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Identification of the main cause of mortality in a commercial *Oreaochromis niloticus* farm: The role of poly-β-hydroxybutyrate as a preventive measure against Edwardsiellosis

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The current study aimed to identify the primary cause of mortality in a commercial *Oreochromis niloticus* (*O. niloticus*) farm. Furthermore, the efficacy of poly-β-hydroxybutyrate (PHB) as a feed additive to prevent mortality was investigated after in-vitro testing. Also, a histopathological examination was carried out. The samples of naturally diseased *O. niloticus* showed swellings and hemorrhages on the body surface. Moreover, the post-mortem examination revealed black fluids with an awful odor, a congested liver, and intestinal gasses. Concerning diagnosis of the main cause of mortality in diseased *O. niloticus,* , there were eight isolates might be *Edwardsiella tarda* based on the biochemical identification results. Also, the identification was confirmed by using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The resulting spectra of two previously identified *E. tarda* strains were then compared to those found in the Bruker database and the two strains were identified as *E. tarda* at the species level. For detection of pathogenicity of identified strains, the virulence *E. tarda* hemolysin (ETHA) gene were detected at band 1078 bp in the eight identified strains. Regarding the in-vitro antimicrobial activity of PHB against *E. tarda*, the antibacterial activity of blood and tissues had been calculated using the Agar and well diffusion procedures Prior to and after the challenge,. Lower bacterial counts and a larger inhibition zone were signs of the tested materials' concentration-dependent antibacterial activity. For in-vivo evaluation of PHB in dietary-fed *O. niloticus*, PHB was effective in preventing Edwardsiellosis, with the lowest mortality rates in the group fed 10 g PHB/Kg feed after being injected with *E. tarda*. In comparison, the group that received a 5 g PHB/Kg feed after receiving an injection of *E. tarda* saw 16.6% mortalities and a 66.7 percent relative survival rate. The control positive group had 50% mortality. The results of the biochemical testes showed that *O. niloticus*'s kidney and liver functions were unaffected by dietary PHB supplementation. Protein, albumin, globulin, urea, creatinine, aspartate transaminase levels were identical to those of the control negative group. According to the current study, the groups fed high and low PHB concentrations had considerably higher levels of immunoglobulin M, complement 5, and interleukin-1 than the control group. The number of total viable bacteria was lower in the gut of PHB-fed groups than in control. Compared to mild histological alterations in those fed with a lower dosage and severe histopathological abnormalities in the control group, the greater dose of PHB effectively prevented Edwardsiellosis in *O. niloticus* with decreased mortality and no histopathological changes.

Key words: Edwardsiellosis, *O. niloticus*, Poly-β-hydroxybutyrate, Antimicrobial activity, Prevention, Histopathological examination.

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Introduction

Tilapias have been deemed an appropriate species for aquaculture due to their rapid development, ability to tolerate poor water quality, hardiness, and disease resistance (Prabu et al., 2019). In tilapia production, bacterial infections are the leading cause of death (Situmorang, 2014) and they were the main challenge to tilapia production in Egypt during the past five years (Mabrok et al., 2020). Moreover, Edwardsiellosis is one of the most common bacterial diseases that cause great economic losses in tilapia production. *Edwardsiella tarda (E. tarda)* found all over the world and affects numerous commercially significant fish species, notably Nile tilapia (*Oreochromis niloticus*) (Park et al., 2012). Furthermore, it causes economic losses in the natural environment and fish aquaculture across the world (Park et al., 2012). Infected fish exhibit pigmentation loss, exophthalmia, eye opacities, abdominal expansion, and petechial bleeding in the fin and skin (Sahoo et al., 1998). Internally, the liver, spleen, and kidneys are clogged, and the abdomen contains fluid and bloody discharges with a foul odor (Miyazaki et al., 1985).

To prevent and manage Edwardsiellosis epidemics, researchers have sought to develop quick ways for recognizing *E. tarda* infection. Although, polymerase chain reaction (PCR)-based technologies were developed to detect harmful bacteria in fish quickly (Sakai et al., 2009), MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) was recently established as a technique for correctly detecting bacterial strains derived from clinical samples (Bizzini et al., 2010). This approach compares the fingerprint of an unidentified isolate's spectral fingerprint with reference database spectra, despite minor between-colony and culture-condition-related changes (Kurokawa et al., 2013).

The economic damages due to Edwardsiellosis epidemic cannot be avoided by fish farms (Kim et al., 2017). To address this problem, antibiotics are commonly used in fish feed. However, because the oral administration of such antibiotics in aqua-feed is restricted in some countries, the development of alternative safe sources of dietary additives to substitute antibiotics has become an essential and continuous demand (Alderman et al., 2003). Among these safe products is poly-β-hydroxybutyrate (PHB), a prebiotic that is generally retained by bacteria and is advantageous to a variety of aquaculture species (Schryver et al., 2010). Many bacteria produce hydroxybutyrate as an internal energy and carbon reserve component (Anderson and Dawes, 1990). PHB was first examined in aquaculture to prevent *Artemia franciscana* larvae from Vibrio infection (Halet et al., 2007; Defoirdt et al., 2007). Since then, its use has been examined effectively on a variety of parameters in various fish species, including gut microbiota, immunology, and disease resistance (Nhan et al., 2010; Schryver et al., 2010; Najdegerami et al., 2017; Sui et al., 2012; Thai et al., 2014). In all of these studies, the use of PHB resulted in a statistically significant positive response (Najdegerami et al., 2017). Furthermore, dietary PHB was beneficial in reducing infection with *Edwardsiella ictaluri* in *O. niloticus* (Situmorang et al., 2015). PHB was not previously documented to be used in the prevention of Edwardsiellosis in *O. niloticus*. Depending on aforementioned data, the aim of this study was to identify the pathogen responsible for *O. niloticus* mortality in a commercial fish farm in Beni-Suef, Egypt. Then, the impact of PHB as a feed additive on prevention of the main cause of mortality in *O. niloticus* was tested following *in-vitro* determination. A histological investigation was also conducted.

Materials and methods

Identification of the main cause of mortality in a commercial Oreochromis niloticus farm

1) Collection of naturally diseased fish 2.2.1.

A total of 150 *O. niloticus* were collected alive

from a commercial fish farm in Beni-Suef, Egypt, with an average body weight of 21 ± 4 g and with skin ulcers and hemorrhages. Fish were examined externally and postmortem, as stated by Noga (2000).

2) Bacteriological isolation and identification

For isolation, a loopful of external musculature lesion, kidney, liver, and spleen were streaked onto trypticase soya agar medium (Oxide) and incubated at 28 degrees Celsius for 24 hours. Purified isolates were used as stocks for further morphological and biochemical identifications. On the other hand, bacterial films were prepared from each probable pure isolate and stained with Gram's stain (Cruickshank et al., 1979) before being examined under a bright field microscope with an oil immersion lens. Furthermore, the separate colonies were biochemically identified by the cytochrome oxidase test (Biomerieux, France), motility, H_2S production, and indole tests by SIM medium (Oxide), urease test, citrate utilisation test, and fermentation of maltose, xylose, lactose, sucrose, and mannitol (Collee et al. 1996 and Austin and Austin 2007). Moreover, the biochemically identified isolates were confirmed by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) based on Abouseada et al. (2016). MALDI-TOF MS analysis was conducted in the positive linear mode under the control of Flex Control software (mass over charge ratio (m/z) ranging from 2,000 to 20,000). Each spectrum was generated by averaging 240 laser shots in 40 shot increments taken in automated mode at the minimal laser power required for sample ionization following the manufacturer's instructions. Spectra were matched to a fingerprint database of 5,623 entries using the Bruker Biotyper 3.1 software and library. The utilized MALDI-TOF MS Identification score criteria were based on the manufacturer's recommendations. A score of ≥2.000 indicated species-level identification, a score of 1.700~1.999 suggested genus-level identification, and a score of <1.700 was considered non-reliable identification. Isolates that did not achieve a score of >1.700 were retested using the protein extraction technique.

