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Combined effects of dietary malic acid and *Bacillus subtilis* on growth, gut microbiota and blood parameters of Nile tilapia (*Oreochromis niloticus*)

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Abstract

The study investigated effect of dietary supplementation with malic acid, *Bacillus subtilis* and a mixture of the two (3×2 factorial trial) on Nile tilapia (*Oreochromis niloticus*) health. Treatment groups (T1–T6) were fed diets containing three levels of malic acid (0.0, 5.0 and 10.0 g/kg), each of which was supplemented with 0 and 1.1×10^5 cfu/g *B. subtilis*, respectively. Each treatment group was assigned randomly to triplicate groups of 35 fish (5.26 ± 0.06 g) in 18 tanks for 84 days. The results indicated that survival was higher in all dietary treatments as compared to the control fed fish. The highest values of FBW, WG and SGR (%), PER, PPV and ER were recorded in groups T4 and T6, which were fed diets supplemented with 5 g malic acid/kg and 1.1×10^5 cfu/g *B. subtilis* and 10 g malic acid/kg and 1.1×10^5 cfu/g *B. subtilis*, respectively. The best value of FCR was obtained in groups T4 and T6. The lowest total bacterial count in the gut and faeces was detected in fish from group T6. The values of haematocrit, haemoglobin, red blood cells, white blood cells, total protein, albumin and globulin were significantly higher ($p < .05$) in fish from groups T4 and T6 (diets supplemented with malic acid and *B. subtilis*). As compared to the control fed fish, te mixture of these substances are promising as immune enhancer in aquacultured fish.

KEYWORDS
Bacillus subtilis, blood parameters, growth, gut microbiota, Nile tilapia, organic acid

1 | INTRODUCTION

Microbial balance and proper pH in the digestive tract eliminate pathogenic microorganisms. This is necessary to maintain fish health in a satisfactory state and to achieve expected production levels. Over the last decade, research has focused on the application of probiotics and prebiotics to replace antibiotic growth promoters in the fish farming industry (Irianto & Austin, 2002; Yanbo & Zirong, 2006) and to enhance the growth and health of the host (Gatesoupe, 1999; Kesarcodi-Watson, Kaspar, Lategan, & Gibson, 2008).

Most commercial probiotics currently being tested in aquaculture are lactic acid bacteria (e.g., *Lactobacillus*), the genus *Bacillus*, photosynthetic bacteria (e.g., *Rhodobacter sphaeroides*) and the yeasts *Pseudomonas* or *Vibrio* (Wang, 2011). *Bacillus* spp. has been isolated from marine fish intestines (Sugita, Hirose, Matsuo, & Deguchi, 1998), among others, and some species of this genus have shown inhibitory activity against various pathogens (Rengpipat, Phianphak, Piyatiratitivorakul, & Menasveta, 1998; Sugita et al., 1998). 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 (Lee, Kim, Choi, & Paik, 2013). It has also been proved that microflora can artificially become dominated by *Bacillus* sp. (up to 50% of the total) in the GI tract if it is added to the water for 20 days (Gullian & Rodriguez, 2002).

Malic acid is a four-carbon dicarboxylic acid that occurs naturally in many fruit organic acids (it was first isolated from apples), and it is synthesized commercially (Sniffen et al., 2006). Malic acid is used in the food industry as an acidity regulator. This organic acid is used in

foods as a flavour enhancer and for pH (1% malic acid solution is about 2.3) and microbial control, because it inhibits the growth of pathogenic bacteria and pathogenic fungi (Ricke, 2003). An exceptionally valuable nutritional property of this acid is that after oral administration, it stimulates the secretion of gastric juices and increases peristalsis. Small doses of malic acid have prebiotic properties, stimulating the development of non-pathogenic, acidophilic and saprophytic bacteria whereas at higher doses it can exhibit bacteriostatic and bactericidal properties (Salou, Leroy, Goma, & Pareilleux, 1991).

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 organic acids. In *in vitro* studies, *Lactobacillus plantarum* exhibited better growth in the presence of malic acid as compared to the control group (Passos, Fleming, Hassan, & McFeeters, 2003). Therefore, in the authors' opinion, the current study is innovative and facilitates a better understanding of the interactions occurring among probiotics and malic acid in the gastrointestinal tract of fish. In summation, the aim of the current investigation was to assess the dietary inclusion of malic acid, *Bacillus subtilis* or a mix of the two on the growth performance, feed utilization, gut microbiota and haematological and biochemical blood parameters of Nile tilapia (*Oreochromis niloticus*; Linnaeus, 1758). After carp, the tilapia species comprise the second most important group of farmed fish in the world, and they are the most widely cultivated of any farmed fish.

2 | MATERIALS AND METHODS

2.1 | Fish and culture technique

Nile tilapia, *Oreochromis niloticus*, were obtained from a private farm (Elfyum Governorate, Egypt). The fish were acclimated to experimental conditions for 2 weeks at the laboratory of fish at the Faculty of Agriculture, Benha University. During the acclimation period, the fish were fed a control diet (299 g/kg crude protein) at a rate of 3% of biomass, which was provided in equal rations at 09:00 and 15:00 for 2 weeks to acclimate them to the artificial diet and experimental conditions. Dechlorinated public utility water was supplied to each tank housed in an artificially illuminated room. About one-third of the water volume in each tank was replaced daily with fresh, aerated water after removing the accumulated excreta.

