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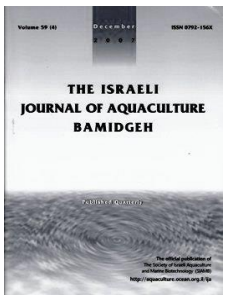

Use of Lactic Acid Bacteria from Nile tilapia *Oreochromis niloticus* as Probiotics for Sustainable Production and Improvement in Fish Welfare

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Key words: natural probiotics, *O. niloticus*, growth, *L. fermentum,* immune responses, fish welfare

Abstract

Two studies were conducted to investigate the effectiveness of lactic acid bacteria (LAB) from the intestine, gills, and skin of Nile tilapia *Oreochromis niloticus* as probiotics to promote growth, bolster the immune system, and improve general fish welfare. Results of the first study including LAB characterization indicated four major strains in the three organs as *Lactobacillus fermentum* (60.0%), *L. brevis* (16.7%), *L. acidophilus* (13.3%) and *L. xylosus* (10.0%). Safety tests of these LAB isolates conducted on some samples of the fish confirmed that they were non-pathogenic. In the second study *L. fermentum*, which showed the greatest promise as a probiotic, was used at different dilution levels to prepare diets for feeding *O. niloticus* juveniles weighing 18.1±0.1g. Diets 1-5 contained 0 colony forming unit (cfu)/g, 10^3cfu/g, 10^5cfu/g, 10^7cfu/g and 10^9cfu/g of *L. fermentum*, respectively. The feeding trials in fish fed diets 1-5, resulted in growth from the initial weight of 18.1g to 32.0^e, 46.0^a, 44.3^b, 43.9^c and 40.8^d g, respectively. Inclusion of *L. fermentum* in the diets increased carcass protein, pack cell volume, hemoglobin, RBC, and reduced glutamate ($P<0.05$). Challenge tests conducted on the fish using pathogenic *Pseudomonas aeruginosa* indicated that the diets supplemented with *L. fermentum* improved fish immune responses. In conclusion, the studies revealed that LAB from *O. niloticus* can act as probiotics to improve growth, immune responses, and fish welfare.

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Introduction

Aquaculture is the fastest growing food industry in the world. In 2012, the total world aquaculture production was 59.7 million metric tons (FAO 2013). Based on the current level of fish production and consumption, this sector is anticipated to supply a total of 80 million tons of fish by the year 2050. Consequently, as aquaculture production intensifies, the incidence of bacterial and disease infections in aquaculture systems increase. This necessitates the use of more chemotherapeutics and antibiotics to keep infections in check. Meanwhile, the current level and quantity of chemotherapeutics and antibiotics used in aquaculture are problematic because they have led to emergence of antimicrobial resistance among pathogenic bacteria (Gatesoupe 2002). The abuse of these antimicrobial agents in the aquaculture industry is of great public health concern as it has resulted in the presence of antibiotic residues in fish, and development of resistant bacteria in the environment (Nwanna, 2010). This has led to proposals for research into the use of probiotics as an alternative. Probiotics are biological control agents that are cheap, reliable, and non-toxic to people who eat the fish (Gatesoupe 2002). The use of probiotics in fish diets may have positive effects on growth due to increased levels of gastrointestinal tract enzymes (GIT) resulting in rapid digestion of carbohydrates, protein, and transformation into energy (Gatesoupe, 2002), diminishing mortality by disease, antagonism to pathogens, better microbial intestinal balance, and for the environment (Thompson et al 2005). LAB are potential probiotics for fish (Irianto & Austin, 2002). However, more information is needed on the effect of specific LAB on Nile tilapia *Oreochromis niloticus* welfare, the site locations, and abundance of specific LAB in particular fish species. Commercial probiotics are expensive and add to the total cost of fish feeds. Therefore, we evaluated the effectiveness of natural probiotic bacteria extracted from various organs of *O. niloticus* for improving growth, immune responses, and welfare of the fish. Site locations and abundance of specific LAB were also considered. Two studies were conducted; the first, to isolate, identify, characterize, and culture, the species of Lactic Acid Bacteria (LAB) from *O. niloticus* as probiotics. The second was to evaluate the effect of the most promising probiotics (from the first study) on growth, and hematological and plasma biochemical properties of the fish, and to determine the ability of the fish fed probiotics to withstand pathogenic attack using *Pseudomonas aeruginosa* in a challenge test.

