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# Effect of Formic Acid and Biotin Supplementation on Growth Performance and Survival of the Carp Fish Challenged with *Vibrio parahaemolyticus* Infection

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## ABSTRACT

The present study was designed to examine the dietary supplementation effect of formic acid, biotin or their combination on growth performance, digestive enzymes activity, some serum biochemical parameters, histopathological alterations and the survival of common carp fish (*Cyprinus carpio*) challenged with *Vibrio parahaemolyticus* infection. Approximately, 150 carps (50±3g) were divided randomly into five experimental groups in triplicate, G1: negative control; without any supplementation; G2: positive control; without any supplementation then challenged with IP injection with 0.2 ml of ($1x10^5$CFU/ml) *V. parahaemolyticus* on day 45; G3: carps were fed a prepared ration supplemented with 0.2% formic acid for 45 days then challenged as previously; G4: carps were fed a prepared ration supplemented with 0.06mg/ kg biotin for 45 days then challenged; and G5: carps were supplemented with both formic acid and biotin for 45 days then challenged. After 45 days of feeding trials, a higher weight gain % and specific growth rate with lower FCR were observed in G5, G3, and G4, respectively, than the control groups. Total protein, albumin, globulin, and digestive enzymes activity were significantly higher in all the supplemented groups when compared with the control, especially G5. After 45 days, decreased levels of ALP, ALT, AST, creatinine, and urea were recorded in all the supplemented groups compared to the control. The survival rate of carps challenged with *Vibrio parahaemolyticus* increased from 46.6% in G2 up to 93.3, 86.6 and 83.3% in G5, G3, and G4, respectively. Histopathological alterations revealed the appearance of normal structure of hepatic, renal and splenic tissue for G5 compared with G3 and G4, respectively. Generally, it could be stated that formic acid and biotin could be good choices as dietary additives targeting the enhancement of growth performance and survival rate.

## INTRODUCTION

Aquaculture, specifically in developing countries, rapidly expands to meet the global demand for dependable food sources that are rich in protein **(FAO *et al*., 2023)**.

Common carp is one of the most valuable fish all over the world. An estimated 4.2 million tons are produced annually, which accounts for approximately 7.7% of the total

fish farming industry **(FAO, 2020)**. Many threats and microbial diseases are commonly linked to intensive carp culture, which negatively impacts sustainable fish farming **(Pucher, 2013)**. Vibriosis (genus *Vibrio* and family Vibrionaceae) is one of these microbial diseases that is the most prevalent in both wild and cultured fish. Massive mortality in fish farming systems across the globe is attributed to vibriosis **(Thompson *et al.*, 2004; Cheng *et al.*, 2005)**. Bacterial species, including *Vibrio parahaemolyticus, V. anguillarum, V. salmonicida, V. alginolyticus, V. fluvialis, V. pelagius,* and *V. vulnificus* are the predominant bacterial pathogens of the genus *Vibrio* that cause significant mortality in numerous fish species **(Sonia & Lipton, 2012; Dhayanithi *et al.*, 2013; Dhayanithi *et al.*, 2015)**. The occurrence of Vibriois outbreaks is predominantly attributed to stressors, such as elevated temperatures, dissolved oxygen deficiency, substandard water quality, dense population densities and other similar environmental conditions **(Fioravanti & Florio, 2017)**. Hemorrhages on the skin and base of fins, pale gills, dark skin, skin ulcers, ascites, exophthalmia, corneal opacity, splenomegaly, and enteritis are some of the common symptoms exhibited by Vibrio-infected fish **(Toranzo *et al.*, 2005)**.

As a result, preventative measures against infectious diseases must be implemented to improve the survival rate, immunity and growth performance of fish. Regrettably, fish farmers frequently resort to the use of chemical disinfectants and antibiotics as a primary method of managing infectious agents. However, the residues in fish flesh that result from the improper and excessive use of chemicals and antibiotics have sparked public health concerns due to their numerous adverse effects and environmental hazards **(Harper, 2007; Brown *et al*., 2021)**.Therefore, resorting to using natural and safe alternatives to antibiotics that stimulate growth and immunity has become an absolute necessity. Increasing the efficiency of the feed is one approach to minimizing these risks **(Takase, 2023)**. In the promotion of fish health and growth, a multitude of compounds have been utilized as feed additives, yielding favorable results **(Marimuthu *et al*., 2022; Vijayaram *et al*., 2023)**. Organic acids are one of these additives utilized in fish culture for this purpose; therefore, dietary acidifier use is a method for improving the health conditions of fish culture **(Jedi *et al.*, 2021)**.

A multitude of investigations have revealed the effects for various organic acids and their salts, including formic acid, butyric acid, fumaric acid, propionic acid, malic acid, lactic acid, sorbic acid, acetic acid, and citric acid, among others, on fish growth efficiency, disease tolerance, gastrointestinal health, and immune function **(Adams & Boopathy, 2013; Reda *et al*., 2016; TranNgoc *et al*., 2018; das Neves *et al*., 2021; da Silva *et al*., 2023)**. The introduction of formic acid or citric acid into the diets of Caspian Sea brown trout (*Salmo trutta caspius*), rainbow trout (*Oncorhynchus mykiss*), sea bream (*Pagrus major*) and Asian seabass (*Lates calcarifer*) improved the growth and bioavailability of minerals, including magnesium, calcium, phosphorus, and iron **(Jun-**

**sheng *et al.*, 2006; Reshahri *et al.*, 2019; Kalantarian *et al.*, 2020; Mohammadian *et al.*, 2020)**.

Formic acid salts have been linked in prior research to disease resistance **(Ramli *et al.*, 2005; Ng *et al.*, 2009)**,and enhanced performance **(Ng *et al.*, 2015; Hoseinifar *et al.*, 2016)**in different cultured fish species.

Clearly, acidification of fish feed decreases the microbial load by decreasing the gut pH; furthermore, cell death results from the diffusion of organic acids into bacterial cells, which decreases the cytoplasmic pH of the bacterial cells **(Booth & Stratford, 2003)**.

Anti-*Vibrio* spp. activity has been attributed to organic acids, including formic acid, which makes them an essential category of additives **(Mine & Boopathy, 2011; Adams & Boopathy, 2013; Da Silva *et al.*, 2013)**.

In addition to organic acids, vitamins have a positive effect on fish health. Biotin, awater-soluble vitamin commonly associated with the vitamin B complex, is involved in a variety of distinct carboxylation and decarboxylation processes **(Zhang *et al.*, 2016)**. Biotin could be used for the enhancement of growth performance and immunity. It functions as a coenzyme for a number of enzymes that fix $CO_2$, including propionyl-CoA carboxylase (PCC), methylcrotonoyl-CoA carboxylase, acetyl-CoA carboxylase, and pyruvate carboxylase (PC), and these enzymes are essential for processes, including gluconeogenesis, fatty acid synthesis & degeneration, and the Krebs cycle **(Shiau & Chin, 1999)**. Therefore, biotin is essential for the regulation of amino acid, carbohydrate and lipid intermediary metabolism **(Albarracin *et al.*, 2008; Larrieta *et al.*, 2012)**.

Biotin serves a pivotal role in systemic processes such as immunity in addition to being a metabolic component and carboxylase prosthetic group **(Báez-Saldaña & Ortega, 2004; Fernandez-Mejia, 2005)**.

The purpose of this trial was to examine the impact of dietary supplementation of biotin, formic acid or their combination on growth performance, digestive enzymes activity, some serum biochemical parameters, survival, and histopathological changes in common carp fish infected with *Vibrio parahaemolyticus*.

