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Research Article

Effect of lemon pomace inclusion on growth, immune response, anti-oxidative capacity, intestinal health, and disease resistance against *Edwardsiella tarda* infection in Nile tilapia (*Oreochromis niloticus*)

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Abstract

This trial aimed to investigate the impact of dietary lemon pomace inclusion on growth, immune response, anti-oxidative capacity, intestinal health, and disease resistance against *Edwardsiella tarda* in the Nile tilapia. The fish weighted (20±5 g) were randomly allocated into three groups and fed diets containing varying amounts of dried lemon pomace powder (0[control], 1, and 2%) for 10 weeks. After this period, the fish were challenged with *E. tarda.* The bacterium was isolated from naturally infected fish from fish farms in the Kafr-Elsheikh governorate, Egypt with a rate of 14%, and its virulence genes (*cds1*, *qse C*, and *pvsA*) were detected using PCR. It was observed that 1 or 2% of lemon pomace dietary addition improved productive performance compared to control. Also, 1 or 2% of lemon pomace dietary addition reduced serum glucose, cortisol, triglycerides and cholesterol concentrations, while increasing the serum catalase and superoxide dismutase activities and improving phagocytic, lysozyme, and bactericidal activities in a dose-dependent manner compared to the control group. Moreover, 1 or 2% of lemon pomace dietary addition increased the length of intestinal villi and goblet cell number in a dose-dependent manner of different intestinal portions compared to the control group. The highest survival (80%) with the lowest mortality (20%), morbidity (56.6%), and re-isolation (30%) rates after *E. tarda* infection was observed in the group fed a diet containing 2.0% lemon pomace followed by 1.0% lemon pomace group, while the worst rates were recorded in the control fish.

Introduction

Nile tilapia is considered the most important fish species in Egypt, accounting for 71.38% of all cultured fish in Africa and 1.54% of total cultured fish worldwide, as a lipid-rich source of animal protein (El-Tawab *et al*., 2021; FAO, 2021). Outbreaks of diseases, particularly those caused by bacterial infections, pose a major and highly significant constraint to the fish industry (Saad, 2002). Bacteria are considered one of the most common causes of diseases in environmentally stressed cultured fish in warm water (Abd El-Kader, 2015). *Edwardsiaella tarda* is a bacterial pathogen that infects various age groups of fish in warm freshwater (Bin *et al*., 2012).

E. tarda is a Gram-negative bacterium that can cause systemic disease in both fish and humans. Recently, the complete genome sequence of a highly pathogenic, multidrug-resistant strain of *E. tarda* found in China revealed the presence of several genes related to virulence and toxin production, enabling its survival within phagocytic cells and its ability to infect a wide range of hosts (Verjan *et al.*, 2013).

E. tarda is also important due to its zoonotic nature. Infected fish that are processed for human consumption can cause gastroenteritis and meningitis, particularly among people with impaired immune systems (Mizunoe *et al*., 2006). Catalase (katB), TTSS regulator (esrB), putative killing factor (mukF), Fimbrial operon (fimA), glutamate decarboxylase (gadB), Citrate lyaseligase (citC), pstS, pstC, type III secretion system (ssrB), astA, isor, ompS2, Hemolysine A (hlyA) and ATPase domain of DNA Gyrase (gyrB) were considered as virulence genes in *E. tarda* (Choresca *et al.,* 2011; Abd El-Kader, 2015). PCR is considered the fastest and most reliable diagnostic tool for the detection of *Edwardsia tarda* in Nile tilapia, utilizing DNA extraction and amplification with GoTaq®, Hot Start® Green Master Mix (GML), and Oligonucleotide (OLN) primers (Iregui *et al.,* 2012; El Seedy *et al.,* 2015).

Enhancing growth and disease control are the primary goals of the fish industry. The use of antibiotics and therapeutics in aquaculture to combat opportunistic bacteria is common. (Austin and Austin, 2007). However, the use of antibiotics can lead to antibiotic resistance bacteria and residue in fish fillet (Esiobu *et al.,* 2002). Herbs or their byproducts have been used in aquaculture feeds in recent years as a substitute for antibiotics.

Herbs and bioactive components have the advantage of not leaving any residue and not adversely affecting the health of fish, humans, or the environment. Medicinal plants or their by-products can be used therapeutically to control diseases and promote growth in fish (Adewole, 2014; Newaj-Fyzul and Austin, 2015). Aquaculture production leads to various complications such as stress factors which reduce fish immunity, the development of infectious diseases and subsequently reduce the economic efficiency of fish production (Harikrishnan *et al.,* 2020). So suitable feed additives may play an important role as growth promoters and immune stimulation in aquaculture.

Lemon is an essential medicinal plant of the genus Citrus of the Rutaceae family (Tirado *et al.*, 1995), it contains different bioactive components such as essential oil, flavonoids, vitamin C and potassium (Garcia Beltran *et al.*, 2017). Lemon is widely used in Egypt daily; almost all parts such as fruit, peels, and leaves are used as a household medicine either alone or in combination with other herbs. Annual lemon production in Egypt was approximately 4.3 million tons (FAO, 2021). There are many by-products obtained during lemon juice processing such as peel, seed and residue pulp which represent about 50% of the whole fruit and can be used as feed additives in different animal feeds (Gonzalez-Molina *et al.*, 2010).

In aquaculture, lemon pomace improved growth and health status in rainbow trout (Chekani *et al.*, 2021) in common carp (Sadeghi *et al.*, 2021) and in gilthead seabream (Garcia Beltran *et al.*, 2017). There is little information here about the effect of lemon pomace powder on growth promoters and immune enhancers of Nile tilapia are available. So the present study aimed to through light on the effect of dietary lemon pomace powder on growth performance, immune response, antioxidant activity, liver health, intestinal morphology and resistance against *Edwardsiella tarda* challenge of Nile tilapia fish.

Material and methods

Collection of fish samples

One hundred Nile tilapia fish samples (apparently healthy and naturally infected fish) were randomly collected from fish farms in the Kafr- Elsheikh governorate and transported quickly to the Animal Health Research Institute lab (kafr- Elsheikh branch).

