Chapter 5

Gut Microbiota and Innate Immune Response of *Macrobrachium vollenhovenii* Infected with *Pseudomonas aeruginosa* and *Aeromonas hydrophila* Fed Diets Supplemented with *Lactobacillus acidophilus*

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Abstract

The use of antibiotics as disease control agents has become contentious due to rise in drug-resistant bacteria such as *Psuedomonas aeruginosa* and *Aeromonas hydrophilla*. Studies have shown antibacterial potentials of some probiotics such as *Lactobacillus acidophillus* as promising alternative. Therefore, effects of diets fortified with *Lactobacillus acidophillus* on gut ecology and health status of African River prawn, *Macrobranchium vollenhovenii* were investigated. Prawns fed diets fortified with *Lactobacillus acidophillus* were challenged with *Psuedomonas aeruginosa* (1×10^7 cfu/mL) and *Aeromonas hydrophilla* (5×10^5 cfu/mL) using bath method for 14 days. Total viable and total enterobacteriaceae counts were determined on plate count agar and McConkey agar, respectively. Haemolymph (mL), total haemocyte count (cells/mL), catalase (mg/g protein), superoxide dismutase (mg/g protein, respiratory burst activity (μmoles) and survival rate (%) were evaluated using standard procedures. Data were analysed using descriptive statistics and ANOVA at $p = 0.05$. Results revealed that *Marobranchium vollenhovenii*-fed supplemented diets had reduced bacteria load, gut flora dominated by beneficial bacteria, enhanced immune system and protection against *Aeromonas hydrophila* at 10^3 cfu/mL inclusion level and could be used as immunodulation against *Aeromonas* infection.

Keywords: gut microbiota, innate immune response, infection, bacteria

1. Introduction

Prawn such as *Macrobrachium vollenhobenii* is an important source animal protein that provides good quality protein and essential minerals and vitamins. It is also regarded as safest animal source of protein which comes from either wild or culture system. The current decline in the status fish from wild has been attributed to overfishing, illegal and irresponsible fishing, habitat destruction and pollution, among other factors. More so, the increase in population and change in the consumption pattern of fish has create huge gap between the demand and supply of fish [1]. One of the ways to bridge this gap is through aquaculture. The act of aquaculture means rearing of aquatic organisms, which entails any form of intervention to improve the production quantity and quality, management, disease prevention and control. Over the years, aquaculture has become the fastest growing sector in the world [2].

Worldwide total crusteceans was 6,915,100 tonnes representing about 7% of total aquaculture production [2]. This achievement was attributed to transformation of farming techniques from simple methods to an improved and intensive aquaculture which promotes high seed quantities, adequate knowledge of stocking density, supply of required fish feeds. However, in spite of the success recorded in the prawn farms in recent times, the performance are far from it demand. The poor performance has been associated with many constraints, such as, modern knowledge in the science of fish farming, government policy, fish feed industry, marketing, distribution, and diseases. One of the major barriers to prawn farming are diseases and their management.

Disease is an establishment of pathogens in prawn tissues which cause disorderliness in physiological function of the fish that result in physical, biological and economical losses. Diseases arise as a result of complex interaction among the fish, pathogen and culture environment [3, 4]. Fish has inbuilt immune systems and defence mechanisms which protect them from being infected with pathogens. But practice of intensive farming system has the possibility of exposing prawn to infections. However, bacteria have been reported to be responsible for about 70–80% of disease infection in fish [5]. Some important bacteria in prawn farming are *Pseudomonas aeruginosa* and *Aeromonas hydrophila. Pseudomonas aeruginosa* and *Aeromonas hydrophila* are gram-negative bacteria in the families of Pseudomonadaceae and Aeromonadaceae respectively. They are ubiquitous, facultative anaerobe, rod-shaped and sugar fermented organism. Studies have reported that *Pseudomonas aeruginosa* and *Aeromonas hydrophila* infection in fish has resulted into haemorrhagic, speticemia, furunculosis and high mortality among others [1].