Materials and Methods

Study 1: *Sample collection, Isolation, and Identification of probiotics bacteria*. Eighty samples of juvenile *O. niloticus*, (18–18.2 g), were taken randomly from the production pond at the Fish farm unit of Department of Wildlife and Fisheries Management, University of Ibadan, Ibadan, Nigeria. They were anesthetized using 150 mg/l solution of MS-222 (Tricaine methanesulfonate). Bacterial strains were then isolated from the intestine, gills, and skin, and evaluated for their probiotic quality. Samples from the intestine, gills and skin of the fish were cultured in de Man Rogosa and Sharpe (MRS) medium and incubated at 30°C until growth was visible. The isolated bacteria were purified by subculture in a maintenance medium consisting of MRS broth with 12% (w/v) glycerol. After the biochemical and morphological analysis, the bacteria were identified according to the methods of Austin and Austin (1993) and AP120E strip system (bioMerieux).

Antimicrobial activity. Antimicrobial activity or antagonistics test were conducted to determine the inhibition zone of the bacteria isolates (*L. fermentum, L. brevis, L. acidophilus, L. xylosus*) against a pathogenic organism *Pseudomonas aeruginosa*, which was obtained from the Microbiology unit, Department of Botany and Microbiology, University of Ibadan, Nigeria. The antagonistic activity of these probiotics was examined using a medium of Mueller and Hinton agar in the well-diffusion assay method according to Schillinger and Lucke, (1989). The inhibition zone was determined according to Schillinger and Lucke (1989). A drop of supernatant of the isolates was introduced into the wells using a sterile dropper. The sterilized plates were seeded with indicator organisms, incubated aerobically at 30°C for 24 h, after which they were checked for the appearance of inhibition zone which was scored as positive if the width of the clear zone was 0.5mm or larger.

Safety tests of the two most promising LAB on O. niloticus. Safety tests were conducted on the two most promising LAB (*L. fermentum, L. brevis*) isolates to determine whether they were pathogenic or non-pathogenic to fish before using them as probiotics for the fish (Fig. 1). This was done using 60 healthy *O. niloticus* fish (18–18.2 g). The fish were acclimatized for two weeks in indoor tanks and later divided into 3 equal groups comprising 20 fish per treatment. They were anesthetized in 150 mg/l solution of MS-222 (Tricaine methanesulfonate). Group 1 served as the control and was injected intra-peritoneally with 0.3ml sterile normal saline solution, while groups 2 and 3 were injected with 0.3 ml of saline containing 10^7 cfu/ml *L. fermentum* and 10^7 cfu/ml *L. brevis.* All groups were observed for two weeks to monitor mortality and morbidity rates. The fish were subjected to laboratory examination and bacterial re-isolation. This test was very important to ensure that the LAB isolated was non-pathogenic.

Multiplication of the most promising LAB (L. fermentum) for fish diets. *L. fermentum,* which demonstrated the most promising probiotic activity after the antagonistic and safety tests (Table 2), was prepared for inclusion in the diet of *O. niloticus*. The preparation of probiotic bacteria was carried out by inoculating MRS broth with the isolates. The broth was then at 30°C for 48 h, then centrifuged at 3000rpm for 30 min. After centrifugation, the bacteria were washed twice with sterile saline. Bacterial suspensions were prepared containing 10^3, 10^5, $10^7$and 10^9 cfu/g bacterial cells per ml of saline solution.

Study 2: Diet preparation/fish feeding trial. The bacterial suspension prepared was added to basal diets containing 30% crude protein and 4.608 KJ/g gross energy (Table 1).

Table 1. Physical and chemical characteristics of strains of LAB from the *O. niloticus*

