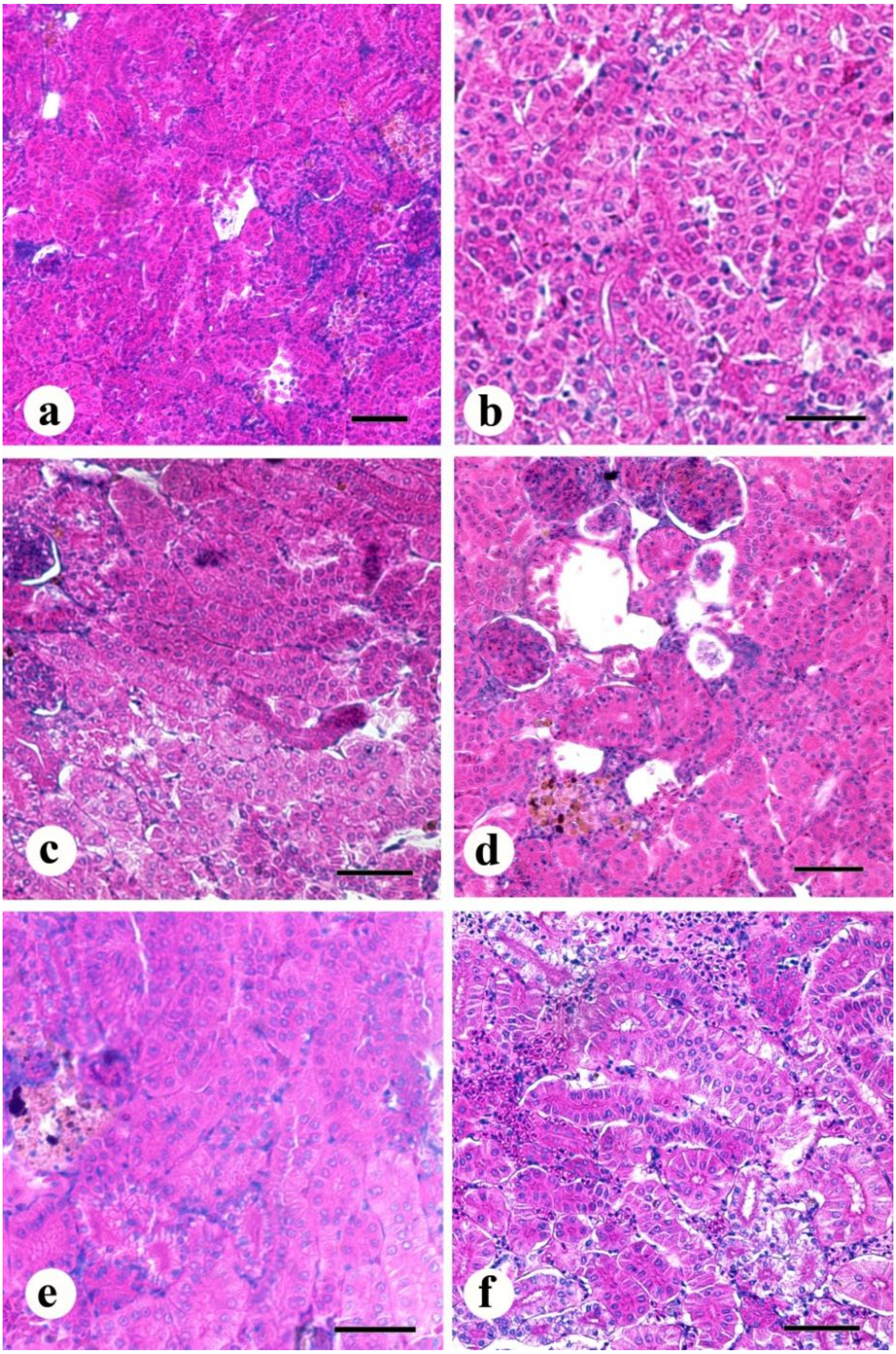

Figure (4): Histomorphology of Nile Tilapia posterior kidney (a) Control group showing focal areas of renal tubular necrosis. (b) QZ Toss™ only group showing normal tubular structure. (c) Bioorgano 250 gm/ton + QZ Toss™ group showing multifocal tubular degeneration and necrosis with mild activation of melano-macrophage centers. (d) Bioorgano 250 gm/ton only group showing focal tubular degeneration and necrosis with mild activation of melano-macrophage centers. (e) Bioorgano 500 gm/ton + QZ Toss™ group showing normal tubule-glomerular structure with activation of melanomacrophage centers. (f) Bioorgano 500 gm/ton only group showing focal mild vacuolar degeneration of renal tubular cells. Hematoxylin & Eosin stain. Scale bar = 50 µm.

