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Using of some phytobiotics and probiotics as promotors to cultured Nile Tilapia

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Abstract
The present study was carried out to investigate the effects of dietary thyme, cinnamon and/or *Bacillus subtilis* probiotic supplementation on growth performance, biochemical parameters, antioxidant activity and protection against *Aeromonas hydrophila* infection of *Oreochromis niloticus*. Total number of two-hundred and ten *O. niloticus* fish were used in our work (60 fish for isolation of *Aeromonas hydrophila* and 150 fish for experimental growth trial were allotted into six equal groups, fed on basal diet supplemented by 0.0 or with 10 g thyme or cinnamon herb/kg diet without or with 0.1g *Bacillus subtilis* probiotic /kg diet respectively for 8 weeks and, subsequently, challenged with *A. hydrophila* previously isolated from local Nile tilapia farms. Obtained data revealed that thyme or cinnamon supplementation significantly (P<0.05) improved total gain, daily gain, gain%, SGR%, total feed intake and FCR throughout the whole experimental period compared with control. *Bacillus subtilis* supplementation alone or with thyme herb supplementation in Nile tilapia fish diet improved total gain, daily gain, gain%, SGR%, total feed intake and FCR, however *Bacillus subtilis* supplementation with cinnamon non-significantly reduced the mentioned parameters compared with fish group fed on the same diet without *Bacillus subtilis* supplementation. Moreover, it was noticed that thyme or cinnamon supplementation increased serum total protein, globulin, SOD and CAT levels and reduced serum ALT, AST, uric acid and creatinine concentrations compared with control, while *Bacillus subtilis* supplementation alone or with thyme herb supplementation in Nile tilapia fish diet increased serum total protein, globulin, SOD and CAT levels and reduced serum ALT, AST, uric acid and creatinine concentrations and opposite result obtained with cinnamon and probiotic addition compared with fish group fed on the same diet without *Bacillus subtilis* supplementation. Inclusion of thyme or cinnamon herbs without or with *Bacillus subtilis* supplementation in Nile tilapia fish diet reduced mortality% and *Aeromonas hydrophila* re-isolation rate compared with control and the best value obtained by fish group fed on diet supplemented by thyme with probiotic or thyme alone. It could be concluded that using thyme with probiotic or alone good due to their high effective growth promoting, antibacterial and positive impact on liver and kidney functions and antioxidant enzymes activities, also using of cinnamon had good antioxidant and antibacterial activity. This study highly recommends the use of thyme and/or probiotic as therapeutic and protective agents in dealing with *Aeromonas hydrophila* infection in fish culture.

Keywords: Phytobiotic, *Bacillus subtilis*, growth performance, serum biochemistry, *Aeromonas hydrophila*, *Oreochromis niloticus*

Introduction
Fish considered the most important economic protein source compared to other sources of animal protein, moreover fish contribute about 30% of the total animal protein consumption in developing countries [1]. Aquaculture contributes more than half of the total fish production in the world [2], while contributes about 77% of the total fish production in Egypt and secures more than 580,000 jobs for workers in this sector [3]. Bacterial pathogens are the most serious disease affecting fish resulting in high mortalities and economic losses among fish and fish farms [4]. *Aeromonas* species are responsible for wide range spectrum of diseases among fish and human, as Motile *Aeromonas Septicemia* (MAS) in fish which is caused by *A. hydrophila* leading to high mortalities and high economic losses. [5]. Virulence genes act as a key component in determining the potential pathogenicity of the micro-organism, acting multi functionally and multi factorially and can be used for virulence typing of *A. hydrophila* isolates [6]**.** The pathogenicity of motile Aeromonads has been linked to some virulence factors including structural features associated with adhesion, cell invasion, resistance to phagocytosis

and extracellular factors such as aerolysin, a spore-forming toxin, which is cytolytic and enterotoxin gene [7]. Haemolysins (haemolysin and aerolysin) belong to a large group of spore-forming bacterial cytolysins, which can cause cytoplasmic content leakage by breaking the cellular membrane, and then cell death [8, 9].

Aeromonas hydrophila infection has been mainly controlled by antibiotics such as oxytetracycline (OTC), sulfadimethoxine and ormetoprim [10]. However, the continuous use of antibiotics has led to the development of drug-resistant bacteria, which has reduced the efficacy of the drugs [11]. To reduce or avoid the dependence of aquaculture on antibiotics, natural products have been considered for use as alternatives to control bacterial infections. Prebiotics, probiotics and their combinations are under extensive investigation for their potential beneficial effects on fish health and growth.

The inclusion of common probiotic strains, such as *Bacillus spp*., in fish feed can also help promote beneficial bacterial on the skin and intestine to outcompete pathogenic bacteria [12]. One indisputable advantage of spore-forming *Bacillus spp*. is that it is stable in the gastric environment as it is not affected by gastric secretions [13]. Also, it has been found that intestinal microflora can artificially become dominated by Bacillus sp. (up to 50% of the total) if it is added to the water for 20 days [14].

Thyme is an herb known since ancient times and used in different areas of life such as, cosmetic industry and medicine. It includes thymol- an essential oil, which has antiseptic properties, rich in potassium, magnesium, vitamins A, C, E and antioxidants [15]. Thymol also has an antimicrobial effect on bacteria, fungi and yeasts [16]. The most important therapeutic properties of thyme extract on fish health are antioxidant, antiseptic and digestion stimulant [17]. The use of cinnamon as a natural food supplement has a beneficial effect on growth, food utilization and resistance to *A. hydrophila* infection. The diet containing 1% cinnamon powder resulted in greater specific growth rate (SGR), feed conversion ratio (FCR), feed efficiency ratio (FER), protein efficiency ratio (PER), apparent protein utilization (APU), and energy utilization (EU), [18].

