Ethiopian Journal of Science and Sustainable Development

e-ISSN 2663-3205 *Volume 11 (1), 2024*

Journal Home Page: www.ejssd.astu.edu.et

Research Paper

Bacterial-resistance, growth and immune competence of *Oreochromis niloticus* fed *Lactobacillus fermentum*

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Article Info

Article History:
Received 18 July 2023
Received in revised form 22 January 2024
Accepted 04 February 2024

Keywords:
gut,
innate immune,
L. fermentum,
Nile Tilapia,
probiotics

Abstract

Aquaculture production can be boosted with enhanced feed efficiency, growth and disease resistance using probiotics. In this study, the effects of *Lactobacillus fermentum* (LF) probiotic on growth, intestine, immune competence of *Oreochromis niloticus*, and the resistance to virulent *Aeromonas hydrophila* were investigated. A basal (LF1) diet (35% crude protein) was formulated and five treatments with *L. fermentum* included at 1 x 10^3 cfu/g (LF2), 1 x 10^5 cfu/g (LF3), 1 x 10^7 cfu/g (LF4), 1 x 10^9 cfu/g (LF5) and 1 x 10^{11} cfu/g (LF6). The six diets were each allotted to three groups of *Oreochromis niloticus* (1.85±0.01g mean weight) for 90 days. After which fish were injected with *A. hydrophilia*, fed and observed for 15 days. At the end of the 90 days, LF diets fed fish had higher ($p<0.05$) mean weight gain. Feed conversion ratio and specific growth rate were superior in fish fed LF3 and LF4. Villi height was significantly high in LF3, while microbial loads were more in the guts of fish fed probiotics. Blood packed cell volume and haemoglobin increased significantly in the groups fed LF. Hydrogen peroxide and malondialdehyde reduced significantly ($p<0.05$) in the liver and kidney of fish fed *L. fermentum*, while glutathione transferase and glutathione peroxidase activities were increased. The challenge test with virulent *Aeromonas hydrophila* showed improved resistance in fish fed probiotic. Thus, the study disclosed improved growth performance, enhanced health status and better survival with the supplementation of *Lactobacillus fermentum* in the diets of *Oreochromis niloticus*.

1. Introduction

Fish disease outbreaks in aquaculture is a major impediment to the continuous growth of fish food producing sector, which has been described as the world's fastest growing (Subasinghe *et al*., 2003; FAO, 2018). This is evident in the reduction in growth rate from the 10.8 and 9.5% recorded in the 80's and 90's, respectively to 5.8 % between 2001 and 2016 (FAO, 2018). It is therefore, important to establish ways to effectively control and treat diseases in cultured fish, so as to ensure increased production, more profit to farmers, and quality fish and fish products.

Bacterial infections are the major causes of mortality in fish (Austin and Austin, 2007) and motile *Aeromonas*, especially *Aeromonas hydrophila*, has been identified in most freshwater fish diseases (Pridgeon and Klesius, 2011). *A. hydrophila* comprises gram-negative motile, straight rods belonging to the family Aeromonadaceae. They are notorious for increased

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https://doi.org/10.20372/ejssdastu:v11.i1.2024.716

capacity to uptake and exchange antimicrobial resistance genes and they exhibit several antibiotics resistance pattern (Ogbonne *et al*., 2020). According to the authors, isolates of *A. hydrophila* were completely resistant to some antibiotics, while some resistance levels vary from 55 to 70%. Antimicrobial resistance is a well acknowledged threat to public health globally, and Nigeria is not left out. According to FMAEH (2017), the emergence of multidrug resistant microbes has resulted in exponential increase in mortality and economic losses to farmers in Nigeria.

The control and treatment of these pathogenic bacteria is achieved in the past by routine application of antibiotics (Lewbart, 2001). However, inappropriate and overuse of these compounds resulted in the development of antibiotic resistance strains of bacteria (Omitoyin *et al*., 2019). A survey on antibiotic resistant disease revealed that *A. hydrophila* and *A. sobria* isolated from tilapia hybrid resisted oxytetracycline, erythromycin and sulfadiazine, which were frequently used in treatments and prevention of bacterial diseases in fish (Wang *et al*, 2003). This, coupled with the build-up of antibiotic residues in cultured species and the disruption of the micro flora and fauna in lakes receiving effluents from ponds (Ringo *et al*., 2004), presented major challenges to the continued use of antibiotics and its eventual ban in several parts of the world.

