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Blood profiles of African catfish *Clarias gariepinus* fingerlings fed diets supplemented with three strains of *Lactobacillus plantarum*

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ABSTRACT

A 10-week feeding trial was carried out to evaluate strain-specific effects of Lactobacillus plantarum on blood profiles of African catfish Clarias gariepinus fingerlings. A catfish diet was formulated and divided into five portions (experimental diets). The first portion served as control (with no probiotic added to it, labeled as NP) while the second portion received 2.5g kg^{-1} multi-species commercial probiotics (CP). The third, fourth and fifth portions were the experimental diets that contained 2×10^{9}cfu/g of L. plantarum strains of ascension numbers LC333558, LC333559 and LC333560, and labeled as LP58, LP59 and LP60, respectively. Fifteen apparently healthy C gariepinus fingerlings (4.86±0.59g) were stocked in an experimental tank of 70 litres capacity, while the experimental feed was fed to the fish in triplicate tanks. Similar rearing conditions were maintained in all experimental tanks. The results showed significant ($p<0.05$) difference between the fish fed control diet and probiotics supplementation diets in some of the haematological parameters examined. Probiotics supplementations did not affect total and differential leukocytes among different experimental groups. Except for RBC counts, fish groups fed with strains LB59 and LB60 had similar effects and appeared to have better haematological values than groups fed with LB58. Strains-specific effect of L. planatrum was also observed in creatinine levels among the experimental groups. Hence, it can be concluded that strains of L. plantarum affected some haematological parameters of C. gariepinus fingerlings positively, while dietary supplementation of L. plantarum LC333559 strain could be employed to improve haematological parameters of C. gariepinus fingerlings.

Introduction

Probiotics is live, dead or component of microbial cells which, when administered via feed or rearing water, benefits the host by improving disease resistance, health status, growth performance, feed utilization, stress response, general vigour, or the host's environmental rearing conditions (Merrifield *et al.*, 2010). The use of probiotics emerged as a potential tool to reduce mortalities and consequently improve growth and well-being of the cultured organism (Gatesoupe, 1999; Gomez-Gil *et al.*, 2000; Verschuere *et al.*, 2000).

African catfish *Clarias batrachus* is one of the popular freshwater fish and it has been cultured worldwide (Aderolu *et al.*, 2017; Marimuthu *et al.*, 2019; Adeniyi *et al.*, 2021). In African catfish aquaculture, several attempts have been made by scientists to evaluate the best bacterial species, mainly those of lactic acid bacteria, for probiotic applications, using indices such as feed efficiency and growth performance, body composition, liver and gut morphology, *in-vitro* antibacterial activities and challenge with pathogens (Al-Dohail *et al.*, 2009; Ogunshe and Olabode, 2009; Al-Dohail *et al.*, 2011; Nwanna and Tope-Jegede, 2017).

Fish blood consists of fluid plasma and particulate entities that are largely blood cells or their remains. Each of the blood components plays different roles in maintaining the health and well being of fish. For example, erythrocytes, packed cell volume and haemoglobin are major and reliable indicators of various sources of stress (Rainza-Paiva *et al*, 2000)

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and these parameters decrease in the presence of anti-nutritional factors (Osuigwe *et al.,* 2007). Neutrophils may be considered to be surveillance cells that sweep through the blood stream, scanning for tissue infections or other inflammatory events (Shah *et al.*, 2017). Neutrophils, as well as monocytes and macrophages, are phagocytes that act to remove irritants, bacteria or damaged cells and tissues during inflammations. According to Ajeniyi and Solomon (2014), blood urea nitrogen (BUN) and creatinine are the most abundant non-protein nitrogen constituents in the body and their determination are the most commonly preferred tests of the kidney's ability to excrete metabolic wastes. Based on the aforementioned roles, fish blood parameters are invaluable bio-indicators or diagnostic tools for assessing the physiological status of fish, as they provide quick indications of any stress-induced changes caused by infectious diseases, environmental pollutions, diets or other unfavourable conditions (Zhou *et al.*, 2009).

