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Influence of Dietary *Bacillus subtilis* and Acidifiers on Nile Tilapia's Growth Performance, Immune Functions, and Antioxidant Capacity

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Abstract

The use of natural feed additives presents an eco-friendly strategy to improve the performance and well-being of Nile Tilapia. A total of 180 juvenile Nile tilapia was randomly assigned to three treatment groups, each with three replicates (20 fish per replicate), and reared in glass aquaria. The groups were as follows: (1) control group, fed the basal diet; (2) probiotic group, fed the basal diet supplemented with 2 g/kg of Bacillus subtilis (2×10^{11} CFU/kg); and (3) acidifier group, fed the basal diet supplemented with 5 g/kg of an organic acid blend. The organic acid and Bacillus subtilis supplementation boosted fingerlings' growth outcomes, protein retention and specific growth rate with superior nutrient digestibility unlike control group. In addition, fish fed the Bacillus subtilis fed groups displayed significant hematological benefits, such as lower serum cholesterol levels and increased high-density lipoprotein cholesterol (HDL-C) levels, distinguishing them from the other treatment groups. The administration of probiotics and acidifiers effectively strengthened the fish's antioxidant system, reducing oxidative stress markers such as malondialdehyde (MDA) and enhancing enzymatic antioxidants including total antioxidant capacity (TAOC) besides superoxide dismutase (SOD) activity. Furthermore, the inclusion of probiotics and organic acidifiers enhanced the immune response, as reflected by increased serum IgM, lysozyme activity, phagocytic activity, and the upregulation of *mucin-2* and *interleukin-10* gene expression in the intestine. Concerning the intestinal microbiota, the *Bacillus subtilis* fortified group exhibited an increased lactobacilli DNA concentration unlike a decreased lactobacilli DNA concentration of Enterobacteriaceae. Overall, *Bacillus* and acidifier inclusion positively influenced performance, immune status, and gut bacterial composition, contributing to the economic viability of tilapia farming.

Introduction

Nile tilapia (*Oreochromis niloticus*) is considered the second most commercially important freshwater fish species cultured globally, following carp fish , recognized for its fast growth rate, efficient yield, and robust resistance to disease (Munguti et al., 2022). Fish culture is one of the fastest-growing food production industries worldwide, providing approximately 50% of the protein consumed by humans (Dawood, Noreldin, & Sewilam, 2021). The recent rapid expansion of the aquaculture industry has led to an increased demand for aquafeed and various feed additives used in the nutrition and health management of aquatic animals (Ruenkoed et al., 2023). Furthermore, incorporating such feed additives into diets can minimize the need for chemical treatments and medications to control infections, helping to safeguard human health and the environment from their negative impacts. Furthermore, probiotics help stimulate the proliferation of beneficial gut bacteria in fish, which supports better growth and overall health (Adel & Dawood, 2021). Moreover, probiotics are valuable feed additives in aquaculture, demonstrating significant benefits in enhancing disease resistance, promoting growth, boosting immune responses, and improving overall health of cultured fish. Extensive research has shown that Bacillus probiotics can effectively hinder the development of harmful microorganisms, including both pathogenic bacteria and toxic phytoplankton, thereby improving the aquatic environment for fish culture (A. Nayak et al., 2023). These beneficial microbes support host health by

fostering the proliferation of advantageous intestinal flora, limiting the presence of disease-inducing pathogens, and preserving a stable gut microbiome (P. Zhou, Chen, Patil, & Dong, 2024). Bacillus species produce a wide array of functional metabolites and bioactive molecules within the gastrointestinal tract, including antimicrobial peptides like Protein E2, lipopeptides, and bacteriocins, as well as hydrolytic enzymes like protease, cellulase, chitinase, , glucanase and alpha-amylase (Kovács, 2019; Yan, Zheng, Chen, Han, & Han, 2013). Bacillus species are regarded as highly promising probiotic agents in aquaculture, largely due to their thermal stability, which enables them to endure the pelletization process. Once ingested, they successfully colonize the fish gastrointestinal tract and secrete various vital digestive enzymes (Tang et al., 2019). In comparison to humans, intestinal acid secretion is generally lower in most fish species (Fabay, Serrano Jr, Alejos, & Fabay, 2022). Acidifiers can reduce gastrointestinal pH, enhance phytate breakdown, suppress harmful gut microbes, accelerate gut transit time, increase pepsin enzyme activity, and improve mineral absorption, overall nutrient digestibility and nitrogen retention (Abdel-Tawwab, Khattaby, & Monier, 2019). Acidifiers have been shown to support protein digestion and enhance the absorption of essential minerals such as phosphorus, calcium, zinc magnesium elements that are crucial for various metabolic functions (Mohtashemipour, Mohammadian, Mesbah, Rezaie, & Mozanzadeh, 2023). Specifically, short-chain organic acids have demonstrated the ability to reshape gut microbial communities, increase the efficacy of antibiotics against common aquaculture pathogens, and lower the microbial burden within the intestines post-consumption (Lathakumari, Vajravelu, Satheesan, Ravi, & Thulukanam, 2024). Supplementing fish diets with combinations like propionic and formic acids, and calcium propionate showed notable improvements in the growth and performance of Nile tilapia (Ali, El-Sayed, Eissa, & Hanafi, 2018). Therefore, both bacillus species and acidifiers are considered effective dietary additives in fish culturing (Salehi, Bagheri, Sotoudeh, Ghasemi, & Mozanzadeh, 2023). The current study designed for investigating the effects of dietary inclusion of probiotics derived from bioactive compounds either individually or in combination with organic acids including propionic, formic, citric, and lactic acids on growth performance, immune responses, biochemical parameters, and gene expression in Oreochromis niloticus.