An extensive review of the literature on this topic revealed that there are no reports on the in vivo kinetics of probiotic bacterial growth in the presence of thyme or cinnamon. In vitro studies, *Bacillus spp*. exhibited higher survival rate in the presence of thyme oil as compared to the cinnamon oil group [19]. Therefore, in the authors' opinion, the current study is innovative and facilitates a better understanding of the interactions occurring among probiotics and thyme or cinnamonin the gastrointestinal tract of fish. In summation, the aim of the current investigation was to assess the dietary inclusion of thyme, cinnamon, *Bacillus subtilis* or a mix of the two on the growth performance, feed utilization, biochemical blood parameters and health status of Nile tilapia.

Materials and methods

Experimental design

Fish

One hundred and fifty of *O. niloticus* weighted (20±5 gm) were obtained from Private fish farm at Kafr EL-Sheikh Governorate and transported in double walled polyethylene bags to the lab. The health conditions of fish were examined for any disease condition (parasitic, bacterial) [20]**.** Fish was placed in well-prepared fiberglass (750L) tanks filled with de-chlorinated water. The water temperature was adjusted to 25±2 °C and the oxygen level was maintained at optimal level using aerators. Fish was fed basal diet at a rate of 3% body weight twice daily. The uneaten food and excreta were siphoned and water exchange of about its third volume was done daily.

Preparation of Herbal plants

Thyme and cinnamon were purchased from local hypermarket in Kafr El-Sheikh Governorate. The dry whole plant of thyme and cinnamon were grinded to fine powder by electrical blinder and preserved at 4 ^{0}C in refrigerator tell use.

Feeding diets and experimental design

The diets were formulated to meet nutrient requirements of *O. niloticus* fish [21], Table (1). Fish were randomly allotted into six equal groups (25 fish per group) received the prepared pelleted experimental diet according to the experimental design Table (2). All dietary ingredients were finally ground, well mixed and pelleted through 2.0 mm diameter. Three diet samples have been collected for proximate analysis**.** One was taken at the beginning, one in the middle and one at the end of the experimental as grab sample from the feed stocks. Feed samples were stored at -4 ^{o}C for later analysis.

Table 1: Ingredients and proximate analysis of the basal diet

Physical composition		Chemical composition	
Ingredients	**%**	**Items**	**%**
		Dry matter (DM)	88.76
		Moisture	11.24
Yellow corn7%	36.61	Crude protein (CP)	30.84
Soybean meal 45.24%	33	Ether extract (EE)	4.96
Fish Meal 60%	12.5	Crude fiber (CF)	3.2
wheat bran 13.3%	1.75	Ash	6.43
Corn gluten 62.56%	10	**NFE	43.33
Soybean oil	4	Methionine	0.759
Di-calcium phosphate	1.5	Lysine	1.85
Salt	0.2	Calcium	0.72
*Vitamins and minerals mixture	0.3	Total phosphorus	0.87
		***Digestible energy (DE)	3425.56 Kcal/Kg
		P/E ratio	90.03

*The used Vitamins and minerals mixture (Multivita Co.) composed of vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10g, vitamin K3 2g, vitamin B1 1g, vitamin B2 5g, vitamin B6 1.5g, vitamin B12 0.01g, Niacin 30g, Biotin 0.050g, Folic acid 1g, Pantothenic acid 10g, Iron 30g, Manganese 60g, Copper 4 g, Zinc 50g, Iodine 1g, Cobalt 0.1g, Selenium 0.1g, calcium carbonate (CaCO3) carrier to 3000g.

**NFE = Nitrogen free extract and calculated by difference {100 – (moisture% + CP% + EE% + CF% + Ash%)}. ***Digestible energy (DE) was calculated (kcal/kg) using formula based on chemical composition of feed stuffs nutrients [21].

Table 2: Outline of the experiment design

Groups No.	Dietary supplementation			
	Basal diet	Thyme herb* (10g/kg diet)	Cinnamon herb** (10g/kg diet)	*B. subtilis* probiotic (0.1g/kg diet)***
1	“””	--	--	--
2	“””	+	--	--
3	“””	--	+	--
4	“””	--	--	+
5	“””	+	--	+
6	“””	--	+	+

*Thyme: Fresh prepared seeds powder. The proximate analysis of Thyme dry biomass as feed base provided: 12.5% moisture, 5.4 crude protein, 4.21 crude fat, 9.6% Ash, 68.29% total carbohydrate, 1.15 % Ca, 2.8% total phosphorus, 2976.8 Digestible Energy.
**Cinnamon: Fresh prepared seeds powder. The proximate analysis of Cinnamon dry biomass as feed base provided: 13.5% moisture, 7.5 crude protein, 6.34 crude fat, 8.25% Ash, 64.41% total carbohydrate, 1.5% Ca, 3.83% total phosphorus, 3117.1 Digestible Energy.
***probiotic: *Bacillus subtilis* spore (ATCCPTA-6737(4b 1823), 1 gm contain $2x10^8$cfu/g. Produced by Kemin-Balgika.

Experimental procedure
The 6 aquaria’s were randomly assigned to one of the six treatments. The fish were fed by hand twice times a day at 9:00 and 14:00 h. Fish were fed to apparent visual satiation and utmost care was taken to assure that all feed supplied was consumed. All fish in each aquarium were weighed at the beginning (W0) and biweekly for a continuous 10 weeks (70 days). Weight gain, was calculated as: Weight gain = (Final body weight- Initial body weight). Gain% = (Total gain/Initial Wt.) X100. Feed Conversion Ratio (FCR) was calculated by dividing total feed intake per aquarium by the total body weight gain per the same aquarium [22], Protein Efficiency Ratio (PER) was calculated. Specific Growth Rate (SGR) was determined as described by [23]. Total body weight gain and average daily gain were also estimated according to [24].

Analytical procedure
Collected feed samples were analyzed for Dry Matter (DM), moisture and ash contents according to [25], crude protein using Kjeldahl method according to [26], and ether extract was determined according to [27], technique as modified by [28].

