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IMPACT OF PROTEIN AND PROBIOTIC SUPPLEMENTATION ON IMMUNITY AND SURVIVAL OF *Clarias gariepinus* UNDER PATHOGEN CHALLENGE IN AQUACULTURE

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ABSTRACT

Bacterial infections, particularly those caused by Pseudomonas aeruginosa, pose a threat to aquaculture and lead to economic losses in African catfish (Clarias gariepinus). This study evaluates the effects of dietary protein and probiotic supplementation on fish health, growth performance, and survival following P. aeruginosa infection, using both in vitro and in vivo approaches. The in vitro assays determined the infective dose of P. aeruginosa and its interactions with probiotics. At the same time, in vivo trials assessed survival, behavioral responses, feed utilization efficiency, and pathological changes over 72 hours. Furthermore, fish were fed varying protein levels (35%, 40%, and 45%) and probiotic concentrations (1:2:3 ratio) for two months before being challenged with bacteria. Higher protein (40–45%) and probiotic supplementation (2–3 mL) significantly improved survival rates (up to 83.33%), reduced weight loss, and mitigated organ damage as opposed to groups with lower supplementation (50% survival), though lower than the uninfected controls (100% survival). The improved survival and reduced pathological damage in probiotic-supplemented fish suggest a potential enhancement of immune defense mechanisms and overall physiological resilience. Probiotics have been reported to modulate immune responses by promoting beneficial microbiota, competing with pathogens, and supporting host immunity, which may contribute to the observed protective effects. Additionally, probiotic-fed groups exhibited improved water quality with lower accumulation of nitrogenous waste in infected tanks. These findings suggest that protein- and probiotic-enriched diets enhance disease resilience, feed efficiency, and water quality in aquaculture, supporting sustainable fish production through nutritional and health improvement. Future research is recommended to investigate immune-related biomarkers to better understand the immune-modulatory effects of these dietary interventions.

KEYWORDS: African catfish; aquaculture; immunity enhancement; probiotic and protein supplementation; *Pseudomonas aeruginosa*

INTRODUCTION

Aquaculture is one of the fastest-growing food production sectors globally, significantly contributing to food security, nutrition, and economic development. However, its sustainability is increasingly challenged by infectious diseases, especially those caused by opportunistic bacterial pathogens, currently responsible for high mortality rates and substantial economic losses in fish farming systems (Irshath *et al.*, 2023). The emergence and persistence of these diseases are influenced by complex interactions among pathogenic microorganisms, host fish, and environmental conditions, underscoring the need for integrated fish health management strategies (Hamed *et al.*, 2018). Despite advancements in disease diagnostics and treatment, there remains a notable gap in developing sustainable, nutrition-based interventions to prevent bacterial outbreaks in intensive aquaculture systems.

Among the bacterial pathogens affecting aquaculture, *Pseudomonas aeruginosa* is recognized as a highly virulent and multidrug-resistant species that poses a serious threat to freshwater fishes, including African catfish (*Clarias gariepinus*) (Derome *et al.*, 2016). This opportunistic pathogen is commonly associated with polluted and temperature-variable aquatic environments. It is capable of causing severe clinical symptoms, such as skin ulcerations, abdomi-

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nal distension, hepatic necrosis, and systemic organ damage, with mortality rates reaching up to 100% during outbreaks (El-Bahar *et al*., 2019; Obisesan *et al*., 2022). The bacterium's ability to colonize fish surfaces and internal organs, persistence in aquatic systems, and spreading antibiotic resistance genes further complicate its control efforts (El-Gamal *et al*., 2018; Oludotun *et al*., 2019; Zada *et al*., 2024; Hussein *et al*., 2023). While the pathogenicity of *P. aeruginosa* is well documented, limited studies have investigated non-antibiotic interventions—particularly nutritional strategies—to enhance host resilience against this pathogen.

Probiotic supplementation has emerged as a promising alternative for disease control, offering benefits such as improved intestinal microbial balance, enhanced immune responses, and competitive exclusion of pathogens (Hai, 2015; Wang *et al*., 2021). In addition, protein-rich diets are known to support optimal fish growth, tissue repair, and immune function, especially under stressful conditions such as exposure to pathogens (Giri *et al*., 2013). However, the individual contributions of dietary probiotics and protein levels to disease resistance in African catfish under *P. aeruginosa* challenge remain underexplored. There is a critical knowledge gap concerning how each of these nutritional factors, when applied separately, influences survival, physiological response, and environmental impact during bacterial infection.

