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Research Article

Interaction effect of vaccine, *curcuma longa*, and *Annona muricata* on growth performance, survival rate, and immuno–health status in *Clarias gariepinus* against *Pseudomonas aeruginosa*

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Abstract

This study examined the effects of a combination of adjuvant monovalent vaccination (AdMV), aqueous monovalent vaccine (AqMV), soursop leaves, and turmeric rhizome on the growth performance, survival rate, and immuno-health status of *Clarias gariepinus* fingerlings following *Pseudomonas aeruginosa* challenge. Twenty *C. gariepinus* fingerlings (4.91 ± 0.01 g) were fed a diets consist of the control (0%), AqMV2 (0%), AdMV3 (0%), (AqMV+TR)4 (1.0%), (AdMV+TR)5 (1.0%), (AqMV+SL)6 (1.0%), (AdMV+TR)7 (1.0%), (AqMV+TR+SL)8 (2.0%), and (AdMV+TR+SL)9 (2.0%) for 84 days. The fish were fed twice daily at 3% body weight. The weight gain (WG), specific growth rate (SGR), white blood cells (WBC), lymphocytes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total protein (TP) levels were examined. *C. gariepinus* fingerlings were induced with 4.85 $\log_{10}$ CFU/mL of *P. aeruginosa* from a 24-hour-old culture intraperitoneally, mortality and relative survival percentage were recorded 28 days post-infection. The findings indicate that WG, and SGR, were better in the control group than all other treated groups except AdMV2. The treated groups showed higher WBC, lymphocyte, TP, and albumin levels when compared to the control. A reduction was noted in the kidney, muscle, and spleen bacterial counts in the treated groups compared with the control, and a drop in the AST and ALT values of the treated groups relative to the control. The treated groups showed lower mortality and better relative percentage of survival. These findings indicate that vaccination, turmeric rhizome, and soursop leaves together enhanced the growth, health metrics, and survival rate of *C. gariepinus* fingerlings against *P. aeruginosa.*

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Introduction

Fish infections frequently occur because of a mix of various pathogens, including bacteria, viruses, fungi, and parasites. Among these, bacterial fish diseases are believed to pose a major problem for the aquaculture industry. According to Willey and Woolverton (2008), bacteria are microscopic, single-celled organisms that are constantly present in water, soil, and air. Cultured freshwater fish, including catfish, salmon, and trout, contain various harmful microorganisms. However, stress factors in aquaculture include handling, parasite infestation, low dissolved oxygen, high ammonia and nitrite, poor nutrition, increased fish density, and weakened fish food. Stress weakens the immune system, rendering fish more vulnerable to bacterial illnesses. Therefore, fish kept in captivity are more susceptible to diseases than those in the wild. *Aeromonas hydrophila*, *Pseudomonas aeruginosa, Cytophaga* (Flexibacter) *columnaris,* and *Pseudomonas fluorescens* are typical opportunistic bacteria that can infect and kill fish. Certain microorganisms are classified as obligatory pathogens because in the absence of stress, they may be the only factor contributing to the *disease.* There are those who believe that *Yersinia ruckeri, Renibacterium salmoninarum, Edwardsiella ictaluri,* and *Aeromonas salmonicida* are obligate pathogens (Durborow and Francis-Floyd, 1996; Todar, 2008; Cornelis, 2008). Medicated feed is a successful treatment for several bacterial diseases in aquaculture. According to Willey and Woolverton (2008), *Pseudomonas aeruginosa* is a gram-negative bacterium responsible for most bacterial infections in Nigeria. Most *Pseudomonas aeruginosa* are classified as obligatory aerobes; however, under specific environmental conditions, bacteria can function as facultative anaerobes. Additionally, it is thought to be a chemoheterotroph because it obtains energy (Cooper *et al.,* 2003; Ryan and Ray, 2004; Willey and Woolverton, 2008). Bacteria with rod-like, straight, or slightly curved shapes belong to *Pseudomonas*. *P. aerug*inosa is a rod-shaped bacterium. Its dimensions are between 0.5 and 1.0 mm in length and 1.5 and 5.0 mm in breadth. A polar flagellum is used by nearly all strains for motility (Todar, 2008). Gram-negative *P. aeruginosa* cells have a gram-negative cell wall comprising three layers: an outer membrane, a thin layer of peptidoglycan, and a plasma membrane (Cornelis, 2008). Humans, plants, and animals can contract *P. aeruginosa* infections. They afflicted immunocompromised people. The permeability barrier provided by the gram-negative outer membrane of *P. aeruginosa* confers inherent resistance to numerous antibiotics (Willey and Woolverton, 2008). Therefore, substitutes are required, such as natural products with antibacterial properties.

Studies involving fish and shellfish have demonstrated the efficacy of medicinal plants as growth promoters, immunostimulants, agents with antibacterial, antiviral, antifungal, and antiparasitic properties, and hunger stimulants (Bello *et al.,* 2012; Syahidah *et al.,* 2015; Olusola *et al.,* 2020). Secondary metabolites derived from medicinal plants, either pure or mixed compounds, are responsible for these biological actions (Radulović *et al.,* 2013). These chemicals may also be sources of

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novel medications to control disorders (Savoia, 2012). Fish farming uses an increasing number of vaccines against bacterial infections (Sommerset *et al.,* 2005). Although their use in aquaculture has not yet been thoroughly explored, medicinal herbs, such as soursop leaves and turmeric rhizomes, may be a possible feed element to improve fish health and growth. Therefore, the purpose of this study was to assess the combined effects of aqueous monovalent vaccination (AqMV), adjuvant monovalent vaccine (AdMV), soursop leaves, and turmeric rhizomes on the growth, health parameters, and survival of *Clarias gariepinus* fingerlings following *Pseudomonas aeruginosa* challenges.