Materials and Methods

Fish Collection and Maintenance

The procedures in this study adhered to the ethical standards for fish reception and care set by the Faculty of Veterinary Medicine, Zagazig University, Egypt (ZU-IACUC/2/F/254/2024).

One hundred eighty healthy Nile tilapia (*Oreochromis niloticus*), averaging 10.70±0.9 g, were purchased from a private farm in Kafr El-Sheikh, Egypt. Fish was acclimated for two weeks in 75-liter plastic aquaria and fed a commercial basal diet containing 30% protein (El Dakahlia Company, Menoufia Governorate).

Preparation and Composition of Experimental Diets

The formulation of the experimental diets followed the nutritional guidelines of (NRC, 1993), Table 1. All feed ingredients were finely ground, thoroughly mixed, and processed into afloat pellets with a diameter of approximately 1 mm using a pelletizer. Once dried by air, the pellets were crushed to appropriate sizes and stored at 20°C til the feeding trial began. Representative feed samples from each dietary treatment were collected and subjected to proximate composition analysis according to the standard procedures described by (AOAC, 2000**)**.

Procedure of Culture

The experimental fingerlings were unbased manner assigned to three experimental treatments, each with three replicates containing 20 fish per replicate, housed in nine 75-liter plastic aquaria filled with continuously aerated dechlorinated tap water using a small air compressor. The aquaria were cleaned daily, with 25% of the water replaced each day. The water temperature was retained between 25 and 27°C. Dissolved oxygen levels stayed at 6.27±0.4 mg/L, measured by DO meter (YSI Pro ODO, Yellow Spring Instruments, USA). The pH of 7.15±0.2 was maintained pH sensor (INESA Device, Shanghai, China). Weekly measurements of water quality parameters included nitrite (0.019±0.004 mg/L), ammonium (0.13±0.025 mg/L), the hardness of water was 9.43±0.20 mg/L, nitrate (0.15±0.05 mg/L), and salinity (0.34±0.05 g/L), following the procedures of (APHA, 2005).

Nutritional Assessment Study

Throughout the 12-week experimental period, fish were provided feed three times per day at scheduled intervals of 8.5:00, 12:00, and 15:00 hours. The second treatment group received the basal diet enriched with 2 g/kg of Aqua-Grow, a probiotic product containing Bacillus subtilis at a concentration of 2×10^{11} CFU/kg, supplied by Canal Aqua Cure Company (Port Said, Egypt). The third group received the basal diet supplemented with 5 g/kg of Aqua-FR, a blend containing for each kg; 50 g each of citric, propionic, formic, and lactic acids (Company of Canal Aqua Cure, Port Said, Egypt). On a biweekly schedule, all fish from each aquarium were weighed, and feed amounts were adjusted based on changes in body weight throughout the experiment, following standard procedures described by (AOAC, 2000).

Growth Performance Assessment

Growth performance was evaluated by measuring key parameters, including weight gain, specific growth rate (SGR), feed conversion ratio (FCR), protein productive value (PPV) and protein retention efficiency (PRE) was estimated according to (Ibrahim et al., 2022).

Hematological Specimens

Upon completion of the 12-week feeding experiment, fish (n=10) were randomly selected from each replicate for blood collection. To minimize stress during sampling, fish were anesthetized via immersion in a 0.1 ppm solution of Ethyl 3-aminobenzoate methane sulfonate (product No. E10521, Sigma-Aldrich Chemie GmbH, Eschenstrasse, Germany). Blood samples were drawn from the caudal blood vessels of each fish (n = 10) without using anticoagulants and collected into plastic Eppendorf tubes. The Sera were then separated by centrifuging the blood samples at 3000 × g for 15 minutes. (Aly, Ahmed, Ghareeb, & Mohamed, 2008). After collection, serum samples were promptly stored at –20°C until used for immunological and biochemical analyses. The remaining blood was transferred into EDTA-coated tubes for hematological examination.

Specimen Sampling

Following blood collection, intestinal tissue samples were harvested from each fish (n=10 per replicate). The samples were gently washed with physiological saline, immediately frozen in liquid nitrogen at –180°C, and stored at –80°C until further analysis of target gene expression, as per standard methodologies (AOAC, 2000), Whole body of experimental Nile tilapia samples were analyzed for CF, NFE, EE, ash, and CP.

Evaluation of Digestive Efficiency

After the feeding trial, fish from each treatment group (18 fish per group, with 6 fish per replicate) underwent a 15-day digestibility test. The experimental diets were supplemented with 5 g/kg chromic oxide as an external marker. The dry matter (DM), crude protein (CP), ether extract (EE), and crude fiber (CF) content of the diets and fecal samples were analyzed according to the methods described by (AOAC, 2000). Fecal samples were collected 5 to 6 hours after feeding using a fine wire mesh net, freeze-dried, and accumulated over the trial period. The levels of chromic oxide in feces and feed were examined corresponding to the technique defined by (Petry & Rapp, 1970).