## MATERIALS AND METHODS

### Experimental design

A sample of 180 common carp fish, seleced from a private fish farm in Kafr El-Sheikh Governorate, appeared to be in good health with no visible lesions and normal behavioral reflexes (50± 3g/fish), was moved to the Tanta Lab of AHRI for acclimatization purposes 14 days prior to the experiment. Fish were introduced into glass aquaria measuring 70×40×30cm and containing chlorine gas-free tap water at a temperature of 25±1°C **(Innes, 1966)** with continual aeration using an air compressor. Carps were fed 3% of their body weight twice daily (at 08:00 and 15:00)**(Eurell *et al.*,**

**1978)**, whereas carps weights were measured weekly for adjusting the amount of feed taken. Twice a week, 30% of the aquarium water was replaced and fish feces were siphoned manually. For a period of 45 days, carps were cultivated in triplicates (10 fish per aquarium) and divided into five groups (G) (30 fish/G). The following effects of formic acid and biotin were estimated using a pilot trial: G1:Negative control; for 45 days, fish were basally fed a commercial pellet, and on day 45, fish were injected intraperitoneally (IP) with 0.1ml of injectable 0.9% sterile saline;G2:Positive control; directly after 45 days of feeding basal ration, fish were IP injected with 0.2mL of the LD50 dose of the *V. parahaemolyticus* strain, which was predetermined ($1x10^5$CFU/fish); G3: For 45 days, fish were provided with a prepared diet supplemented with 0.2% formic acid, on day 45, the fish were subsequently inoculated with *V. Parahaemolyticus*; G4: Fish were infected on day 45 after being fed a prepared ration supplemented with 0.06mg/kg biotin for a period of 45 days, and G5: After 45 days of feeding a prepared diet supplemented with 0.2% formic acid and 0.06mg/kg biotin, the fish were inoculated on day 45 with *V. Parahaemolyticus*.

Daily examinations of the experimentally infected carps were conducted after infection, and a record of the clinical signs, mortalities, and necropsy findings was maintained for a period of 15 days. The *V. Parahaemolyticus* pathogen was re-isolated from the liver, spleen, and kidney of experimentally infected fish that had become morbid to confirm that the infection was the cause of mortality.

***Vibrio parahaemolyticus* and challenge test**

A previously identified virulent strain of *V. parahaemolyticus* was obtained from Poultry and Fish Diseases Department, Fac. Vet. Med. Alex. Univ. Egypt. After 45 days of feeding, infected groups were IP injected with 0.2mL of $LD_{50}$dose of *V. parahaemolytics* strain ($1x10^5$CFU/fish), which was previously determined, 10 days before the time of injection **(Reed & Muench, 1938)**. The trial documented the mortality and survival rates of fish for a duration of two weeks following the infection.

**Growth parameters and survival rate calculations**

The fish's weight was recorded on days 0 and 45; to determine growth parameters, all fish were stopped feeding for 24h before weighing, and each fish individual was then weighed. Parameters were calculated as follows:Weight gain (g) = final fish wt (g) −initial fish wt(g); Weight gain (%) = (final fish wt−initial fish wt)/initial fish wt × 100;Specific growth rate (SGR) = 100 × [(Ln final fish wt) − (Ln initial fish wt.)]/days fed;Feed conversion ratio (FCR) = feed intake (g)/wt gain (g), and Fish survival %= 100 × final number/initial number.

**Blood samples**

Blood samples were taken three times throughout the period of the experiment from all groups; on the 25th & 45th days, as well as post-challenge (at the end of the

experiment). According to **Stoskopf (1993)**, carps were not fed for the preceding 24 hours before being sampled. A 50mg/L solution of benzocaine was utilized to anesthetize fishes (9 fish/G). Blood was collected from the caudal vein of the fish utilizing a clean, dry centrifuge tube free from any anticoagulant drugs. For serum collection, the collected blood was centrifuged at 5000rpm for 5 minutes at room temperature; it was then frozen at -20°C for subsequent assays.

Some serum biochemical investigations were measured by enzymatic methods using an automated analyzer such as hepatic and kidney health indicators:Albumin **(Reinhold, 1953)**, alanine aminotransferases and aspartate (ALT and AST) activities **(Reitman & Frankel, 1957)**, urea **(Batton & Crouch, 1977)**, total protein (TP) **(Doumas *et al.*, 1981)**, creatinine **(Houot, 1985)**,and , and alkaline phosphatase (ALP) **(Tietz, 1995)**.The mathematical determination of globulin required the albumin value to be subtracted from the TP. Subsequently, the albumin/globulin ratio was approximated through the division of the two values.

Commercial kits (Spectrum, ELITech, BioSystems and Biomed Companies, Egypt) were utilized to determine all tests in strict adherence to the guidelines provided by the manufacturers.

**Determination of digestive enzymes**

To assess the effects of formic acid and biotin on digestive enzymes, in each group, nine fish were anesthetized with eugenol (1:10,000; Shanghai Reagent Corp., Shanghai, China). Three fish were selected per tank; tissues were immediately dissected, including the intestine and hepatopancreas. This step was followed by the procedures described in the succeeding lines. The intestine was emptied directly and subjected to three washes using ice-cold phosphate buffer (pH 7.0, 200mM).The intestine and hepatopancreas were subsequently removed and homogenized with an electric homogenizer (XHF-D, Xinzhi, China). Following homogenization, the homogenate was centrifuged at 5000rpm for 5 minutes while cooled. The lipase and amylase activities were determined using the supernatant in accordance with the protocols outlined by **Caraway (1959)** and **Moss and Henderson (1999)** utilizing kits supplied by spectrum (Cairo, Egypt) and biodiagnostic (Giza, Egypt), respectively.

**Histopathological sampling and examination**

On day 45 and post-challenge, liver, spleen, and kidney samples were obtained from three fish per group; these samples were inspected for gross abnormalities prior to fixation in 10% neutral buffered formalin. After dehydration, clarification, and cleaning, the fixed samples were paraffin embedded. At a thickness of 5 microns, the paraffin blocks were sectioned. According to **Bancroft and Layton (2012)**, the sections were stained using hematoxylin and eosin solutions before being examined under a light microscope (Olympus BX50, Japan).

### Water quality parameters

Water samples were collected at two-weekly intervals in order to measure the following water quality parameters. Salinity measurement was assessed by utilizing a salinity meter (YSI Eco Sense EC300 Salinity/Conductivity 151, China); water temperature by utilizing a digital thermometer; dissolved oxygen, utilizing a digital meter (HI 9142, HANNA, China), and via utilizing PH indicator strips, the PH (MColorpHast-Germany) was measured, while kits were used for determining the concentration of unionized ammonia (NH3) and total ammonia in water (USA, Virginia Company, lot. No.201134).

In accordance with the recommended standard guidelines, all water quality parameters should be within the acceptable limits **(APHA, 1998)**.

### Statistical analysis

Statistical tests were conducted using the one-way ANOVA test to determine the optimal treatment, with significance considered at $P< 0.05$. Data analysis was performed using SPSS 20 software and presented as mean ± SE (n = 5)**(Petrie & Watson, 1999)**.

## RESULTS

### Clinical signs

Carps from G1 exhibited no external lesions or mortalities throughout the experiment. However, within the first week of infection, experimentally infected carps with *V. parahaemolyticus* exhibited internal and external symptoms that were similar to those observed in naturally occurring infections (Fig. 1a- g), such as loss of reflexes, anorexia, showing hemorrhagic areas on skin and fins (Fig.1a-b), hemorrhagic vent, abdominal distention (Fig.1c, d), points of hemorrhage, skin ulcerations and erosions with scales detachment. The P.M. lesions were oedema, ascites, liver enlargement and paleness **(Fig.1e-f)**, kidney enlargement and congestion of the gills and spleen (Fig.1f-g). To validate the etiological agent responsible for the infection, *V. parahaemolyticus* was re-isolated from the spleen, liver, and kidney of infected and freshly died fish.