The problems of increase antibiotic resistance bacteria, residual effect and environmental unfriendly experienced in the use of antibiotics could ameliorate by probiotics application. Therefore, current studies have moved toward 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 utilisation, modulate gut flora, inhibit the growth of pathogenic bacteria and improve growth and immune system of the fish as reported in the previous studies [6, 7]. Several probiotics have been used in

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aquaculture but probiotics from lactic acid bacteria (LAB) and *Bacillus* species are often used [7, 8].

Lactobacillus acidophilus is bacteria belonging to the genus Bacillus. It probiotics benefit as member of LAB group to improve non-specific immune response and disease resistance in in *Macrobrachium vollenhovenii* has not been fully elucidated hence the need for this study. Therefore, this study investigated the effect of diets fortified with *Lactobacillus acidophilus* on gut microbiota and innate immune response of *Macrobrachium vollenhovenii* Infected with *Pseudomonas aeruginosa* and *Aeromonas hydrophila.*

2. Materials and methods

2.1. Preparation of experimental diets

Feed ingredients were purchased from a reliable store Melbourne, Australia. *Lactobacillus acidophilus* ATCC 4356 was obtained from a laboratory and prepared into 0, 10^1, 10^2, 10^3, 10^4 and 10^5 cfu/mL (**Table 1**). Soya bean was further prepared by toasting in a hot plate for 15 min at 100°C. Ingredients were ground in hammer mill and mixed together to formulate 43% crude protein (**Table 1**) calculated according to Pearson Square Method [9]. The mixed ingredients were pelleted through pelleting machine. The feeds were packed in polythene bags and stored in a cool dry place at room temperature until use with labels on them. The crude proteins of the ingredients were: fish meal (72%); toasted soybean (46.2%) and white maize (9.3%) [10]. The feeds were reproduced after 21 days to avoid nutrients depletion.

2.2. Experimental design and procedure

Macrobrachium vollenhenvonii juveniles (mean weight = 18.02 ± 0.11 g; n = 360; 180 prawns for each pathogen) were obtained from a reputable farm in Melbourne, Australia and acclimatised for 2 weeks in glass aquaria tanks before the experiment. Prawns were weighed and distributed into 18 glass aquaria tanks (35 × 30 × 20 cm^3) in a completely randomised design with three replicates. Each tank contained 20 prawns. The tanks were constantly connected to aerator (Model: AP-60) with air blowers. The prawns that were fed experimental diets were further subjected to *Pseudomonas aeruginosa* and *Aeromonas hydrophila* infection for 2 weeks to examine their innate immune performances and survival. The diets were fed to the *Macrobrachium vollenhovenii* to satiation throughout the experimentation. Measurements of the weight changes were measured using sensitive scale (**Model: M1207)**.

2.3. Evaluation of gut microbiota

Three prawns were collected from each experimental unit before and after the trial for gut microbiota evaluation. Each prawn was deactivated in freezer at −20°C for 10 min and sterilised using formalin (50 ppm). The guts were aseptically collected and weighed into sterile universal bottles containing peptone water (0.1%) to release the available bacteria for a period of 2 h. 1 mL was taken from each sample bottle and diluted 10-folds and subsequently serially

Ingredients (%)	***Lactobacillus acidophilus* inclusion levels cfu/mL**					
Groundnut cake	25.00	25.00	25.00	25.00	25.00	25.00
Soya meal	24.00	24.00	24.00	24.00	24.00	24.00
Fish meal	22.15	22.15	22.15	22.15	22.15	22.15
DCP	1.00	1.00	1.00	1.00	1.00	1.00
Salt	1.00	1.00	1.00	1.00	1.00	1.00
Flour	0.89	0.89	0.89	0.89	0.89	0.89
Vegetable oil	0.50	0.50	0.50	0.50	0.50	0.50
Maize	24.96	24.96	24.96	24.96	24.96	24.96
Premix*	0.50	0.50	0.50	0.50	0.50	0.50
LA	0	10^1	10^2	10^3	10^4	10^5
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition						
Moisture	9.16	9.16	9.16	9.16	9.16	9.16
Crude protein	40.00	40.04	40.10	40.11	40.12	40.12
Ether extract	9.63	9.63	9.54	9.63	9.66	9.65
Total ash	8.27	8.27	8.27	8.27	8.27	8.27
Carbohydrates	20.17	20.13	20.16	20.06	20.02	20.03
Crude fibre	12.77	12.77	12.77	12.77	12.77	12.77