Criteria	*L. fermentum*	*L. brevis*	*L. acidophilus*	*L. xylosus*
Gram reaction	+ve[1]	+ve	+ve	+ve
Cellular morphology	Rod	Rod	Rod	Rod
catalase	-ve[2]	-ve	-ve	-ve
Oxidase	-ve	-ve	-ve	-ve
Indole test	-ve	-ve	-ve	-ve
Starch	-ve	-ve	-ve	-ve
Motility	-ve	-ve	-ve	-ve
MR Methyl Red	+ve	+ve	+ve	+ve
H_2S production	-ve	-ve	+ve	-ve
Glucose	+ve	+ve	+ve	+ve
Lactose	-ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve
Salicin	-ve	+ve	+ve	-ve
Fructose	+ve	-ve	+ve	+ve
Xylose	+ve	+ve	-ve	+ve
Maltose	+ve	+ve	+ve	+ve
Ribose	-ve	-ve	+ve	+ve
Raffinose	+ve	+ve	+ve	+ve
Melibiose	-ve	+ve	+ve	-ve
Rhamanose	-ve	-ve	+ve	-ve
Trehalose	-ve	-ve	+ve	+ve
Sonbose	-ve	+ve	-ve	-ve
Mannitol	+ve	+ve	-ve	+ve
Galactose	+ve	+ve	+ve	+ve
NH_3 from Arginine	+ve	-ve	+ve	+ve
Growth on TC@4%	+ve	-ve	-ve	+ve
Growth on TC@15%	+ve	-ve		+ve
Growth on TC@ 45%	+ve	-ve	-ve	+ve
Homo (HM)/Hetero Fermentation (HE)	HE	HE	HE	HM
Growth @ 4% NaOH	-ve	-ve	-ve	-ve

[1.] means positive that is present
[2.] means negative that is absent

Table 2. Safety test of *L. fermentum* and *L. brevis* on *O. niloticus* after 2 weeks observation

Isolates	*Control (0.9% SS[a])*	*L. fermentum (10^7 cfu/ml)*	*L. brevis (10^7 cfu/ml)*
No. of fish injected	20	20	20
Route of injection	IP[b]	IP	IP
Ave. wt. of fish (g)	18.1±0.93	18.0±0.04	18.0±0.01
Dosage	0.3ml	0.3ml	0.3ml
Mortality	1	0	0
Survival (%)	95	100	100

[a] Saline solution
[b] Intraperitoneally

The suspensions were added to the basal diet to make up five treatments, (T1-T5) including basal diet without *L. fermentum*, (DL_0 - control), basal diet containing 10^3 cfu/g (DL_1), 10^5 cfu/g (DL_2), 10^7 cfu/g ($DL_{3)}$ and 10^9 cfu/g (DL_4), respectively (Table 3). The experimental diets were passed through a clean, sterile, auger Spaghetti Machine with 2mm dice for proper pelletization. The diets were kept at an ambient temperature for cooling and drying before being packed into black plastic bags. They were stored at −4°C This preparation procedure was repeated every two weeks.

Table 3. Gross composition of the diets (g/100g)

Ingredients	*Diets*				
	DL_0	*DL_1*	*DL_2*	*DL_3*	*DL_4*
Fish Meal	23.0	23.0	23.0	23.0	23.0
Soya bean meal	33.5	33.5	33.50	33.5	33.5
Yellow Maize	37.0	37.0	37.00	37.0	37.0
Vegetable Oil	3.00	3.00	3.00	3.00	3.00
Vitamin and mineral premix[1]	0.40	0.40	0.40	0.40	0.40
Methionine	0.40	0.40	0.40	0.40	0.40
Starch	0.70	0.70	0.70	0.70	0.70
Probiotic concentration(CFU/g)	-	10^3	10^5	10^7	10^9
N.F.E	36.4	37.2	36.8	37.4	37.8

g/Kg diet Vitamin and Minerals: Vitamin A –10,000,000 I.U.; D3- 2,000,000 I.U.; E –23,000mg; K3 – 2,000 mg; B1 – 3,000 mg; B2- 6,000 mg; Nacin– 50,000 mg; Calcium Pathonate – 10,000 mg; B6 – 5,000 mg; B12-25.0 mg; Folic acid 1,000 mg; Biotin- 50.0 mg; Choline chloride – 400,000 mg; Manganese – 120,000 mg; Iron- 100,000 mg; Copper– 8,500 mg; Iodine – 1,500 mg; Cobalt-300 mg; Selenium-120 mg; Antioxidant 120,000 mg.
DL_0 = diet without *Lactobacillius fermentum*
DL_1 = diet with 10^3 *L. fermentum*
DL_2 = diet with 10^5 *L. ferm*entum
DL_3 = diet with 10^7 *L. fermentum*
DL_4 = diet with 10^9 *L. fermentum*