This study aims to separately evaluate the effects of different levels of dietary protein (35%, 40%, and 45%) and probiotic concentrations (1 mL, 2 mL, and 3 mL per kg feed) on the health, immune response, and survival of *Clarias gariepinus* experimentally challenged with *P. aeruginosa*. The bacterium was characterized through in vitro tests, while in vivo trials monitored clinical symptoms, behavioral changes, feed utilization, pathological lesions, and water quality indicators over 72 hours post-infection. After separate evaluations, the most effective protein level and probiotic concentration were identified and comparatively assessed to determine their relative effectiveness in enhancing fish resilience. Ultimately, this research seeks to inform nutrition-based, eco-friendly interventions for bacterial disease control in catfish aquaculture, while addressing the current evidence gap on the separate roles of dietary protein and probiotics in fish disease mitigation following standard procedures (Ringø *et al*., 2020).

MATERIALS AND METHODS

Study area

The study was conducted at Jimma University, Jimma, located 353 km southwest of Addis Ababa, Ethiopia. Experimental fish were fed diets supplemented with protein and probiotics within an aquaponics system in a greenhouse. *In vitro* experiments were carried out in the Microbiology Laboratory, Department of Biology, while *in vivo* trials were performed in the Aquaculture and Fisheries Laboratory using glass aquariums.

Experimental design and treatment protocols

This study investigated the independent effects of dietary protein and probiotic supplementation on growth, immune response, and survival of African catfish (*Clarias gariepinus*) following *Pseudomonas aeruginosa* exposure. The experiment had two phases: (i) a 60-day pre-exposure feeding trial testing graded protein (FT1–FT3) or probiotic (PT1–PT3) treatments separately, and (ii) a 96-hour post-exposure pathogen challenge. Protein and probiotic effects were evaluated independently, with no combined treatments, ensuring experimental separation. After feeding, all fish were exposed simultaneously to *P. aeruginosa* under identical conditions to standardize infection and assess disease resistance across groups.

Experimental fish, diets, and treatments

Uniform-sized *Clarias gariepinus* were obtained from holding tanks and acclimatized under controlled laboratory conditions. Fish were randomly assigned to two main dietary treatment groups: protein supplementation and probiotic supplementation. For the Protein Supplementation (FT) group, diets were formulated using locally available aquaculture feed ingredients with 35% (FT1), 40% (FT2), and 45% (FT3) crude protein. For the Probiotic Supplementation (PT) group, probiotics were added at 1 mL/kg (PT1), 2 mL/kg (PT2), and 3 mL/kg (PT3) of feed. The probiotic strains (Lactobacillus, Lactococcus, and Leuconostoc) were previously isolated from the gut of *C. gariepinus* and selected for their immune-stimulatory and health-promoting effects. Each treatment was conducted in duplicate, resulting in a total of 12 tanks (6 for protein and 6 for probiotics), with 6 fish per tank. Fish were fed 5% of their body weight twice daily for 2 months. At the end of the feeding trial, individuals with similar average body weights were selected for the pathogen challenge experiment.

In vitro pathogen-probiotic assay

To determine the infective dose of *Pseudomonas aeruginosa* and evaluate its interaction with probiotics, the pathogen was cultured in nutrient broth and Pseudomonas Isolation Agar, and turbidity was standardized to 0.5 McFarland. Various volumes of *P. aeruginosa* (0.5–3 mL) were co-incubated with different probiotic doses (1–3 mL) in triplicate for 72

hours. Interactions were assessed via turbidity, pigment production, and hydrogen sulfide formation, while disk diffusion and growth inhibition assays evaluated probiotic antagonism.

Growth and survival of *P. aeruginosa* were inferred from pigment intensity and turbidity, reflecting metabolic activity under aerobic conditions. Turbidity was attributed solely to pathogen proliferation, as the medium did not support probiotic growth. These in vitro results indicate pathogen behavior in isolation: in aquaculture, probiotics suppress pathogens via competitive exclusion, antimicrobial production, and immunomodulation, which are not captured in this assay. The *P. aeruginosa* concentration showing maximal infectivity was selected for subsequent in vivo challenge.

In vivo growth, health, and pathogen challenge trials

Phase I: Growth and health evaluation

As described above, fish were maintained on their respective dietary treatments for two months under controlled conditions. Growth performance and health parameters of *C. gariepinus* were regularly monitored throughout the period. This phase provided baseline data for the subsequent pathogen exposure experiment.

