



## Effect of Protease and Prebiotic mixtures with Free Fishmeal Diets on Physiological Responses and Histological Examinations of the Red Tilapia, *Oreochromis* sp.

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### ABSTRACT

The present study was designed to investigate the dietary effects of protease and prebiotic mixtures with free fishmeal diets on red tilapia (*Oreochromis* sp.). One hundred and fifty fish weighing  $7.40 \pm 0.05$ g were randomly divided into four experimental treatments in 3 replicates. Four experimental diets were designed containing different inclusion levels of protease as 0%, 0.10%, 0.15%, and 0.20% named T1, T2, T3 and T4, respectively beside control with prebiotic mixtures was 2 g kg<sup>-1</sup>. All fish were fed 3% of the body weight daily for 84 days. Liver and visceral weights were measured to determine relative gut length, hepatosomatic index (HSI) and viscerosomatic index (VSI) indices. HSI and VSI were higher in the groups fed with protease with free fishmeal diets compared to the control group especially in T4 and T3, respectively. The results showed significant increase in ALT, AST, ALP, TG and TP in T3 and T4 groups compared to the control group. In addition, significant enhancement was observed in T4 of villi numbers, goblet cells numbers and villi width. Furthermore, the control group showed the highest villi length but the higher muscularis layer thickness was recorded for T2 group. By intestinal investigation, the results show an improvement of T2, T3 and T4 groups but T1 group has villi shortening and broadening. The results of liver histology show an improvement of T3 and T4 and mild improvement with moderate inflammation in some tissues in T1 and T2 groups. The results of spleen histology show an improvement of T2, T3 and T4 groups with some congestion in some tissues but T1 group has a hemorrhage and some congestion in some tissues. In conclusion, the increasing protease level with prebiotic mixtures can effectively improve the physiological status and health of red tilapia (*Oreochromis* sp.).

### INTRODUCTION

The development of feeds for aquaculture has been traditionally based on fishmeal as the main protein source because of its high level of protein content and essential amino acid profile (Tacon, 1993; Watanabe, 2002; El-Saidy and Gaber, 2004). However, the global fishmeal has increased in price more than twice in recent

years (FAO, 2016). In the year 2012, it was estimated that 24.3 million tons of aquaculture species which fed commercially manufactured aquaculture feed. Aquaculture feed production has been growing at an average year rate of 10.3% since year 2000 and there is and expected consumption of aquaculture diets of 65.4 million tons by 2020 and 87.1 million tons by 2025 (Tacon and Metian, 2015).

The probiotics represent a new era in aquaculture and both commercial and scientific interest in this topic is increasing. Indeed, probiotics are commonly used as therapeutic and prophylactic supplements (Nayak, 2010; Hoseinifar *et al.*, 2014). Probiotics when administered in suitable amounts; give benefits to the health of the animal by improving the balance of the microbiota in the intestine (Verschuere *et al.*, 2000). Probiotics are becoming an internal part of aquaculture practices to obtain high production. Probiotics offer benefits with regard to improving immune status and fish production (Cerezuela *et al.*, 2011).

The addition of prebiotics to diets has been applied in aquaculture practices and it plays a role in promoting growth, digestive enzyme activity, immune responses and the composition of beneficial bacteria in the fish digestive tract (Zhou *et al.*, 2010; Soleimani *et al.*, 2012; Zhang *et al.*, 2012; Akrami *et al.*, 2013). Prebiotics and probiotics demonstrated positively modulate the intestinal microflora and could improve fish health. The use of these products in aquaculture is widely accepted. However, some results on their efficiency from a few studies have been conflicting (Gatesoupe, 2005; Grimoud *et al.*, 2010).

Supplementation with enzymes is effective to eliminate the anti-nutritional factors and improve the utilization of feeds energy and amino acids, resulting in improved fish growth performance (Lin *et al.*, 2007) and the intestinal health (Castillo and Gatlin, 2015). Exogenous proteases may compensate for the deficiency of endogenous enzymes and help in the breakdown of proteins improving digestibility (Shi *et al.*, 2016) and carbohydrates are used to help in the breakdown of hemicelluloses which are part of the cell wall (Ebringerová, 2005). Enzyme can improve nitrogen and amino acid utilization by increasing the access to proteins for digestive proteases (Tahir *et al.*, 2008) and the addition of beta mannanase decreases the immune related signal caused by beta mannans (Arsenault *et al.*, 2017).

Proteases are primary enzymes which have been isolated from various parts of Nile tilapia digestive tract (Tengjaroenkul *et al.*, 2000; Hinsui *et al.*, 2006). On the other hand variations in the quality and quantity of nutrients used in diets may modify enzymatic profile and activity in the animal's digestive tract (Lundstedt *et al.*, 2004). Thus, feed composition might induce biological adaptations, including an increase in nutrient absorption (Moraes and Bidinotto, 2000). Digestive enzymes have been investigated to understanding nutritional requirements and the effects of feed composition on enzyme activity in order to reduce feeding costs in fish rearing (Caruso *et al.*, 1996).

Many researchers stated that the products which improve feed efficiency are particularly important since feed costs are a major expense in aquaculture production. Non-nutritive feed additives are being used in aquatic feeds to ensure digestion, ingestion and absorption of feeding nutrients. Additives of feed may be both nutritive and non-nutritive ingredients and acts by either direct or indirect methods on the animal's system (Barrows and Hardy, 2000; Nates, 2016).

The herbal supplements used as prebiotic will aid to improve shrimp health and positively reflect on the product quality. Herbs possess other interesting properties like non-toxic, biodegradable and biocompatible substances (Citarasu *et al.*, 2003). Herbs have been used for controlling shrimp and fish diseases (Düğenci *et al.*, 2003). There are many aromatic plants such as, garlic and fennel in worldwide, especially in the Mediterranean area (Kadri *et al.*, 2011). The extracted essential oil from these plants is usually used for antioxidant digestive stimulant (Botsoglou *et al.*, 2004). Fennel, *Foeniculum vulgare*, is a biennial medicinal plant belonging to the family Apiaceae (Umbelliferae). Essential oils of fennel have liver protective effects (Ozbek *et al.*, 2003) as well as anti-inflammatory and antioxidant activities (Choi and Hwan, 2004). Therefore, the objective of the present study was to investigate the effects of exogenous enzymes (protease) and prebiotic mixtures on red tilapia physiological status and histological examination of intestine, liver and spleen.

