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EXPLORING THE INTERACTIVE EFFECTS OF THYMOL OIL AND *PEDIOCOCCUS ACIDILACTICI*: MOVING TOWARDS AN ENHANCED PERFORMANCE, HEMATOLOGY, IMMUNITY AND GUT HISTOLOGY OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Abstract

An 84-day experimental trial was conducted to investigate the effects of dietary thymol and/or *P. acidilactici* on growth performance, intestinal digestive enzymes, bacterial counts, intestinal histomorphometric indices, hemato-biochemical indices, and antioxidant responses in Nile tilapia, *Oreochromis niloticus*. A basal diet (30.34 g kg^{-1} of crude protein) and isocaloric diet (19.21 MJ kg^{-1} gross energy) was used to form the experimental treatments. Control group was the basal diet without additives and the other three formulated diets were supplemented with 2.32 thymol mg kg^{-1} diet or *P. acidilactici* at 2×10^9 CFU kg^{-1} (probiotic; pro), and their mixture. A total number of 300 healthy fingerlings (initial average weight 4.51±0.01 g) were randomly allocated into four groups (25 fish for each group in triplicates). The results showed that the best values of growth and feed conversion ratio (FCR) were recorded in fish fed diet containing a mixture of thymol + *P. acidilactici*. The activity of endogenous enzymes including amylase, lipase, trypsin, and chymotrypsin was substantially boosted (P≤0.05) by diets supplemented with thymol + *P. acidilactici*. Furthermore, fish fed diet supplemented with thymol + *P. acidilactici* had greater villi width, villi height, goblet cells, absorption area, muscularis mucosa, and muscularis. Diets supplemented with a mixture of thymol + *P. acidilactici* substantially improved hematological markers. The diets supplemented with thymol + *P. acidilactici* improved the activities of catalase (CAT), glutathione peroxidase (Gpx), superoxide dismutase (SOD), and total antioxidant capacity (TAC) versus basal diet. In conclusion, the synergetic effect between thymol + *P. acidilactici* ameliorated the growth, feed efficiency, intestinal digestive enzymes, intestinal histological morphometric, hemato-biochemical indices, and antioxidant responses of Nile tilapia.

Key words: thymol, growth, digestive enzymes, bacterial counts, intestinal histomorphometric indices, hemato-biochemical indices, antioxidant response, Nile tilapia

The growth of aquaculture industry goes in parallel with the growth of global population to fulfill human demand for seafood products. Recently, intensive strategy of aquaculture has been adopted all over the world. However, this strategy is accompanied by many challenges, specifically the excessive need for feed, possibility of disease outbreaks, pathogen infection that consequently has negative impacts on aquaculture industry (Li et al., 2019; Ali et al., 2023; Soltan et al., 2023). These challenges have attracted the attention of scientists to conduct more research to adjust feed formulation and incorporate feed additives to enhance growth and boost immune system response to cope with various stressors. At the present time plenty of researches is undertaken to achieve aquaculture sustainability and assure aquatic animals' well-being via enhanced growth and immune system response of fish (Saleh et al., 2022; Mohammady et al., 2023 a). One of the useful techniques is to incorporate bioactive compounds derived from medicinal plants as prebiotics, phytochemicals, essential oils and immunostimulants for fish health and growth management that shows promising protocols for improving aquaculture industry (Jagruthi et al., 2014; Gültepe et al., 2015; Wang et al., 2016; Muqier et al., 2017; Hassaan et al., 2021 a, b, c). Previous research has revealed that probiotics as *P. acidilactici* isolates are viable probiotic candidates for aquafeed production (Banerjee et al., 2017; Dawood et al., 2019; Hassaan et al., 2014, 2021 a, b). Moreover, Castex et al. (2009) stated that prawns fed diets supplemented with *P. acidilactici* increase weight gain, survival rate, FCR, and the activity of oxidative enzymes. Also, Ferguson et al. (2010) found that tilapia (*Oreochromis niloticus*) fed diets enriched with *P. acidilactici* diet showed an improvement of growth, survival rate, and immune system response. In addition, *Lactobacillus* bacteria (LAB) have a vital role to boost the growth, immune system and health of the aquatic animal by defending them from harmful bacterial pathogens through producing sufficient amounts