Serum biochemical parameters
Biochemical examinations of the *O. niloticus* were performed on surviving fish. The body surfaces were cleaned and blotted dry with adsorbent paper. Blood samples, collected 2ml/fish from three fish of each group at the end of the experiment from caudal vein using disposable 3-cc syringes and 21-gauge needles according to [29]. One ml of blood was collected by syringe containing EDTA anticoagulant for total leucocytes count were counted [30] using haemocytometer and differential leucocytes count [31]. Another Blood samples were transferred into Eppendorf tubes without anticoagulants for serum separation for biochemical determination [32]. Serum total protein was determined [33], while serum albumin was detected [34]. Moreover, serum globulin was calculated by subtract the total serum albumin from total serum protein [35, 36]. Activities of ALT (GPT) and AST (GOT) were detected [37]. Urea and creatinine were determined [38, 39] respectively. SOD and CAT activities were measured [40].

Isolation and identification of *Aeromonas hydrophila* for experimental challenge
A number of 60 apparently healthy and naturally infected *Oreochromis niloticus* fish were collected from Kafr El-Sheikh governorate farms. The diseased fish showed petechial hemorrhages externally over the body and fins. The peritoneal cavity was swollen with bloody ascetic fluid with visible hemorrhages on the internal organs of the affected fish. The fish samples were kept in tanks partially filled with the same water of the pond then transported to the Lab.

Swabs were taken aseptically using a sterile loop from kidney, liver, spleen, skin, and gills and inoculated into trypticase soy broth (TSB; Oxoid) and incubated at 37 °C for 24hr. A loop ful of the obtained broth was streaked on Tryptic soya agar, MacConkey’s agar media and *Aeromonas* Medium Base (Oxoid, Ltd.) with its supplement (Ampicillin), followed by incubation at 37 °C 24 hr. The purified colonies were morphologically characterization such as shape, Gram character and motility test. Biochemical characters such as oxidase, oxidative-fermentative (OF), acid and gas production from sugars (glucose, lactose, maltose, sucrose and manitol), esculin hydrolysis and 0/129 test were done to confirm their generic and specific natures. Physiological characters were studied by observing the growth of each isolate at temperatures of 24, 37 and 40 °C in different concentrations of NaCl as 0, 1, 2, 3.5 and 4% to confirm the identification of the *A. hydrophila* bacteria [41].

Detection of some virulence genes in *Aeromonas hydrophila* using polymerase chain reaction (PCR)

- **DNA extraction.** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.
- **Oligonucleotide Primer:** Primers used were supplied from Metabion (Germany) are listed in Table (1).
- **PCR amplification:** Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp 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 Applied biosystem 2720 thermal cycler.
- **Analysis of the PCR Products:** The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) and generuler 100 bp DNA ladder (Fermentas, thermos, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 3: Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Aerolysin (*Aero*)	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	326	94 ˚C 5 min.	94 ˚C 30 sec.	52 ˚C 30 sec.	72 ˚C 30 sec..	72 ˚C 7 min.	Singh *et al.*, 2008
Haemolysin (*hly*)	CTATGAAAAAACTAAAAATAACTG CAGTATAAGTGGGGAAATGGAAAG	1500	94 ˚C 15 min.	94 ˚C 1 min.	55 ˚C 1 min.	72 ˚C 1.5 min.	72 ˚C 15 min.	Yousr *et al.*, 2007

Experimental challenge protocol

One of the virulent *A. hydrophila* strain that was previously isolated from *O. niloticus* was grown overnight on tryptone soy broth at 28 °C, then centrifuged at 3000 ×g for 10 minute at 4 °C; the pelleted cells were washed twice and re-suspended in sterile physiological saline and adjusted to 1×10^7 CFU/ml. At the end of the experiment at the 8th week, fish from each treated and control groups were injected intra-peritoneal (IP) with 0.2 ml suspension of *A. hydrophila*. Mortalities were monitored over 2 weeks after challenge. Clinical signs and post mortem findings in dead and moribund fish were recorded.

Feeding and observation activities during challenge

All tilapia groups were maintained on their treatment diets as in the growth trial for the previous 8 weeks. During the challenge, feed was offered to fish and un-eaten feed and fecal wastes were siphoned out of each aquarium as needed. Each treatment had its own set of equipment, such as nets and siphoning hose, and was disinfected after every use to avoid cross contamination. Fish were observed twice daily and, moribund and dead fish were removed, counted and post mortem lesions of was detected [42] during each observation period. For each treatment, 10 moribund were necropsied and samples from liver, kidney, intestine, spleen and gills were streaked on TSA plates for bacterial isolation [43]**.** At the end of the experiment, all surviving fish were counted, euthanized and properly disposed.

Statistical analysis

Statistical analysis was made using Analysis of Variance (ANOVA) one-way analysis of variance for study the effect of different treatment groups on the different studied variables studied that includes (growth performance parameters, hematological and biochemical) variables using [44].

Results

Growth performance and feed utilization:

Effect of dietary thyme or cinnamon herb and *Bacillus subtilis* supplementation and their interaction on growth performance and feed utilization parameters in Nile tilapia fish is presented in Table (4). The obtained data indicated that thyme or cinnamon supplementation significantly (P<0.05) improved total gain, daily gain, gain%, SGR%, total feed intake and FCR throughout the whole experimental period compared with control. *Bacillus subtilis* supplementation alone in Nile tilapia fish diet significantly (P<0.05) improved total gain, daily gain, gain%, SGR%, total feed intake and FCR compared with control, while *Bacillus subtilis* with thyme herb supplementation non-significantly improved the mentioned parameters but *Bacillus subtilis* supplementation with cinnamon non-significantly reduced the mentioned parameters compared with fish group fed on the same diet without *Bacillus subtilis* supplementation. The highest values of FBW, WG and SGR (%) and PER were recorded in group fed on diet supplemented by both thyme herb and *Bacillus subtilis*.