Bacteriological examination

Fish were aseptically dissected and kidney, liver, spleen and gills were swabbed and inoculated into trypticase soy broth and incubated at 37^0 C for 18-24 hrs. Then streaked onto *Salmonella-Shigella* agar media and incubated at 37^0C for 24-48hrs (Lima *et al*., 2008). The suspected colonies were preserved in semisolid agar, and tested for Gram reaction, and cultural, morphological and biochemical characteristics (Xiao *et al.,* 2008).

Molecular identification and detection of some virulence genes of E.tarda by PCR

Seven *E.tarda* isolates were confirmed and tested for some virulence genes (cds1, qseC and pvsA) using PCR at Biotech Research Unit at N.L.Q.P. at Animal Health Research Institute (AHRI).

DNA extraction

DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH).

Oligonucleotide Primer. Primers were supplied from Metabion (Germany) as described in Table 1. PCR amplification. primers were used in a 25- µL reaction containing 12.5 µL EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µL of each primer of 20 pmol concentration, 4.5 µL of water, and 6 µL of DNA template. The reaction was performed in an Appliedbio system 2720 thermal cycler. Analysis of the PCR products. The yield of PCR was separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 40 µL of the products were loaded in each gel slot.

Generuler 100 bp DNA ladder (Fermentas, Thermo, Germany) was used in the determination of the fragment sizes. Gel photographed through gel documentation system (Alpha Innotech, Biometra) and the obtained data was analyzed using the available computer software.

Table 1: Sequences of primers, target virulence genes, amplicon sizes and cycling instructions

Target gene	Primers sequences	Amplified segment	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
E. tarda GyrB1	GCATGGAGACCTTCAGCAAT GCGGAGATTTTGCTCTTCTT	415 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Park *et al*., 2014
Cds1	TCTCCACCCATAATGCCACG CAAACGGCGTCGTGTAGTCG	435 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
qseC	CAGCAGTAGCAGGATCACCA ATGGACGTATGCTGCTCAAC	260 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Castro *et., al*. 2016
pvsA	CTGGAGCAGTACCTCGACGG CGATGCTGCGGTAGTTGATC	313 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

Lemon pomace preparation

Lemon pomace was obtained from a local lemon juice factory in Egypt. The pomace was dried under shade at room temperature. Dried lemon pomace was powered and passed through a 2mm screen and chemically analyzed before inclusion in the experimental diets (lemon pomace powder ‘LPP’ contains: 6.9% moisture, 7.8% crude protein, 5.7% ash, 0.45% phosphorus and 0.66% calcium).

Feeding diets and experimental design

The basal diet was formulated and prepared to meet the nutrient requirements of the Nile tilapia fish according to the NRC (2011) and presented in Table 2. One hundred and thirty five of *O. niloticus* weighted (24±5 gm) were allocated into three equal groups and each group containing 45 fish was distributed to three replicates. The health conditions of fish were examined for any disease condition (parasitic and bacterial). The basal diet was formulated without LPP inclusion (group, 1) and considered as control, while groups 2 and 3 were fed on the basal diet with LPP inclusion at 1.0% and 2.0% respectively. All ingredients component was fine-grinding and thoroughly mixed, then warm water (400 ml/kg) was added and pressed to produce 2mm pellets. The obtained pellets were dried in a hot air oven set at 45 ^{0}c and stored in airtight bags prior to use.

Experimental design and procedure

Fish in each group were fed to satiation twice (at 9:00 and 14:00) with care to ensure that all added feed was consumed. Fish were weighed at the start of the experiment (W0) and then biweekly weighed for 8 weeks.

Table 2: Physical and chemical composition of the experimental diets

Ingredients%	Lemon pomace powder inclusion rate		
	0.0	**1.0**	**2.0**
Yellow corn	22.8	21	18.3
Corn gluten	9	9	9
Fish meal	12	12	12
Soybean meal (44%)	33	33	33
Lemon pomace powder	0	1	2
Wheat bran	10	9.5	9.5
Wheat grain	8	9.3	11
Sunflower oil	2	2	2
di-calcium phosphate	0.5	0.5	0.5
Choline chloride	0.1	0.1	0.1
methionine	0.1	0.1	0.1
salt	0.2	0.2	0.2
Vitamin mixture*	0.15	0.15	0.15
Mineral mixture**	0.15	0.15	0.15
Carboxy methyl cellulose	2	2	2
Chemical composition:			
Moisture	10.8	11.2	10.6
Crude protein	29.7	29.9	29.5
Ether extract	4.5	4.4	4.6
Ash	5.8	5.6	6.1
Crude fiber	6.1	6.2	5.9
NFE	43.1	42.7	43.3
Calcium	0.77	0..74	0.76
Total phosphorus	0.67	0.71	0.66
DE***	3215.9	3202.5	3222.4

*Vitamins mixture- each one Kg contains: vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10 g, vitamin K3 2 g, vitamin B1 1 g, vitamin B2 5 g, vitamin B6 1.5 g, vitamin B12 0.01 g, vitamin C 250 g, Niacin 30 g, Biotin 0.050 g, Folic acid 1 g and Pantothenic acid 10 g and carrier to 1000 g.

**Mineral mixture - each one Kg contains: Manganese 60 g, Copper 4 g, Zinc 50 g, Iron 5 g, Iodine 1 g, Cobalt 0.1 g, Selenium 0.1 g, calcium carbonate (CaCO3) carrier to 1000 g.

*** Digestible energy (DE) was calculated (kcal/kg) according chemical composition of used feedstuffs (NRC 2011).

Calculations

Weight gain was calculated as follows:

Weight gain = (Final body weight- Initial body weight)

Gain% was calculated as follows:

Gain%= (Total gain/Initial Wt.) X100

Specific Growth Rate (SGR) was calculated as follows:

SGR=(lnWf - lnWi x100)/t

Where InWf = the natural logarithm of the final weight,InWi = the natural logarithm of the initial weight, andt= time (days) between lnWf and lnWi

Feed Conversion Ratio (FCR) was calculated as follows:

FCR = Feed intake (FI) per aquarium/weight gain per the same aquarium

Protein Efficiency Ratio (PER) was calculated as follows:

PER=Weight gain/Protein intake

Efficiency of Energy Utilization (EEU) was calculated as follows:

EEU=digestible energy (DE) intake/weight gain

Sampling

Blood samples were collected from nine fishes of each group (three of each replicate) at the end of the feed trial and a certain amount of the samples were divided into tubes containing sodium citrate for hematological parameters and phagocytosis, while the other part of the blood was transferred into tubes without anticoagulant for serum separation. The blood specimens were left to coagulate at room temperature and then centrifuged at 3000g for 15 min and the supernatant was removed.The obtained serum was kept at −18°C to measure the lipid profile, glucose, cortisol, antioxidant and immunological parameters.