Materials and methods

Plant *source and identification*

Turmeric rhizomes were purchased from Oja Oba market in Ondo, Ondo State Nigeria and Soursop leaves were obtained from a farm in Oka-Akoko, Nigeria. These plants were taken to the Department of Biological Sciences (Botany Programme), Olusegun Agagu University of Science and Technology, Okitipupa for identification and authentication.

Processing of plant materials

The turmeric rhizome was washed with sterile distilled water and allowed to air-dry at ambient temperature (25°C) for one hour. The dry outer coverings of the turmeric rhizome were manually peeled off, washed, and dried for one week, ground into a fine powder, and stored (4°C) until required. The soursop leaves were air – dried for 4 weeks, ground with a hammer mill to fine powder, and stored at 25°C until required.

Bacterial and vaccine preparation

Bacterial (*Pseudomonas aeruginosa*) isolated from diseased *C. gariepinus* were obtained from a known research institution that was isolated from diseased *C. gariepinus.* Vaccines were prepared as described by Olusola *et al.* (2024) and Zheng *et al*. (2012). The bacterial stock was streaked onto tryptic soy broth (TSB) agar plates supplemented with 2.5% NaCl. After incubation at 37°C for 24 h, single colonies were picked up into liquid TSB supplemented with 2.5% NaCl and incubated under rotation at 110 to 120 r/min for another 28 h. The cells were collected by centrifugation (4°C, 1397.5 xg 5 min), washed three times with phosphate-buffered saline (PBS, pH 7.2), and resuspended to a final concentration of 1×10^9 cells/mL. Then, 1% (v/v) formalin (formaldehyde 38% w/v, Panreac, Spain) was added to the bacterial suspension, and the bacteria were inactivated after incubation at 37°C for 48 h under shaking at 110 to 120 r/min. Inactive cells were collected by centrifugation (5000 rpm, 5 min) and washed 3 times with PBS (pH 7.2) to remove formalin. The cells were resuspended in sterile distilled water to obtain their original volume. To obtain aqueous monovalent bacteria (AqMV), the bacterial culture was centrifuged (4°C, 1397.5 xg, 5 min), the supernatants were discarded, and the pellets were re-suspended in buffered phosphate-buffered saline (PBS, pH 7.2). Vaccine absorbance (A ¼ 580 nm) was adjusted to 1 (which represents a concentration of 1×10^9 cells ml/L) in PBS (pH 7.2).

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Preparation of the propolis adjuvant vaccine

Propolis (Beehive) was purchased from Rotex herbal medicine in Ondo City, Ondo State, Nigeria. Adjuvant vaccine was prepared as described by Olusola *et al.* (2024). Fifteen grams (15 g) propolis was dissolved in 60 mL 95% ethanol, and the solution was incubated in a water bath at 25°C for 24 h under shaking and then filtered through a sterile membrane with a microporous diameter of 0.22 mm to remove any impurities. The concentration of the filtered solution was adjusted to 30 mg/mL using phosphate-buffered saline (PBS, pH 7.2). To prepare monovalent vaccine adjuvant (AdMV), the aqueous bacterin was concentrated by centrifugation and mixed with a natural plant propolis adjuvant at a ratio of 30:70 to obtain a stable fluid emulsion with a final concentration of 1×10^9 cells mL/L.

Procedures of vaccination, experimental system and feeding trials

Fish (20) with aqueous monovalent vaccine (AqMV) and adjuvant monovalent vaccine (AdMV) were immersed in 10 mL (1×10^8 cells) of 10 liters of water v/v for 2 h. In addition, the control group (20 *C. gariepinus* was immersed in 10 mL of PBS (pH 7.2) containing 10 L of water for 2 h. After an incubation period of 2 h, the water containing the bacterial suspension and PBS (pH 7.2) was removed and 40 L of fresh water was added to the experimental tank with a capacity of 45 L. The experiment was carried out in 18 experimental tanks for 8 weeks of feeding trials and four weeks for challenge tests in the Department of Fisheries and Aquaculture Laboratory, Olusegun Agagu University of Science and Technology, Okitipupa, Nigeria. Each treatment had two replicates, 20 fish per replicate with a mean initial body weight of (4.91±0.01 g) and uniform-sized fish were selected from 450 fingerlings. The tank was weighed and distributed throughout the experimental tank. The fish were acclimated for 14 days in glass aquaria before the experiment. All fish were starved for 24 h prior to vaccination. The fish were fed 3% body weight daily at 9:00 am and 5:00 pm. Weight changes were measured fortnightly and the feeding rate was adjusted based on the new body weight. The water in each tank was replaced every three (3) days throughout the experiment to prevent fouling that may have resulted from feed residues. All applicable international, national and institutional guidelines for the care and use of animals were followed by the authors. All experimental protocols were approved by the Bioethical Committee of the Olusegun Agagu University of Science and Technology, Okitipupa, Nigeria.

Preparation of the experimental diet

After preparation, the feed ingredients were mixed to formulate a 40% crude protein diet. Each diet mixture was extruded through a 1/4 mm die mincer of a Hobart A-200T pelleting machine to form a noodle-like strand that was mechanically broken into suitable sizes for the *Clarias gariepinus* juveniles. The pelleted diets were sun-dried, packed in labeled polythene bags, and stored in a cool, dry place to prevent mycotoxin formation (Table 1).