An approach to disease prevention is the reduction or total elimination of pathogenic microbes from cultured fish. This is mostly achieved by feeding live microbial adjunct that are beneficial to the host through the modification of ambient microbial population, enhanced feed utilization and nutritional value, and improved host response towards disease and modulating mucosal and systemic immunity (Vine *et al*., 2004). Bryan (2015) stated that not all probiotics produce the same effects for all fish species; thus, more work is required to establish the effect of specific probiotics (Nwanna *et al*., 2013). *Lactobacillus fermentum* is a probiotic candidate which is reported to produce bacteriocins that inhibits the growth of some pathogens *in vitro* (Ng *et al*., 2009). A number of works have been reported on the use of *L. fermentum* to improve fish resistance to diseases, causing better growth and boosting of immune response (Balcazar *et al.*, 2006; Balcazar *et al.*, 2007). Lactic acid bacteria have been isolated from the skin, gill and gut of fish and used as probiotics (Nwanna *et al*., 2013). Gewaily *et al* (2021) reported improved performance in Nile Tilapia fed on *L. plantarum* diets. *L. fermentum* supplementation resulted in increased weight and nutrient utilization, and improved immune system in common carp, *Cyprinus carpio* (Govindaraj *et al*., 2020). The variations in strains, species and the source of isolation of bacteria, factors coupled with the fish a probiotic is administered to, will most likely influence the results from each trial. This study examines effects of *Lactobacillus fermentum* isolated from cheese-milk on the growth, gut micro flora and immune response of an important freshwater fish in Nigeria and Sub-Saharan Africa, the Nile Tilapia.

2. Materials and Methods

2.1. Experimental site and procedure

The study was carried out in the Department of Aquaculture and Fisheries Management, Wet Laboratory, University of Ibadan, Nigeria. Four hundred and fifty fingerlings of *O. niloticus* (1.85±0.01g) were procured and acclimatized for 14 days to laboratory condition during which they were fed commercial food. Fish were distributed randomly into eighteen 50cm x 34cm x 27cm rectangular plastic aquaria after acclimatization at 25 fish per tank.

2.2. Isolation, identification of *Lactobacillus fermentum*, diet preparation and feeding

Cheese produced from milk was procured in Ibadan and 500 µL of the liquid cheese sample was inoculated to MRS broth (10 µL) and incubated to obtain enriched culture for 48 h at 37 °C. One ml of the culture was thereafter inoculated into 10 ml PBS (pH 2.5) (Erkkila and Petaja, 2000) and allowed to incubate for 3 h. At 2500 g, the culture was centrifuged for 5 min and the survived organisms inoculated in 10 ml MRS broth before incubating at 37ºC for 24 h.

The modified method of Gilliland *et al* (1984) was used for screening against bile salt. MRS broth was used to inoculate the overnight culture and incubated at 37 ºC for 4 h. The enriched culture of the acid-/bile resistant cultivation was serially diluted and thereafter, 0.01ml of 10^{-5} dilution spread into MRS-agar plates and allowed to incubate for 24 h at 37 °C. A number of single colonies were selected at random and then incubated in

MRS broth (10 mL). Isolates were screened using morphological evaluation of the single clones. Films were prepared using the pure isolates earlier obtained, and Gram's stain carried out for morphological characterization under the microscope. Suspected isolates were biochemically identified according to Quinn *et al.* (1994). The *Lactobacillus fermentum* isolates were sub-cultured using MRS broth.

A diet was formulated to have 30% crude protein using Pearson Square Method. Feed ingredients (Table 1) were ground, weighed, thoroughly mixed and made into dough by making it moist using the proper amount of oil and water. The dough was left for an hour and autoclaved at 121 ^{o}C for 25 min, cooled and the sub-cultured *L. fermentum* suspended in oil was added to the basal diet (control, LF1), at 1 x 10^3 cfu/g (LF2), 1 x 10^5 cfu/g (LF3), 1 x 10^7 cfu/g (LF4), 1 x 10^9 cfu/g (LF5) and 1 x 10^{11} cfu/g (LF6) and pelletized using a hand pelletizer with 2 mm die size. Prior to feeding, the viability of the putative probiotics was ascertained (Folorunsho, 2010). A gram of each feed sample was dissolved in 9 ml of distilled water. This was diluted serially from 10^1 – 10^9 cfu/ml. One millilitre was taken from 10^2, 10^4, 10^6, 10^8 and 10^{10} cfu/ml concentration and poured on the MRS agar already prepared in a Petri dish under a sterile environment. These were incubated in an anaerobic environment at 37 ^{0}C for 48 h and the total count of colonies done.

For 90 days under a semi-static condition, triplicate groups of fish were fed the six diets to satiation daily (8.00-8.20 a.m. and 3.00-3.20 p.m.). Water quality was monitored throughout the trials and weight was measured biweekly.

Table 1: Gross composition of the diet for the control and the five treatments

Feed ingredient	Amount (g/100g)
Fish meal	17.13
Soya bean meal	17.13
Ground nut cake	8.56
Yellow maize	26.50
Biscuits waste	26.50
Fish oil	2.00
Fish Premix*	0.50
Lysine	0.60
Methionine	0.50
Vitamin C	0.08
Salt	0.50

*Composition kg^{-1}: Vit. A – 1,000,000 UI; Mg – 2,600 mg; Vit. B1 – 2,500 mg; Zn – 14,000 mg; Fe – 10,000 mg; Vit. B2 – 2,500 mg; Cu – 1,400 mg; Co – 20 mg; I – 60 mg; Vit. B6 – 2,500 mg; Se – 60 mg; Vit. D3 – 400.00 UI; Pantothenic acid – 5,000 mg; Vit. E – 10,000 mg; Vit. K3 – 500 mg; Biotin – 80,000 mcg; Vit. B12 – 3,000 mcg; Vit. C – 35,000 mg; Folic acid – 500 mg; Niacin – 10,000 mg; Chorine – 200,000 mg; Methionine – 130 g; Instill – 5,000 mg; Etoxiquin – 15,000 mg. LF = *Lactobacillus fermentum*

2.3. Chemical analysis

Water quality was monitored using Combined Probe Model 57 (YSI) for dissolved oxygen and temperature, digital pH meter for pH and commercial test kits for ammonia and nitrite. The proximate analysis of the experimental diets and fish, after feeding experiment, on dry matter basis were carried out in accordance with AOAC (2005) in duplicates (Table 2).