2.2 | Water quality

Water temperature was recorded daily at 13:00 with a mercury thermometer. Dissolved oxygen (DO) was measured at 07:00 with a YSI 56 oxygen meter (YSI Company, Yellow Springs Instrument, Yellow Springs, OH, USA). Total ammonia was measured twice weekly with a DREL 2000 spectrophotometer (Hash Company, Loveland, CO, USA). Water pH was estimated in the morning with a pH meter (Orion pH meter, Abilene, TX, USA). The water temperature ranged from 27.20 to 29.25°C; dissolved oxygen (DO) from 5.32 and 6.81 mg/L; pH values from 8.04 and 8.30; and total ammonia from 0.18 to 0.2 mg/L in the various treatments throughout the experiment (84 days). All

tested water quality criteria (temperature, pH value, DO and total ammonia) were suitable and within acceptable limits for rearing Nile tilapia, *Oreochromis niloticus* fingerlings (Boyd, 1979). The positive water quality was reflected in good growth performance as there were no mortalities in any of the treatments. A photoperiod of 12hr light/12hr dark (08:00–20:00) was used with fluorescent ceiling lamps supplying illumination.

2.3 | Experimental design

A 3 × 2 factorial experiment was designed to study the main effects of short-chain malic acid (dicarboxylic acid), the probiotic *Bacillus subtilis* and their combined impact on growth, feed utilization, stomach and gut pH, gut microbiota flora, haematological and biochemical blood parameters and the proximate analysis of whole Nile tilapia, *Oreochromis niloticus*. A total of 630 fish with an average initial weight of (5.26 ± 0.06 g) were used in the trial. Thirty-five fish were stocked randomly into plastic tanks (0.5 m³ each) representing the six diet treatment groups (group T1, the control diet [Table 1] was not supplemented with malic acid or *B. subtilis*; group T2—0 g/kg malic acid and 1.1×10^5 cfu/g *B. subtilis*; group T3—5 g/kg malic acid and 0 cfu/g *B. subtilis*; group T4—5 g malic acid/kg diet and 1.1×10^5 cfu/g *B. subtilis*; group T5—10 g malic acid/kg diet and 0 cfu/g *B. subtilis*; group T6—10 g malic acid/kg diet and 1.1×10^5 cfu/g *B. subtilis*) each in three replicates (*n* = 3).

2.4 | Preparation of *Bacillus subtilis* inocula and diets

The *B. subtilis* strain was obtained in powdered form from the National Organization for Drug Control and Research in Cairo, Egypt. The *B. subtilis* culture was prepared by adding 10 mg of strain powder to 100 ml of specific media (peptone 5 g/L and beef extract 3 g/L, pH 7.0), which was incubated at 37°C. After 24 hr, 1 ml was inoculated into 100 ml fresh specific media broth that was incubated for a further 48 hr at 37°C. After incubation, the cells were collected by centrifugation (3,000 *g* for 10 min). The basal diet (Table 1) was supplemented dropwise with the collected cells to obtain 0.00 and 1.1×10^5 cfu/g, respectively (Shelby, Lim, Yildirim-Aksoy, & Delaney, 2006).

Bacillus subtilis was counted by serial dilution of the material in sterile distilled water and plating on tryptic soy agar. The bacterial counts, in cfu of the inoculated diet, were determined using the spread plate method. A volume of 0.2 ml from each dilution was spread onto triplicate nutrient agar plates. The plates were incubated at room temperature (25°C) for 48 hr, and plates containing from 30 to 300 cfu/plate were used to count the number of *B. subtilis*.

The basal diet was formulated to contain approximately 289.90 g/kg crude protein and 18.44 MJ/kg diet of gross energy, which has been shown to be sufficient to support the optimal growth of Nile tilapia, *Oreochromis niloticus* (Table 1). Soybean meal contributed the bulk of dietary protein with fish meal, corn gluten, yellow corn and wheat bran. The diets were prepared to confirm the control diet. The basal diets were supplemented separately with 0.00 g, 5 g and 10 g malic acid/kg (Pharmaceutical Company Adoia, Cairo, Egypt), and each level

TABLE 1 Ingredient and proximate composition of the experimental diets (g/kg dry diet)

	Diet (malic acid g/kg/*Bacillus subtilis* cfu/g)					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Ingredient	(0/0)	(0/1.1 × 10^5)	(5/0)	(5/1.1 × 10^5)	(10/0)	(10/1.1 × 10^5)
Fish meal	50	50	50	50	50	50
Soybean meal	450	450	450	450	450	450
Corn gluten	20	20	20	20	20	20
Yellow corn	260	260	260	260	260	260
Wheat bran	160	160	155	155	150	150
Soya oil	40	40	40	40	40	40
Malic acid	0	0	5	5	10	10
Vita & M[a]	20	20	20	20	20	20
Chemical analysis						
Dry matter	893.30	893.00	888.80	888.30	884.40	884.50
Crude protein	289.90	289.50	289.50	289.20	289.50	289.40
Crude lipid	52.70	53.00	52.20	52.10	52.20	52.10
Ash	49.00	49.10	48.30	48.50	48.20	48.20
Fibre content	53.70	53.50	53.10	53.00	52.90	52.80
NFE[b]	554.70	554.90	556.90	557.20	557.20	557.50
ME[c]	16.09	16.10	16.10	16.10	16.11	16.11