Dry Matter Apparent Digestibility Coefficient Each Diet Was Estimated

Digestion coefficient= (indicator percentage in faeces) — (indicator percentage in feed) / indicator percentage in faeces) x 100. (NRC, 1993)

Table 1. Formulated Feed Composition for Nile Tilapia used in experiment

Feeding Ingredient	Inclusion levels %
soybeans meal	32.50
Yellow maize	26.00
Corn gluten meal	8.00
wheat screenings	10.00
meat meal	7.00
rice bran	6.00
Fish meal	1.00
sunflower	1.70
soya oil	3.00
oil	3.00
lysine	0.20
methionine	0.35
Premix*	0.25
Sodium chloride	0.50
$CaCo_3$	0.50
Calculated composition	
Dry matter, %	88.44
Ether extract, %	10.06
Crude protien, %	30.40
Crude fiber, %	4.77
Ash, %	5.12
Nitrogen free extract, %	38.10
Digestible energy, Kcal/ kg die	3093.36

* Each kilogram of the premix included 50,000 mg of vitamin E, 10,000 mg of vitamin K_3, and 5,000 mg of folic acid, along with 100 mg of biotin and 50,000 mg of pantothenic acid. It also contained a substantial amount of niacin at 100,000 mg. The B-complex group was well represented, with 20,000 mg each of vitamins B_1 (thiamine), B_2 (riboflavin), and B_6, and 20 mg of vitamin B_{12}. The formulation provided high doses of fat-soluble vitamins as well, including 5,500,000 IU of vitamin A and 1,000,000 IU of vitamin D_3. Mineral components included 15,000 mg of zinc in the form of zinc methionine, 30,000 mg of iron from iron sulfate, and 10,000 mg of manganese sulfate. The premix also offered 5,000 mg of iodine from potassium iodide and 5,000 mg of copper from copper sulfate. Cobalt and selenium were present in trace amounts as 200 mg of cobalt sulfate and 300 mg of sodium selenite, respectively. Additionally, magnesium was supplied at 40,000 mg via magnesium oxide. To standardize the mixture and ensure even distribution, calcium carbonate was used as a carrier to bring the total weight to 1,000 grams per kilogram of premix.

Blood Profile Assessment

The assessment of hematological parameters, including concentration of hemoglobin (Hb), total white blood cell (WBC) count, hematocrit (Hct)and red blood cell (RBC) count, was conducted utilizing the Hema Screen 18 automated hematology analyzer (Hospitex Diagnostics, Sesto Fiorentino, Italy). All measurements were performed in accordance with the manufacturer's standardized operating procedures to ensure accuracy and consistency. To assess disparity of leukocytes, including lymphocytes and heterophils, smears from peripheral blood were generated, followed by fixation using absolute methanol. The fixed slides were then stained employing Giemsa stain to facilitate morphological examination of blood cells (Lewis, Bain, & Bates, 2006). Serum levels of total protein, albumin, total cholesterol (TC), triacylglycerol (TAG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), and alanine aminotransferase (ALT) were measured using a commercial diagnostic kit (SPINREACT, Santa Coloma, E-17176, Spain), following the manufacturer's instructions.

Immunity and Antioxidant Assays

Total antioxidant capacity (TAOC) was assessed based on the protocol established by (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993), which evaluates the antioxidant potential of serum samples. Serum lysozyme activity was quantified through an agarose gel lysis assay, conducted in accordance with the method originally described by (Schultz, 1987). Malondialdehyde (MDA) content were determined as followed via Chanda and Dave (2009). The activity of serum superoxide dismutase (SOD) was determined according to the methodology described by (Cowell, Dowman, Lewis, Pirzad, & Watkins, 1994) Author Name or Reference], enabling the evaluation of enzymatic defense against superoxide radicals. Phagocytic activity (PP) was evaluated using an indirect assay method, as outlined by Martins et al. (2008), which quantifies the ability of phagocytes to engulf and internalize foreign particles (Martins et al., 2008). The concentration of immunoglobulin M (IgM) was quantified using a commercially available fish-specific ELISA kit (Cusabio Biotech Co., Ltd., USA), following the protocol provided by the manufacturer.

Quantitative Analysis of Genes

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to evaluate the expression levels of specific genes in intestinal tissue, with a focus on interleukin-10 (IL-10) and mucin-2 (MUC-2) as target transcripts. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer's protocol, and RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Real-time PCR was performed using the Rotor-Gene Q2plex technique (Qiagen Inc., Valencia, CA, USA). The thermal cycling conditions included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 2 minutes. All reactions were run in triplicate, and relative gene expression levels were calculated using the $^{2^{-\Delta\Delta}}$Ct method. β-actin was used as the internal control for normalization of target gene expression (Livak & Schmittgen, 2001). Sequences of the primers utilized for the RT-qPCR assays are detailed in Table 2.

Quantification of Bacterial Load Via DNA Copy Number Determination

DNA copy number quantification of Enterobacteriaceae and Lactobacillus spp. was performed on intestinal tissue specimens using quantitative PCR techniques. Bacterial genomic DNA was isolated from samples employing the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the protocol provided by the manufacturer. The quality and concentration of the extracted DNA were assessed spectrophotometrically using a Spectrostar NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantification of bacterial DNA

Table 2. The sequence of Primers employed for real-time PCR assessment

Gene	Sequence	Accession No/ Reference
Mucin-2	F- AAAGACTCATGTGCCTGCGA R- TCCAGGGTGGGTATCGGATT	XM_025902524.1
Interleukin -10	F-CTGCTAGATCAGTCCGTCGAA R-GCAGAACCGTGTCCAGGTAA	XM_013269189.3/
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	(Bartosch, Fite, Macfarlane, & McMurdo, 2004)
Lactobacillus species	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	(Walter et al., 2001)
β-actin	F- AGCAAGCAGGAGTACGATGAG R- TGTGTGGTGTGTGGTTGTTTTG	XM_003443127.5/
TATA-binding protein	F-GTCCACGGTGAATCTTGGTT R-GCGCAGTAGTACGTGGTTCTC	Acc:8484

was performed using a Stratagene MX3005P real-time PCR system in 96-well polypropylene plates. The specific primer sequences used are listed in Table 2. A standard curve was generated to quantify bacterial DNA copy numbers. DNA extracted from pure bacterial cultures was subjected to 10-fold serial dilutions and analyzed in the same RT-PCR run. Ct (cycle threshold) values corresponding to each dilution were used to construct standard curves. DNA copy numbers in the samples were calculated by interpolating sample Ct values onto the standard curves and expressed as the logarithm base 10 of colony-forming units (CFU) per gram of sample.