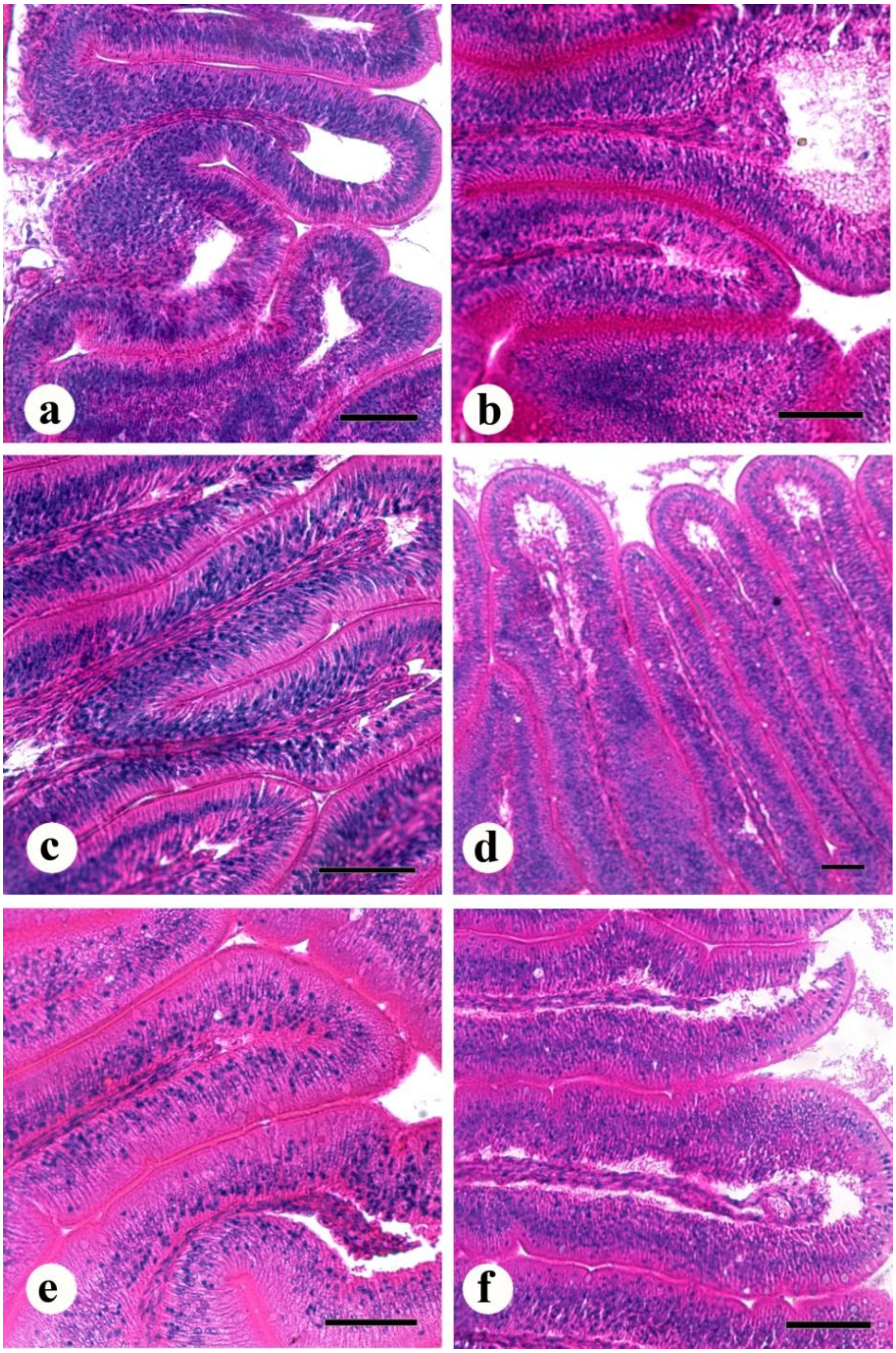

Figure (5): Histomorphology of Nile Tilapia Intestine (a) Control group showing moderate sub-epithelial edema. (b) QZ Toss™ only group showing mild sub-epithelial edema. (c) Bioorgano 250 gm/ton + QZ Toss™ group showing normal healthy epithelial structure with obvious increase in villar height. (d) Bioorgano 250 gm/ton only group showing normal healthy epithelial structure with obvious increase in villar height. (e) Bioorgano 500 gm/ton + QZ Toss™ group showing normal healthy epithelial structure with obvious increase in villar height. (f) Bioorgano 500 gm/ton only group showing normal healthy epithelial structure with obvious increase in villar height. Hematoxylin & Eosin stain. Scale bar = 50 μm.

One hundred eighty *O. niloticus* fish were distributed randomly in 6 concrete tanks, 30 fish / tank which filled with aerated deep well water. Fish in the 1st tank were fed regular feed till the end of experiment (30 days) and act as negative control. Fish in 2nd tank were fed regular feed, as well as QZ Toss™ in a dose of 2 g/ m^3 was added to the water, after that QZ Toss™ was added again as 1 g/m3 each week till the end of experiment. Fish in the 3rd tank were fed on ration containing 250 gm/ ton bioorgano with addition of QZ Toss™ exactly like the 2nd group. Fish in the 4th tank were fed on ration containing 250 gm/ ton bioorgano without application of QZ Toss™. Fish in the 5th tank were fed on ration containing 500 gm/ ton bioorgano with addition of QZ Toss™ exactly like the 2nd group. Fish in the 6th tank were fed on ration containing 500 g/ ton bioorgano without application of QZ Toss™ till the end of experiment.