Table 2: Gross composition of the diets (g/100g)

Ingredient	Composition (% Dry Mass)					
	LF1	LF2	LF3	LF4	LF5	LF6
Crude protein	35.70±1.0	34.25±2.0	34.05±1.0	35.10±2.0	35.10±1.0	34.30±2.0
Ash	8.70±1.0	9.30±0.5	8.50±1.0	8.10±1.0	8.50±2.0	8.40±1.0
Ether extract	7.70±1.0	8.10±1.5	7.90±1.0	7.70±2.0	8.20±2.0	7.80±1.0
Crude fiber	3.40±1.5	3.20±1.2	3.50±1.6	3.30±1.3	3.10±1.2	3.20±1.2
Moisture	7.80±0.5	7.71±1.1	7.59±0.7	8.24±1.4	7.82±0.4	8.25±1.2

2.4. Growth evaluation

After 90 days of feeding, the biweekly weight and feed intake recorded were used to calculate the growth parameters including treatment means for weight gain (MWG), growth rate (SGR), percentage weight gain (PWG), food conversion ratio (FCR),protein efficiency ratio (PER) according to Castell and Tiews (1980) and percentage survival estimated. Feed intake (g) is sum of fed during experimental period.

$$\textbf{MWG}\ (g) = W2 - W1$$

$$\textbf{SGR} = \{[\mathrm{Log_e}W2 - \mathrm{Log_e}W1] \div [T2 - T1]\} \times 100$$

$$\textbf{FCR} = \text{Feed Intake (g)} \div \text{Weight gain(g)}$$

$$\textbf{PER} = \text{Mean weight gain} \div \text{Protein intake}$$

Survival rate =

$$[\text{No. of fish at Tz} \div \text{No. of fish at Ta}] \times 100$$

Protein intake =

$$\text{Feed intake} \times \%\text{Protein in diets}$$

where: W2 = final weight,
W1 = initial weight,
$\mathrm{Log_e}$= Natural log,
Tz – Ta = experimental period in days,

2.5. Analysis of gut microflora

At the start of the trial, 5 fish were picked randomly after acclimatisation for gut bacterial analysis. Fish were stunned and aseptically gutted using sterile scissors and forceps. Guts (intestine) was kept in sterile plates and taken for isolation, characterisation and identification of bacterial colonies (Ogunbanwo *et al.,* 2003; Ogunshe *et al*., 2007). This process was carried out for six randomly picked fish samples from each treatment after 90 days of feeding.

2.6. Analysis of Blood indices in fish fed experimental diets

Nine fish randomly selected per treatment group were tranquilized in 150 mg/l of Tirana Methane Sulphonate for blood collection. Hematological parameters were determined by the standard methods (Kelly, 1979; Schalm, *et al*., 1975; Adeyemo *et al*., 2007). Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) activities were determined using methods described by Reitman and Frankel (1957), while the colorimetric method of Tietz *et al* (1983) was used to measure alkaline phosphate (ALP).

2.7. Evaluation of gut-morphometric features in experimental fish

The gut-morphometric was carried out as described by Oladele *et al* (2012). Before measurements, villus must have its base clearly embedded in the sub mucosa (10x magnification) and there must not be discontinuity or fold in the body (4x magnification). The villus should also have simple columnar epithelium present at the tip (40x magnification). Five villi were picked randomly from each section and measured in each slide as a field. Five fields were used and the villi lengths, widths and cryptal depths were measured (microns converted to cm) with the aid of software in an Am scope camera (MU900).

2.8. Oxidative Stress

Excised kidney and liver tissues were washed in ice-cold saline solution (normal) and then blotted with filter paper, weighed and homogenized (Teflon homogenizer). Homogenates resulting were cold-centrifuged for 10 min at -4 ˚C and 10,000 rpm to obtain a post mitochondrial fraction (PMF). Biochemical analyses were done using the supernatants collected. Protein concentrations of samples were determined using the Biuret method described by Gornal *et al.* (1949). For evaluation of nitrogen oxide (NO) production, the level of Nitrite (an indication of NO) in the liver and kidney tissues were measured using Griess reagent (Olaleye *et al.*, 2007). The activity of superoxide dismutase (SOD) in samples was determined according to Misra and Fridovich (1972), while glutathione (GSH) and glutathione peroxidase (GPx) were estimated following the method of Beutler *et al*., (1963). Reduced glutathione level was evaluated through the method of Ellman (1959). To determine lipid peroxidation, the formation of thiobarbituric acid reactive substances (TBARS) were measured (Varshney and Kale, 1990). The malondialdehyde (MDA) level was calculated according to Adam-Vizi and Seregi (1982), while Gluthathione s-transferase activity was determined according to Habig *et al.* (1974).