Statistical Assessment

Study findings are presented as mean ± standard deviation (SD). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 22.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine significant differences among groups, with significance set at P<0.05.

Results

Evaluation of Growth Performance Indices

Table 3 presents the data on growth performance. Fish fed diets supplemented with either probiotics or organic acids displayed significantly (P≤0.05) greeter final body weight (FBW), body weight gain %, protein efficiency ratio (PER), and SGR in comparison with the control one. Additionally, feed conversion ratio (FCR) showed a significant improvement (P≤0.05) in mutually probiotic and acidifier groups relative to the control. Among all treatments, probiotic-supplemented diet yielded the utmost protein production. However, no significant differences (P≥0.05) were observed in the condition factor (K) between the three experimental groups.

Assessment of the Body Chemical Composition Indicators

Table 4 illustrates the impact of dietary supplementation of probiotics and organic acids on the body composition of fish. The inclusion of probiotics and organic acids in the diet resulted in a statistically significant increase (P≤0.05) in dry matter content when correlated to the control one. Furthermore, crude protein levels were significantly elevated (P≤0.05) in both supplemented groups, indicating improved protein retention in fish muscle. The group receiving probiotics exhibited a marked rise (P≤0.05) in ether extract levels, suggesting greater lipid accumulation relative to the control and organic acid-treated fish. Conversely, ash content was significantly reduced (P≤0.05) in fish fed both additives relative to the regulator group. Overall, these results indicate that together probiotic and acidifiers supplementation may meaningfully alter the chemical composition of Nile tilapia, influencing parameters such as moisture, protein, fat, and mineral content.

Table 3. Role of supplemental B. subtilis and Organic acids on Nile tilapia's growth performance

Parameter	Experimental diets				
	Control	Organic acids	*B. subtilis*	P-value	SEM
Initial body weight, g	10.73	10.74	10.69	0.95	
Final body weight, g	31.32^{c}	37.22^{b}	39.15^{a}	< 0.01	
Feed conversion ratio	2.05^{a}	1.89^{b}	1.78^{c}	0.003	
Feed intake, g	42.31^{b}	50.22^{a}	50.89^{a}	0.003	
Speific growth rate %	1.19^{c}	1.39^{b}	1.43^{a}	< 0.01	
Body gain %	192.05^{c}	247.03^{b}	266.41^{a}	0.002	
Protein Production value	0.22^{b}	0.31^{a}	0.31^{a}	0.01	
Body gain, g	20.57^{c}	26.52^{b}	28.45^{a}	0.005	
Condition Factor	2.28	2.22	2.28	< 0.70	
Protein retention	0.04^{b}	0.05^{a}	0.07^{a}	< 0.01	
Protein efficiency ratio	1.61^{c}	1.74^{b}	1.86^{a}	< 0.01	

[a-c] Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

Table 4. Role of supplemental B. subtilis and Organic acids on Nile tilapia's body composition

Paramter	Experimental diets					
	At the beginning of experiment	Control	Organic acids	*B. subtilis*	P-value	SEM
Dry matter	24.13^{b}	23.97^{b}	28.33^{a}	28.26^{a}	< 0.001	
Ether extract	17.10^{c}	18.18^{b}	13.37^{d}	21.40^{a}	0.02	
Crude protein	57.21^{b}	57.50^{b}	58.57^{a}	57.68^{b}	< 0.01	
Ash	16.98^{a}	15.40^{b}	12.22^{d}	14.02^{c}	< 0.01	

[a-c] Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

Nutrients Digestibility

As presented in Table 5, fish that received the diet enriched with probiotics demonstrated a significant improvement (P≤0.05) in the digestibility of DM, CP, lipids, and CF, as reflected by the elevated digestion coefficient values. Although the group fed with organic acid supplementation also showed enhanced digestibility relative to the control group, the extent of improvement was less substantial compared to the probiotic-fed fish. These findings highlight the superior effectiveness of probiotics in promoting nutrient assimilation in Nile tilapia.

Measurement of Hematological Parameters

According to the data in Table 6, fish receiving diets supplemented with either probiotics or organic acids exhibited a significant increase (P≤0.05) in hemoglobin concentration, hematocrit levels, and red blood cell (RBC) counts correlated to the control one. However, the percentage of lymphocytes and heterophils in the probiotic-fed group remained statistically unchanged (P≥0.05) when related to the control one, indicating no notable outcome of the probiotic on these specific immune cell populations.

Serum Biochemical Parameters

Table 7 shows that fish fed the probiotic-enriched diet exhibited a significant reduction (P≤0.05) in serum total cholesterol relative to both organic acid and control groups. Additionally, this group recorded a notable increase (P≤0.05) in high-density lipoprotein (HDL) levels relative to the control. Low-density lipoprotein (LDL) concentrations were also significantly lower (P≤0.05) in the probiotic group compared to the control. However, no significant variances (P≥0.05) were detected among the three groups regarding triglycerides (TG), total protein (TP), albumin, or globulin levels.