Several studies have demonstrated the immunological and haematological stimulation of fish's defense mechanisms by probiotic bacteria (Brunt *et al.*, 2008; Pieters *et al.*, 2008). However, following increasing understanding of genomic diversity among microorganisms, two strains from the same species may exert different and sometime opposite effects (Aureli *et al.*, 2011; Siezen and van Hylckama Vlieg, 2011). In other word, each strain has unique properties and differs greatly in its modes of action, making it improper to extrapolate the effects of one bacterial strain on other strains of the same bacterial species (Boyle *et al.*, 2006; Pineiro and Stanton, 2007). It is therefore expedient to evaluate the specific beneficial effect of any bacterial strain intended for use as probiotics so as to avoid confusion arisen from wrong strain identification. The work presented here evaluated the effect of three strains of *Lactobacillus plantarum* on blood profiles of African catfish, *Clarias gariepinus* fingerlings.

Materials and Methods

Pre-experimental conditions

The three strains of *Lactubacillus plantarum* used in this experiment were previously isolated from fermented maize, sorghum and soyabeans, and genotypically characterized with accession numbers LC333558, LC333559 and LC333560 (Diyaolu *et al.*, 2018). Each bacterial strain was maintained in deMan Rogosa and Sharpe (MRS) broth and kept in refrigerator. Prior to use, 1 ml of each broth was added to 9 ml of freshly prepared sterile MRS broth, and incubated anaerobically for 24 hours. 0.1 ml of the broth was examined under a light microscope (400X magnification) for purity, after which 1 ml was inoculated into 500 ml sterile MRS broth and incubated for 24 hours. The bacterial cells harvested after centrifugation were enumerated by pour-plating method and used for diet preparation.

Two hundred and fifty hatchery-bred *C. gariepinus* fingerlings were obtained from a farm in Akure, Nigeria, and brought to the Teaching and Research Farm, Federal University of Technology, Akure, Nigeria. The fish were acclimatized for a period of two weeks, during which they were fed with commercial diets (Coppens, 2mm size for one week) and control diets (for another one week) in order to prepare their gastro-intestinal tract for the experiment. Rearing water was exchanged every two days

The dry feed ingredients were procured from a local animal feed mill in Akure, Nigeria. The coarse ingredients (maize. soya bean meal, groundnut cake, bone meal) were ground to small particles and sieved in order to obtain a fine particle size of each ingredient. They were stored in a cool dry place prior to use.

Experimental procedures

The experimental diets were formulated to contain 40 % crude protein, 11.6 % crude lipid, and gross energy 461 kJ g^{-1} (Table 1). Prior to pelleting, the dietary ingredients (excluding starch) were mixed thoroughly and divided into five portions. The first portion had no probiotic added to it (non probiotic control, NP) while the second portion received 2.5g kg^{-1} multi-species commercial probiotic (CP, containing *Bacillus subtilis* ($2x10^{11}$cfu), *Lactobacillus lactis* ($1.5x10^{11}$cfu); *Lactobacillus acidophilus* ($1.2x10^{11}$cfu); *Lactobacillu sporogenes* ($1.2x10^{11}$cfu); *Pediococcus acidilactici* ($2x10^{11}$cfu); *Candida utilis* ($1x10^{11}$cfu); amylase (1.000 iu); protease (1.000 iu); β-glucanase (800 iu); cellulose (500 iu); lipase (500 iu)). The third, fourth and fifth portions contained $2x10^{9}$cfu/g of *L. plantarum* strains of accession numbers LC333558, LC333559 and LC333560, labeled as LP58, LP59 and LP60 respectively. This dose was selected based values obtained from previous probiotic studies on other fish species (Panigrahi *et al.*, 2004; Batista *et al.*, 2016). The starch (binder) was prepared with hot water, allowed to cool to room temperature and added to the separate portions of the experimental diets. Each portion was then mixed thoroughly, moistured appropriately to form homogenous dough, pelleted into 2 mm sizes using a pelleting machine and air-dried for two day. The feeds were packed in air tight polythene bags and kept in a cool dry place until required for use.

Groups of 15 fish were weighed and randomly distributed into fifteen rectangular glass tanks of 70 litres capacity filled with 60 litres of bore hole water. Each feed (treatment) was maintained in triplicate tanks. The fish were fed daily at 5 % body weight in two portions at 8:00 and 18:00 hours for 10 weeks. The fish in each tank were batch-weighed bi-weekly using electronic weighing balance. The weight obtained was used to adjust the feed offered to the fish for a period of two weeks. Rearing water was drained and replaced with fresh water every two days. Feeding ratios were adjusted biweekly based on the

weight of fish. Water quality parameters, such as temperature, dissolved oxygen and pH, were monitored prior to draining to determine if probiotics supplemented diets had any effect on rearing water, using Hanna multiparameter (Model Hi-98194).