Table 4: Growth performance of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Items	Herbs addition	*Bacillus subtilis* supplementation	
		Without	With
Initial weight (g/fish)	Control	23.08 ± 1.76^{ax}	22.26 ± 1.87^{ax}
	Thyme	23.07 ± 1.55^{ax}	22.69 ± 1.50^{ax}
	Cinnamon	22.52 ± 1.81^{ax}	22.98 ± 1.38^{ax}
Final weight (g/fish)	Control	45.80 ± 3.11^{ax}	50.19 ± 3.00^{ax}
	Thyme	51.37 ± 1.68^{ax}	52.23 ± 2.45^{ax}
	Cinnamon	48.62 ± 2.97^{ax}	47.60 ± 2.63^{ax}
Total gain (g/fish)	Control	22.72 ± 2.10^{by}	27.93 ± 1.47^{ax}
	Thyme	28.30 ± 0.76^{ax}	29.55 ± 1.21^{ax}
	Cinnamon	26.10 ± 1.34^{ax}	24.62 ± 1.24^{bx}
Daily gain (g/fish)	Control	0.41 ± 0.03^{by}	0.50 ± 0.02^{ax}
	Thyme	$0.51\pm0..01^{ax}$	0.53 ± 0.01^{ax}
	Cinnamon	0.47 ± 0.02^{ax}	0.44 ± 0.02^{bx}
Gain%	Control	98.45 ± 6.67^{by}	125.47 ± 6.11^{ax}
	Thyme	122.70 ± 6.03^{ax}	130.22 ± 5.08^{ax}
	Cinnamon	115.91 ± 3.50^{ax}	107.17 ± 2.28^{bx}
SGR%	Control	0.53 ± 0.03^{by}	0.63 ± 0.03^{ax}
	Thyme	0.62 ± 0.02^{ax}	0.65 ± 0.02^{ax}
	Cinnamon	0.60 ± 0.01^{ax}	0.57 ± 0.01^{bx}
TotTotal feed intake (g/fish)	Control	48.25	53.82
	Thyme	53.43	55.02
	Cinnamon	53.48	49.26
Average FCR*	Control	2.12 ± 0.15^{ax}	1.93 ± 0.16^{ay}
	Thyme	1.89 ± 0.06^{bx}	1.86 ± 0.09^{ax}
	Cinnamon	2.10 ± 0.11^{abx}	2.00 ± 0.12^{ax}
Average PER**	Control	1.53 ± 0.14^{ax}	1.68 ± 0.09^{ax}
	Thyme	1.72 ± 0.07^{ax}	1.74 ± 0.07^{ax}
	Cinnamon	1.58 ± 0.11^{ax}	1.62 ± 0.11^{ax}

Values are means ± standard error. Mean values with different letters at the same column (a - c letters) or row (x – z letters) differ significantly at (P<0.05). *FCR (Feed Conversion Ratio). **PER (Protein Efficiency Ratio)

Blood serum units

Supplementation of thyme or cinnamon herbs in fish diet significantly (P<0.05) increased serum total protein, albumin and globulin concentrations in compression with control. On the other hand, *Bacillus subtilis* supplementation alone or with thyme herb in Nile tilapia fish diet significantly (P<0.05) improved the mentioned serum units, while significantly (P<0.05) reduced in fish group fed on diet supplemented by *Bacillus subtilis* and cinnamon herb when compared with fish group fed on the same diet without *Bacillus subtilis* supplementation. The highest values of serum total protein, albumin and globulin concentrations were recorded in group fed on diet supplemented by both thyme herb and *Bacillus subtilis*.

Table 5: Blood serum units of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Parameters	Herbs addition	Probiotic upplementation	
		Without	With
Total protein (g/dl)	Control	5.31 ± 0.09^{by}	5.45 ± 0.08^{bx}
	Thyme	5.57 ± 0.12^{ay}	6.09 ± 0.06^{ax}
	Cinnamon	5.43 ± 0.14^{abx}	5.13 ± 0.08^{cy}
Albumin (g/dl)	Control	4.56 ± 0.21^{by}	4.89 ± 0.13^{bx}
	Thyme	5.23 ± 0.12^{ax}	5.23 ± 0.05^{ax}
	Cinnamon	4.71 ± 0.07^{bx}	4.56 ± 0.22^{cx}
Globulin (g/dl)	Control	0.22 ± 0.02^{cy}	0.72 ± 0.04^{bx}
	Thyme	0.75 ± 0.09^{ay}	1.20 ± 0.05^{ax}
	Cinnamon	0.42 ± 0.04^{bx}	0.34 ± 0.02^{cy}

Values are means ± standard error. Mean values with different letters at the same column (a – c letters) or row (x – z letters) differ significantly at (P<0.05).

Kidney and liver functions related serum parameters & antioxidant enzyme activity

Table (6) shows that thyme or cinnamon herbs inclusion in Nile tilapia fish diet significantly (P<0.05) reduced blood serum urea, creatinine, AST and ALT concentrations while, significantly (P<0.05) increased serum SOD and CAT activities compared with control. Moreover, *B. subtilis* supplementation alone or with thyme herb addition significantly (P<0.05) reduced blood serum urea, creatinine, AST and ALT concentrations while, significantly (P<0.05) increased serum SOD and CAT activities compared with fish group fed on the same diet without *B. subtilis* supplementation. However, *B. subtilis* supplementation with cinnamon herb addition significantly (P<0.05) increased blood serum urea, creatinine, AST and ALT concentrations while, significantly (P<0.05) decreased serum SOD and CAT activities compared with fish group fed on the same diet without *B. subtilis* supplementation.