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

	Control (0%)	AqMV 2 (0%)	AdMV 3 (0%)	(AqMV +TR)4 (1%)	(AdMV+TR)5 (1%)	(AqMV+SL)6 (1%)	(AdMV+SL)7 (1%)	(AqMV+SL+TR)8 (2%)	(AdMV+SL +TR)9 (2%)
Fish meal	21.25	21.25	21.25	21.25	21.25	21.25	21.25	21.25	21.25
Soybean	42.49	42.49	42.49	42.49	42.49	42.49	42.49	42.49	42.49
Yellow maize	28.26	28.26	28.26	28.26	28.26	28.26	28.26	28.26	28.26
Starch	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vegetable oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Dicalcium phosphate (DCP)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Salt	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vit- min premix	2.00	2.00	2.00	1.00	1.00	1.00	1.00	-	-
Soursop leaves	-	-	-	-	-	1.00	1.00	1.00	1.00
Turmeric rhizome	-	-	-	1.00	1.00	-	-	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Moisture	7.01	6.88	7.02	6.87	6.89	7.02	7.06	7.06	7.04
Crude protein	40.10	40.09	40.08	40.18	40.16	40.14	39.95	40.12	40.06
Crude fibre	8.12	7.89	7.96	7.87	7.91	7.86	7.96	7.84	7.88
Ether extract	8.40	6.38	6.98	6.48	6.86	6.78	6.86	6.78	6.79
Ash	17.95	19.28	19.48	19.98	19.88	19.89	18.98	19.67	18.85
NFE	18.42	19.48	18.48	18.62	18.30	18.31	19.19	18.53	19.38
Gross energy (KJ/g)	1594.98	1533.19	1539. 46	1524.47	1533.51	1530.04	1543.86	1533.36	1546.97

* Vitamin mineral premix. Each 2.kg of premix contain; 12.5 million international unit (MIU); D_3, 2.5 MIU;E, 40 g; K_3 2 g; B1,5.5g;BB6,5g; Niacin 55 g; Calcium Pantothenate 11.5 g; Chlorine chloride 500 g; Folic acid, Biotin,0.08g;Manganese, 120 g; Iron, 100 g; Zinc, 80 g, Copper,8.5g; Iodine, 1.5g;Cobalt,0.3g;Selenium, 0.12 g; Anti-oxidant, 120 g. ***Proximate composition of experimental diet using standard methods of AOAC, (2005). Gross energy (kJ/g) = (protein content × 23.6) + (lipid content × 39.5) + (carbohydrate content × 17.2)

Biological evaluation

The fish were evaluated after eight weeks of feeding trials as follows:

$$\text{Weight gain (\%)} = \frac{100\,(\text{final body weight} - \text{initial body weight})}{\text{initial body weight}}$$

$$\textit{Specific growth rate (SGR)} = \frac{100\,(\log \textit{final body weight} - \log \textit{initial body weight})}{\text{Time (days)}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry weight of feed fed (g)}}{\text{fish weight gain (g)}}$$

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$$\text{Nitrogen metabolism (g)} = \frac{(0.549)(a+b)h}{2}$$

Where, 0.549 = constant value, a = Initial mean weight of fish, b = Final mean weight of fish, h = Experimental periods in days

Bacterial counts

Three *C gariepinus* fingerlings were taken from each experimental tank, anaesthetized with 2 – phenoxy ethanol (50 mL/L), and dissected at 0 and 28 days post–infection. Fish samples (muscle, spleen, and kidney) were collected for bacteriological examination via asepsis (medical examinations or procedures that prevent contamination or infection of microbes). According to the American Public Health Association, 1 g of fish sample was ground in 9 mL sterile peptone water in a mortar. One milliliter of this suspension was diluted with peptone water to 10^{-4}. Each diluent (1 mL) was poured into three petri dishes for plate count agar with OADC enrichment. The incubation period was 48 h at 37°C. After incubation of the fish sample dishes, the colonies were counted using a colony counter and the mean average colony size was calculated and recorded as described by Olusola *et al.* (2024).

Challenge test

To determine the 50% lethal dose (LD_{50}), three different dilutions of bacteria were used to infect five fish. LD_{50} calculations were performed using the method of Reed and Muench (1938), and an LD_{50} of. 4.85 $\log_{10}$ CFU/mL was observed. Two hundred and seventy (270) *C. gariepinus* (15 from each treatment) were challenged by the intraperitoneal route with 4.85 $\log_{10}$ CFU/mL *Pseudomonas aeruginosa* for 24 h. The challenged fish were kept under observation for 28 days. Mortality was recorded and the relative percentage of survival (RPS) among the challenged fish was determined as described by Olusola and Nwokike (2018). All experimental protocols were approved by the Bioethical Committee of Olusegun Agagu University of Science and Technology, Okitipupa, Nigeria:

$$RLP = 1 - [percentage\ of\ mortality\ in\ treated\ group]\ x\ 100$$
$$[percentage\ of\ mortality\ in\ control\ group]$$

Haematological and biochemical analysis

This was performed before and after the experiment at the institution's laboratory within 30 min of sampling. Three to four centimeters from the genital opening of each fish were punctured and wiped with dry tissue paper to avoid contamination with mucus. The needle was inserted at a right angle to the vertebral column of the fish and gently aspirated during penetration. Blood was collected under gentle aspiration until approximately 1 cm^3 was obtained. Thereafter, the needle was gently withdrawn and the blood was gently transferred into heparinized plastic containers. The samples were then gently and thoroughly mixed. Plasma was obtained from blood samples by centrifugation, drawn into a 1 cm^3 plastic syringe, and transferred into a universal bottle in the refrigerator for later use in

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biochemical analysis. A standard hemocytometer was used for both erythrocyte and leukocyte counts according to the methods of Blaxhall and Daisley (1973), using modified hyme's dilution fluid. The collected blood was introduced into an improved Neubauer Counting Chamber, and the cells were counted under a microscope at 100× magnification. Blood samples for biochemical analysis were centrifuged for 5 minutes at 503.1 xg using a Hawsley minor bench centrifuge. The derivatives were stored at – 20°C and analyzed at the institution's laboratory for glucose concentration, total protein, total lipid, albumin, globulin, and albumin and globulin ratios.