Fish feeding, culture system and experimental design. *O. niloticus* juveniles (18.1 ± 0.1 g), were obtained from a production pond in the fish farm unit of Department of Wildlife and Fisheries Management, University of Ibadan, Nigeria. The fish were acclimatized in an indoor concrete tank for two weeks, and then randomly stocked at a rate of 18 fish per 100-l glass tanks. Each tank was supplied with fully aerated tap water, flow rate of 1.5 l/min. The fish were fed to apparent satiation twice daily for 90 days. The experiment consisting of 5 treatments which represented varying levels of probiotic inclusion in the diet as follows: (DL_0) basal diet (control), basal diet containing 10^3 cfu/g (DL_1); 10^5 cfu/g (DL_2); 10^7 cfu/g (DL_3) and 10^9 cfu/g (DL_4) respectively, was replicated 3 times. Fish were weighed bi-weekly and growth parameters were calculated. At the end of the experiment fish were counted and weighed. The growth parameters and feed utilization indices were calculated as follows: Weight gain = Final wt. – initial wt. Specific growth rate (SGR) = 100 (Ln W_2 − Ln W_1)/T where W_1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period; Feed conversion ratio (FCR) = Feed intake (g)/Weight gain (g); Protein efficiency ratio (PER) = Weight gain (g)/Protein intake (g).

Proximate composition analysis. Proximate composition of the diets and fish (whole body) from each treatment, were analyzed according to the standard methods of AOAC (1990).

Hematological Assessment. This was carried out to determine the effect of the bacteria on the blood parameters as a means of assessing the health status of the fish. After the

feeding trials, five fish from each tank were randomly selected. They were anesthetized using 150 mg/l solution of MS-222 (Tricaine methanesulfonate). Blood samples were collected with a sterile syringe, from the caudal vein, using EDTA-disodium as an anticoagulant. The blood samples were used for determining the erythrocyte-count and hemoglobin content (Stoskopf, 1993). The hematocrit value (HCT) was calculated according to Stoskopf's method (1993). Plasma was obtained by blood centrifugation at 3000 rpm for 15 min. The plasma was stored at -70^{o}C for further biochemical analyses.

The total protein-content was determined by buiret method described by Reitman and Frankel (1957). Plasma Albumin was estimated colorimetrically, while plasma globulin was calculated by mathematical subtraction of the albumin value from total protein. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and glutamate were determined according to methods of Reitman and Frankel (1957).

After the feeding trials, 20 fish were randomly selected from each treatment group for challenge tests using *P. aeruginosa* bacteria, conducted on *O. niloticus* fed diets with *L. fermentum*. They were anesthetized using 150 mg/l solution of MS-222 (Tricaine methanesulfonate). The test was conducted according to the method of Austin *et al.* (1995). The fish were injected intra-peritoneally with 0.3 ml of 10^7 cells/ml pathogenic *P. aeruginosa*. The fish were observed for 14 days to record any abnormal clinical signs and mortality.

Statistical analysis. All data were analyzed using descriptive statistics and analysis of variance (ANOVA). Duncan's multiple range test (Duncan, 1955) was used to separate the means among different treatments at (P=0.05) significant levels.

Results

Bacteriological examinations revealed the suspected probiotic bacterial isolates as lactic acid bacteria. Twenty-five lactic acid bacteria isolates were identified and then categorized into four main strains (*Lactobacillus fermentum*, *L. brevis*, *L. acidophilus* and *L. xylosus*) (Fig. 1). Most of these strains were isolated from the gills of the fish (Fig. 2).The physical and biochemical characteristics of these strains are presented in Table I.

The in-vitro probiotic activity of the LAB isolates (Fig. 3) confirmed inhibitory effects against *P. aeruginosa*. Inhibitory activity is used to confirm organisms as probiotics. The largest inhibition zone was produced by *L. fermentum,* followed by *L. brevis*, *L. acidophilus* and *L. xylosus* in that order. The LAB isolates from the present study demonstrated strong probiotic qualities and *L. fermentum* which had the largest inhibition zone (best probiotic activity) was selected for growth trials in the second study.