Table 6: Kidney and liver functions related serum parameters & antioxidant enzyme activity of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Parameters	Herbs addition	Probiotic supplementation	
		Without	With
Urea (mg/dl)	Control	23.65 ± 0.048^{ax}	20.74 ± 0.30^{by}
	Thyme	18.38 ± 0.09^{cx}	17.29 ± 0.11^{cy}
	Cinnamon	21.49 ± 0.62^{by}	23.04 ± 0.28^{ax}
Creatinine (mg/dl)	Control	0.36 ± 0.01^{ax}	0.07 ± 0.01^{by}
	Thyme	0.07 ± 0.01^{cx}	0.03 ± 0.01^{cy}
	Cinnamon	0.23 ± 0.01^{by}	0.30 ± 0.01^{ax}
AST (u/l)	Control	66.00 ± 1.02^{ax}	51.00 ± 1.54^{by}
	Thyme	46.00 ± 1.53^{cx}	35.00 ± 1.03^{cy}
	Cinnamon	54.00 ± 1.54^{bx}	58.00 ± 1.02^{ax}
ALT (u/l)	Control	14.00 ± 1.02^{ax}	8.00 ± 0.51^{by}
	Thyme	7.00 ± 1.02^{cx}	5.00 ± 0.51^{cy}
	Cinnamon	11.00 ± 1.02^{bx}	12.00 ± 0.51^{ax}
SOD (u/l)	Control	187.5 ± 1.63^{by}	562.5 ± 3.29^{ax}
	Thyme	562.5 ± 2.04^{ax}	562.5 ± 2.81^{ax}
	Cinnamon	562.5 ± 1.29^{ax}	178.5 ± 1.52^{by}
CAT (u/l)	Control	13.46 ± 1.23^{by}	72.67 ± 1.69^{bx}
	Thyme	74.02 ± 1.24^{ay}	125.17 ± 1.11^{ax}
	Cinnamon	72.67 ± 1.54^{ax}	56.53 ± 1.03^{cy}

Values are means ± standard error. Mean values with different letters at the same column (a - c letters) or row (x – z letters) differ significantly at (P<0.05).

Total and differential leukocytes count

Total and differential leukocytes count of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis* is presented in Table (7). It was observed that herbs without or with *B. subtilis* supplementation had no significant effect on total WBCs counts, while increased neutrophil, monocyte and lymphocyte percentages compared with control. Positive interaction between thyme or cinnamon herb and *Bacillus subtilis* on total and differential leukocytes count of Nile tilapia fish was observed.

Table 7: Total and differential leukocytes count of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Parameters	Herbs addition	Probiotic supplementation	
		Without	With
WBCs	Control	27.25±0.76^{ax}	25.50±0.41^{ax}
	Thyme	29.00±1.29^{ax}	26.50±1.52^{ax}
	Cinnamon	26.25±0.88^{ax}	25.75±1.78^{ax}
Neutrophil%	Control	53.17±1.61^{bx}	55.55±1.75^{bx}
	Thyme	56.25±0.99^{ay}	62.67±1.31^{ax}
	Cinnamon	54.00±3.05^{abx}	53.74±1.94^{bx}
Basophil%	Control	8.00±0.53^{ax}	6.25±0.23^{ax}
	Thyme	3.70±0.10^{bx}	3.50±0.22^{bx}
	Cinnamon	7.00±0.35^{ax}	7.40±0.33^{ax}
Eosinophil%	Control	1.60±0.05^{ax}	1.10±0.09^{abx}
	Thyme	0.90±0.15^{cx}	0.90±0.11^{bx}
	Cinnamon	1.20±0.16^{bx}	1.30±0.13^{ax}
Monocyte%	Control	2.40±0.20^{cy}	5.40±0.22^{ax}
	Thyme	5.80±0. 16^{ax}	6.40±0.34^{ax}
	Cinnamon	3.70±0.13^{bx}	3.70±0.16^{bx}
Lymphocyte%	Control	29.93±1.81^{by}	32.38±1.11^{bx}
	Thyme	33.00±1.53^{ay}	38.24±1.76^{ax}
	Cinnamon	31.20±0.94^{abx}	30.00±0.99^{bx}

Values are means ± standard error. Mean values with different letters at the same column (a - c letters) or row (x – z letters) differ significantly at (P<0.05).

Bacteriological, morphological and biochemical characterization of the isolates used for experimental challenge Table (8)

A total 10 bacterial isolates were recovered from 60 apparently healthy and naturally infected fish, and *A. hydrophila* was identified based on the morphological, conventional, and biochemical analysis. The isolates gave rise to yellowish opaque, round, convex and semi-translucent colonies on TSA plates and pale colonies on MacConkey's agar media. While on *Aeromonas* agar they give green colonies darker in center than emerging. The results of their morphological, biochemical and physiological tests are shown in Table 8. On the basis of their growth in Vibrio static agent 0/129 the present isolates were confirmed to be *Aeromonas sp.*, and on the basis of their esculin hydrolysis characters they were confirmed to be *A. hydrophila.*

Table 8: Incidence and characteristicsof *A. hydrophila* isolated and used for experimental challenge.

Items		Isolates characters
Incidence		
No. of samples		60
Positive samples no.		10
Positive samples%		16.67
Characteristics		
Gram stain		-
motility		+
Oxidase		+
OF test		Fermentative
Acid and gas production from sugar	Maltose	+
	Sucrose	+
	Dextrose	+
Acid production	Arabinose	+
Growth in Vibriostatic agent 0/129		+
Esculin hydrolysis		+
Growth at	24 °C	+ optimum
	37 °C	+
	40 °C	-
Growth in NaCl solution	0%	+
	1%	+
	2%	-
	3%	-
	4%	-