**Fig.1.(a-g)**Experimentally infected common carp fish with *Vibrio parahaemolyticus* showing: **a.** Hemorrhagic patches on skin with ascites,**b.** Hemorrhagic areas on tail fin, **c-d.** Abdominal ascites with hemorrhagic vent, **e.** Paleness and enlargement of liver with necrotic area, **f.** Congestion and enlargement of kidney, and **g.** Paleness and enlargement of the different internal organs, including liver, kidney and, spleen

**Table 1.**Effect of formic acid and biotin or their combination on growth performance and survival rates (Mean ± SE)

| Item | G1 | G2 | G3 | G4 | G5 |
|---|---|---|---|---|---|
| **Intial weight (g)** | **49.66±0.88$^{a}$** | **50.66±1.45$^{a}$** | **50.33±1.2$^{a}$** | **50.33±0.66$^{a}$** | **50±1.0$^{a}$** |
| **Final weight (g)** | **61.33±0.66$^{c}$** | **61.66±2.02$^{c}$** | **69±0.57$^{ab}$** | **66.66±0.88$^{b}$** | **71±0.57$^{a}$** |
| **Weight gain (g)** | **11.6±0.33$^{d}$** | **11±0.57$^{d}$** | **18.6±0.66$^{b}$** | **16.3±0.33$^{c}$** | **21±0.57$^{a}$** |
| **WG%** | **23.52±1.01$^{d}$** | **21.68±0.52$^{d}$** | **37.19±2.24$^{b}$** | **32.45±0.57$^{c}$** | **42.07±1.96$^{a}$** |
| **SGR(%/day)** | **0.46±0.01$^{c}$** | **0.43±0.0$^{c}$** | **0.7±0.03$^{b}$** | **0.62±0.0$^{b}$** | **0.78±0.03$^{a}$** |
| **Feed intake (g feed/ fish)** | **70.66±0.88$^{b}$** | **72.33±2.33$^{ab}$** | **75.33±1.2$^{ab}$** | **75±1.0$^{ab}$** | **75.66±1.33$^{a}$** |
| **FCR** | **6.06±0.24$^{a}$** | **6.58±0.13$^{a}$** | **4.04±0.19$^{c}$** | **4.59±0.08$^{b}$** | **3.6±0.15$^{c}$** |
| **Survival %** | **100** | **46.66** | **86.66** | **83.33** | **93.33** |

The various letters in the same raw indicate statistically significant differences when $P< 0.05$. G1: Negative control, G2: Positive control, G3: Formic acid, G4: Biotin, G5: Formic acid+ biotin.

**Growth performance and survival rates**

As indicated in Table (1), all the values of growth performance (weight gain, weight gain%, SGR and final body weight) were significantly increased in all supplemented groups than the control on days 25 and 45. Moreover, they were arranged in the following descending order: G5, G3, G4, G1 and G2. Significantly lower FCR was observed in the supplemented groups compared to the control group. The carp's survival rate challenged with *Vibrio parahaemolyticus* increased from 46.6% in G2 up to 93.3, 86.6 and 83.3% in G5, G3 and G4, respectively. The best results of growth performance and survival rate were obtained in G5.

**Serum biochemical parameters**

As shown in Table (2), albumin, globulin, and total protein after 25 and 45 days of formic acid and biotin supplementation were significantly higher in all supplemented groups than the control group (G1). This increase was particularly notable after 45 days of supplementation, with the levels following a descending order: G5, G4, and then G3. Post challenge, the levels of TP, albumin, and globulin in all groups were arranged in the following descending order: G1, G5, G4, G3 then G2. The levels of G2 were significantly less than those of the other groups. On other hand, supplementation of formic acid and biotin insignificantly affected the albumin/ globulin (A/G) ratio in all groups.

Regarding AST, ALT, and ALP activities, after 25 days of supplementation, the AST and ALT activities in G3 were not significantly different from the control, while

there was an increase in ALP activity. On the other hand, in G4 and G5, the activities of ALT, AST, and ALP were significantly lower than the control.

The activities of ALT, AST, and ALP results after 45 days revealed a decrease in their levels in all supplemented groups than the control, in the following ascending sequence: G5, G3, and G4. The enzymatic activities increase subsequent to the challenge, particularly in G2 was arranged descendingly as follows: G5, G3, G4, and G2. Regarding urea and creatinine concentrations, the results also revealed a decrease in their levels than the control, especially after 45 days in the following ascending order: G5, G3, and G4. Post challenge, all supplemented groups elicited a significant increase than G1,with G2 showing the highest increase, followed by G5, G3, and G4 in an ascending order.

**Table 2.** Effect of formic acid and biotin or their combination on some of serum biochemical parameters (Mean ± SE).

| G | Period | TP (g/dl) | Albumin (g/dl) | Globulin (g/dl) | A/G ratio | AST (U/L) | ALT (U/L) | ALP (U/L) | Urea (mg/dl) | Creatinine (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|---|
| G1 | At 25 days | 2.86± 0.05 $^{c}$ | 1.09± 0.01 $^{b}$ | 1.77± 0.05 $^{cd}$ | 0.61± 0.01 $^{b}$ | 86.13± 0.05 $^{c}$ | 49.33± 0.05$^{a}$ | 35.67± 0.05$^{ab}$ | 8.63± 0.05$^{a}$ | 0.17± 0.01 $^{a}$ |
| | At 45 days | 2.90± 0.05 $^{c}$ | 1.17± 0.01 $^{d}$ | 1.73± 0.01 $^{d}$ | 0.67± 0.01$^{bc}$ | 85.05± 0.01 $^{b}$ | 47.66± 0.01$^{b}$ | 36.09± 0.01$^{b}$ | 8.90± 0.05 $^{a}$ | 0.18± 0.01 $^{a}$ |
| | Post challenge | 2.93± 0.01 $^{a}$ | 1.15± 0.05 $^{a}$ | 1.78± 0.05 $^{a}$ | 0.64± 0.01 $^{a}$ | 84.86± 0.05 $^{e}$ | 47.27± 0.05 $^{e}$ | 34.12± 0.05 $^{e}$ | 8.86± 0.01 $^{e}$ | 0.17± 0.01 $^{d}$ |
| G2 | At 25 days | 2.76± 0.05 $^{c}$ | 1.07± 0.01 $^{c}$ | 1.69± 0.05 $^{d}$ | 0.63± 0.01 $^{a}$ | 87.16± 0.01 $^{a}$ | 48.34± 0.01$^{b}$ | 38.33± 0.01 $^{a}$ | 8.73± 0.01 $^{a}$ | 0.18± 0.01 $^{a}$ |
| | At 45 days | 2.8± 0.01 $^{d}$ | 1.47± 0.01 $^{b}$ | 1.33± 0.01 $^{e}$ | 1.10± 0.05 $^{a}$ | 85.23± 0.01 $^{a}$ | 49.67± 0.01$^{a}$ | 32.66± 0.01$^{c}$ | 8.83± 0.01$^{a}$ | 0.19± 0.01$^{a}$ |
| | Post challenge | 1.96± 0.01 $^{d}$ | 0.77± 0.01 $^{b}$ | 1.19± 0.05 $^{c}$ | 0.64± 0.01 $^{a}$ | 153.51± 0.01 $^{a}$ | 101.6± 0.05 $^{a}$ | 68.03± 0.01 $^{a}$ | 15.66± 0.01 $^{a}$ | 0.28± 0.01 $^{a}$ |
| G3 | At 25 days | 3.13± 0.01 $^{b}$ | 1.23± 0.01 $^{a}$ | 1.90± 0.05 $^{bc}$ | 0.64± 0.01 $^{a}$ | 86.34± 0.01 $^{b}$ | 48.38± 0.01$^{b}$ | 38.92± 0.01 $^{a}$ | 8.20± 0.05 $^{c}$ | 0.14± 0.01 $^{b}$ |
| | At 45 days | 3.23± 0.01 $^{b}$ | 1.24± 0.01 $^{c}$ | 1.99± 0.01 $^{c}$ | 0.62± 0.01 $^{c}$ | 80.08± 0.01 $^{d}$ | 42.15± 0.01$^{d}$ | 32.16± 0.01$^{d}$ | 7.96± 0.01 $^{c}$ | 0.12± 0.01 $^{c}$ |
| | Post challenge | 2.60± 0.05 $^{c}$ | 1.10± 0.05 $^{a}$ | 1.50± 0.05 $^{b}$ | 0.73± 0.01 $^{a}$ | 96.30± 0.05 $^{c}$ | 62.31± 0.01$^{c}$ | 46.73± 0.05 $^{c}$ | 11.63± 0.01 $^{c}$ | 0.22± 0.01$^{b}$ |
| G4 | At 25 days | 3.13± 0.01 $^{b}$ | 1.09± 0.01$^{b}$ | 2.04± 0.01 $^{ab}$ | 0.53± 0.01 $^{d}$ | 83.27± 0.01$^{d}$ | 46.14± 0.01 $^{c}$ | 35.18± 0.01 $^{ab}$ | 8.33± 0.01$^{b}$ | 0.17± 0.01 $^{a}$ |
| | At 45 days | 3.30± 0.01 $^{b}$ | 1.25± 0.01 $^{c}$ | 2.05± 0.01$^{b}$ | 0.60± 0.05 $^{c}$ | 82.13± 0.01 $^{c}$ | 44.03± 0.01 $^{c}$ | 36.72± 0.01 $^{a}$ | 8.16± 0.01$^{b}$ | 0.15± 0.01$^{b}$ |
| | Post | 2.73± | 1.13± | 1.60± | 0.70± | 112.16± | 73.66± | 50.08± | 12.80± | 0.23± |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **challenge** | **0.01 [b]** | **0.01 [a]** | **0.05 [b]** | **0.05 [a]** | **0.01 [b]** | **0.01 [b]** | **0.01 [b]** | **0.05 [b]** | **0.01 [b]** |
| **G5** | **At 25 days** | **3.33± 0.01 [a]** | **1.24± 0.01 [a]** | **2.09± 0.01[a]** | **0.59± 0.01 [c]** | **80.61± 0.01 [e]** | **42.08± 0.01 [d]** | **33.15± 0.01 [b]** | **7.93± 0.01 [d]** | **0.13± 0.01 [b]** |
| | **At 45 days** | **3.70± 0.01 [a]** | **1.61± 0.01 [a]** | **2.09± 0.01 [a]** | **0.77± 0.01 [b]** | **78.38± 0.01 [e]** | **40.24± 0.01 [e]** | **31.67± 0.01 [e]** | **7.73± 0.01 [d]** | **0.11± 0.01 [c]** |
| | **Post challenge** | **2.86± 0.01 [a]** | **1.08± 0.01 [a]** | **1.78± 0.01 [a]** | **0.60± 0.05 [a]** | **89.31± 0.01 [d]** | **58.67± 0.01 [d]** | **42.34± 0.01 [d]** | **10.16± 0.01 [d]** | **0.19± 0.01 [c]** |