Immunological and Antioxidant Responses

As shown in Table 8, fish receiving diets supplemented with probiotics or organic acids exhibited significantly elevated (P≤0.05) levels of immunoglobulin M (IgM) and enhanced lysozyme activity compared to the control group. Both treatment groups also demonstrated a significant increase (P≤0.05) in phagocytic function, reflected by higher phagocytic percentages and index. Additionally, malondialdehyde (MDA) levels were significantly reduced (P≤0.05) in the probiotic and organic acid groups, indicating lower lipid peroxidation. Superoxide dismutase (SOD) activity was notably elevated (P≤0.05) in the probiotic-fed fish. Although both supplemented groups showed an upward trend in total antioxidant capacity (TAC), while the increase was not statistically different (P≥0.05).

Intestinal Microbiota and Gene Expression

As illustrated in Figure 1, fish receiving the probiotic-supplemented diet exhibited a significant decrease in Enterobacteriaceae counts, alongside a marked elevation in lactobacilli populations, compared to other groups. Figure 2 further demonstrates that the expression levels of *mucin-2* besides *IL-10* mRNA were significantly increased (P<0.05) in the probiotic group, with the acidifiers group also showing a moderate increase, whereas the control group exhibited no such enhancement.

Discussion

The use of probiotics and organic acids or their salts has become increasingly common in aquaculture due to their benefits in enhancing nutrient absorption,

Table 5. Role of supplemental B. subtilis and Organic acids on Nile tilapia's on digestibility % of nutrients

Paramter	Experimental diets				
	Control	Organic acids	*B. subtilis*	P -value	SEM
Dry matter, %	91.88^{c}	92.36^{b}	94.32^{a}	0.005	
Lipids%	97.95^{c}	99.47^{b}	99.74^{a}	<0.01	
Crude protein, %	94.52^{c}	95.35^{b}	96.47^{a}	0.01	
Crude fiber, %	65.21^{c}	69.95^{b}	77.54^{a}	0.01	

$^{a-c}$ Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

Table 6. Role of supplemental B. subtilis and Organic acids on Nile tilapia's blood parameter

Parameter	Experimental diets				
	Control	Organic acids	probiotics	P -value	SEM
Heterophil %	40.00	41.69	41.05	0.099	
Hemoglobin (g/dl)	10.14^{c}	12.34^{a}	12.32^{b}	0.045	
Lymphocytes %	33.34	31.68	34.68	0.166	
RBCs (10^{6}/µL)	1.45^{b}	1.79^{a}	1.75^{a}	0.042	
Hematocrite	15.71^{b}	23.87^{a}	24.03^{a}	0.012	

$^{a-c}$ Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

Table 7. Role of supplemental B. subtilis and Organic acids on Nile tilapia's biochemistry

	Experimental diet				
Paramter	Control	Organic acids blend	*B. subtilis*	P -value	SEM
Total protein	3.52	3.59	3.25	0.386	
High density lipoproteins (mg/dl)	68.64^{b}	85.03^{a}	72.33^{ab}	0.033	
Triglycerides (mg/dl)	120.16^{a}	104.68^{a}	101.17^{a}	0.327	
Very low density lipoproteins (mg/dl)	24.04	20.94	20.23	0.327	
Albumin(mg/dl)	1.83	1.74	1.73	0.776	
Globulin(mg/dl)	1.70	1.89	1.52	0.124	
Total cholesterol (mg/dl)	125.16^{a}	124.36^{a}	107.17^{b}	0.007	
low density lipoproteins (mg/dl)	32.44^{a}	18.43^{b}	14.62^{b}	0. 008	

$^{a-c}$ Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

Table 8. Role of supplemental B. subtilis and Organic acids on Nile tilapia's antioxidant and immunological parameters indices

		Experimental diet				
Paramter		Control	Organic acids	*B. subtilis*	P -value	SEM
Lysozyme activity Unit / ml		66.63^{c}	86.09^{a}	97.38^{b}	0.049	
IgM(µg/ml)		4.44^{b}	5.17^{a}	5.06^{a}	0.041	
Phagocytic activity	Phagocytic %	25.30^{a}	25.35^{a}	26.35^{a}	0.005	
	Phagocytic index	1.04^{b}	1.19^{a}	1.18^{a}	<0.004	
Antioxidant activity	Superoxide dismutase (U/L)	72.06^{a}	83.17^{b}	120.69^{a}	0.007	
	Malondialdehyde (nmol/L)	118.31^{a}	109.96^{b}	112.38^{b}	0.019	
	Total antioxidants capacity (µmol/L)	272.63^{b}	316.34^{a}	322.07^{a}	0.049	

$^{a-c}$ Means inside the similar row with dissimilar superiors are considerably altered (P<0.05).