[a]Vitamin and mineral mix (mg or g/kg diet): MnSO4, 40 mg; MgO, 10 mg; K2SO4, 40 mg; ZnCO3, 60 mg; KI, 0.4 mg; CuSO4, 12 mg; ferric citrate, 250 mg; Na2SeO3, 0.24 mg; Co, 0.2 mg; retinol, 40,000 IU; cholecalciferol, 4,000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.
[b]NFE (nitrogen-free extract) = 100−(crude protein + lipid + ash + fibre content). *B. licheniformis* was prepared to obtain (1.0 × 10^{10} CFU/g approximately.
[c]Metabolizable energy (kJ/g), calculated based on the physiological fuel values (Jauncey 1982).

of malic acid was supplemented with 0.0 or 1.1 × 10^5 cfu/g *B. subtilis*; therefore, six experimental diets were formulated. All dry ingredients were thoroughly mixed with soybean oil and a vitamin–mineral mixture, and then passed through a laboratory pellet mill (2-mm die; CMP California Pellet Mill, San Francisco, CA, USA) at the National Institute of Oceanography and Fisheries, Cairo Governorate, Egypt.

2.5 | Growth performance and feed utilization parameters

All fish were fed at rate of 3% of the total biomass daily. During the 84 days of the experiment, the daily ration was divided into two equal amounts and offered two times a day (9:00 and 15.00 hr). All fish in each tank were weighed biweekly and the amount of daily allowance feed was adjusted accordingly. The amount of feed presented daily was weighed. Uneaten pellets were collected and counted and this information was used to estimate the total feed intake of the fish.

Live body weight (g) was determined in all fish from each tank every 14 days during the experiment. Growth performance parameters were measured with the following equations: Condition factor (K) = $W/L3 × 100$, where W = weight of fish in g; L = total length of fish in cm; weight gain (WG) = final weight (g)−initial weight (g); specific growth rate (SGR, %) = $(LnW2−LnW1)/t × 100$, where Ln = the natural

log, $W1$ = first fish weight, $W2$ = subsequent fish weight in grams, t = period in days; feed conversion ratio (FCR) = feed ingested (g)/ weight gain (g); protein efficiency ratio (PER) = weight gain (g)/protein ingested (g); protein productive value (PPV, %) = protein gain of whole fish (g)/protein intake (g) × 100; energy retention (ER, %) = energy gain (g)/(energy intake) × 100.

2.6 | Sample collection

At the start of the experiment, 10 fish were collected and kept frozen at −20°C for subsequent initial proximate analysis. At the end of the experiment, six fish were selected randomly from each treatment and anesthetized with t-amyl alcohol, sacrificed and homogenized in a blender to determine proximate composition. The remaining fish in the tank were maintained for the subsequent analysis of gut microbial flora and blood sampling. The fish were pooled for each tank, oven-dried, ground and stored at −20°C for subsequent analysis.

2.7 | Blood sampling and haematological and biochemical indices

At the end of the experiment, the fish were anesthetized with t-amyl alcohol, and then, blood samples were drawn from the caudal veins of

five fish from each treatment. The samples were divided into two portions. The first portion was collected with the anticoagulant 100 g/L ethylenediaminetetraacetate (EDTA) to measure haematocrit (Ht), haemoglobin (Hb), red blood cells (RBC) and white blood cells (WBC). Ht was determined as described by Brown (1988), Hb was determined using the standardized cyanomethaemoglobin procedure with haemoglobin kits, and the total WBC count was performed according to the method described by Svesbodora, Fravda, and Palakova (1991). The second portion of the blood samples were allowed to clot at 4°C and centrifuged at 3,000 *g* for 10 min. The non-hemolysed serum was collected and stored at −20°C until analysis.

Levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to the method described by Reitman and Frankel (1957), and serum creatinine was measured with calorimetric and enzymatic determination methods as described by Henary, Cannon, and Winkleman (1974). Total serum protein, albumin and globulin were determined according to Doumas, Bayse, Carter, Peters, and Schaffer (1981).

2.8 | Analytical method

At the initiation and termination of the trial, a random sample of five fish was collected from each tank, and then oven-dried at 70°C to a constant weight to calculate weight loss. The chemical analysis was conducted according to AOAC (1995) and stored at −20°C for subsequent analysis. Proximate analysis was conducted on both diet and fish samples. The crude protein, lipid and ash contents were all determined with standard methods (AOAC 1995). Crude protein was determined with the micro-Kjeldhal method, N × 6.25 (using a Kjeltec 1030 auto analyser, Tecator, Höganäs, Sweden) according to method number 984.13, and lipid content was determined with Soxhlet extraction with diethyl ether (40–60°C) (AOAC 1995), according to method number 920.39. Ash content was determined by incineration at 550°C for 12 hr (AOAC 1995), according to method number 942.05. Fibre content was determined using the method described by Van Soest, Robertson, and Lewis (1991)). Nitrogen-free extract was computed by subtracting the sum of the values for crude protein, crude lipid, crude fibre and ash from 1000.

The diet and stomach and gut content pH were measured according to the methodology described by Baruah et al. (2005). Five grams of the commercial feed was macerated in a porcelain mortar and mixed with 50 ml of deionized water for 1 min using a magnetic stirrer. After homogenization, the diet solution pH was measured using a digital pH meter (Orion pH meter).

