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Research Article

Beneficial Effects of Dietary *Bacillus subtilis* and Citric Acid Supplementation on Growth Performance, Feed Efficiency and Body Composition of Juvenile Red Hybrid Tilapia (*O. Niloticus×O. mosambicus*)

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Abstract

Background and Objective: Citric acid and *Bacillus subtilis* are commonly used as dietary supplements to improve growth performance of fish. Here we investigated the effects of dietary supplementation with *Bacillus subtilis* and citric acid on growth performance, feed efficiency and body composition of juvenile red hybrid tilapia. **Materials and Methods:** Over a 90-day experimental period, growth performance, feed utilization and body composition were measured and intestinal morphometric and histopathological examinations were performed for 120 fish divided into four groups (D1-D4). D1 received the control diet. For groups D2-D4, control diet was supplemented with: D2: 10 mL kg^{-1} diet *B. subtilis*; D3:10 mL kg^{-1} *B. subtilis* and 5 g citric acid kg^{-1} diet and D4:10 mL kg^{-1} *B. subtilis* and 10 g citric acid kg^{-1} diet. **Results:** Fish fed diets containing citric acid and *B. subtilis* exhibited significant improvements in body weight gain, specific growth rate, condition factor, feed conversion rate, feed efficiency, protein efficiency ratio, protein productive value and chemical composition relative to fish fed the control diet. Fish in D3 and D4 showed incremental increases in villi length in the duodenum and jejunum. **Conclusion:** Citric acid and *B. subtilis* supplementation had a synergistic effect to improve feed efficiency, growth performance and body composition as well as morphometric characteristics of juvenile red hybrid tilapia.

Key words: *Bacillus subtilis*, citric acid, feed efficiency, growth performance, hybrid tilapia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Red hybrid tilapia is gaining popularity in many countries due to its similarity to other species such as sea bream and sea bass. Red hybrid tilapia have both high growth performance and feed conversion ratio. Fish production methods involve ecological conditions that expose fish to stresses and diseases that can lead to significant economic losses[1,2]. In Egypt, government-approved antibiotic therapies are used to defend against and treat infectious aquatic diseases. However, bacterial strains that are resistant to anti-microbial scan rapidly emerge[3] and lead to antibiotic-resistant infections in fish[4].

Probiotics are microorganisms that have beneficial actions and can help reduce reliance on antibiotics. Many commercially available probiotics are used as feed additives in fish production[5,6]. *Bacillus subtilis* is one potential probiotic that is used to formulate fish feed[7]. Dietary *B. subtilis* supplementation is safe for mammals and produces high amounts of beneficial secondary metabolites such as antibiotics, enzymes and proteins[8].

Addition of organic acids to fish diets decreases the pH in the gut, which enhances phytate hydrolysis, kills pathogens and improves mineralization and nutrient absorption. Due to its unique flavor and high buffering capacity, citric acid is a widely used organic acid for diet acidification[9]. Daily supplementation with citric acid of diets fed to tilapia (*O. niloticus*× *O.* aureus)[10] and rainbow trout (*Oncorhynchus* mykiss)[11] was associated with enhanced growth and food digestibility. In this study we explored the effect of *B. subtilis* and citric acid supplementation on growth performance, feed efficiency and body composition of juvenile red hybrid tilapia (*O. niloticus*× *O. mosambicus*).

MATERIALS AND METHODS

Preparation of *Bacillus subtilis*: *Bacillus subtilis* E5 was a gift from Dr. Mohamed Hassan (Minufiya University, Egypt). Its molecular identification was confirmed by PCR amplification and sequencing of a 1,465 bp of a consensus 16S rRNA gene as described previously by Hassan and Belal[12]. The sequencing results were assessed using DNASTAR software (Laser gene, Madison, WI, USA). *Bacillus subtilis* E5 solution was prepared as described previously by Belal *et al.*[13] Briefly, nutrient broth was inoculated with 1 mL *B. subtilis* E5 cell suspension (10° CFU mL^{-1})and incubated overnight at 30°C with 150 rpm shaking. The bacterial cell concentration was determined by plating serial dilutions of nutrient broth medium on nutrient agar medium.