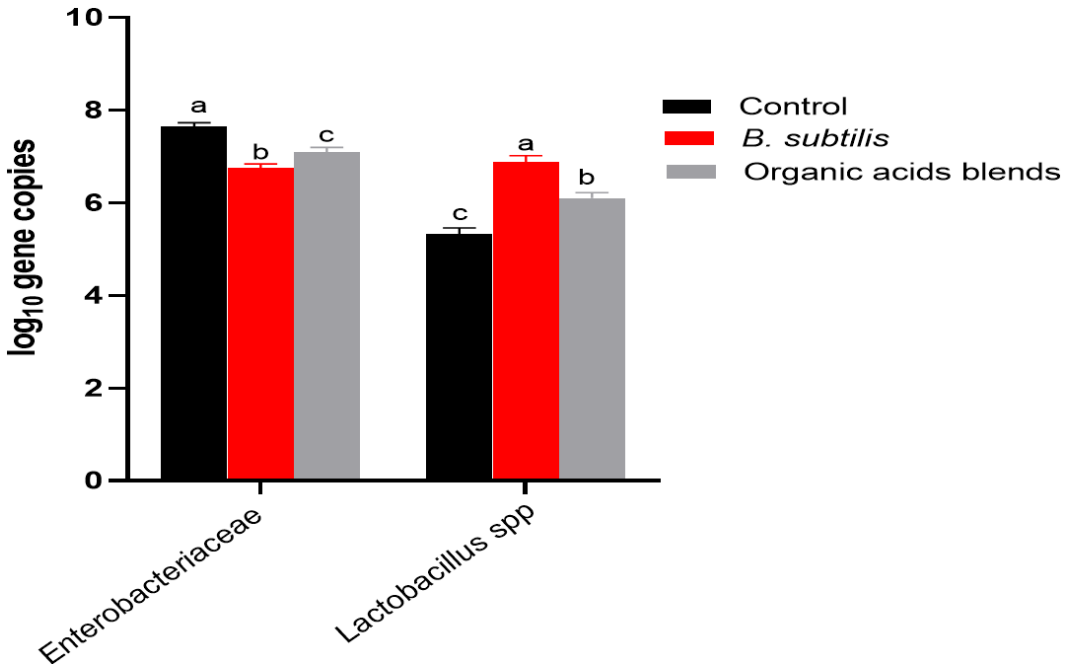

Figure 1. Influence of the tested dietary treatments on the number of intestinal bacterial DNA copies (expressed as $\log_{10}$ cfu/g) in Nile tilapia. Data are presented as mean values±standard error (SE). Different superscript letters within a row indicate statistically significant differences (P<0.05).

strengthening immune defenses, minimizing disease incidence, and improving growth and endurance rates (Thakur et al., 2025). In this study, dietary inclusion of either 2 g/kg of probiotics bacteria or 5 g/kg of an organic acid blend significantly improved the growth, feed conversion efficiency, and protein utilization in Nile tilapia when compared to the un-supplemented control group. These findings align with those of Lukkana, Jantrakajorn, and Wongtavatchai (2016), who demonstrated that *Bacillus* species contribute positively to growth and nutrient assimilation in Nile tilapia. Similarly, El-Son et al. (2022) observed that probiotics may enhance digestive enzyme activity and thereby support growth potential in fish. A study by (Agouz, Soltan, & Meshrf, 2015) demonstrated that incorporating organic salts such as calcium lactate with calcium propionate into the diet of *Oreochromis niloticus* significantly enhanced body weight gain and feed efficiency, which were closely associated with improved growth performance metrics. Furthermore, these additives contributed to higher apparent digestibility of nutrients and minerals, suggesting more efficient protein utilization. This optimization of nutrient use could lead to reduced feed requirements and, consequently, lower production costs. Supporting this, Fabay et al. (2022) noted that dietary acidifiers improve pepsin activity and mineral absorption, ultimately enhancing both growth and feed efficiency. In agreement, Reda et al. (2016) reported that supplementing Nile tilapia diets with a combination of formic - propionic acids, with calcium propionate at levels of 0.1% and 0.2% improved fish health, growth rates, and feed conversion. Probiotics play a crucial role in improving intestinal health by fostering the proliferation of beneficial microbes and suppressing pathogenic ones (Liu et al., 2017). When the digestive tract is functioning well, it enhances the absorption and breakdown of nutrients, which translates into better growth metrics like weight gain and specific growth rate (Iwashita, Nakandakare, Terhune, Wood, & Ranzani-Paiva, 2015). Certain Bacillus species have the ability to establish themselves in the intestinal lining of fish, where they contribute to organic acid production and stimulate digestive enzyme activity. This activity boosts the degradation of dietary components and allows nutrients to be more easily absorbed. As a result, fish experience improved development and better gut morphology (Addo et al., 2017), Furthermore, probiotics are capable of producing enzymes that assist in nutrient digestion (Balcázar et al., 2006), making these nutrients more accessible, ultimately leading to increased weight gain, feed intake, protein efficiency, and feed conversion (Dawood & Koshio, 2016). The current findings suggest that organic acids have the potential to suppress harmful Enterobacteriaceae in the gut. These compounds contribute to lowering intestinal pH levels (Elala & Ragaa, 2015), enhancing digestive enzyme function, and inhibiting the growth of pathogenic bacteria (Koh, Romano, Zahrah, & Ng, 2016). By promoting nutrient uptake and improving digestibility, organic acids support better growth outcomes, albeit slightly less effectively than probiotics (Hassaan, Wafa, Soltan, Goda, & Mogheth, 2014). The use of organic acid salts in Nile tilapia diets has been linked to improved growth by fostering beneficial gut microbiota and limiting harmful microorganisms, which results in a more balanced intestinal environment and heightened enzymatic activity, ultimately enhancing feed utilization and nutrient absorption. Additionally, the current results show an increase in lactobacilli populations with organic acid supplementation, consistent with findings reported by (Agouz et al., 2015). Diets combining acidifiers with probiotics yielded superior growth performance may be due to a greater secretion of