The various letters in the same raw indicate statically significant differences when $P< 0.05$.G1: Negative control, G2: Positive control, G3: Formic acid, G4:Biotin, G5: Formic acid+ biotin.

**Table 3.** Effect of formic acid and biotin or their combination on amylase and lipase activities (Mean ± SE).

| Group | Period | Amylase (U/L) | Lipase(U/L) |
|---|---|---|---|
| **G1** | **At 25 days** | **52.67±0.01[e]** | **34.30±0.01[d]** |
| | **At 45 days** | **54.33±0.01[d]** | **35.66±0.01[c]** |
| | **Post challenge** | **55.66±0.01[a]** | **37.81±0.01[a]** |
| **G2** | **At 25 days** | **53.63±0.01[d]** | **36.92±0.01[c]** |
| | **At 45 days** | **52.55±0.01[e]** | **34.33±0.01[c]** |
| | **Post challenge** | **40.34±0.01[e]** | **27.67±0.01[e]** |
| **G3** | **At 25 days** | **60.98±0.01[b]** | **38.55±0.01[b]** |
| | **At 45 days** | **63.88±0.01[b]** | **41.40±0.23[b]** |
| | **Post challenge** | **47.67±0.01[c]** | **29.63±0.01[c]** |
| **G4** | **At 25 days** | **58.33±0.01[c]** | **38.6±0.05 [b]** |
| | **At 45 days** | **60.62±0.01[c]** | **40.64±0.01[b]** |
| | **Post challenge** | **43.40±0.01[d]** | **29.27±0.01[d]** |
| **G5** | **At 25 days** | **64.08±0.01[a]** | **41.85±0.01[a]** |
| | **At 45 days** | **68.88±0.01[a]** | **43.71±0.93[a]** |
| | **P. challenge** | **49.67±0.01[b]** | **31.33±0.01[b]** |

The various letters in the same raw indicate statically significant differences when $P< 0.05$.G1: Negative control, G2: Positive control, G3: Formic acid, G4: biotin, G5: Formic acid+ biotin.

**Digestive enzymes activities**

Table (3) provides a summary of the results regarding the evaluation of the activity of lipase and amylase, at the 25- and 45-day marks of the experiment, lipase and amylase activities were elevated in all supplemented groups especially in G5 and G3 than G1, which indicates that formic acid had more influence on lipase and amylase activities

than biotin, and their activities were proportionally increased with increasing the duration of supplementation of 0.2% formic acid up to 45 days. Post challenge, all supplemented groups recorded a significant good difference in the activities of amylase and lipase than G2.

## Histopathological alterations

### *Microscopic examination of hepatopancreas samples*

G1: Normal control showed apparent intact basophilic pancreatic acinar cells with luminal zymogenic granules (blue star). Evidently intact hepatic parenchyma, accompanied by copious records of intact hepatocytes exhibiting intact nuclear and subcellular characteristics (arrow)(Fig.2A.B**)**. However, post infection, G2 challenged with *V. parahaemolyticus* showed the same records as G1, with moderate higher records of congested hepatic BVs (blue star) (Fig. 2C, D). Moreover, G3 supplemented with formic acid and challenged showed more organized histological features of hepatic parenchyma with apparent higher records of intact hepatocytes with minimal records of degenerative changes (Fig. 2E, F). Furthermore, G4 supplemented with biotin and challenged showed the same records as G1, with mild congested hepatic BVs (blue star) (Fig. 2G, H). In addition, G5 supplemented with formic and biotin and challenged showed apparent intact basophilic pancreatic acinar cells with luminal zymogenic granules (blue star). Apparent intact hepatic parenchyma with abundant records of intact hepatocytes displayed intact nuclear and subcellular details (arrow)(Fig. 2I,J).

### *Microscopic examination of renal samples*

G1: Normal control showed many records of apparent intact nephronal segments with an intact epithelial lining showing intact subcellular details (black arrow) with focal figures of melanomacrphage (blue star).(Fig. 3A, B). Post challenge, G2 challenged with *V. parahaemolyticus* and without any supplementation showed moderate tubular epithelial degenerative changes with focal records of nuclear pyknosis or vacuolated cytoplasm (black arrow), accompanied with significant increase of interstitial lymphoreticular tissue (blue star) with higher intraepithelial lymphocytic infiltrates (Fig. 3C, D). Moreover, G3 suppemented with formic and challenged showed an apparent intact tubular epithelium with intact subcellular details (black arrow), with a depletion of interstitial lymphoreticular tissue (Fig. 3E, F). Furthermore, G4 supplemented with biotin and challenged showed marked records of tubular damage and loss (black arrow) with a severe dilatation and congested BVs (blue star), and a significant depletion of interstitial lymphocytic infiltrates (red star) (Fig. 3G, H). However, G5 supplemented with formic and biotin and challenged showed a higher tubular protective efficacy with a mild persistent damage and loss (black arrow), along with persistent records of higher interstitial infiltrates (blue star) (Fig. 3I, J)

***Microscopic examination of splenic samples***

All samples from different groups post challenge showed normal organized histological features of a splenic tissue with intact white pulp and red pulp (Fig. 4A, C, E). For Group2 (infected) and Group4, which was supplemented with biotin only and challenged, only mild higher figures of melanomacrophages (black arrows) were observed compared to the other samples (Fig. 4B, D).

Samples taken from liver, kidney, and spleen directly after 45 days of supplementation of formic acid, biotin, or their combination were the same as G1, which indicates that these additives don't cause pathological alterations or hazards for them.