Phase II: Pathogen challenge trial

Following the growth phase, fish with similar body weights were redistributed into standardized glass aquariums (120 cm × 30 cm × 40 cm; 10 L water volume), with 6 fish per tank, according to the treatment combinations described in Table 1. Each treatment group was replicated twice. Before stocking, fish were labeled on their caudal fins for identification, and baseline water quality parameters were recorded to ensure consistency. Aquariums were equipped with aerators and heaters to maintain optimal environmental conditions. The experimentally determined infective dose of *P. aeruginosa* at 3 ml/L was administered to the designated treatment groups, while the control groups received an equivalent volume of sterile saline. Post-infection, fish were monitored for 96 hours to assess survival, behavioral

Water quality and histopathological evaluation

Water quality parameters including dissolved oxygen (DO), temperature, pH, electrical conductivity (EC), total dissolved solids (TDS), salinity, and nitrogenous compounds (NH_4^+, NH_3-N, NO_2^-, and NO_3^-) were measured every 24 hours over 72 hours using a Palintest Photometer 7500 Bluetooth device (by Palintest Ltd, UK). Fish were closely observed for signs of disease or stress, such as ulceration, erratic swimming, or respiratory difficulty. At the conclusion of the 96-hour observation period, fish were humanely euthanized. Tissues from the gills, liver, kidneys, and intestines were collected and processed for histopathological analysis following established protocols. Sampling and preparation were performed according to standard fish tissue histology methods, ensuring proper fixation and sectioning (Maftuch *et al*., 2017; Fiedler *et al*., 2023). Visual documentation was performed for both water quality parameters and pathological findings.

Data collection

Data were collected from both in vitro and in vivo experiments. In vitro assays involved measuring turbidity, pigment production, hydrogen sulfide (H_2S) levels, and secondary metabolite production at 24-hour intervals over a 72-hour incubation period. In vivo trials included observations of growth performance such as weight gain or loss and survival rate; physiological responses including changes in skin pigmen-

Table 1. Fish groups and treatment combinations in phase II pathogen challenge following probiotic and protein supplementation

Aquarium Treatments	Composition	Number of Fish	Treatment Description
AT1	3 fish from 35% protein + 3 fish from 1 mL probiotic	6	Low protein + low probiotic
AT2	3 fish from 40% protein + 3 fish from 2 mL probiotic	6	Medium protein + medium probiotic
AT3	3 fish from 45% protein + 3 fish from 3 mL probiotic	6	High protein + high probiotic
AT0 (Control)	Equal numbers from all groups, no pathogen exposure	6	Sterile saline (negative control)

Key: Aquarium treatment (AT)

tation, mucus secretion, gill coloration, and eye clarity; behavioral responses like alterations in swimming patterns, feeding behavior, and visible signs of stress; and anatomical observations of internal organ abnormalities detected during post-mortem examinations (Manchanayake, 2023).

Data analysis

Statistical analyses were conducted using SPSS version 20.0, with significance set at $p < 0.05$. Growth performance data, including weight gain or loss and survival rate, were analyzed using one-way ANOVA followed by Tukey's post-hoc test to determine the significance of differences among treatment groups. Data on pathogen-probiotic interactions were analyzed using a repeated-measures ANOVA to account for time-dependent measurements, while survival probabilities were compared using the Kaplan-Meier method with the log-rank test. Water quality parameters were analyzed by two-way ANOVA to assess treatment effects over time. Morphological and anatomical observations were graded either qualitatively or semi-quantitatively as appropriate.

RESULTS AND DISCUSSION

In vitro antagonistic activities of probiotics against *p.aeruginosa*

The in vitro assays demonstrated that gastrointestinal probiotics significantly inhibited the growth and virulence of *P. aeruginosa* in a dose-dependent manner. Turbidity measurements (FTU/ml, Table 2) showed a clear reduction in bacterial growth with increment in probiotic concentrations (1, 2, and 3 mL/kg feed). The highest turbidity value (337.50 ± 159.42 FTU/mL) of the pathogen was recorded at the lowest probiotic dose (1 mL), while the lowest turbidity (261.88 ± 83.24 FTU/mL) was observed at the highest probiotic dose (3 mL), confirming strong antagonistic activity of the probiotics. The initial turbidity controls included distilled water (0 FTU/mL), nutrient broth (18 FTU/mL), MRS broth (22 FTU/mL), 24-hour activated *P. aeruginosa* (52 FTU/mL), and probiotics alone (320 FTU/mL). Among all treatments, the highest infective dose of Pseudomonas aeruginosa was established as 3 mL/L of overnight culture, adjusted to approximately $1 \times 10x$ CFU/mL by comparison with the 0.5 McFarland turbidity standard ($\approx 1.5 \times 10x$ CFU/mL). This dosage corresponded to the highest turbidity, intense blue-green pigment production, and hydrogen sulfide (H_2S) formation, while probiotic colony growth was minimal or absent. Furthermore, the characteristic green pigment of *P. aeruginosa* diminished with higher probiotic doses and disappeared upon water agitation. This suggests that probiotic secretions interfere with pigment biosynthesis or stabilization, likely by disrupting quorum sensing mechanisms essential for bacterial virulence expression. These finding align with previous report made by Abu-Bakar *et al*. (2025) and Chávarri *et al*. (2021), who linked reduced turbidity to suppressed bacterial growth through probiotic metabolite activity, as well as reports by Srivastava *et al*. (2022) and Ringø *et al*. (2020) highlighting the role of probiotics in quorum sensing inhibition.