2.9 | Microbial analysis

Freshly expelled faecal samples were collected daily by siphon from each tank for 1 week before the end of the experiment for determinations of microbial counts and bacterial identification. The gut was excised aseptically and cut open, and the gut contents were removed and washed gently three times with sterile, distilled water

to remove non-adherent surface bacteria. The collected faecal and gut samples were then homogenized separately in a mortar that had been rinsed with 96% ethanol. The homogenate samples were suspended in 10 ml of saline solution and diluted serially to 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} in 9 ml saline solution (Al-Harbi & Uddin, 2004). Bacterial counts (colony-forming units, cfu) of the faecal and gut samples were determined using the spread plate method. A volume of 0.5 ml from each dilution was spread onto triplicate nutrient agar plates. The plates were incubated (25 ± 1°C) for 48 hr. Plates containing between 30 and 300 cfu were used to count the number of total viable bacteria.

2.10 | Statistical analysis

All the data were analysed with SAS version 6.12 software. One-way analysis of variance (one-way ANOVA) was used to determine whether there was significant variation among the treatments. When overall differences were found, differences between means were tested by Duncan multiple range test. Two-way ANOVA was used for analysing the individual effects of malic acid and *B. subtilis* and the interaction between them. All differences were considered significant at $p < .05$, and the results are presented as means with pooled standard error of the mean.

3 | RESULTS

3.1 | Growth performance and feed utilization

The effects of malic acid, *Bacillus subtilis* and their interaction on the growth performance and feed utilization of the treatment groups are presented in Tables 2 and 3. The results indicate that survival (S) after 84 days was significantly higher in all dietary treatments in comparison with the control group T1 ($p < .05$). The final body weight (FBW), weight gain (WG), specific growth rate (SGR (%), condition factor (K), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV, %) and energy retention (ER) were significantly higher in all dietary treatments (T2–T6; $p < .05$) in comparison with the control group T1. The highest values of FBW, WG and SGR (%), PER, PPV and ER were recorded in groups T4 and T6 that were fed diets containing 5 g malic acid/kg and 1.1×10^5 cfu/g *B. subtilis* and 10 g malic acid/kg and 1.1×10^5 cfu/g *B. subtilis*, respectively. The best FCR values were obtained in groups T4 and T6 (diets supplemented with malic acid and *B. subtilis*).

3.2 | Diet, stomach and gut pH

The pH of the diet was decreased significantly after supplementation with malic acid ($p < .05$), but it was not affected by supplementation with *B. subtilis* or by the interaction between levels of malic acid and *B. subtilis* in comparison with the control group (Table 4; $p > .05$). The lowest pH levels in the diets were recorded in groups T5 and T6, which received high levels of malic acid. The pH of the stomach and

TABLE 2 Growth performance of Nile tilapia (*Oreochromis niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days

T	Malic acid g/kg	B. subtilis cfu/g	S (%)	Body weight		WG (g/fish)	SGR (% per day)	K
				IBW (g)	FBW (g)			
T1	0	0	91.43 b	5.26	34.12 c	28.86 c	2.12 c	1.97 b
T2	0	1.1×10^5	94.29 a	5.23	35.26 bc	30.03 b	2.20 b	2.07 b
T3	5	0	95.72 a	5.27	35.16 bc	29.89 b	2.19 b	2.06 b
T4	5	1.1×10^5	95.72 a	5.26	38.08 a	32.82 a	2.32 a	2.31 a
T5	10	0	95.72 a	5.26	35.99 b	30.73 b	2.20 b	2.29 a
T6	10	1.1×10^5	95.72 a	5.28	38.73 a	33.45 a	2.32 a	2.10 a
Pooled *SE*			1.16	0.48	0.67	0.53	0.02	0.07
Two-way ANOVA (*p*-value)								
Malic acid			.091	.968	.001	.004	.002	.002
B. subtilis			.362	.874	.001	.001	.001	.178
Malic acid × *B. subtilis*			.043	.623	.012	.021	.012	.002

Data are presented as means ± pooled standard error (*SE*). Means followed by different letters in each column are significantly different (*p* < .05). S, survival; IBW, initial body weight (g); FBW, final body weight (g); WG, weight gain; SGR, specific growth rate; K, condition factor; SR, survival rate; FI, feed intake (g); FCR, feed conversion ratio; PER, protein efficiency ratio.

TABLE 3 Feed utilization of Nile tilapia (*Oreochromis niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days

T	Malic acid g/kg	B. subtilis cfu/g	Feed intake and nutrient utilization				
			FI (g/fish)	FCR	PER	PPV (%)	ER (%)
T1	0	0	52.66	1.92 a	1.80 c	33.74 f	22.62 b
T2	0	1.1×10^5	52.14	1.76 b	1.97 b	36.11 e	21.88 c
T3	5	0	51.53	1.74 b	1.99 b	36.85 d	21.13 d
T4	5	1.1×10^5	52.24	1.58 c	2.19 a	39.15 a	24.25 a
T5	10	0	51.58	1.73 b	1.99 b	38.72 b	23.38 a
T6	10	1.1×10^5	52.32	1.58 c	2.19 a	39.29 a	24.33 a
Pooled *SE*			1.06	0.21	0.32	1.32	1.11
Two-way ANOVA (*p*-value)							
Malic acid			.968	.001	.004	.002	.002
B. subtilis			.874	.012	.001	.001	.178
Malic acid × *B. subtilis*			.623	.015	.021	.012	.002

Data are presented as means ± pooled standard error (*SE*). Means followed by different letters in each column are significantly (*p* < .05) different. FI, feed intake (g); FCR, feed conversion ratio; PER, protein efficiency ratio; PPV, protein productive value; ER, energy retention.

gut decreased significantly after the addition of malic acid and *B. subtilis* in comparison with the control group (*p* < .05).