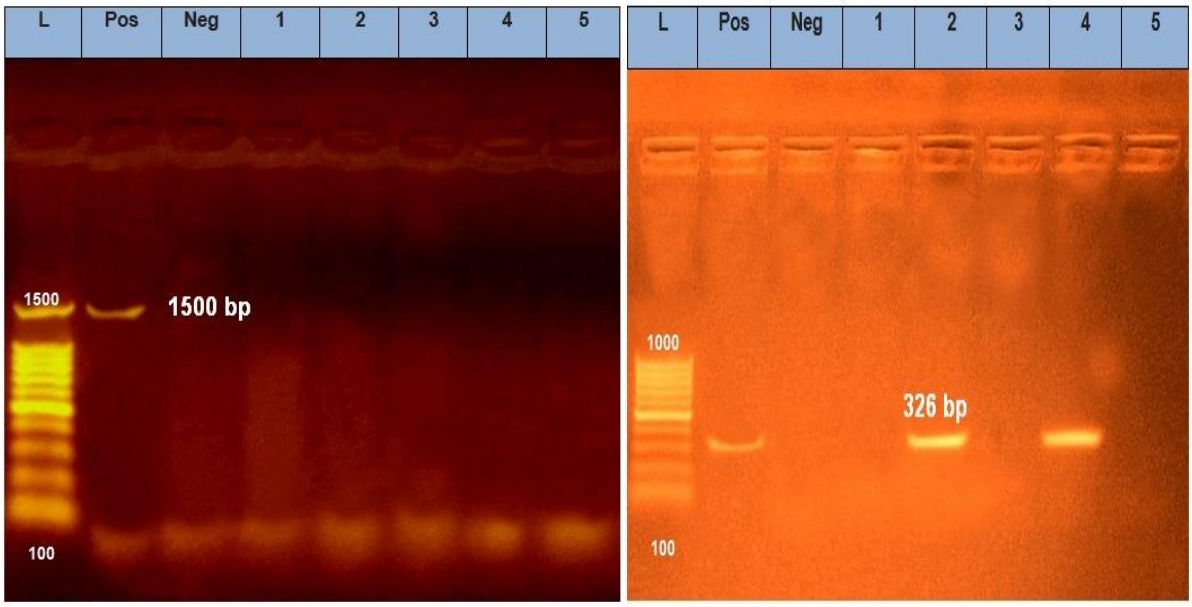

Fig 1: "Right" Agarose gel electrophoresis of PCR amplification products of *hly* gene for characterization of *A. hydrophila.* Lane L: Molecular size marker (100-1500 bp). Lane Pos. and Neg.: Positive and negative controls. Lane1, 2, 3, 4, &5: Negative *A. hydrophila* strains for *hly* gene at 1500bp.Fig (2):"left" Agarose gel electrophoresis of PCR amplification products of *Aero* gene for characterization of *A. hydrophila.* Lane L: Molecular size marker (100-1000 bp). Lane Pos. and Neg.: Positive and negative controls.Lane 2&4:Positive *A. hydrophila* strains for *Aero* gene at 326 bp.

***Aeromonas hydrophila* re-isolation rate after experimental challenge:**

Inclusion of thyme or cinnamon herbs in Nile tilapia fish diet reduced *A. hydrophila* re-isolation% from 90% of control to 10% and 30% respectively (Table, 9). *Bacillus subtilis* supplementation reduced re-isolation% from 90% to 20% while, *B. subtilis* with cinnamon addition increased from 30% to 40%. The lowest re-isolation% (10%) was obtained by

thyme herb addition alone or with *B. subtilis*.

Table 9: *Aeromonas hydrophila* re-isolation rate after challenge of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Parameters	Herbs addition	Probiotic supplementation	
		Without	With
Total No. of fish	Control	10	10
	Thyme	10	10
	Cinnamon	10	10
No. of fish that have bacteria isolate	Control	9	2
	Thyme	1	1
	Cinnamon	3	4
Re-isolation rate	Control	90	20
	Thyme	10	10
	Cinnamon	30	40

Mortality rate after experimental challenge

Inclusion of thyme or cinnamon herbs in Nile tilapia fish diet reduced mortality% from 48% of control to 20% and 36% respectively (Table, 10). *Bacillus subtilis* supplementation alone or with thyme herb reduced mortality % from 48% and 20% to 28% and 12% respectively while, *B. subtilis* with cinnamon addition had no effect on mortality% in comparison with fish group fed on cinnamon supplemented diet without *B. subtilis*. The lowest mortality % (12%) was obtained by thyme herb addition with *B. subtilis*.

Table 10: Mortality rate after experimental challenge of Nile tilapia fish fed on diets supplemented by thyme or cinnamon herbs without or with *Bacillus subtilis*

Parameters	Herbs addition	Probiotic supplementation	
		Without	With
Total No. of fish	Control	25	25
	Thyme	25	25
	Cinnamon	25	25
No. of dead fish	Control	12	7
	Thyme	5	3
	Cinnamon	9	9
Mortality rate %	Control	48	28
	Thyme	20	12
	Cinnamon	36	36
Survival rate %	Control	52	72
	Thyme	80	88
	Cinnamon	64	64

Clinical signs and Post Mortem Findings post experimental challenge

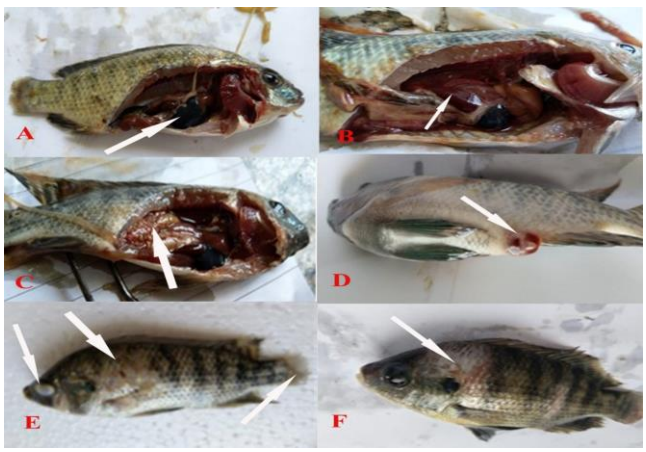

Fig 2: A. Distended gall bladder. B. Congested liver and spleen. C. Hemorrhages in all internal organs and abdomen filled with bloody fluid D. Protrusion of anal opening. E. Ulcer and hemorrhages on skin and corneal opacity. F. Ulcer on skin and erosion on dorsal fin and tail.

The clinical examination of fish post challenge (Fig. 2) exhibited hemorrhages all over the fish body at the ventral part of abdomen, base of the fins, and around the anal opening. Some fish showed congestion in the fins and loss of fin membrane. Others showed opaque eye, skin ulceration, Protrusion of anal opening and abdominal distention. Internally these fishes showed congested liver and kidneys, distended gall bladder, congested and enlarged spleen and hemorrhage in internal organs.

Discussion

Fish and its products are of a great importance for human nutrition in worldwide and provides good health benefits [45]. However, fish may act as a vehicle for pathogenic bacteria naturally occurring in aquatic environments or derived from polluted waters or from post-capture contamination [46].