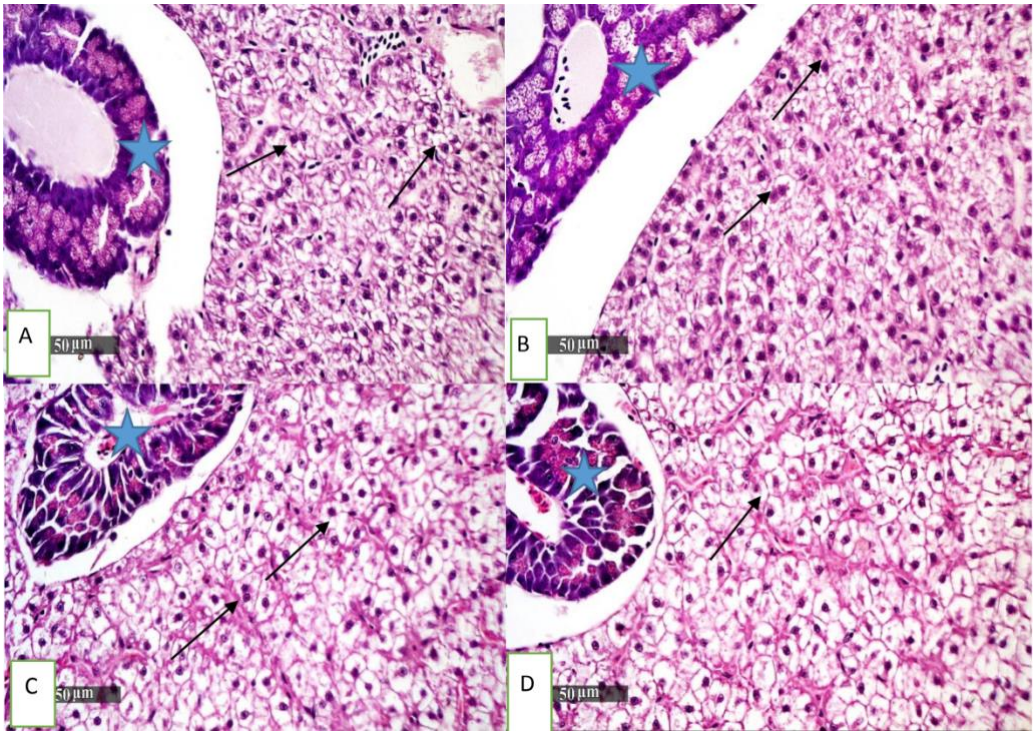


**Fig. 2.(A, B)** Hepatopancreas of G1 showed normal control apparent intact basophilic pancreatic acinar cells with luminal zymogenic granules (blue star). Apparent intact hepatic parenchyma with abundant records of intact hepatocytes showing intact nuclear and subcellular details (arrow)(H&E).**(C, D)** Hepatopancrease of G2 challenged with *V.parahaemolyticus* showed the same records as G1, with moderate higher records of congested hepatic BVs (blue star)(H&E)

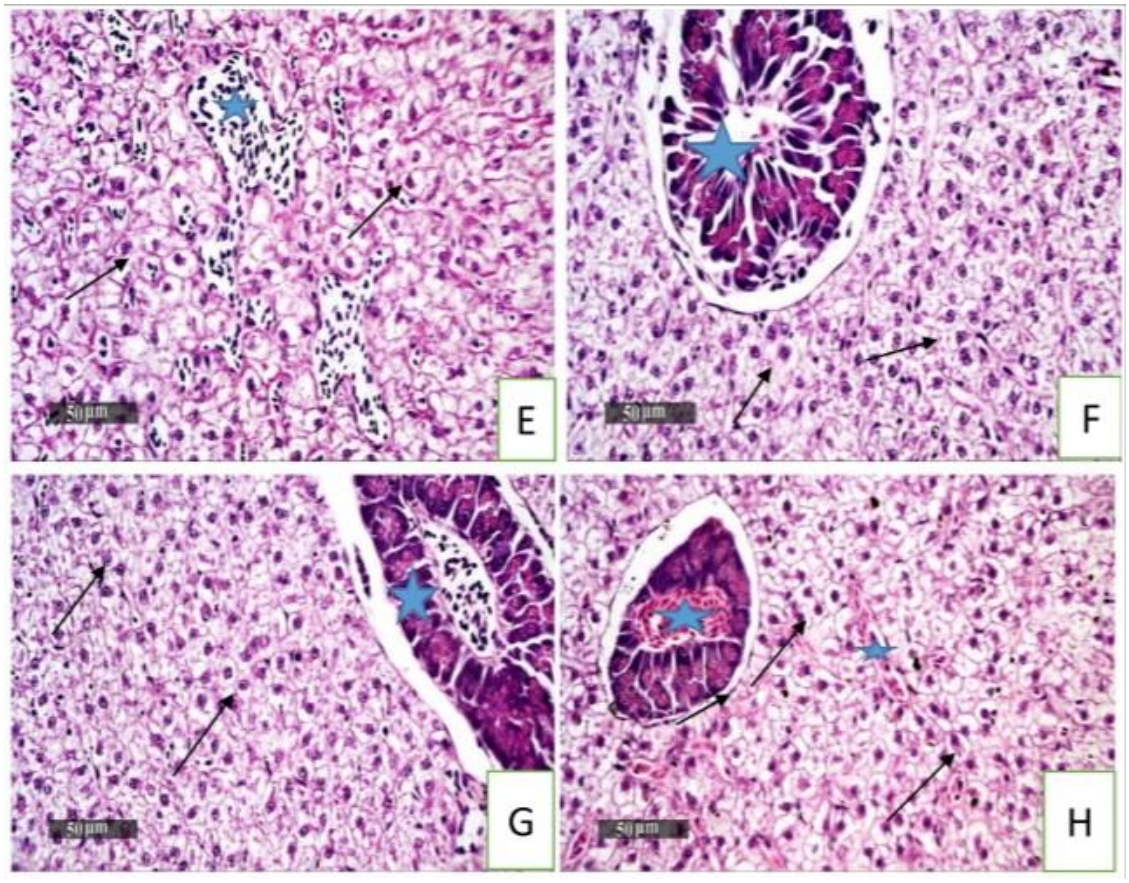


**Fig. 2.(E, F)** Hepatopancrease of G3 treated with formic acid showed more organized histological features of hepatic parenchyma with apparent higher records of intact hepatocytes with minimal records of degenerative changes(H&E).**(G,H)** Hepatopancrease of G4 treated with biotin showed the same records as G1 with mild congested hepatic BVs (blue star)(H&E)

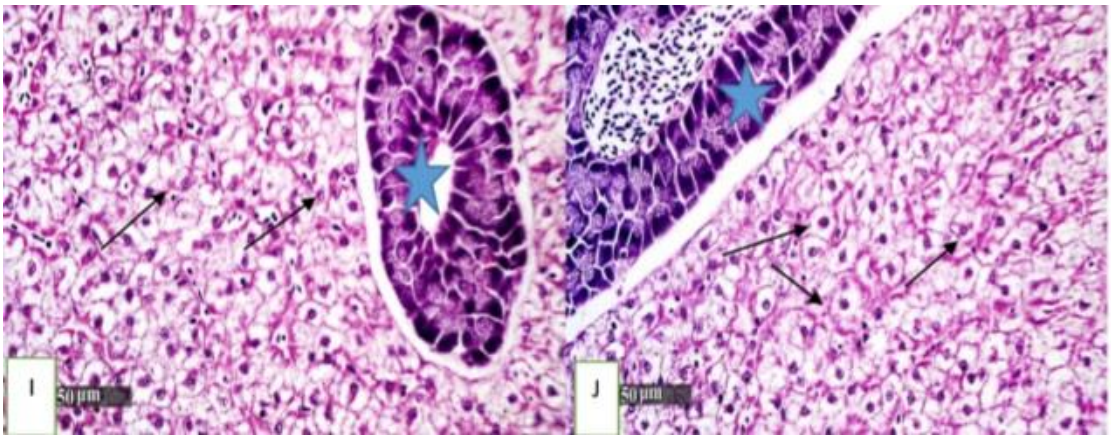


**Fig. 2.(I, J)** Hepatopancreas of G5 treated with formic and biotin showed apparent intact basophilic pancreatic acinar cells with luminal zymogenic granules (blue star). Apparent intact hepatic parenchyma with abundant records of intact hepatocytes showing intact nuclear and subcellular details (arrow)(H&E)

