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RESEARCH PAPER

Effect of Biotronic® Top Liquid Supplementation on the Growth Performance and Disease Resistance of Nile Tilapia (*Oreochromis niloticus* L.)

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Abstract

The present trial was conducted to evaluate whether Biotronic® Top Liquid (BTL) supplementation would benefit during the nursing of Nile tilapia (*Oreochromis niloticus*) fry in terms of survival, growth, and disease resistance. Isonitrogenous (22.9±0.2% CP) diets were prepared by supplementing 0.0, 0.5, 1.0, 1.5, 2.0 to 2.5 L/ton of BTL diet i.e. T1 to T6, respectively. Each of the 18 aquaria (100 L) was stocked with 45 fish (16.17±0.40g, Mean±SE) having three replicates per treatment. During the trial, fish biomass increased linearly with the increase in BTL dose i.e. 230g (10.3%) of fish biomass/L of BTL. Results showed that higher dose of BTL/ton of feed resulted in to increase growth rate, with an indication of improved growth beyond the tested level i.e. >2.5 L/ton. Bacterial challenge test using *Aeromonas hydrophila* showed that the highest BTL dose could maintain average fish survival rate above 65%, which was 25% higher than that of control. Average survival improved linearly i.e. (y)=8.857x+40.317 (R^2=0.64) with a nearly 9% increase in survival per liter of BTL supplementation. BTL improved SGR, DWG, and PER by 3.4, 1.6 and 2.1 times, respectively as compared to the control when the fish were challenged by bacteria. Therefore, the dose of 2.5 L/ton of feed is recommended based on the present study.

Introduction

Tilapias have become the second largest and most widely farmed aquaculture species group which are playing a critical role in achieving food security and improved nutrition globally (Bhujel, 2014). Tilapia farming is spreading all over tropical and subtropical areas. They are hardy fish and if co-cultured with shrimp or in rotation, may serve as solutions to diseases of shrimp (Duy et al., 2013). Although, tilapia was once considered disease resistant, nowadays its farming is suffering by frequent occurrence of devastating diseases mainly due to the use of excessive feeds, chemicals and increase in intensification (Wu et al., 2013). Tilapias are often stocked over 100 fish/m^3 of cage volume or overcrowding and periodic handling, and also affected sudden rise and fall in temperature, poor water quality and poor nutritional status leading to stress or immunosuppressant causing increased susceptibility to infection by parasite, bacterial and viral diseases e.g., *Streptococcus* sp., *Aeromonas* sp., *Edwardsiella* sp., *Pseudomonas fluorescens, Vibrio anguillarum, Flavobacterium columanare,* Tilapia Lake virus etc. (Plumb, 1997; Belton et al. 2009; Bhujel, 2014; Rico et al. 2014; Reverter et al. 2014).

Aeromonas hydrophila is one of the most prevalent bacteria in freshwater fish. It causes hemorrhagic septicemia, dark skin and hyperemia of the fin base (Yardimci and Aydin, 2011). The optimum temperature for most of the pathogens, e.g., *Aeromonas hydrophila*

is 28°C (Maalej et al. 2004), which is optimal for tilapia as well. Various antibiotics and other chemicals are used to control these pathogens in tilapia farms, but the use of antibiotics as immune stimulants can be harmful to animals, consumers, and the environment (Alderman and Hasting, 1998; Watts et al. 2017). The most used antibiotics is oxytetracycline which was found to be used by 45% of the farmers followed by enrofloxacin (6%) and sulfa-dimethoxine (6%) in Northern Thailand (Lebel et al. (2013), Whereas in Central Thailand have found five groups of chemicals: namely, ß-lactams, quinolones, sulfonamides, tetracyclines, diaminopyrimidines (Rico et al. 2014). In these areas, the most used antibiotics were enrofloxacin (59% of the interviewed farmers), followed by oxytetracycline (48%), amoxicillin (28%), and sulfadiazine or trimethoprim (28%). On an average, at least two different antibiotics are used per farm: but in 24% cases 4-7 different antibiotic ingredients. Excessive use of antibiotics will eventually create antibiotic resistance and hazards. Therefore, there is an urgent need to carry out more research on alternative and sustainable solutions.

The feed supplement Biotronic® Top Liquid (BTL) is a combination of formic, propionic and acetic acid with a Permeabilizing Complex™ mixture, a natural blend of flavoring substances. The product targets bacteria in general but with greater efficacy on Gram-negative bacteria. The Permeabilizing Complex™ blend has previously displayed to be an enhancer of antimicrobial activity against *E. coli* and Salmonella of both the *Enteritidis* and *Typhimurium serovar* (Riemensperger et al. 2012). Formic acid has been used successfully to inhibit the growth of *Vibrio* spp. in vitro (Adams and Boopathy, 2013), and to improve survival of shrimps (*Litopenaeus vannamei*) after challenge with *Vibrio parahaemolyticus* (Chuchird et al. 2015). The combination of formic acid with propionic proved to be effective in lowering the total bacteria counts in the gastrointestinal tract of olive flounder (*Paralichthys olivaceus*), and especially the counts of *Vibrio* spp. could be reduced (Park et al. 2011). Further, for formic, acetic, and propionic acid, various studies showing that they are able to support digestion by improving feed digestibility and enhance availability of minerals like P, Mg and Ca (Sugiura et al. 2006; Sarker et al. 2012; Silva et al. 2013). An attractive and important advantage of natural products are absence of residues and also any negative effects on fish health or to human and environment (Baba et al. 2016). Addition of a feed additive containing the Permeabilizing Complex™ blend to the diet of weaned piglets led to a significantly increased average daily weight gain and final weight (Riemensperger et. al. 2012). BTL is commercially available and has been applied both in shrimp and fish farming settings. However, few scientific studies have been published so far investigating its efficiency in aquatic organisms.

Maintaining fish health and maximizing fish survival and growth to increase profitability or return on investment is a key objective of farming. At the same time, reduction in cost of production could also help maximize the profit. Use of feed additives could help reduce feed cost, which incurs often 60-70% of the total production cost (Lara-Flores et al. 2003). The main objective of the present study was to evaluate the efficacy of BTL in Nile tilapia fingerlings and determine an optimum dose based on the relationship of its dose with survival, growth, feed conversion efficiency, and protein efficiency in a normal culture condition, and also when challenged with *Aeromonas hydrophila*.