of aggressive compounds toward pathogens (Ferguson et al., 2010). Alongside, using of probiotic beside prebiotics has a positive impacts on aquaculture industry. Phytogenics compounds in comparison to antibiotics and growth hormones are safer and healthier that have been approved by WHO since 2006. Ahmadifar et al. (2021, 2022) and Terzi et al. (2023) reported that using of antibiotic growth promoters showed a risk of producing strains resistant to pathogenic bacteria, while hormones might accumulate in fish and shrimp tissue and subsequently negatively affect human health. One of the promising strategies to improve performance and health of aquatic animals is using of dietary supplements such as the prebiotics and probiotics in aquafeeds (Terzi et al., 2023). Prebiotics as phytogenic compounds and essential oils (EOs) have gained considerable attention in animal science for their potential to improve animal health, performance, and manipulate gut microflora community (Ferguson et al., 2010). Thymol is a phytogenic compound of the essential oils (EOs) from thyme herbs (Hala et al., 2012) used to boost the growth and feed efficiency via improving the digestive and oxidative enzymes secretion and activity that improves growth performance, health and controls disease outbreaks in fish (Acar et al., 2015; Ahmadifar et al., 2021, 2022; Terzi et al., 2023). Previous studies have shown that dietary incorporation of thymol has shown growth-promoting effects on growth of poultry and some fish species (Ahmadifar et al., 2021). The effects of thymol mainly depends on the dose and species (Ahmadifar et al., 2021). A combination of thymol and carvacrol improved the growth of rainbow trout (*Oncorhynchus mykiss*) and European sturgeon (*Huso huso*) (Ahmadifar et al., 2021). Moreover, Merrifield et al. (2010 a, b) found that inclusion of essential oils in rainbow trout (*Oncorhynchus mykiss*) enhanced the growth and immune system through improving the levels of blood leucocyte, and enhanced the histology of gastrointestinal tract. On the opposite side, inclusion of thymol did not stimulate growth of channel catfish (*Ictalurus punctatus*) (Zheng et al., 2009). Moreover, Giannenas et al. (2012) did not find significant improvement of weight gain of rainbow trout fed a diet supplemented with 6000 ppm thymol. Overall, the studies above highlight the importance of recognizing the proper phytogenic compounds and doses that result in growth enhancement in different fish species. A mixture of probiotics and essential oil can greatly amplify the benefits not only in terms of immunity but also as powerful antioxidants and growth promoters. Presently, there is shortage of information on the impact of essential oil as thymol on the growth, feed efficiency, digestive and oxidative enzymes activity and gut morphology of Nile tilapia (*Oreochromis niloticus*). Thus, the purpose of this study is to determine the synergistic effects of essential oil and probiotic (*Pediococcus acidilactici*) administration on Nile tilapia growth performance, intestinal digestive enzymes, feed utilization, intestinal histomorphometric indices, hemato-biochemical indices, and antioxidant status.

Material and methods

Probiotic preparation and thymol source

Solvent tolerant strains of *Pediococcus acidilactici* (1887^{AL}) used in the present study were obtained from Microbiological Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Inoculum was prepared by inoculating a loopful of stock culture of strains *P. acidilactici* in the medium which contained (gL^{-1}): casein peptone, tryptic digest 10.00; meat extract, 10.0, yeast extract 5.00, glucose 20.00, tween (80) 1.00, K_2HPO_4 2.00, Na-acetate 5.00, $(NH_4)_2$ citrate 2.00, $MgSO_4 \times 7\ H_2O$ 0.20, $MnSO_4 \times H_2O$ 0.05, distilled water (1000.00 ml) and adjusted to pH 6.20–6.50. The incubation was done at 37°C by using incubator (Binder, Tuttlingen, Germany). The overnight grown culture was used as inoculum. *P. acidilactici* was prepared to obtain (1.0×10^{10} CFU g^{-1}) approximately. Thymol (>95% purity) was supplied from Sigma-Aldrich Chemical Co., USA.

Experimental design and diets

Four isonitrogenous (303.4 g kg^{-1} of crude protein) and isocaloric (19.21 MJ kg^{-1} gross energy) diets were formulated from practical ingredients (Table 1). The first diet was the control diet without supplementation of either thymol or *Pediococcus acidilactici*. The other three formulated diets were supplemented with 5 mg thymol kg^{-1} or *Pediococcus acidilactici* (*P. acidilactici*) at 2×10^9 CFU kg^{-1} (probiotic; pro), and their mixture 5 mg thymol kg^{-1} + 2×10^9 *P. acidilactici* (CFU kg^{-1}). Addition of probiotic source *P. acidilactici* to diets was according to Hassaan et al. (2018). Formulated diets were prepared using a laboratory pellet mill (0.5-mm die) according to Azaza et al. (2010) and Hassaan et al. (2018) and kept at 4°C until usage. The AOAC (1995) method was used to determine the dry matter, ash, crude protein, and crude lipid.

Fish rearing technique

Mono-sex Nile tilapia, *Oreochromis niloticus* L., were transported from a private farm (Kafer El-sheekh Government, Egypt) to the Fish Nutrition Laboratory, Faculty of Agriculture, Benha University. They were kept in two cement ponds (3 × 4 × 2.25 m) for 15 days to acclimatize for the experimental circumstances and fed a commercial diet (30% crude protein and 18.90 MJ kg^{-1} gross energy) before the feeding trial. Following acclimation, 300 healthy fish (initial average weight 4.51±0.01 g) were allocated into four groups with three replicates, with a stocking density of 25 fish per plastic tank (0.5 m^3). For the duration of the experiment, all tanks received compressed air to provide the oxygen they required. To monitor their growth, the experimental fish were fed three times daily at 09:00, 12:00, and 15:00. Each day, new water was provided to each tank after the accumulated feces were syphoned off, restoring roughly 20% of the water. The water quality parameters were monitored during the experiment and were found to be within the permitted range for tilapia (Boyd, 1990).