Experimental design: The current study was conducted at the Fish Experimental Station, Department of Animal Production, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt. A total of 120 juvenile red hybrid tilapia fish (*O. niloticus*×*O. mosambicus*) were purchased from the Kilo 21 seawater fish hatchery (Alexandria Governorate, Egypt). After two weeks accommodation period, fish were randomly distributed into four experimental dietary groups in 4 ponds and stocked at a density 10 fish $m^{-1\ 3}$. The average initial weight of the fish was 0.2 g $fish^{-1}$. The first group (D1) received a control diet (Table1) containing about 30% crude protein and 4,700 kcal kg^{-1} gross energy. The second group (D2) received the control diet supplemented with a suspension of *B. subtilis* probiotic (10° CFU mL^{-1})at 10 mL kg^{-1} diet. The third group (D3) received the control diet with 10 mL *B. subtilis* and 5g citric acid kg^{-1} diet. The fourth (D4) group received the control diet with 10 mL *B. subtilis* and 10 g citric acid kg^{-1} diet. Fish were fed the diet for 6 days/week with two equal portions given at 9 am and 2 pm. Every two weeks during the experimental period, fish in each tank were weighed and the amount of feed was adjusted according to the new fish biomass[14]. All experimental tanks were equipped with an aeration system connected to a 5 hp air pump.

Growth performance parameters: Fish growth performance, weight gain, average body weight gain (BWG), condition factor (K) and specific growth rate (SGR) were determined according to Ricker[15] and Castell and Tiews[16] as:

$$\text{BWG} = \text{Final weight (g)-Initial weight (g)}$$

$$\text{K} = \frac{\text{Final body weight (g)}}{\text{Final body length (mL)}} \times 100$$

$$\text{SGR} = (\{\ln \text{final wt}\}\text{-}\{\ln \text{initial wt}\}/\text{days}) \times 100$$

Feed efficiency parameters: Feed intake (FI) was calculated as:

$$\text{FI} = \text{Fish weight} \times (\text{Feeding level}/100) \times \text{Number of days}$$

The feed conversion ratio (FCR) was calculated according to Tacon[17] and expressed as the proportion of dry food consumed per unit live weight gain of fish:

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

Table 1: Basal diet composition and proximate analyses

Components (%)	D1	D2	D3	D4
Fish meal	8	8	8	8
Gluten	20	20	20	20
Soybean meal	20	20	20	20
Wheat bran	7	7	7	7
Corn	35	35	35	35
*Vit and min. mix	2	2	2	2
Citric acid (g kg^{-1} diet)	0	0	5	10
Bacillus subtilis (mL kg^{-1} diet)	0	10	10	10
Linseed Oil	6	6	6	6
Carboxy methyl cellulose (CMC)	2	2	2	2
Proximate analysis of diets				
Dry matter (DM) %	91.27	90.92	90.82	90.68
Crude protein (CP) %	29.09	29.05	29.03	29.01
Ether extract (EE) %	12.99	13.38	13.00	13.22
Crude five (CF) %	4.44	4.42	4.43	4.41
Ash%	8.32	6.89	7.00	8.18
**NFE%	44.00	45.20	45.50	44.10
***GE (kcal/100 g)3	470.51	479.11	476.20	472.53
****DG (kcal/100 g)3	352.88	359.33	357.15	354.40