Analytical methods

The experimental feed and fish were analyzed for proximate composition before the experiment. Samples (50 g) were taken from each feed sample and four (4) fish were taken before the experiment and five (5) fish were taken after the experiment from each treatment and analyzed for their proximate composition according to the methods of the Association of Official Analytical Chemists (AOAC, 2005).

Statistical analysis

Growth performance and nutrient utilization, gut morphometry, bacteria count hematology, and biochemical analysis results from the experiment were subjected to one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences 2006 version 15.0). Duncan's multiple range tests at a significance level of $p<0.05$ were used to compare differences between individual means.

Results

Growth performance and nutrient utilization

The results of the biological evaluation revealed that there was a general increase in the values of all the treatments, and the results showed that the diets were better utilized and absorbed by the fish. There were significant differences ($p<0.05$) in final body weight, weight gain, percentage weight gain, specific growth rate, feed conversion ratio, nitrogen metabolism, and survival rate among the dietary groups. However, the control group is better than all other treatments except AdMV 2 (Table 2).

Haematological parameters

The hematology results showed that there were variations in the values obtained for all parameters. The packed cell volume, hemoglobin, red blood cell count, mean cell hemoglobin concentration, and lymphocytes were not significantly different ($p>0.05$) among the treated groups, whereas white blood cell platelets, mean cell hemoglobin, mean cell volume, neutrophils, monocytes, and eosinophils were significantly different among the treated groups which the control had the highest values (Table 3).

Blood performance and biochemical of C. gariepinus on aqueous and adjuvant vaccine, soursop leaves and turmeric rhizome meal-based diets

The results of blood performance showed that the control had the highest value (11.38) and the treated groups, (AqMV+TR)4, had the lowest value (10.91), and there was a significant difference ($p<0.05$) among the dietary groups.

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Table 2: Growth performance and nutrient utilization of *C. gariepinus* fed experimental diets for 56 days.

	Control (0%)	AqMV2 (0%)	AdMV3 (0%)	(AqMV+TR)4 (1%)	(AdMV+TR)5 (1%)	(AqMV+SL)6 (1%)	(AdMV+SL)7 (1%)	(AqMV+SL+TR)8 (2%)	(AdV+SL+TR)9 (2%)
Initial body weight (g)	4.91±0.01^{a}	4.91±0.01^{a}	4.91±0.00^{a}	4.91±0.01^{a}	4.91±0.01^{a}	4.91±0.00^{a}	4.91±0.01^{a}	4.91±0.01^{a}	4.91±0.01^{a}
Final body weight (g)	23.53±0.40^{c}	36.45±0.62^{f}	16.78±0.85^{bc}	21.15±0.22^{d}	19.96±0.88^{d}	13.43±0.10^{a}	13.19±0.69^{a}	17.18±0.03^{c}	15.05±0.77^{ab}
Weight gain (g)	18.62±4.00^{f}	31.54±0.08^{g}	11.87±0.85^{cd}	16.24±0.22^{e}	15.05±0.88^{e}	8.52±0.10^{ab}	8.28±0.68^{a}	12.27±0.03^{d}	10.14±0.76^{bc}
Percentage weight gain (%)	379.65±8.85^{g}	629.35±7.95^{h}	241.65±7.25^{d}	331.10±14.40^{f}	307.40±0.50^{e}	168.80±4.10^{b}	134.90±0.20^{a}	250.50±0.90^{d}	205.75±4.35^{c}
Specific growth rate(g)	1.04±0.02^{bc}	1.22±0.41^{c}	0.68±0.05^{ab}	0.91±0.21^{abc}	0.86±0.15^{abc}	0.41±0.18^{ab}	0.39±0.16^{a}	0.71±0.08^{abc}	0.57±0.07^{ab}
Feed conversion ratio	0.65±0.01^{a}	1.04±0.51^{ab}	2.26±0.16^{c}	1.02±0.27^{ab}	1.09±0.21^{ab}	1.95±0.44^{bc}	2.00±0.41^{bc}	1.41±0.12^{abc}	2.16±0.16^{c}
Nitrogen metabolism	437.03±6.00^{e}	635.63±21.05^{f}	333.34±12.99^{bc}	400.52±0.06^{d}	382.15±4.12^{d}	281.70±0.72^{a}	279.34±5.24^{a}	339.41±5.68^{c}	306.67±1.84^{ab}
Survival rate	70.00±5.00^{a}	80.00 ±5.00^{a}	75.00±10.00^{a}	77.50±2.50^{a}	80.00±10.00^{a}	85.00±5.00^{a}	85.00±.00^{a}	87.50±2.50^{a}	87.50±7.50^{a}

Means (n =2) in the same row with similar superscripts are not significantly different (p>0.05), SL= Soursop leaves; TR = Turmeric rhizome; AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine.