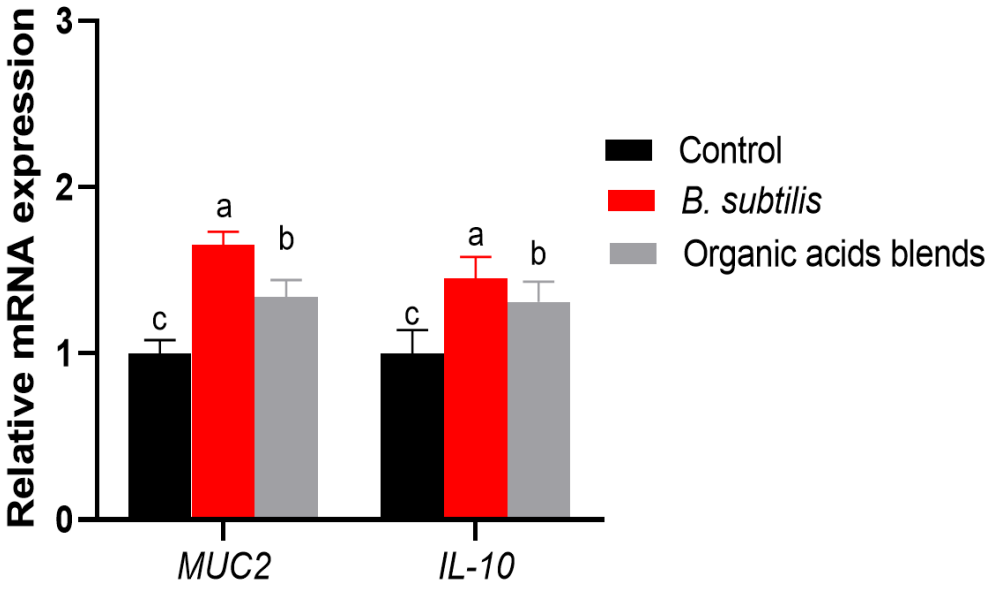

Figure 2. Impact of dietary treatments on the expression levels of intestinal mucin-2 (MUC-2) and interleukin-10 (IL-10) genes in Nile tilapia. Results are shown as mean±standard error (SE). Superscripts that differ within the same row denote significant differences (P<0.05).

digestive enzymes that aid in the breakdown and assimilation of nutrients. Beyond growth, these supplements contribute to overall health improvements by boosting metabolic activity and refining intestinal structure(El-Kady, Magouz, Mahmoud, & Abdel-Rahim, 2022; Irianto & Austin, 2002).

The inclusion of 0.2% formic and propionic acid or their salts in the diet of Nile tilapia resulted in higher retention of both protein and fat in the fish's body. This effect is likely due to improved digestion and assimilation of nutrients, as suggested by Nuez-Ortin (2011). Moreover, dietary supplementation with malic acid and/or *Bacillus subtilis* influenced the fish's body composition, with a noticeable reduction in lipid levels compared to those fed a standard diet (Hassaan, Soltan, Jarmołowicz, & Abdo, 2018). Similarly,(Reda, Mahmoud, Selim, & El-Araby, 2016) observed that a 0.2% inclusion of formic and propionic acid salts enhanced protein and fat deposition in tilapia. Our findings support these observations, showing that administering 2g/kg of probiotics or 5g/kg of a blend of organic acids significantly altered the fish's body makeup, particularly by raising dry matter and protein concentrations factors that contribute to better growth and more efficient dietary protein use. Interestingly, the group that received organic acid supplementation showed reduced body fat, suggesting a potential effect on lipid metabolism. Fish provided with diets containing organic acid salts exhibited the lowest fat levels and the highest concentrations of protein and ash in their body tissues when compared to the control group (Agouz et al., 2015). Fish that received diets enhanced with probiotics exhibited reduced fat levels and increased concentrations of crude protein and dry matter. This effect is associated with heightened activity of key digestive enzymes as protease, lipase, and amylase which are stimulated by probiotic supplementation. These enzymes play essential roles in breaking down proteins, lipids, and carbohydrates, allowing probiotics to enhance both the quantity and quality of fish production (Adorian et al., 2019). In our study, fish fed a diet containing 2g/kg of probiotics showed greeter obvious digestibility factors for DM, CP, fat, crude fiber, and ash demonstrating higher nutrient assimilation unlike both control group and those given acidifiers in their diets. Although the inclusion of 5g/kg of acidifiers improved performance relative to the control group, it was still less effective than the probiotic-enriched feed. These findings align with (Daboor, Esmael, & Lall, 2010) who reported that probiotics can significantly enhance the breakdown of proteins, carbohydrates, and lipids due to increased enzymatic activity—contributing to better growth outcomes and feed conversion. Supporting this, incorporating dietary acidifiers into fish feed has been shown to enhance the overall productivity of Nile tilapia (Elala & Ragaa, 2015). Diets fortified with 3 g/kg of probiotics demonstrated higher apparent nutrient digestibility compared to un-supplemented control diets, as observed by Ramos et al. (2017). In our findings, both probiotics and organic acid additives had a beneficial impact on the hematological profile of Nile tilapia. Similarly, the inclusion of probiotics and/or malic acid at concentrations of 5 or 10 g/kg in the diet led to notable increases in these blood parameters (Hassaan et al., 2018; Meshrf, 2014). Diets enhanced with acidifiers at 2 g/kg were particularly effective at boosting white blood cell counts, while higher doses of organic acids or their salts were necessary to stimulate lymphocyte and neutrophil production (Reda et al., 2016). Our findings demonstrated that diets supplemented with probiotics led to a marked decrease in total cholesterol levels, whereas those enriched with organic acids notably raised HDL concentrations. However, no statistically significant changes were observed in triglycerides, total protein, albumin, globulin, VLDL, or LDL across the treatment groups. Previous research by (Hassaan et al., 2018) also reported elevated levels of total protein, albumin, and globulin in fish fed diets containing *Bacillus subtilis* or malic acid. Globulin, in particular, plays a crucial role in immune function, as it contributes to antibody production and serves as an indicator of immune response. Both probiotics and organic acids have been associated with improvements in non-specific immune markers, including increases in albumin, globulin, and total protein (Ibrahim, Rahman, et al., 2024). Meanwhile, (Guo et al., 2022) noted no significant alterations in total protein, HDL, or LDL levels in fish receiving *B. subtilis* supplementation. Nonetheless, *B. subtilis* has been linked to reductions in blood lipid concentrations such as cholesterol and triglycerides, potentially enhancing the immune system of Nile tilapia (El-Son, Elshopakey, Rezk, Eldessouki, & Elbahnaswy, 2022). The observed cholesterol-lowering effect in fish given probiotic diets could be due to the microbial fermentation of undigested carbohydrates in the gut, which produces short-chain fatty acids. These fatty acids may suppress hepatic cholesterol synthesis and simultaneously promote the uptake of cholesterol from the bloodstream by the liver, ultimately reducing overall lipid levels in circulation (Elbahnaswy et al., 2024).