*Vitamin and mineral mixture kg^{-1} premix: Vitamin D: 0.8 million IU; A: 1.33 g, D3: 1.68 g, E: 6.66 g, C: 16.8 g, k: 0.8 g, B1: 0.4 g, Riboflavin: 3.75 g, B6: 2.45 g, B12: 0.33 mg, NI: 9.42 g, Pantothenic acid: 12.42 g, Folic acid: 0.68 g, Biotin: 16.6 mg, BHT: 0.5 g, Mn: 14.7 g, Zn: 31.6 g, Fe: 18.3 g, 1: 0.62 g, Selenium: 0.22 g and Co: 6.8 mg. **Calculated by differences [Nitrogen free extract (NFE) = [100-(CP+EE+ CF+Ash)]. ***Gross energy value was calculated from their chemical composition as 5.64, 9.44 and 4.11 kcal g^{-1} for protein, l ipid and NFE, respectively. ****Digestible energy, using digestible energy = gross energy×0.75. *B. subtilis* was supplemented at 10 mL kg^{-1} diet of a 10^8 CFU mL^{-1} suspension

Protein efficiency ratio (PER) was calculated according to Davies and Morris[18]:

$$\text{PER} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

Feed efficiency (FE) was calculated as:

$$\text{FE (\%)} = \frac{\text{Weight gain (g)}}{\text{Feed intake (g)}}$$

Protein productive value (PPV) was calculated according to the following equation from Marais andKissil[19]:

$$\text{PPV (\%)} = \frac{\text{PR1 PR0}}{\text{PI}} \times 100$$

where, PR1 is the total fish body protein (on a dry matter basis) at the end of the experiment.; PR0 is the total fish body protein (on a dry matter basis)at the start of the experiment and PI is the protein intake.

Histopathological and morphometric assessment of intestinal villi: Samples were collected from different regions of the intestine and fixed in 10% neutral buffered formalin. Following dehydration and clearance, the tissues were fixed in paraffin wax and sectioned into 5 µm thick serial sections that were then stained with hematoxylin and eosin[20].

Morphometric assays were performed using Image J analysis software (National Institutes of Health, USA). The villus height was measured from the tip of the villus to the villus-crypt junction and the villus width was measured from the middle of the villus. Crypt depth was measured from the crypt-villus junction to the crypt base.

Body composition analysis: At the beginning of the feeding trial, 10 fingerlings were netted, weighed and immediately stored in a deep freezer (-18°C) for chemical analysis at baseline. At the end of the experimental period, five fingerlings were used to isolate final samples for each treatment. Tissue samples from each treatment were dried at 65°C for 24 h and homogenized using a mixer. Representative samples were chemically analyzed according to AOAC[21] methods and energy contents were calculated according to the NRC[22].

Statistical analysis: SPSS software (SPSS version 13.0, IBM, Chicago, IL, USA) was used for data analysis with one way ANOVA and Scheffe's protected least significant difference test was applied with $p<0.05$.

RESULTS

Growth performance: Juvenile red hybrid tilapia fish (*O. niloticus*× *O. mosambicus*) fed diets supplemented with *B. subtilis* and citric acid showed significant ($p\leq 0.05$) increases in final body weight, total weight gain and SGR% as well as condition factors relative to the group that received the control diet (Table 2). Although addition of *B. subtilis* alone significantly improved growth performance parameters, supplementation with *B. subtilis* and citric acid at 5 g kg^{-1} or 10 g kg^{-1} diet produced the largest increases relative to the control diet. For the D4 group that received diet supplemented with *B. subtilis* and 10 g kg^{-1} citric acid, values of5.0±0.01 g, 4.8±0.87, 0.08±0.48 and 4.21±0.48 were seen for final body weight, total weight gain, average daily gain and SGR%, respectively (Table 2).

Feed utilization: Feed utilization of red hybrid tilapia (*O. niloticus*× *O. mosambicus*) fingerlings was significantly ($p \leq 0.05$) increased for groups fed diets containing *B. subtilis* alone or in combination with citric acid compared with the control diet group (Table 3). This increase was further enhanced by both *B. subtilis* and citric acid supplementation at 5 or 10 g kg^{-1} diet and no significant differences were seen between the two citric acid concentrations.