The value of total protein was highest in (AqMV + TR)4 (81.00) and the lowest was obtained before the experiment (69.00); albumin values were highest in (AqMV+TR)4 (42.00) and lowest in the previous experiment (30.00). The globulin had the highest value in (AqMV+TR+SL)8 (43.00) and the lowest in (AdMV+TR+SL)9 and AqMV2 (34.00). In addition, the highest albumin/globulin ratio was obtained in AqMV2 (1.10) and the lowest in the prior experiment, (AdMV+SL)7 and (AqMV+TR+SL)8 (0.80), respectively. There were significant differences (p<0.05) in total protein, albumin, and globulin among the dietary groups, with no significant difference (p>0.05) in the albumin/globulin ratio among the dietary groups (Table 4).

Blood serum indices

The highest value of aspartate aminotransferase (AST) was recorded in the control (11.00) and the lowest in (AqMV+TR)4, while the control (13.00) recorded the highest value in alanine aminotransferase (ALT) and the lowest in (AqMV+TR)4 (3.00). In addition, the highest value of glucose was recorded in the control (6.80) and the lowest in (AqMV+TR)4 (4.00).

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Table 3: Mean post-challenge test haematology of *C. gariepinus* fed experimental diets for 28 days.

	PCV	HB	RBC	WBC	PLAT	MCV	MCH	MCHC	NEUTRO	LYMPH	MONO	EOSINO
Before	42.00±0.01^{a}	14.00±0.00^{a}	4.70±0.03^{a}	13.80±0.05^{d}	35.90±0.02^{e}	89.00±0.80^{d}	29.80±0.02^{de}	33.30±0.01^{a}	71.00±0.03^{ab}	29.00±0.05^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Control	42.00±0.03^{a}	14.00±0.06^{a}	4.60±0.02^{a}	18.40±0.07^{g}	34.10±0.10^{c}	91.30±0.01^{e}	30.40±0.08^{e}	33.30±0.09^{a}	70.00±0.05^{ab}	30.00±0.01^{a}	0.00±0.04^{a}	0.00±0.00^{a}
AqMV2	40.00±0.02^{a}	13.30±0.01^{a}	4.70±0.05^{a}	17.60±0.01^{f}	30.20±0.04^{b}	85.10±0.03^{c}	28.30±0.01^{b}	33.30±0.07^{a}	71.00±0.01^{b}	27.00±0.03^{a}	1.00±0.01^{ab}	1.00±0.01^{b}
AdMV3	41.00±0.06^{a}	13.20±0.04^{a}	4.80±0.01^{a}	17.90±0.03^{f}	30.70±0.90^{b}	85.40±0.10^{c}	28.50±0.01^{b}	33.40±0.02^{a}	75.00±0.12^{ab}	25.00±0.01^{a}	0.00±0.00^{a}	0.00±0.00^{a}
(AqMV + TR)4	40.00±0.00^{a}	12.70±0.01^{a}	4.80±0.00^{a}	10.30±0.08^{b}	30.60±0.03^{b}	79.20±0.20^{a}	26.50±0.03^{a}	33.40±0.01^{a}	69.00±0.08^{ab}	30.00±0.01^{a}	0.00±0.00^{a}	1.00±0.00^{b}
(AdMV + TR)5	40.00±0.05^{a}	13.00±0.06^{a}	4.60±0.01^{a}	11.60±0.02^{c}	29.40±0.01^{a}	84.00±0.15^{c}	28.30±0.01^{b}	33.30±0.04^{a}	72.00±0.09^{ab}	28.00±0.02^{a}	0.00±0.00^{a}	0.00±0.00^{a}
(AqMV + SL)6	41.00±0.01^{a}	13.70±0.04^{a}	4.70±0.02^{a}	17.10±0.01^{f}	30.10±0.02^{b}	81.60±0.01^{b}	29.20±0.02^{cd}	33.40±0.01^{a}	68.00±0.01^{a}	30.00±0.01^{a}	2.00±0.01^{ab}	0.00±0.00^{a}
(AdMV + SL)7	40.00±0.02^{a}	13.30±0.05^{a}	4.90±0.04^{a}	16.50±0.07^{e}	30.50±0.01^{ab}	81.60±0.05^{b}	29.10±0.04^{c}	33.30±0.03^{a}	65.00±0.04^{ab}	30.00±0.03^{a}	4.00±0.00^{ab}	1.00±0.01^{b}
(AqMV + SL + TR)8	41.00±0.07^{a}	13.70±0.07^{a}	4.70±0.01^{a}	17.10±0.09^{f}	24.50±0.05^{c}	93.40±0.02^{c}	31.90±0.02^{f}	33.40±0.01^{a}	70.00±0.05^{ab}	30.00±0.02^{a}	0.00±0.00^{a}	0.00±0.00^{a}
(AqMV + SL + TR)9	42.00±0.08^{a}	14.30±0.01^{a}	4.70±0.03^{a}	9.50±0.07^{a}	35.90±0.04^{d}	102.40±0.78^{g}	34.10±0.01^{g}	33.30±0.02^{a}	70.00±0.06^{ab}	28.00±0.04^{a}	2.00±0.01^{ab}	0.00±0.00^{a}

Means (n = 2) in the same column with similar superscripts are not significantly different (p>0.05)**,** SL= Soursop leaves, TR = Turmeric rhizome, AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine, PCV=Packed cell volume, Hb= hemoglobin, RBC= red blood cell, WBC=White blood cell, MCV=Mean cell volume, MCH=Mean cell hemoglobin, MCHC=Mean cell hemoglobin concentration, Lymph=Lymphocytes, Mono=Monocytes, Eosino=Eosinophils.