Table 1. Ingredients (g kg^{-1} diet) and proximate chemical analysis of the experimental diets (% on dry matter basis)

Ingredients	g kg^{-1}
Fish meal	50
Soybean meal	470
Corn gluten	40
Yellow corn	220
Wheat bran	160
Soya oil	40
Vita & M[1]	20
Proximate analysis (g kg^{-1})	
dry matter	89.30
crude protein	30.34
crude lipid	7.01
ash	4.85
fiber content	5.46
NFE[2]	52.34
GE (MJ kg^{1}) [3]	19.21
thymol mg kg^{-1}	2.32

[1]Vitamin and mineral mix (mg or g/kg diet): $MnSO_4$, 40 mg; MgO, 10 mg; K_2SO_4, 40 mg; $ZnCO_3$, 60 mg; KI, 0.4 mg; $CuSO_4$, 12 mg; ferric citrate, 250 mg; Na_2SeO_3, 0.24 mg; Co, 0.2 mg; retinol, 40000 IU; cholecalciferol, 4000 IU; α-tocopherol acetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg. *P. acidilactici* was prepared to obtain (1.0×10^{10} CFU g^{-1} approximately, Microbiological Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt).

[2]NFE (nitrogen-free extract) = 100 – (crude protein + lipid + ash + fiber content).

[3]Gross energy calculated using gross calorific values of 23.63, 39.52 and 17.15 kJ/g for protein, fat and carbohydrate, respectively according to Brett (1973).

Growth and feed efficiency

At the end of the feeding trial, fish were removed from each tank, assigned a number, and their final body weight was recorded. The formulas in Table 2 footnote were used to calculate growth and feed efficiency.

Endogenous enzymes activities

Middle intestine samples from each treatment (five fish from each tank) were immediately homogenized in 10 volumes (w v^{-1}) of ice-cold physiological saline solution and centrifuged at 5000 g for 15 minutes at 4°C before being stored for endogenous enzyme activity analysis (Furné et al., 2008). Amylase activity was assessed using the technique provided by Bernfeld (1951), lipase activities were estimated using the technique given by Zamani et al. (2009), and chymotrypsin and trypsin activities were assessed according to Bernfeld (1951).

Intestinal total bacterial count

At the end of the experiment, the intestine and gut of three fish from each replicate were aseptically removed and cleaned three times with sterile distilled water to eliminate non-adherent surface germs. Then they were cleaned separately in 96% ethanol and homogenized with a mortar and pestle. The homogenate samples were suspended in 10 mL of saline solution and diluted to 10^5 in 9 mL of saline solution (Al-Harbi and Uddin, 2004). Each dilution was put onto duplicate nutrient agar media and standard nutrient agar medium in a 0.5 mL volume. The plates were incubated for 48 hours at 25±1°C. Following incubation, the colonies in each plate were counted (only plates with a count of 15 to 300 colonies were approved).

Table 2. Growth performance and feed utilization of Nile tilapia, *O. niloticus* fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Initial body weight (g fish $^{-1}$)	4.50±0.01	4.49±0.02	4.49±0.03	4.46±0.02	0.567
Final body weight (g fish $^{-1}$)	28.64±0.87 c	30.29±0.39 b	30.63±0.18 b	32.20±0.14 a	0.021
Weight gain (g fish $^{-1}$)	24.13±0.23 c	25.82±0.89 b	25.32±0.95 b	27.73±0.81 a	0.031
Specific growth rate (%day $^{-1}$)	2.06±0.01 c	2.13±0.01 b	2.14±0.02 b	2.20±0.01 a	0.025
Feed intake (g $fish^{-1}$)	35.02±0.14 c	36.71±0.13 b	36.83±0.58 b	38.58±0.39 a	0.001
Feed conversion ratio	1.45±0.03 c	1.44±0.02 a	1.41±0.02 a	1.39±0.01 b	0.001
Protein efficiency ratio (g)	2.56±0.89 c	2.59±0.79 b	2.54±0.56 b	2.63±0.63 a	0.004
Protein productive value (%)	41.36±1.39	41.50±1.2	41.16±1.89	41.28±2.12	0.003
Fat retention (%)	74.55±1.28 b	75.15±1.89 a	74.63±1.02 b	71.99±1.25 c	0.001
Energy retention (%)	26.90±0.87	26.72±0.59	26.58±0.33	26.51±0.56	0.014
Fish survival (%)	94.00±1.58 c	96.00±1.30 b	96.00±1.11 b	98.00±1.87 a	0.001

Means followed by different letters in the same row are significantly different ($P<0.05$).

Weight gain (WG) = final weight (g) – initial weight (g); Specific growth rate (SGR) = LnW2 – LnW1/t (days), where, Ln = the natural log; W1 = initial fish weight, W2 = the final fish weight in grams and t = period in days; Feed conversion ratio (FCR) was calculated according to the equation: FCR = Feed intake (g)/weight gain (g); Protein efficiency ratio (PER) = weight gain (g)/protein ingested (g).

Intestinal histological techniques

After the feeding trial ended, five fish were chosen at random from each treatment, and the livers and intestinal midsections were removed. The experimental fish's liver and intestine were dissected and fixed in Bouin's solution for 24 hours and the histology measures were estimated according to Wassef et al. (2016) and Ibrahim et al. (2021 a).