Detection of pathogenicity of identified strains by presence of *E. tarda* hemolysin (ETHA) gene

The ETHA virulence gene was utilized to validate the isolates' pathogenicity. The PCR approach described by Hirono et al. (1997) was used to detect the *E.tarda* ETHA gene using a commercial kit (ECL Rapid Detection System, Amersham) and the manufacturer's protocol. The forward primer for the *E. tarda* ETHA gene was 5′-GAT AAT TAA CCA GAA CTA CCG -3′, while the reverse primer was 5′-TGT TCA CCA CCC GAT CGT CGA TG -3′. The PCR conditions were 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C. PCR products were seen in an ultraviolet transilluminator after 20 minutes of staining with ethidium bromide on agarose gel 1%.

Preventive measure of the cause of mortality by PHB

1) Source of PHB

PHB was purchased from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany), containing a pamphlet and supplemented with all-structure product details.

2) In-vitro PHB antimicrobial activity estimation against the causative agent of mortality

(1) Serum bacterial and bactericidal count

For serum bacterial count, sterile microtubes were used to incubate 40 µl of serum at pre- and post-challenge at lower and higher PHB doses, as well as the presence of control negative and positive serum, with the same volume of 40 µl of the previously prepared bacterial suspension with McFarland tube at 1.5×10^8 CFU/mL for 1 hour at 37°C. After 1 hour, about 10 µl of the incubated serum was distributed on tryptone soya agar (TSA) and incubated at 37°C for 24 hours. The CFU were manually counted, and the results were represented as a bacterial count in CFU values. On

the other hand, the serum bactericidal activity was performed as the same previously mentioned method but with equation application. Serum bactericidal concentrations were calculated as the percentage of CFU in the test group to that in the positive group: 1-CFU/positive (%).

(2) Serum and tissue agar well diffusion

The McFarland opacity tube was used to estimate bacterial counts at 1.5×10^8 CFU/mL. Following pouring serum into the excised wells after media solidification, the bacterial zone of inhibition was determined using Muller Hinton Agar medium. After 20 minutes of thorough heating and media sterilization at 121°C, the media was allowed to cool to 45°C before 0.25 mL of the produced bacterial suspension was added to the 250 mL media, mixed well, and poured onto the plates until solidification. Then, a pourer of 150 µl size was employed for poring, and serum pre-challenge, post-challenge, and control negative serum were poured to the excised pores in the same amount. Finally, the plates were incubated for 24 hours at 37°C to estimate the inhibition zone. Similarly, the tissue agar well diffusion was performed but the tissue had to be prepared first. One gram of each tissue sample was obtained and completely homogenized in 4 mL of distilled water. The homogenized tissue was then centrifuged for 15 minutes at 3000 revolutions per minute. The supernatant was transferred to sterile containers for concentration testing. The produced tissue was then put into the previously removed wells and incubated for 150 minutes.

In-vivo prevention of causative agent of mortality in *O. niloticus*

(1) Diet Preparation

A mortar was used to compress the commercial pelleted fish food into a fine powder. Three fish diets were made by combining two different PHB concentrations with the previously generated fine powder. Diet 1 had no additives (control), diet 2 had 5 g PHB/Kg feed, and diet 3 had 10 g PHB/Kg feed (Magdalena et al 2016; Schryver et al 2010). To obtain a homogenous mixture, the contents of each fish diet were mixed with distilled water. The mixture was applied to a manual hand-minced meat processing machine (Italy) to produce extruded strings, which were then air-dried for 48 hours before being broken down to roughly 2-mm long pellets (Rattanachaikunsopon and Phumkhachorn, 2010).

(2) Collection and management of experimental fish

Three hundred and eighty four healthy *O. niloticus* with an average body weight of 18±3 g were collected alive from the Abo-Saleh fish hatchery in Beni-Suef, Egypt. The gathered fish were brought to Egypt's Beni-Suef University's Faculty of Veterinary Medicine's wet laboratory of the Fish Diseases and Management Department. The fish were housed in three 500-L fiberglass aquariums with chlorine-free tap water and continuous aeration. For 14 days, the fish were acclimatized in these fiberglass tanks and given a 3% body weight pelleted commercial fish meal (Brsiek factory, Egypt, Table 1). After acclimatization, the fish were placed in 90×25×40 cm glass aquaria with a water capacity of 30 L. Through an air blower, each tank got continuous artificial aeration (1 air stone). In the experimental aquaria, the water exchange rate was 10% per day. Fish were given 3% of their body weight twice daily. During the trial experiments, water quality factors such as water temperature (26°C 1°C) was measured with a water thermometer (Yellow Spring Instrument Co., USA), dissolved oxygen (DO; 6.5 2 mg/L) was measured with a D.O meter (Yellow Spring Instrument Co., USA), and pH (7-8) was measured with pH indicator paper (Yellow Spring Instrument Co., USA) (were monitored twice weekly.

(3) Experimental infection and determination of LD_{50} of bacterial isolate in healthy *O. niloticus*

After acclimation, a total of 120 healthy *O. niloti-*

Table 1. Chemical composition (g/kg on dry weight basis) of diets of experiments

Ingredients	g/kg
Soybean meal (42.7% CP)	300
Fish meal (65.0% CP)	400
Corn meal*	100
Wheat gluten	50
Corn oil	20
Rice bran	60
Fish oil	10
Mineral Premix**	30
Vitamin Premix***	30
Total	1000
Proximate chemical composition	
Crude protein	462
Dry matter	93.2
Crude fibers	49
Crude lipids	135
Ash	103

*corn meal: 100 in Diet 1, 95 in Diet 2, 90 in Diet 3
**Mineral premix: Zn 4 g, Mn 1.4 mg, Fe 14 g, Mg 10 g, Co 30 mg, Cu 350 mg, Se 35 mg, I 40 mg.
***Vitamin premix include (/kg in premix): vitamin A 67 IU, vitamin E 7.4 g, vitamin D 16.2 IU, vitamin K3 340 mg, vitamin B1 670 mg, vitamin B2 1000 mg, vitamin B6 800 mg, vitamin B12 1.4 mg, vitamin C 10 g, D-pantothenic acid 2.65 g, folic acid 330 mg, nicotinamide 5.35 g, choline chloride 35 g, biotin 34 mg, inositol 8 g.

cus were divided into five groups of eight fish each, with three replicates. The isolate was cultured overnight at densities of 3×10^6, 3×10^5, 3×10^4, and 3×10^3 CFU/mL. Each dilution was intraperitoneally injected into a fish group at 300 μL/fish, while the fish in the fifth group served as controls, receiving 300 μL of physiological saline per fish. All fish groups were intensively monitored for 2 weeks. Daily deaths were recorded, and organs were aseptically streaked on *Salmonella Shigella* agar for re-isolation and re-identification.

(4) Prevention scheme

Following acclimation, 144 *O. niloticus* were split into four groups of 12 fish each, each with three replicates. Diet 1 was provided to the fish in the first and second groups (control negative and positive, respectively), whereas diet 2 was fed to the fish in the third group. Furthermore, the fish in the fourth group were fed with diet 3. All groups were given food at 3% of their body weight with their particular diet twice a day for 30 days throughout the experiment. The first (control negative) group with its replicates was intraperitoneally injected with 300 μL physiological saline per fish at the end of feeding period (30 days). The second (control positive), third, and fourth groups and their replicates were intraperitoneally injected with causative agent of bacteria at 300 μL/fish of 3×10^4 CFU/mL. The injected groups were kept in a separate glass aquaria for 2 weeks. The mortalities were noticed and the relative percent survival (RPS) was calculated according to Amend (1981) using the following: RPS = 1 − (% of mortality in treated groups/% of mortality in control group) × 100.