2.9. Challenge Test

After the feeding experiment, challenge test was conducted by exposing the survived fish to virulent bacteria "*Aeromonas hydrophila"* following the procedures of Austin *et al*., (1995) with 20 fish selected per treatment. The fish per treatment were divided into two sets and the first sets injected inter-peritoneally with 1 x 10^7 cell/ml (Omitoyin *et al*., 2019) virulent *Aeromonas hydrophila* carried in 0.5 ml phosphate buffer saline solution (PBS) while the second sets were injected with 0.5 ml PBS. Feeding with experimental diets continued for 15 days. Mortality was monitored to ascertain clinical signs and survival rate of the experimental fish.

2.10. Statistical Analysis

Homogeneity of variance in data collected was confirmed using Bartlett's test. Data were analysed using one- way analysis of variance (ANOVA) to establish the effect of *L. fermentum* on fish. Differences in mean were separated using Duncan's test at the 5 % probability level. The optimum inclusion levels were determined using the Polynomial regression with the aid of IBM Statistical Package for Social Science (SPSS) version 20.

3. Results and Discussion

3.1. Results

3.1.1 Water quality and Fish carcass composition

Pooled mean of water quality parameters throughout the 90-day experimental period for dissolved oxygen, pH, temperature, ammonia and nitrite were 5.1 mg/l, 7.39, 28.09 ^oC, 2.00 mg/l and 0.20 mg/l, respectively. The composition of carcass in fish fed diets shows significantly higher crude protein in the carcass of fish fed *L. fermentum* (Table 3). The highest value of 74.8 % crude protein was recorded in LF3 fish and the least value of 62.1 % was observed in LF1. Similarly, higher fat deposits were observed in probiotic-fed fish when compared with LF1. While fibre content was statistically similar across treatments.

3.1.2 Nutrient utilization and fish growth indices

The indices of growth in tilapia fingerlings fed *L. fermentum* supplements for 90 days are presented in Table 4. Significantly high final weights were observed in LF3, LF4 and LF5, with LF3 having the highest value of 6.23 g. Mean weight gain (MWG) and percentage weight gain followed this same pattern. MWG was significantly high in LF3 and LF4, with values of 4.19 and 4.15g, respectively, and the least value of 3.09 g recorded in LF1. Feed conversion ratio (FCR) ranged from 2.24 (LF3) to 3.00 (LF1) and results shows better FCR values in LF3 and LF4 compared to other treatments. Specific growth rate (SGR) was highest in LF4 followed by LF3. These two groups had significantly higher protein efficiency ratios (PER) with the least value of 0.95 observed in LF1.

Table 3: Proximate Analysis of Fish Fed *L. fermentum* Supplemented Diet

Parameter (%)	LF1	LF2	LF3	LF4	LF5	LF6
Crude protein	62.14 ± 0.17^a	65.15 ± 0.32^b	74.79 ± 0.36^d	73.37 ± 0.59^d	66.77 ± 0.44^c	74.16 ± 0.35^d
Ash	6.50 ± 0.15^d	6.20 ± 0.17^c	4.90 ± 0.15^b	4.50 ± 0.11^a	6.30 ± 0.15^c	5.10 ± 0.2^b
Ether extract	6.43 ± 0.11^a	6.63 ± 0.15^{ab}	7.80 ± 0.11^d	7.4 ± 0.11^{cd}	6.8 ± 0.15^b	7.5 ± 0.15^{cd}
Crude fibre	0.02 ± 0.01^a	0.02 ± 0.01^a	0.02 ± 0.01^a	0.03 ± 0.01^a	0.02 ± 0.01^a	0.03 ± 0.01^a
Moisture	54.53 ± 1.9^a	59.41 ± 2.7^{ab}	60.58 ± 1.1^b	56.64 ± 5.5^{ab}	61.32 ± 0.5^b	57.63 ± 3.4^{ab}
Dry matter	45.47 ± 1.7^b	40.59 ± 2.7^{ab}	39.41 ± 1.1^a	43.36 ± 5.5^{ab}	38.68 ± 0.5^a	42.37 ± 3.4^{ab}

Means with the same superscripts across row are not significantly different (p>0.0)

Table 4: Growth response and nutrient utilization of *Oreochromis niloticus* fed experimental diet