Table 1. Gross and proximate composition of experimental diets (g/100g).

Ingredients	Composition (%)
Maize	20.00
Full fat soya	24.50
Groundnut cake	24.50
Fish meal	20.00
Vitamin/mineral premix*	4.00
Fish oil	2.00
DL-methionine	1.00
L-lysine	1.00
Starch	3.00
Total	100.00
Proximate composition	
Moisture	11.46±0.52
Ash	7.08±0.44
Crude protein	40.71±3.89
Fat	11.63±0.35
Crude fibre	4.12±0.02
Carbonhydrate	25.01±0.37
Gross energy	461.86±6.25

Vitamin-Mineral premix* consisting of Vitamin A, 1600 IU; vitamin D, 2400 IU; vitamin E, 160 mg; vitamin K, 16 mg; thiamin, 36 mg; riboflavin, 48 mg; pyridoxine, 24 mg; niacin 288 mg; panthotenic acid, 96 mg; folic acid, 8 mg; biotin, 1.3 mg; cyanocobalamin, 48 mg; ascorbic acid, 720 mg; choline chloride, 320 mg; calcium 5.2 g; cobalt, 3.2 mg; iodine, 4.8 mg; copper, 8 mg; iron, 32 mg; manganese, 76 mg; zinc, 160 mg; Endox (antioxidant) 200 mg. Manufactured by DSM Nutritional Products Limited, Basle, Switzerland

Blood collection

At the end of the experimental (10 weeks) period, blood samples (10 mL) were collected by syringe from caudal vein of three catfish fingerlings per tank (nine fish per treatment) for the evaluation of haematological and serum biochemical parameters. Ethylene di-amine tetra-acetic acid (EDTA) at 1.0 mg/mL of blood was used as anticoagulant in samples meant for haematology, while no anticoagulant was added to samples meant for serum biochemical analysis.

Haematological analysis

Total erythrocyte and total leukocyte levels were determined using a haemocytometer following the methods described by Svobodova *et al.* (1991). Haematocrit was determined according to Brown (1988) using micro-haematocrit method and reported as percentage packed cell volume (% PCV). Sahli's method was used for the estimation of haemoglobin levels (Blaxhall and Daisley, 1973). Derived blood parameters were estimated as described by Dacie and Lewis (2001) as follows:

Mean corpuscular volume (MCV): This is average volume of red cells, and is expressed as:

$$\text{MCV (cu. } \mu m^3) = \frac{Haematocrit\ (\%) \times 10}{RBC\ count\ in\ million}$$

Mean corpuscular haemoglobin (MCH): This is the average haemoglobin content (by weight) of a red cell, and is expressed as:

$$\text{MCH (pg)} = \frac{\text{Haemoglobin} \times 10}{\text{RBC count in million}}$$

Mean corpuscular haemoglobin concentration (MCHC): This was obtained using the formula:

$$\text{MCHC (pg)} = \frac{\text{Haemoglobin}}{\text{PCV}} \text{x100}$$

Serum biochemical analysis

The Gomal's biuret method was used for serum protein determination. The serum was fractionized to determine albumin and total protein, while the globulin content was taken to be the difference between the total protein and albumin. Blood urea and creatinine were determined using Nesselerization method and Jaffe spectrophotometric techniques respectively (Aitken *et al.* 2003).

Statistical analysis

All values obtained in this study were recorded as means ± standard deviation. For haematological and serum biochemical values, one-way analysis of variance (ANOVA) was used to determine the existence of significant differences among mean treatments, and where differences existed, Duncan Multiple Range test at $P<0.05$ was performed to separate them. The recorded bi-weekly fish weights in all experimental treatments were plotted as a chart.