Evaluation of PHB on the health status of *O. niloticus*

(1) Serum and intestinal collection and histopathological examination

After acclimation, 120 *O. niloticus* were split into four groups of ten fish each, each with three replicates. Fish groups were fed and challenged in the same way as the previously indicated preventative strategy was. At the end of the feeding session, three fish from each group were caught and anaesthetized with tricaine methane sulfonate (MS222, Sigma-Aldrich Chemical Co. Germany). The serum from the sedated fish was collected for biochemical and immunological tests. Another three fish were drugged with their counterparts in all feeding categories in order to collect gut for bacterial count. The remaining four fish in each group were netted and anaesthetized for histopathology. For serum separation, blood was drawn from caudal veins without the use of an anticoagulant. The serum was collected and kept at -20°C. Alanine trans-

aminase (ALT) and aspartate transaminase (AST) were measured (BioSystems S.A. Costa Brava, 30. 08030 Barcelona, Spain), and total protein and albumin (SPINREACT, S.A./S.A.U. Ctra. SantaColoma, Spain) were estimated for liver functions, as well as urea and creatinine (BioSystems S.A. Costa Brava, 30) for kidney function.

Interleukin 1 (IL-1) levels were measured using test kits (CUSABIO, Fish Interleukin 1 ELISA Kit Catalog Number. CSB-E13259Fh, China). Immunoglobulin (IgM) levels were evaluated using commercial test kits as directed by the manufacturer (CUSABIO, Fish immunoglobulin M ELISA Kit Catalog Number. CSB-E12045Fh, China). Complement 5 (CUSABIO, Fish Complement 5 (C5 ELISA Kit Catalog Number. CSB-F13502F, China) was also evaluated.

For estimation of intestinal total bacterial count, the intestinal microbial flora of the experimental fish was enumerated after a 24-hour fast. Five grams of collected intestine were homogenized and serially diluted aseptically. The spread plate method was used to count bacteria using Trypticase soya agar as a general medium (Krieg & Holt, 1984).

For histopathological examination, the gills, liver, spleen, and kidney of anaesthetized fish in each group were removed separately before and 96 hours after injection. MS222 was used to anaesthetize the fish, and tissue specimens were collected from the six groups under study and promptly preserved in Bouin's solution for 24 hours at room temperature. Routine histological methods were used on the specimens (dehydration, clearing, and paraffin embedding at 56 °C in a hot air oven). The resulting 4-6-m thick slices were stained with hematoxylin and eosin (H&E) and inspected under light microscopy (Bancroft Gamble 2008).

Statistical Analyses

All data were analyzed statistically using one-way analysis of variance (post hoc test; Dunnet's test) in Advanced Models 16.0 software (SPSS, Tokyo, Japan). P-values less than 0.05 were deemed statistically significant.

Results

Abnormalities in clinical and post-mortem *O. niloticus* samples

The naturally infected *O. niloticus* displayed scale-loss in several body areas, as well as congestion and hemorrhages. Exophthalmia, opacity, and skin darkening were noticed on the body's surfaces, as well as swellings with bleeding (Fig. 1, A, B, C, D). The postmortem found a variety of abnormalities, including black fluids with a foul odor, a congested liver, and intestinal gases (Fig. 1, E).

Biochemical and MALDI-TOF-MS identification

The results of the manual biochemical tests indicated that eight of the isolates were *E. tarda*. All putative isolates were Gram-negative, cytochrome oxidase-negative, motile, and positive for H_2S, indole, and citrate, but negative for urea hydrolysis. In addition, they could ferment maltose but not xylose, sucrose, lactose, or mannitol.

Two previously identified *E. tarda* strains were evaluated using MALDI-TOF MS, and the resulting spectra were compared to those in the Bruker database. At the species level, the two strains were identified as E. tarda (Table 2).

Presence of ETHA virulence gene

The presence of the ETHA virulence gene at band 1078 bp was confirmed by agarose gel electrophoresis of PCR results from the eight tested strains (Fig. 2). The results clearly demonstrated the presence of the virulence gene (ETHA).

In-vitro antimicrobial activity of PHB against *E. tarda*

Fig. 3 demonstrates that the positive (serum-free) group had the highest values, as evidenced by the

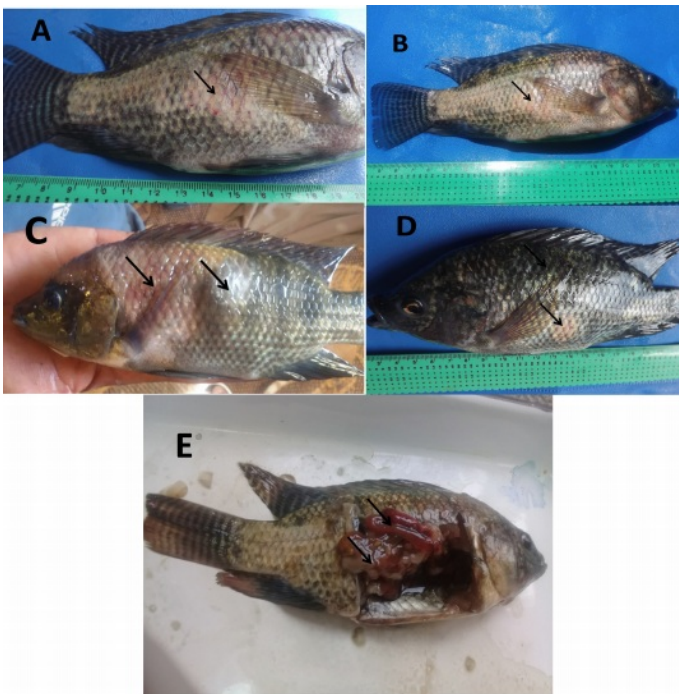

Fig. 1. Naturally infected *O. niloticus* showed scales-loss from some areas of the body, congestion and hemorrhages all over the fish body (A). Swellings with hemorrhage at the body sides were seen (C). Exophthalmia, opacity and skin darkening were seen (D). There were congested liver and gasses in the intestine (E).

Table 2. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) for *E. tarda* identification

Analyte Name	Analyte ID	Organism (best match)	*Score Value	Organism (second best match)	*Score Value
1 (+++) **(B)	Ed	*Edwardsiella tarda*	2.535	*Edwardsiella tarda*	2.478
2 (+++) (B)	Ed	*Edwardsiella tarda*	2.443	*Edwardsiella tarda*	2.411

**B = Genus Consistency: Genus Consistency: The best match was classified as 'green' or 'yellow'. Further 'green' or 'yellow' matches have at least the same genus as the first one.
*Meaning of Score Values

Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	Green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	Green
1.700 ... 1.999	probable genus identification	(+)	Yellow
0.000 ... 1.699	Not reliable identification	(-)	Red

greatest bacterial growth, because no serum can eliminate germs, in contrast to the control negative group, which had no bacterial growth both pre- and post-challenge. However, bacterial growth was reduced in the before challenge (BC) and after challenge (AC) groups due to bactericidal capacity in serum, primarily in the AC group (p 0.05), which had the lowest bacterial growth. The bacterial count in the BC group's serum was low, but the AC group demonstrated full killing at the higher than the lower dose, with increased bacterial growth in the control positive serum-free dose.

Table 3. Biochemical analysis and immunological parameters of control negative and experimental feeding groups

Blood parameters	Control negative	1.0% PHB/kg feed	2.0% PHB/kg feed
ALT (µ/l)	25.10±0.2	23.27±0.03	20.93±0.12
AST (µ/l)	28.01±0.06	27.22±0.02	24.99±0.04
Protein (g/dl)	3.7^{c}±0.05	4.02^{b}±0.04	4.83^{a}±0.02
Albumin (g/dl)	2.61^{a}±0.22	2.16^{a}±0.22	1.21^{b}±0.21
Globulin (g/dl)	1.09^{c}±0.22	1.54^{b}±0.22	2.48^{a}±0.20
A/G ratio	2.49^{a}±0.79	1.43^{b}±0.37	0.49^{c}±0.13
Urea (mg/dl)	19.9±0.01	19.7±0.06	19.1±0.04
Creatinine (mg/dl)	0.57±0.02	0.53±0.003	0.52±0.003
IgM (µg/ml)	2.07±0.03	$2.9±0.024^{b}$	$3.01±0.019^{a}$
Complement 5	0.90±0.04	$1.88±0.01^{b}$	$2.01±0.01^{a}$
IL-1β (pg/mL)	3.43±0.01	$4.76±0.03^{b}$	$5.88±0.001^{a}$

Data are expressed as mean ± SE (n = 3) with different superscript letters (a,b,&c) in the same row are significantly different at $P<0.05$.