Blood hemato-biochemical indices

Six fish were picked from each treatment at the end of the experiment and anesthetized with 3-aminobenzoic acid ethyl ester (MS 222, 100 mg/L, Sigma Aldrich, Egypt) for blood sample collection. Blood has been drawn from the anesthetized fish and separated into two sections. The first was collected with the anticoagulant 10% EDTA to assess hematocrit (Htc), hemoglobin (Hb), red blood cells (RBCs), and white blood cells (WBCs). Htc was measured by using the Hrubec et al. (2000) guideline, Hb count was assessed using hemoglobin kits, and total WBC count was performed using Martins et al. (2004) indirect approach. The non-hemolyzed serum was obtained by centrifuging the blood sample at 3000 rpm for 10 minutes to determine aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum by using the technique established by Reitman and Frankel (1957). Total serum protein and albumin were determined using the methods of Coles (1974). According to Coles (1974), globulin was calculated by subtracting total albumin from protein.

Oxidative enzymes activity in liver

Hepatic samples (three fish livers per replicate) were weighed, rinsed, and homogenized in ice-cold phosphate buffer (1:10; phosphate buffer: pH 7.4, 0.064 M). The activity of superoxide dismutase (SOD) was assessed by using Peskin and Winterbourn's (2000) method. The measurement of total antioxidant capacity (TAC) activity was carried out using a modified version of Moin (1986) approach. Glutathione peroxidase (Gpx) was measured using Giustarini et al. (2013) method.

Statistical analysis

A normality test is used to determine whether sample data has been drawn from a normally distributed population and to assure they are from the same population. One-way ANOVA analysis was used to show the different significant variation among the treatments using SAS program (SAS, 1993). When overall differences were found, differences between means were tested by Duncan (1955) new multiple range test. All differences were considered significant at $P<0.05$ and the results are presented as means with standard error of the mean.

Results

Water quality

The water quality parameters were within acceptable range for Nile tilapia as temperature, pH, dissolved oxygen, and total ammonia in the water were between 27 and 30°C, 7.5, and 8.2 on the pH scale, 5.8 mg L^{-1} or higher, and 0.16 to 0.2 mg L^{-1}, respectively

Growth and feed efficiency

Table 2 shows the effects of thymol 2.32 g/kg, *P. acidilactici*t 2×10^9 CFU kg^{-1}) and their interaction on fish growth performance and feed utilization efficiency response. Dietary thymol, probiotic, and their interaction improved final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER). The combination provided the highest values of growth parameters and best FCR. There were no significant ($P>0.05$) variations in energy retention between fish given a control diet, probiotic, or thymol and their combination. The survival rate of fish given diets enriched with a combination of thymol + *P. acidilactici* was considerably ($P<0.05$) higher than the other diets (Table 2).

Table 3. Total viable bacterial count in gut and intestine of Nile tilapia, *O. niloticus* fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Gut	5.10×10 a	4.35×10 a	5.30×10 a	3.50×10 a	0.023
Intestine	5.25×10 a	4.20×10 a	5.20×10 a	3.75×10 a	0.003

Means followed by different letters in the same row are significantly different ($P<0.05$).

Table 4. Digestive enzymes activity of Nile tilapia, *O. niloticus* fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Amylase	2.35±0.13 c	3.15±0.17 b	3.45±0.11 b	6.30±0.12 a	0.002
Lipase	2.60±0.11 c	3.45±0.17 b	4.35±0.12 b	5.00±0.23 a	0.001
Trypsin	11.80±0.98 c	4.35±0.88 d	19.35±1.07 b	28.00±1.10 a	0.011
Chymotrypsin	7.85±0.51 c	5.00±0.38 d	9.25±0.36 b	11.00±0.98 a	0.001

Means followed by different letters in the same row are significantly different ($P<0.05$).

Table 5. Intestinal histomorphometry of Nile tilapia fingerlings fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Villi width (mm)	272.50±3.96 c	353.00±6.39 b	359.00±8.33 b	376.00±9.55 a	0.001
Villi height (mm)	554.50±5.68 c	806.00±3.69 b	798.00±5.39 b	860.50±2.33 a	0.001
Goblet cells (no.)	21.50±0.69 c	30.00±0.89 b	31.50±0.39 b	34.00±0.91 a	0.011
Absorption area (mm)	8.16±0.25 c	9.13±0.36 a	8.89±0.36 b	9.19±0.25 a	0.001
Muscularis mucosa (mm)	53.50±0.99 c	55.00±0.81 b	57.00±0.36 b	62.00±1.12 a	0.001
Muscularis height (mm)	253.50±1.28 c	262.00±2.00 b	263.50±2.89 b	265.50±2.98 a	0.001

Means followed by different letters in the same row are significantly different (P<0.05).