Determination of hematological parameters

The white blood cells (WBC, (x 10^3/mm^3) were counted using a hemocytometer. Red blood cells (RBC, (x 10^6/mm^3), PCV % and hemoglobin (Hb, g/dl) were determined by using the method by (Dacie and Lewis, 1996). Neutrophils and leucocyte counts were obtained using peripheral blood smears stained by Giemsa (Beutler *et al*., 2001).

Determination of serum biochemical parameters

Serum glucose, cortisol, triglyceride, cholesterol, HDL and LDL concentrations and also, serum superoxide dismutase (SOD), catalase (CAT), Malondialdehyde (MDA) activities were estimated using commercial kits produced by Bio Diagnostic (Diagnostic and Research Reagents).

Determination of immunological parameters:

Serum lysozyme and bactericidal activities were estimated according to Sahu *et al.* (2006) and Rainger and Rowley (1993), respectively. Moreover, phagocytic index and activity were calculated according to (Kawahara *et al*., 1991).

Histopathology examination

The anterior, middle and posterior sections of intestines and heptosplenic tissue of three fish were collected and directly fixed in 10% formalin solution, dehydrated with ethanol (100%), embedded in paraffin, sectioned at 3µm, and stained by hematoxylin and eosin stain. The hepatosplenic and intestinal sections were examined for histological changes as mentioned by Bancroft *et al.* (2013).

Feed sample collection and chemical analysis

Feed samples from each experimental diet were collected at the start, middle and at the end of experimental period and stored at -4°C for later proximate analysis. Dry matter, ash, crude protein and ether extract were determined by standardized methods of the Association of Official Analytical Chemists (AOAC, 1995). Calcium and phosphorus were analyzed according to Slavin (1968) and Geriche and Kurmies (1952), respectively.

Preparation of the bacterial strain for experimental challenge

E. trada virulent isolates were sub-cultured on *Salmonella-shigella* agar (S.S agar) plates and incubated at 37⁰C for 24 hrs. A typical colony of *E. trada* was picked up and inoculated in tryptic soya broth for 24 hrs at 37°C then centrifuged at 3000 rpm for 4 minutes and the bacterial cells were re-suspended in phosphate buffered saline and adjusted to (1.2x10^8 cfu/mL) by using

McFarland standard tube according to Cruickshank *et al*. (1982).

Protocol of experimental challenge

At the end of the trial 30 fish from each group were intraperitoneal (I/P) injected with 0.2 ml of the prepared bacterial strain (1.2×10^8 cfu/mL) and kept for 10 days after injection under observation Nagy *et al*. (2018). Clinical signs and mortality rates for challenged fish were followed up and recorded. Gills, liver and kidney samples were collected from 10 fish (dead and survivors) in each group for bacteriological examination and re-isolation.

Statistical analysis

Statistical analysis of the obtained data was made using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. Results obtained were illustrated as mean±standard error of the mean (SEM) and the differences were considered significant at $p<0.05$.

Results

Clinical signs of naturally infected fish

Clinical signs and postmortem lesions of naturally infected *O. niloticus* are presented in Figures 1 and 2.

Figure 1: ulcer and hemorrhages on skin, and opaque eye (left) and opaque eye and tail erosions (right) in Nile tilapia fish

Figure 2: Distended gall bladder, congestion and hemorrhages in all internal organs of Nile tilapia fish.

Isolation and identification of E. tarda

Isolates of *E. tarda* were detected in 14% of examined Oreochromis niloticus. Isolated colonies were identified and characterized as Colony morphology, culture and microscopic characters were identified as described by Fang *et al.* (2006) and Xiao *et al.* (2008).

Molecular identification and detection of some virulence genes of E. tarda using PCR

It was detected that 415 bp fragment of the gyrB gene of *E. tarda* shown in Figure 3 and virulence genes of *E.* tarda which are *qseC*, *pvsA* and *cds1*gene as shown in Figures 4 to 6.

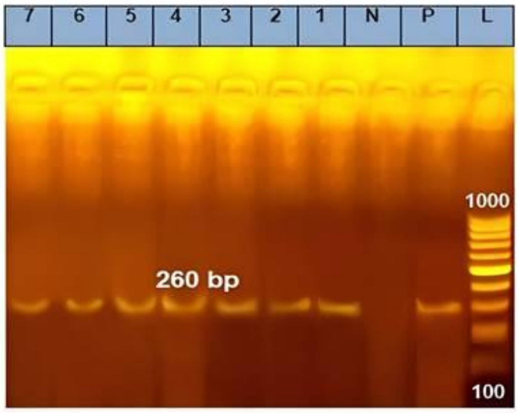

Figure 3: PCR amplification products of *qseC* gene for characterization of *E. tarda.* Lane L: marker (100-1000 bp). Lane P. and N.: control Positive and negative. Lane1-7: Positive for *E. tarda* strains at 260 bp.

Growth performance and feed efficiency parameters

The growth performance of fish is illustrated in Table 3. It was noticed that 1.0% or 2.0% of LPP inclusion in fish diet non-significantly ($p \geq 0.05$) increased final body weight and total gain by about (6.4% and 19.3%) and (9.04% and 31.6%) respectively, but 1.0% of LPP addition non-significantly ($p \geq 0.05$) improved gain% and SGR value and high LPP (2.0%) inclusion significantly ($p<0.05$) improved gain% and SGR value compared to the control group. Moreover, 1.0% or 2.0% of LPP inclusion in the Nile tilapia diet increased total feed intake and addition non-significantly ($p \geq 0.05$) improved average FCR, PER and EUU throughout the whole experimental period compared to the control group.