Results

The results of the blood analyses (Table 2) revealed that probiotic supplementation had remarkable effects on haematological parameters of *C. gariepinus* fingerlings. The packed cell volume (PCV) was significantly ($P<0.05$) higher in all diets supplemented with strains of *L. plantarum* when compared with non probiotic (NP) and commercial probiotic (CP) diet groups. Similar trends were observed for haemoglobin (Hb) and red blood cells (RCB). Significantly lower values ($p<0.05$) were recorded for PCV, Hb, red blood cells (RBC) and mean corpuscular volume (MCV). Similarly, highest values were recorded for mean corpuscular haemoglobin concentration (MCHC) in commercial probiotics supplemented (CP) groups.

For leukocyte counts (Table 3), total leukocyte or white blood cells (WBC) counts were not significantly differed ($p>0.05$) among the experimental groups. Similarly, there was no significant variation ($p>0.05$) in some differential leukocyte counts, including lymphocyte, monocyte and eosinophil counts. In contrast, strain dependent effects were observed for neutrophil counts among the experimental groups, with significantly higher values ($p<0.05$) occurring in LP59 and LP60 groups.

The results of the serum biochemical analysis (Table 4) of the experimental fish showed that serum urea, calcium and globulin were not significantly ($p>0.05$) affected by probiotic supplementation, although numerical differences were recorded for these parameters. Serum total protein and albumin were significantly higher ($p<0.05$) in LP60 groups than in others probiotic treatments, while creatinine was found to be significantly lower ($p>0.05$) in groups fed with the three strains of *L plantarum* than in the control (NP) and CP groups.

The biweekly weight curves (Figure 1) of *C. gariepinus* fingerlings fed diets supplemented with three strains of *L. plantarum* showed that fish groups fed with the three strains of *L. plantarum* had higher weight than those without probiotic supplementation (control, NP) and commercial probiotic supplemented diets (CP) at the 10th week of the experiment. However, the ascending order for fish weight among all experimental groups was LP59<LP60<LP58<CP<NC.

It was observed that water quality parameters in all experimental tanks were significantly different from inflow water. However, experimental diets did not impact rearing water differently, as observed parameters in all experimental tanks were not statistically different from each other (Table 5).

Table 2. The haematological parameters of *C. gariepinus* fingerlings fed diets supplemented with three strains of *L. plantarum*.

Treatment Group	PCV (%)	HB (g/l)	RBC (10^6mm^3)	MCV (cu. µm^3)	MCH (pg)	MCHC (pg)
NP	38.12±3.56^{b}	12.65±1.47^{bc}	4.61±0.29^{b}	82.60±8.74^{a}	27.39±3.11^{b}	33.15±7.89^{b}
CP	35.00±3.25^{a}	10.39±1.88^{a}	4.33±0.72^{a}	81.32±9.55^{a}	23.95±3.49^{a}	29.42±8.57^{b}
LP58	42.58±4.79^{c}	11.63±1.72^{b}	4.81±0.55^{c}	87.50±6.33^{b}	24.16±5.76^{a}	27.61±5.09^{a}
LP59	44.16±2.91^{c}	13.36±2.01^{c}	4.90±0.34^{c}	89.75±9.59^{b}	26.53±6.01^{a,b}	29.54±5.45^{a,b}
LP60	45.15±3.66^{c}	12.00±1.59^{b}	4.79±0.61^{c}	95.74±11.68^{c}	25.53±5.19^{a,b}	26.66±6.27^{a}

Values were recorded from three replicate trials as means ± standard deviation. Means with different superscripts on the same column are significantly different ($p < 0.05$).

Table 3. The total (white blood cells) and differential leukocyte counts of *C. gariepinus* fingerlings fed diets supplemented with three strains of *L. plantarum*.

Treatment groups	Total leukocytes (10^3 mm^3)	Differential leukocytes			
		Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)
NC	8.21±0.35	48.38±4.20^{a}	40.00±6.88^{a}	8.82±0.57^{a}	1.75±0.32^{a}
CP	8.39±0.66	49.73±8.11^{a}	40.30±11.84^{a}	8.65±0.43^{a}	1.60±0.37^{a}
LP58	8.33±0.42	51.17±5.09^{b}	40.91±6.36^{a}	9.00±0.03^{a}	1.65±0.02^{a}
LP59	8.24±0.71	51.81±4.25^{b}	39.96±10.73^{a}	8.71±0.55^{a}	1.55±0.06^{a}
LP60	8.27±1.76	52.03±4.13^{b}	40.14±8.91^{a}	8.95±0.62^{a}	1.62±0.07^{a}

Means with different superscripts on the same column are significantly different ($p < 0.05$).