Material and Methods

Experimental Fish and System

The experiment was conducted at the Aqua-Centre facility of the Asian Institute of Technology (AIT), Thailand located 42 km north of Bangkok for a total of 12 weeks. All-male Nile tilapia (*Oreochromis niloticus*) fingerlings were obtained from the AIT' tilapia hatchery, which were in the earthen pond system, for the trial. The experimental fish were passed through an adaptation period of two weeks during which they were fed with control diet containing 22.9±1.3% crude protein, (CP9931, Charoen Pokphand Food Public Co. Ltd) three times daily until apparent satiation. Acclimated fish (13.81±0.23 g, mean ± SE,) were randomly distributed to 18 glass tanks (45 fish per aquarium) with 100 L volume of dechlorinated freshwater. Each tank unit was composed by an individual biological filter system including shells, gravels, and cotton sheets. During the experiment, average temperature was 28.4±0.1°C with 12-12 light-dark photoperiod cycle using fluorescent tubes as a light source. Each aquarium was supplied with compressed air diffused through air-stones connected to a pump. Settled fish wastes were siphoned out daily from each aquarium by reducing 50% of water, then new water was added to maintain the original volume. Fish were transitioned to a control diet twice daily at an initial feeding rate of 4% of biomass for two weeks, before using them for the 10 weeks trial.

Test Diets

Commercial floating pellets (22.9±1.3% crude protein, CP9931, Charoen Pokphand Food Public Co. Ltd.) mixed with Biotronic® Top Liquid (BTL, BIOMIN Holding GmbH, Austria). Three replications were used for six treatments of commercial diets supplemented at varying level of BTL i.e. 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 L of BTL per ton of feed which are named as Treatment 1 to 6 i.e. T1 to T6. A total 120kg (20kg /treatment) pellets were prepared by mixing different dose of BTL liquid. The diets were oven dried at 50°C oven (UN55) until the moisture was around 10% and stored in plastic sealed bags in a refrigerator (4-10°C) for further use. These treatments were randomly allocated in 18 aquaria using complete randomized design (CRD). Detailed

experimental plan and the period are presented in Table 1. After diets were prepared, 1 kg feed was sampled per treatment diets for proximate analysis of crude protein, lipid, ash and fiber and BTL recovery. Chemical compositions of all the six experimental diets are presented in Table 2.

Proximate Analysis of Diets and Fish

The test diets and whole fish body from each treatment at the beginning and at the end of the experiment were analyzed for proximate composition (moisture, crude protein, total lipid, total ash, and total fiber). Moisture content was estimated by drying the sample to constant weight at 105^{0}C under air oven method (UN55). Total ash was determined by combusting dry sample in muffle furnace at 550^{0}C. Nitrogen content was measured using a Micro-Kjeldahl apparatus after digestion with Conc. H_2SO_4 acid (FOSS Tecator Digestor) and crude protein was estimated by multiplying the nitrogen content by 6.25 (Tecator Manual, 1987). Total lipid content was determined by Soxhlet method (Tecator Manual, 1980). Crude fiber was analyzed following the Weende method by using the Fibertec system (Tecator Manual, 1978).

Fish Growth and Feed Utilization

Survival and growth of fish were monitored once in two weeks by counting and weighing of all the fish (total biomass) from each replicate tank. The following performance indicators were considered to determine the impacts of feed supplement:

- Fish survival rate (%) = (Final number / Initial number) × 100 %
- Biomass gain = (Final weight (g) – Initial weight (g)
- Specific growth rate (SGR, %/day) = ((L_n Weight at harvest – L_n Weight at stocking) ×100/days
- Feed conversion ratio (FCR) = Feed intake in Dry matter/Wet weight gain
- Feed conversion Efficiency (FCE) = Wet weight gain*100/ Feed intake in dry matter basis
- Protein Efficiency ratio (PER) = Wet weight gain /Protein intake in feed

Blood Sampling

Six fish from each aquarium (45 fish/group) were randomly selected end of the feeding trial and anesthetized using 60 mg/L of MS222 (Ethyl 3-aminobenzoate methanesulfonate). Hematological studies were done collecting blood samples in a sterile 1 mL syringe from the caudal vein of each fish. Serum was separated from the bloods centrifuging at 3500 g for 15 min and stored at -20^{0}C until use. All blood and serum samples were sent to Thai Vet Lab Co. Ltd to check CBC (cells blood count), total protein and SGPT (alanine aminotransferase). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were calculated (Table 5).

Fish Gut Analysis

Three fish from each replicate aquarium out of 45 fish were randomly selected after 10 weeks of feeding for dissection. Gastro-intestinal tract was removed from each fish with sterile dissecting instruments. An anterior part of the intestine was removed and washed with a sterile saline solution for 2-3 min. Then it was macerated and transferred to tubes containing 9.0 mL of 0.85% (w/v) NaCl solution and 15-20 glass beads, agitated with a mixer for 2 minutes. The homogenate was serial diluted to 10^{-9} in 9 mL volume of sterile 0.85% (w/v) of saline solution. Nutrient agar plates were prepared and kept in refrigerator at 4^{0}C before starting incubation.

Table 1. Experimental period with Biotronic® Top Liquid and bacterial challenge test

Adaptation period (Control diet)	Feeding experiment with test diets April 5-June 14 (10 weeks)					Bacterial challenge test
March 20-April 4 (14 Days)	Week1-2	Week3-4	Week5-6	Week7-8	Week9-10	June 20-July 4

Table 2. Proximate composition (% on dry matter basis) of the experimental diets (Mean±SD)

Proximate composition	0.0 L/ton (T1)	0.5 L/ton (T2)	1.0 L/ton (T3)	1.5 L/ton (T4)	2.0 L/ton (T5)	2.5 L/ton (T6)
Dry matter	90.87±0.1^a	92.62±0.1^b	93.37±0.20^c	93.49±0.0^d	94.62 ±0.05^e	94.85±0.1^f
Ash	8.05 ±0.04^b	6.76±0.3^a	7.95±0.03^b	7.26 ±0.04^{ab}	7.78±0.01^b	7.79±0.01^b
Lipid	4.55 ±0.4^a	6.14 ±0.7^{ab}	6.35 ±0.5^b	6.71±0.4^{bc}	7.72±1.1^{bc}	11.46 ±0.4^c
Fiber	4.33±0.3^a	4.54±0.4^a	5.17±2.7^a	5.04±4.7^a	5.071±3.3^a	5.61±2.6^a
Protein	22.86±1.3^a	22.89±2.1^a	22.86±0.6^a	24.37±2.9^a	24.55±0.7^a	24.94±1.2^a
[1]NFE	51.08±1.6^a	52.27±2.5^a	48.86±0.9^a	47.92±1.6^a	48.08±0.7^a	46.09±3.4^a
[2]GE (kcal/kg)	3821±27^a	4022±44^a	3989±70^a	4070±104^a	4100±78^a	4174±121^a

Note: All values are Mean ± SE, calculate from three replicates. Values for each experiment group in the same row followed by different superscripts are significantly (P<0.05) different. DM=Dry matters (%),[2]GE=gross energy.