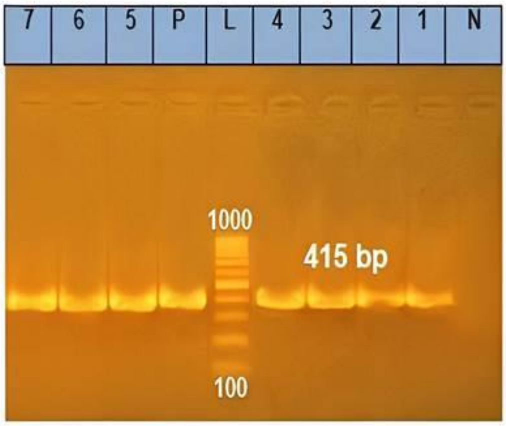

Figure 4: PCR amplification products of *gyrB1*gene for identification of *E. tarda.* Lane L: Marker (100-1000 bp). Lane P. and N.: controls positive and negative. Lane1-7: Positive for *E. tarda* strains at 415 bp.

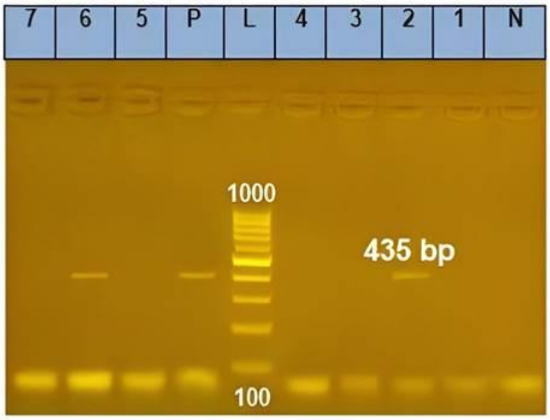

Figure 5: PCR amplification products of *cds1*gene for characterization of *E. tarda.* Lane L: marker (100-1000 bp). Lane P. and N.: control positive and negative. Lane2 and 6 : Positive for *E. tarda* strains at 435 bp.

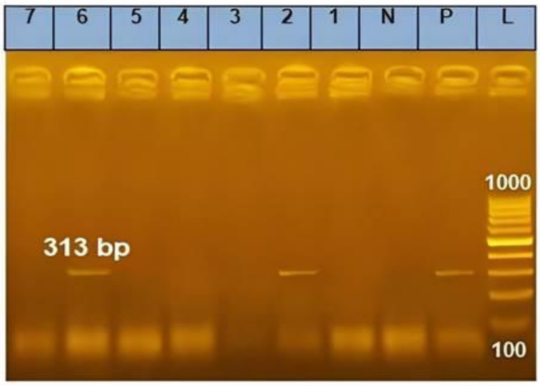

Figure 6: PCR amplification products of *pvsA* gene for characterization of *E. tarda.* Lane L: marker (100-1000 bp). Lane P. and N.: control positive and negative. Lane2 and 6 : Positive for *E. tarda* strains at 313 bp.

Hematological parameters

Hematological results (Table 4), it was found that LPP inclusion at both levels in the Nile tilapia diet had no significant effect on RBCs, Hb%, PCV%, monocyte%, eosinophil% and basophil% while, significantly increasing WBCs and neutrophil% compared to the control group.

Table 3: Growth performance of Nile tilapia fish fed different levels of lemon pomace powder

Parameters	**Dietary lemon pomace powder inclusion levels**			***p*-Value**
	0.0% (control)	**1.0%**	**2.0%**	
Initial weight (g/fish)	24.59±1.13	24.17±0.71	24.64±1.14	0.936
Final weight (g/fish)	34.95±1.64	37.20±1.31	38.11±1.45	0.294
Total weight gain (g/fish)	9.78±0.99	11.67±0.83	12.87±0.66	0.051
Gain%	39.87±3.62^{b}	44.90±2,83^{ab}	51.65±2.62^{a}	0.041
SGR (%/day)[1]	0.26±0.004^{b}	0.28±0.003^{ab}	0.32±0.002^{a}	0.028
Total feed intake (g/fish)	32.79±0.0^{b}	34.93±0.53^{a}	33.81±0.17^{ab}	0.0002
FCR[2]	3.35±0.23	2.99±0.36	2.63±0.24	0.0672
PER[3]	0.99±0.12	1.11±0.11	1.27±0.08	0.0864
EEU[4]	9.96±0.89	8.89±1.03	7.80±0.67	0.0671

Different letters represent statistical difference between different LPP levels. Significant difference considered at (p<0.05).

[1] SGR=specific growth rate.

[2] FCR = feed conversion ratio.

[3] PER= protein efficiency ratio.

[4] EEU= efficiency of energy utilization.

Table 4: Hematological results of Nile tilapia fish fed different levels of lemon pomace powder

Parameters	**Dietary lemon pomace powder inclusion levels**			***p*-Value**
	0.0% (control)	**1.0%**	**2.0%**	
RBCs (x 10^6/mm^3)*	1.66±0.18	1.69±0.19	1.83±0.03	0.401
WBCs (x 10^3/mm^3)**	26.6±0.79^{b}	29.27±0.88^{a}	31.80±1.09^{a}	0.0001
Hb%***	8.31±0.88	8.43±1.05	9.13±0.17	0.401
PCV%****	27.40±3.12	27.82±4.03	30.14±0.56	0.412
Neutrophil%	31.27±0.82^{c}	35.40±0.31^{b}	37.67±0.76^{a}	0.0001
Lymphocyte%	53.60±0.91^{a}	48.50±1.12^{b}	47.60±0.59^{b}	0.0001
Monocyte%	5.97±0.67	6.53±0.73	6.33±0.79	0.565
Eosinophil%	1.83±0.11	1.87±0.12	1.67±0.14	0.719
Basophil%	7.33±0.98	7.70±0.47	6.63±0.45	0.159

Different letters represent statistical difference between different LPP levels. Significant difference considered at (p<0.05).

*RBCs = red blood cells.

** WBCs = white blood cells.

*** Hb = hemoglobin.

****PCV = packed cell volume.

Serum glucose and cortisol

The serum glucose and lipid profile of the Nile tilapia fish fed different levels of LPP is presented in Table 5. Dietary LPP at 1.0 or 2.0% significantly (p<0.05) reduced serum glucose concentration, while a low level of LPP addition non-significantly (p≥0.05) reduced serum cortisol level and 2.0% of LPP inclusion significantly (p<0.05) reduced serum cortisol concentration compared to the control group.