## MATERIALS AND METHODS

### 1. Experimental Fish and Study Technique

A total of 150 red tilapia fingerlings were equally distributed in 15 glass aquaria (70 X 40 X 60) with total capacity of 100 liters at Mariculture Research Center, North Sinai Governorate, Egypt. Ten fingerlings per aquarium were stocked with an average initial weight  $7.40 \pm 0.05$ g and an average initial length  $7.30 \pm 0.24$ cm. The fish were acclimatized for two weeks and fed commercial diet. This study was conducted as four five treatments including the control treatments with three replicates. The fish were fed a diet contain  $30.14 \pm 0.08\%$  crude protein and isocaloric  $4430.11 \pm 0.5$ Kcal/kg twice a day at 10:00 am and 16:00 pm at a rate of 3% of total body weight form March to June for 84 days (12 weeks). All aquaria were siphoned once a day to remove fecal materials then replaced by aerated clean water. Each aquarium was supplied with compressed air. Total fish weight in each aquarium was determined every two weeks to evaluate their growth and adjust the feeding rate.

### 2. Water Quality Parameters

Water temperature, salinity, pH, dissolved oxygen (DO) and total ammonia nitrogen (TAN) were measured twice weekly. Water salinity and temperature were recorded using conductivity-temperature meter (SET model 315i, Weilheim, WTW GmbH, Germany). DO was measured by oximeter (SET model 315i, Weilheim, WTW GmbH, Germany). The pH was measured using a pH-meter (SET model 315i, Weilheim, WTW GmbH, Germany). TAN was measured by ammonia nesslerization method (Eaton *et al.*, 1992). During the experimental period, means of the aquaria water temperature were  $28.3 \pm 0.21^\circ\text{C}$  using under water heaters, water salinity was  $20.8 \pm 0.26$  mg L<sup>-1</sup>, dissolved oxygen was  $6.8 \pm 0.05$ mg L<sup>-1</sup>, pH was  $7.97 \pm 0.04$  and TAN was  $0.16 \pm 0.02$  mg L<sup>-1</sup>.

### 3. Feeding Diets

The feeding diets were composed of protease enzyme and prebiotic mixtures (125 g fennel+250 g marjoram+375 g fenugreek+250 g ginger) per kilogram with diets free fishmeal. The control group feeding diet without any additives (Table 1). T1 was the group feeding on diet containing prebiotic mixtures without protease. Treatments of T2, T3 and T4 were fed on diets containing protease enzyme at 0.10, 0.15 and 0.20 g kg<sup>-1</sup>

plus prebiotic mixtures, respectively. The protease (5000 U g<sup>-1</sup> product, supplied by Huvepharma, Antwerp, Belgium) was added to the diets to provide three concentrations of 500 U g<sup>-1</sup> (0.10 mg kg<sup>-1</sup>), 750 U g<sup>-1</sup> (0.15 mg kg<sup>-1</sup>) and 1000 U g<sup>-1</sup> (0.20 mg kg<sup>-1</sup>) as shown in Table 1.

Activity of protease enzyme was assayed according to the method from the **Committee on Food Chemicals Codex (1996)**. One protease unit was the amount of enzyme that releases 1.0 µg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per minute at pH 7.5 and 40°C. The analyzed activity of protease was 4395 U g<sup>-1</sup>. Using a laboratory pellet mill (California Pellet Mill, San Francisco, CA, USA), the ingredients were mixed and manufactured as pellets and dried at 37°C overnight. The pellets were subsequently stored at -20°C until use according to the methods of **Shi *et al.* (2016)**.

**Table 1.** Formulation and proximate analysis of the experimental diets (on dry matter basis).

	Control	T1	T2	T3	T4
Fish meal (60%)	10	0	0	0	0
Soya bean meal (45%)	28	27	27	27	27
Rice bran (14.4%)	16	15	15	15	15
Yellow corn (8.5)	13	9	9	10	10
Wheat bran (16.4)	13	17	16.9	14.85	14.8
Gluten (60%)	10	20	20	21	21
Prebiotic mixtures <sup>(1)</sup>	0	2	2	2	2
Protease Enzyme <sup>(2)</sup>	0	0	0.1	0.15	0.2
Linseed oil	2	2	2	2	2
Sun flower oil	2	2	2	2	2
Fish oil	2	2	2	2	2
Vitamin and minerals mixture <sup>(3)</sup>	4	4	4	4	4
<b>Proximate analysis</b>					
Crud protein	30.14	30.20	30.02	30.20	30.19
Either extract	2.73	2.19	2.18	2.07	2.06
Crude fiber	5.62	5.93	5.92	5.69	5.69
Ash	5.63	4.56	4.55	4.43	4.43
NFE	55.88	57.12	57.33	57.61	57.63
GE <sup>(4)</sup>	4196.09	4198.06	4195.34	4206.31	4205.61
DE <sup>(5)</sup>	3707.60	3706.30	3703.75	3712.65	3711.95
ME <sup>(6)</sup>	2287.94	2266.96	2262.46	2265.16	2264.29
P/E <sup>(7)</sup>	1317.34	1332.18	1326.87	1333.24	1333.31

<sup>1</sup>Prebiotic mixtures (125g fennel + 250g marjoram + 375g fenugreek + 250g ginger)/kg according to **El-Badwy (2016)**.

<sup>2</sup>The protease (5000 U g<sup>-1</sup> product, supplied by Huvepharma, Antwerp, Belgium).