Growth performance and feed utilization

Our study has shown, for the first time, the synergistic effect of thyme herb and the probiotic *B. subtilis*, which produced the best results in all the parameters analyzed (growth performance, body composition, blood parameters, liver enzymes, antioxidant enzymes and protection against *A. hydrophila* experimental challenge).

The largest increases in body weight and feed utilization were observed in fish group that received diet with a mixture of thyme herb and *B. subtilis* probiotic; however, the addition of thyme or cinnamon herb or *B. subtilis* alone also significantly improved the growth performance of Nile tilapia fish. This improvement of body weight gains maybe found due to the active materials in the herbal plants which increased the efficiency of utilization of feed, resulting in enhanced growth. Thymol and carvacrol from thyme are active material in the plant, which are considered as appetizer and stimulating of digestion, in addition to their antimicrobial activity against intestinal bacteria resulting of enhancing health status and growth [47]. One factor that may affect the effectiveness of the herbal adjuvant as a growth stimulant might also be the period in which the supplemented diet is applied. That supplementing diets with thyme herb stimulates growth has been reported previously in Nile tilapia [48, 49], also, these results agreed with [50] who reported that fish received thyme and rosemary treated diets showed a significant increase ($P < 0.05$) in weight gain rate, specific growth rate, and average body weight at 2nd and 4th weeks. Feed conversion ratio revealed significant increase at 4thweeks for thyme treated group compared to the control, [51] documented that *Oreochromis niloticus* fed diets supplemented with thyme (1% and 2%) levels had positive effect on growth performance. On the other hand, [52] showed that there was no enhancement in growth performance of *stellatus* juvenile after feeding 1% of dietary thyme, [53] studied that the tilapias supplemented with the probiotic showed significantly better final weight, body length, specific growth rate, weight gain, feed intake, feed conversion ratio and protein efficiency ratio than those fed the basal diet. Probiotics regulate digestion by facilitating increases in beneficial microbes and microbial enzyme activity. They also improve intestinal microbiological balance, and consequently, they improve digestion, food absorption and feed utilization [54, 55]. Moreover, probiotics may lead to improved gut microvilli morphology and

consequently result in the enhanced absorption of nutrients [56]. The synergistic effect of organic acid and the probiotic *B. subtilis* on fish growth was previously reported by [57].

Reduction in body weight and feed utilization were observed in fish group that received diet with a mixture of cinnamon herb and *B. subtilis* probiotic compared to fish group fed on diet supplemented by cinnamon herb or *B. subtilis* probiotic alone. These data indicated that cinnamon herb had antagonistic effect on *B. subtilis*. Cinnamon oil has been reported to be able to inhibit both Gram-positive and Gram-negative bacteria, including *Escherichia coliO157:H7*, *Helicobacter pylori*, *Listeria monocytogenes*, and *Salmonella choleraesuis* [58].

Blood serum units, Kidney and liver functions related serum parameters & antioxidant enzyme activity

Plasma enzyme activity provides important information on the functional state of different organs as their activities might be altered by disease, chemical exposure to toxic or stress conditions. ALT and AST are two of the important enzymes in the hepatocytes as the indicators of hepatotoxicity. Increased activity of ALT and AST in serum is associated with hepatocytes or tissue damage caused by environmental pollution and leaching of these enzymes in blood. [59] Reported that even low-levels of organic contamination lead to increased hepatocytes enzymes activities in fish. In this study fish fed diets containing thyme or cinnamon herbs and/or *B. subtilis* exhibited a significant decrease in serum urea, creatinine concentrations and also decreased ALT and AST activity as well as improved values of total protein, albumin and globulin compared with fish fed the control diet. The role of probiotics in modulating metabolic enzymes has also been investigated and reviewed in a few other aquatic organisms. [60] Reported that a diet supplemented with *B. subtilis* could decrease the ALT and AST activity of Nile tilapia. More advanced studies in higher vertebrates and mammals suggest that probiotics might have a therapeutic role in the modulation of the gut–liver axis, because intestinal microflora might be involved as a cofactor of chronic liver damage [61]. Bacteria can regulate the activation of Kupffer cells and the production of nitric oxide and cytokines. Moreover, in in vitro research, some *lactobacilli* reduce lipid peroxidation and attenuate acute liver injury [62, 61]. Increases in protein gram levels are thought to be associated with a stronger innate response in fish [63]. Globulin level is very often used as an indicator of immune responses and a source of antibody production. Some studies have demonstrated that probiotics can stimulate certain aspects of the non-specific immune response such as total protein, albumin and globulin [64]. [65] demonstrated that *Bacillus* sp. provided disease protection by activating humoral and cellular immune defenses in tiger shrimp (*Penaeus monodon*). The enhancement of immune response associated with dietary acidification and/or probiotic supplementation could be due to their inhibitory effects against the pathogenic microorganisms throughout the gastrointestinal tract. Administering probiotics (with or without malic acid) reduces the incidence and duration of diseases by enhancing colonization resistance and/or direct inhibitory effects against pathogens [66]. These results can be attributed to flavonoids and phenolic compounds present in the essential oils, which may cause stabilized cell membrane and protect the hepatocytes against deleterious agents and toxic compounds to cells that is reflected in alteration of serum enzymes. Another way thyme essential oil may exert antioxidative effects is by increasing the bioavailability of vitamin C and E in fish body, thus increasing the body's natural antioxidant [67].

Our results showed that dietary supplementation of thyme or cinnamonand probiotic improved the antioxidant status (SOD & CAT activities) of the fish which suggest improved immunity and health status. Similarly, [68] reported that phytogenic (carvacrol and thymol) inclusion in diets significantly decreased malondialdehyde formation of O. mykiss fillet compared to the control fish. Some common phytobiotics, such as oregano essential oils, have major antioxidant action [69], and this is due to their content of total phenolic compounds [70]. [71] stated that phytogenic products can scavenge free radicals by their antioxidant capacity through single-electron transfer. [72] found that dietary supplementation with cinnamaldehyde or thymol significantly reduced (P <0.05) the malondialdehyde (MDA) formation and increased glutathione reductase (GR) in the muscle of tilapia fingerlings. Significant interaction between thyme or cinnamon herbs and *B. subtilis* probiotic on antioxidant enzymes activities were observed. The synergistic effects between thyme and *B. subtilis* probiotic, while antagonistic effect between thyme and *B. subtilis* probiotic.