Serum lipid profile

The serum lipid profile as affected by different levels of LPP in the Nile tilapia fish diet is presented in Table 5. Dietary inclusion of LPP at 1.0 or 2.0% non-significantly ($p \geq 0.05$) decreased serum triglycerides, total cholesterol, LDL, vLDL concentrations and CHO/HDL ratio but significantly ($p<0.05$) increased serum HDL concentration compared to the control group.

Table 5: Serum glucose and lipid profile of Nile tilapia fish fed different levels of lemon pomace powder

Parameters	**Dietary lemon pomace powder inclusion levels**			***p*-Value**
	0.0% (control)	**1.0%**	**2.0%**	
Glucose (mg/dl)	102.54±12.32^{b}	84.35±2.17^{a}	77.14±1.02^{a}	0.001
Cortisol (ng/mL)	22.70±1.29^{a}	20.63±1.05^{ab}	19.27±0.71^{b}	0.002
Triglycerides (mg/dl)	324.88±9.88	280.44±51.89	303.55±18.64	0.247
Cholesterol (mg/dl)	285.80±54.74	259.7±28.17	261.38±17.47	0.611
HDL (mg/dl)[1]	52.30±1.65^{b}	61.70±1.52^{a}	62.13±3.11^{a}	0.0001
LDL (mg/dl)[2]	168.53±56.11	141.29±34.35	138.54±14.82	0.574
VLDL (mg/dl)[3]	64.97±2.00	56.09±10.29	60.71±3.71	0.247
CHO/HDL ratio[4]	3.22±1.02	2.26±0.50	2.22±0.17	0.158

Different letters represent statistical difference between different LPP levels. Significant difference considered at ($p<0.05$).
[1] HDL=high density lipoprotein.
[2] LDL=low density lipoprotein.
[3] VLDL=very low density lipoprotein.
[4] CHO/HDL=cholesterol/ high density lipoprotein.

Serum SOD, CAT and MAD enzymes activities

Dietary inclusion of 1.0% LPP in the Nile tilapia fish diet non-significantly ($p \geq 0.05$) increased serum SOD and CAT enzymes activities, while 2.0% addition of LPP significantly ($p<0.05$) increased serum CAT activity only compared to the control. Moreover, LPP decreased MAD enzyme activity in a dose-dependent manner compared to control (Table 6).

Immune response

The effect of different levels of LPP on phagocytosis, lysozyme and bactericidal activity of the Nile tilapia is presented in Table 7. Phagocytic activity, phagocytic index, and lysozyme activity were non-significantly ($p \geq 0.05$) improved in the fish group fed on 1% LPP, but phagocytic and lysozyme activities were significantly ($p<0.05$) stimulated in the fish group fed 2% LPP compared to the control. Moreover, both fish groups fed 1 or 2% LPP significantly ($p<0.05$) improved bactericidal activity compared to the control group.

Table 6: Serum antioxidants enzymes activity of Nile tilapia fish fed different levels of lemon pomace powder.

Parameters	**Dietary lemon pomace powder inclusion levels**			***p*-Value**
	0.0% (control)	1.0%	2.0%	
SOD (U/mL)[1]	1374.60±367.61	1675.73±316.l7	1641.03±297.74	0.697
CAT (U/L)[2]	148.47±33.76^{b}	191.73±43.26^{ab}	284.93±77.26^{a}	0.015
MAD (nmol/mL)[3]	9.78±0.47^{a}	8.62±0.47^{ab}	7.78±00.29^{b}	0.0001

Different letters represent statistical difference between different LPP levels. Significant difference considered at ($p<0.05$). [1]superoxide dismutase (SOD). [2]catalase (CAT). [3]Malondialdehyde (MDA)

Table 7: phagocytosis, lysozyme and bactericidal activity of Nile tilapia fish fed different levels of lemon pomace powder

Parameters	Dietary lemon pomace powder inclusion levels			*p*-Value
	0.0% (control)	1.0%	2.0%	
Phagocytic activity	57.70±4.22^{b}	62.63±2.05^{ab}	70.43±5.53^{a}	0.003
Phagocytic index	2.33±0.22^{a}	2.30±0.08^{a}	2.26±0.19^{a}	0.899
Lysozyme activity	0.30±0.06^{b}	0.53±0.12^{ab}	0.55±0.08^{a}	0.022
Bactericidal	79.17±2.69^{b}	64.80±8.71^{a}	62.47±4.06^{a}	0.028

Different letters represent statistical difference between different LPP levels. Significant difference considered at ($p<0.05$).

Intestinal morphology

The impact of LPP on the intestinal morphology of Nile tilapia fish is presented in Table 8 and Figures 7 to 9. Inclusion of LPP at 1.0 or 2.0% in the Nile tilapia fish diet significantly ($p<0.05$) increased villi length and goblet cell number in a dose-dependent manner of the anterior, middle and posterior intestinal portions compared to the control. The villi width significantly ($p<0.05$) decreased in the anterior portion of the intestine with LPP inclusion at different levels while no effect on villi width in the middle and posterior portions of the intestine compared to the control. The inclusion of LPP in the diet showed no notable impact on the inter-villi space in the anterior section of the intestine. However, in the middle and posterior sections, the dietary addition of LPP significantly reduced the inter-villi space compared to the control group (P<0.05).

Hepatopancrease histopathology

In all experimental groups, the histopathological analysis revealed changes in the liver (hepatocytes), spleen (splenocytes) and pancreatic tissues, as shown in Figure 10.

The challenge against Edwardsiella tarda infection Mortality started on the second day after the challenge in the control group. After ten days, the survival, mortality, morbidity, and re-isolation rates in the fish group fed a 2.0% LPP-containing diet were reported as 80%, 20%, 56.66%, and 30% respectively and in the group fed on 1.0% LPP-containing diet were reported as 63.33%, 36.66%, 63.33% and 50% respectively, but the lower rate of survival (40%) and higher rates of mortality, morbidity, and re-isolation (40%, 60%, 80% and 90% respectively) were recorded in the control fish group as shown in Table 9.

Table 8: Intestinal morphology of Nile tilapia fish fed different levels of lemon pomace powder.