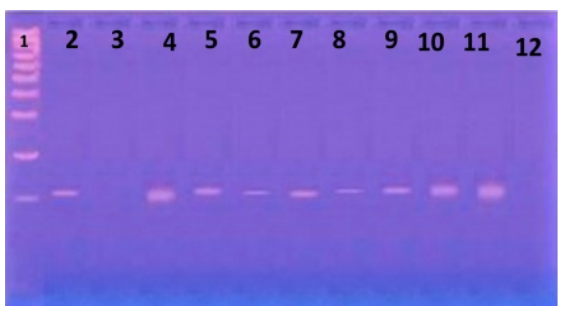

Fig. 2. PCR amplification of *E. tarda* hemolysin (ETHA) gene (1078bp). Lane (1): Ladder "Marker, 1000 bp", Lane (2): positive control. Lane (3): negative control. Lanes (4–11): examined samples, Lane 12: empty.

After applying the previously mentioned equation, the positive group (serum-free) displayed zero activity once there was no serum, but the bacterial activity increased in the BC and AC groups, mainly in the AC group ($p<0.05$), as indicated by the higher serum bactericidal activity profile, as shown in Fig. 3.

Fig. 4 showed that the estimated inhibition zone also indicated serum bactericidal activity, with the larger zone of inhibition observed more in the high concentration of the tested materials compared to the lower concentration and control in the AC group, and the standard doxycycline antibiotic showing the larg-

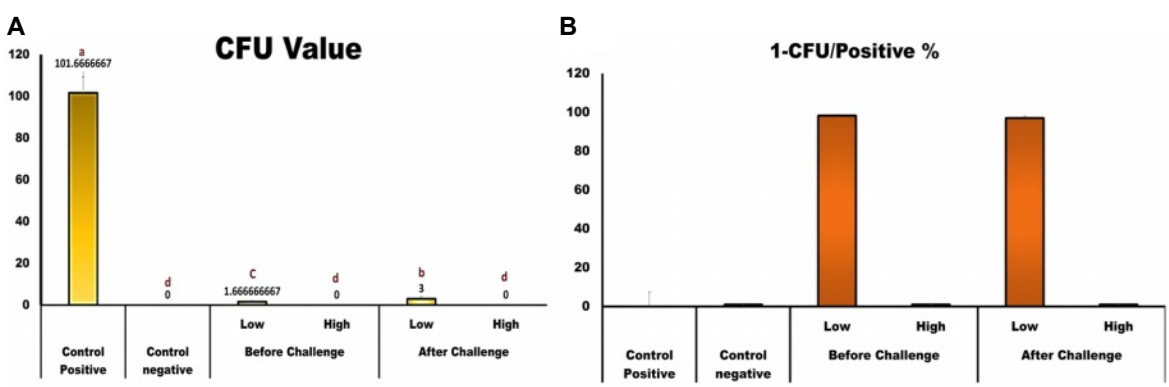

Fig. 3A. CFU value (Mean ± SD) of PHB at low and high dose after challenge trial. Significant differences were indicated by different letters ($p<0.05$), (replicates=3).

Fig. 3B. Serum bactericidal activity (1 - CFU/positive %) (Mean ± SD) of PHB after challenge trial. Significant differences are indicated by different letters ($P<0.05$), (replicates=3).

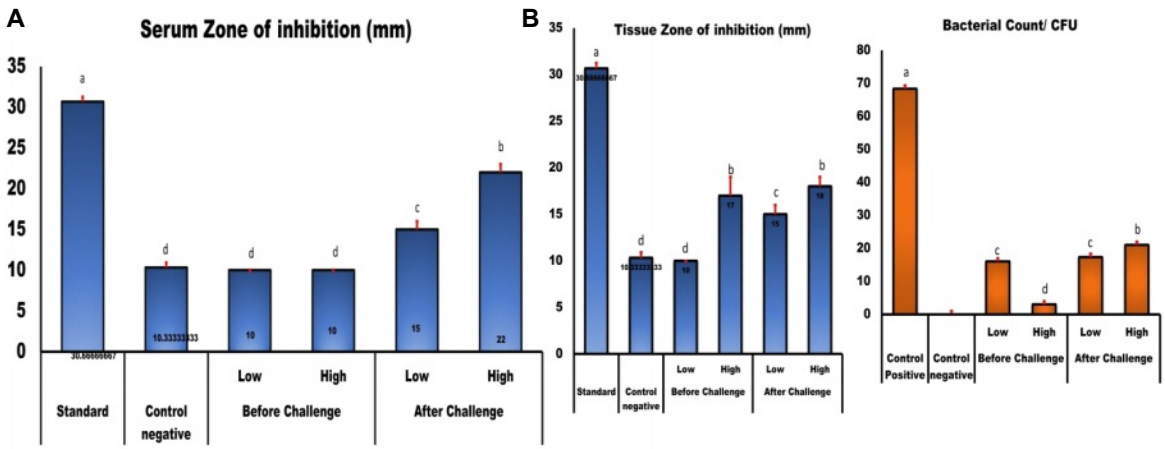

Fig. 4. A. Serum zone of inhibition (Mean ± SD) of PHB AC& BC. Significant differences are indicated by different letters ($P<0.05$), (replicates=3). Figure 4. B. Tissue zone of inhibition and the bacterial count (Mean ± SD) of PHB AC& BC. Significant differences are indicated by different letters ($P<0.05$), (replicates=3)

est inhibition zone. With full killing, no bacterial growth was seen in the BC group. The inhibitory zone for the tissue was assessed, and no zone was found in the control negative. The clear zone of inhibition was larger at the high concentration than in the low concentration. In compared to the low PHB dosage and the control, the high PHB dose in the tissue considerably reduced the bacterial count.

In–vivo prevention of Edwardsiellosis in *O. niloticus*

After observing the experimentally infected *O. niloticus* for 2 weeks, the fish died in the first week of the experiment, with LD_{50} of 3×10^4 CFU/mL.

The results of preventive measure revealed that, the PHB-fed groups resembled those on the control diet with no supplement in appearance and behavior. Furthermore, there were no mortality and 100% RPS in the groups that were fed a normal food after being injected with physiological saline (control negative). In contrast, after being injected with *E. tarda*, there was 8.33% mortality and 83.4 RPS in a higher PHB concentration. The group that was fed a lower PHB concentration and then injected with *E. tarda* had a 16.6% mortality rate and a 66.7 RPS. The mortality rate in the control positive group was 50%.

The impact of PHB on the health status of *O. niloticus*

(1) Biochemical and immunological parameters and intestinal total bacterial count

The biochemical study demonstrated that dietary PHB supplementation had no effect on *O. niloticus* kidney and liver functioning. The ALT and AST levels, as well as protein, albumin, globulin, urea, and creatinine levels, did not differ from the control group (Table 3).

After 30 days of feeding, the IgM, complement 5, and IL-1 levels in the fish fed at varied PHB concentrations increased significantly compared to the control ($p<0.05$) (Table 3).

Regarding intestinal total bacterial count, the total viable intestinal bacterial counts of *O. niloticus* fed 5 g and 10 g of PHB/kg feed- for 30 days were $1.1 \times 10^6 \pm 8.8 \times 10^3$ and $8.6 \times 10^5 \pm 8.7 \times 10^5$ CFU g^{-1}, respectively, in comparison with $1.6 \times 10^6 \pm 6.02 \times 10^4$ CFU g^{-1} in the control group.