[1]Nitrogen free extract (NFE) = 100 – (crude protein % + crude lipid% +crude fiber %+ total ash %).

Samples of 0.6 mL of homogenized gut and dilutions of these saline solutions were spread on plates under sterile environment. Finally, number of total bacteria in gut was estimated using dilution method.

Bacterial Challenge Test

Aeromonas hydrophila was isolated from infected Nile tilapia and freshly prepared using Tryptone soya broth (TSB). Bacteria culture was incubation at 25°C overnight and adjusted to 1 x 10^8 CFU/mL in phosphate buffer saline (PBS). After conducting feeding trial (after 10 weeks), a set of 10 fish were randomly sampled from each of the 18 replicate aquaria for bacterial challenge test. Another set of 10 fish were continued in normal conditions in aquaria without bacterial addition (mock-Infected). Each aquarium of 30 L each and stocked with those 10 fishes (50.4 ± 3.31 g, mean weight ± SE) in each aquarium. Volume of each was reduced to 10 L and added 1 x 10^8 CFU/mL *A. hydrophila*, prepared previously for each of the 18 aquaria for immersion challenge method. Fish were and kept for 3 hours and water was added to increase the volume to 30 L. Another 18 aquaria without bacteria were subjected to the same protocol, however using only 0.85% (w/v) NaCl without bacteria. Cumulative mortality and external observation of fish were noted daily for 14 days. Dead fish once appeared in aquaria were recorded and removed.

Water Quality Analysis

Water pH, temperature and dissolved oxygen (DO) were recorded daily at 0830h. Dissolved oxygen was measured with a dissolved oxygen meter (Cyberscan DO 110 RS232 model). Temperature and pH were measured using pH meter (Cyberscan pH11 model). Ammoniacal nitrogen concentration was monitored weekly using Phenate method (AOAC, 2003). The water temperature range during the running of this experiment was 26.2 – 31.6°C with 12-12 light–dark photoperiod cycle using fluorescent tubes as a light source. Dissolved oxygen concentration ranged from 3.87 – 5.96 mg/L, the pH ranged 6.8 – 7.6 and the ammonia concentration ranged 0.03 – 1.52 mg/L. All the water quality parameters acceptable ranges for fish growth. Water quality parameters were not significantly different among the treatments; therefore, did not have apparent effects on the survival and growth of fish as well as bacteria.

Data Analysis

The SPSS 22.0 statistical package for social sciences (SPSS Inc.) was used for multiple comparisons among several means, all at 5% significance level. All means are presented with ± standard error (SE). One-way analysis of variance (ANOVA) was carried out to see the effects of BTL dose followed by Tukey HSD for comparisons. Regression analysis was used to see the relationship between BTL dose with the survival and growth parameters.

Results

Performance During Feeding Period

Growth parameters of the feeding experiment are presented in Table 3. During the 10 weeks of experimental period, average survival of tilapia ranged from 74.8 to 84.4%. However, the values were not significantly ($P>0.05$) different among the treatments. Initial mean weight of fish was 13.81±0.23 g and there was no significant ($P>0.05$) difference at the start of the experiment.

The fish biomass (g/tank) increased linearly (Figure 1, $y = 229.68x+2229.7$, $R^2=0.93$) with the increase in BTL dose. It is indicating that every unit (L) increase nearly 230 g i.e. 10.3% from its initial biomass when applied in the conditions used in the experiment. Biomass results were significant difference among the treatment and highest biomass was related to higher dose of BTL. Improvements were also seen in specific growth (SGR, $y=0.0964x^2-0.1706x+2.2716$, $R^2=0.82$) and daily weight gain (DWG, $y=0.0567x^2-0.0911x+0.763$, $R^2=0.90$) with the increase in the liquid dose (Figure 2). DWG was significant among the treatment and T6 was showed highest DWG compared to T2. The relationship was quadratic in nature, which showed lowest SGR and DWG

Table 3. Growth performance and nutrient utilization of Nile tilapia (*Oreochromis niloticus*) fed different dose of BTL for 10 weeks period

Parameters	Growth performance of fish for different treatments						P-value
	0.0 L/ton (T1)	0.5 L/ton (T2)	1.0 L/ton (T3)	1.5 L/ton (T4)	2.0 L/ton (T5)	2.5 L/ton (T6)	
IW (g)	13.7^{a}±0.1	13.66^{a}±0.1	13.64^{a}±0.2	13.6^{a}±0.4	13.51^{a}±0.5	13.8^{a}±0.0	0.284
FW (g)	67.7^{a}±2.9	62.8^{a}±4.4	66.7^{a}±2.3	67.2^{a}±5.0	69.15^{a}±3	76.4^{a}±1.2	0.260
BG (g/fish)	54.02^{a}±2.8	49.2^{ab}±4.4	53.1^{ab}±2.2	52.7^{ab}±4.6	55.6^{ab}±3.1	62.7^{b}±3.6	<0.05
Survival (%)	74.8^{a}±7.1	83.7^{a}±4.9	81.5^{a}±2.7	84.4^{a}±2.2	83.7^{a}±4.86	84.4^{a}±2.6	0.620
FCR	1.24^{b}±0.1	1.19^{b}±0.0	1.14^{ab}±0.1	1.11^{ab}±0.1	1.07^{ab}±0.1	0.93^{b}±0.2	<0.05
FCE	0.81^{a}±0.1	0.84^{a}±0.0	0.88^{a}±0.1	0.91^{ab}±0.1	0.95^{ab}±0.1	1.08^{b}±0.0	<0.05
PER	3.37^{a}±0.3	3.43^{a}±0.3	3.6^{a}±0.2	3.54^{a}±0.4	3.65^{a}±0.4	4.10^{a}±0.1	0.482
SGR (%)	2.28^{ab}±0.1	2.17^{a}±0.1	2.27^{ab}±0.0	2.18^{a}±0.1	2.33^{ab}±0.1	2.45^{b}±0.1	<0.05

Note. All values are Mean ± SE, calculate from three replicates. Values for each experiment group in the same row followed by different superscripts are significantly ($P<0.05$) different. IW= Initial weight: FW= final weight; BG=biomass gain; FCR=Feed conversion ratio; FCE=Feed conversion efficiency; PER= protein efficiency ratio; SGR=specific growth rate.