<sup>3</sup>Vitamin and minerals kg<sup>-1</sup> of mixture contains: 4800 IU Vit A, 2400 IU cholecalciferol (Vit D), 40g Vit E, 8g Vit K, 4g Vit B12, 4g Vit B2, 6g Vit B6, 4g pantothenic acid, 8g nicotinic acid, 400mg folic acid, 20mg biotin, 200mg choline, 4g copper, 0.4g Iodine, 12g Iron, 22g manganese, 22g zinc, 0.04g selenium folic acid, 1.2mg niacin, 12mg d-calcium pantothenate, 26mg pyridoxine HCl, 6mg riboflavin, 7.2mg thiamin HCl, 1.2mg sodium chloride (NaCl, 39% Na and 61% Cl), 3077mg ferrous sulfate (FeSO<sub>4</sub>·7H<sub>2</sub>O, 20% Fe), 65mg manganese sulfate (MnSO<sub>4</sub>, 36 % Mn), 89 mg zinc sulfate (ZnSO<sub>4</sub>·7H<sub>2</sub>O, 40 % Zn), 150mg copper sulfate (CuSO<sub>4</sub>·5H<sub>2</sub>O, 25 % Cu) and 28mg potassium iodide (KI, 24 % K and 76 % I).

<sup>4</sup>GE (Kcal/kg) = 5.65 (CP %) + 9.45 (EE %) + 4.0 (NFE %) according to **Viola *et al.* (1981)**.

<sup>5</sup>DE (Kcal/kg) = 5 (CP %) + 9 (EE %) + 3.5 (NFE %) according to **NRC (1993)**.

<sup>6</sup>ME (Kcal/kg) = 3.9 (CP %) + 8 (EE %) + 1.6 NFE (%) according to **Philips and Brockway (1959)**.

<sup>7</sup>P/E (mg/ Kcal) = (mg Protein/ME Kcal) X 100 according to **Wee and Tuan (1988)**.

#### 4. Morphometric Parameters

At the end of the experiment, after 84 days, randomly five fish were collected from each aquarium to withdrawn blood for biochemical analysis from each aquarium were collected and then sacrificed to obtain final length, final weight, liver and visceral weights in order to determine relative gut length (RLG), hepatosomatic index (HSI) and viscerosomatic index (VSI) indices as follow: relative gut length (RGL) = absolute gut length (cm)/Total body length (cm) according to **Al-Hussini (1947)**.  $HSI = 100 \text{ [liver weight (g)/total body weight (g)]}$  according to **Schreck and Moyle (1990)**;  $VSI = 100 \text{ [visceral weight (g)/total body weight (g)]}$  according to **Ricker (1979)**.

#### 5. Biochemical Blood Indices

Blood samples from the other five fish from each aquarium were collected from the caudal vein. Serum was separated at 3000 rpm for 15 minutes using digital centrifuge, and kept in plastic vials well stoppered at  $-20^{\circ}\text{C}$  until analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined calorimetrically according to **Reitman and Frankel (1957)**, total protein was determined according to the method of **Dumas (1975)**, triglycerides were assessed according to the method of **Fossati and Prencipe (1982)**. Alkaline phosphates (ALP) was determined according to **German Society for Clinical Chemistry (1972)**.

#### 6. Histological Examinations

At the end of the experiment, the intestine, liver and spleen were removed from three fish from each aquarium in isotonic saline solution, after which, they were fixed in Bouin's solution for about 24 hrs. The specimens were then preserved in 70 % ethyl alcohol, dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax as usual. Sections of 4-6  $\mu\text{m}$  thickness were mounted on chemically clean glass slides. The sections were prepared then stained with Harri's Haematoxylin and Eosin according to **Pearse (1972)**. Each section of intestine was examined to measure the biometric indices including, the number of villi in section, muscularis layer thickness ( $\mu\text{m}$ ), the goblet cells numbers, villi length ( $\mu\text{m}$ ) and villi width ( $\mu\text{m}$ ) using micrometer slide according to the method of **Kuitunen et al. (1982)**.

#### 7. Statistical Analysis

Data were tested using the one way ANOVA by the General Linear Models (GLMs) procedures using SAS (**SAS, 2004**). Where a significant difference was observed for a measured value, mean separated using Duncan's multiple range test (**Duncan, 1955**) at the 5% level.

## RESULTS

#### 1. Morphometric Parameters

Relative gut length (RGL), Hepatosomatic index (HSI) and viscerosomatic index (VSI) of fish were calculated (Table 2). Data in Table 2 show insignificantly differences in relative gut length (RGL) between treatments. Values of hepatosomatic index and viscerosomatic index for all tested fish groups were significantly different  $P \leq 0.05$ . The highest HSI value was recorded for T4 group and the lowest value was recorded for T3 group. The highest VSI was recorded for T2 group and the lowest one was recorded for T4 group.

**Table 2.** Morphometric measurements of red tilapia fingerlings as affected by addition of protease and prebiotic mixtures with diets free fishmeal.

*Items	Experimental diets				
	Control	T1	T2	T3	T4
HSI	2.46 <sup>ab</sup> ± 0.64	2.78 <sup>ab</sup> ± 1.01	2.66 <sup>ab</sup> ± 0.67	2.30 <sup>b</sup> ± 0.10	3.03 <sup>a</sup> ± 0.49
VSI	4.94 <sup>ab</sup> ± 1.15	5.34 <sup>ab</sup> ± 1.68	5.96 <sup>a</sup> ± 1.22	5.58 <sup>ab</sup> ± 2.21	4.64 <sup>b</sup> ± 1.31
RGL	4.42 ± 0.87	4.23 ± 0.87	5.02 ± 1.10	4.75 ± 1.22	5.03 ± 0.92

\*Data are presented as means ± SD. Means followed by different letters in each row are significantly different  $P \leq 0.05$ .

## 2. Biochemical Blood Indices

Data in Table 3 show significantly different  $P \leq 0.05$  between treatments of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides (TG) and total protein (TP). The highest value of ALT was recorded for T4 group while the lowest value was recorded for control group. The highest value of AST was recorded for T3 group while the lowest one was recorded for control group. The highest value of ALP activity was recorded for T1 and T3 groups and the lowest value was recorded for T4 group. T3 group was recorded the highest value of triglycerides and T4 group was recorded the lowest value. The highest value of total protein was recorded for T3 group and the lowest value was recorded for T2 group.