The water in tanks receiving QZ Toss™ remained without change till the end of experiment, while the water in the tanks without QZ Toss™ was changed daily. The amount of ration was re-adjusted every week according to the fish body weight. Fish were kept under observation for any up normal signs. The level of ammonia in each tank was determined by ammonia kits at the end of experiment. At the end of the experiment the fish were weighted to estimate the growth rate.

2.5. Sampling

At the end of the experiment, the fish was immobilized on absorbent paper towel and kept motionless. The body surface was then cleaned and blotted dry. The blood samples were collected from the caudal vein on EDTA to determine haemoglobin and white blood cell count. Other blood samples were collected without anticoagulants for serum separation. The serum samples were stored at −20 °C for biochemical analysis.

After complete necropsy of the fish, fresh tissue specimens were collected from hepatopancreas, posterior kidney, intestine and gills were rapidly fixed in Davidson's fixative for 24 hours then transferred to 70% ethanol till processing proceeds, for histopathological examination.

2.5.3. Determination of some biochemical parameters

The serum samples were used to measure alanine aminotransferase (Pulkkinen et al.) and aspartate aminotransferase (AST), they were determined colorimetrically according to the methods described by Reitman and Frankel (1957). Serum urea and creatinine were determined colorimetrically according to the methods described by Fawcett and Scott (1960) and Bartles et al. (1972), respectively.

2.5.4. Total leucocyte count (TLC)

The total leucocyte count was determined by haemocytometry.

2.5.5 .Histopathological examination

The fixed tissue specimens were processed through the conventional paraffin embedding techniques (Suvarna et al., 2018). Paraffin blocks were cut as 4 µm-thick tissue sections. Then 2 replicates from the same section were mounted on slides then processed for Hematoxylin-Eosin (H&E) staining, cover-slipped then visualized by Light Microscope (Olympus BX43).