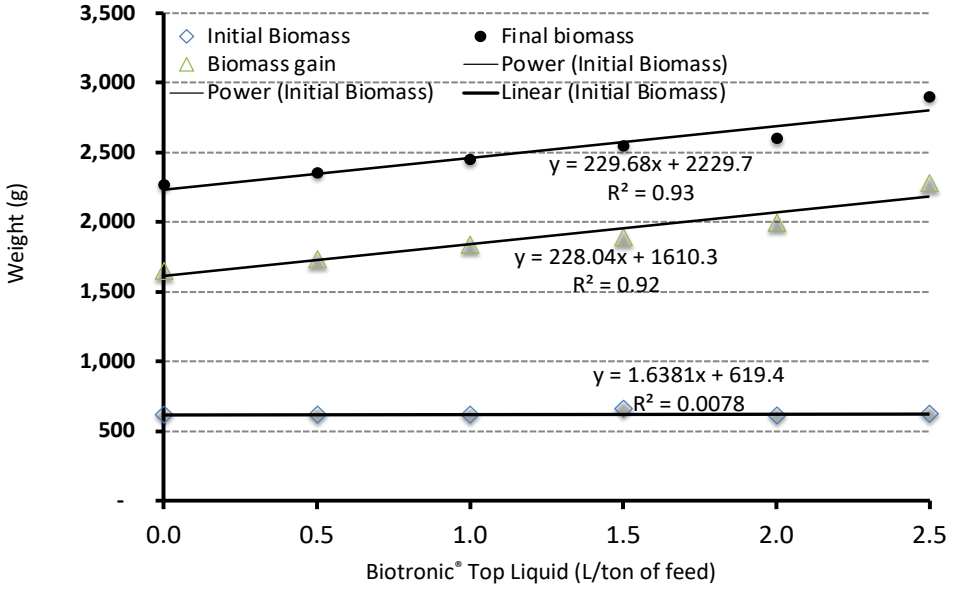

Figure 1. Linear relationship of Initial and Final biomass and Biomass gain (g in Y-axis) with the increased doses of Biotronic® Top Liquid in Nile tilapia (*O. niloticus*) during the feeding trial.

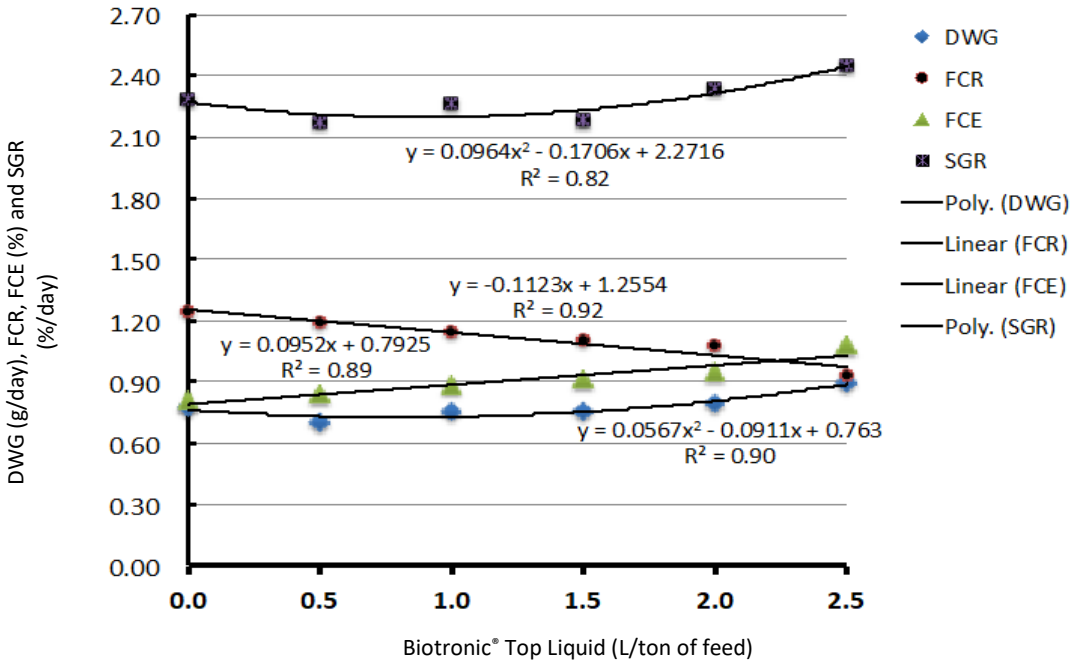

Figure 2. Relationships of daily weight gain i.e. DWG (polynomial)), Feed conversion ratio i.e. FCR (negative linear), Food conversion efficiency i.e. FCE% (positive linear) and Specific growth rate i.e. SGR%/day (polynomial) of Nile tilapia (*O. niloticus*) with varying levels of the Biotronic® Top Liquid obtained during the feeding trial.

at estimated dose of 0.885 and 0.803 L/ton with respective values of 2.2% per day and 0.73 g per day; but increasing trend was seen at higher doses after 1.5 L/ton. Biomass has increased linearly, FCR (y=-0.1123x+1.2554, R^2=0.92) and feed conversion efficiency (FCE, y=0.0952x+0.7925, R^2=0.89) showed the similar trend.

Results of proximate analysis (Table 4) of whole body of fish on dry mater basis also showed that, crude protein, crude lipid and ash levels increased with the increase in the doses of BTL. Crude protein content of the fish increased linearly which ranged from 43% to 57% i.e. an increase of 32% at the highest dose as compared to the control. Figure 3 shows the relationship i.e. crude protein (y)=4.7846x+43.852 (R^2 = 0.87) that means every liter increase in BTL would increase 4.78% in crude protein level in the fish body on dry matter basis. Similarly, lipid content of whole body of Nile tilapia ranged from 9.97 to 19.95% showing more than 100% increment by the highest dose of BTL as compared to the control. The relationship was quadratic i.e. Crude lipid (y)=-2.6109x^2+9.3897x+12.09 (R^2 =

0.8491, Figure 3). The ash content increased with the increase in dose of BTL ranging from 2.39 to 2.85%; with lowest from control treatment and highest at T6 i.e. 2.5 L/ton dose.

Challenge Test with *Aeromonas hydrophila*

The survival rate and parameters of growth performance after challenged test are shown in Figure 4 and 5. In the group with bacterial challenge test, BTL showed very clear effects on survival right from the 6th hour after exposure (Figure 4).