(2) Histopathological findings

The histopathological findings of control positive

group showed that abnormal alterations in gills, liver, spleen, and kidneys of the experimentally infected *O. niloticus* with *E. tarda.* Severe branchial blood vessel congestion, degenerative primary lamellae changes, and sloughed secondary lamellae were observed (Fig. 5A). Severe vacuolar hepatocyte degeneration, portal vein congestion, and dilated congested hepatic sinusoids were noticed (Fig. 5B), as well as inflammatory cells infiltration and edema in the hepatic parenchyma (Fig. 7C) were observed. The spleens showed severe lymphocyte depletion of the splenic pulps and blood vessel dilatation, and congestion surrounded by numerous melanomacrophage center accumulation (Fig. 5D) and necrotic areas. Degenerative changes in renal tubules, leucocytic infiltration in the interstitial, and congestions of renal blood vessels were detected in the examined kidney (Fig. 5E). On contrarily, the histopathological findings of control negative group revealed that the gills had a normal histological picture of primary and secondary lamellae with the normal arrangement (Fig. 6A). The hepatopancreas showed normal histological architecture of the central veins, the portal areas, the radiating hepatocytes, and pancreatic tissues in the hepatic parenchyma (Fig. 6B). Spleen showed a normal histological structure of splenic pulps (red and white) and melanomacrophage centers (Fig. 6C). The kidney showed the normal histological structure of the corpuscles and tubules (Fig. 6D). Regarding the histopathological findings of *O. niloticus* group fed 5 g PHB/Kg feed and injected

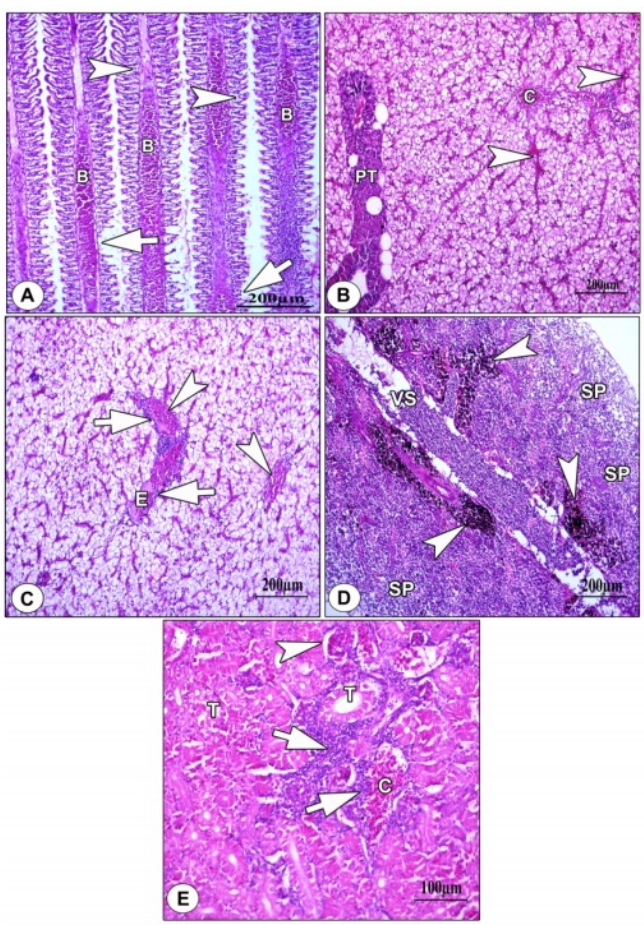

Fig. 5. photomicrograph of a control positive group of *O. niloticus* showing: A) Severe congestion of branchial blood vessels (B), degenerative changes of primary lamellae (arrowheads) and sloughed secondary gill lamellae (arrows), B) Congestion of portal vein (C), dilated congested hepatic sinusoids (arrowheads) and normal pancreatic tissues (PT), C) Inflammatory cells infiltration (arrows), congestions of hepatic blood vessels (arrowheads) and edema (E) in the hepatic parenchyma, D) Severe depletion of lymphocytes of splenic pulps (SP) with congestion and dilatation of the blood vessel (Vs), E) Degenerated renal tubules (T), leucocytic infiltration in the interstitium (arrows), congestion of renal blood vessel (C) and normal renal corpuscle (arrowhead). H&E stain, scale bars: 200 µm, 200 µm, 200 µm, 200 µm and 100 µm, respectively (replicates=3).

with *E. tarda*, the gills, spleen, and kidney showed no histopathological alternations (Fig. 7A, 7C, and 7D). Mild degenerative changes in hepatocytes of the hepatopancreas were observed (Fig. 7B). On the other hand, the group of *O. niloticus* fed 5 g PHB/Kg feed and injected with physiological saline showed no his-

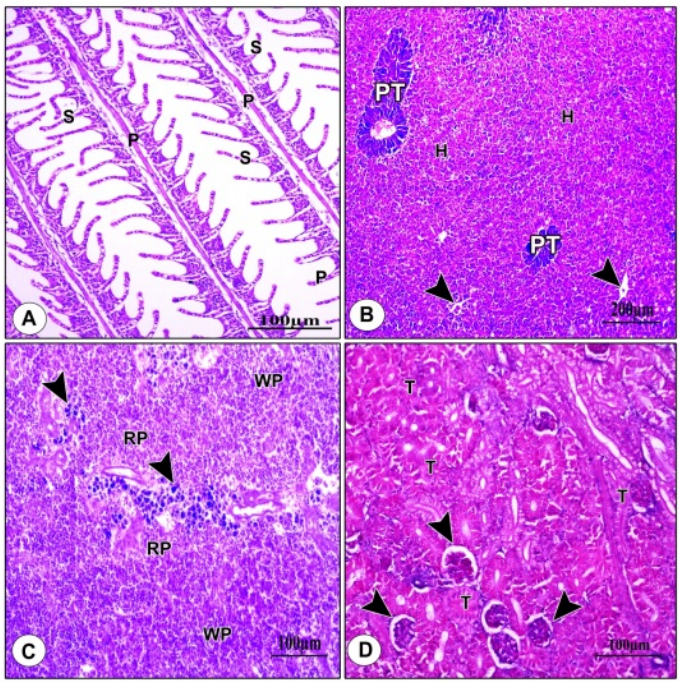

Fig. 6. photomicrograph of a control negative group of *O. niloticus* showing: A) Normal histological picture of primary (P) and secondary (S) gill lamellae, B) Normal histological architecture of the central veins (arrowheads), hepatocytes (H) and pancreatic tissues (PT), C) Normal splenic pulps "red (RP) and white (WP)" and melanomacrophage centers (arrowheads), D) Normal histological structure of renal tubules (T) and corpuscles (arrowheads). H&E stain, scale bars: 100 µm, 200 µm, 100 µm and 100 µm, respectively (replicates=3).

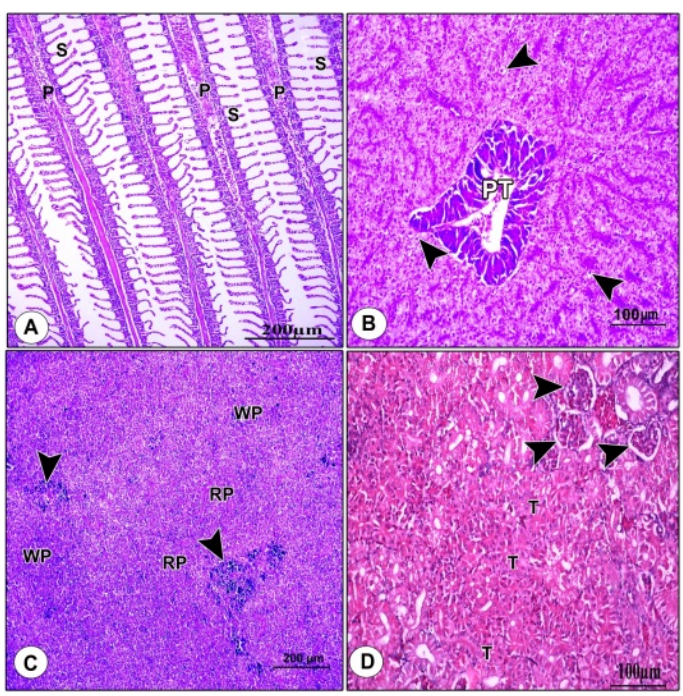

Fig. 7. photomicrograph of *O. niloticus niloticus* fish fed 5 g PHB/Kg feed then injected with *E. tarda* showing; A) Normal histological picture of gill lamellae "primary (P) and secondary (S)", B) Mild degenerative changes of hepatocytes (arrowheads), C) Normal red (RP), white (WP) pulps and melanomacrophage centers (arrowheads), D) Normal histological structure of renal tubules (T) and corpuscle (arrowheads). H&E stain, scale bars: 200 µm, 100 µm, 200 µm, 100 µm, respectively (replicates=3).