Body composition analysis: Whole body dry matter values for the supplemented groups showed significant differences ($p \leq 0.05$)compared to the D1(control)group (Table 4). Addition of *B. subtilis* alone significantly improved DM%, CP% and ash% values and further significant increases were seen for D4 that received *B. subtilis* with 10 g kg^{-1} citric acid (DM%: 30.93±0.93, CP%: 64.44±0.22 and ash %: 11.06±0.43). Meanwhile, the ether extract percentage was highest for the D1 control group (36.88%±0.11) and lowest (24.0%±0.60) for the D4 group (*B. subtilis* with 10 g kg^{-1} citric acid) (Table 4).

Morphometric assessment of intestinal villi absorptive capacity: Morphometric assessment of intestine tissue samples showed significant in creases in mucosal and villi length in the duodenum, jejunum and ileum among all treatment groups, with the exception of mucosal length in the duodenum for D2. The highest values were seen for the D4 group (*B. subtilis* and 10 g kg^{-1} diet citric acid), followed by the D3 group (*B. subtilis* and 5 g kg^{-1} diet citric acid) (Table 5).

Histopathology assessment of intestinal villi: Histopathology examination of intestine samples showed that the duodenum, jejunum and ileum of fish from the D1 control group and fish that received *B. subtilis* supplementation only (D2) had normal villi and normal mucosal lining (Fig. 1 a-f). Fish in the D3 and D4 groups showed increases in villi length, number of branches in the jejunum and normal mucosal lining in the ileum (Fig.1 g-l).

Table 2: Effect of *B. subtilis* and citric acid diet supplementation on growth performance of red hybrid Tilapia (*O. niloticus*× *O. mosambicus*)

Parameters	D1	D2	D3	D4
Initial weight (g)	0.20±0.03	0.20±0.05	0.20±0.02	0.20±0.01
Final weight (g)	2.20±0.44^{b}	4.00±1.03^{ab}	4.60±0.81^{a}	5.00±0.87^{a}
Total weight gain (g)	2.00±0.44^{b}	3.80±1.03^{ab}	4.40±0.81^{a}	4.80±0.87^{a}
Avg. daily gain (g)	0.03±0.008^{b}	0.06±0.01^{ab}	0.07±0.01^{a}	0.08±0.02^{a}
SGR (%/day)	3.46±0.17^{b}	4.04±0.25^{a}	4.14±0.39^{a}	4.21±0.48^{a}
Condition factor (K)	1.55±0.09^{b}	1.81±0.02^{a}	1.84±0.11^{a}	1.81±0.22a

D1: Control diet, D2: 10 mL diet^{-1} *B. subtilis* suspension, D3: 10 mL *B. subtilis* +5 g kg^{-1} citric acid, D4: 10 mL *B. subtilis* +10 g kg^{-1} citric acid, Different letters indicate the same raw mean significant differences. SGR: Specific growth rate

Table 3: Effect of *B. subtilis* and citric acid dietary supplementation on feed utilization of red hybrid tilapia (*O. niloticus*× *O. mosambicus*)

Parameters	D1	D2	D3	D4
Feed conversion ratio (g fish^{-1})	4.38±0.75^{a}	2.37±0.63^{b}	2.20±0.84^{b}	2.19±0.72^{b}
Feed efficiency (g fish^{-1})	0.23±0.05^{b}	0.44±0.03^{ab}	0.51±0.02^{a}	0.56±0.01^{a}
Protein retention (PR%)	0.33±0.07^{b}	0.62±0.09^{ab}	0.88±0.07^{a}	0.64±0.03^{a}
Protein efficiency ratio (g fish^{-1})	0.78±0.09^{b}	1.49±0.19^{ab}	1.72±0.11^{a}	1.88±0.13^{a}
Protein productive value (%)	13.06±1.89^{b}	24.39±2.00^{ab}	24.71±1.59^{a}	28.68±1.90^{a}

D1: Control diet; D2: 10 mL diet^{-1} *B. subtilis* suspension, D3: 10 mL *B. subtilis* +5 g kg^{-1} citric acid, D4: 10 mL *B. subtilis* +10 g kg^{-1} citric acid; Different letters indicate the same raw mean significant differences