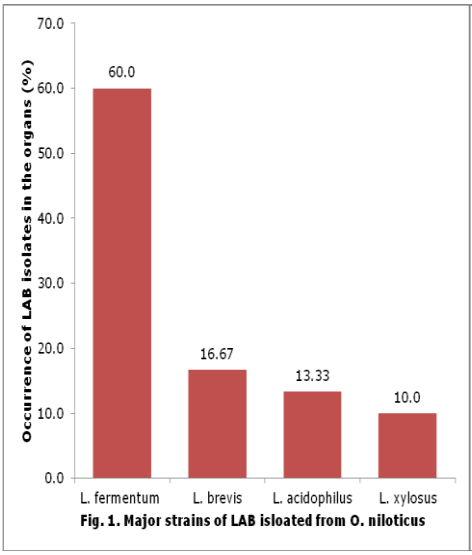

Fig. 1. Major strains of LAB isloated from O. niloticus

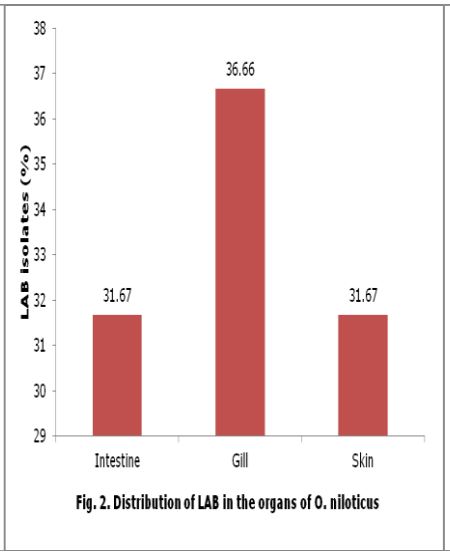

Fig. 2. Distribution of LAB in the organs of O. niloticus

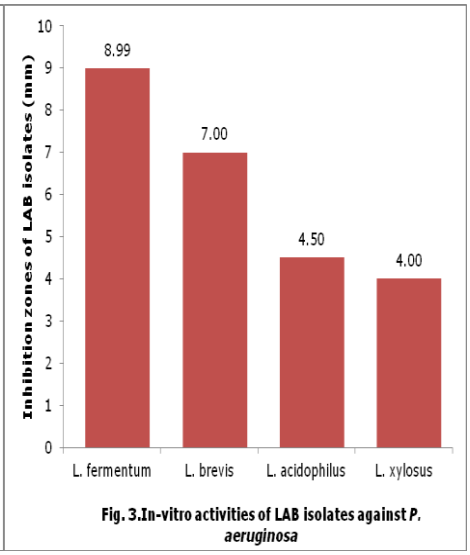

Fig. 3.In-vitro activities of LAB isolates against *P. aeruginosa*

The physical and biochemical characteristics of the LAB isolated from *O. niloticus* confirm the isolates as lactic acid bacteria, because they are rod shaped and gram positive (+ve) in reaction. They showed no reaction with NaOH medium because they are known for their increased tolerance to low pH. These characteristics makes LAB superior to other bacteria in the gastrointestinal tract (GIT) of fish which is acidic in nature and during processes of natural fermentation LAB withstand increased acidity from organic acid production.

Safety tests of *L. fermentum* and *L. brevis* showed that *L. fermentum* and *L. brevis* were harmless to *O. niloticus* since no clinical signs or mortalities were observed (Table 2). In the test using saline solution as the control, survival was 95%, and the 5% mortality recorded could be due to stress.

The composition of the experimental diets was identical except for the amount of *L. fermentum* added (Table 3). This was reflected in the proximate composition of the diets as the values were very closely related.

The growth response and nutrient utilization, i.e. the final weight, mean weight gain, specific growth rate, food conversion efficiency, and protein efficiency ratio of *O. niloticus* fed diets supplemented with *L. fermentum* significantly improved in relation to fish fed a diet without *L. fermentum* (Table 4). No significant difference ($P>0.05$) in the mean weight gain, SGR, and PER, was seen between the treatment diets containing 10^3, 10^5 and 10^7 cfu/g of the bacteria. But the mean weight gain and PER of fish in these groups were significantly higher ($P<0.05$) than those of the fish fed diets without the bacteria or diets containing 10^9 cfu/g of the bacteria. Growth performance of the fish fed a diet 10^3 cfu/g of bacteria was either statistically or numerically best indicating that this level could be optimal for fish growth. The growth performance of the fish decreased as dilution levels of the *L. fermentum* added to the diets increased.