Parameters	Dietary lemon pomace powder inclusion levels			*p*-Value
	0.0% (control)	1.0%	2.0%	
Interior portion				
Villi length (µm/mm)	193.53±10.75^{c}	250.36±16.79^{b}	329.68±14.47^{a}	0.0001
Villi width (µm/mm)	90.29±2.52^{a}	77.61±5.39^{b}	73.99±5.23^{b}	0.0006
Inter villi space	74.33±5.27	74.97±5.71	71.36±5.25	0.679
Goblet cells No./mm2	20.67±1.28^{c}	27.67±2.12^{b}	43.67±1.65^{a}	0.0001
Middle portion				
Villi length (µm/mm)	343.64±11.88^{c}	472.18±15.37^{b}	582.26±28.44^{a}	0.0001
Villi width (µm/mm)	79.99±0.48^{a}	71.86±2.09^{b}	78.62±1.59^{a}	0.0001
Inter villi space	88.74±4.41^{a}	69.96±3.82^{b}	51.86±2.65^{c}	0.0001
Goblet cells No./mm2	37.33±1.52^{c}	48.33±1.29^{b}	68.67±2.59^{a}	0.0001
Posterior portion				
Villi length (µm/mm)	122.19±5.88^{c}	243.56±12.94^{b}	376.91±18.82^{a}	0.0001
Villi width (µm/mm)	104.65±5.29^{ab}	95.85±5.29^{b}	132.59±18.06^{a}	0.0072
Inter villi space	120.15±8.23^{a}	85.74±3.53^{b}	75.42±3.53^{b}	0.0001
Goblet cells No./mm2	13.00±1.47^{b}	23.00±2.35^{a}	25.67±2.06^{a}	0.0001

Different letters represent statistical difference between different LPP levels. Significant difference considered at ($p<0.05$).

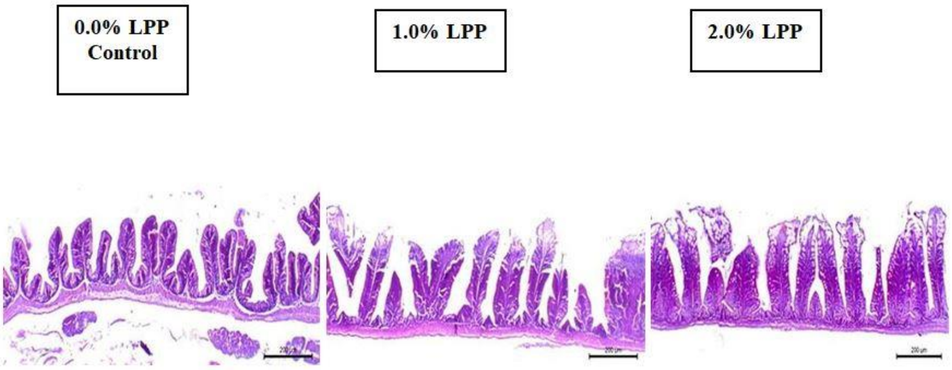

Figure 7: intestinal morphology (anterior portion) of Nile tilapia fish showing normal villi of the control group and the fish group fed 1.0% LPP containing diet, while showing increased villi length of fish group fed 2.0% LPP containing diet.

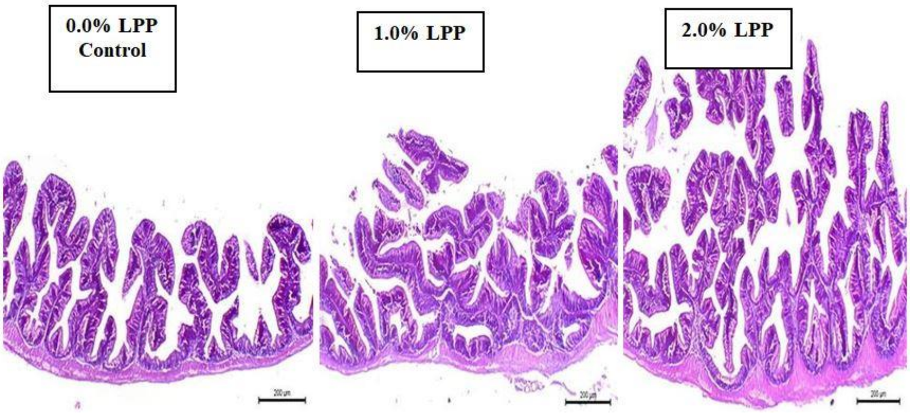

Figure 8: intestinal morphology (middle portion) of Nile tilapia fish showing long and branched villi lined with pseudostratified epithelium of the control group, while showing increased villi length of the fish group fed 1.0% LPP containing diet and showing marked increase of villi length of fish group fed 2.0% LPP containing diet.

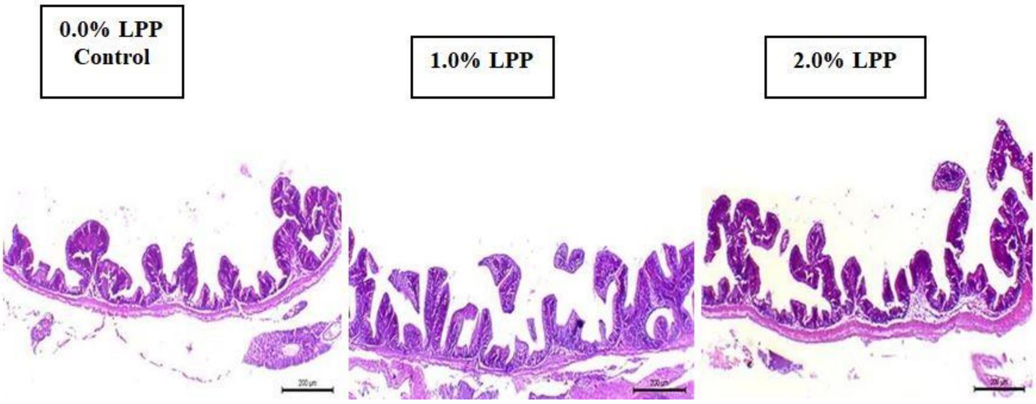

Figure 9: intestinal morphology (posterior portion) of Nile tilapia fish showing normal mucosal folds of the control group, while showing normal mucosal folds of the fish group fed 1.0% LPP containing diet and showing increased villi length of fish group fed 2.0% LPP containing diet.