Survival rate dropped right from Day 1 and reached below 40% in case of control and T2 i.e. 0.5 L/ton dose whereas highest dose of BTL (T6) maintained the survival above 80% until Day 7, and above 65% until it stabilized reaching at that level from 11-14th day. Average survival improved linearly i.e. (y)=8.8571x+40.317 (R^2=0.64) with the increase in the dose of supplementation (Figure 5, top). It shows an increase of almost 9% survival for every liter of BTL dose. Interestingly, when the fish were not exposed to bacteria, survival remained high (>90%) and there is no clear sign of improved survival due to the supplementation (Figure 5, right). Similarly, the weight/biomass gain increased by 81% (P<0.05) over control at the highest dose of BTL in the case of bacterial challenged group whereas in the case of without bacterial challenged group biomass gain due to the highest dose of BTL was only 42% higher than that of control group (Figure 6). Specific growth rate (SGR), daily weight gain (DWG), protein efficiency ratio (PER) and overall food conversion efficiency (FCE) increased with the increase in the dose of BTL in both the conditions (with and without bacterial challenges) (Figure 6). Based on the slope of regression lines, increase in average SGR per liter of BTL in the case of bacterial challenge is 0.35% (Figure 6, left), which is 3.5 times higher than the SGR increment rate of fish when they were not challenged with bacteria (0.10%, Figure 6, right). Similarly, average daily weight gain (DWG) of the challenged fish with addition of BTL was 1.6 times, PER 2.1 times and FCE is 1.8 times higher than the respective indicators when fish were reared in the bacterial exposure.

Clinical Findings and Blood Composition

In the bacterial challenge groups, after 3 hours of bacterial immersion all fishes were observed to be docile and often gathering in a corner of each aquarium.

Table 4. Proximate composition (% dry matter basis) of the experimental fish (whole body)

Components	Initial fish	Final fish					
		T1	T2	T3	T4	T5	T6
Dry matter	9.72±1.4^{ab}	9.13±1.4^{ab}	8.25±0.7^{ab}	10.90±0.6^{a}	9.11±0.2^{ab}	8.92±1.1^{ab}	9.04±2.2^{b}
Ash	2.39±0.1^{a}	2.58±0.1^{a}	2.25±0.1^{b}	2.53±0.1^{c}	2.66±0.0^{c}	2.36±0.0^{d}	2.85±0.0^{e}
Lipid	9.97±3.9^{a}	10.82±2.5^{a}	18.38±1.8^{a}	19.04±2.5^{a}	18.99±1.8^{a}	19.87±2.5^{a}	19.95±2.2^{a}
Protein	43.24±0.5^{a}	45.523±0.8^{a}	45.55±1.5^{a}	48.44±2.9^{a}	48.029±3.9^{a}	54.41±5.6^{b}	57.04±0.6^{b}
NFE	34.69±2.3^{b}	31.95±2.1^{b}	25.56±3.2^{bc}	19.08±1.6^{bc}	21.20±2.4^{bc}	14.44±8.2^{ab}	11.12±4.1^{a}

Notes: Values within the same row with different superscripts are significantly different at (P<0.05).

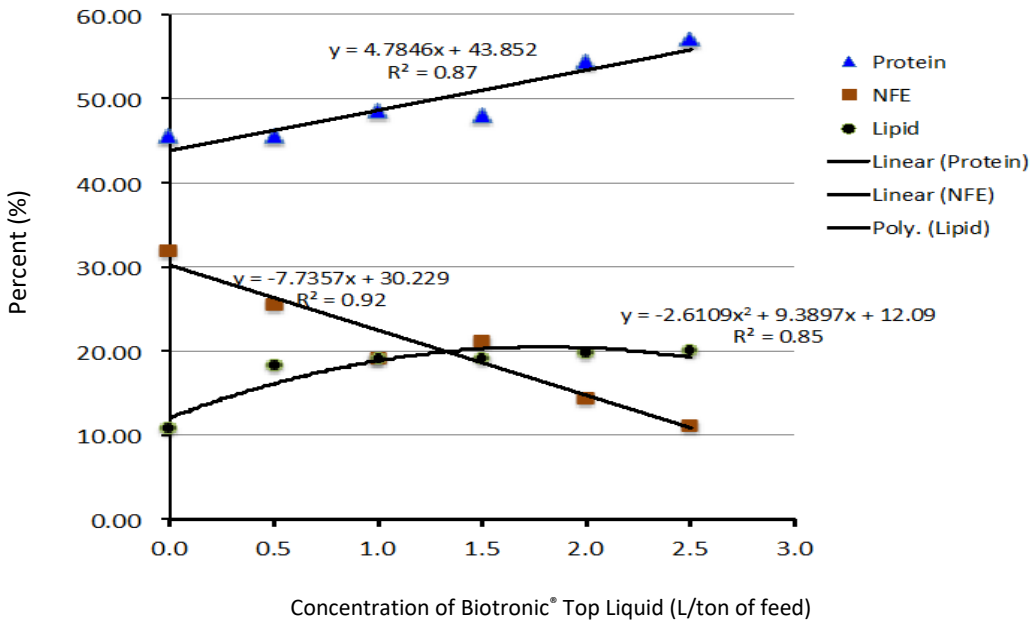

Figure 3. Effects of increasing dose of Biotronic® Top Liquid (L/ton of feed) in crude protein (linear), NFE (polynomial) and whole-body lipid composition of Nile tilapia (negative linear) after feeding experimental diets for 10 weeks.