**Table 3.** Biochemical indices of red tilapia fingerlings at the end of the experiment.

*Items	Experimental diets				
	Control	T1	T2	T3	T4
ALT UI <sup>-1</sup>	60.26 <sup>d</sup> ± 0.25	97.03 <sup>c</sup> ± 0.15	105.10 <sup>b</sup> ± 0.52	45.30 <sup>e</sup> ± 0.30	164.0 <sup>a</sup> ± 0.20
AST UI <sup>-1</sup>	142.36 <sup>e</sup> ± 0.78	649.03 <sup>b</sup> ± 1.15	230.16 <sup>d</sup> ± 1.26	729.02 <sup>a</sup> ± 0.75	535.13 <sup>c</sup> ± 0.32
ALP UI <sup>-1</sup>	45.30 <sup>b</sup> ± 0.61	70.46 <sup>a</sup> ± 0.50	23.00 <sup>c</sup> ± 0.20	70.23 <sup>a</sup> ± 0.21	23.06 <sup>c</sup> ± 0.21
TG Umg <sup>-1</sup>	263.03 <sup>c</sup> ± 0.45	58.00 <sup>d</sup> ± 0.40	316.06 <sup>b</sup> ± 0.40	680.06 <sup>a</sup> ± 0.21	23.47 <sup>e</sup> ± 0.45
TP g dl <sup>-1</sup>	4.17 <sup>b</sup> ± 0.21	3.97 <sup>bc</sup> ± 0.15	3.45 <sup>c</sup> ± 0.40	6.10 <sup>a</sup> ± 0.36	4.27 <sup>b</sup> ± 0.23

\*Data are presented as means ± SD. Means followed by different letters in each row are significantly different  $P \leq 0.05$ .

## 3. Histological Examinations

### 3.1. The intestine

Data in Table 4 show significantly different  $P \leq 0.05$  between treatments of intestine for villi numbers in section, muscularis layer thickness (µm), goblet cells numbers, villi length (µm) and villi width (µm). The highest villi number, goblet cells numbers and villi width were recorded for T4 group. The least values of villi numbers in

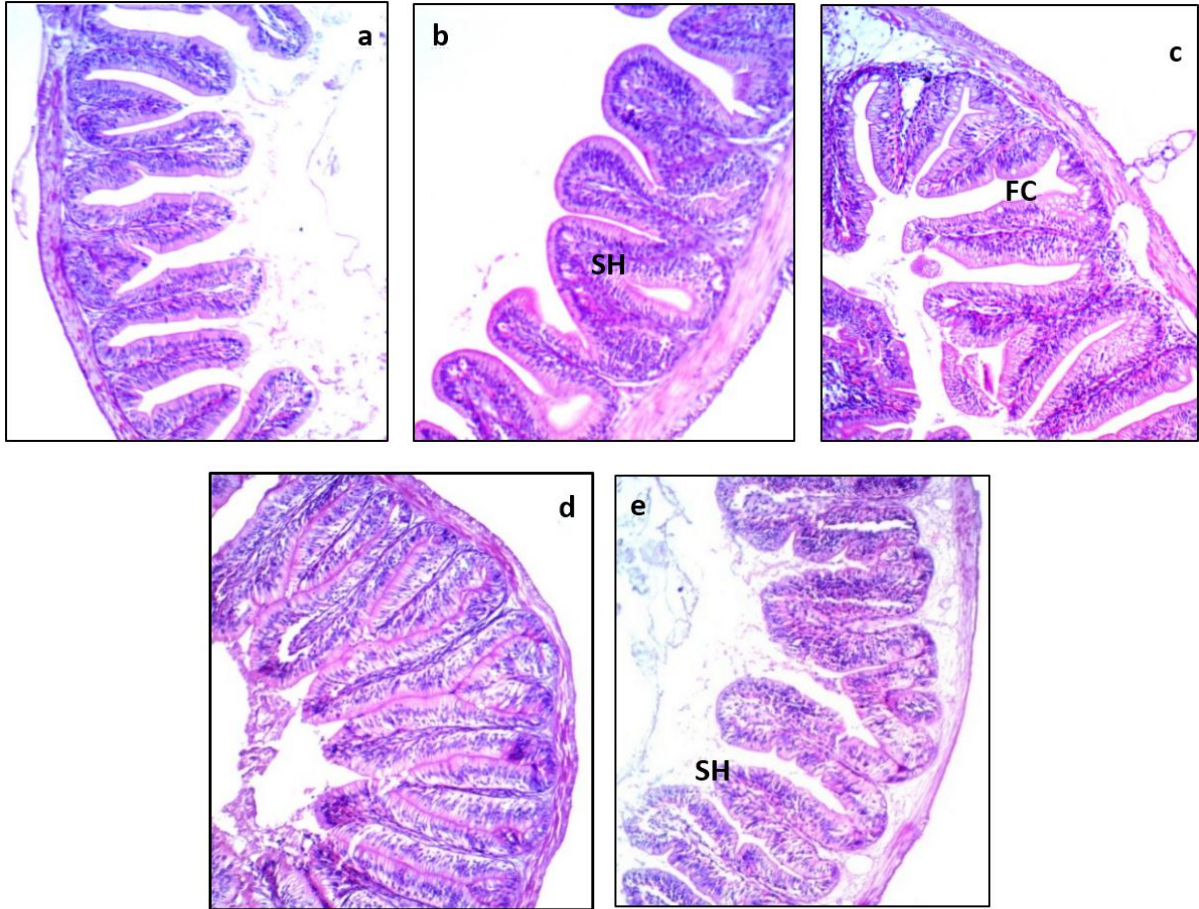
section, goblet cells numbers, villi length ( $\mu\text{m}$ ) and villi width ( $\mu\text{m}$ ) were recorded for T2 group. The highest value for the muscularis layer thickness was recorded for T2 group and the lowest value was recorded for T4 group. The lowest value of goblet cells numbers was recorded for T2 group. The longest villi was recorded for control group and the shortest villi was recorded for T2 group.