Growth parameters	LF1	LF2	LF3	LF4	LF5	LF6
Initial Weight (g)	1.85±0.01	1.97±0.01	1.98±0.05	1.70±0.2	1.98±0.01	1.94±0.01
Final Weight (g)	4.94±0.02^{a}	5.20±0.03^{a}	6.23±0.02^{c}	5.85±0.03^{b}	5.62±0.02^{b}	5.16±0.03^{a}
Weight gain (g)	3.09±0.01^{a}	3.23±0.02^{a}	4.19±0.03^{c}	4.15±0.02^{c}	3.64±0.01^{b}	3.22±0.01^{a}
% Weight gain	167.0±2.0^{a}	163.9±1.0^{a}	199.5±2.0^{c}	244.1±2.0^{d}	183.8±3.0^{b}	165.9±3.0^{a}
Feed Intake (g)	9.28±0.00^{a}	9.39±0.00^{bc}	9.51±0.00^{d}	9.38±0.00^{b}	9.39±0.00^{bc}	9.43±0.03^{c}
Feed conversion ratio	3.00±0.00^{d}	2.90±0.05^{c}	2.24±0.00^{a}	2.26±0.01^{a}	2.57±0.00^{b}	2.92±0.03^{c}
Specific growth rate	1.09±0.00^{b}	1.07±0.00^{a}	1.27±0.00^{d}	1.37±0.00^{e}	1.16±0.00^{c}	1.08±0.00^{ab}
Protein efficiency ratio	0.95±0.00^{a}	0.98±0.00^{b}	1.27±0.00^{d}	1.26±0.00^{d}	1.09±0.01^{c}	0.98±0.00^{b}

Means with the same superscripts across the rows are not significantly different at p<0.05

3.1.3 Fish gut morphometry

As shown in Figure 1, there was no significant variation in the villi height in the intestine of fish across treatments. And for the exception of the LF3 group with a significantly higher villi width, others were statistically similar.

3.1.4 Microbial Assay of the gastrointestinal tract of *O. niloticus* fed diets

The pH for sensitivity test ranged from 6.53 to 6.84 as shown in Table 5. Microbial load in the gut of experimental fish varied significantly (p<0.05) and suggests a dose dependent response with the least load of 3.84 x 10^{5} cfu/g recorded in LF1 group and the highest load of 8.27 x 10^{8} cfu/g in LF6.

While Table 6 shows the identified bacteria in fish guts. *L. fermentum* was present in experimental fish irrespective of the assigned treatment. The LF6 group recorded occurrence of 9 bacteria while LF1 to LF3 had 7 each, LF4 had 8 and LF5 had 5. All the isolates were gram positive and short rod.

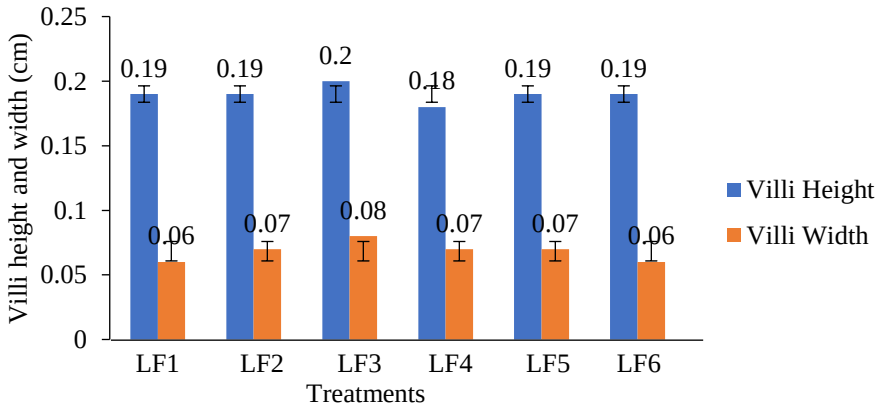

Figure 1. Histomorphometry of *O. niloticus* intestine fed *L. fermentum* supplemented diets

Table 5: Microbial Assay of Gastro-Intestinal Tract of *O. niloticus* fed experimental diets

Treatments	LF1	LF2	LF3	LF4	LF5	LF6
Bacterial Count (CFU/g)	0.0038±0.03 x10^{8a}	4.77±0.05 x10^{8b}	5.22±0.04 x10^{8c}	6.16±0.04 x10^{8d}	7.61±0.02 x10^{8e}	8.27±0.05 x10^{8f}
pH sensitivity test	6.84±0.04	6.73±0.03	6.64±0.02	6.53±0.02	6.61±0.01	6.58±0.01

Means with the same superscripts down the column are not significantly different at p<0.05

Table 6: Bacteria identified in the gut of fish fed *L. fermentum* diets for 90 days

Sample	*L. delbrueckii*	*L. fermentum*	*L. casei*	*S. faecalis*	*L. plantarum*	*L. hilgardii*	*S. lactis*	*S. thermophiles*	*L. cremoris*	*L. leichmannii*	*L. coagulans*	*L. salivarius*	*L. cellobiosus*	*L. coryniformis*
LF1	-	+	+	-	-	-	+	-	-	-	+	+	+	+
LF2	-	+	+	-	-	-	+	-	-	-	+	+	+	+
LF3	+	+	-	+	+	+	-	+	-	+	-	-	-	-
LF4	+	+	+	+	+	-	-	+	+	+	-	-	-	-
LF5	-	+	-	-	-	-	+	-	-	-	-	+	+	+
LF6	-	+	-	+	+	+	+	+	+	+	+	-	-	-

+ means present; – means absent

3.1.5 Haematology of *O. niloticus* fingerling fed different level of *L. fermentum*

Values for packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV and, mean corpuscular haemoglobin (MCH) were significantly higher ($p<0.05$) in groups fed *L. fermentum* compared to the control (Table 7). The reverse is observed in the results of red blood cells, where the control group had a significantly higher RBC. White blood cells including the granulocytes constituents of neutrophils, eosinophils and basophils and the non-granulocytes lymphocytes and monocytes were not affected by the probiotic inclusions and levels in diets. Values for aspartate aminotransferase and alanine aminotransferase were significantly lower in fish fed probiotics.