Table 4. Some serum biochemical parameters of *C. gariepinus* fingerlings fed diets supplemented with three strains of *L. plantarum*.

Treatment groups	Albumin (g/l)	Globulin (g/l)	Total protein (g/l)	Urea (nmol/l)	Calcium (nmol/l)	Creatinine (nmol/l)
NP	14.52±2.77^{a}	25.02±3.11^{a}	39.57±6.07^{a}	1.64±0.08^{a}	2.43±0.12^{a}	47.31±7.23^{a}
CP	15.62±1.93^{a}	23.23±4.75^{a}	38.82±5.13^{a}	1.63±0.02^{a}	2.34±0.09^{a}	46.00±7.53^{a}
LP58	16.24±2.56^{a,b}	24.38±4.05^{a}	40.51±5.99^{a}	1.58±0.04^{a}	2.36±0.11^{a}	50.05±6.96^{b}
LP59	15.63±2.08^{a}	23.90±3.47^{a}	39.86±6.11^{a}	1.62±0.04^{a}	2.36±0.09^{a}	48.25±6.36^{a,b}
LP60	18.25±1.59^{b}	24.22±2.98^{a}	42.47±5.53^{b}	1.63±0.05^{a}	2.35±0.08^{a}	49.95±7.18^{b}

Means with different superscripts on the same column are significantly different ($p < 0.05$); column means with no superscripts are not significantly different ($p > 0.05$).

Table 5. Water quality parameters in rearing tanks of *C gariepinus* fed diets supplemented with three strains of *L.plantarum*

Treatment	Dissolved Oxygen (mg/l)	Temperature (^{0}C)	pH
Inflow water	6.19±0.70^b	27.75±0.35^a	6.83±0.44^a
NP	5.16±0.52^a	27.82±0.35^a	8.67±0.37^b
CP	5.16±0.20^a	27.60±0.60^a	8.52±0.20^b
LP58	5.13±0.30^a	27.70±0.50^a	8.38±0.03^b
LP59	5.15±0.20^a	27.63±0.50^a	8.27±0.06^b
LP60	5.15±0.05^a	27.55±0.45^a	8.47±0.21^b

Values in the same column superscripted with different letters are significantly different ($p<0.05$)

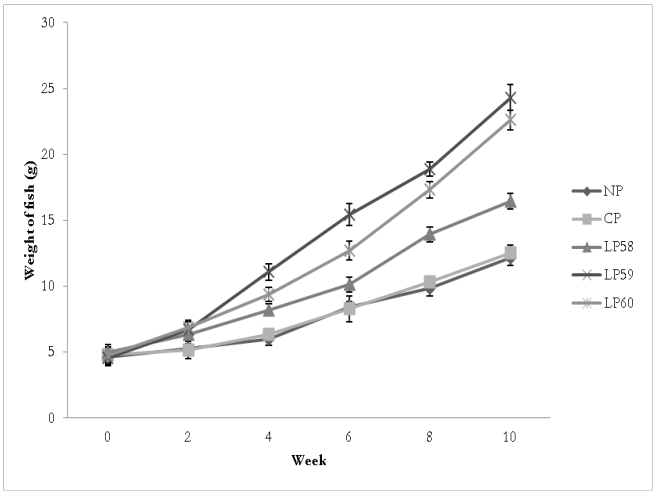

Figure 1. Weight curves of *C. gariepinus* fingerlings fed with diets supplemented with three strains of *L. plantarum* for 10 weeks.

Discussion

In this study, dietary supplementation of three strains of *L. plantarum* was observed to affect blood parameters of the experimental fish differently. Haemoglobin concentration was found to be significantly higher in fish groups fed with *L. plantarum* supplemented diets than the controls. Similarly, higher number of circulating erythrocytes (RBC) was observed in these fish groups. Increasing numbers of heamoglubin concentration and red blood cells observed in this study could imply that probiotic supplementation improve fish activeness. According to Eisler (1965), there was a correlation between heamoglobin concentration and activities of fish, as the more active fishes tend to have higher heamoglobin values than the sedentary ones. High values of erythrocyte counts recorded in this study could also indicate increased oxygen absorption and transportation capacity of the cells of *C. gariepinus* fingerlings fed with probiotics-supplemented diets, as opined by Akintayo *et al.* (2008) and Fagbenro *et al.* (2013).