**Table 4.** Histological biometric measurement for intestine of red tilapia fingerlings at the end of the experiment.

*Items	Experimental diets				
	Control	T1	T2	T3	T4
Villi numbers	18.50 <sup>d</sup> $\pm 0.50$	23.00 <sup>b</sup> $\pm 1.00$	11.50 <sup>e</sup> $\pm 0.50$	20.10 <sup>c</sup> $\pm 0.17$	25.17 <sup>a</sup> $\pm 1.26$
Muscularis thickness ( $\mu\text{m}$ )	29.29 <sup>c</sup> $\pm 0.53$	28.33 <sup>c</sup> $\pm 0.36$	44.43 <sup>a</sup> $\pm 1.97$	32.01 <sup>b</sup> $\pm 0.64$	27.40 <sup>c</sup> $\pm 0.61$
No. Goblet cells	19.00 <sup>c</sup> $\pm 1.00$	26.00 <sup>b</sup> $\pm 1.00$	8.00 <sup>d</sup> $\pm 1.00$	34.17 <sup>a</sup> $\pm 1.04$	35.80 <sup>a</sup> $\pm 0.26$
Villi length ( $\mu\text{m}$ )	355.24 <sup>a</sup> $\pm 0.41$	266.78 <sup>c</sup> $\pm 2.39$	156.33 <sup>e</sup> $\pm 1.64$	226.35 <sup>d</sup> $\pm 1.05$	325.74 <sup>b</sup> $\pm 0.38$
Villi width ( $\mu\text{m}$ )	122.95 <sup>b</sup> $\pm 3.88$	121.55 <sup>b</sup> $\pm 0.72$	76.61 <sup>d</sup> $\pm 0.58$	84.49 <sup>c</sup> $\pm 0.45$	130.65 <sup>a</sup> $\pm 0.59$

\*Data are presented as means $\pm$ SD. Means followed by different letters in each row are significantly different  $P \leq 0.05$ .

Fig. 1a (control group) shows the normal structure of fish intestine with normal length of villi and goblet cells numbers. Fig. 1b (T1 group) shows no improvement, marked shortening and blunting of villi with degeneration of surface epithelial cells and moderate broadening of villous cores. Fig. 1c (T2 group) shows marked improvement, almost normal villous architecture with minimal residual mild focal spacing. Fig. 1d (T3 group) shows marked improvement of villi length and arrangement with almost normal villous architecture. Fig. 1e (T4 group) shows mild improvement with residual moderate villous shortening and blunting. Some specimen showed no improvement, marked shortening and blunting of villi with degeneration of surface epithelial cells.