Table 4. Growth response and nutrient utilization of O. niloticus fed the experimental diets

Growth parameters	Diets/Treatments				
	DL_0	*DL_1*	*DL_2*	*DL_3*	*DL_4*
Initial mean wt (g)	18.1±0.09	18.1±0.36	18.1±0.13	18.2±0.21	18.1±0.18
Final mean wt (g)	32.0±0.45^{e}	46.0±0.32^{a}	44.3±0.49^{b}	43.9±0.36^{c}	40.8±0.32^{d}
Mean wt gain	13.9±0.01^{c}	27.9±0.02^{a}	26.1±0.03^{a}	25.8±0.03^{a}	22.7±0.03^{b}
Weight gain (%)	76.9±0.01^{d}	159.0±0.02^{a}	143.3±0.03^{b}	142.1±0.01^{b}	125.0±0.03^{c}
Feed conversion ratio	3.57±0.02^{a}	2.50±0.00^{ab}	2.58±0.02^{a}	2.61±0.03^{a}	2.60±0.02^{a}
Specific growth rate	0.63±0.04^{b}	1.04±0.21^{a}	0.99±0.05^{a}	0.98±0.04^{a}	0.91±0.12^{a}
Protein efficiency ratio	12.2±0.03^{c}	24.2±0.21^{a}	22.8±0.04^{a}	22.5±0.12^{a}	19.8±0.02^{b}

Mean values on same row with similar superscripts are not different ($P>0.05$)

The proximate composition of the experimental (whole) fish indicated that the crude protein of fish fed diets with *L. fermentum* was significantly higher than in fish fed a diet without *L. fermentum.* The ash, moisture, and lipid contents were not statistically different, and demonstrated no specific trend (Table 5).

Table 5. Proximate composition of whole body of *O. niloticus* fed experimental diets

Diets	*Crude protein (%)*	*Crude fat (%)*	*Moisture (%)*	*Ash (%)*
DL_0 (Control	50.3±0.02	4.35±0.028^{a}	7.47±0.14^{a}	4.28±0.007^{a}
DL_1(10^3CFU/g)	52.9±0.06^{a}	4.57±0.01^{a}	6.35±0.04^{a}	4.21±0.02^{a}
DL_2(10^5CFU/g)	52.6±0.01^{a}	4.54±0.01^{a}	6.95±0.01^{a}	4.29±0.01^{a}
DL_3(10^7CFU/g)	52.7±0.01^{a}	4.53±0.01^{a}	7.04±0.01^{a}	4.26±0.03^{a}
DL_4 (10^9CFU/g)	52.2±0.01^{a}	4.45±0.01^{a}	7.04±0.04^{a}	4.25±0.01^{a}

Mean values in the same column with similar superscripts are not different ($p>0.05$)

The hematological profile, PCV, Hb, and RBC, of all the fish fed diets supplemented with *L. fermentum* showed significant improvement over the fish fed a diet without *L. fermentum*. The levels decreased with increasing dilution levels of *L. fermentum* in the diets, while the WBC of all the fish was not affected, suggesting that inclusion of *L. fermentum* into the diets did not cause additional stress in the fish (Table 6).

Table 6. Hematology of O. niloticus fed the experimental diets

	Parameters			
	Pack cell volume (%)	*Hemoglobin (g/dl)*	*Red blood cells($x10^6$)*	*White blood cells ($x10^3$ µl)*
DL_0	22.2±0.02[c]	4.50±0.03[c]	1.80±0.04[c]	2.55±0.13[a]
DL_1	27.6±0.02[a]	7.35±0.03[a]	2.86±0.04[a]	2.60±0.01[a]
DL_2	25.9±0.02b	7.05±0.01[a]	2.64±0.02[a]	2.56±0.03[a]
DL_3	24.5±0.04[b]	6.90±0.03[b]	2.57±0.03[b]	2.55±0.03[a]
DL_4	23.9±0.03[c]	6.80±0.12[b]	2.47±0.00[b]	2.55±0.01[a]

Mean values in the same column with similar superscripts are not significantly different (P>0.05)

The plasma biochemistry indicated that diets supplemented with *L. fermentum* greatly reduced the glutamate contents of the fish. The addition of *L. fermentum* to the diets also caused a marginal increase in the concentration of aspartate aminotransferace, total protein, globulin, and alanine aminotransferace (Table 7).

After the challenge test the control group had the highest mortality rate (100%) during the 2 weeks observation period. Survival rate decreased with increasing dilution levels of probiotics in the diets (Table 8). No apparent clinical signs were observed in fish given probiotic diets after the challenge test, but fish in the control group had lesions all over the body, their gills were ulcerated, and part of the fins were removed.

