

The effect of *Lactobacillus acidophilus* as a dietary supplement on nonspecific immune response and disease resistance in juvenile common carp, *Cyprinos carpio*

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Abstract

Effect of diet fortified with *Lactobacillus acidophilus* at 0.0, 0.2, 0.4 and 0.6% on nonspecific immune response and disease resistant common carp juvenile infected with *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were examined. Immune response parameters (total protein, albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, urea, creatinine and immunoglobulin), percentage mortality and relative protection level were determined on days 1, 14, 28, 42 and 56. Fish fed *Lactobacillus acidophilus* fortified diet had significantly improved nonspecific immune parameters especially group treated 0.4% inclusion with lower mortality rate indicating a better relative protection level against *Pseudomonas aeruginosa* and *Aeromonas hydrophila*.

Keywords

Diseases resistance
Immune response
Lactobacillus acidophilus
Pseudomonas aeruginosa
Aeromonas hydrophilla

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Introduction

The need for aquaculture became necessary following the tremendous decrease in supply from captured fisheries due to over-fishing, unreported, illegal and irresponsible fishing, habitat destruction and pollution among other factors. Aquaculture has been accountable for the remarkable and progressive growth in the fish supply following geometric increase in fish farming. Despite the achievement recorded in the aquaculture sector, some constraint such as disease outbreak has hindered the industry. Disease in fish is an establishment of pathogens in fish tissues which causes disorderliness in physiological function of the fish that result in to physical, biological and economical loses. Diseases arise as result of complex interaction among the fish, pathogen and culture environment (Ajani *et al.*, 2011). Antibiotics are one of the common means of control and management of fish diseases. However, use of Chemotherapeutics in aquaculture as disease control agents has become debatable due to rise of drug resistant bacteria, residual effects and restraint the immune system of the fish (Ulukoy *et al.*, 2017) leading to search for alternative such as probiotics.

Probiotics are life microbial feed supplements that improve health host by modify the gastrointestinal tract of the fish. Fish, being a hydrophilic animal rely solely on the environment (water) which

filtering through the body and gill as fish performs it physiological function would benefit from use of probiotics. Probiotics enhance the nutrient utilization, modulate gut flora, inhibit the growth of pathogenic bacteria and improve growth and immune system of the fish as reported in the previous studies (Nayak, 2010, Ulukoy *et al.*, 2017). Several probiotics have been used in aquaculture but probiotics from lactic acid bacteria (LAB) and Bacillus species are often used (Wang *et al.*, 2013; Muno-Atienza *et al.*, 2013; Ulukoy *et al.*, 2017).

Lactobacillus acidophilus is bacteria belonging to the genus *Lactobacillus* which significant present in gut of common carp (*Cyprinos carpio*). It has been isolated from piglet, chicken dung etc. It probiotics benefit as member of LAB group to improve non-specific immune response and disease resistance in *Cyprinos carpio* has not been fully elucidated hence the need for this study. The aim of this study was to determine the effect dietary supplementation of *Lactobacillus acidophilus* nonspecific immune response and disease resistance in juvenile Common carp, *Cyprinos carpio*.

Materials and Methods

Culture and isolation of *L. acidophilus*

Chicken manure were obtained from a University of Ilorin Teaching and Research Farm (Poultry

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Table 1. Primer used for *Lacidophilus acidophilus* identification

Primers	Length	Primer sequence	Amplification product bp
La-F	22	5'CCTTTCTAAGGAAGCGAAGGAT 3'	199
La-R	22	5'AATTCTCTTCTCGGTCGCTCTA 3'	199

Source: adapted from Amin *et al.* (2011).

Unit), Nigeria and added to a selective medium of de Man-Rogosa Sharpe (MRS) broth (LAB M) and sodium azide (0.02% w/v). The medium was transported to the Department of Aquaculture and Fisheries, University of Ilorin, Nigeria in an ice box immediately and incubated at 37°C for 24 hr (Al-Dohail *et al.*, 2009). The medium was streaked on MRS agar prepared plates at 37°C for 48 hr. The plates which showed typical colony morphology of *Lactobacillus acidophilus* and sub-cultured to obtained pure colony.