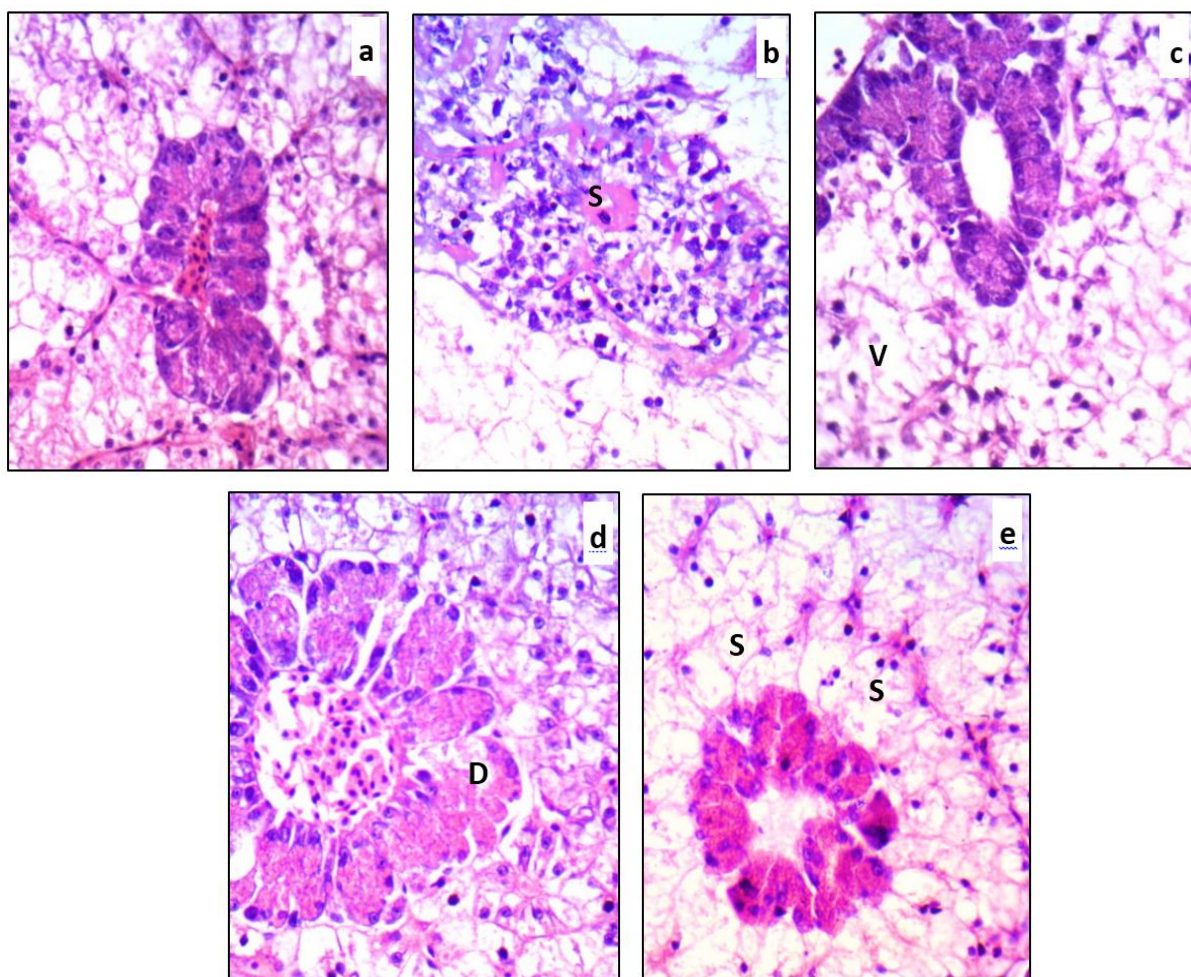


**Fig. 1** Transverse sections of fish intestine, a is control group shows normal structure of villi architecture. b is T1 group shows no improvement, marked shortening (SH) and blunting of villi and moderate broadening of villous cores. c is T2 shows marked improvement, almost normal villous architecture with minimal residual mild focal spacing (FC) of villi. d is T3 shows marked improvement of villi length and arrangement with almost normal villous architecture. e is T4 shows mild improvement with residual moderate villous shortening (SH) and blunting with no degeneration of surface epithelial cells. Some specimen showed no improvement, marked shortening and blunting of villi with degeneration of surface epithelial cells H & E, 100X.

### 3.2. The liver

Fig. 2a (control group) shows the normal structure of fish hepatopancreas. Fig. 2b (T1 group) shows mild improvement and residual moderate swelling of cells with clearing of cytoplasm with moderate inflammatory cells infiltrating pancreatic island. Fig. 2c (T2 group) shows mild improvement with residual marked vacuolation of hepatocytic cytoplasm and focal mild inflammatory infiltrate with mild congestion. Fig. 2d (T3 group) shows marked improvement with residual mild vacuolar degeneration of hepatocytes. The pancreatic islands showed mild congestion. Fig. 2e (T4 group) shows marked improvement with inflammation with residual mild swelling of cells with clearing of cytoplasm. The pancreatic islands are normal.

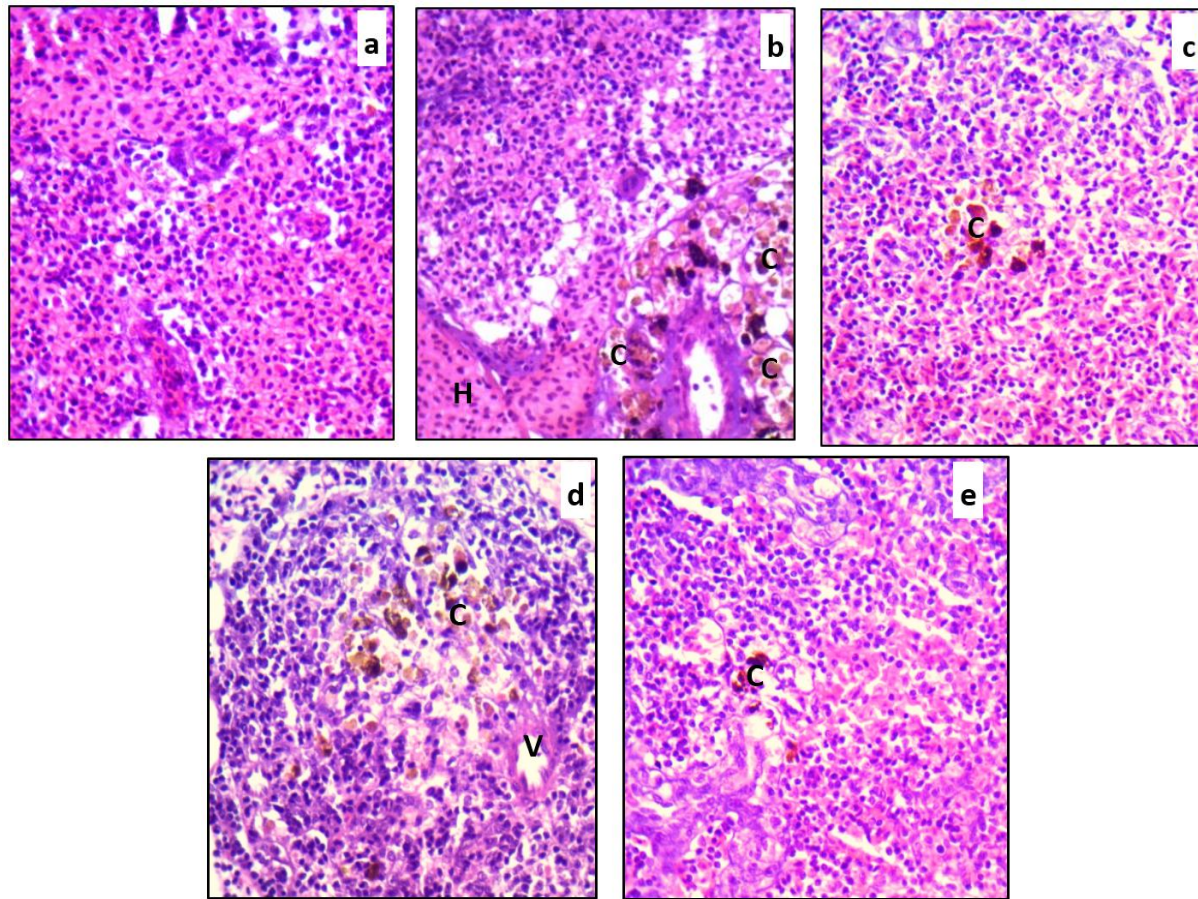




**Fig. 2** Transverse sections of fish liver, a is control group shows normal structure of hepatopancreatic. b is T1 group shows mild improvement and residual moderate swelling (S) of cells with clearing of cytoplasm with moderate inflammatory cells infiltrating pancreatic island. c is T2 shows mild improvement with residual marked vacuolation (V) of hepatocytic cytoplasm and focal mild inflammatory infiltrate with mild congestion. d is T3 shows marked improvement with residual mild vacuolar degeneration (D) of hepatocytes. The pancreatic islands showed mild congestion. e is T4 shows marked improvement and inflammation with residual mild swelling (S) of cells with clearing of cytoplasm. Pancreatic islands are normal H & E, 400X.