Table 6. Hematological indices of Nile tilapia fingerlings fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Hb (g dl^{-1})	8.05±0.36 b	7.60±0.32 c	7.35±0.35 c	10.00±0.23 a	0.002
Hct (%)	14.95±0.36 b	16.85±0.98 a	16.65±0.56 a	13.30±0.12 c	0.002
RBCs×10^6/µl	1.24±0.23 b	1.63±0.36 a	1.59±0.23 a	1.27±0.02 b	0.011
WBC×10^3 mm^{-3}	26.50±0.33 d	49.05±1.3 b	33.65±0.69 c	71.65±0.98 a	0.001
Granulocytes (%)	9.49±0.32 c	13.91±0.21 b	17.22±0.23 a	15.68±0.21 a	0.015
Lymphocyte %	87.01±5.31a	80.69±1.36 b	77.22±2.61 b	80.60±2.21 b	0.002
Monocyte %	3.15±0.23 c	4.30±0.23 ab	5.11±0.36 a	3.76±0.32 c	0.001

Means followed by different letters in the same row are significantly different (P<0.05).

Table 7. Serum biochemical indices of Nile tilapia fingerlings fed experimental diets for 84 days

Items	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Alanine aminotransferase (UL^{-1})	39.50±0.66 a	33.50±0.23 c	37.00±0.36 b	38.50±0.56 b	0.001
Aspartate aminotransferase (UL^{-1})	17.50±0.23 a	11.00±0.22 c	18.50±0.36 b	17.50±0.22 b	0.001
Total protein (g dL^{-1})	2.54±0.36 c	2.90±0.36 b	2.73±0.36 b	3.10±0.23 a	0.001
Albumin (g dL^{-1})	1.38±0.12 c	1.75±0.33 a	1.43±0.32 bc	1.52±0.03 b	0.01
Globulin (g dL^{-1})	1.15±0.03 c	1.15±0.03 c	1.31±0.02 b	1.59±0.02 a	0.002
Cholesterol (mg dL^{-1})	107.50±0.89 b	98.50±1.12 a	94.00±1.11 a	90.50±1.11 a	0.002
Triglyceride (mg dL^{-1})	44.50±0.33 a	35.50±0.23 b	30.50±0.36 c	27.00±0.31 d	0.002
LDL-C (mg dL^{-1})	17.50±0.02 a	13.50±0.02 b	12.00±0.02 b	13.00±0.03 b	0.002
HDL-C (mg dL^{-1})	80.50±1.02 a	76.50±1.14 b	76.40±1.36 b	72.46±1.11 b	0.031

Means followed by different letters in the same row are significantly different (P<0.05).

Table 8. Oxidative stress assay (U mg^{-1} protein) of Nile tilapia, *O. niloticus* fed experimental diets for 84 days

	Experimental treatments				P value
	control	thymol	*P. acidilactici*	thymol + *P. acidilactici*	
Catalase	36.00±1.12 c	39.55±1.01 b	41.00±1.07 b	52.50±0.98 a	0.002
Glutathione peroxidase	40.50±0.89 c	46.50±0.98 b	64.00±0.99 a	62.50±1.23 a	0.001
Superoxide dismutase	40.00±0.88 d	52.00±0.102 b	45.50±0.54 c	60.50±0.98 a	0.001
Total antioxidant	35.50±0.66 c	47.50±0.36 b	38.50±0.87 c	72.50±0.39 a	0.027

Means followed by different letters in the same row are significantly different (P<0.05).

Endogenous enzymes activities and total intestinal and gut bacterial counts

The activity of endogenous enzymes including amylase, lipase, trypsin, and chymotrypsin was substantially boosted ($P<0.05$) by supplemental diets with thymol, probiotic, and a combination of thymol + *P. acidilactici* (Table 4). The synergetic effect of thymol + *P. acidilactici* resulted in the maximum activity of amylase, trypsin, and chymotrypsin. The highest total intestinal and gut bacterial counts were detected in fish fed a diet supplemented with probiotic (Table 3).

Histomorphometric indices

In comparison to the control diet, fish given thymol, probiotic, and pro-thym had considerably enhanced villi width, villi height, goblet cells, absorption area, muscularis mucosa, and muscularis height (Table 5). When compared to other diets, the fish given diet supplemented with thymol + *P. acidilactici* had the greatest significant ($P<0.05$) records of the aforesaid indicators.

Hemato-biochemical parameters

Diets supplemented with thymol, probiotic, and pro-thym substantially improved hematological markers (Table 6). The pro-thym treatment had higher hemoglobin (Hb) and white blood cell (WBC) counts ($P\leq0.05$) than the other treatments. The greatest percent of hematocrit, granulocytes and monocyte were seen in fish fed a probiotic-supplemented diet (Table 6).

Serum of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes were lowered in fish fed diets supplemented with thymol compared to the control diet (Table 7). Serum total protein and globulin levels were greater ($P<0.05$) in fish fed diets containing thymol + *P. acidilactici*, while fish fed thymol recorded the highest value of albumin compared to control diet (Table 7). However, supplementation of thymol, probiotic, and pro-thym, lowered the levels of cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (Tale 7).