Identification of *L. acidophilus*

All suspected isolates were subjected to both biochemical and molecular characterizations. Biochemical tests comprises gram stain, motility test, catalase test and sugar (Fructose, Galactose, Lactose, Maltose, Mannitol, Rhamnose, Sucrose and Xylose) fermentation (Pyar and Peh, 2014) while molecular characterization was carried out using Polymerase Chain Reaction (PCR) using specific primer (Table 1).

The DNA extract of *L. acidophilus* isolates

The isolates were suspended in 1.5 ml of enriched MRS broths, grown on a shaker for 48 hours at 48°C and pelleted by centrifugation at 6000 rpm (4600 x g) for 5 mins. The pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37°C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed (LQAD, 2008). The suspension was incubated for 10 mins at 65°C and kept on ice for 15 mins. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 mins and centrifugation at 7200 x g for 20 mins. The aqueous phase was transferred to a new tube, isopropanol (1: 0.6) was added and DNA was precipitated at -20°C for 16 hr. DNA was collected by centrifugation at 7200 x g for 10 mins, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately 3 hours and finally dissolved in 50

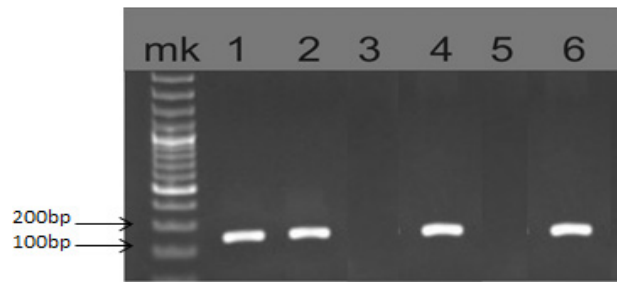


Figure 1. PCR image of *Lactobacillus acidophilus* showing positive bands on Lanes 1, 2, 4 and 6. Lane 1 = Positive control of *Lactobacillus acidophilus* (ATCC4356) while Lanes 2, 4, and 6 are identified isolates that amplified at 199 bp.

µl of TE buffer (LQAD, 2008).

Polymerase chain reaction (PCR) condition

Polymerase chain reaction (PCR) analyses were carried at -70°C in skim milk. One to two loop of the bacteria with phenotypically analysis grown on MRS agar, were resolved in TE (Trace EDTA) buffer and boiled at 100°C for 15 mins. The PCR conditions were initial denaturation of 94°C for 5 mins followed by 30 cycle of denaturation of 94°C for 30 s, annealing of 52°C for 30s for cas-ITS specific primer (45°C for 45 s) and final extension at 72°C for 10 mins using a thermocycler (eppendorf). The integrity of the amplified band genes fragment was checked on a 1.0% Agarose gel ran at 110V for 1 hour to confirm amplification and pictures were taken under gel electrophoresis (Amin *et al.*, 2011). The confirmed isolates were shown in Figure 1.

Experimental diets

The experimental diets were formulated to meet the nutritional requirements of common carp, containing 32% protein comprises (Lim *et al.*, 1979). The diets were 0% (control diet), 0.2% *L. acidophilus* (T1), 0.4% *L. acidophilus* (T2) and 0.4% *L. acidophilus* (T3) (Table 2). The feed ingredients were mixed thoroughly with distilled water and cultured isolates. The resulting dough was made into pellets, which were air-dried and stored in airtight containers at room temperature to prevent mould growth until required.

Experimental design

Two hundred and forty (240) healthy common carp (21.34±1.85 g) were obtained from a commercial farm in Jos, Nigeria. The fish were acclimatized for 2 weeks in 60 cm x 38 cm x 27 cm rectangular plastic tanks and fed commercial diets twice daily before experimentation. For the feeding trial, the fish were allotted to 12 tanks in a completely randomized

design containing 20 fish per tank in triplicate. The fish were fed experimental diets twice per day at 3% body weight for 56 days. The blood samples were obtained from the caudal vein on days 1, 14, 28, 42, and 56 of the trial periods.