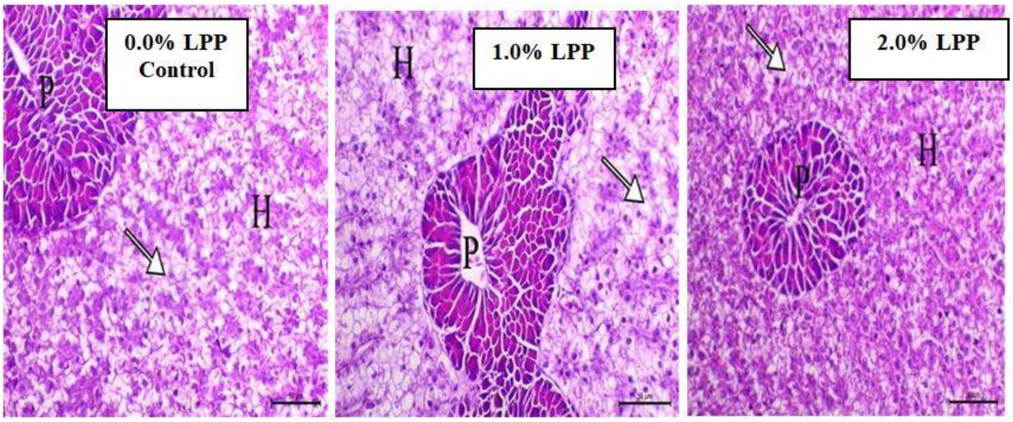

Figure 10: Hepatopancrease histopathology of Nile tilapia fish showing normal hepatic and pancreatic portions (H indicates hepatocytes, P indicates pancreatic tissues and arrowhead indicates normal hepatocytes with moderate vacuolation of the control group and mild vacuolation of the fish fed on 1% or 2% LPP containing diets.

Table 9: *Edwardsiella tarda* re-isolation rate after experimental challenge

Parameters	**Dietary lemon pomace powder inclusion levels**		
	0.0% (control)	**1.0%**	**2.0%**
No. of fish before challenge	30	30	30
No. of fish after challenge	12	19	24
Mortality rate			
Number	18	11	6
Percentage	60	36.66	20
Survival rate			
Number	12	19	24
Percentage	40	63.33	80
Morbidity rate			
Number	24	19	17
Percentage	80	63.33	56.66
Re-isolation rate			
Number	9/10	5/10	3/10
Percentage	90	50	30

Discussion

Edwardsiellosis is a severe systemic disease caused by *Edwardsiella tarda*. It affects both wild and cultured fish. *Edwardsiella* is one of the most harmful pathogens in aquaculture, with a wide host range and high economic losses for aquaculture industries around the world (Hou *et al.*, 2009; Park *et al.*, 2012) including humans (Xie *et al*., 2014). In our study, naturally infected O.niloticus had petechial hemorrhages and hemorrhagic patches on the flank area, scale detachment, skin discolouration, protruded hemorrhagic vent, eye exophthalmia and corneal opacity. These findings are similar to the ones mentioned by some of the authors (Han *et al.* (2006), Padros *et al.* (2006), Shabaan (2007), and Ibrahim *et al.* (2011).

The postmortem lesions of naturally infected *O. niloticus* showed heavy congestion with hemorrhagic spots on the body, exophthalmia, and ascites. There was heavy congestion of the internal organs (Liver, Kidney, Spleen, and intestine). Blood ascetic fluid was present in the abdomen cavity. These results was like those recorded by Ibrahem *et al*. (2011), Fatma Korui, 2012; Carlos *et al*., 2012).

In our study *E.tarda* isolation rate was 14%, which was isolated from internal organs (liver, kidney, spleen and gills) of apparently healthy and naturally infected fish collected from fish farms in the Kafr-Elsheikh governorate. This percentage almost agreed with that of Korni *et al*. (2012) who recorded an isolation rate of13.3% and higher than those mentioned by El-Seedy *et al.* (2015) and Abd El-Tawab *et al*. (2021) who recorded an isolation rate of 4.3%and 8%, respectively and less than that reported by Nagy *et al.* (2018) who isolated it with a percentage of 28%.

DNA gyrase gyr B is the principle for the replication and qseC genes (sensor protein included in quorum sensing which enable *E. tarda* to reach Quorum sensing (QS) to harmonize cellular responses to environmental changes by intercellular complex communication systems and regulates cellular behaviors like biofilm formation, bioluminescence and expressing virulence factors managing the expression of flagellar genes and motility besides the secretion system improving the pathogenicity of *E.tarda* (Xin *et al*., 2011; Weigel and Demuth, 2015). Both DNA gyrase gyr B and qseC genes were detected in all examined isolates, this typically agreed with El-Seedy *et al*. (2015) while, chondroitinase enzymes gene (cds1) destroys fish cartilage in case of chronicity (Xu *et al*., 2014) and vibrioferrin (pvsA) gene which is a siderophores type that provides *E. tarda* with iron which is a necessary factor for growth in the host and virulence factors expression aiding in survival and replication of *E. tarda* (Kokubo *et al.,* 1990) was detected in two isolates (2&6) which means heterogenicity of the tested isolates, this disagreed with Castro *et al.* (2016) who detected them in all isolates.

In this study, dietary inclusion of LPP at both levels (1.0 or 2.0%) non-significantly ($p \geq 0.05$) improved FBW, TG, FCR, PER and EUU of Nile tilapia fish. The growth-positive effect of LPP may be due improvements in intestinal health and nutrient utilization (Milos *et al*., 2000)**.** This improvement may be related to the active components of LPP, which act as antibacterial, antioxidant, and growth promoters for fish (AL-Jabri and Hossain, 2014; Xi *et al*., 2017). The obtained results are in harmony with very recent studies in *L. victorianus, Lates calcarifer*, *S. aurata, O. mossambicus*, and *O. niloticus* treated with different levels of orange and lemon pomace supplementation diets against pathogens (Baba *et al*., 2016; Shiu *et al*., 2016; García Beltran *et al*., 2017; Doan *et al*., 2018; Laein *et al.,* 2021).

Serum glucose and cortisol levels are considered good indicators involved in energy management and are used as a monitor of fish health and stress conditions (Messina *et al.*, 2013). It is well known that serum cortisol levels increased during stress exposure, and consequently, fish increased serum glucose concentration to compensate for the energy demand (Martinez-Porchas *et al.*, 2009). Decreasing serum levels of glucose and cortisol may be related to the ability of LPP to reduce the adverse effect of stress factors in the Nile tilapia, and that positive anti-stress effect may be due to the presence of many active compounds in LPP that have sedative and analgesic effects (Mercier *et al*., 2009). Similar serum glucose and cortisol decreasing levels with dietary LPP inclusion were reported in tilapia (Acar *et al.,* 2015) and in *L. victorianus* (Ngugi *et al.,* 2017).