### 3.3. The spleen

Fig. 3a (control group) shows the normal structure of spleen. Fig. 3b (T1 group) shows residual mild congestion, focal moderate hemorrhage with hemosiderin laden macrophages. Fig. 3c (T2 group) shows marked improvement with residual mild congestion. Fig. 3d (T3 group) shows marked improvement with residual mild vacuolation and focal congestion, some has hemosiderin laden macrophages. Fig. 3e (T4 group) shows moderate improvement with residual moderate congestion and focal hemorrhage.



**Fig. 3** Transverse sections of fish spleen, a is control group shows normal structure of its cells. b is T1 group shows residual mild congestion (C), focal moderate hemorrhage (H) with hemosiderin laden macrophages. c is T2 shows marked improvement with residual mild congestion. d is T3 group shows marked improvement with residual mild vacuolation (V) and focal congestion (C); some has hemosiderin laden macrophages. e is T4 group moderate improvement with residual moderate congestion (C) H & E, 400X.

## DISCUSSION

### 1. Morphometric Parameters

The present study indicated that the HSI and VSI of red tilapia fed the experimental diets were normal with no observable irregularity. HSI and VSI were affected by the different levels of additives in the diets. Values of HSI and VSI for all tested fish groups were significantly different  $P \leq 0.05$  may be due to the combination of (protease+prebiotic mixtures) and the absence of fishmeal.

HSI was slightly higher for fish fed with T4 group. This result are similar to **Ahmad *et al.* (2012)** who reported that the hepatosomatic indexes and viscerosomatic indices increased with the increase of dietary carbohydrate level similar results were found by **Adeoye *et al.* (2016)** in *Oreochromis niloticus* fed probiotics and a complex of enzymes. On contrary **Ranjan *et al.* (2018)** reported no differences in hepatosomatic index in *Labeo rohita* supplemented exogenous enzymes to fermented and non-fermented



de-oiled rice bran. Relative gut length was not affected by different diets this might be due to slight differences between the final weights of fish.

## 2. Biochemical Blood Indices

Aminotransferases mainly aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the major enzymes in the basal metabolism of cell (Samsonova *et al.*, 2003). These aminotransferase enzymes act as a link between carbohydrate and protein metabolism (Shamna *et al.*, 2015) and are involved in the transfer of amino nitrogen among amino acid to form new amino acids (Tok *et al.*, 2016). Hassaan *et al.* (2018) reported that ALT and AST belong to the non-plasma specific enzymes which are localized within tissue cells of liver, heart, gills, kidneys, muscle and other organs. Gaudet *et al.* (1975) reported that in blood plasma may give specific information about organ dysfunction. Casillas *et al.* (1983) addition that moreover AST and ALT are transferases concerned with nonessential amino-acid metabolism and gluconeogenesis.

Data showed low ALT and AST in control diet and the highest values was in diets free fishmeal with protease and prebiotic mixtures it might be due to use plant only in diets and absence of some essential amino acid or there may be some anti-nutritional substances to be shown later in the studies by researches. On the other side T4 showed lowest ALP and TG than control group it might be due to plant protein appears act to lower cholesterol level, in the present work total lipid is significantly decreased by increase fenugreek in fish diet, because fenugreek seed would be considered as effective agent for lipid lowering purposes (Abu Saleh *et al.*, 2006) and it is rich protein (26%) has added advantage is a good source of protein as well as fiber (48%) and it might exert a lipid lowering effect moreover, the ability of fenugreek alkaloids treatment to reduce blood serum lipids and total lipids (Sharma, 1986). In this study T3 group showed the highest total protein. Proteins are the most important compounds in the serum, with albumin and globulin being the major serum proteins, which play a very important role in the immune response Zhou *et al.* (2014). Although Hassaan *et al.* (2018) showed that fish fed diets containing malic acid and/or *B. subtilis* exhibited a significant decrease in transaminases ALT and AST activity as well as improved values of total protein, compared with fish fed the control diet. Also similar results were obtained by El-Dakar (2004) who found a significant lower  $P \leq 0.05$  in levels of ALT and AST in Nile tilapia, *O. niloticus*, fed fennel seed meal supplemented with diet. Soltan and El-Laithy (2008) found that ALT and AST levels were significantly decreased when Nile tilapia fed diets supplemented with 1% garlic powder or 1% fennel seed with compared to other diets.

## 3. Histological Examinations

Zhang *et al.* (2012) indicated that protease enzyme has ability to the improvement of intestinal structure this is in agreement with the present study where data showed that T4 was the highest villi numbers and width.

In current results the highest value for the muscular layer thickness of intestine was recorded in the intestine of fish of T2 group. In the same way the goblet cells numbers in the intestine of the red tilapia was higher in T4 and T3 groups than control group. Data shows the longest villi was in control group and the shortest one was in T2 group. It does not matter much villi length or shortness, the results agreed with those of Borgeson *et al.* (2006) who reported that villi length decreased with declining fishmeal levels but the most important is number of villi and surface area exposed to enzymes.

Intestine showed a great enhancement in structure due to (protease+prebiotic mixtures) in diets. Results are agreeing with **Song (2017)** who found dietary protease significantly increased protease activities in stomach and intestine and enhanced nutrient digestibility. **Chen *et al.* (2009)** found an improvement of the histological structure of digestive tract. The improved intestinal histology by dietary protease was also reported in rainbow trout (**Zhang *et al.*, 2012**). Thus, it seems that the composition of basal diet is an important factor affecting the action of (protease + prebiotic mixtures).

**Uran *et al.* (2008)** demonstrated similar effects in common carp who found there was evidence that intestinal villi shrunk and appeared irregular in fish fed SBM-based diets while no negative effects were seen in the control group. **Enes *et al.* (2012)**, however, presented opposing results and cited no differences in intestinal morphology, height or density of intestinal villi of white sea bream fed FM diets and treatments supplemented with graded level NSP. On the contrary, **Mathlouthi *et al.* (2002)** found that xylanase/ $\beta$ -glucanase cocktail counteracted NSP effects of a rye-based diet and increased small intestine villi length of broilers. In terms of gastrointestinal morphology, there was no significant difference in mid-intestine with respect to perimeter ratios, goblet cells levels, but significantly higher microvilli density (a measure of absorptive intestinal surface area) was observed in tilapia fed the phytase and carbohydrase supplemented diets.