Blood and serum analyses

The blood was collected from the caudal vein with the aid of 5 ml sterile syringe and needle inserted gently at 45°C. The blood was gently transferred into lithium heparinized anticoagulant bottles at about 25°C. The serum was collected with a pipette following a centrifugation at 3,000 rpm for 30 mins.

Red blood cells count (RBC) and White blood cells count (WBC)

The RBC was determined using differential method: the samples were diluted 1:200 with Rees and Ecker's diluent fluid. Few drops were expelled out, and then introduced under cover slip on an improved Neubauer count chamber (Neubauer improved bright line Marienfeld, Germany 0.100 mm, 0.0025 mm²) count with x 10 objective. The amounts were calculated as $RBC \times 10^{12} L = \text{Numbers of cell counted} \times \text{Depth} \times \text{Dilution} \times \text{Area}$ (Oresegun and Alegbeleye, 2001). The WBC was counted in a Neubauer counting chamber (Schaperclaus *et al.*, 1991; Ulukoy *et al.*, 2017) in triplicates.

Lymphocytes and heterocytes counts

The lymphocytes were determined by observing the smear under the light microscope (x 100 objective lens). The small cells containing nuclei, showing granular appearance were observed and expressed as percentage (Oyewale, 2011). While heterocytes cell with an average of 6-9µ with circular shape were observed with the aid of light microscope. The cells with rod shape with central granules with irregular size and not closely grouped, colourless to pink with bilobed or multilobed were observed and expressed as percentage (Oyewale, 2011).

Nitroblue tetazolium (NBT) counts

The NBT was measured by determine the respiratory burst activity (Anderson *et al.*, 1992). The blood samples were incubated at 25°C for 30 mins and washed with 0.067 mM sodium phosphate buffer (pH 6.4). A single drop of 0.2% of NBT solution placed on a slide and incubated for 30 mins at 25°C. The NBT cells were counted under the microscope (x 100 objective lens) in triplicates.

Immunoglobulin M (IgM)

The total immunoglobulin M (IgM) were

Table 2. Gross composition of experimental diets, with or without probiotic, formulated to contain 32% protein and fed to common carp.

Ingredients	T0 (Control)	T1 (0.2%)	T2 (0.4%)	T3 (0.6%)
Fish meal	25.24	25.24	25.24	25.24
Soybean	25.24	25.24	25.24	25.24
Maize	43.52	43.52	43.52	43.52
Premixes*	2.00	2.00	2.00	2.00
Starch	1.00	0.80	0.60	0.40
Di-calcium phosphate	2.00	2.00	2.00	2.00
Vegetable oil	1.00	1.00	1.00	1.00
Probiotic	0.00	0.20	0.40	0.60
Total	100.00	100.00	100.00	100.00

* Premixes = HI-MIX®AQUA (Fish) each one kilogram (1 kg) contains; vitamin A, 4,000,000 International Unit (IU); vitamin D3, 8,00,000 IU; vitamin E, 40, 000 IU; vitamin K3, 1,600 mg; vitamin B1, 4,000 mg; vitamin B2, 3,000 mg; vitamin B6, 3,800 mg; vitamin B12, 3 mcg; Nicotinic acid 18000 mg; Pantothenic acid, 8000 mg; Folic acid, 800 mg; Biotin, 100 mcg; Choline chloride 120,000 mg; Iron, 8000 mg; Copper, 800 mg; Manganese, 6000 mg; Zinc, 20,000 mg; Iodine, 400 mg; Selenium, 40 mg; Vitamin C C(coated), 60,000 mg; Inositol, 10,000 mg; Colbat, 150 mg; Lysine, 10,000 mg; Methionine, 10,000 mg; Antioxidant, 25,000 mg.

determined by enzyme-linked immunosorbent assay (ELISA) using immunoglobulin M (IgM) ELISA kit for fish in the chemical pathology Laboratory of the Faculty of Veterinary Medicine, University of Ilorin, Nigeria following the manufacturer's instruction.