2.5.6 . Statistical analysis

All data were statistically analyzed using one-way Analysis of Variance (ANOVA) using GraphPad Prism 5 (San Diego, USA). All declarations of significance depended on ($p < 0.05$).

3. Results

3.1. Growth performance

The growth performance of *O. niloticus* fish fed on different concentrations of Bioorgano is summarized in Table (Mart et al.). The results revealed that both bioorgano supplemented groups showed increase in live body weight gain which was apparently significant in bioorgano with QZ Toss™™ group compared to control one.

3.2. Ammonia levels in water

Inclusion of QZ Toss™ in fish tanks water decreased ammonia levels in water as showed in figure (Mart et al.), where all groups of QZ Toss™ (QZ Toss™ only and bioorgano with QZ Toss™) showed zero levels of ammonia in water compared to a mean level of (0.5) ammonia in groups without QZ Toss™.

3.3. Haematogram and serum parameters

All experimental fish groups have normal haematological and serum biochemical findings (Figure 2).

Liver enzymes showed no significant different for AST, while bioorgano groups showed a slight non-significant decline in ALT activity as compared to control group (Figure 2). Indicating that there are no harmful effects of using QZ Toss™ and different concentration of Bioorgano on liver function.

For the assessment of the normal functioning of the kidneys, renal function tests are frequently used. Creatinine and Urea are end products of creatine phosphate and protein catabolism respectively. their accumulation in the body indicates impairment of kidney function.

Renal function tests represented in urea and creatinine showed normal values as the control group, excluding any drawbacks of bioorgano supplementation on kidneys function (Figure 2).

3.4. Histopathological findings

We performed histopathological analysis following Bioorgano and QZ Toss™ supplementation in various organs.

In the intestine (Figure 5), our results revealed that Carvacrol and thymol content of Bioorgano increased intestinal villi length regardless of the concentration used compared to the control group.

increase villi length is associated with increasing surface area and food absorption. That may explain at least in part the observed induction of fish growth rate.

In the posterior kidney (Figure 4), the main observed histopathological changes in Bioorgano supplemented group was the activation of melanomacrophages centers (MMCs) which are the aggregation of phagocytes and melanin pigment. their activation is linked to enhancement of fish immune response.

4. Discussion

With the increase of aquaculture production, Fish diseases have become a major problem in the fish farming industry (Das et al., 2013).The excessive use of broad-spectrum chemotherapeutics without regulations has led to the spread of drug-resistant pathogens (Das et al., 2013; Soliman et al., 2014).

Probiotics have been proposed as an alternative to antibiotics since it has the ability to reduce pathogenic organisms in the gastrointestinal tract of fish through their antagonistic activity in colonization site on the host's intestine (Mohammadian et al., 2018). Additionally, an increasing number of studies highlighted the fundamental role of probiotic bacteria on fish immunity (Nandi et al., 2017; Perez-Sanchez et al., 2011). Here we investigated the health effects of combing probiotics and plant extracts in Nile Tilapia.

In our experiment, Dietary supplementation of Bioorgano significantly enhanced growth rate in Nile Tilapia. that may be attributed at least in part to the flavouring effect of carvacrol which has been frequently used as a natural appetizer(Knowles et al., 2005) (Wadikar and Premavalli, 2011). Importantly, combining QZ Toss™ and Bioorgano gave the highest growth rates, that may indicate that both QZ Toss™ and Bioorgano enhance growth in different mechanisms and both synergizing each other.

Water quality is determined by various parameters like temperature, pH, carbon dioxide, alkalinity, hardness, ammonia, nitrite, and nitrate (Bhatnagar and Devi, 2013). Among these parameters, the ammonia level playing a fundamental role in fish aquaculture because of its toxicity and the ammonotelic nature of many fishes (excrete nitrogenous waste in the form of ammonia, not urea as in animals); meaning that there is a continuous production of toxic ammonia that needs to be eliminated (Hargreaves and Tucker, 2004; Ip and Chew, 2010).