However, the pH of the stomach and gut of Nile tilapia was not affected by the interaction between malic acid and *B. subtilis* (Table 4; *p* > .05). The lowest pH level of the stomach and gut was recorded in fish from groups T4, T5 and T6.

3.3 | Total bacterial counts

Total bacterial counts in the gut and faeces were significantly (*p* < .05) affected by dietary supplementation with malic acid (*p* < .05), and a significant interaction was observed between malic acid and *B. subtilis*

(Table 4). The lowest total bacterial count in the gut and faeces was detected in fish from group T6, while the highest total bacterial count in the gut and faeces was noted in group T1.

3.4 | Haematological blood indices

Changes in the haematological parameters of Nile tilapia were affected significantly (*p* < .05) by malic acid levels, *B. subtilis* and the mix of them (Table 5). Haematocrit (Ht), haemoglobin (Hb), red blood cell (RBC) and total white blood cell (WBC) values were significantly higher in fish from groups T4 and T6 in comparison with the other treatments (*p* < .05), while the lowest values were noted in fish from

TABLE 4 The pH of diet and gastrointestinal tract, and total bacterial count (cfu/g) in faeces and gut of Nile tilapia (*Oreochromis niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days

| T | Malic acid g/kg | *B. subtilis* cfu/g | pH | | | Total count bacteria ($\times 10^7$) cfu/g | |
			Diet	Stomach	Gut	Gut	Faeces
T1	0	0	5.87^a	1.93^a	6.80^a	4.10^b	4.30^a
T2	0	1.1×10^5	5.87^a	1.89^b	6.50^b	4.47^a	4.20^a
T3	5	0	5.80^b	1.75^c	6.27^c	3.43^c	3.57^b
T4	5	1.1×10^5	5.79^b	1.72^d	6.13 ^{cd}	4.20^b	3.50^b
T5	10	0	5.71^c	1.68^e	6.20 ^{cd}	3.20^d	3.27^c
T6	10	1.1×10^5	5.69^c	1.64^f	6.07^d	3.20^b	1.63^d
Pooled *SE*			0.01	0.006	0.05	0.07	0.04
Two-way ANOVA (*p*-value)							
Malic acid			.001	.001	.001	.001	.002
B. subtilis			.272	.001	.001	.027	.018
Malic acid × *B. subtilis*			.545	.481	.167	.054	.002

Data are presented as means ± pooled standard error (*SE*). Means followed by different letters in each column are significantly (*p* < .05) different.

TABLE 5 Two-way ANOVA results of dietary malic acid, *B. subtilis* and their interaction on haematological and biochemical blood parameters of Nile tilapia, *O. niloticus*

| | Probability (*p*-value) | | |
Parameters	Malic acid	*B. subtilis*	Malic acid × *B. subtilis*
Haematocrit (%)	.004	.028	.042
Haemoglobin (g/dl)	<.001	.012	.015
Red blood cells ($\times 10^{-6}$ µl)	.004	<.001	.021
White blood cells ($\times 10^{-3}$ µl)	.002	<.001	.012
Alanine aminotransferase (U/L)	<.001	<.001	<.001
Aspartate aminotransferase (U/L)	.004	.005	.005
Alkaline phosphatase (U/L)	.004	<.001	.016
Total protein (g/dl)	.003	<.001	.033
Albumin (g/dl)	.002	.002	.046
Globulin (g/dl)	.04	.006	.037

group T1 that received a diet that was not supplemented with malic acid or *B. subtilis* (Figures 1–3; *p* < .05).

3.5 | Biochemical blood indices

The alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities of the serum were significantly affected by malic acid and *B. subtilis*, and significant interaction was observed between malic acid and *B. subtilis* (Table 5). Over the experimental period, fish from groups T4 and T6 exhibited the lowest values of ALT and AST (Figures 4 and 5). The highest value of ALP was noted in fish from groups T4 and T6 (Figure 6). No significant differences were noted in ALT, AST or ALP among groups T2, T3 or T5.

Total protein (TP), albumin (AL) and globulin (GL) were significantly (*p* < .05) affected by malic acid, *B. subtilis* and the mix of the two (Table 5). High TP, Al and GL values were recorded in groups T4 and T6, while the lowest contents of TP, Al and GL were noted in fish from

group T1 that were fed a diet not supplemented with malic acid or *B. subtilis*. There were no significant differences between the fish from groups T2, T3 or T5 (Figure 7).