Note: DCP = Dicalcium phosphate; LA = *Lactobacillus acidophilus*.*Premixes = HI-MIX®AQUA (Fish) each 1 kg contains; vitamin A, 4000,000 International Unit (IU); vitamin D3, 8,00,000 IU; vitamin E, 40, 000 IU; vitamin K3, 1600 mg; vitamin B1, 4000 mg; vitamin B2, 3000 mg; vitamin B6, 3800 mg; vitamin B12, 3 mcg; Nicotinic acid 18,000 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.

Table 1. Ingredients and chemical composition (%; on dry matter basis) of experimental diets containing graded levels of *Lactobacillus acidophilus*.

diluted with dilution factor of 10^{-4}. 2 mL was taken from each diluted sample and dispensed into two Petri dishes (1 mL to each). The first dish received plate count agar (PCA, LAB M, LAB149) for total viable count (TVC), while the second Petri dish received MacConkey agar (LAB M, LAB002) for total enterobactereceae count (TEB) using the pure plate count method [11–12]. Each dilution was overlaid, respectively, with PCA and MacConkey that have been cooled to 50°C. At this temperature, agar is still in liquid form [12].

The dishes were then gently swirled to mix the bacteria with the liquid agar. The mixtures were allowed to harden. When the mixture was hardened, the individual cells were fixed in place and incubated (Newlife Laboratory Incubator NL-9052-1) for 24 h at 37°C to allow distinguished colonies to form. The colonies formed were counted using Wincom Colony Counter (16 W, 220 V ± 10%, 50 Hz). The experiments were replicated three times. The TVC and TEB were expressed in Log_{10}CFU/g [12].

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2.4. Bacterial challenge

To evaluate prawn resistance to disease infection, a challenge test using the pathogenic bacteria, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* was performed following 84 days feeding trial. Prior to the challenge test, a preliminary experiment was performed to determine the LD_{50} (lethal dose) of the pathogenic bacteria. For the challenge test, 10 prawn were selected from each replicate tank and transferred into another tank filled with dechlorinated freshwater. *Pseudomonas aeruginosa* was grown nutrient agar broth and *Aeromonas hydrophila* on PBS broth for 24 h at 35°C in an incubator in Microbiology Laboratory. Bacterial cells were then centrifuged at 3000× g for 30 min to form pellets. The pellets were re-suspended in 1 mL of 0.1% peptone water and after which 100 mL of the suspension were diluted at 4:10 with water from experimental tank. The prawn were exposed to bacterial solution of both *Pseudomonas aeruginosa* (1×10^7 CFU/mL) and *Aeromonas hydrophila* (5×10^5 CFU/mL) for 30 min and returned into experimental set up [13, 14]. The fish were fasted for 24 before infection and feeding with experimental diets resumed 12 h later. All prawn groups were kept under observation for 14 days to record any abnormal clinical signs and the daily mortality.

2.5. Determination of innate immune response parameters

Three prawns were randomly selected from each experimental unit. The haemolymph was collected from the ventral part of the haemocoel of the second abdominal segment with the aid of a sterilised syringe and a 21-gauge disposable hypodermic needle containing 1 mL of Alserver's solution and was transferred into anticoagulant bottle (EDTA). The plasma was prepared by centrifuged the haemolymph at 300× g for 10 min at 4°C. The haemocytes were suspended and adjusted to a concentration of 5×10^6 cells/mL in an ice-cold.

The innate immune parameters were measured using the diagnostic reagent kits (Randox® Laboratories, Crumlin, County Antrim, UK). Superoxide dismutase (SOD) activity was measured spectrophotochemically by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU/mL xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm. Catalase (CAT) activity was determined by measuring the decrease of H_2O_2 concentration at 240 nm according to [15]. The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and 10.6 mM H_2O_2 freshly prepared.