The mean turbidity levels in cultures with varying concentrations of *P. aeruginosa* combined with different probiotic doses are summarized above (Table 2). Turbidity levels increased proportionally with higher *P. aeruginosa* concentrations, indicating enhanced bacterial growth. However, growing probiotic doses—particularly at 2–3 mL/kg feed—significantly reduced turbidity, demonstrating the antagonistic effect of probiotics in suppressing pathogen proliferation.

In general, based on turbidity, pigment production, hydrogen sulfide (H_2S) formation, and inhibition by probiotics, an infective dose of 3 mL *P. aeruginosa* was established for subsequent in vivo challenge assays. Quantitative assessment of key virulence markers—namely blue-green pigment intensity, secondary metabolite secretion, and H_2S production—demonstrated a dose-dependent increase with rising *P. aeruginosa* concentrations (Figure 1). These virulence traits are consistent with the bacterium's pathogenic potential, particularly its ability to produce pyocyanin and other phenazine compounds,

Table 2. Mean turbidity of the in-vitro test of probiotics and *P. aeruginosa* per treatment

Probiotic Treatments	Volume of pathogen (*P. aeruginosa)* and the corresponding mean turbidity					
	T1 (0.5 ml)	T2 (1 ml)	T3 (1.5 ml)	T4 (2 ml)	T5 (2.5 ml)	T6 (3 ml)
T1 (1 ml)	274.38 ± 94.02	292.50 ± 96.81	304.38 ± 108.02	319.38 ± 157.24	322.50 ± 135.83	337.50 ± 159.42
T2 (2 ml)	265.00 ± 76.53	295.00 ± 87.46	301.25 ± 93.61	306.88 ± 113.80	311.88 ± 128.76	327.50 ± 132.83
T3 (3 ml)	261.88 ± 83.24	288.75 ± 83.91	293.75 ± 80.35	294.38 ± 102.66	301.88 ± 100.14	313.75 ± 93.46

which contribute to oxidative stress and host tissue damage (Hall *et al*., 2016). Interestingly, these virulence parameters were significantly suppressed in probiotic-treated samples, suggesting an inhibitory effect of probiotic-derived metabolites on *P. aeruginosa* pathogenicity. The observed destabilization of the green pigment upon agitation further supports the potential of probiotic secretions—such as bacteriocins or organic acids—in interfering with pigment stability and biofilm-associated traits. The dose-dependent antagonistic interaction implies a competitive dynamic where probiotics exert antimicrobial effects, possibly through niche exclusion, quorum quenching, or direct inhibition of virulence gene expression (Maldonado-Gómez *et al*., 2016). Finally, when mixed cultures of *P. aeruginosa* and probiotics were plated on MRS agar, an inverse relationship between pathogen load and probiotic colony formation was confirmed at high densities, and the reciprocal enhancement of probiotic viability at higher probiotic doses. Pseudomonas aeruginosa produces characteristic pigments, such as pyocyanin (blue-green), which are visible in culture media, as shown in test tube A. These secondary metabolites are not essential for growth but enhance survival by providing ecological advantages, including antimicrobial activity against competing microorganisms.

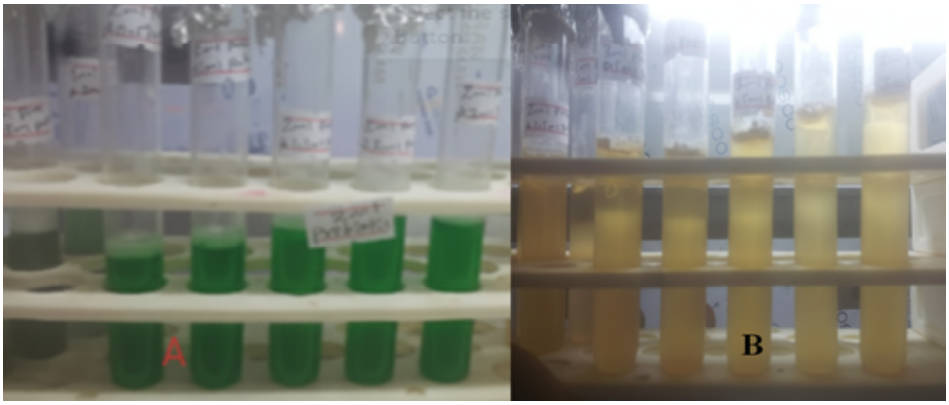

Figure 1. Illustration of (A) blue-green pigment, and (B) secondary metabolites and H_2S.