3.6 | Proximate composition

The proximate composition of tilapia fed diets supplemented with malic acid, *B. subtilis* or the mix of the two is presented in Table 6. Dietary supplementation with *B. subtilis* alone did not have a significant (*p* > .05) impact on protein, lipid or ash content. Dry matter was not affected by dietary malic acid, *B. subtilis* or the mix of the two supplements. The protein content of the fish from group T5 was significantly higher than that noted in the other treatments. The highest lipid content was detected in fish from group T1 that received a diet that was not supplemented with malic acid or *B. subtilis*. Ash content was significantly higher (*p* < .05) in fish from groups T3, T4, T5 and T6 in comparison with the other treatments.
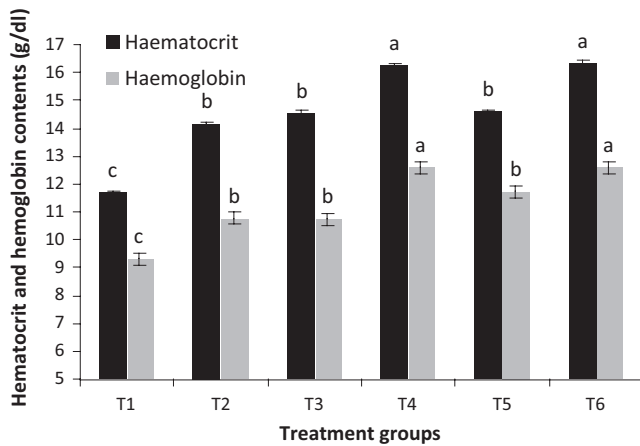
FIGURE 1 Haematocrit and haemoglobin of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups (*p* < 0.05)
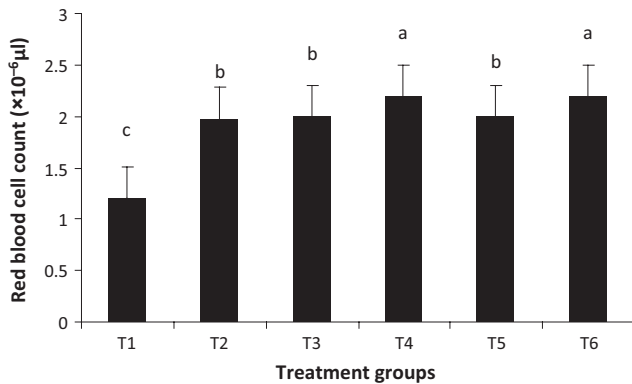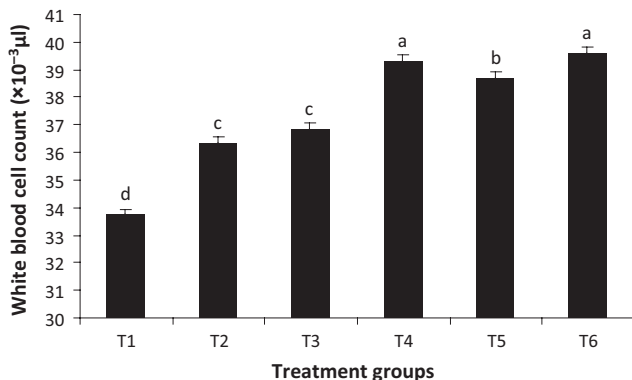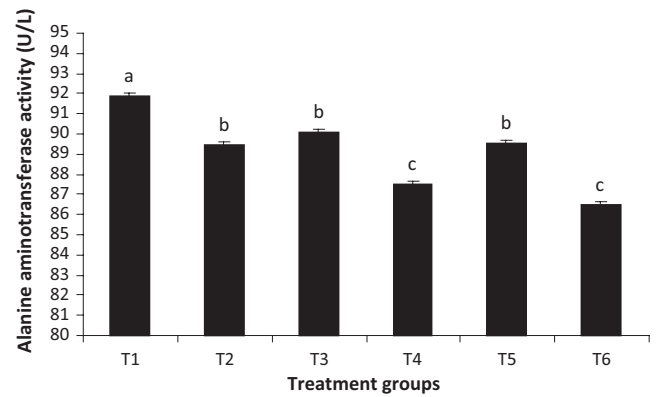

FIGURE 2 Red blood cell count of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups (*p* < 0.05)
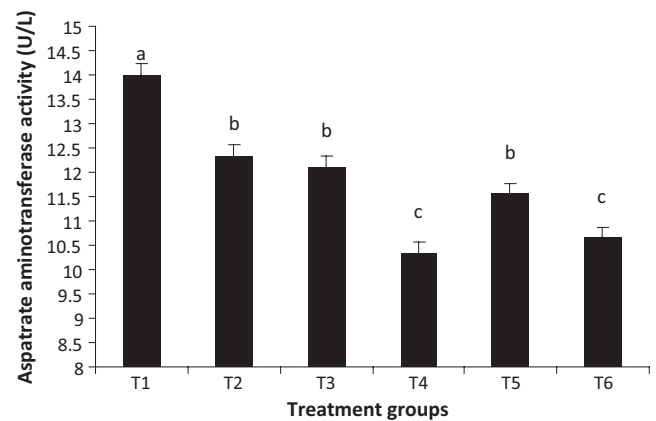

FIGURE 3 White blood cell count of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups (*p* < 0.05)

FIGURE 4 Alanine aminotransferase of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups (*p* < 0.05)

FIGURE 5 Aspartate aminotransferase of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups (*p* < 0.05)

4 | DISCUSSION

Our study has shown, for the first time, the synergistic effect of malic acid and the probiotic *B. subtilis*, which produced the best results in all the parameters analysed (growth, body composition, intestine pH, bacteria count, blood parameters, liver enzymes).