The respiratory burst activity was measured using diagnostic reagent kits (Randox, London, UK) as described by [16]. Respiratory burst activity was quantified by the nitroblue tetrazolium (NBT) assay which measures the quantity of intracellular oxidative free radicals; according to [17], with some modification. Briefly, 100 mL of the haemocytes were added to each well of a 96 well microtitre plate (Nalge-Nunc, Hereford, UK). The plate was incubated at 25°C, for 2 h to allow attachment of cells. Unattached cells were washed off three times using fresh L-15 medium. L-15 medium was then supplemented with NBT (1 mg/mL) and phorbol 12-myristate 13-acetate (PMA, SigmaeAldrich; 1 mg/mL) dissolved in dimethyl sulphoxide (DMSO, Sigma), and 100 mL added to each well of the microtitre plate and incubated for 1 h at

room temperature. After incubation, the supernatant removed from the plate and NBT reduction fixed with 100% methanol for 10 min. The plate was then washed with 70% methanol, and left to air dry. A mixture of 120 mL of 2 M potassium hydroxide and 140 mL DMSO was added to dissolve the resulting formazan blue crystals. The NBT reduction was measured using the microplate reader (Optica, Mikura Ltd., UK) at 630 nm, and respiratory burst activity was expressed as NBT reduction.

Total haemocyte count (THC) was performed in a haemocytomter using microscope. The phenolosidase activity (PO) was evaluated by measuring the formation of dopachrome L-dihydrophenylclamine (L-DOPA) at 490 nm with the aid of spectrophotometer. While reactive oxygen intermediates (ROI) were used to measure H_2O_2 by horseadish peroxidase (dependent oxidation of phenol red) while chemiluminescence was used to measure the light emission from reactive oxygen intermediates [18]. Lysozyme activity of fish sera was determined by using lysoplate technique [19]. In brief, 0.60 mg/mL *Micrococcus luteus* was cast in 1% agarose gel (Difco, USA) with 50 mM phosphate buffer (pH 6.2). Wells (6 mm) were created nutrient agar plates and were filled 25 μL of serum samples and incubated for 20 h at 25°C. Lysozyme activity was calculated from a standard curve prepared with lysozyme from chicken egg white. The respiratory burst activity was measured using diagnostic reagent kits (Randox, London, UK) as described by Chiu et al. (2007). Relative protection level (RPL) was estimated as RPL = [(1−%mortality in treatment)/% mortality in control) × 100] [20].

2.6. Statistical analysis

The results were presented as mean ± SE of three replicates. Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. The homogeneity of variances among different treatments was tested using Bartlett's test. Then, data were subjected to one-way ANOVA to evaluate effects of *Lactobacillus acidophilus* supplementation. Differences between means were tested at the 5% probability level using Duncan test. The optimum *Lactobacillus acidophilus* level was determined using polynomial regression analysis. All the statistical analyses were done using SPSS program version 20 (SPSS, Richmond, VA, USA).

3. Results

The effects of *Lactobacillus acidophilus* as a supplement on gut microbiota of *Macrobranchium vollenhovenii* are presented in **Table 2**. The total viable bacteria count (TVC) was significantly decreased in the gut samples of prawn fed with feed supplemented diets ($p < 0.05$). Similarly, total enterobactereceae (TEB) was reduced significantly in fish fed fortified diets. The highest TVC and TEB were recorded in fish fed control diet.

Table 3 depicts microbiota composition of *Macrobranchium vollenhevenii* fed diets fortified with *Lactobacillus acidophilus*. The results indicated the species composition of the prawn varied significantly with respect to levels of inclusion of *Lactobacillus acidophilus.* The dominated species were *Pseudomonas aeruginosa, Aeromonas hydrophila, Bacillus and Staphylococcus* species.

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***Lactobacillus acidophilus* inclusion levels (cfu/mL)**	**Parameters (log_{10}cfu/mL)**	
	TVC*	**TEB***
Control	6.32 ± 0.03[a]	6.12 ± 0.02
10^1	6.21 ± 0.04	5.96 ± 0.11
10^2	6.18 ± 0.20	5.72 ± 0.38
10^3	6.01 ± 0.16	5.62 ± 0.12
10^4	5.91 ± 0.09	5.43 ± 0.13[a]
10^5	5.81 ± 0.45	5.40 ± 0.90[a]

Means (Log_{10} cgu/mL) with different superscripts are significantly different ($p < 0.05$), while, absence of letters means no significantly different ($P > 0.05$). TVC = Total viable bacteria count; TEB = Total enterobacterceae.