3.1.6 Oxidative Assay of the Liver and Kidney

Results of the assay of oxidative stress indicators analysed in the kidney presented in Table 8 reveals that total protein showed no significant difference ($p>0.05$) across groups. There was significant reduction in hydrogen peroxide levels with inclusion of *L. fermentum*. The highest value of 48.01µmol/mg was recorded in LF1, while LF4 had the least value of 41.29 µmol/mg. MDA was reduced significantly in fish fed diets LF2, LF3 and LF4 with the least value observed in LF3. Glutathione peroxidase (GPx), glutathione s-transferase (GST), superoxide dismutase (SOD) and NO values increased significantly with probiotic addition. In the liver, TP was significantly increased in the LF3 to LF6 groups, however, hydrogen peroxide and MDA reduced significantly with inclusion of probiotic (Table 9). There were significant increases in the values of GSH, GPx, GST, SOD and NO with the inclusion of *L. fermentum*.

3.1.7 Challenge Test

The control group recorded 95 % (19 out of 20) mortality or 5% survival after 15 days challenge test with *Aeromonas hydrophila*. No mortality was recorded in the groups fed *L. fermentum* diets.

3.2. Discussion

The experimental diets fed to fish for 90 days met the dietary requirement for *Oreochromis niloticus* for optimal growth and performance (Britz, 2008). Similarly, water parameters in all experimental tanks fall within the recommended range for tropical fish culture (Ajani *et al*, 2011). The use of probiotics as dietary supplements has been reported to cause a colonization of host's gut and thus increase feed utilization through the synthesis of growth factors, co-factors, amino and fatty acids and augmentation of the immune response of the cultured species (Farzanfar 2006; Talpur *et al*., 2013).

Table 7: Haematological parameter of *Oreochromis niloticus* fingerling fed with diet of different level of probiotics

Indicators	LF1	LF2	LF3	LF4	LF5	LF6
PCV	24.77±0.10^{a}	26.33±0.00^{c}	27.39±0.06^{d}	26.09±0.00^{b}	26.33±0.00^{c}	26.38±0.04^{c}
Hb	7.87±0.00^{a}	8.50±0.05^{b}	9.26±0.03^{e}	8.71±0.04^{c}	8.95±0.05^{d}	9.143±0.01^{e}
RBC	3.53±0.08^{e}	2.74±0.00^{bc}	2.66±0.02^{ab}	2.57±0.00^{a}	2.95±0.00^{d}	2.80±0.00^{c}
WBC	16.60±0.15^{c}	7.14±0.05^{a}	16.81±0.04^{c}	16.66±0.49^{c}	14.88±0.72^{b}	14.13±0.15^{b}
Platelet	176.00±1.0^{b}	169.00±1.0^{ab}	193.00±4.0^{c}	194.00±4.0^{c}	163.00±1.0^{a}	189.50±0.5^{c}
Lymphocyte	61.91±0.24^{c}	61.45±0.45^{bc}	61.38±0.2^{a}	67.83±0.16^{d}	60.38±0.29^{b}	57.50±0.50^{a}
MCV	79.61±0.39^{a}	103.17±0.05^{e}	97.95±0.37^{c}	106.39±0.4^{f}	95.96±0.13^{b}	99.35±0.27^{d}
MCHC	33.85±0.05^{d}	32.09±0.01^{a}	32.19±0.01^{b}	32.32±0.00^{c}	34.04±0.03^{e}	34.66±0.00^{f}
MCH	26.74±0.00^{a}	33.59±0.01^{d}	31.51±0.22^{b}	35.53±0.01^{f}	32.43±0.02^{c}	34.44±0.01^{e}
Neutrophils	33.00±0.00^{c}	34.01±0.01^{d}	32.00±0.00^{b}	29.67±0.34^{a}	32.99±0.00^{c}	38.00±0.01^{e}
Monocytes	2.68±0.01^{c}	2.33±0.00^{b}	3.27±0.06^{e}	3.06±0.06^{d}	2.72±0.05^{c}	2.01±0.01^{a}
Eosinophil	2.32±0.01^{d}	1.33±0.00^{a}	2.00±0.00^{c}	1.68±0.01^{b}	2.99±0.01^{f}	2.68±0.01^{e}
Basophil	0.33±0.01^{a}	0.33±0.01^{a}	0.33±0.02^{a}	0.00	0.67±0.01^{a}	0.33±0.01^{a}
AST	215.15±0.17^{e}	201.66±0.46^{b}	193.50±0.50^{a}	218.33±0.34^{f}	213.11±0.42^{d}	206.99±0.01^{c}
ALT	32.83±0.16^{e}	24.32±0.00^{b}	25.37±0.04^{c}	25.99±0.01^{d}	20.58±0.25^{a}	23.94±0.05^{b}
ALP	206.50±0.5^{c}	214.67±0.22^{e}	198.50±0.50^{a}	213.43±0.23^{d}	202.70±0.30^{b}	205.71±0.06^{c}

Means with the same superscripts across the rows are not significantly different at p<0.05

PCV, packed cell volume; Hb, haemoglobin; RBC, red blood cell; WBC. White blood cell; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; MCH mean corpuscular haemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphate.