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Table 4: Mean post-challenge test of blood performance and biochemical of *Clarias gariepinus* fingerlings fed experimental diets for 28 days.

TRT	Blood performance	Total Protein	Albumin	Globulin	A: G Ratio
Before	11.04±0.01^{c}	69.00±0.08^{a}	30.00±0.00^{a}	39.00±0.05^{ab}	0.80±0.00^{a}
Control	11.38±0.03^{e}	74.00±0.06^{abc}	35.00±0.04^{ab}	39.00±0.03^{ab}	0.90±0.01^{a}
AqMV2	11.28±0.02^{d}	72.00±0.10^{ab}	38.00±0.01^{bc}	34.00±0.02^{a}	1.10±0.03^{a}
AdMV3	11.32±0.00^{de}	76.00±0.04^{bc}	39.00±0.02^{bc}	37.00±0.01^{ab}	1.10±0.00^{a}
(AqMV + TR)4	10.84±0.02^{b}	81.00±0.15^{c}	42.00±0.06^{c}	39.00±0.04^{ab}	1.10±0.03^{a}
(AdMV + TR)5	10.83±0.01^{b}	73.00±0.07^{ab}	34.00±0.02^{ab}	39.00±0.08^{ab}	0.90±0.02^{a}
(AqMV + SL)6	11.28±0.03^{d}	72.00±0.01^{ab}	36.00±0.05^{abc}	36.00±0.01^{ab}	1.00±0.04^{a}
(AdMV + SL)7	11.30±0.02^{d}	75.00±0.02^{abc}	33.00±0.01^{ab}	42.00±0.03^{b}	0.80±0.00^{a}
(AqMV + SL + TR)8	11.32±0.04^{de}	75.00±0.08^{abc}	32.00±0.00^{ab}	43.00±0.09^{b}	0.80±0.01^{a}
(AdMV + SL + TR)9	10.75±0.02^{a}	73.00±0.03^{ab}	39.00±0.09^{bc}	34.00±0.01^{a}	1.20±0.06^{a}

Means (n =2) in the same row with similar superscripts are not significantly different (p>0.05), SL= Soursop leaves; TR = Turmeric rhizome; AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine.

There were significant differences (p<0.05) in the values of ALT and glucose among the dietary groups, and no significant difference (p>0.05) in AST among the dietary groups (Table 5).

Table 5: Mean post-challenge test of blood serum indices of *Clarias gariepinus* fingerlings fed experimental diets for 28 days.

	AST	ALT	GLUCOSE
Before	4.00±0.04^{a}	9.00±0.08^{ab}	5.30±0.01^{bcd}
Control	11.00±0.20^{a}	13.00±0.04^{b}	6.80±0.25^{e}
AqMV2	9.00±0.02^{a}	7.00±0.01^{ab}	4.90±0.03^{b}
AdMV3	7.00±0.01^{a}	5.00±0.00^{a}	5.10±0.01^{bc}
(AqMV + TR)4	4.00±0.00^{a}	3.00±0.03^{a}	4.00±0.04^{a}
(AdMV + TR)5	5.00±0.03^{a}	7.00±0.06^{ab}	4.20±0.05^{a}
(AqMV + SL)6	6.00±0.04^{a}	7.00±0.05^{ab}	4.10±0.01^{a}
(AdMV + SL)7	7.00±0.06^{a}	8.00±0.90^{ab}	5.90±0.07^{d}
(AqMV + SL + TR)8	6.00±0.01^{a}	7.00±0.01^{ab}	5.80±0.08^{d}
(AdMV + SL + TR)9	8.00±0.06^{a}	7.00±0.02^{ab}	5.60±0.04^{cd}

Means (n =2) in the same row with similar superscripts are not significantly different (p>0.05), SL= Soursop leaves; TR = Turmeric rhizome; AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine.

Challenge test

The results showed that the highest mortality was recorded in the control (13.00) and the lowest in (AqMV+TR)4, (AqMV+SL)6, and (AdMV+SL)7 (1.00), respectively. The percentage mortality was highest in the control (86.67) and lowest in (AqMV+TR)4, (AqMV+SL)6, and (AdMV+SL)7 (6.67), while the highest relative percentage of survival was recorded in (AqMV+TR)4, (AqMV+SL)6, and (AdMV+SL)7 (92.00%), respectively, and lowest in the control (0%). There was a significant difference (p<0.05) in the percentage of mortality and relative percent of survival among the dietary groups (Table 6).

Bacterial counts in the muscle, kidney and spleen of C. gariepinus after 28 days post-inoculation

The results revealed that there was a general reduction in the bacterial counts of the muscle, kidney, and spleen at 28 days post-

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infection, and there was a significant difference (p<0.05) among the dietary groups (Table 7).

Table 6: Challenge test with *Pseudomonas aeruginosa*, induced via the intraperitoneal route, was conducted on *C. gariepinus* for 28 days .