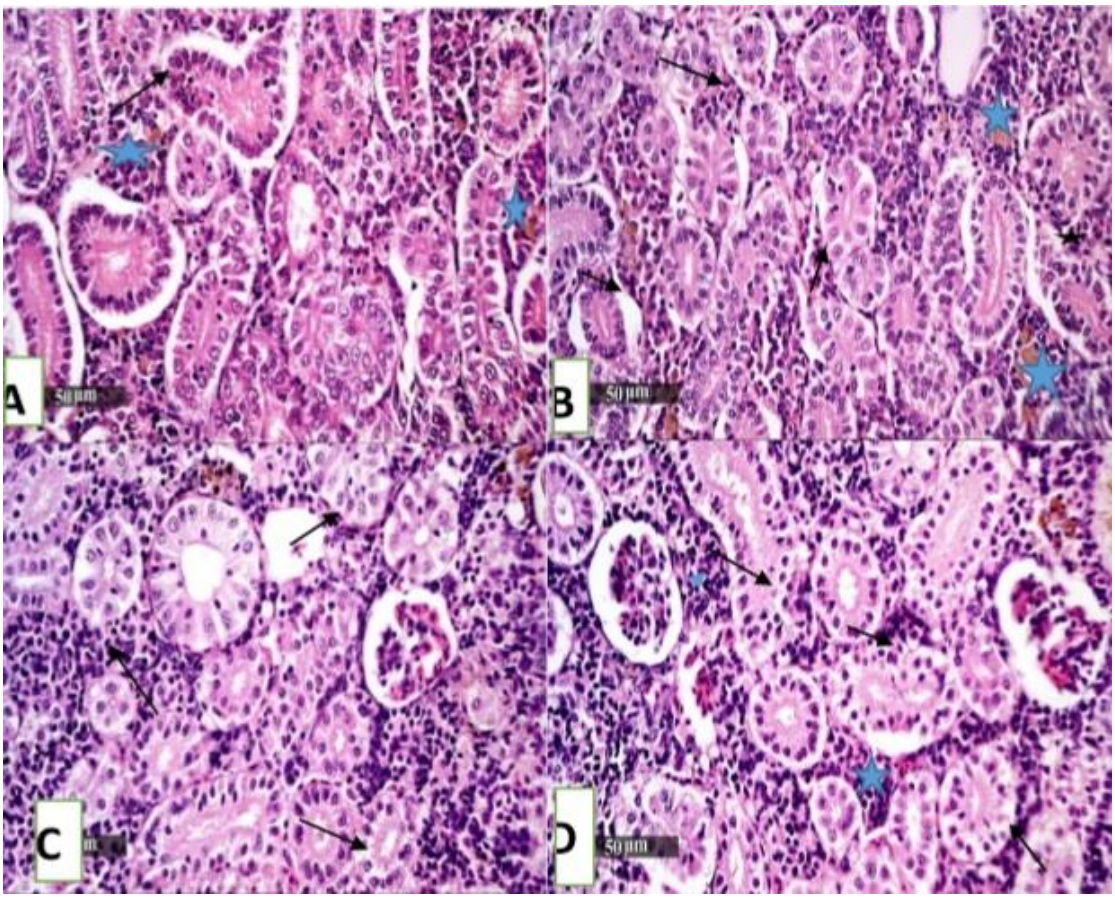


**Fig.3.(A, B)** Kidney of G1 normal control showed many records of apparent intact nephronal segments with intact epithelial lining showing intact subcellular details (black arrow) with focal figures of melanomacrphage (blue star)(H&E). **(C, D)** Kidney of G2 challenged with *V.parahaemolyticus* showed moderate tubular epithelial degenerative changes with focal records of nuclear pyknosis or vacuolated cytoplasm (black arrow) accompanied with significant increase of interstitial lymphoreticular tissue (blue star) with higher intraepithelial lymphocytic infiltrates (H&E)

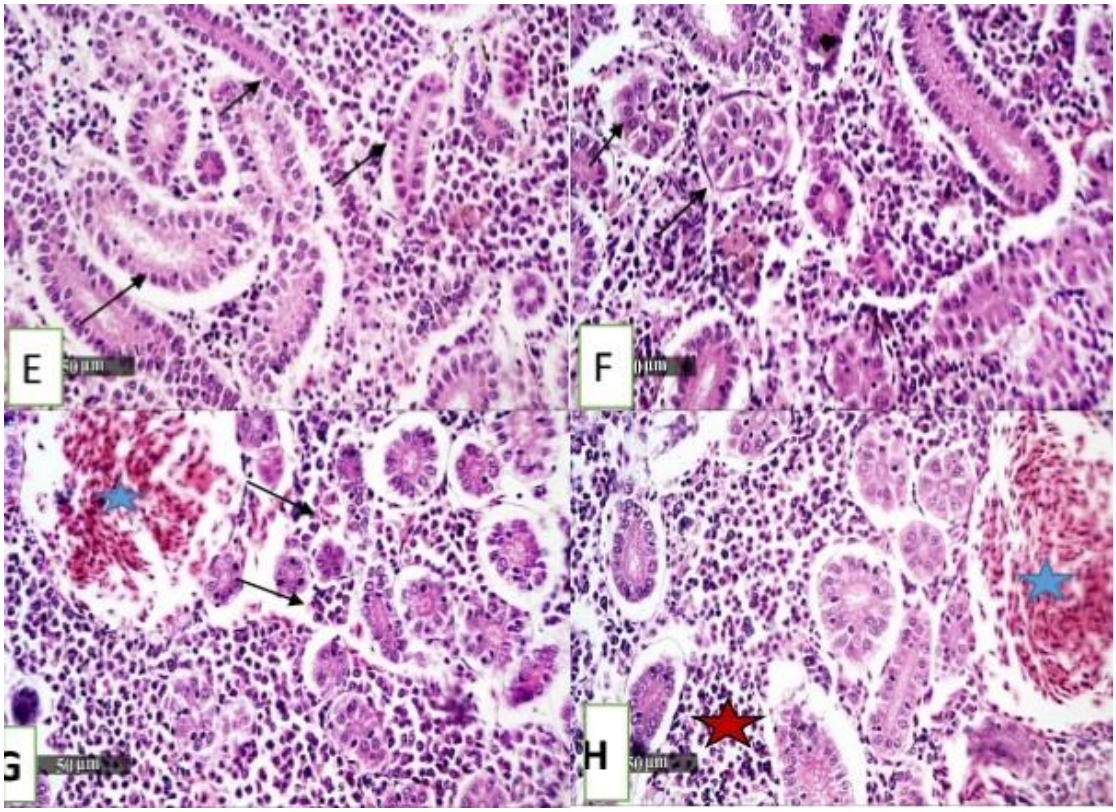


**Fig. 3.(E, F)** Kidney of G3 treated with formic showed apparent intact tubular epithelium with intact subcellular details (black arrow) with depletion of interstitial lymphoreticular tissue.(H&E). **(G, H)** Kidney of G4 treated with biotin showed marked records of tubular damage and loss (black arrow) with sever dilatation and congested BVs(blue star). Also, significant depletion of interstitial lymphocytic infiltrates were observed (red star)(H&E)

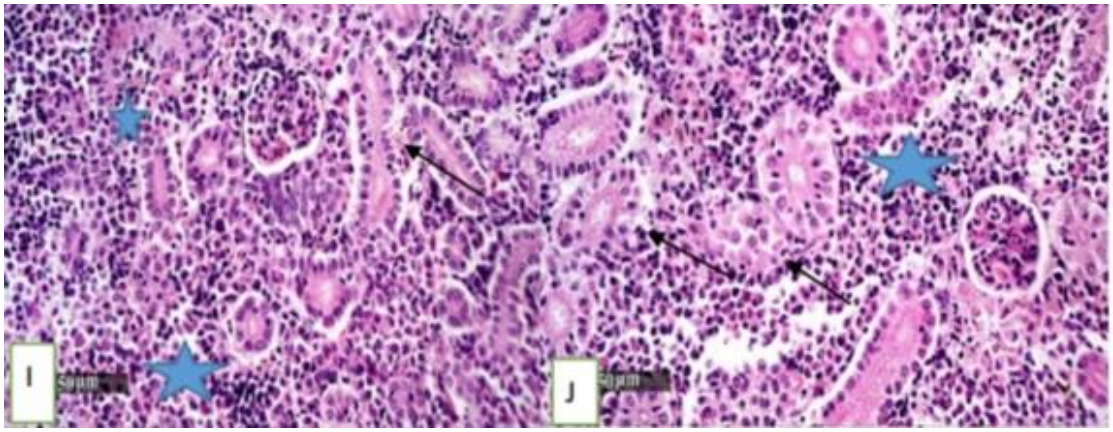


**Fig. 3. (i, j)** Kidney of G5 treated with formic acid and biotin showed higher tubular protective efficacy with mild persistent damage and loss (black arrow), along with persistent records of higher interstitial infiltrates observed (blue star) (H&E)