The largest increases in body weight and feed utilization were observed in groups that received diet with a mixture of malic acid and the probiotic; however, the addition of the acid or *B. subtilis* alone also significantly improved the growth performance of the fish. That supplementing diets with organic acid or salts stimulates growth has been reported previously in Nile tilapia (Hassaan, Soltan, Agouz, & Badr, 2013; Hassaan, Soltan, & Ghonemy, 2014; Hassaan, Wafa, Soltan, Goda, & Mogheth, 2014; Meshrf, 2014; Reda, Mahmoud, Selim, & El-Araby, 2016) and rainbow trout, *Oncorhynchus mykiss* (De Wet, 2005). Increased growth in fish fed diets supplemented with a probiotic has also been observed in trout (Gatesoupe, 1991), rohu, *Labeo rohita* (Bairagi, Sarkar Ghosh, Sen, & Ray, 2004), grass carp, *Ctenopharyngodon idella* (Wang, 2011) and tilapia (Aly, Ahmed, Ghareeb, & Mohamed,
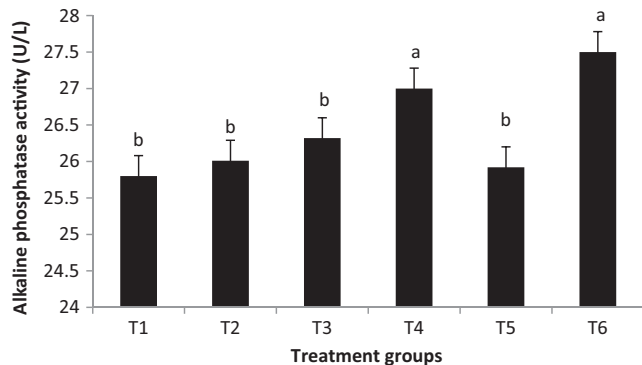
FIGURE 6 Alkaline phosphatase of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups ($p < 0.05$)
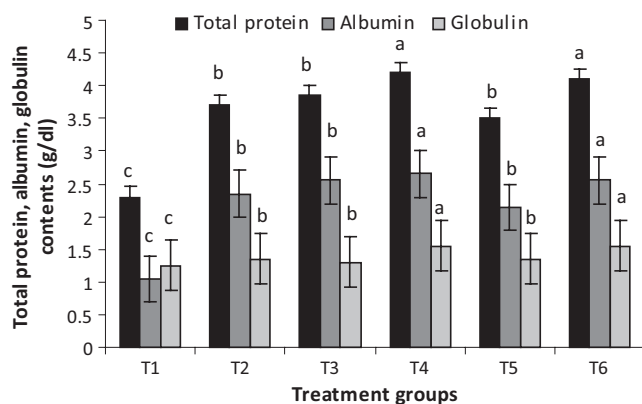

FIGURE 7 Total protein, albumin and globulin of Nile tilapia (*O. niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days. Different letters indicate significant differences between groups ($p < 0.05$)

2008; Hassaan, Soltan et al., 2014; Hassaan, Wafa et al., 2014). 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 (El-Haroun, Goda, & Chowdhury, 2006; Mohapatra et al., 2012). Moreover, probiotics may lead to improved gut microvilli morphology and consequently result in the enhanced absorption of nutrients (Zhang et al., 2015).

The modes of action of organic acids appear to be different. Several hypotheses have been suggested to explain the effects of organic acids on enhancing nutrient utilization in terrestrial livestock, which include the following: lowering gastric pH leading to increased pepsin activation; lowering intestinal pH that might increase mineral solubilization resulting in increased mineral absorption; or as a result of decreased intestinal microbial activity that might otherwise utilize nutrients now spared for the host animal (De Wet, 2005).

The reduction in total bacterial count stems primarily from the diffusion of organic acids into the bacterial cells, the dissociation of these acids inside the cells, the consequent decrease in cytoplasmic pH and eventual cell death as described by Booth and Stratford (2003). We hypothesize that in our experiment, the lower pH and the addition of *B. subtitles* could have led to a reduction in total count bacteria in the Nile tilapia gut. Explaining the phenomenon of qualitative and quantitative changes in the ecosystem of fish intestinal microflora requires further clarification and deeper microbiological analysis.

Obtaining such promising results could have stemmed from *B. subtilis* successfully colonizing fish gastrointestinal tracts. However, malic acid could have supported the activity of the probiotic by either inhibiting or stimulating the growth of intestinal bacteria. For example, malic acid was shown to have antibacterial activity against strains of the pathogenic bacteria *Enterobacter sakazakii* (Back, Jin, & Lee, 2009). The effect of malic acid on beneficial probiotic bacteria is not yet clear. Salou et al. (1991) observed that the addition of this acid to the

TABLE 6 Body composition of Nile tilapia (*Oreochromis niloticus*) fed the diets with graded levels of dietary malic acid and *B. subtilis* after 84 days (g/kg)

T	Malic acid g	*B. subtilis* cfu/g	Proximate analysis of whole body of fish			
			Dry matter	Protein	Lipid	Ash
T1	0	0	261.00	598.30[b]	217.90[a]	134.30[b]
T2	0	1.1×10^5	258.00	595.70[b]	197.20[b]	132.20[b]
T3	5	0	259.90	588.00[bc]	172.80[c]	153.90[a]
T4	5	1.1×10^5	264.30	622.30[a]	191.20[b]	149.60[a]
T5	10	0	263.80	575.70[c]	171.50[ab]	149.30[a]
T6	10	1.1×10^5	262.70	619.70[bc]	169.90[ab]	149.90[a]
Pooled *SE*			0.45	0.41	0.48	0.55
Two-way ANOVA (*p*-value)						
Malic acid			0.007	0.009	0.003	0.002
B. subtilis			0.905	0.149	0.631	0.579
Malic acid × *B. subtilis*			0.131	0.003	0.001	0.044

Data are presented as means ± pooled standard error (*SE*). Means followed by different superscript letters in each column are significantly ($p < .05$) different.

medium resulted in faster growth of the lactic acid bacteria *Leuconosto oenos* up to 75 mM, whereas a slight inhibitory effect was observed above this value.