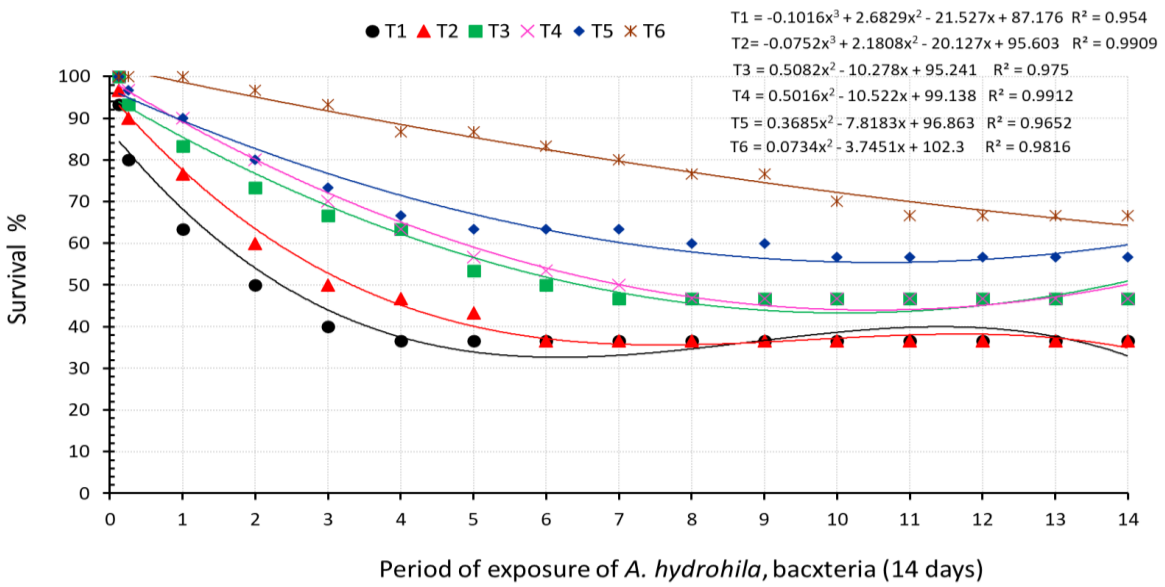

Figure 4. Polynomial relationship between the period of exposure and percent survival (Y-axis) of Nile tilapia over the period (3rd hour to 14th days) after challenged with *Aeromonas hydrophila.*

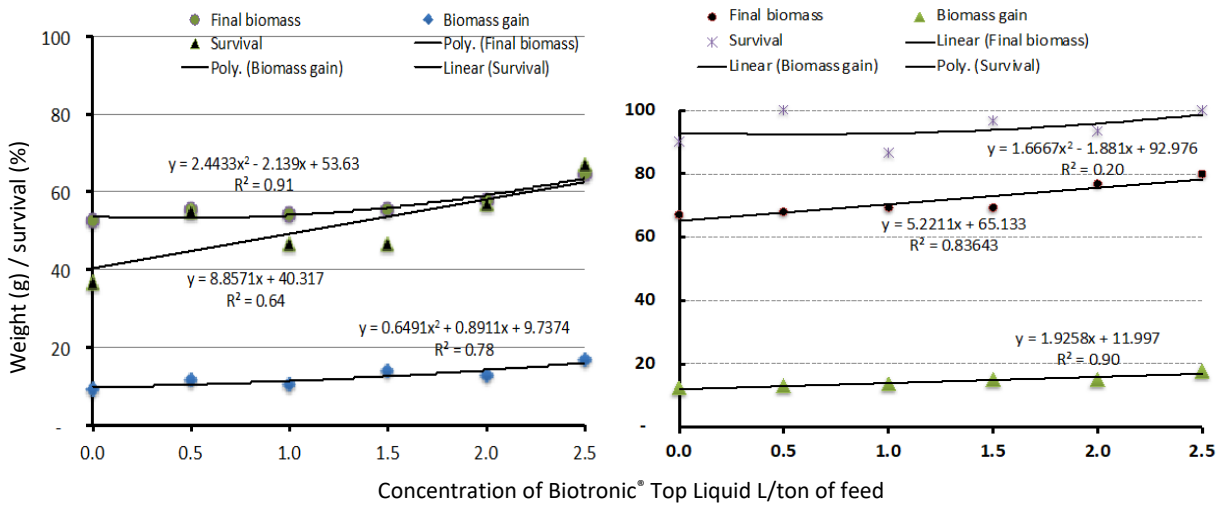

Figure 5. Linear and polynomial relationship between BTL concentration and fish biomass (g) and survival of fish (%) over the period (3 hour – 14 days) when fish were challenged (left) and not challenged (right) with *A. hydrophila.*

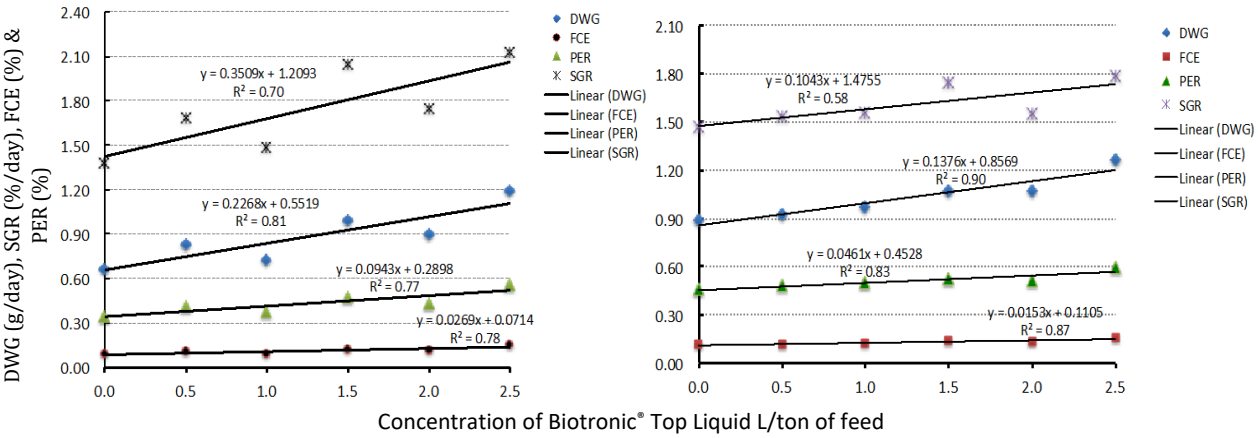

Figure 6. Linear relationship between BTL concentration and Daily weight gain (DWG, g/day), Specific Growth Rate (SGR, %/day), Feed conversion efficiency (FCE%) and Protein Efficiency Ratio (PER%) of fish for 3 hour – 14 days when fish were challenged (left) and not challenged (right) with *A. hydrophila.*

After 6–12 hours mortality started to occur. By 1st day, reluctance to eat, decreased feed intake and darkening of skin were observed. From the 3rd day fin rot especially on the tips of the pectorals were observed and hyperemia of the fin bases were also seen. From day 7 until day 14 after challenge, feed intake decreased considerably, and almost all the fish gathered at the bottom of each aquarium. Fish were unstable to swim, and their skin became darker and fin rot was clearer. Macroscopic findings of the skin, gill, liver, kidney, heart, and stomach were almost dissolved, and hyperemia was clearer on the last day or challenge.

In blood samples RBC (red blood cells), Hb (hemoglobin), Hct (Hematocrit Blood Test) and total protein (TP) increased with the increase in the dose of BTL i.e., increased from T1 to T6. The trend is more prominent ($P<0.05$) in case of bacterial exposure. SGPT (alanine aminotransferase) values decreased with the increase of BTL from T1 to T6 (Table 5).