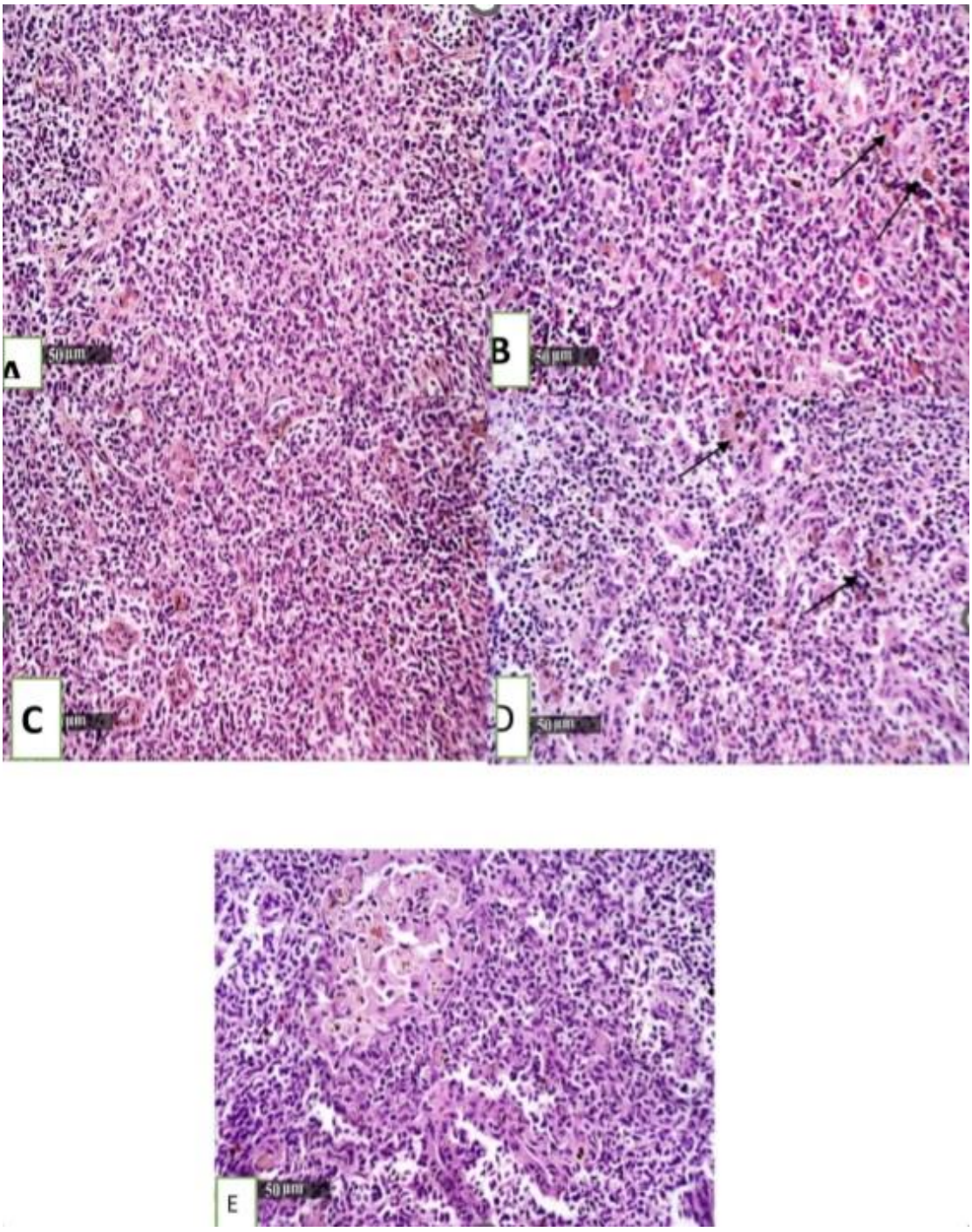


**Fig. 4. (A, C, E)** Spleen from different groups showing normal organized histological featutres of splenic tissue with intact white pulp and red pulp (H&E). **(B, D)** Spleen of G2 (infected) and G4 treated with biotin showed only mild higher figures of melanomacrophages (black arrows) compared with other samples (H&E)

**Water quality parameters**

Parameters of water were: unionized ammonia (NH3) from 0.05±0.1mg/L, total ammonia from 0.18±0.02mg/L, dissolved oxygen 8±0.5mg/L, pH 7.5±0.5, temperature 25±1°C,and salinity ‰ 10 to 12.

## DISCUSSION

Vitamins and organic acids are frequently utilized as additives in animal feed. Despite being commonly used as feed additives to promote animal and poultry growth, organic acids are also being studied for their efficacy in various aquatic organisms. Research, including studies on hybrid red tilapia **(Ng *et al.*, 2009)** and Pacific white shrimp **(Da Silva *et al.*, 2013; Su *et al.*, 2014)** has shown that supplementation with organic acids or their salts has the potential to exert beneficial effects on growth performance and disease resistance.

Fish require an essential water-soluble B-complex vitamin **(Mohamed *et al.*, 2000, Halver, 2002)**. Biotin, an essential water-soluble vitamin, is vital for all animal and fish growth and health **(Yossa*etal*., 2013)**.

This trial examined the impact of formic acid, biotin, or a combination of the two, when administered with diet, on growth performance digestive enzymes, some serum biochemical parameters, histopathological alterations and survival of common carp fish challenged with *Vibrio parahaemolyticus*, compared with carps fed the control diet without any supplements.

Results in this investigation revealed that both formic acid and biotin induce the growth performance (increased weight gain, final body weight, weight gain% and SGR with reduced FCR) and digestive enzymes activity (amylase and lipase), especially in G5 followed by G3 then G4. Additionally, the results recorded an increase in the survival rates of carps supplemented with formic acid and biotin then challenged with *Vibrio parahaemolyticus* from 46.6% in the control group (without any supplementation) up to 93.3, 86.6 and 83.3% for G5, and G4, respectively.

Growth rate is determined by feed utilization, which is predominately influenced by the fish's absorptive and digestive capacities **(Hakim *et al.*, 2006)**.

The impact of organic acid application on the performance of fish is extremely variable and depends on an extensive number of factors, such as the age, species, type, and concentrations of organic acids utilized **(Tran-Ngoc *et al.*, 2018)**. The observed growth enhancement in fish may be attributed, at least in part, to the enhancement in the stimulation of digestive enzyme activity; this stimulation may be facilitated by the decrease in gut pH following organic acid supplementation; for instance, intestinal enzyme activities (Phosphatases and leucin-aminopeptidase) and pepsin activity, pancreatic enzyme activities (lipase, amylase and trypsin) were all observed to be greater in fish fed organic acids **(Castillo *et al.*, 2014)**. In addition, the promotion of advantageous acid-tolerant bacteria proliferation, such as LAB (Lactic acid bacteria**)** was

detected **(Luckstadt, 2008)**. Furthermore, an enhancement was recorded in mineral and nutrient digestibility, which may consequently enhance nutrients absorption and subsequently the growth rate **(Ng *et al.*, 2015; Hoseinifar *et al.*, 2016; Omosowone *et al.*, 2018)**.