Generally, better haematological values were observed in fish groups supplemented with the three strains of *L. plantarum* than control groups, while slight differential effects were also observed among groups fed with strains of *L. plantarum*. Except for RBC counts, fish groups fed with strains LC333559 and LC333560 had similar effects and appeared to have better haematological values than groups fed with LC333558. Although the mechanism for haematological stimulation in this study is uncertain, the positive effects observed might be due to the release of some vitamins or growth factors by different strains of *L. plantarum*. For instance, Leblanc *et al.* (2011) stated that certain lactic acid bacteria (LAB) can synthesize water-soluble vitamins such as the B-group (e.g. folates, riboflavin and vitamin B12) that are essential for fish's wellbeing. Production of these essential nutrients could vary from one strain to another, all of which might be responsible for strain-dependent effects observed in this study.

Modulation of the innate humoral and cellular defences is one of the benefits of probiotic treatment described in fish (Nayak, 2010), since the innate immune system is the first line of host defence against pathogenic organisms (Sinyakov *et al.*, 2002). The neutrophils, being the most abundant immune cell type of circulating leukocyte in most vertebrates, are the body's first line of defence against foreign invaders and constitute the major cell type involved in acute and some forms of chronic inflammation (Zhang *et al.*, 2017). Transmigration of neutrophils through the vascular endothelial walls into the inflamed tissues is a critical defense mechanism of the innate immune system against infection and injury caused by sepsis, trauma, ischemia-reperfusion and other acute or chronic inflammatory diseases (Hirano *et al.*, 2016). In the present study, significantly higher neutrophil values observed in fish fed diets supplemented with all tested strains of *L. planatrum* could be an indication of increased immunological functions of *C. gariepinus* by these bacterial strains (Shah *et al.*, 2017). However, total leukocytes, monocytes, lymphocytes and eosinophil counts appeared not to be affected by probiotics

supplementations among experimental groups. Studies by Hoseinifar *et al.* (2017) reported similar effects when rainbow trout (*Oncorhynchus mykiss*) were fed with probiotics, prebiotics and combinations of both. Our observation in this study was not supported by the report of Al-Dohail *et al.* (2009) who observed increased total leukocyte values when *Lactobacillus acidophilus* was supplemented in diets of *C. gariepinus* fingerlings. The contradictory results may be attributed to difference in species of probiotics used.

The measurement of serum biochemical parameters is of considerable diagnostic value in fish, as it relates to general nutritional status as well as the integrity of the osmoregulatory system and proper functioning of some vital organs including liver and kidney (Ajeniyi and Solomon, 2014). For instance, serum calcium and other electrolytes levels indicate the operation of a variety of homeostatic mechanisms in the body (Douglas and Jane, 2010). In this study, strains-dependent effect of *L. planatrum* was noticed only in albumin and creatinine levels in the experimental fish, while other serum parameters examined were not affected by probiotic dietary supplementations. It should be noted that, although high creatinine level could indicate excessive muscle formation or stress (Ajeniyi and Solomon, 2014), all values observed were within the ranges for normal healthy *C. gariepinus* as reported by Myburgh *et al.* (2008).

Fish weight was found to be higher in groups fed diets supplemented with three strains of *L. plantarum* than in the control groups at week 10 of the experiment, with the highest weight being recorded *L. plantarum* LC3335559 strain. Earlier study by Nwanna and Tope-Jegede (2017) reported similar improvement in growth indices (including weight gain and specific growth rate) of African catfish juveniles fed with *L. plantarum*. The results obtained in the present study supports the view that each strain can elicit different effect on the host, thus the need to assess the performance of any proposed species to strain level during evaluation of candidate probiotics

Conclusion

The present research demonstrated the existence of strain-specific effects of *L. plantarum* on the blood profiles of African catfish fingerlings. There was improvement in the heamotological and serum biochemical values of *C. gariepinus* fingerlings fed diet supplemented with *L. plantarum* LC333559 strain. Further research is warranted to evaluate other benefits of this strain before they can be employed as probiotics in African catfish aquaculture.

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