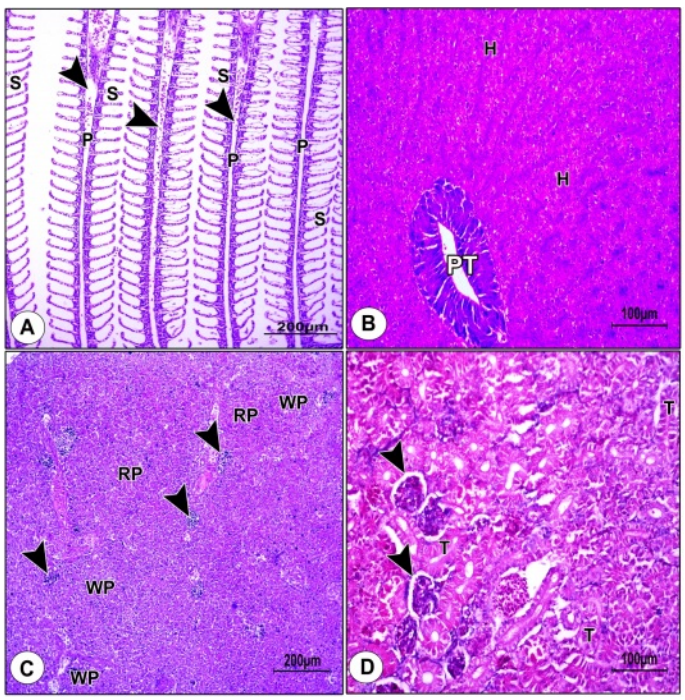

Fig. 8. photomicrograph of *O. niloticus* fed 10 g PHB/Kg feed 30 days then injected with *E. tarda* showing normal histological architecture of: A) Primary (P) and secondary (S) gill lamellae, B) central veins (arrowheads) and hepatocytes of the hepatopancreas, C) splenic pulps (red "RP" and white "WP") and melanomacrophage centers (arrowheads), D) Renal tubules (T) and corpuscles (arrowhead). H&E stain, scale bars: 200 µm, 100 µm, 200 µm and 100 µm, respectively (replicates=3).

topathological changes were observed in all examined organs.

Concerning group of *O. niloticus* fed 10 g PHB/Kg feed and injected with physiological saline, the gills, hepatopancreas, spleen, and kidney showed no histopathological abnormalities. Similarly, the group of *O. niloticus* fed 10 g PHB/Kg feed and injected with *E.* tarda had normal gills, hepatopancreas, spleen, and kidney (Fig. 8A, 8B, 8C, and 12D).

Discussion

Edwardsiellosis is a serious bacterial disease that affects a wide range of commercially important wild and cultured fish species (Mohanty and Sahoo, 2007). *E. tarda*'s growing fish host range exacerbates infection severity (Mohanty and Sahoo, 2007). The most severe epizootics have been seen in catfish and Nile tilapia (Darwish et al., 2000). Although *E. tarda* is a fish-borne bacterium, it infects people and poses a public health danger in addition to having significant economic relevance due to the bacteria's ugly appearance and unpleasant rotten odor (Noga, 2000).

Antibiotics have been utilized extensively in aquaculture during the last two decades to treat a wide range of bacterial diseases (Dfoirdt et al., 2011). Many various types of bio-control research have recently been conducted in attempt to establish alternative disease prevention strategies in aquaculture (Sihag, 2012). The current work determined the cause of death in farmed *O. niloticus* and then applied dietary PHB to avoid that causative factor after in-vitro determination. Also, histological studies were performed to evaluate the dietary inclusion of PHB for Edwardsiellosis prevention in *O. niloticus*. According to our findings, naturally infected *O. niloticus* presented substantial clinical abnormalities. The septicemic clinical signs and post-mortem lesions of naturally infected *O. niloticus* may be due to extracellular *E. tarda* products such as ETHA (Hirono et al., 1997), which were detected by PCR in the current study.

Bacterial culture and routine biochemical tests are currently less reliable and time-consuming diagnostic approaches for recognizing *E. tarda* in diseased fish.

PCR methods may be used to specifically and swiftly identify *E. tarda*, resulting in fast and appropriate management measures and treatments for affected fish (Sakai et al., 2009). The identity of *E. tarda* was validated in this study by detecting ETHA in eight isolates, which is unique for *E. tarda* identification and pathogenicity. According to a recent functional study, ETHA is essential for invasion in vivo and in vitro, and is regulated by the two-component system EsrA-EsrB and the nucleic protein HhaEt (Wang et al., 2010). ETHA was responsible for esrB's increased cell-invasion abilities. Furthermore, ETHA expression in esrB was temperature-dependent, and the nucleoid protein HhaEt was discovered to drive ETHA expression by directly binding to its promoter (Wang et al., 2010). These findings demonstrated that the virulence determinant ETHA was absolutely required for *E. tarda* invasion abilities and was directed by a sophisticated and well regulated network designed for *E. tarda* invasion, colonization, and infection in fish (Wang et al., 2010).

MALDI-TOF MS has recently been established as a technology for identifying bacterial strains isolated from clinical samples with high accuracy (Bizzini et al., 2010). In the current study, two isolated strains of *E. tarda* were successfully identified with a high degree of confidence using a MALDI Biotyper. Quynh et al., (2016) demonstrated that utilizing a MALDI Biotyper, all *E. ictaluri* isolates from striped catfish were successfully identified with a high degree of confidence. Furthermore, Bizzini et al., (2010) and Kurokawa et al., (2013) demonstrated MALDI-TOF MS as a high-throughput screening method that permits simultaneous detection of numerous bacteria within 1 hour in recent publications on several dangerous bacteria. As a result, MALDI-TOF MS appears to be a viable approach for identifying *E. tarda*.

Because it may be used to evaluate innate defense mechanisms and because numerous substances or processes can impact or regulate immune system responses, serum bactericidal activity is an essential metric in immunology research (Biller-Takahashi et al., 2013). The rise in this parameter seen in this study is due to pathogen identification by the innate system, which activated acute phase proteins to protect fish from invading microorganisms. First, the bactericidal activity of fish serum was discovered, and an important technique for immunology research in tested fish was created. Globulin levels showed an increase in protective proteins following the challenge, which is connected to serum bactericidal activity (Murray and Fletcher, 1976; Arason, 1996; Ellis 2001; Magnadottir, 2006; Maqsood et al., 2009).

The serum bactericidal activity of examined substances is used to calculate fish responses in percentages (Fig. 2). When compared to fish that had not been experimentally infected, the challenged fish displayed a better ability to remove microorganisms. Due to the insufficient serum in the protocol test, the positive group's bactericidal activity is weak in comparison to the control and challenge groups. In the positive group, the bacteria were simply suspended in a PBS solution. Misra et al., (2006) used the bactericidal activity assay to examine the effect of β-glucan in *Labeo rohita* fingerlings, whereas Misra et al., (2009) used the assay to assess bactericidal activity following a long-term dose of levamisole to the same fish, as previously estimated for the role of the immune system on bactericidal activity for different materials used in fish. Das et al., (2009) employed the same technique in their research on the influence of *Euglena Viridis* on the immunological response of *L. rohita*.

In this investigation, the increased detected blood bactericidal activity after the challenge (Fig. 2) demonstrates that serum protective proteins are raised after a natural infection, such as during disease outbreaks, or after experimental infections, such as vaccination and challenge. Acute phase proteins and other immune system markers can also rise as a result of changes in the hepatic, neuroendocrine, and immune systems that occur quickly after infection or damage.

Inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF-), are proteins synthesized by the liver, encephalon, or immune cells that act in tissue reparation, homeostasis maintenance, and innate and acquired system modulation, and are influenced by the presence of injury (cell membrane damage, resulting in the release of tissue factors and arachidonic acid metabolites) or pathogen (Bayne and Gerwick, 2001; Magnadottir et al., 2011).

In-vivo experiments confirmed the in-vitro antibacterial findings, as higher PHB concentrations inhibited Edwardsiellosis in *O. niloticus* with lowest mortality rate. In contrast, 16.66% and 50% mortality were seen in groups of fish administered 5 g PHB/kg feed for 30 days after being injected with pathogenic *E. tarda* strainsand in the control positive group, respectively. Situmorang et al., (2016) found that feeding *O. niloticus* larvae PHB-enriched *Artemia nauplii* resulted in a 20% higher survival rate compared to challenged control larvae by *Edwardsiella ictaluri* gly09R. Situmorang et al., (2014) discovered higher survival rate of sea bass larvae fed just on PHB particles (in comparison to starvation). Their findings revealed that PHB resistance was linked to intestinal synthesis of the monomer 3-hydroxybutyrate (3-HB), which worked as an anti-pathogenic molecule (Bonartsev et al., 2019).

The current study found that fish fed different PHB doses had considerably higher IgM, complement 5, and IL-1 levels than controls. PHB may improve fish immunology by raising IgM, C5, and IL-1 levels. Ponnusamy et al., (2013) discovered that feeding PHB-containing diets from *Bacillus Thuringian* to *Oreochromis mossambicus* had a significant immunostimulatory effect on both nonspecific (higher lysozyme, total peroxidases, and antiprotease activity) and specific (higher antibody response) immunity.

The current study found that groups fed PHB had a lower total viable bacterial count in the intestine than controls. The synthesis of short-chain fatty acids (SCFA) in the gut as a result of prebiotic compound (PHB) fermentation, which results in acidification of the intestinal environment, is of particular interest (Defoirdt et al., 2007).. SCFA, for example, inhibits the development of numerous potentially hazardous bacteria, including Salmonella spp. and Vibrio spp (Defoirdt et al., 2007). The acid's un-dissociated form, which may permeate the bacterial cell membrane, is assumed to cause the growth inhibitory action (Defoirdt et al., 2007). Once inside, the acid causes protons (H+) to be released in the neutral cytoplasm, reducing intracellular pH and driving bacteria to transfer energy to the efflux pathway of surplus protons, thereby straining the cell metabolism and resulting in cell growth suppression or cell death (Situmorang et al., 2014; Kato et al., 1992).

Conclusions

E. tarda was the cause of mortality in cultured *O. niloticus,* as confirmed by PCR and MALDI-TOF, which are quick, accurate, and low-cost techniques for identifying microorganisms. PHB exhibits significant antibacterial activity against pathogenic *E. tarda* in a dose-dependent manner. A higher concentration of PHB prevented Edwardsiellosis in *O niloticus* with a low mortality rate and without histological abnormalities. In contrast, in the lower concentration-fed group, 16.66% mortality and mild histopathological changes occurred, while in the positive control group, 50% mortality and severe histopathological changes occurred.

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Declarations

Funding

No funding was received.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

All experiments were approved by the Beni-Suef Institutional Animal Care and Use Committee (BSU-IACUC) of the Faculty of Veterinary Medicine, Beni-Suef University, Egypt. The number of ethical approve is 022-333 under old title of "Role of poly-β-hydroxybutyrate against Edwardsiellosis in Nile tilapia, *Oreochromis niloticus".*

Competing interests

Not applicable

Availability of data and material: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Code availability (software application or custom code): Not applicable

Author contributions:- Fatma M. M. Korni: Applied experimental scheme, management of fish throughout the experimental period, prevention experiments, writing - review & editing.

Fatma I. Abo El-Ela: Applied the *in-vitro* study, biochemical testes, writing, reviewing.

Usama K. Moawad: Applied histopathological study, writing, reviewing.

Consent for publication: All authors have consented to publish the data in a scientific journal.

References

Abouseada, N, May M., Passent M. (2016). Impact of MALDI TOF in Diagnostic Microbiology, 25 , (3): 31-35.

Alderman, David & Hastings, T. S., Alderman DJ, Hastings TS.(2003). Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks. International Journal of Food Science & Technology. 33. 139-155. 10.1046/j.1365-2621.1998.3320139.x.

Amend, D.F. (1981). Potency testing of fish vaccines. Developments in Biological Standardization 49: 447-454.

Anderson A and Dawes E. (990). Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 1;54: 450-472.

ARASON, G.J. (1996). Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol., v.6, p.277-289.

Austin, B. and Austin, D. A. (2007): Bacterial fish pathogen. Diseases of farmed and wild fish. 4th Ed. Ellis Harwood Limited. New York, London. page 30.

Bancroft J, Gamble A. (2008). Theory and practice of histological techniques. 6th ed. Edinburgh, London, Melbourne, New York: Churchill-Livingstone.

BAYNE, C.J.; GERWICK, L. (2001). The acute phase response and innate immunity of fish. Dev. Comp. Immunol., v.25, p.725-743.

Biller-Takahashi, J.D.; Takahashi, L.S.; Pilarski, F.; Sebastião , F.A.; Urbinati, E.C.(2013). Serum bactericidal activity as indicator of innate immunity in pacu Piaractus mesopotamicus (Holmberg, 1887). Arq. Bras. Med. Vet. Zootec., v.65, n.6, p.1745-1751.

Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. (2010). Performance of matrix-assisted laser desorption ionizationtime of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol. 48, 1549-1554.

Bonartsev AP, Bonartseva GA, Reshetov IV, Kirpichnikov MP, Shaitan KV. (2019). Application of Polyhydroxyalkanoates in Medicine and the Biological Activity of Natural Poly(3-Hydroxybutyrate). Acta Naturae. 11(2):4-16. doi:10.32607/20758251-2019-11-2-4-16.

Collee, J. G.; Duguid, J. P.; Fraser and Marmion, B. P. (1996). Mackie and McCartney practical medical microbiology. 14th Ed. The English language book society and Churchill Livingstone Edinburgh and New York.

Cruickshank R., Duguid J.P., Marmian B.P. and Swain R.H.A. (1979). The practice of medical Microbiol 12th ed., Churchill Livingstone, Edinburgh, London. Medical Microbiol.(2) 121-123.

Darwish A, Plumb, J. A. and Newton, J. C. (2000). Histopathology and pathogenesis of experimental infection with Edwardsiella tarda in channel catfish; J. Aqua. Anim. Hlth.., 12: 255-266.

DAS, B.K.; PRADHAN, J.; SAHU, S. (2009). The effect of Euglena viridis on immune response of rohu, Labeo rohita (Ham.). Fish Shellfish Immunol., v.26,

p.871-876.

ELLIS, A.E. (2001). Innate host defense mechanism of fish against virus and bacteria. Dev. Comp. Immunol., v.25, p.827-839.

Defoirdt T, et al. (2007). The bacterial storage compound poly-b-hydroxybutyrate protects Artemia franciscana from pathogenic Vibrio campbellii. Enviro Microbiol. 09:445-452.

Dfoirdt, T.; Sorgeloos, P. (2011). Bossier, P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr. Opin. Microbiol. 14, 251-258

Halet D, et al. (2007). Poly-b-hydroxybutyrate-accumulating bacteria protect gnotobiotic Artemia franciscana from pathogenic Vibrio campbellii. FEMS Microbiol Ecol. 60:363-369.

Hirono, I. Nahoko Tange and Takashi Aoki (1997). Iron-regulated haemolysin gene from Edwardsiella tarda. Molecular Microbiology (1997) 24(4), 851-856.

Kato N., Konishi H., Shimao M. & Sakazawa C. (1992.) Production of 3 hydroxybutyric acid trimer by Bacillus megaterium B-124. J Ferment Bioeng 73: 246-247.