Formic acid's antimicrobial activity against *Vibrio* spp. contributed to an additional increase in the survival rate. Additionally, it was documented *in vitro* **(Adams & Boopathy, 2013; Da Silva *et al.*, 2013)**. Antimicrobial activity has been observed in organic acids toward a variety of pathogenic bacteria, including *Vibrio* spp., *Salmonella* spp., and *Escherichia coli* **(Ricke, 2003; Papatsiros & Billinis, 2012; Da Silva *et al.*, 2013)**. This may be the result of undissociated organic acid forms being capable of penetrating bacterial cell membranes and dissociating into H+ and anions within the cytoplasm. Upon entering bacterial cells, a decrease was deteced in the intracellular pH causing disruptions to various processes, such as protein synthesis, genetic material, and metabolic enzymes **(Yowaphui *et al.*, 2016)**. Furthermore, the bacterial cell's capacity to regulate pH homeostasis is compromised as organic acids depleting ATP levels, which is necessary for the pumping of excess H+ out of the cell **(Ricke, 2003; Lückstädt & Mellor, 2011)**. Nevertheless, not every organic acid exerts an impact on bacteria, organic acids that exhibit distinct antimicrobial properties are, in fact, short-chain acids (C1-C7), including formic, citric acetic, butyric, propionic, lactic, malic, and tartaric acids **(Dibner & Buttin, 2002; Papatsiros & Billinis, 2012)**.

The performance and survival outcomes of this trial are consistent with those of numerous other studies which indicated that, supplementation of formic and organic acids increase the growth rates and weight gain of rainbow trout fingerlings and *Oncorhynchus mykiss* **(de Wet, 2005)**, juvenile red drum **(Castillo *et al.*, 2014)**, *O. niloticus* **(Eid, 2012; Hassaan *et al.*, 2014)**, and common carp fish **(Albassam *et al.*, 2021)**. Other studies, however, have failed to identify any growth-promoting properties of organic acids **(Gislason *et al.*, 1996; Ng *et al.*, 2009; Ebrahimi *et al.*, 2017; Asriqah *et al.*, 2018; Omosowone *et al.*, 2018)**, which seem to depend on the fish species.

Several reports have proven the improvement of survival rates of different fish after supplementation of formic and its salts, such as increasing the survival rate of Pacific white shrimp infected with hybrid tilapia challenged with *V. anguillarum***(Ramli *et al.*, 2005)**, and *V. parahaemolyticus* **(Yowaphui *et al.*, 2016)**.

Additionally, vitamins assist significantly to growth and survival by functioning mainly as cofactors for enzymes that are responsible for sustaining optimal metabolic processes and fish health **(Bender, 2003)**; therefore, insufficient provision results in decreased enzymatic activities, which subsequently induce susceptibility to infections and diseases, poor growth, and impaired survival **(Halver, 2002; NRC, 2011)**.

A supplemention of biotin is essential for promoting healthy fish growth, as it functions as a coenzyme for various carboxylases that are involved in the synthesis of fatty acids, gluconeogenesis, and the metabolism of amino acids **(Zempleni *et al.*, 2009)**.

Holocarboxylase synthetase catalyzes the reaction by which biotin is bonded to the e-amino group of a particular lysine moiety in carboxylases (E.C 6.3.4.10) **(Rodriguez-Melendez & Zempleni, 2003; Combs, 2008)**. Vital metabolic processes involving carbohydrates, proteins, and lipids are catalyzed by biotin-dependent carboxylases in all animal species, including mammals and fish **(Mohamed *et al*., 2000; Rodriguez-Melendez & Zempleni, 2003; Li *et al*., 2010)**.

The findings of this research align with those of **Zhao *et al.* (2012)** who reported that, biotin significantly enhanced the development of juvenile Jian carp through mechanisms including the optimization of intestinal microbiota, the improved absorption and digestion and the increased activities of digestive and brush border enzymes. Additionally, juvenile hybrid tilapia exhibited an increase in growth in response to biotin **(Shiau & Chin, 1999)** and the same observation was recorded for the Japanese seabass (*Lateolabrax japonicus* C.) **(Li *et al.*, 2010)**.

Hematology constitutes one significant determinant that can be considered in the assessment of the nutritional value of a fish diet **(Hari *et al.*, 2004)**.

In the current trial, a significant positive impact of both formic acid and biotin occurred on serum albumin, globulin, and total protein concentrations detected at 25 and 45 days, when compared with the control.

The well-balanced diets consumed by fish indicate their healthy immune systems, as evidenced by the elevated concentrations of albumin and total protein in their blood **(Mohammadiazarm *et al*., 2021)**. In line with this, prior research has indicated that, supplementing fish diets with an organic acid mixture, which contains taurine and medium-chain fatty acids, may result in increased levels of blood proteins in different fish species, such as the Nile tilapia (*O. niloticus*), yellow catfish (*Pelteobagrus fulvidraco*) **(Zhang *et al*., 2018; Abd El-Naby *et al*., 2019)**, as well as the common carp (*Cyprinus carpio*) **(Magouz *et al*., 2020)**.

AST and ALT enzymes are significant biomarkers of liver function; elevated levels of AST and ALT in the bloodstream indicate damage in liver cells **(Guardiola *et al*., 2014; Jafarpour & Nekuie, 2016)**.

The present data after 25 days of supplementation showed that fish in G3 exhibited AST and ALT activities that were not significantly affected compared to the control, while there was an increase in ALP activity. Meanwhile, in G4 and G5, the activities of ALP, ALT, and AST were significantly lower than the control.

Conversely, there was a decrease in their levels after 45 days. Furthermore, the liver exhibited no signs of damage in tissues or hemorrhages, suggesting that the administration of dietary supplements containing formic acid and biotin did not pose any risks. A similar outcome was achieved by **Ng *et al.*(2009)**, **Sherif and Doaa (2013)**, **Abu Elala and Ragaa (2014)** and **Ng *et al.*(2015)**, with acidifiers supplementation.

Post challenge, there was a significant elevation in ALP, AST, and ALT, particularly in G2, compared to G1, indicating a liver dysfunction caused by *Vibrio parahaemolyticus* infection.

The results of this research demonstrated a reduction in urea and creatinine levels at 25 and 45 days in the groups that received the supplements in comparison with the control group.The kidneys exhibited no signs of tissue damage or hemorrhaging, suggesting that dietary supplementation with biotin and formic acid did not pose any adverse effects. However, there was an increase in their levels post challenge by *Vibrio parahaemolyticus* infection.

It is worthy to note that, creatinine concentration is a crucial indicator of renal function; creatinine levels in the blood rise when renal filtration is deficient; whereas, the increased urea concentration in the infected carps could be the result of gill dysfunctions since urea is primarily excreted via the gills **(Murray *et al.*, 1990)**.

The observed decline in kidney and liver function indices provides further evidence for the protective effects of biotin and formic acid.

Similiarly, **Sobhy *et al.* (2018)** and **Hussein *et al.* (2023)** observed a decline in the AST and ALT concentrations of gilthead sea bream and the Nile tilapia that were provided with diets supplemented with organic acids.

To examine the influence of stress on fish health, a comprehensive assessment of histopathological alterations in fish tissues is necessary **(Georgieva *et al.*, 2021)**. The pathological examination revealed a multitude of lesions in the spleen, liver and kidney of the fish that were subjected to *V. parahaemolyticus* during the course of this investigation. The microorganism's presence may induce these lesions by generating intracellular reactive oxygen species (ROS), which impairs intracellular equilibrium, resulting in cellular death and DNA damage **(Liu *et al.*, 2021)**, with the exception of the addition of formic acid and biotin; dietary formic acid supplementation did not provide tissue protection against the effects of stress. Hepatic damage was documented in red hybrid tilapia and the Nile tilapia that were provided with an excessive dietary intake of organic acids by **Romano *etal.* (2016)** and **Ebrahimi *etal.* (2017)**. In a similar vein, moderate histopathological changes were observed in the intestine, kidney, and liver of the Nile tilapia fed on organic acid diets **(Rabea *et al.*, 2023)**. On the contrary, the administration of dietary formic acid and biotin led to the development of moderate lesions. These lesions were attributed to the improved immune-antioxidant parameters and decreased ROS release, which protected the tissues from the detrimental effects of stress.

## CONCLUSION

The findings of this research indicate that fish fed a prepared ration supplemented with 0.1% formic acid or 0.06mg/kg biotin or their combination experience an

improvement in the growth performance, digestive enzymes activity, some serum biochemical parameters, and the histopathological alterations, aligned with the survival of common carps challenged with *Vibrio parahaemolyticus* infection. In general both formic acid and biotin could be good choices as additives in fish diet.

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