In our study, intestinal pH and the total number of bacteria decreased significantly in all the groups that were fed diets supplemented with either organic acid or the *B. subtilis* and organic acid blend. Similar observations were noted by Ng, Koh, Sudesh, and Siti Zahrah (2009) in the intestinal tract of red hybrid tilapia, *Oreochromis* sp. fed diets supplemented with organic acid.

In our study, body composition was affected by dietary malic acid and/or *B. subtilis*, which was particulary associated with a significant decrease in lipid content compared with the control diet. In other studies, Bagheri, Hedayati, Yavari, Alizade, and Farzanfar (2008) observed elevated protein levels in rainbow trout fed diet supplemented with *B. subtilis* and *B. licheniformis* and Azarin, Aramli, Imanpour, and Rajabpour (2015) with the same probiotic in Kutum (*Rutilus frisii kutum*) fry. They concluded that greater protein values of carcass may be due to proteins secreted by members of genus *Bacillus* (Rosovitz, Voskuil, & Chambliss, 1998). The body composition of fish mainly depends on the nutrient concentration and quality of the diet. The production of fish with more protein and less fat represents an important achievement, and it is a very desirable feature for fish farmers and consumers.

Dietary supplementation with malic acid (5 or 10 g/kg diet) and/ or probiotic significantly improved the values of Hb, Hct, RBC and WBC of tilapia. Similar findings have been reported for Indian major carp, *Labeo rohita* (Kumar, Mukherjee, Prasad, & Pal, 2006), Juvenile beluga (*Huso huso*) (Khajepour & Hosseini, 2012), Nile tilapia (Hassaan, Soltan et al., 2014; Hassaan, Wafa et al., 2014; Meshrf, 2014; Reda et al., 2016) and *Catla catla* (Renuka, Venkateshwarlu, & Naik, 2014) that received organic acid, organic salts or a probiotic. To date, there is no exact clarification on how probiotics and organic acids stimulate the blood parameters. However, it is well known that white blood cells are important components of the innate immune response that regulates immune function. Increases in the numbers of immune cells facilitate fish defences against infections (Ballarin et al., 2004; Jalali, Ahmadifar, Sudagar, & Takami, 2009). Our results also indicated that the application of malic acid and/or probiotics improves the Nile tilapia hematopoietic system, and they are safe as feed supplements.

Fish fed diets containing malic acid and/or *B. subtilis* exhibited a significant decrease in transaminases ALT and AST activity as well as improved values of total protein, albumin and globulin compared with fish fed the control diet. Another study on Nile tilapia reported the same effect of metabolic enzymes and proteinogram when the fish were fed a diet containing a blend of malic and oxalic acids at 5 g/kg diet (Meshrf, 2014). The role of probiotics in modulating metabolic enzymes has also been investigated and reviewed in a few other aquatic organisms. Soltan and El-Laithy (2008) 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 (Adawi, Ahrné, & Molin,

2001). 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 (Adawi et al., 2001; Lin & Chang, 2000). Increases in proteinogram levels are thought to be associated with a stronger innate response in fish (Jha, Pal, Sahu, Kumar, & Mukherjee, 2007). Globulin level is very often used as an indicator of immune responses and a source of antibody production. Some studies have demonstrated that probiotics and organic acids can stimulate certain aspects of the non-specific immune response such as total protein, albumin and globulin (Ferguson et al., 2010; Gómez & Balcázar, 2008; Hassaan, Soltan et al., 2014; Hassaan, Wafa et al., 2014; Rahmani & Speer, 2005). Rengpipat, Rukpratanporn, Piyatiratitivorakul, and Menasaveta (2000) demonstrated that *Bacillus* sp. provided disease protection by activating humoral and cellular immune defences 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. It is widely known that body immune response originates is the intestines, among other places. The presence of pathogenic bacteria weakens the local immune system, which is highly mobilized to fight intruders. 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 (Balcázar et al., 2006).

The most important aspect of using advantageous bacteria in aquaculture is the necessity of gaining knowledge of intestinal microbiology, preparing effectively and evaluating probiotic safety. Moreover, a better understanding of probiotic rationale, preparation and safety could be of interest to commercial (Wang, Li, & Lin, 2008) and environmentally sustainable aquaculture.

In the current study, we have demonstrated a marked reduction in total bacterial counts in the faeces of fish fed diets supplemented with malic acid. Additionally, we have demonstrated that administering probiotics and organic acids together resulted in a strong synergistic effect on all of the parameters analysed in Nile tilapia. The malic acid and *B. subtilis* mixture enhances both growth and health through its positive impact on the GI tract, liver function, blood parameters and non-specific immune responses. The mix of these substances could be used in intensive aquaculture production. The optimal dose determined in our study for Nile tilapia is 5 g of malic acid/kg and 1.1×10^5 *Bacillus subtilis* cfu/g. In subsequent studies, the authors will strive to increase their understanding of the dependence between the microbial composition of the fish gastrointestinal tract and fish immune response, including that originating in the intestines.

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