Antioxidant enzymes activities

The supplemental diets with thymol, probiotic, and pro-thym improved the activities of catalase (CAT), glutathione peroxidase (Gpx), superoxide dismutase (SOD), and total antioxidant capacity (TAC) compared with the control diet (Table 8). The greatest levels of CAT, SOD, and TAC activity were seen in fish fed a pro-thym diet.

Digestive enzyme activity

Fish fed diets supplemented with either probiotic, thymol and their combination improved the activities of amylase, lipase, trypsin, chymotrypsin compared with the control and other diets (Table 4). The greatest levels of digestive enzyme activity were seen in fish fed a combination of thymol and *P. acidilactici* group.

Discussion

Probiotics and phytochemicals, particularly thymol, are strengthening the body's antioxidant capacity, improving immunity, lowering the risk of infectious diseases, and improving aquatic animal performance (Zhou et al., 2015; Xie et al., 2018; Li et al., 2019; Hassaan et al., 2021 c). In the present study, diet supplemented with a combination of thymol + *P. acidilactici* significantly improved WG, SGR, PER and FCR of Nile tilapia. The present results are consistent with Diao et al. (2015) who found that animals fed diets supplemented with thymol had improved feed efficiency, nutrient digestion and assimilation, and performance along with enhanced intestinal microbiota population. Moreover, Ahmadifar et al. (2021) reported juvenile rainbow trout fed diets supplemented with 2.0 or 3.0 g thymol oil improved growth performance parameters (FBW, FE, DGR and SGR) and survival rate and enhanced chemical body composition (protein and lipid content). Alike, Zheng et al. (2009) reported that thymol has a positive impact and enhanced survival rate and performance of channel catfish subjected to infection of *Aeromonas hydrophila*. Furthermore, Amer et al. (2018) found that inclusion of thymol and cinnamaldehyde at two levels (1 and 2 mL kg^{-1} diet) has positive impacts on growth performance and blood parameters of Nile tilapia, and they found that the use of 1 mL thymol kg^{-1} in the Nile tilapia diet improved the growth performance significantly compared to the control and other treated groups. In addition, Sönmez et al. (2015) found that rainbow trout fed diet supplemented with 1.5 g kg^{-1} of thymol oil had significantly enhanced specific growth rate, weight gain and feed conversion ratio by 11.7%, 20.3% and 20.5%, respectively, compared with the control group. Recently, thymol oil supplemented at 0.5 mg kg^{-1} feed to rainbow trout basal diet enhanced the weight gain, length gain and specific growth rate by 30.9%, 12.6% and 18.36%, respectively, compared with the control group (Zargar et al., 2019). Also, Ibrahim et al (2022) found that tilapia fed diets supplemented with a combination of thymol and thymoquinone displayed significantly enhanced growth rate and feed conversion ratio. Unlike the present study, Yousefi et al. (2018) stated that there is no significant impact on performance of rainbow trout by feeding thyme extracts. The contradiction between researches could be attributed to the dose of thymol, fish species, size and lab conditions. The enhancement of growth and feed efficiency could be related to several scenarios such as: i) thyme improves the metabolism and assimilation of the nutrients in the gut (Yousefi et al., 2018), ii) thymol has the ability to increase the survival rate and reduce mortalities and disease via its antibacterial function in the gastrointestinal tract in fish (Hamdan et al., 2016), iii) thymol oil enhanced the digestive enzymes activity and the nutrients digestibility and modulated intestinal microbiota activity and decreased the counts of pathogen bacteria (Khalil et al., 2020), iv) the effect of probiotics and thy-

mol in stimulating appetite (Hamdan et al., 2016; Hassaan et al., 2018), and v) allowing better release of the digestive enzymes such as protease, amylase, and lipase via their significant impact on the gastrointestinal histology and morphology (Ghafarifarsani et al., 2022), which consequently improved nutrients digestion, assimilation, thus improving growth and feed efficiency (Hamdan et al., 2016), vi) moreover, probiotics and/or thymol break down the toxic compounds or promote the production of beneficial metabolites and/or intestinal microbes, which may improve nutrient and growth efficiency (El-Rhman et al., 2009; Sankar et al., 2017; Shabbir et al., 2021), vii) the hormone-like functions of thymol as flavonoid that can promote the growth of animals (Baker, 1998).

Digestive enzymes play an important role in digesting nutrients in fish, directly enhancing the digestive ability (Wen et al., 2009; Adeoye et al., 2016). The activities of intestinal enzymes vary in their distribution and intensity according to feeding habits and intestinal morphology. In the present study, the activity of amylase, lipase, trypsin and chymotrypsin was higher with the addition of thymol + *P. acidilactici*. The present results are in parallel with Ibrahim et al. (2022) who found that Nile tilapia fed diets enriched with thymol and thymoquinone displayed significantly upregulated expression of the genes encoding digestive enzymes (pepsinogen, chymotrypsinogen, α-amylase and lipase). However, more research is necessary to clarify their mechanism of action. The improvement of digestive enzymes could be attributed to: i) the role of probiotic (*P. acidilactici*) in producing nutrients (amino acids, vitamins, and fatty acids) that could be involved in digestive processes and assimilation of diet components (Bolasina et al., 2006; Shan et al., 2008; Ray et al., 2012) and ii) the role of thymol as an essential oil with the potential to bind nutrients and promote quick digestion in Nile tilapia by lowering digesta viscosity in the fish's gut (Ray et al., 2012).