Effects of probiotics and dietary protein on fish survival and behavior

Fish weight and survival were significantly influenced by varying levels of probiotic supplementation (PT) and dietary protein (FT) following *P. aeruginosa* challenge (Table 3). Infected fish generally exhibited weight loss, reduced nutrient uptake, and compromised health, whereas uninfected controls maintained steady growth with 100% survival. Survival rates were lowest in the fish fed the lowest-protein diet (FT1, 35% protein) at 50%, followed by intermediate survival in the FT2 (40% protein) and FT3 (45% protein) groups, each recording 66.67% survival. Probiotic treatment improved outcomes in a dose-dependent manner: PT2 and PT3 groups (2–3 mL/kg feed) achieved the highest survival (~83–90%), while PT1 (1 mL/kg feed) showed moderate improvement with 66.67% survival. These findings corroborate earlier studies by Giri *et al*. (2013), which documented that both probiotics and optimal dietary protein levels

Table 3. Morphological observations and survival rates of protein-fed and probiotic-treated fish

Aquarium Treatment	Fish Source	Initial Weight (g)	Final Weight (g)	Weight Change (±g)	Stocked Fish	Dead Fish	Survival Rate (%)
AT1	PT1	118.67±8.04	97.17±47.75	-21.5±27.9	6	2	66.67
	FT1	121.67±8.17	60.67±66.61	-61±37.39	6	3	50.00
AT2	PT2	126.00±2.28	123.50±3.21	-2.5±2.75	6	1	83.33
	FT2	130.00±10.22	83.83±65.10	-46.17±37.66	6	2	66.67
AT3	PT3	122.17±8.54	118.00±5.83	-4.17±7.18	6	1	83.33
	FT3	130.67±4.55	102.00±50.25	-28.67±27.4	6	2	66.67
Control	P1,2,3	113.33±6.35	116.00±6.96	+2.67±6.66	6	0	100.00
	F1,2,3	121.17±6.56	123.67±6.12	+2.5±6.34	6	0	100.00

Key: Aquarium treatment (AT), Probiotics treatment (PT), Feed treatment (FT), Probiotics control (PC), and Feed control (FC).

bolster fish immune resilience and improve survival under pathogen-induced stress. The negative number in Table 3 indicates that the mean weight was reduced from each treatment.

The Kaplan–Meier survival analysis revealed significant differences ($p < 0.05$) among treatment groups, demonstrating the importance of dietary protein and probiotic supplementation in enhancing disease resistance in African catfish (*Clarias gariepinus*) (Figure 2). The uninfected control group exhibited 100% survival, confirming that mortality was solely due to pathogen challenge. Among the treated groups, fish supplemented with probiotics at moderate (PT2) and high (PT3) levels achieved the highest survival (83.33%), with only one death in each group. In contrast, protein-treated groups recorded lower survival rates, with FT1 showing the highest mortality (50% survival), while FT2 and FT3 reached 66.67%. Mortality occurred earliest and was most severe in fish fed low-protein diets without probiotic support, highlighting the detrimental effect of poor nutrition on immunity. These results underscore that probiotic supplementation, particularly at 2–3 mL/kg feed, provided greater protection than protein enrichment alone, likely by improving gut health, inhibiting pathogen colonization, and stimulating immune responses. Overall, the findings align with the existing literature, which emphasizes that optimized nutrition combined with effective probiotics synergistically enhances fish immunity and resilience against bacterial infections, supporting sustainable disease management in aquaculture (Hoseinifar *et al.*, 2024).