Table 2. Gut microbiota of *Macrobranchium vollenhovenii* fed diets fortified with *Lactobacillus acidophilus*.

Parameters (log_{10}cfu/mL)	***Lactobacillus acidophilus* inclusion levels (cfu/mL)**					
	Control	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**
Pseudonomas aeruginosa	6.02 ± 0.09	5.92 ± 0.06	5.63 ± 0.26	5.57 ± 0.28a	5.32 ± 0.04a	5.15 ± 0.32a
Aeromonas hydrophila	6.06 ± 0.29a	5.49 ± 0.03a	5.38 ± 0.07	5.20 ± 0.22	4.90 ± 0.19	4.85 ± 0.04
Bacillus species	4.98 ± 0.07a	6.08 ± 0.58	6.14 ± 0.02	6.15 ± 0.03	6.17 ± 0.41	6.26 ± 0.21
Staphylococcus species	6.24 ± 0.03	6.22 ± 0.91	6.20 ± 0.15	6.19 ± 0.33	6.18 ± 0.21	6.18 ± 0.05

Means (Log_{10} cgu/mL) with different superscripts are significantly different ($p < 0.05$), while, absence of letters means no significantly different ($P > 0.05$).

Table 3. *Gut microbiota composition of Macrobranchium vollenhovenii fed diets fortified with lactobacillus acidophilus*.

Pseudomonasa aeruginosa and Aeromonas hydrophila were reduced significantly ($p < 0.05$) while *Bacillus* species was greatly increased. However, there was no significantly difference in the load of *Staphylococcus* species. The highest *Pseudomonas aeruginosa, Aeromonas hydrophila* and *Staphylococcus* species were recorded in prawn fed control diet while the least was observed in prawn fed diet fortified with 10^5 cfu/mL *Lactobacilus acidophilus*. However, the highest *Bacillus* species was obtained in prawn fed diet supplemented with 10^5 cfu/mL *Lactobacilus acidophilus*.

Effects of *Lactobacillus acidophilus* based diets on innate immune responses of *Macrobranchium vollenhovenii* was presented in **Table 4**. The immune response of the prawn was stimulated by *Lactobacillus acidophilus* supplementation. The SOD, CAT, RBA, THC, PO, ROI and lysozyme activity were significantly different ($p < 0.05$) among the treatments. Highest SOD, CAT, RBA, THC, PO, ROI and lysozyme activity were recorded in prawn fed 10^5 cfu/mL diet; meanwhile, the lowest values were obtained in the control group.

Parameters (log_{10}cfu/mL)	*Lactobacillus acidophilus* inclusion levels (cfu/mL)					
	Control	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**
SOD (mg/g protein)	0.41 ± 0.01a	2.45 ± 0.02ab	3.62 ± 0.05b	6.33 ± 0.15	6.57 ± 0.55	6.82 ± 0.22
CAT mg/g protein)	1.51 ± 0.02a	2.04 ± 0.04a	2.74 ± 0.01	3.06 ± 0.20	3.47 ± 0.21	3.85 ± 0.04
RBA	165.4 ± 2.17a	163.1 ± 3.13a	172.8 ± 1.11b	188.0 ± 2.15b	215.4 ± 2.46	243.7 ± 2.85
THC	12.06 ± 0.26a	13.11 ± 0.05	13.53 ± 0.l8	14.02 ± 0.17	14.65 ± 0.78	14.82 ± 0.16
PO (U/mL)	3.24 ± 0.43a	3.51 ± 0.07	4.07 ± 0.16	4.33 ± 0.09	4.89 ± 0.31	5.21 ± 0.34
ROI	5.27 ± 0.21a	6.02 ± 0.15	6.31 ± 0.34	6.38 ± 0.21	6.54 ± 0.23	6.79 ± 0.29
Lysozyme activity	8.13 ± 0.62a	11.20 ± 0.38a	13.05 ± 0.03	13.46 ± 0.78	13.78 ± 0.19	14.02 ± 0.18

Means with different superscripts are significantly different ($p < 0.05$), while, absence of letters means no significantly different ($P > 0.05$).