	Control (0%)	AqMV 2 (0%)	AdMV 3 (0%)	AqMV + TR)4 (1%)	(AdMV + TR)5 (1%)	(AqMV + SL)6 (1%)	(AdMV + SL)7 (1%)	(AqMV+ SL+ TR)8 (2%)	(AdMV+ SL +TR)9 (2%)
No. of fish injected	15	15	15	15	15	15	15	15	15
Mortality	13^{d}	2^{ab}	2^{ab}	1^{a}	4^{c}	1^{a}	1^{a}	2^{ab}	3^{bc}
Percentage mortality	86.67±0.09^{d}	13.33±0.01^{ab}	13.33±0.02^{ab}	6.67±0.02^{a}	26.67±0.04^{c}	6.67±0.02^{a}	6.67±0.01^{a}	13.33±0.00^{ab}	20.00±0.01^{bc}
Relative Percent of survival	0.00±0.00^{a}	85.00±0.08^{d}	85.00±0.06^{d}	92.00±0.04^{e}	69.00±0.07^{b}	92.00±0.04^{e}	92.00±0.08^{e}	85.00±0.06^{d}	77.00±0.40^{c}

Means (n =2) in the same row with similar superscripts are not significantly different (p>0.05), SL= Soursop leaves; TR = Turmeric rhizome; AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine.

Table 7: Bacterial counts (log_{10} CFU/g) in the muscle, kidney and spleen of *Clarias gariepinus* after 28 days post-inoculation.

Treatment	Muscle		Kidney		Spleen	
	0 day	28 days	0 day	28 days	0 day	28 days
Control (0%)	7.89±0.01^{a}	8.07±0.03^{e}	6.92±0.02^{a}	7.21±0.09^{g}	5.41±0.04^{a}	6.17±0.03^{f}
AqMV2 (0%)	7.89±0.01^{a}	2.78±0.05^{a}	6.92±0.02^{a}	3.03±0.01^{ab}	5.41±0.04^{a}	2.85±0.06^{a}
AdMV3 (0%)	7.89±0.01^{a}	3.06±0.07^{c}	6.92±0.02^{a}	3.54±0.05^{e}	5.41±0.04^{a}	2.98±0.02^{b}
(AqMV + TR)4 (1%)	7.89±0.01^{a}	3.19±0.02^{d}	6.92±0.02^{a}	3.67±0.01^{f}	5.41±0.04^{a}	3.13±0.02^{d}
(AdMV + TR)5 (1%)	7.89±0.01^{a}	3.11±0.01^{c}	6.92±0.02^{a}	3.61±0.04^{f}	5.41±0.04^{a}	3.24±0.10^{e}
(AqMV + SL)6 (1%)	7.89±0.01^{a}	3.07±0.03^{c}	6.92±0.02^{a}	3.12±0.06^{cd}	5.41±0.04^{a}	3.08±0.03^{cd}
(AdMV + SL)7 (1%)	7.89±0.01^{a}	3.10±0.05^{c}	6.92±0.02^{a}	3.18±0.08^{d}	5.41±0.04^{a}	3.11±0.01^{d}
(AqMV + SL + TR)8 (2%)	7.89±0.01^{a}	2.72±0.07^{a}	6.92±0.02^{a}	3.01±0.02^{a}	5.41±0.04^{a}	3.04±0.04^{bc}
(AdMV + SL + TR)9 (2%)	7.89±0.01^{a}	2.88±0.03^{b}	6.92±0.02^{a}	3.09±0.00^{bc}	5.41±0.04^{a}	3.07±0.01^{cd}

Means (n =2) in the same row with similar superscripts are not significantly different (p>0.05), SL= Soursop leaves; TR = Turmeric rhizome; AqMV = Aqueous Monovalent Vaccine, AdMV = Adjuvant Monovalent Vaccine.

Discussion

Medicinal plants have long been believed to be the original form of human and animal medicine. According to Bello *et al*. (2013), it offers extensive potential for managing health and promoting growth. Biological evaluation indicated that the weight of the fish increased across all food categories on average. Improved feed utilization and dietary ingredient absorption were the causes of growth gain in *C. gariepinus* fingerlings administered an aqueous monovalent vaccination and a supplemented diet of herbs. The results of the study

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revealed that growth performance in the control group was better than other treated groups except AdMV2. In comparison, aqueous monovalent vaccination performed better in terms of the specific growth rate, weight gain, weight gain percentage, and nitrogen metabolism. A significant difference was observed between the treated groups. This research negates the findings of Fawole *et al*. (2022) and Raissy *et al*. (2022), who found that feeding mixed medicinal plants to *C. gariepinus* fingerlings and *Cyprinus carpio* improved their growth performance relative to control animals.

Ichthyo-hematology would be helpful in evaluating feed and feed mixtures, assessing fish conditions, determining the harmful effects of substances, and determining illnesses. The packed cell volume data revealed variations in values between 38.00 and 43.00% among the treated groups; however, these values fell within the range recommended by Abulu's recommended range of 40 to 54% (Abulu, 2021). The values did not differ significantly between the treated groups. When comparing the hemoglobin content result at the conclusion of 28 days post-infection to the value recorded in the control and the value acquired prior to the experiment, it was found that all except (AdMV+TR+SL)9 showed a decrease in hemoglobin content. A significant difference was observed between the dietary groups. The results of Das *et al*. (2009), who showed a decrease in hemoglobin content following a challenge test, are supported by the current investigation. Compared to the control, the RBC values of the treated groups were higher; however, these values fell within Abulu (2021) suggested 4.7–6.1 × 10^{12}/L for RBC. The fish in the treated groups did not exhibit any signs of anemia, as indicated by these values, which did not differ significantly across the dietary groups. This finding may justify the use of plant immunostimulants as safe alternatives for fish farming.