Fish liver is described as multifunctional organ acting in detoxification, production of vitellogenin as well as the deposition and metabolism of carbohydrates and fat (**Bruslé and Anadon, 1996**). In most teleost fish, the liver is divided into lobes located cranially and ventrally in the body cavity with a reddish-brown color (**Bruslé and Anadon, 1996**). Hepatopancreatic tissue in control shows normal structure all treatments T1, T2, T3 and T4 were in agreement with **Ostaszewska *et al.* (2005)** who found in trout, the soybean containing diets can cause disturbances in intracellular digestion in enterocytes, pathological changes in pancreatic secretory epithelium and metabolic disturbances in the liver. But there was minor inflammatory in T1 and congestion in T2 might be due to amount of ALT and AST has been demonstrated that when protease inhibitors bind to proteases, it causes the pancreas to secrete greater amounts of digestive enzymes to overcome the inhibitors and digest the dietary protein (**Haard *et al.*, 1996**). Our results show that the liver does not have symptoms and this may be due to the presence of (corn gluten+protease+prebiotic mixtures) this was in agree with **Pereira *et al.* (2002)** they showed that the brassica by-products and gelatinized starch can modify liver histology of rainbow trout.

The spleen has been considered the central component of the immune system and plays an important role in responses against pathogen invasion. It also is a selective filter of the vascular system (**Press and Evensen, 1999**). The spleen also performs hemocateresis in several teleosts and promotes the maturation of the lymphocytes in both humoral and cellular defenses (**Press and Evensen, 1999**).

In the present study, in spleen, T1 and T3 show little congestion in addition to appearance of some hemosiderin-laden macrophage, which indicates that there macrophage cells, which play an important role in the fish immunity the addition of the mixture (protease and prebiotic mixtures) led to the emergence of immune cells the reduction of melanomacrophage centers, pigmented macrophages subtype found in teleosts, particularly in the kidney, liver, spleen and occasionally gills, brain and gonads

(Wen, 2016). Moreover, the presence of congestion could be also related to an adaptation of fish to the recirculation system, resulting in of the natural stress condition. In T2 and T4 groups, the spleen tissue show marked improvement might be due to using of protease and prebiotic mixtures.

## CONCLUSION

Increasing the protease enzyme with prebiotic mixtures in fish diets without fishmeal improved the physiological status of fish and help in health of red tilapia (*Oreochromis sp.*). Histologically there are an improvement of T2, T3 and T4 groups with little histopathological observations.

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### Arabic Summary

تأثير أنزيم البروتياز ومخلوط البريبوتيك لعلائق خالية من مسحوق السمك على الاستجابات الفسيولوجية والفحص النسيجي لأسماك البلطي الأحمر

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صممت الدراسة الحالية لفحص تأثير أنزيم البروتياز ومخلوط البريبوتيك لعلائق خالية من مسحوق السمك على أسماك البلطي الأحمر. وزعت 150 سمكة بمتوسط وزن  $7.40 \pm 0.05$  جرام عشوائياً على أربع معاملات بثلاث مكررات. تم تجهيز أربعة علائق تجريبية محتوية على إنزيم البروتياز بنسبة 0.10%، 0.15% و 0.20% للمعاملات الأولى والثانية والثالثة والرابعة على التوالي بالإضافة إلى 2 جرام/كجم مخلوط البريبوتيك لكل العلائق. غذيت الأسماك على 3% من وزنها يومياً لمدة 84 يوم. وتم قياس وزن الكبد وكذلك الأمعاء لحساب الطول النسبي للأمعاء، معامل وزن الكبد بالنسبة لوزن الجسم ومعامل وزن الأمعاء بالنسبة للكبد. ولوحظ زيادة في معامل وزن الكبد والأمعاء بالنسبة لوزن الجسم في الأسماك المغذاة على علائق غير المحتوية على مسحوق السمك مقارنة بالمجموعة الضابطة وخاصة للمعاملة الرابعة والثالثة على التوالي المحتوية على نسبة عالية من إنزيم البروتياز. كما لوحظ أن هناك زيادة معنوية للإنزيمات الكبدية ألانين أمينوترانزفيريز، أسبرتات أمينوترانزفيريز، ألكالين فوسفاتيز، مستوي الدهون الثلاثية في الدم والبروتين الكلي للمعاملات الثالثة والرابعة مقارنة بالمجموعة الضابطة. بالإضافة إلى وجود زيادة ملحوظة للمعاملة الرابعة في عدد الخملات، عدد الخلايا الكأسية وعرض الخملات. علاوة على ذلك حققت المجموعة الضابطة أكبر طول للخملات بينما حققت المعاملة الثانية أكبر سمك للطبقة العضلية. كما يتضح من الفحص النسيجي للأمعاء الأسماك تحسن لقطاعات المعاملة الثانية والثالثة والرابعة بينما المعاملة الأولى أوضحت قصر وزيادة عرض الخملات. واتضح من الفحص النسيجي لكبد الأسماك تحسن للمعاملة الثالثة والرابعة وتحسن متوسط مصحوباً بالتهابات متوسطة لبعض أنسجة المعاملات الأولى والثانية. أما نتائج الفحص النسيجي للطحال أوضحت تحسن لقطاعات المعاملات الثانية والثالثة والرابعة مصحوباً ببعض الاحتقانات لبعض الأنسجة. ولوحظ أن الطحال للمعاملة الأولى بها نزيف وبعض الاحتقانات لبعض الأنسجة. خلاصة القول فإن زيادة مستوي إنزيم البروتياز مع مخلوط البريبوتيك يعمل على تحسن للحالة الفسيولوجية لصحة أسماك البلطي الأحمر.