Table 8: Oxidative Stress Assay of *O. niloticus* Kidney Fed *L. Fermentum* Supplemented Diet

Parameter	LF1	LF2	LF3	LF4	LF5	LF6
Total protein (U/mg)	0.33±0.01^{a}	0.36±0.01^{a}	0.37±0.01^{a}	0.35±0.02^{a}	0.31±0.01^{a}	0.30±0.02^{a}
H_2O_2 (µmol/mg)	48.01±1.5^{a}	47.14±2^{a}	44.42±1^{c}	41.29±1.2^{b}	41.52±2.1^{b}	45.98±1^{c}
MDA (U/mg)	2.79±0.2^{a}	1.65±0.01^{cd}	1.59±0.01^{d}	1.86±0.02^{c}	2.44±0.01^{b}	2.61±0.01^{ab}
GSH (U/mg wet tissue)	67.58±0.2^{b}	67.56±3.2^{a}	68.27±2.2^{a}	68.08±2.1^{b}	71.52±2.3^{a}	69.05±2^{a}
GP_X (U/mg wet tissue)	561.20±5.4^{a}	579.87±6.3^{b}	638.09±5.3^{c}	624.62±6.1^{c}	668.19±4^{d}	716.36±5^{e}
GST (U/mg wet tissue)	0.15±0.01^{b}	0.05±0.01^{d}	0.24±0.03^{b}	0.48±0.03^{a}	0.63±0.02^{c}	0.45±0.04^{a}
SOD (U/mg tissue)	19.75±5^{b}	23.58±2^{ab}	21.29±4^{a}	24.57±4^{a}	25.79±3^{a}	21.79±3^{b}
NO (µmol/mg)	0.48±0.04^{c}	0.50±0.01^{c}	0.63±0.02^{a}	0.68±0.011^{a}	0.83±0.01^{b}	0.67±0.02^{a}

Means with the same superscripts across row are not significantly different at p<0.05

H_2O_2, hydrogen peroxide; MDA, malonaldehyde; GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione s-transferase; SOD, superoxide dismutase; NO, nitrogen oxide

Table 9: Oxidative Stress Assay of *O. niloticus* Liver Fed *L. Fermentum* Supplemented Diet

Parameter	LF1	LF2	LF3	LF4	LF5	LF6
Total protein (U/mg)	0.17±0.03^{b}	0.17±0.02^{b}	0.23±0.01^{a}	0.19±0.02^{c}	0.19±0.02^{c}	0.21±0.01^{d}
H_2O_2 (µmol/mg)	48.81±3.2^{a}	35.33±2^{b}	39.5±1.2^{c}	39.06±1.4^{c}	34.29±1^{b}	44.33±1^{d}
MDA (U/mg)	5.88±0.03^{b}	4.13±0.01^{a}	4.62±0.03^{a}	5.12±0.03^{b}	4.79±0.01^{a}	4.46±0.02^{a}
GSH (U/mg wet tissue)	88.17±3.2^{a}	95.61±5.1^{b}	95.56±4.3^{b}	93.84±4.6^{c}	98.77±3.1^{d}	93.68±2.3^{c}
GP_X (U/mg wet tissue)	1002.19±125^{a}	1263.57±102^{b}	1174.68±78.5^{c}	1187.14±98^{c}	1377.13±86.3^{d}	1112.99±117^{c}
GST (U/mg wet tissue)	0.04±0.01^{d}	0.43±0.04^{a}	0.92±0.12^{c}	0.72±0.14^{b}	0.77±0.2^{b}	0.49±0.31^{a}
SOD (U/mg tissue)	35.05±2.1^{a}	43.98±2.2^{b}	40.19±2^{b}	41.23±1.2^{b}	47.21±3.2^{c}	38.85±4^{a}
NO (µmol/mg)	1.69±0.14^{a}	1.61±0.02^{a}	1.87±0.04^{b}	1.33±0.02^{c}	0.88±0.01^{d}	1.54±0.02^{e}

Means with the same superscripts across row are not significantly different at $p<0.05$

H_2O_2, hydrogen peroxide; MDA, malonaldehyde; GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione s-transferase; SOD, superoxide dismutase; NO, nitrogen oxide