More liver cells were damaged showing the similar trend. Other indicators show decreasing trend, after challenged by bacteria e.g., SGPT value; T6 had significantly lower ($P<0.05$) than in the fish raised without Bacteria. More bacteria colonies or count showed decreased numbers gradually with the increase in BTL treatment from T1 to T6 (Figure 7). Both sampling results showed that there was significant difference ($P<0.05$) in bacterial colonies between T6 and control.

Discussion

During the first 10 weeks of growth period, survival of tilapia remained 75% or higher and did not differ with the dose of BTL indicates that culture conditions and health of the fish remained within the comfort zone when BTL was included up to 2.5 liter per ton of feed. However, linear increment in biomass, and feed conversion efficiency as well as linear decrease in FCR values indicated that BTL had positive effects on growth and feed utilization. Quadratic relationship of the feed supplement with daily weight gain and percent specific growth rate showed an indication that supplementation requires at least 1 liter of BTL or higher doses per ton of feed to show positive effects. Further increase in supplementation of BTL up to 2.5 L/ton of feed is proved to be beneficial. The limiting factor for higher BTL dosage would be the increased cost of the final feed. Growth improvement was also evident in terms of protein deposition in the body of fish without increase in dry matter. This indicates that the BTL might have helped protein intake, digestion, and overall protein metabolism. Similar findings have been reported by Ng and Koh, (2016). Various studies are available showing that formic, acetic, and propionic acid, are able to support digestion in aquaculture by improving feed digestibility in general and enhance availability of minerals such as calcium (Ca), Phosphorous (P) and magnesium (Mg) (Sugiura et al. 2006; Sarker et al. 2012; Silva et al. 2013). However, more research is needed to determine the optimum dose, which may fall above the tested level, for tilapia in various culture conditions, considering extended growth periods, especially six months or more as practiced by the farmers.

More interesting part of this study is that lipid content of the whole body of fish, which has doubled at highest dose compared to the control treatment. There seems to have double functions; the BTL itself contains high level of lipid, and at the same time, increased appetite in fish might have positive impacts on lipid intake and its metabolism. Nevertheless, it indicates that supplementation of BTL and lipids is reflected in the fish body. However, what type of lipids were increased was not clear as lipid analysis was not done due to the lack of laboratory facility. Therefore, further studies should be done to analyze lipid classes and fatty acids especially omega-3 and 6. As tilapias have been criticized to have high level of omega-6 and low level of omega-3 fatty acid as compared to marine species, there might be opportunity of raising omega-3 through the supplementation for some organic acids (De Souza et al. 2007).

When challenged with *A. hydrophila* in immersion, presence of bacteria in the body and there was increased mortality in case of no BTL supplementation or at low doses. It clearly showed that feeding BTL helps reduce bacterial growth and improves disease resistance. Present finding is in line with many researchers who have found that organic acids used as feed additives help to control the bacterial disease such as *Vibrio* spp. (Park et al. 2011; Adams and Boopathy, 2013). Although, most of these researchers used injection method rather than immersion method applied in the present study. From this study, disease resistance was also evidenced from the hematocrit (Hct) and WBC values. The variation degree on the hematological response is an important tool to fish health diagnosis and may vary according to stressor stimulus, treatment, parasitic or infectious diseases (Silveira-Coffigny et al. 2004). It is regarded that Hct values reflect the health status of fish, hence nutritional deficiency and diseases are associated with low levels of Hct (El-Asely et al. 2014). Hemoglobin results are also associated with fish health. Higher hemoglobin values were shown to have better level of fish health. The treatment with the highest dose (T6) showed the best hematological values as compared to all other treatments.

Infection of *A. hydrophila* occurs when the common predisposing factors are present such as abrupt temperature changes, and stress due to handling, crowding, and inadequate feed and oxygen (Yardimci and Aydin, 2011). Therefore, suggestions to maintain stable water quality and smooth handling fish during farming activities. However, in most of the cases, these are not easy to control, and the fish are usually exposed to those pre-disposing factors in most culture environments. Therefore, application of BTL as a prevention measure sounds very promising, as it, is

Table 5. Hematological changes (mean±SD) of *Orechromis niloticus*

	WBC/µL	RBC 10^6/µL	Hb (g/dL)	Hct (%)	MCV	MCH	MCHC	SGPT (IU/L)	TP (g/dL)
T1-B	1467±501^{a}	2.1±0.4^{a}	10.8±1.8^{a}	30.8±5.4^{a}	145.3±12^{b}	50.7±4.0^{a}	35.0±0.4^{a}	63.6±23.3^{b}	2.8±0.6^{a}
T1-A	3000±2951^{a}	2.0±0.2^{a}	10.1±0.8^{a}	28.9±3.8^{a}	142.4±3^{b}	50.0±1.2^{a}	35.2±0.7^{a}	77.8±50.8^{a}	2.7±0.6^{a}
T2-B	1550±363^{a}	2.3±0.3^{a}	11.1±1.1^{a}	31.6±3.1^{a}	139.4±3^{ab}	48.9±1.3^{ab}	35.1±0.1^{a}	54.1±22.7^{bc}	3.0±0.4^{ab}
T2-A	1308±372^{a}	2.1±0.1^{ab}	10.7±0.6^{ab}	30.4±1.6^{ab}	141.9±3^{a}	49.8±1.3^{a}	35.1±0.4^{a}	64.7±39.9^{a}	2.9±0.7^{a}
T3-B	1550±756^{a}	2.5±0.5^{a}	11.6±2.0^{a}	33.2±5.5^{a}	135.5±6^{a}	47.4±2.2^{a}	35.0±0.1^{a}	54.1±27.0^{bc}	3.1±0.4^{ab}
T3-A	2192±195^{a}	2.3±0.3^{ab}	11.1±1.4^{ab}	31.6±4.0^{ab}	139.7±2^{a}	49.0±0.9^{a}	35.0±0.5^{a}	65.8±50.4^{a}	3.1±0.5^{a}
T4-B	1606±122^{a}	2.5±0.5^{a}	12.1±2.1^{ab}	34.4±6.0^{a}	136.9±5^{a}	48.1±1.7^{a}	35.2±0.4^{a}	40.6±36.6^{ab}	3.4±0.5^{ab}
T4-A	2086±229^{a}	2.4±0.3^{ab}	11.6±1.1^{ab}	32.9±3.0^{ab}	139.3±5^{a}	48.9±1.9^{a}	35.2±0.2^{a}	51.2±42.7^{a}	3.3±0.9^{a}
T5-B	1610±736^{a}	2.5±0.3^{a}	12.3±1.2^{a}	35±3.2^{a}	138.9±5^{a}	48.8±1.8^{ab}	35.2±0.4^{a}	38.9±26.4^{ab}	3.3±0.5^{ab}
T5-A	1950±969^{a}	2.4±0.3^{b}	11.8±1.4^{b}	33.6±4^{b}	140.4±7^{a}	49.2±2.5^{a}	35.0±0.1^{a}	44.5±28.3^{a}	3.3±0.4^{a}
T6-B	1767±483^{a}	2.6±0.2^{a}	12.5±0.8^{a}	35.5±2.1^{a}	136.6±4^{a}	47.9±1.4^{a}	35.1±0.2^{a}	27.3±18.0^{a}	3.5±0.3^{b}
T6-A	1679±847^{a}	2.5±0.3^{b}	12.0±1.2^{b}	34.1±3.6^{b}	139.6±5^{a}	49.0±1.8^{a}	35.0±0.1^{a}	41.5±23.6^{a}	3.3±0.3^{a}