Table 4. Innate immune response parameters of *Macrobranchium vollenhovenii* fed diets fortified with *Lactobacillus acidophilus*.

In addition, the exposure of prawn fed fortified diets to *Pseudomonas aeruginosa* infection was presented in **Table 5**. The result indicated that there was significant difference ($p < 0.05$) in survival rate and relative protection of prawn fed fortified diets. Highest survival rate and relative protection were recorded in group fed 10^5 cfu/mL *Lactobacilus acidophilus* inclusion level and least were recorded in control group.

Table 6 reveals the resistant of *Macrobranchium vollenhovenii* to *Aeromonas hydrophila* infection. Survival and relative protection of prawn to *Aeromonas hydrophila* were significantly different ($p < 0.05$). Prawn fed 10^5 cfu/mL *Lactobacilus acidophilus* inclusion level had highest survival and relative protection rates while the prawn treated control diet had the lowest survival and relative protection rates.

Lactobacillus acidophilus inclusion levels (cfu/mL)	Parameters (log_{10}cfu/mL)		
	Initial stock	**Survival (%)**	**Relative protection**
Control	30	23.3 ± 0.03a	0.00 ± 0.00a
10^1	30	40.0 ± 0.13b	21.74 ± 0.15b
10^2	30	73.3 ± 0.23c	65.22 ± 0.16c
10^3	30	86.7 ± 0.71	82.61 ± 0.37
10^4	30	93.3 ± 0.11	91.30 ± 0.21
10^5	30	96.7 ± 0.42	95.65 ± 0.02

Means with different superscripts are significantly different ($p < 0.05$), while, absence of letters means no significantly different ($P > 0.05$).

Table 5. Resistant of *Macrobranchium vollenhovenii* fed diets fortified with *Lactobacillus acidophilus* to *Pseudomonas aeruginosa* infection.

Lactobacillus acidophilus inclusion levels (cfu/mL)	Parameters (log_{10}cfu/mL)		
	Initial stock	Survival (%)	Relative protection
Control	30	10.0 ± 0.01a	0.00 ± 0.00a
10^1	30	30.0 ± 0.01b	22.22 ± 0.03b
10^2	30	36.7 ± 0.15b	29.63 ± 0.11b
10^3	30	83.3 ± 0.21	81.48 ± 0.16
10^4	30	90.0 ± 0.13	88.89 ± 0.80
10^5	30	93.3 ± 0.04	92.59 ± 0.51

Means with different superscripts are significantly different ($p < 0.05$), while, absence of letters means no significantly different ($P > 0.05$).

Table 6. Resistant of *Macrobranchium vollenhovenii* fed diets fortified with *Lactobacillus acidophilus* to *Aeromonas hydrophila* infection.

4. Discussion

The result of the gut flora revealed that there were reductions in the bacteria load of the prawn fed fortified diets. The decrease in the in observed could be attributed to the activities of *Lactobacillus acidophilus* as probiotics which modulate the gut pH to its favour and outcompete the pathogenic organisms. According to International Commission on the Microbiological Specification of Foods [21] the acceptable level of bacterial load in fish tissue should be lower than 5.70 Log_{10}cfu/g. In this study, the TVC and TEB of prawn fed fortified diets were within the recommended values except the control and fish fed 10^1 cfu/mL of *Lactobacillus acidophilus*. The reduction in the load of bacteria especially TEB signifies that the probiotics improve the gut of the prawn by outcompete the pathogenic bacteria. The gut flora comprises *Pseudomonas aeruginosa, Aeromonas hydrophila,* Bacillus species and *Staphylococcus* species. Fish fed *Lactobacillus acidophilus* based diets had higher gut flora dominated by *Bacillus* species that the fish fed control diet and reduction of pathogenic organisms were observed. The findings of this study are in agreement with the work of [22] who reported that probiotics enhanced the population of beneficial bacteria and suppressed the growth of pathogenic bacteria.