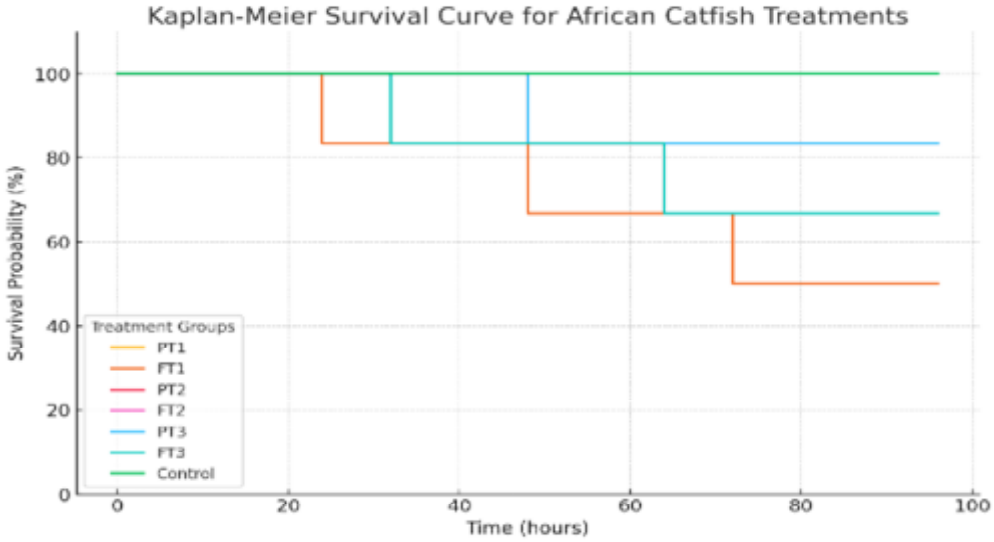

Figure 2. Kaplan-Meier Survival Curve of *C. gariepinus* under protein and probiotic supplementation following challenge with *P. aeruginosa.*

Clinical signs and histopathological alterations

Following *P. aeruginosa* infection, fish lacking probiotic or protein supplementation exhibited pronounced clinical signs within 24–48 hours, including hemorrhagic lesions, skin depigmentation, abdominal swelling, lethargy, fin rot, and ulcerative skin erosions (Figure 3). Internally, severe pathological changes were evident, particularly in vital organs such as the liver, kidneys, intestines, and gills—sites commonly targeted during septicemia. Histopathological examination revealed extensive gill necrosis, hepatic vacuolization, renal enlargement, and intestinal mucosal inflammation. These alterations are consistent with previous findings by Oludotun *et al.* (2015), who described similar tissue damage in fish subjected to bacterial septicemia (Figure 5). In contrast, fish groups receiving probiotic supplementation and/or high-protein diets exhibited significantly milder clinical symptoms and reduced tissue degeneration. Probiotic-treated fish showed higher activity levels and improved external appearance, with fewer hemorrhages and less pigmentation loss. Histological assessments confirmed reduced necrosis in the gills and less extensive inflammation in hepatic and intestinal tissues. These observations support the immunomodulatory, anti-inflammatory, and tissue-protective roles of probiotics and dietary protein, as previously reported by Rodrigues *et al.* (2019) and Hassan *et al.* (2023). The gills, which serve as a primary site for both pathogen entry and host defense, responded particularly well to probiotic intervention, underscoring the importance of maintaining mucosal integrity for aquatic disease resistance.

The pathological findings in this study demonstrated that *P. aeruginosa* infection induced severe

damage to critical internal organs of *C. gariepinus*, characterized by necrosis and structural degradation of the gills, hepatomegaly with liver necrosis, kidney enlargement with necrosis, and intestinal swelling accompanied by hemorrhaging (Figure 4). These findings are consistent with previous reports highlighting the multi-organ impact of *P. aeruginosa* infection in African catfish, including gill lamellar epithelial damage, liver congestion, and kidney pathology (Ahmed *et al.*, 2023). Notably, the severity of these pathological changes was most pronounced in fish fed with the low-protein diet (FT1), suggesting that insufficient dietary protein may exacerbate tissue vulnerability and compromise immune defenses. In contrast, fish receiving probiotic supplementation and the control group exhibited significantly reduced tis-

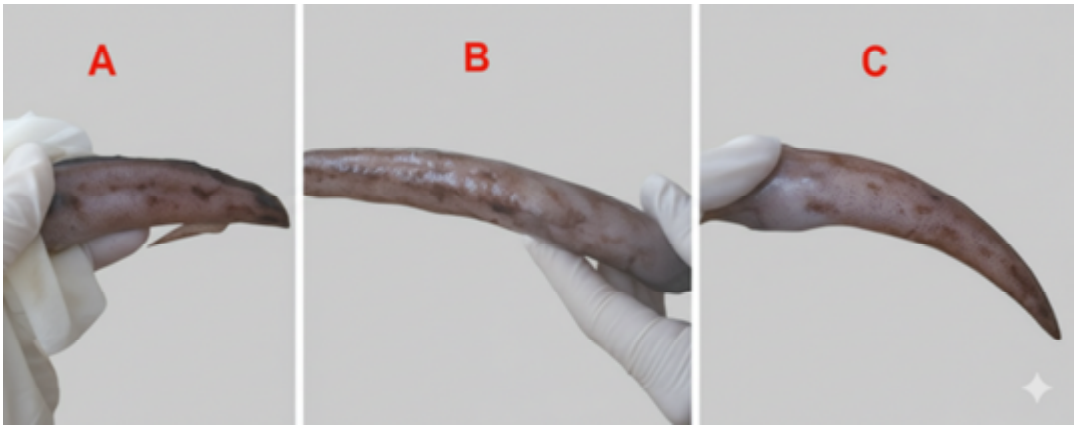

Figure 3. Clinical manifestations in infected fish: (A) Eroded skin, (B) Ulcerated skin, (C) Abdominal swelling and hemorrhages.