Bacteria challenge

The juvenile common carp fed experimental diets for 56 days were challenged with *Pseudomonas aeruginosa* and *Aeromonas hydrophila* to examine its resistance against the pathogens for 14 days. The fish were intraperitoneally injected with 1.0×10^7 cfu/mL of *P. aeruginosa* and *A. hydrophila* broth (making 2 groups). Each group contain 120 fish (10 fish per tank) randomly allotted to 4 treatments in triplicates in a completely randomized design. Relative protection level (RPL) was calculated as follows.

$RPS = (1 - (\% \text{ mortality in treatment}) / (\% \text{ mortality in control})) \times 100$ [1] (Amend 1981)

Statistical analysis

Data obtained were analysed by one-way analysis of variance (ANOVA) at $p = 0.05$. The means among the treatments were compared using Duncan's Multiple Range Test using SPSS statistical package

Table 3. Red Blood Cells (RBC), White Blood Cells (WBC), Lymphocytes, Heterocytes and Nitroblue Tetrazolium (NBT) in blood samples of juvenile common carp fed diets fortified with different *Lactobacillus acidophilus* inclusion levels

Parameters	Treatments	Days				
		1	14	28	42	56
Red Blood Cells ($\times 10^6/\mu\text{L}$)	Control	1.05 \pm 0.03	1.04 \pm 0.06 ^a	1.06 \pm 0.01 ^a	1.05 \pm 0.04 ^a	1.09 \pm 0.11 ^a
	T1 (0.2%)	1.11 \pm 0.07	1.13 \pm 0.05 ^{ab}	1.13 \pm 0.04 ^{ab}	1.16 \pm 0.01	1.18 \pm 0.02
	T2 (0.4%)	1.03 \pm 0.10 ^A	1.15 \pm 0.01	1.20 \pm 0.00	1.22 \pm 0.20	1.27 \pm 0.30
	T3 (0.6%)	1.15 \pm 0.07	1.16 \pm 0.03	1.19 \pm 0.01	1.28 \pm 0.22	1.31 \pm 0.11
White Blood Cells ($\times 10^6/\mu\text{L}$)	Control	6.30 \pm 0.81 ^a	6.27 \pm 0.23 ^a	6.31 \pm 0.10 ^a	6.32 \pm 0.03 ^a	6.35 \pm 0.13 ^{aA}
	T1 (0.2%)	6.47 \pm 0.09 ^A	6.82 \pm 0.56 ^B	7.50 \pm 0.12 ^C	7.61 \pm 0.08 ^C	8.36 \pm 0.20 ^D
	T2 (0.4%)	7.12 \pm 0.21 ^A	7.63 \pm 0.03	7.84 \pm 0.20	8.13 \pm 0.21	8.94 \pm 0.27 ^C
	T3 (0.6%)	6.49 \pm 0.05	7.40 \pm 0.08 ^B	8.21 \pm 0.03 ^C	8.97 \pm 0.57 ^{CD}	10.57 \pm 0.01 ^{DE}
Lymphocytes (%)	Control	54.33 \pm 1.23 ^{aA}	57.13 \pm 1.00	55.17 \pm 0.82 ^{aB}	60.00 \pm 0.39 ^a	62.33 \pm 0.23 ^a
	T1 (0.2%)	62.47 \pm 1.03 ^A	65.67 \pm 1.34	67.33 \pm 1.90	69.17 \pm 1.21	71.67 \pm 1.98 ^B
	T2 (0.4%)	63.67 \pm 1.68 ^A	65.37 \pm 1.61	66.67 \pm 0.29	69.33 \pm 0.88	72.03 \pm 4.21 ^A
	T3 (0.6%)	69.00 \pm 0.87 ^B	66.13 \pm 1.18 ^{aA}	69.13 \pm 1.40	72.45 \pm 0.37	78.17 \pm 1.38 ^B
Heterocytes (%)	Control	30.23 \pm 1.02 ^a	24.00 \pm 1.06 ^a	25.17 \pm 1.33 ^a	25.33 \pm 1.26 ^a	26.00 \pm 1.25
	T1 (0.2%)	29.17 \pm 1.11	26.13 \pm 0.59 ^A	26.45 \pm 1.02 ^{aB}	27.11 \pm 1.00	27.17 \pm 1.62
	T2 (0.4%)	26.32 \pm 1.13 ^A	25.67 \pm 1.07 ^A	27.33 \pm 1.22 ^{aB}	27.67 \pm 0.85	28.10 \pm 0.76
	T3 (0.6%)	25.33 \pm 0.76 ^{aA}	27.33 \pm 0.92 ^{aB}	28.23 \pm 1.01	29.33 \pm 1.06	30.67 \pm 1.00
Nitroblue Tetrazolium (%)	Control	3.02 \pm 0.02 ^{aA}	2.86 \pm 0.09 ^{aB}	2.35 \pm 0.04 ^a	1.96 \pm 0.03 ^a	1.73 \pm 0.04 ^a
	T1 (0.2%)	3.10 \pm 0.04 ^{abA}	3.13 \pm 0.03 ^A	3.15 \pm 0.10 ^B	3.18 \pm 0.01 ^B	3.19 \pm 0.21 ^B
	T2 (0.4%)	3.24 \pm 0.11 ^A	3.25 \pm 0.05 ^A	4.27 \pm 0.12 ^B	4.60 \pm 0.03	4.95 \pm 0.33
	T3 (0.6%)	3.22 \pm 0.06 ^A	3.47 \pm 0.13 ^a	3.98 \pm 0.08	4.72 \pm 0.21	5.04 \pm 0.05 ^B
Immunoglobulin ($\mu\text{g/mL}$)	Control	62.14 \pm 3.12 ^{aA}	65.02 \pm 2.19 ^a	65.43 \pm 1.65 ^a	71.24 \pm 7.02 ^a	76.53 \pm 5.90 ^a
	T1 (0.2%)	71.01 \pm 4.20 ^B	63.78 \pm 3.07 ^{aA}	81.06 \pm 2.06 ^B	83.50 \pm 1.53 ^B	87.39 \pm 7.44 ^a
	T2 (0.4%)	93.53 \pm 3.67 ^{aA}	94.48 \pm 7.02 ^a	101.28 \pm 1.77 ^C	135.11 \pm 6.07	135.45 \pm 2.51
	T3 (0.6%)	121.89 \pm 5.09 ^{aA}	130.72 \pm 1.54	135.03 \pm 3.15 ^D	137.97 \pm 4.13	141.62 \pm 4.03