Total and differential leukocytes count

The increase in WBC counts, following feeding of thyme or cinnamon herbs of Nile fish diets, demonstrates the immune stimulatory effects of mentioned herbs which is in line with [73] who obtained increased WBC in juvenile *beluga* after feeding with ginger diet. The increasing trend in WBC count could be related to a stimulation of the immune system with thyme essential oil due to phenolic compounds such as eugenol (2-methoxy-4-(2-propenyl) phenol), thymol and carvacrol present in essential oils. Similar to the present study, [73] reported that supplementary diets with suitable doses of Coriolus versicolor polysaccharides enhanced the WBC count in crucian carp when challenged with *Aeromonas hydrophila*. Neutrophil, lymphocyte and monocyte levels showed an increase in fish fed with thyme or cinnamon herbs containing diet. Similar to the results of [74] who reported that total leucocytes and differential leukocytic count were significantly increased in thyme treated group at 6th weeks, [75] who found that fish fed 1% of thyme supplementation for 45 days had significantly increase lymphocyte, neutrophil and monocyte percentages compared with control and also with [76] recorded significant increase in WBCs count in tilapia fed on herbal supplemented diet.

Bacteriological and morphological characteristics of the isolates used for experimental challenge

As evident from our results, the bacteriological examination of the samples revealed that 10 out of 60 samples were positive for *A. Hydrophila* with a percentage of 16.66% of the examined fish. This percentage is lower than finding mentioned by [77] who isolated *A. hydrophila* from *O. niloticus with a percentage of* 40.8% and [78] who obtained *A. hydrophila* from *O. niloticus* with a percentage of 29%. Our percentage is higher than that obtained by [79] could isolate *A. hydrophila* from cultured Nile tilapia samples with the percentage of 12% and [9] who found that only 12 isolates out of 197 isolates with a percentage of 6% confirmed as *Aeromonas hydrophila*.

The morphological characteristics of the colonies, Gram

staining and the biochemical profile of Aeromonas species was similar to those previously reported by [80]. A PCR was done for detecting haemolysin (*hly*) as well as aerolysin (*Aero*) genes as genetic markers for virulence determinants. Hence for the brief work the Polymerase chain reaction (PCR) application for the aerolysin gene and haemolysin gene were carried out for the detection of pathogenic strains *A. hydrophila*. The role of both genes in *Aeromonas* pathogenicity has already been demonstrated [81]**.** The results of PCR for amplification of *hly*gene in *A. hydrophila* (Fig. 1) showed that the *hly* gene was not amplified in *A. hydrophila* studied strains. These results disagreed with [79].

In addition, the results of PCR for amplification of *Aero* gene in *A. hydrophila* (Fig. 2) showed that, the *aero* gene was 2 out of 5 with a percentage of 40% and giving product of 326 bp. The obtained results were consistent with [82] who identified aero gene in 52.6% of *A. hydrophila*. And these results disagreed with that obtained by [79] who showed that, the *Aero*gene was amplified in 9 out of 10 *A. hydrophila* with a percentage of 90%. Also disagreed with that recorded by [83]**.**

Clinical signs and Post Mortem Findings post experimental challenge

The clinical examination of fish post challenge exhibited hemorrhages all over the fish body at the ventral part of abdomen, base of the fins, and around the anal opening. Some fish showed congestion in the fins and loss of fin membrane. Others showed opaque eye, skin ulceration, Protrusion of anal opening and abdominal distention. Internally these fishes showed congested liver and kidneys, distended gall bladder, congested and enlarged spleen and hemorrhage in internal organs. These results were similar to that reported by [83]**.**

Re-isolation and mortality rate post experimental challenge

In our results, we observed that the re-isolation rate decreased in groups treated with thyme, cinnamon and probiotic compared to the control group. The mechanism of the antimicrobial activity of both thyme and cinnamon on *A. hydrophila* is still unknown. However, several possible mechanisms have been proposed. Cinnamaldehyde may damage and destroy the bacterial cell surface [84], inhibit amino acid decarboxylase activity [85] and decrease cellular glutathione levels [86]. The carbonyl group of cinnamaldehyde is thought to be responsible for antimicrobial action by binding to cellular proteins, preventing them from functioning properly [87]**.** Moreover, addition of *B. subtitles* could have led to a reduction in total count bacteria in the Nile tilapia gut and consequently reduce the re-isolation rate of *A. hydrophila* after challenge [57]**.**

The mortality rate decreased in groups treated with thyme, probiotic and cinnamon compared to the control group fed the basal diet. This agreed with those obtained by [74] who revealed that groups of *O. niloticus* fed on 1% thyme and rosemary showed reducing in mortality rates (10%) compared to (90%) controls; [87] who reported that *O. Mossambicus* fed on thyme and rosemary incorporated diets exhibited reduction in cumulative mortality in treated groups. According to [88], the effectiveness of probiotics in terms of protection against infection is often attributed to enhanced immunity.

The lowest *A. hydrophila* re-isolation% and mortality% was obtained through synergetic interaction between thyme herb dietary inclusion with *B. subtilis* supplementation, while better to supplement cinnamon herb or *B. subtilis* probiotic separately in fish diet. Sustainable aquaculture development requires the use of safe and effective solutions to overcome the challenges in this industry. There is increasing evidence that natural products such as herbs and probiotics could have applications in aquaculture, to achieve a good feed efficiency, growth promotion, animal health, and increase resistance to bacterial infection.

Conclusion

The results of the present study have established that dietary thyme or cinnamon or B. *subtilis* probiotic separately supplementation can improve growth performance, liver function, antioxidant status, immunity of *O. niloticus* and increase resistance to bacterial infection. The results also indicated that there is synergistic effect of thyme herb and the probiotic *B. subtilis* on fish performance and health status, while cinnamon and *B. subtilis* combination not recommended as fish feed additive.

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