Values for each experiment group in the same row followed by different superscripts are significantly (P<0.05) different. (B=before challenge test; A=After challenge test)

**T1-B = treatment 1 before challenge test ,T2-B = treatment 2 before challenge test , T3-B = treatment 3 before challenge test , T4-B = treatment 4 before challenge test , T5-B = treatment 5 before challenge test , T6-B = treatment 6 before challenge test , T1-A = treatment 1 after challenged test, T2-A = treatment 2 after challenged test, T3-A = treatment 3 after challenged test, T4-A = treatment 4 after challenged test, T5-A = treatment 5 after challenged test, T6-A = treatment 6 after challenged test.

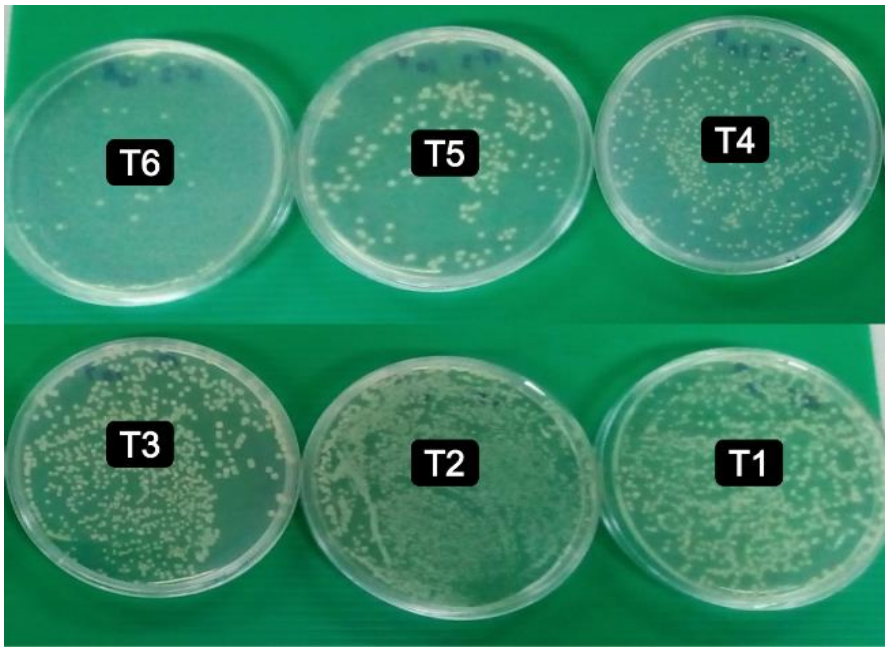

Figure 7. Bacterial count in samples from the fish treated with Biotronic® Top Liquid at 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 L/ton of diet as coded as T1 to T6 respectively.

effective, relatively cheap, and easy to use. Furthermore, the growing awareness from consumers and producers of aquaculture species has resulted in calls for responsible and sustainable aquaculture. Public opinion and regulation authorities in most export countries, especially in Europe and US, focus now on the uses and misuses of antibiotics in aquaculture due to antimicrobial resistance (AMR) (Santos and Ramos, 2018). Due to public awareness and attention, there is a call to shift production methods without the use of antibiotics. It has been a big challenge, especially, for aqua culturists to save their fish or aquatic animals during their culture when ban on the use of antibiotics is becoming a must by the rules. EU has banned, with effect of January 2006, all antibiotic growth promoters (AGP) from livestock production since the use of low levels of these antibiotics in animal feeds increases the bacterial resistance in the species pathogenic for animals but also in humans. It is expected that more countries will follow the same policy, and in the case of Asian countries which export seafood to EU, they will need to comply with European recommendations and their standards. Due to the above-mentioned facts, alternatives strategies needed to be explored. The good result of the present research with BTL showed that it could be a promising alternative for the use of in-feed antibiotics commonly applied in aquaculture. More research should also be done for other species of fish in addition to Nile tilapia, and shrimp/prawn exposing to various other types of bacteria and pathogens, which have been the hurdles in tilapia industry, and overall aquaculture expansion for food security of the people.

Conclusion and Recommendation

BTL enhances growth, feed conversion efficiency, protein efficiency ratio, and reduces feed conversion ratio when supplanted at the dose ranging from 1.5 to 2.5 L/ton. Its effects are prominent in presence of bacteria, especially *A. hydrophila* at the doses higher than 1 L/ton of feed. Higher the doses of BTL supplementation lower will be the bacterial counts. When fish are challenged with bacteria, BTL supplementation increased overall survival of Nile tilapia by about 25% with the rate of nearly 9% per liter of BTL. The supplementation of BTL resulted in 3.4 times higher specific growth rate, 1.6 times daily weight gain and 2.1 times protein efficiency ratio of the fish with bacterial challenged group as compared to that of the group without challenge. The trend indicates that the higher doses than tested i.e. 2.5 L/ton could still be beneficial for growth; however, more research is needed to confirm it. At the same time, whether increased level would be economic needs to be tested. Nevertheless, the 2.5 L/ton, the highest tested dose, is recommend for supplementation based on the present study. More research is also needed to test the product for different culture conditions and extended periods.

Ethical Statement

All applicable national and institutional guidelines for the care and use of animals were followed.

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Author Contribution

Ram C. Bhujel: Conceptualization, Writing -review and editing; Anusha D. Perera: Data Curation, Formal Analysis, Investigation, Methodology, and Writing - original draft; Antonia Tacconi: Funding Acquisition, Project Administration, Resources, Writing -review and editing; and Rui A. Gonçalves: Supervision, Writing - review and editing.

Conflict of Interest

Authors declare that there is no conflict of interest.

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