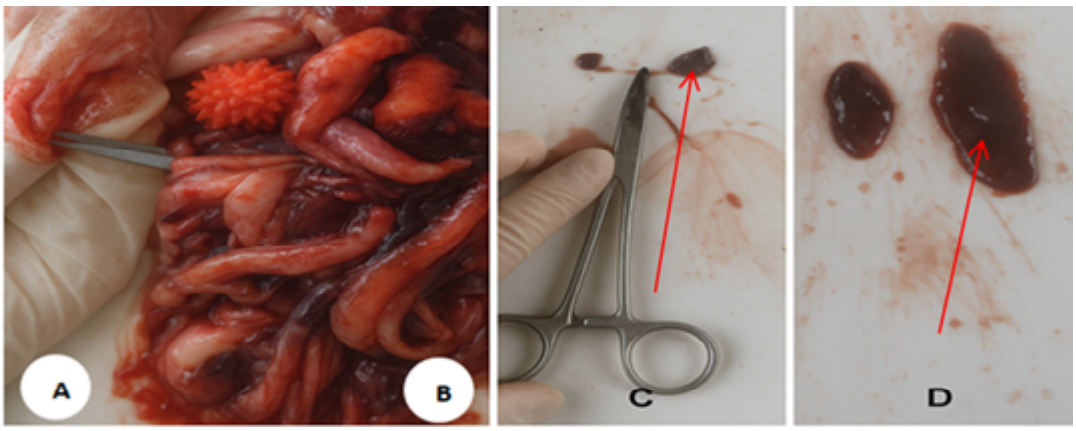

Figure 4. Histopathological alterations in infected fish.

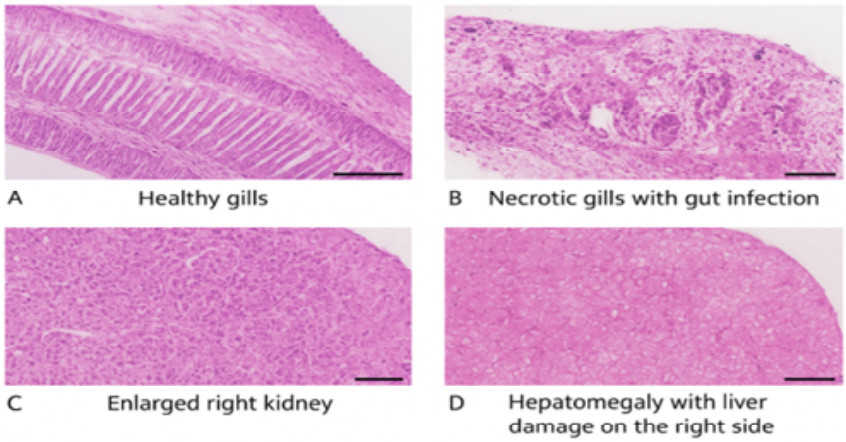

Figure 5. Histopathological alterations in infected fish: (A) healthy gills, (B) necrotic gills with gut infection, (C) enlarged right kidney, (D) hepatomegaly with liver damage on the right side.

sue damage and better maintenance of organ integrity, which likely reflects the immune-modulatory and protective effects of probiotics in mitigating bacterial infection. These results underscore the importance of adequate nutrition and probiotic administration in enhancing disease resistance and maintaining tissue health during bacterial challenges in aquaculture.

Water quality and nitrogenous waste accumulation

Water quality parameters, including dissolved oxygen (DO), pH, electrical conductivity (EC), total dissolved solids (TDS), and salinity, were routinely monitored alongside toxic nitrogenous compounds such as ammonium (NH_4^+), ammonia nitrogen (NH_3-N), nitrite (NO_2^-), and nitrate (NO_3^-) (Table 4). Significant variation in water quality was observed across treatment groups, particularly in relation to dietary protein content and probiotic supplementation. Infected fish reared without probiotic or adequate protein support, especially those fed the lowest protein diet (FT1, 35%), exhibited the poorest water quality. These groups showed significantly elevated concentrations of ammonium, nitrite, and nitrate, indicating inefficient nitrogen utilization, impaired digestion, and microbial imbalance. The resulting accumulation of toxic nitrogenous wastes likely exacerbated environmental stress and contributed to higher mortality. Conversely, groups supplemented with probiotics, particularly at higher dosages (PT2 and PT3), maintained superior water quality, with significantly lower levels of toxic nitrogenous compounds. Higher-protein diets (FT3, 45%) played a protective role by supporting better nutrient assimilation and minimizing catabolic waste. These results are consistent with those of Li *et al*. (2020), who reported that probiotic supplementation enhances microbial equilibrium and water purification processes in aquaculture. Additionally, Strzy¿ewska *et al*. (2016) demonstrated that reduced feed waste and improved metabolic efficiency, facilitated by probiotics and optimal nutrition, significantly limit environmental ammonia accumulation—thereby mitigating stress and reducing the risk of secondary infections in intensive fish farming systems.