Table4: Effect of *B. subtilis* and citric acid supplementation on whole body chemical composition of red hybrid tilapia (*O. niloticus*× *O. mosambicus*)

Proximate composition	Initial	D1	D2	D3	D4
Dry matter (DM) (%)	28.58±0.02	29.85±0.50^{b}	30.13±0.13^{ab}	30.90±0.20^{a}	30.93±0.93^{a}
Crude protein (CP) (%)	55.00±0.03	51.78±0.60^{d}	52.93±0.50^{c}	55.08±0.42^{b}	64.44±0.22^{a}
Ether extract(EE) (%)	30.54±0.05	36.88±0.11^{d}	35.61±0.50^{c}	32.00±0.56^{b}	24.00±0.60^{a}
Ash (%)	10.56±0.01	9.98±0.60^{b}	10.44±0.44^{ab}	10.71±0.71^{a}	11.06±0.43^{a}

D1: Control diet, D2: 10 mL diet^{-1} *B. subtilis* suspension, D3: 10 mL *B. subtilis* +5 g kg^{-1} citric acid, D4: 10 mL *B. subtilis* +10 g kg^{-1} citric acid; Different letters indicate the same raw mean significant differences

Table 5: Morphometric assessment of intestinal villi from red hybrid tilapia (*O. niloticus*× *O. mosambicus*).

	Duodenum		Jejunum		Ileum	
Groups	Mucosal length	Villi length	Mucosal length	Villi length	Mucosal length	Villi length
D1	381.93±38.60[c]	250.95±39.55[c]	416.45±31.02[c]	370.04±47.32[c]	217.09±35.95[b]	136.14±30.01[b]
D2	372.60±37.76[c]	297.19±26.45[c]	575.02±34.24[bc]	476.54±49.07[bc]	274.66±31.53[b]	175.08±36.43[b]
D3	603.21±47.55[b]	490.06±34.73[b]	745.11±27.05[ab]	640.02±40.27[ab]	435.80±28.87[a]	347.01±31.21[a]
D4	612.94±37.41[b]	815.32±31.92[a]	809.35±40.58[a]	686.09±50.16[a]	462.13±21.52[a]	351.94±30.04[a]

D1: Control diet, D2: 10 mL diet^{-1} *B. subtilis* suspension; D3: 10 mL *B. subtilis* +5 g kg^{-1} citric acid, D4: 10 mL *B. subtilis* +10 g kg^{-1} citric acid, Different letters indicate the same raw mean significant differences