Additionally, our study suggests that both probiotics and organic acids may contribute to immune enhancement, either by directly activating immune mechanisms or by fostering the growth of beneficial gut microbiota that, in turn, support immune function. This was reflected in elevated levels of immunoglobulin M (IgM), increased lysozyme activity, and enhanced phagocytic response. In particular, fish fed probiotic-enriched diets showed increased expression of anti-inflammatory cytokine IL-10 and upregulation of the mucin gene—followed closely by those receiving organic acid supplementation—highlighting their immunostimulatory effects. Phagocytosis plays a vital role in fish immunity, especially given their physiological sensitivity to low temperatures (Magnadóttir, 2006). Previous research has also shown that phagocytic

responses in fish can be significantly enhanced by dietary inclusion of *Bacillus* species (S. K. Nayak, 2010). Probiotics and dietary acidifiers may contribute to enhanced immune function by suppressing the growth of harmful microorganisms within the gastrointestinal tract. The intestine plays a central role in initiating immune responses, and its effectiveness can be compromised in the presence of pathogenic bacteria. These invaders weaken local immune defenses, which are normally activated to protect the host. Supplementation with probiotics, either alone or combined with acidifiers, can strengthen this defense by promoting beneficial microbial colonization and directly inhibiting pathogen development, thereby reducing disease incidence and severity (Hassaan et al., 2018). Studies have shown that tilapia fed probiotic-enriched diets display heightened lysozyme activity and increased phagocytic response, particularly under conditions of high stocking density (Telli et al., 2014). Similarly, the use of *Bacillus subtilis* as a dietary additive has been found to significantly boost the phagocytic capacity of Nile tilapia (Aly et al., 2008). In this study, fish groups receiving *Bacillus*-based probiotics showed significantly elevated serum lysozyme activity compared to the control group. The probiotic-treated fish also demonstrated enhanced antioxidant defenses, as evidenced by higher TAOC and SOD activity, while reduced levels of MDA, suggesting that probiotics may exert or stimulate antioxidant effects. Research by (Galagarza et al., 2018) confirmed that *Bacillus subtilis* can enhance both localized and systemic immune functions in tilapia. Lysozymes were known for its broad-spectrum antibacterial action, acts as a crucial part of the innate immune response, defending the host against bacterial and viral pathogens (Li, Xu, Jin, & Li, 2015). Moreover, the highest probiotic dosage resulted in the greatest lysozyme and immunoglobulin M (IgM) concentrations in both serum and skin mucus. Moreover, (Abarike et al., 2018) also reported elevated SOD and catalase (CAT) activity in the serum of all probiotic-treated groups compared to controls. These antioxidant enzymes are essential in combating oxidative damage SOD converts reactive oxygen species (ROS) into hydrogen peroxide, which is then splatted into water and oxygen by CAT (Ibrahim, Pet, et al., 2024; Marwicka & Zięba, 2021). Similar findings were reported by (Mohamed Ibrahim Kord et al., 2021), where probiotic supplementation resulted in considerable increases in both CAT and SOD activity. Under heat stress conditions, fish that received probiotic-supplemented diets exhibited significantly higher levels of immunoglobulin M (IgM), as well as enhanced phagocytic capacity and lysozyme activity, compared to un-supplemented fish (J. Zhou et al., 2010). Furthermore, the inclusion of *Bacillus* probiotics in tilapia diets was shown to reduce MDA formation and enhance SOD and CAT function in vital organs like the liver and kidneys (Elbahnaswy et al., 2024). In all probiotic-treated groups, SOD activity was elevated and MDA levels were lower than those observed in the control group (Mohamed I Kord et al., 2022). Supplementing diets with *B. subtilis* also resulted in a significant rise in blood SOD and total antioxidant capacity (TAC) in Nile tilapia (Liu et al., 2017).

Conclusion

Supplementing the diet of Nile tilapia with 2 g/kg of Bacillus subtilis and organic (5 g/kg) acid blend can pointedly improve growth performance and nutrient digestibility. Furthermore, this dietary strategy may improve immune function, boost antioxidant defenses, positively influence body composition, and beneficially modulate intestinal microbial populations. Collectively, the inclusion of probiotics in tilapia diets suggests substantial benefits for enhancing health and productivity in aquaculture practices.

Ethical Statement

This study follows the ethics guidelines for arrival reception and fish management of the Faculty of Veterinary Medicine, Zagazig University, Egypt (the protocol was approved with ethics approval number; ZU-IACUC/2/F/254/2024).

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Author Contribution

Conceptualization, EAA, SAA, ATYK, ESH.Methodology, Software, Validation EAA, SAA, ATYK, ESH. Formal analysis, Investigation Resources EAA, SAA, ATYK, ESH.Data Curation Writing, Original Draft Writing EAA, SAA, ATYK, ESH. Review & Editing Visualization, Supervision EAA, SAA, ATYK, ESH. Methodology, Validation EAA, SAA, ATYK, ESH.

Conflict of Interest

The authors declare no conflict of interest.

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