Table 4. Water quality parameters and levels of nitrogenous waste in the glass aquaria

Aquarium Treatments	DO (mg/L)	pH	EC (µS/cm)	TDS (mg/L)	Salinity (ppt)	NH_4 (mg/L)	NH_3-N (mg/L)	NO_2 (mg/L)
T0 (Initial)	6.88	7.18	94.00	59.50	0.03	0.10	0.03	0.02
AT1	0.94	7.74	654.00	426.50	0.30	0.94	0.73	1.13
AT2	0.96	7.58	663.50	430.50	0.32	0.96	0.75	1.18
AT3	1.03	7.47	676.50	439.50	0.34	1.05	0.81	1.33
Control	1.30	7.43	620.00	396.50	0.26	1.17	0.92	1.60

Implications for aquaculture health management

This study underscores the critical role of gastrointestinal probiotics and dietary protein optimization in enhancing the resilience of African catfish (*C. gariepinus*) to *P. aeruginosa* infection. Both in vitro and in vivo findings demonstrated the independent efficacy of these interventions in mitigating disease impacts. In vitro, probiotic strains exhibited vigorous antagonistic activity against *P. aeruginosa*, evidenced by reduced turbidity, suppression of characteristic green pigment production, and inhibition of hydrogen sulfide (H, S) formation—indicators of diminished virulence. Moreover, probiotics reduced *P. aeruginosa*-induced colony inhibition and favored their own growth when co-cultured. In vivo, probiotic (3 mL/kg feed) and high-protein (45%) diets significantly enhanced fish survival, health behavior, and histopathological integrity. These groups exhibited lower mortality, reduced clinical signs (e.g., hemorrhages, fin rot, and tissue necrosis), and improved organ recovery, particularly in the gills, liver, and kidneys. Kaplan–Meier survival analyses confirmed significant survival benefits ($p < 0.05$) for fish receiving optimal interventions. Additionally, water quality metrics improved markedly with these treatments. Probiotic and high-protein groups maintained lower concentrations of toxic nitrogenous wastes (ammonium, nitrite, and nitrate), suggesting enhanced nitrogen cycling, microbial balance, and waste assimilation. These improvements reduce environmental stress and the risk of secondary infections—critical factors in intensive aquaculture operations. Although this study did not explicitly test combined effects, the findings identify optimal thresholds for individual interventions: 3 mL/kg of feed probiotics and 45% dietary protein. These strategies serve as effective, eco-friendly alternatives

to antibiotics, supporting current global efforts to reduce antimicrobial resistance and promote sustainable aquaculture practices (FAO, 2022; Ringø *et al.*, 2020).

CONCLUSION

This study demonstrates that the strategic integration of probiotics and protein-rich diets significantly enhances the health, immunity, and survival of African catfish (*C. gariepinus*) when challenged with *P. aeruginosa*. The established infective dose (3 mL/L) effectively induced infection, as indicated by increased turbidity, blue-green pigmentation, and elevated hydrogen sulfide levels. Notably, higher probiotic concentrations markedly inhibited *P. aeruginosa* proliferation both in vitro and in vivo, leading to improved survival outcomes and reduced clinical signs of infection, including diminished tissue damage, lethargy, and mortality. These findings underscore the importance of dietary optimization —specifically maintaining protein levels at 40–45% and probiotic supplementation at 2–3 mL — as a sustainable, non-antibiotic approach to disease management in aquaculture. Probiotic inclusion not only mitigated infection severity but also enhanced host resilience, reinforcing its potential as a practical alternative to antibiotics in fish farming. To translate these promising results into broader aquaculture practice, future research should focus on field-level implementation, long-term impacts on fish physiology and water quality, and economic feasibility. Ultimately, integrating balanced nutrition with targeted probiotic use presents an ecologically responsible strategy to enhance fish welfare, reduce antimicrobial dependence, and promote the sustainability of aquaculture systems. Integrated trials assessing the synergistic effects of combined probiotic and dietary interventions are warranted. Such investigations could optimize fish health, improve system-wide sustainability, and inform the development of precision aqua-feed formulations and holistic health management protocols for commercial aquaculture operations.

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