Table 7. Plasma biochemistry of *O.niloticus* fed the experimental diets

	Parameters						
	Creatinine (mg/dl)	*AST[w] (IU/L)*	*ALT[x] (IU/dl)*	*GLU[y] (mg/dl)*	*Tt. Prot[z] (g/dl)*	*Albumin (g/dl)*	*Globulin (g/dl)*
DL_0	1.22±0.03[a]	74.0±6.04[a]	2.20±0.04[a]	81.5.0±0.02[a]	3.70±0.02[a]	1.60±0.02[a]	2.10±0.12[b]
DL_1	1.18±0.03[a]	78.9±6.04[a]	2.70±0.03[a]	66.5±0.03[b]	5.75±0.04[a]	1.50±0.03[a]	4.25±0.03[a]
DL_2	1.25±0.05[a]	78.5±5.02[a]	2.50±0.12[a]	67.0±0.02[b]	4.80±0.10[a]	1.54±0.02[a]	3.60±0.04[b]
DL_3	1.26±0.04[a]	77.9±6.01[a]	2.48±0.03[a]	61.0±0.03[c]	4.60±0.03[a]	1.56±0.12[a]	3.40±0.13[b]
DL_4	1.17±0.02[a]	75.0±4.02[a]	2.45±0.12[a]	69.0±0.03[a]	4.25±0.02[a]	1.58±0.04[a]	3.00±0.03[b]

[w]Aspartase
[x]Alanine aminotransferase
[y]Glutamate
[z]Total protein

Table 8. Challenge test on O. niloticus fed the experimental diets

	Diets				
	DL_0	*DL_1*	*DL_2*	*DL_3*	*DL_4*
No. of fish injected	20	20	20	20	20
Route of injection	IP[a]	IP	IP	IP	IP
Dosage of bacteria (cfu/ml)	0.3ml of 10^7	0.3ml of 10^7	0.3ml of 10^7	0.3ml of 10^7	0.3ml of 10^7
Mortality (No.)	20	0	1	2	2
Survival (%)	0	100	95	90	90

[a] intraperitoneally

Discussion

Based on the physical and biochemical characterization according to Austin and Austin (1993) the isolated bacteria from gills, skin, and intestine of the *O. niloticus*, were determined to be lactic acid bacteria (LAB) of the species *Lactobacterium fermenttum, L. brevis*, *L. acidophilus* and *L. xylosus*. The highest concentration of LAB was in the gills, followed by intestine, and skin. This indicates that LAB is distributed in most parts of the fish, not only in the gastrointestinal tract (GIT). In vitro assay showed that all of the isolated LAB, showed an inhibitory zone against *P. aeruginosa*. *Lactobacillus* species have been isolated from the intestine of common carp *Cyprinus carpio*, and the isolates also produced an inhibitory effect against *Aeromonas species* (Hagi and Hoshino, 2009). *Lactobacillus* species have been isolated from the intestinal microbiota of rainbow trout

Onchorhynchus mykiss and the probiotic properties of these isolates against several pathogens were characterized (Balcázar et al., 2008). Lactic acid bacteria are not pathogenic and may be used as probiotics for controlling infectious diseases in fish (Hagi & Hoshino, 2009). The safety tests of the isolated bacteria (*L. fermentum* and *L. brevis*) in this study also confirmed them as probiotics. The inhibitory effect of the isolated LAB against *P. aeruginosa* could be attributed to their natural adherence to the GIT of fishes.

The outstanding growth performance and nutrient utilization of the fish fed diets supplemented with *L. fermentum* over those fed diets without the bacteria has been confirmed using different types of probiotics individually or symbiotically (Mohapatra et al., 2012). This effect of *L. fermentum* may be linked to improvement in the intestinal microbial balance of fish (Bucio *et. al.*, 2004), that out-compete the pathogenic bacteria by boosting the gastrointestinal tract LAB, and hence reducing the pathogenic flora thus leading to increased food absorption (Hagi and Hosino 2009). The inclusion of probiotics in diets may significantly help to stimulate appetite and improve nutrition through production of vitamins, detoxifying toxic compounds in the diets, and also aid the breaking down of indigestible compounds (Hagi and Hosino, 2009). Probiotics increase the protease, amylase, and cellulose activities in grass carp (*Ctenopharyngodon idella*) and hence improve diet digestibility, which might in turn explain improved growth in fish fed diets supplemented with probiotics (Wang, 2011). Probiotics have reduced the incidence of vertebral compression in rainbow trout, thus strengthening the vertebral column leading to firm attachment of fish muscles, increased weight gain in fish, and improved fish growth (Aubin et al., 2005). This information regarding growth performance of the fish confirms *L. fermentum* as a probiotic which can promote growth in *O. niloticus*. The *Lactobacillus* species can be used as probiotic agents to enhance the growth of *O. niloticus* and *C. carpio* (Abdel Hamid, 2008). The improved feed conversion efficiency in fish fed diets supplemented with *L. fermentum* suggests that probiotics can enhance feed utilization (Lara-Flores et al., 2003). The enhanced protein efficiency ratio in all the fish fed diets with *L. fermentum* suggests that the bacteria may be involved in optimizing the use of dietary protein. This could reduce wastage since more than 70% of the cost of fish feeds is protein. Probiotics have been found to improve growth, protein efficiency ratio, and feed conversion ratio, in rohu, *Labeo rohita* (Mohapatra, et al., 2012). Improvement in the biological value of probiotic supplemented diets enhances conversion of dietary protein to flesh in fish (Gatesoupe, 2002).