The control group had a greater white blood cell (WBC) count than the treated groups, and the WBC values of the treated groups were able to stop the spread of infections. A significant difference was observed between the dietary groups. According to Yin *et al*. (2008), when *Cyprinus carpio* were fed a combination of four Chinese herbs, Rheum officinale, *Andrographic paniculata*, *Isatis indigotica*, and *Lonicera japonica*, they showed improved white blood cell counts. The results of this study support these findings. However, Fawole *et al*. (2022) showed that Hb, RBC, WBC, and lymphocytes were better in *C. gariepinus* fingerlings administered a polyherbal mixture, which was comparable to the current findings. According to the study report, there was a significant difference in the MCV and MCH values among the dietary groups in the post-infection test of the treatment groups compared to the control group. This study was in line with the report of Osuigwe *et al*. (2005), who recorded decreases in the MCH and MCV of *C gariepinus* and *Heterobranchus longifilis* fed different dietary levels of raw and boiled jackbean (*Canavalia ensiformes*) seed meal. However, MCHC values were relatively similar among the dietary groups and did not show any significant differences among the dietary groups. Better immune response against the fish disease *P. aeruginosa* may

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be the cause of reduced MCV and MCH values.

This study implies that vaccination with medicinal plants such as soursop leaves and turmeric rhizomes may improve the non-specific immune response. With the exception of (AqMV+SL)6, (AdMV+SL)7, and (AqMV+SL+TR)8, the lymphocyte values of the treated groups were lower than those of the control group, with no discernible difference. Among the dietary groups, soursop leaves, turmeric rhizomes, and aqueous monovalent vaccinations have immunomodulatory effects that improve *P. aeruginosa* disease resistance and non-specific immune responses. These effects are supported by the lower values observed in the treated groups.

Blood performance results indicate that the combination of the two vaccines, the aqueous and adjuvant, along with the medicinal plants (turmeric rhizome and soursop leaves) improves blood performance compared to the administration of the two vaccines alone or separately to *C gariepinus* fingerlings. Compared to the control group, which showed the lowest blood performance, this study showed that the combination of an aqueous monovalent vaccine and turmeric rhizome had the best results. A significant difference was observed between the dietary groups.

A possible explanation for these findings is that medicinal herbs have antibacterial properties that improve protection against foreign antibodies. The present investigation aligns with the findings of Fawole *et al*. (2022), which indicated superior blood performance in fish administered a polyherbal mixture in contrast to the control group. This study agrees with Das *et al*. (2009), who reported higher values of total protein and albumin in *Labeo rohita* fed with *Euglena viridis*. The results showed that the treated groups had higher total protein and albumin levels than the control group. A significantly higher value was obtained when the aqueous monovalent vaccine was combined with turmeric rhizome. Furthermore, globulin levels in the treated groups were greater than those in the control group, and there was a significant difference between the dietary groups. These increased total protein values could be a consequence of increased meal consumption and protein synthesis. However, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were generally higher in the control groups than in the treated groups. The decreased values in the treatment groups suggest improved liver function, which may be due to the high concentration of phytoconstituents, such as flavonoids and saponins, in the plants, strengthening the non-specific immune response and disease resistance of *C gariepinus* fingerlings against *P. aeruginosa* infection.

This research supports the findings of Fawole *et al*. (2022), who found that the total protein, AST, and ALT levels of the treated groups were higher than those of the control group. When comparing the control group to the treated groups, a significantly higher glucose value was found. Glucose level is a useful indicator of oxidative stress. *C. gariepinus* fingerlings exposed to *P. aeruginosa* showed less stress in the treated groups. Combining aqueous monovalent vaccination with turmeric rhizomes produced better results. In this study, aqueous monovalent vaccination combined

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with turmeric rhizome improved the values of albumin, total protein, albumin/globulin ratio, AST, ALT, and glucose. The present investigation bolsters the findings of Fawole *et al*. (2022), who documented increased levels of total protein and glucose in *C. gariepinus* fed a herbal mixture as a defense against bacterial infections.

According to the study's findings, there was a significant difference between the dietary groups, and the control group had a higher percentage of mortality (13.00 %) than the treated groups. The values for the control group ranged from 1.00 to 4.00. Additionally, there was a significant difference in the relative percent of survival between the dietary groups, with the highest percentage in the (AqMV+TR)4 (92.00%) and the lowest in the control group (0%). The results of this study corroborate those of Yin *et al*. (2008), who found that a mixture of medicinal plants improved fish survival following *Aeromonas hydrophila* assault. The results of this study demonstrate that feeding fish a mixture of medicinal herbs, such as the rhizome of turmeric and the leaves of soursop, can stimulate the non-specific immune system, increasing the resistance of fish to *P. aeruginosa* infection.

When comparing the values acquired before the experiment to those obtained 28 days post-infection, the bacterial counts indicated a general decrease in the values of the kidney, muscle, and spleen. However, the control group showed higher values in the kidneys, muscles, and spleen. This increase may have been caused by the reduced ability of the control diet to suppress the immune system and prevent microorganism growth. Additionally, it is possible that the presence of phytoconstituents in the diets of the treated groups contributed to the decline in the kidney, muscle, and spleen values of *C. gariepinus* fingerlings. The kidneys, muscles, and spleens differed significantly across the dietary groups. This result is consistent with the findings of Olusola *et al*. (2024), who found that 28 days after infection, the vaccinated *C. gariepinus* juveniles had fewer bacteria in their bodies than the control group.

Conclusions

This study suggests that a combination of vaccine, turmeric rhizome, and soursop leaves could be used in fish farming as an alternative therapeutic measure to promote growth performance, survival rate, and immune responses, and inhibit bacterial infection of *C gariepinus* fingerlings. Therefore, it is recommended that a combination of an aqueous monovalent vaccine and turmeric rhizome will produce an optimum result and better productivity in organic fish farming.

Conflicts of interest

The authors declare that no conflict of interest was observed during the study.

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