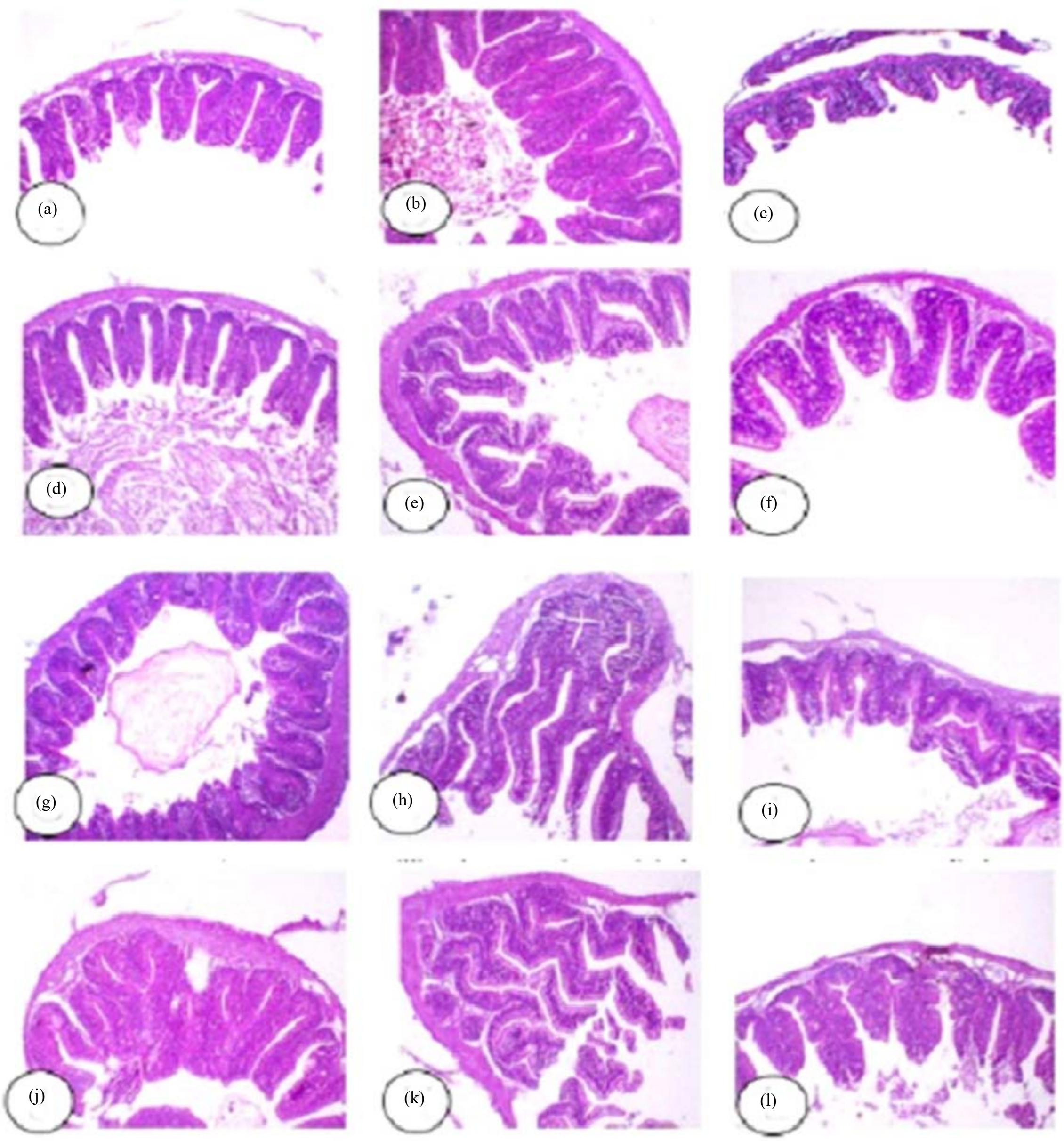

Fig. 1(a-l): Histopathological examination of intestine tissue from red hybrid tilapia (*O. niloticus*× *O. mosambicus*). H and E staining of (a-c) D1 control group, (d-f) D2, (g-I) D3 and (j-l) D4 duodenum, jejunum and ileum tissues, respectively

D1: Control diet, D2: 10 mL diet^{-1} *B. subtilis* suspension; D3: 10 mL *B. subtilis* +5 g kg^{-1} citric acid, D4: 10 mL *B. subtilis* +10 g kg^{-1} citric acid

DISCUSSION

In aquaculture, antibiotics are often used as feed additives to improve survival rates and to enhance growth and feed conversion rates. However, due to concerns about the development of antibiotic resistance, the usage rate of supplements with probiotics and organic acids is increasing[23]. In this study, we demonstrated that supplementation of juvenile red hybrid tilapia fish (*O. niloticus*× *O. mosambicus*) diets with *B. subtilis* and citric acid significantly improved the total body gain, SGR% and condition factor (K) compared to the control group (D1) that had no supplementation. There were no significant differences between D3 and D4 groups that received *B. subtilis* and citric acid at 5 and 10 g kg^{-1} diets, respectively. Feed utilization parameters (FCR, FE, FER and PPV)were significantly improved by addition of both *B. subtilis* and citric acid (D3 and D4) compared to the group that received *B. subtilis* alone (D2) and the control diet (D1).