The function of probiotics is to bio-degrade anti-nutritional factors in feeds thereby improving their nutritional quality. This may have improved the carcass quality of the fish in this study, resulting in deposition of more protein and reduction in moisture content. Appreciable variation in the protein and fat contents of the fish body may be attributed to changes in their synthesis and deposition rate (Irianto and Austin, 2002). This suggests that *L. fermentum* (probiotics) may be involved in protein synthesis. *Bacillus subtilis* enhanced protease levels resulting in increased feed absorption and deposition of protein in common snook fish, *Centropomus undecimalis* (Kennedy et al., 1998).

Blood parameters are good bio-indicators or diagnostic tools for studying the effects of diets on organ function, thus providing vital information for health assessment, and management of cultured fish. Elevated levels of PCV, RBC, and Hb, in fish fed diets supplemented with *L. fermentum* provide evidence that probiotics improve the immune system of the fish. Similar increases in RBC of fish fed with diets fortified with probiotic bacteria have been reported (Irianto and Austin, 2002). Probiotic bacteria may have an impact on the specific and innate immunity of *O. mykiss* (Nikoskelainen et al*.,* 2003). Non-statistical values of WBC in this study indicated that *L. fermentum* in diets was not stressful to fish health. The hematological parameters showed improved fish health. Blood parameters of Indian major carp Rohu *Labeo rohita* were stimulated as a result of *B. subtilis* supplementation in fish diets (Rajesh et al., 2006).

Changes in the biochemical profile of plasma can be used to monitor stress, health status, or the internal environment of fish (Edsall, 1999). The almost identical activity of aspartate aminotransferase, and creatinine kinase, observed in both the control group and the treatment groups*,* indicates that the bacteria did not cause any damage to the muscle tissues, or change the permeability and integrity of the cell membranes (Masopust, 1998). The systematic reduction in glutamate infish fed diets supplemented with *L. fermentum*, as compared to the control group, suggests that the bacteria did not

affect glutamate activity. Potential probiotic lactic acid bacterial strains *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* in mice did not affect the enzyme glutathione peroxidase, a component of the antioxidative defence system (Zhou et al., 2000). The increase in plasma total proteins and globulin from the group of fish fed diets with *L. fermentum*, indicates improved health status of the fish. When Japanese eels were injected with *Vibrio anguillarum*, a pathogenic bacterium, total plasma protein was reduced (Cruz et al., 1989). However, increased alanine activity in *C. carpio* after exposure to *Cyanobacteria* extract could be due to the severe damage of some organs such as liver, spleen, muscle and kidney (Palikova et. al., 2004). In the present study, increased alanine aminotransferase of the fish fed diets containing *L. fermentum* could not be explained as there were no observable physiological or pathological effects on the fish.

Using *P. aeruginosa* for the challenge test, fish fed diets supplemented with *L. fermentum*, demonstrated that *L. fermentum* actually boosted the immune system of the fish. Probiotics also enhanced the non-specific immune parameters such as lysozyme activity, migration of neutrophils, and plasma bactericidal activity of tilapia, enhancing resistance to *Edwardsiella tarda* infection in the fish (Abd El-Rhman et al*.,* 2009). Reduced cumulative mortality was found in atlantic cod *Gadus morhau* fed probiotic diets and challenged with pathogenic *V. anguillarum* (Gilberg et al., 1997). Resistance of *O. mykiss* was enhanced following oral administration of *B. subtilis* and *B. licheniformis* (Raida et al., 2003).

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