Values are means of triplicates groups and presented as mean \pm SD. Values in the same column having different superscript small letters are significantly different ($p < 0.05$); Values in the row having different superscript capital letters. The lack of manuscript letters indicates no significant differences among the treatments.

(Version 20.0, IBM SPSS Inc., NY, USA).

Results

The effect of *Lactobacillus acidophilus* on non-specific immune response parameters of common carp are presented in Table 3. There were significantly increased in the values of red blood cells, white blood cells, lymphocytes, heterocytes and nitroblue tetrazolium in treated groups when compared to control group. However, red blood cells were progressively increase with increase in treatment days in treated groups except on day 1 of fish treated 0.4% *Lactobacillus acidophilus* fortified diet. Highest white blood cells, heterocytes and nitroblue tetrazolium were observed on day 56 ($p < 0.05$). There was increase in the parameters values in treated groups than the control.

There was statistically significant increase in the values of immunoglobulin in groups fed with fortified diets when compared with control group.

However, significantly higher immunoglobulin was recorded in groups treated 0.4 and 0.6% *Lactobacillus acidophilus* based diets ($P < 0.05$). Also, noticeable increases in the immunoglobulin values were obtained with increase in the trial period until day 42 where the values were relatively stable.

The challenged results indicated that fish fed basal diet had better higher mortality rates against *L. acidophilus* and *A. hydrophila* when compared to treated groups with fish exposed to *A. hydrophila* showed higher mortality between the two pathogens (Table 5). Significant reduction in mortality rate in fish fed 0.4 and 0.6% *L. acidophilus* fortified diets. Progressive relative protection against *P. aeruginosa* and *A. hydrophila* were observed in fish fed *L. acidophilus* fortified diets with higher percentage in fish fed 0.4 and 0.6% fortified diets.