Gut flora have continuous and dynamic effect on the host's gut and systemic immune systems. The bacteria are key in promoting the early development of the gut's mucosal immune system both in terms of its physical components and function. The bacteria stimulate the lymphoid tissue associated with the gut mucosa to produce antibodies to pathogens [23]. The immune system recognises and fights harmful bacteria, but leaves the helpful species alone, tolerance developed in juveniles [24]. Recent findings have shown that gut bacteria play a role in the expression of toll-like receptors (TLRs) in the intestines, molecules that help the host repair damage due to injury. The TLRs are one of the two classes of pattern-recognition receptors (PRR) that provide the intestine the ability to discriminate between the pathogenic and commensal bacteria [25]. These PRRs identify

the pathogens that have crossed the mucosal barriers and trigger a set of responses that take action against the pathogen [26].

Also, [27] claims that different species of gut flora could influence the development of key cells of the immune system, by increasing or decreasing the level of tolerance against foreign entities. Once the host immune system is developed, it regulates the bacterial composition in the gut. One of the regulation mechanisms—immune exclusion—is mediated through the neutralisation of secreted immunoglobulin A (IgA) [28].

Harmful bacteria species, such as *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, are unable to grow excessively due to competition from helpful gut flora species adhering to the mucosal lining of the intestine; thus, fish without/with low gut flora are infected very easily [28]. The barrier effect protects hosts from both invading species and species normally present in the gut at low numbers, whose growth is usually inhibited by the gut flora [29]. Helpful bacteria prevent the growth of pathogenic species by competing for nutrients and attachment sites to the epithelium of the colon. Also, symbiotic bacteria are more at home in this ecological niche and are thus more successful in the competition. Probiotics also produce bacteriocins, which are proteinaceous toxins that inhibit growth of similar bacterial strains, substances that kill harmful microbes and the levels of which can be regulated by enzymes produced by the host. Also, the process of fermentation produces lactic acid and different fatty acids, which lowers the pH in the colon, preventing the proliferation of harmful species of bacteria and facilitating that of helpful species [30].

Immune responses of the prawn fed fortified diets were higher than the control. There higher SOD, CAT RBA, THC, PO, ROI and lysozyme activity in prawn fed the *Lactobacillus acidophilus*-based diets than in those fed control diet. Similar observations were made by [22, 31] in fish fed probiotics diets. The SOD, CAT RBA, THC, PO, ROI and lysozyme activity have important roles in the innate immune defence system. For instance, it was reported that lysozyme activity plays a significant role in innate humoral immune factors that control intrusiveness of pathogens by destroying the cell walls of the bacteria [1]. The SOD catalyses the dismutation of the superoxide anions to molecular oxygen and hydrogen peroxide, which further catalyses to water and oxygen. The SOD and CAT play an important role as cellular antioxidants against reactive oxygen species. In this study, prawn fed fortified diets had increased values of SOD, CAT, RBA and lysozyme activity, which could be answerable for the increased concentration of hydrogen peroxide in the oxidation process. The chemicals are released from immune cells and enzymes, as they come in contact with pathogens cumulating into improved immunity.

The investigation into the resistance level of *Macrobranchium vollenhovenii* fed diets fortified with *Lactobacillus acidophilus* revealed that the prawn were protected that the group fed control diet. In this study, prawns were significantly protected against *Pseudomonas aeruginosa* and *Aeropmonas hydrophila* infection with relative protection up to 96%. Similarly, [32–33, 7] reported that there was significant increase in the survival rate of fish fed *Lactobacillus acidophilus* fortified diets. The protection of prawn against the pathogens could be attributed to the gut serves as an entry point through feeding and thus inhibit the growth and survival of the pathogens.

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5. Conclusion

Macrobranhium vollenhovenii fed diets supplemented with *Lactobacillus acidophilus* had reduced total enterobactericeae in the gut, enhanced innate immune response parameters and suggest it usage as immunomodulation and protective agent against *Pseudomonas aeruginosa* and *Aeromonas hydrophila* infection.

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