Probiotics are known to stimulate appetite, modify the population distribution of gut microorganisms and improve absorption of nutrients that in turn translates to improved feed efficiency and growth performance[24,25].

Here we showed that fish fed a diet supplemented with *B. subtilis* had increased percentages of dry matter, crude proteins and ash; these increases were particularly notable for groups that also received citric acid supplementation. Moreover, values for the ether extract percentage, which reflects the lipid content in tissues, were decreased for the D4 group that received 10 g kg^{-1} citric acid relative to the other groups.

The ash% is an indicator of the mineral content of bones and muscles. Fish fed diets supplemented with citric acid showed increased ash contents of muscle[26] and reduced fecal ash contentin Rohu[27].

Our results were consistent with those of He *et al.*[28] for hybrid tilapia, Bairagi *et al.*[29] for Labeorohita (*Hamilton*), Telli *et al.* [30] for Nile tilapia, Ng *et al.*[31] for red hybrid tilapia and Khajepour and Hosseini[32] for Beluga (*Husohuso*). Liu *et al.*[33] showed that Epinephelus coioides fish fed diets containing 10^4, 10^6 and 10^8 CFU g^{-1} *B. subtilis* exhibited significant enhancements in growth performance and feed conversion ratio. Studies conducted by Goda[34]and Mogheth[35]on Nile tilapia reported a negative relationship between protein content and ether extract that reflects the fat content.

Citric acid can increase weight gain and specific growth rates of fish, which is consistent with our results. Meanwhile, citric acid can reduce the feed conversion ratio forBeluga[32] and Rohu[36]. Organic acids such as citric acid likely manifest their beneficial effects by suppressing growth of dangerous microorganisms to balance the microecosystem[37] and adjusting intestinal pH to enhance growth of favorable bacteria[38].

Our results demonstrated that the D2, D3 and D4 groups had significantly increased mucosal and villi length compared to the D1 control group. D4, which received 10 g kg^{-1} diet citric acid, had the longest villi length among the groups. This effect could be attributed to the ability of citric acid to promote epithelial cell proliferation. Indeed, Sakata *et al.*[39] reported that organic acid supplementation supported proliferation of epithelial cells in the pig gastrointestinal mucosa.

In this study we showed that *B. subtilis* and citric acid have synergistic, positive correlation with increases in growth performance, feed efficiency and body chemical composition of tilapia. Daniels *et al.*[40] demonstrated that a diet containing *Bacillus* spp.+ mannan oligosaccharides (MOS)given to larval *Homarus gammarus* significantly improved ($p<0.01$) weight gain, the weight to carapace length ratio, carapace length, food conversion ratio (FCR), specific growth rate (SGR) and post-larval conditions. Khajepour and Hosseini[32] reported that addition of citric acid to the diet of *Husohuso* beluga was associated with increased growth performance (i.e., final weight, weight gain and specific growth rate) and feed utilization (protein efficiency ratio and decreased feed conversion ratio as well as increases in protein and phosphorus digestibility). Su *et al.*[41] indicated that white shrimp fed diets supplemented with citric acid exhibited increased weight gain and decreased feed conversion ratio.

CONCLUSION

Our results suggested that *B. subtilis* and citric acid supplementation of diets fed to juvenile red hybrid tilapia fish (*O. niloticus*× *O. mosambicus*) had a synergistic effect on morphometric characteristics of the intestine, growth performance, feed utilization and body composition.

SIGNIFICANCE STATEMENT

Bacillus subtilis and citric acid supplementation can have synergistic beneficial effects on growth performance, feed efficiency and body composition for red hybrid tilapia (*O. niloticus*× *O. mosambicus*) juveniles. The use of probiotic and organic dietary supplements can promote increases in growth rate and more efficient feed utilization that enhances productivity while reducing the incidence of antibiotic resistance associated with use of antibiotics to promote growth.

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