Discussion

Continuous debate on the use of chemotherapeutic

Table 4. Resistance of common carp juveniles challenged with *Pseudomonas aeruginosa* (1.0×10^7 cfu/mL) and *Aeromonas hydrophila* and fed with different levels of *Lactobacillus acidophilus*.

Pathogen	Treatments	Number of fish		RPL
		infected	Mortality (%)	
<i>Pseudomonas aeruginosa</i>				
(1.0 × 10 ⁷ cfu/mL)	Control	30	63.33	0.00
	T1 (0.2%)	30	36.67	42.11
	T2 (0.4%)	30	20.00	68.42
	T3 (0.6%)	30	13.33	78.95
<i>Aeromonas hydrophilla</i>				
(1.0 × 10 ⁷ cfu/mL)	Control	30	76.67	0.00
	T1 (0.2%)	30	43.33	43.48
	T2 (0.4%)	30	13.33	82.61
	T3 (0.6%)	30	16.67	78.26

Note: RPL = Relative protection level

has ushered in the application of probiotics in aquaculture to enhance the immune system of fish. Various probiotics have been used to immune response of the fish such as *Bacillus* species (Guzel-Seydim *et al.*, 2011), *Lactobacillus* species (Ulukoy *et al.*, 2015); *Lactobacillus fermentum* (Akanmu *et al.*, 2016); *Saccharomyces cerevisiae* (Akanmu *et al.*, 2016) and Kefir (Ulukoy *et al.*, 2017) among others but there dearth of studies on the effect *Lactobacillus acidophilus* on the immune system of common carp.

Previous studies have revealed that measurements of non-specific immune parameters in fish are valuable information on the health status of the fish. The results of this study depicted that haematological parameters such as red blood cells, white blood cells, lymphocytes, heterocytes and nitroblue tetrazolium were significantly increased in common carp fed diets fortified *Lactobacillus acidophilus*. The results obtained are in accordance with the findings of Faramarzi *et al.* (2011) in rainbow trout, Al-Dohail *et al.* (2009) in African catfish fed diets contain *Lactobacillus acidophilus*.

The major components of the immune response against pathogen are immunoglobulin and were examined in this study. The results of this study revealed a significantly increase in the level of immunoglobulin in the fish fed *Lactobacillus acidophilus* fortified diets when compared to control group. Previous studies of Panigrahi *et al.* (2004), Al-Dohail *et al.* (2009), Can *et al.* (2012), Akanmu *et al.* (2016) and Ulukoy *et al.* (2017) showed an increase in the immunoglobulin in fish fed probiotic using

Lactobacillus rhamnosus, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and Kefir respectively. These results in terms of trend share similar view with the present study.

An investigation into the survival rate of common carp fed *Lactobacillus acidophilus* and challenged with *Pseudomonas aeruginosa* and *Aeromonas hydrophila* showed significantly increase survival rates in treated groups than the control which is in agreement with previous (Gildberg *et al.*, 1997; Nikoskelainen *et al.*, 2001; Son *et al.*, 2009; Faramarzi *et al.*, 2011; Pereze-Sanchez *et al.*, 2011; Geng *et al.*, 2012; Abumourad *et al.*, 2013; Araujo *et al.*, 2015).

Similarly, fish fed *Lactobacillus acidophilus* were relatively protected against the pathogens based on the survival and inclusion level. Ulukoy *et al.* (2017) observed similar protection trend in fish fed Kefir based diets.

In conclusion, common carp fed with diets fortified with *Lactobacillus acidophilus* increased the non-specific immune parameters of the fish and other haematological parameters. Also, fish fed diets fortified with *Lactobacillus acidophilus* relatively protect fish against pathogenic *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Therefore, diets fortified with *Lactobacillus acidophilus* at 0.4% is recommended to improve the non-specific immune response of common carp and elevate its protection again pathogenic bacteria.

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