Fish carcass recorded increased crude protein and fat with *Lactobacillus fermentum* inclusion in this study. This shows improved protein synthesis and enhanced lipid production caused by the presence of probiotic in fish diets. According to Fountoulaki *et al* (2003), higher nutrient deposit is usually associated with increase efficiency of metabolism. Growth as indicated by final weight and the mean weight gain were significantly highest in LF3 and LF4 groups, with a decrease observed at higher concentration of *L. fermentum* as seen in LF5 and LF6 groups. Feed intakes were also higher in the groups fed probiotics and were equally well transformed into flesh showing by the superior feed conversion ratio in the *L. fermentum* groups. The results of the study may be attributed to improved appetite shown in increased feed intake and better digestibility and absorption of nutrients evident in the food to flesh conversion and thus better growth (Eshaghzadeh *et al.*, 2015). Stimulation of growth in many aquatic animals fed *L. fermentum* based diets, have been reported; this includes white shrimp (*Litopenaeus vannamei),* Giant freshwater prawn (*Macrobrachium rosenbergii),* Orange-spotted grouper (*Epinephelus coioides) and* Red Sea bream (*Pagrus major)* (Kongnum and Hongpattarakere 2012; Dash *et al*, 2015; Son *et al*., 2009; Dawood *et al*., 2016). Similar result was reported when Nile tilapia was fed probiotic diets containing *Steptococcus faecium* and *Lactobacillus acidophilus* (Lara-Flores *et al*., 2003). In the contrary, insignificant effect of probiotics on fish growth were observed in Fagundes *et al* (2016) and Tachibana *et al* (2012) when Nile tilapia was fed diets containing *L. plantarum* at concentrations of 10^4, 10^6 and 10^8 CFU/g and *B. subtilis,* respectively.

The villi morphometric presented in Figure 3 revealed statistical similarities in all treatments except for the LF3 group with significantly higher dimension. Higher villi height and width will translate to greater areas of absorption and improved nutrient utilization (Omitoyin *et al*., 2019). It has also been hypothesised that Lactobacilli produces fatty acids that are short-chained as products of carbohydrate metabolism and serves as main energy source in intestinal epithelial cells. Pirarat *et al* (2011) suggested that this production in the cells causes an increase in villi height in the digestive tract and subsequently improves nutrient absorption by providing a greater absorptive surface area.

There were variations in the gut intestinal bacterial load, revealing a dose dependent pattern with the highest load observed in the LF6 group. Some of the identified bacteria include *L. fermentum, L. brevis and L casei*.

According to Olanrewaju *et al* (2018), blood is described as a pathos-physiological reflector and the count of its indices give an indication of the health status of fish. In the current study, the effects of probiotic, *L. fermentum* on haematological parameters of *Oreochromis niloticus* was examined. The PCV, Hb,

MCV and MCH were higher in fish fed probiotic supplemented diets, indicating better health status. Increase in Hb levels of Nile tilapia fed *Saccharomyces cerevisiae* and *Bacillus* spp. treated diets were earlier reported (Selim and Reda, 2015; Abu-Elala *et al*., 2013). Red blood cells which are essential component of the innate and also the adaptive immune response systems were significantly lower in fish fed *L. fermentum* in the study. This is contrary to the findings of Opiyo *et al* (2019) where increased RBC and WBC were recorded in fish fed *S. cerevisiae* or *B. subtilis*. The differences in the findings may be due to variations in the probiotic species and the applied dosages. There were also reductions in the aspartate aminotransferase and alanine aminotransferase in the serum of fish fed *L. fermentum* in this study. According to Omitoyin *et al* (2019), the release of these blood enzymes suggests cell damage and lose of cellular integrity. Therefore, the fed probiotic confers better health status and cell integrity to fish.

The innate immune system of the fish is considered very important, as it normally present the first line of defence against stressors. The immune competence of a fish can be ascertained using the oxidative biomarkers which act as first line of antioxidant defence (Carvalho *et al*., 2012). Hydrogen peroxide and malondialdehyde levels in the kidney and liver were reduced with *L. fermentum* inclusion in fish diets. Lipid peroxidation of cell membranes is reported to result in the production of metabolites like malondialdehyde (Borkan and Schwartz, 1989), which are measured and used as indirect markers of oxidant-induced injury (Halliwell and Gutteridge, 1990). The reduction in MDA in this study is an indication of lower occurrence of free radicals in the groups fed probiotics, and therefore reduced peroxidation of lipids, suggesting improved innate immune system in fish fed probiotic. In contrast, the values recorded for GPx, GST, SOD and NO increased significantly with probiotic addition, which shows the activation of antioxidant protection needed in all fish including the healthy ones during their life cycle (Omitoyin *et al.* (2019). The effect of all these was tested via a challenge test with *Aeromonas hydrophila* for fifteen days. Fish fed diets supplemented with *L. fermentum* recorded no mortality indicating that the immune system was boosted. The use of probiotics was reported to improve the non-specific immune of tilapia thus, enhancing resistance to Edwardsville trade infection (Abd El-Rhman *et al*., 2009).

4. Conclusion

Lactobacillus fermentum resulted in increased growth performance of *O. niloticus* fingerling, and there was also an increase in the gut microbial load. This study also revealed better immune system in fish fed diets supplemented with *L. fermentum* resulting in the resistance of virulent *Aeromonas hydrophila.* An inclusion level of 1 x 10^5 cfu/g is recommended when all parameters measured are considered.

Acknowledgement: The authors would like to thank all participants in this study for their collaborative support during the study.

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