Kim, H. S., Lee, K.W., Jung, H.S., Kim, J., Yun, A., Cho, S.H. & Kwon, M. (2017). Effects of dietary inclusion of yacon, ginger and blueberry on growth, body composition and challenge test of juvenile rockfish (*Sebastes schlegeli*) against *Edwardsiella tarda. Aquaculture nutrition*. 24, 1048-1055.

Krieg, N. R. & Holt, J. G. (1984). Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore.

Kurokawa S, Kabayama J, Fukuyasu T, Hwang SD, Park CI, Park SB, del Castillo CS, Hikima J, Jung TS, Kondo H, Hirono I, Takeyama H, Aoki T. (2013). Bacterial classification of fish-pathogenic Mycobacterium species by multigene phylogenetic analyses and MALDI Biotyper identification system. Mar Biotechnol (NY), 15, 340-348.

Najdegerami EH, et al. (2017). Dietary effects of poly-ÃƒÂŸ-hydroxybutyrate on the growth performance, digestive enzyme activity, body composition, mineral uptake and bacterial challenge of rainbow trout fry (*Oncorhynchus mykiss*). Aqua Nutri.; 23: 246-254.

Nhan DT, et al. (2010). The effect of poly ÃƒÂŸ-hydroxybutyrate on larviculture of the giant freshwater prawn Macrobrachium rosenbergii. Aqua.; 302: 76-81.

Noga, E. J. (2000). Fish disease diagnosis and treatment (Iowa: Iowa State University Press).

Mabrok, Mahmoud & Algammal, Abdelazeem & Fathi, Mohamed & Tawfiek, Basma & Hozzein, Wael & el kazzaz, Waleed. (2020). Molecular Typing, Antibiogram and PCR-RFLP Based Detection of *Aeromonas hydrophila* Complex Isolated from *Oreochromis niloticus*.

Magnadottir B. (2006). Innate immunity of fish (overview). Fish Shellfish Immunol., v.20, p.137-151,.

Magdalena Lenny Situmorang, Peter De Schryver, Kristof Dierckens, Peter Bossier (2016). Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia *Oreochromis niloticus* juveniles, Veterinary Microbiology, Volume 182, 2016, Pages 44-49, ISSN 0378-1135, https://doi.org/10.1016/ j.vetmic.2015.10.024.

MAGNADOTTIR, B.; AUDUNSDOTTIR, S.S.; BRAGASON, B.T.H. et al. (2011). The acute phase response of Atlantic cod (Gadus morhua): Humoral and cellular responses. Fish Shellfish. Imunol., v.30, p.1124-1130.

MAQSOOD, S.; SAMOON, M.H.; SINGH, P. (2009). Immunomodulatory and growth promoting effect of dietary levamisole in Cyprinus carpio fingerlings against the challenge of Aeromonas hydrophila. Turk. J. Fish. Aquatic Sci., v.9, p.111-120.

MISRA, C.K.; DAS, B.K.; MUKHERJE, S.C.; PATTNAIK, P. (2006). Effect of multiple injections of bglucan on non-specific immune response and disease resistance in Labeo rohita fingerlings. Fish Shellfish Immunol., v.20, p.305-319.

MISRA, C.K.; DAS, B.K.; MUKHERJEE, S.C. (2009). Immune response, growth and survival of Labeo rohita fingerlings fed with levamisole supplemented diets for longer duration. Aquaculture Nutr., v.15, p.356-365.

Miyazaki T, Kaige N: (1985). Comparative histopathology of Edwardsiellosis in fishes. Fish Pathol. 20: 219-227.

Mohanty, B. & Sahoo, P K. (2007). Edwardsiellosis in fish: a brief review. Journal of Biosciences. 32. 10.1007/s12038-007-0125-x.

MURRAY, C.K.; FLETCHER, T.C. (1976). The immunohistochemical localization of lysozyme in plaice (Pleuronectes platessa L.) tissues. J. Fish Biol., v.9, p.329-334, 1976.

Park S P., Takashi Aoki1,2 and Tae Sung Jung1. (2012). Pathogenesis of and strategies for preventing Edwardsiella tarda infection in fish. Park et al. Veterinary Research 2012, 43:67 http://www.veterinaryresearch.org/content/43/1/67

Ponnusamy, Suguna & Ramesh, Binu & Periyasamy,

Abirami & Saranya, Viswanathan & Kkani, Poornima & Veluchamy, Rajeswari & Shenbagarathai, Rajaiah. (2013). Immunostimulation by Polyhydroxybutyrate-hydroxyvalerate (PHB-HV) from *Bacillus thuringiensis* in *Oreochromis mossambicus*. Fish & shellfish immunology. 36. 10.1016/j.fsi.2013.10.012.

Quynh, Truong & Park, Seong Bin & Kim, Si Won & Lee, Jung & Im, Se & Lazarte, Jassy Mary & Seo, Jong & Lee, Woo-Jai & Kim, Jae & Jung, Tae Sung. (2016). MALDI-TOF MS-based identification of *Edwardsiella ictaluri* isolated from Vietnamese striped catfish (*Pangasius Hypothalamus*). Journal of veterinary science. 0;17(3):377-83. doi: 10.4142/jvs.2016.17.3.377

Prabu E, Rajagopalsamy CBT, Ahilan B, Jeevagan IJMA, Renuhadevi M. (2019). Tilapia–An Excellent Candidate Species for World Aquaculture: A Review. Annu Res Rev Biol. 31(3): 1-14.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2010). Assessment of synergistic efficacy of carvacrol and cymene against Edwardsiella tarda in vitro and in Tilapia (*Oreochromis niloticus*). African Journal of Microbiology Research. 4, 420-425.

Sakai, Takamitsu & Yuasa, Kei & Sano, Motohiko & Iida, Takaji. (2009). Identification of Edwardsiella ictaluri and E. tarda by Species-Specific Polymerase Chain Reaction Targeted to the Upstream Region of the Fimbrial Gene. Journal of aquatic animal health. 21. 124-32. 10.1577/H08-061.1.

Sahoo PK, Mukherjee SC, Sahoo S.K. (1998). *Aeromonas hydrophila* versus *Edwardsiella tarda*: a pathoanatomical study in *Clarias batrachus*. J Aquacult. 6: 57-66.

Sakai T, Yuasa K, Sano M, Iida T. (2009). Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene. J Aquat Anim Health 21, 124-132.

Schryver PD, et al. (2010). Poly-Ã*f*ÂŸ-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, Dicentrarchus labrax. Appl Microbiol Biotechnol; 86: 1535-1541.

Sihag, R.C. (2012). Probiotics: The New Ecofriendly Alternative Measures of Disease Control for Sustainable Aquaculture. Journal of Fisheries and Aquatic Science. 7. 72-103. 10.3923/jfas.2012.72.103.

Situmorang ML, De Schryver P, Dierckens K, Bossier P. (2016). Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia Oreochromis niloticus juveniles. Vet Microbiol. 182: 44-9. doi: 10.1016/j.vetmic.2015.10.024. Epub 2015 Oct 31. PMID: 26711027

Situmorang, M.L.; Dierckens, K.; Mlingi, F.T.; Van Delsen, B.; Bossier, P.(2014). Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. Dis. Aquat. Org. 109, 23-33.

Sui L, et al. (2012). Effect of poly-b-hydroxybutyrate on Chinese mitten crab (*Eriocheir sinensis*) larvae challenged with pathogenic Vibrio anguillarum. J Fish Dis; 35: 359-364.

Thai TQ, et al. (2014). Poly-Ã*f*ÂŸ-hydroxybutyrate content and dose of the bacterial carrier for Artemia enrichment determine the performance of giant freshwater prawn larvae. Applied Micro and Biotech; 98: 5205-5215.

Wang, Xin & Wang, Qiyao & Xiao, Jingfan & Liu, Qin & Wu, Haizhen & Zhang, Yuanxing. (2010). Hemolysin EthA in Edwardsiella tarda is essential for fish invasion in vivo and in vitro and regulated by two-component system EsrA-EsrB and nucleoid protein Hha(Et). Fish & shellfish immunology. 29. 1082-91. 10.1016/j.fsi.2010.08.025.

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