Nile tilapia fed on LPP-containing diets exhibited lower serum triglycerides and cholesterol concentrations compared to the control. This reduction may be due to LPP containing many active compounds such as limomene and insoluble fiber which have hypolipidemic effects in animals (Youssef *et al.,* 2014) and fish. Cholesterol level reduction induced by LPP feeding may be related to inhibition of de novo cholesterol biosynthesis (Ngugi *et al*., 2017). The present data are in harmony with Mohamed *et al. (*2021) stating that orange or lemon essential oils addition to the Nile tilapia diet significantly (p<0.05) reduced serum cholesterol and triglycerides levels compared to the control.

Antioxidant enzyme activities are very important in alleviating the adverse effect of oxidative stress (Ameur *et al.,* 2012). In the current study, LPP addition increased serum SOD and CAT activity with a reduction of serum MAD activity compared to the control, indicating that LPP had an antioxidant effect due to its active biological component (AL-Jabri and Hossain, 2014; Xi *et al.*, 2017). Our data are supported by Abdel Rahman *et al.* (2019) reported a significant increase in serum antioxidant enzyme activities of the Nile tilapia and African catfish fed on a 1.0 or 2.0% LPP-containing diet, except serum SOD activity not affected by LPP addition in the Nile tilapia diet. Also, serum SOD, CAT, and GPX activities increased in common carp fed on probiotics and LPP-containing diets (Harikrishnan *et al*., 2020; Sadeghi *et al*., 2021). Chekani *et al.* (2021) found that the addition of LPP at 0.5, 1.0 or 2.5% in the rainbow trout diet increased SOD and CAT serum activities compared to the control. In contrast, García Beltran *et al.* (2017) found no effect of LPP on the antioxidant enzyme activities in sea bream liver. This difference may be related to different fish species and the experimental conditions of each trial.

Phagocytosis and lysozyme activities are essential components of fish immunity and pathogen control. Lysozyme is an antibacterial agent through several degenerative enzymes, which lead to cell death (Saurabh and Sahoo, 2008). Phagocytosis is the process by which a phagocyte surrounds and destroys bacteria (Bogdan, 2001). In the current study, phagocytic, lysozyme and bactericidal activities were improved in the Nile tilapia fish fed on LPP-containing diets on a dose-dependent basis. The enhancement of lysozyme activity may be related to increased WBCs and neutrophil% in fish groups fed on LPP-containing diets (Ngugi *et al*., 2017) which consequently improves phagocytosis. Also, the immune stimulant effect in the Nile tilapia fed dietary LPP could be attributed to the higher concentration of LPP in essential oils (Vieira *et al*., 2018; Abdel-Latif *et al.*, 2020). Also, the present data agreed with

Mohamed *et al.* (2021) stating that lemon essential oil supplementation enhances the immunity of tilapia compared to the control. Generally, the efficacy of feed additives on health status is evaluated by hematological and serum biochemical changes (El Basuini *et al*., 2022). The higher WBCs and neutrophil% in LPP-fed fish compared to control reflected a specific innate-complex defense against pathogenic bacteria (Abdel Rahman *et al.,* 2019).

Nutrient absorption of the fish depends on intestinal villi length (especially the anterior portion) and goblet cell numbers (Elsabagh *et al.*, 2018). Moreover, villi length, number of goblet cells, and inter villi space affect the capacity of the absorptive area and intestinal health (Khojasteh, 2012). In the current study, dietary LPP increased villi length, which may have improved nutrient utilization and consequently reflected growth and feed utilization. To our knowledge, scarce literature studied the effect of LPP on intestinal morphology. A similar result was obtained by Mohamed *et al*. (2021) indicating that lemon essential supplementation in the Nile tilapia diet increased villi length compared to the control. Reduction of space between intestinal villi and high mucus secretion by goblet cells acting as a natural barrier against pathogen penetration of intestinal mucosa to cause inflammation, consequently, LPP protects fish from pathogen infection and improves intestinal health compared to control. Zhuo *et al.* (2021) stated that fermented LPP inclusion in the Asian sea bass diet increased intestinal health compared to the control.

The challenges against some infectious diseases can be applied in the experimental trial for the evaluation of medicinal plants as an antibiotic alternative to counteract against the adverse effects of fish diseases (Ahmadifar *et al.,* 2019). *Edwardsiella tarda* infection causes a high mortality rate, reduced growth performance, and severe economic losses in the fish (Park *et al.,* 2012). In the current study, the lower mortality and morbidity rates and enhancement of immunity and antioxidative response confirmed the beneficial role of LPP against *Edwardsiella tarda* infection in Nile tilapia. The present data are supported by Sadeghi *et al.* (2021) reported that LPP can increase the resistance against *A. hydrophilia* infection of common carp. Similarly, citrus essential oil addition reduced mortality in *O. mossambicus* challenged by *Streptecoccus iniae* (Acar *et al.,* 2015), and in *Labeo victorianus* challenged by *A. hydrophila* (Ngugi *et al.*, 2017). Also, Zheng *et al.* (2009) reported that oregano essential oil supplementation reduced mortality on *A. hydrophla* challenge in *Ictalurus punctatus*.

Lemon peel essential oil had potent in vitro antibacterial activity against *A. hydrophila* (Öntaş *et al.*, 2016). Also, the survival rate of Mozambique tilapia feeding on LPP essential oil and challenged with *Edwardsiella tarda* has been improved (Baba *et al.,* 2016). All these positive effects may be related to active components in LPP such as limonene, alkaloids, essential oils, dietary *fi*ber, and others (AL-Jabri and Hossain, 2014; Xi *et al.,* 2017).

Conclusions

It can be summarized that dried lemon pomace inclusion of up to 2% in tilapia fish diet improves growth performance, antioxidant capacity, and immune response. LPP reduces stress and improves the serum lipid profile and intestinal health of the Nile tilapia fish. Moreover, LPP increases resistance against the *E. tarda* challenge through the reduction of morbidity and mortality rates.

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Conflicts of interest

The authors declare that they have no competing interests.

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