Another main source of ammonia in fish ponds is the decomposition of organic matter in fish water system such as excess fish food and algae. thus, carefully managing the ammonia level is fundamental for fish health (Hargreaves and Tucker, 2004).

In this study, our results revealed that inclusion of QZ Toss™ in fish tanks significantly reduced ammonia level either with or without Bioorgano supplementation. This is reasonable because Bacillus strains (the main component of QZ Toss™) have the ability to convert ammonia to N_2 under aerobic conditions (Kim et al., 2005). However, addition of Bioorgano have no effect on ammonia level and this is an important point to consider. the aim of combing probiotics and various plant extracts in this study is to diversify and maximize the beneficial health effects.

Regarding the biochemical results, we didn't find any significant changes in in ALT and AST level compared to the control group, indicating that Biorgano treatment do not affect liver function tests (Canbek et al., 2008). However, Bioorgano supplementation slightly reduced ALT level but that reduction was not significant according to *P*-value.

Previous reports have shown that Carvacrol (one of the components of bioorgano) treatment progressively declined urea and creatinine level following gentamycin treatment (Ahmadvand et al., 2016), that highlighting the fundamental role of carvacrol as a kidney protector. However, our analysis showed normal levels of urea and creatinine as compared to the control group. That maybe because we didn't study the protective role of Bioorgano following exposure to a cytotoxic agent, we just compared between Bioorgano treated and control groups. Additionally, neither Biorgano nor QZ Toss™ elevated WBCs count in the blood. In our research, we tried to measure many health and immunity parameters in order to identify the ways by which Bioorgano enhance fish health.

The increase in intestinal Villi length has been associated with better nutrient absorption and feed efficiency. In contrast, shorter and thinner villi were associated with toxins (Awad et al., 2006). Because increased villus height means an increase in the absorptive surface area, expression of brush border enzymes, and nutrient transport systems which affect the digestive and absorptive function of the intestine (Awad et al., 2009).

longer villi usually indicate activation of intestinal cell proliferation, mitosis, and cellular turnover that reflects on its function (Fan et al., 1997; Samanya and Yamauchi, 2002).

In poultry, the effect of using probiotics on intestinal villi length and viability have been intensively studied (Awad et al., 2009). However, there is no much research on fish. A previous report in Tilapia showed that the inclusion of probiotic bacteria Bacillus subtilis and Bacillus toyoi promoted the epithelial layer thickness of the middle intestine (Nakandakare et al., 2013).

Here we tried to study not only the histopathological effect of using probiotics and plant extracts on intestinal epithelium and villi length in the intestine but also the histopathology effect on various organs.

Importantly, our results showed that the plant extracts in bioorgano progressively increased intestinal villi length compared to the control group. That induction was obvious in all bioorgano supplemented groups either at low or high concentration or with or without the inclusion of QZ Toss™.

From these results, we can conclude that the observed increase in growth rate following Bioorgano supplementation is not only due to the flavoring effect of carvacrol but also villi length and subsequent increase absorption surface area may be involved.

The histopathological images of other organs showed activation of melanomacrophage centers, especially in the posterior kidney.

Melanomacrophage centers (MMCs) are highly pigmented phagocytes composed of macrophage-like cells and fragments derived from phagocytosed cells, mainly erythrocytes, and pigments such as melanin, haemosiderin, and lipofuscin. MMCs usually present in haemolymphopoietic organs (liver, spleen, and kidney) of various non-mammalian vertebrates such as reptiles, amphibians, and fish (Passantino et al., 2014; Steinel and Bolnick, 2017).

The main role of MMCs is destruction, detoxification or recycling of endogenous and exogenous material, thus it acts as a defense mechanism. MMCs are indicators of Fish immune response and humoral immunity (Steinel and Bolnick, 2017).

Activation of MMCs following Bioorgano supplementation either alone or in combination with QZ Toss™ indicates that Bioorgano activates the fish immune system.

According to our results in this study, Bioorgano induces fish immunity by enhancement of expression of immune-related genes and by activation of MMCs in the posterior kidney.

Competing Interests

The authors have no conflict of interest.

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