It is well known that the gastrointestinal microbial population (GImp) is controlled by dietary chemical composition (Jiang et al., 2014; Adeoye et al., 2016; Hu et al., 2016). There have been no studies of thymol with probiotics individually or in combination and their effects on gastrointestinal microbial population, and further research is needed to determine their impact on the overall amount of bacteria in fish gut and intestine. In the present study, a combination of thymol and probiotics recorded the lowest total count bacteria in the gut and intestine. Sankar et al. (2017) explained the function of thymol and probiotics and their effect to reduce GImp as follows: i) probiotics prevent pathogenic bacteria from attaching to the gut mucosa through acetic and lactic acid secretion (Ray et al., 2012), ii) probiotics produce beneficial bacteria that compete with pathogens (Bolasina et al., 2006), iii) thymol as an essential oil produces proteinaceous chemicals that act as local antibiotics and reduce the production of pro-inflammatory cytokines (such as IFN-γ, IL-12, and TNF-α) (Bolasina et al., 2006; Shan et al., 2008; Ray et al., 2012), iv) thymol possesses anti-inflammatory capabilities as well as the capacity to reduce the prevalence of *E. coli* and Proteobacteria in mice (Ju et al., 2018), v) thymol may destroy any toxic compounds, alter the beneficial microbiota, and promote the production of intestinal microbes, which may improve nutrient and growth efficiency (Shabbir et al., 2021), and vi) thymol oil enhanced modulated intestinal microbiota community and decreased the counts of pathogen bacteria, besides the antibacterial function of thymol (Khalil et al., 2020).

The intestinal mucosal histomorphology varies depending on diet chemical composition (Jha and Berrocoso, 2016). The formation of this crucial layer may be positively impacted by probiotics (Hassaan et al., 2021 a). In the present data, the intestinal histomorphometry, the administration of *P. acidilactici* and/or thymol had a good effect on the villi width and height, goblet cells, absorption area, muscularis mucosa, and muscularis height, notably in the middle of the gut. The greatest values were seen in fish fed a combination of dietary *P. acidilactici* and thymol. The present results are consistent with Ibrahim et al (2021 b) who found that administration of quercetin nanoparticles in Nile tilapia diet improved the mucosal barriers and gastrointestinal integrity. Furthermore, Abd El-Hamid et al. (2021) found that dietary cinnamaldehyde nanoemulsion improved the histology of gastrointestinal tract of Nile tilapia. Following the same patterns, Ibrahim et al (2021 a) found that supplementing garlic nanohydrogel optimized gastrointestinal integrity of broiler chickens. Furthermore, Ibrahim (2021 b) found that inclusion of thymol nanoemulsion promoted broiler gastrointestinal barrier and bacterial community. These changes in gastrointestinal structure may be due to: i) the antimicrobial action of *P. acidilactici* and/or thymol, which reduces the development and colonization of pathogenic bacteria and stimulates the beneficial microflora proliferation in the gut, resulting in better nutrient absorption and intestinal digestion (Pereira et al., 2018, 2019). This mechanism lowers the infectious and inflammatory processes in the intestinal mucosa, resulting in enhanced villus size and secretory function (Pereira et al., 2019). Hematological indices are used as an indicator to assess fish health (Hassaan et al., 2018). The present study showed that Nile tilapia fed *P. acidilactici* and/or thymol had improved hematological parameters. The present results showed that the values of Hb, Ht RBCs and WBCs rose in fish fed thymol and/or probiotics. The present results are in parallel with previous research which reported the beneficial effects of dietary thymol on enhancing the hematological variables including RBC, Hct, Hb, WBC, lymphocytes, monocytes, and granulocytes of various fish species including Nile tilapia (*O. niloticus*) (Ibrahim et al., 2021 a, 2022; Latif et al., 2021), rohu (*Labeo rohita*) (Ibrahim et al., 2021 b), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) (Taheri-Mirghaed et al., 2020), Asian sea bass (*Lates calcarifer*) (Abdelwahab and El-Bahr, 2012) and channel catfish (*Ictalurus punctatus*) (Zheng et al., 2009).

In addition, the present results showed decreasing of ALT, AST and ALP values of fish fed diets supplemented with thymol and/or probiotics. The present results are consistent with Zargar et al. (2019) who found that inclusion of thymol oil in the rainbow trout diet caused a significant improvement of blood parameters through reduction of ALP, AST and ALT levels. Furthermore, Valladão et al. (2019) stated that the addition of 1% thyme oil in the Nile tilapia diets increased significantly lymphocytes and leucocytes counts, however, no significant effect was detected in the majority of blood biochemical like TP, ALB, GLOB, and ALT and AST levels. Moreover, the AST and ALT levels were significantly decreased when rainbow trout fed diets contained thyme extracts (10 g kg^{-1} diet) compared with the control (Yousefi et al., 2018).

This finding in the present results might be linked to several reasons: i) thymol acts as an antiradical and antioxidant, preventing lipid peroxidation of cell membranes and, as a result suppressing the release of liver-damaging enzymes like AST and ALT into plasma (Cho et al., 2012; Akrami et al., 2015), ii) probiotics may have a therapeutic effect in modulating the gut-liver axis and consequently reduce lipid peroxidation and prevent liver damage (Lin and Chang, 2000; Adawi et al., 2001), iii) probiotics have the ability to control the activation of Kupffer cells as well as the generation of nitric oxide and cytokines (Akrami et al., 2015).

Similarly, serum protein, albumin, and globulin levels are shown to be enhanced here in Nile tilapia by thymol and/or probiotic compared to control diet, which concurs with the findings of Dawood et al. (2015) in red sea bream offered probiotic. Correspondingly to the present results, supplementation of probiotics (*P. acidilactici*) in Nile tilapia, *Oreochromis niloticus* and goldfish (*Carassius auratus*) diets enhanced ($P \leq 0.05$) the serum of total protein, albumin, and globulin (Fadl et al., 2013; Mehdinejad et al., 2019). Moreover, this boost herein may be connected to the increasing action of essential oil thymol on ribosome production and stimulation of DNA and protein synthesis in liver tissues, which results in a rise in serum albumin concentration, indicating that fish are immunologically robust. There have been no studies on the effect of thymol on serum protein, albumin, and globulin levels, as well as their combined effect with probiotics, and further research is needed in this area.

Concerning cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride, the current findings showed that supplementation with thymol and/or probiotic reduced their levels in tilapia, which may be useful in avoiding fatty liver pathological changes and also could be due to the presence of thymol compound as an essential oil compound (Youssef et al., 2014; Zhao et al., 2017). Furthermore, comparable effects of thymol on blood cholesterol and triglyceride or hepatic lipids have already been revealed in rats (Padma et al., 2012), and chicken (Qureshi et al., 2011). The present results are in parallel with previous research which reported the beneficial effects of dietary thymol on enhancing the hematological variables including HDL-C, HDL-C, cholesterol and triglyceride of various fish species including Nile tilapia (*O. niloticus*) (Ibrahim et al., 2021, 2022; Latif et al., 2021), rohu (*Labeo rohita*) (Ibrahim et al., 2021), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) (Taheri-Mirghaed et al., 2020), Asian sea bass (*Lates calcarifer*) (Abdelwahab and El-Bahr, 2012) and channel catfish (*Ictalurus punctatus*) (Zheng et al., 2009).

Antioxidant defense systems include antioxidant enzymes and protect tissues from oxidative damage (Halliwell and Gutteridge, 1990; Sharawy et al., 2017). The current findings demonstrated enhancement in the activities of T-AOC, SOD, and CAT by the effect of thymol and/or probiotic that are in complete concordance with Ibrahim et al. (2022) who found that Nile tilapia fed diets supplemented with a combination of Thy and ThQ displayed significantly improved intestinal antioxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase). Moreover, the present findings are in agreement with Magouz et al. (2022), Amer et al. (2018) and Khondoker et al. (2016), who reported that dietary inclusion of thymol to Nile tilapia increased the expression levels of catalyze and super oxide dismutase genes suggesting the antioxidant characteristics of thymol. The current findings are linked to the addition of the phytochemical thymol, which may improve antioxidative status, inhibit free radical formation, reduce lipidic superoxide damage, and modulate the immune responses in fish (Zhou et al., 2015). Alike at 56 days, the addition of flavonoids from *Allium mongolicum* to the diet greatly improved the *C. argus'*s liver SOD and CAT activities (Li et al., 2019). Furthermore, the antibacterial characteristics of probiotic (*P. acidilactici*) and its encouragement of the formation of good metabolites and/or gut microorganisms are the most likely causes of the enhancement in antioxidant enzymes in the current data (El-Rhman et al., 2009; Sankar et al., 2017; Shabbir et al., 2021). In this context, several studies demonstrated that adding probiotics as *P. acidilactici* or *Lactobacillus plantarum* or *Bacillus subtilis* in shrimp, *Litopenaeus stylirostris*, *Labeo rohita* in Nile tilapia diets enhanced the activity of CAT and SOD (Castex et al., 2009; Giri et al., 2013; Ali et al., 2020). Also, the SOD was enhanced by *Lactobacillus* sp. and *B. amyloliquefaciens* supplementation in Nile tilapia diets (Ridha and Azad, 2012). Further investigation is needed to explore the mechanism by which the combination of thymol and probiotics modulate antioxidant capacity.

Conclusion

It is possible to conclude that supplementing the Nile tilapia diet with thymol and/or probiotics improved the fish's health and development. The synergistic impact of thymol and probiotics had a superior influence on growth, intestinal digestive enzymes, feed efficiency, in-

testinal histomorphometric indices, hemato-biochemical state, and antioxidant status of *Oreochromis niloticus*. Thymol has been shown to have tremendous promise as an immunostimulant, enhancing fish immunological capabilities and productivity in fish farming. Furthermore, more research is needed to fully understand the mechanisms of thymol's impact on Nile tilapia when combined with probiotics.

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