Vol. 3 No. 3 (2025)

ONLINE ISSN :3007-309X
PRINT ISSN :3007-3081

Journal of Medical & Health Sciences Review

AEROMONAS HYDROPHILA- INDUCED PATHOLOGICAL CHANGES IN KEY ORGANS OF THE COMMON CARP (CYPRINUS CARPIO)

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ARTICLE INFO:

Key words: Common carp, Aeromonas hydrophila, Disease resistance, Fish health, Probiotic supplementation

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Article History:
Published on 19 July 2025

ABSTRACT

The importance of aquaculture as a sustainable protein source highlights the need to address challenges like disease outbreaks and poor fish performance. Global difficulties confronting aquaculture include food quality, disease control, water quality and environmental factors. This study aims to evaluate the effectiveness of *Lactobacillus*-derived probiotic supplements in improving the well-being and illness tolerance of common carp towards *Aeromonas hydrophila* an infectious microbe that causes considerable losses in aquaculture. Experimental groups of fish were provided with a meal enriched with *Lactobacillus*-based probiotics, whereas control groups were given a standard diet. After encountering *A. hydrophila*, the functional as well as structural health of essential organs heart, kidney, gut and gills was determined using histopathological and biochemical investigations. Results demonstrated that probiotic-treated fish displayed less tissue damage, enhanced immunological responses and elevated survival rates relative to non-supplemented controls. The probiotic administration significantly alleviated the detrimental effects of bacterial illness safeguarding the functioning of organs and general fish health. The findings indicate the possibility of Lactobacillus-derived beneficial bacteria as an organic sustainable substitute for antibiotics in disease prevention within aquaculture.

INTRODUCTION:

Aquaculture is the cultivation of aquatic organisms. Around 6000 BC, cultivation of fish in ponds was first documented in China. These methods were crude, handcrafted and devoid of scientific understanding. Around the turn of the 1970s, aquaculture helps maintain global food requirements. Aquaculture is one of the most important sources of food. The extensive distribution and outbreaks of several pathogenic microbes cause fish disease and high mortality. As aquaculture increases internationally and drastically reducing productivity and causing significant financial damage. Biofilms and bacterial microenvironments that allow pathogenic bacteria to survive in adverse environmental conditions and resist different treatments. Probiotics and their byproducts have assembled significant interest in the fish industry in recent years as a means of countering harmful bacteria's survival strategy (Rahman *et al.,* 2021).

Aquaculture provides a significant quantity of food globally, because it is the largest source of protein. Global fish production is rapidly increasing in response to rising demand. The output of aquatic animals for human consumption jumped from 21.8 million tons to 156.4 million tons between 1960 and 2018. Asia is the predominant region, contributing 69% in 2018, with China representing 35% of worldwide output (Schar *et al*., 2021).

Aquatic animals are used as a protein source for world's food production and its security.

Nutrients of terrestrial animals, milk and eggs resulted in 76,966 Kt crude protein compared with 13,950 Kt or 15.3% from aquatic animals in 2018. Aquaculture produced a great number of aquatic animals and fisheries resulted in 7,135 Kt crude protein while aquaculture produced 6,815 Kt. Common carp used on current consumption calculated as in order to fulfill the demands of the larger population. Output of aquaculture would need to rise about 82,087 Kt in 2018-2019 to 129,000 KT around 2050. (Boyed *et al.*, 2022).

The common carp, *Cyprinus carpio*, belonging to the *Cyprinidae* family, is a freshwater fish species indigenous to Europe and Asia. They have been extensively disseminated over several regions globally and are currently present in many nations. Common carp exhibit considerable adaptability and resilience in their behavior. They can endure a diverse array of aquatic conditions and frequently inhabit sluggish rivers, ponds, and lakes. The common carp is extensively utilized as an experimental fish species for assessing the impacts of environmental toxins, owing to its minimal needs, extensive compatibility, and significant dispersion, in addition to its economic and ecological significance (Soltani *et al*., 2017).

Common carp is a major protein source. Carp has spread over the world with more noticeable ecological consequences because of its economic significance in this business. Common carp fish is a member of the Cyprinidae loved ones which includes about 220 genera, seven subfamilies and about 20,000 established species. When rearing carp especially tilapia a polyculture method is more frequently utilized in order to optimize growth rates, limit feed conversion proportions and decrease the quantity of fat in the remains. Common carp monoculture has been associated with agro-ecologically beneficial agriculture. The secret to pond aquaculture's successful development was crop growing. Fish production helps to meet the growing demand for fish and marine products around the world. It has contributed significantly to Egypt's recent success in supplying reasonably priced animal protein. Egypt ranked first among African nations and seventh worldwide in terms of fish farming production (Muhammad *et al*., 2024).

Cyprinus carpio is among the highest extensively grown nonsaline fish species. It can aid in mitigating issues associated with overpopulation specifically by offering an economical and sustainable protein source for expanding populations. The potential for increased production from aquaculture and fisheries may have its limits. The economic effects of shifts in supply and demand those who brought about by adjustments in food costs, household income and buyer tastes are frequently disregarded in estimates of future food security. This study examines the demand and availability for fish until 2030 from an economic perspective, focusing on aquaculture (Bjorndal *et al.*, 2024).

Histopathology is frequently utilized in both aquaculture & wild fish populations to identify and monitor pathological abnormalities, assess the impact of contaminants, and facilitate disease surveillance initiatives. It also improves our comprehension of the processes governing relations between hosts and pathogens and the physiological effects of diverse stressors on fish health (Thophon et al., 2003). Histopathology, the microscopic examination of sick tissue, is an essential instrument for diagnosing and comprehending the health condition of aquatic creatures, such as common carp (Cyprinus carpio). The common carp, one of the most extensively cultivated freshwater fish species globally, functions as a model organism in fish farming and environmental toxicity (FAO, 2020).

In common carp, essential organs like the gills, liver, kidneys, skin, and intestines are frequently examined to identify pathological alterations. These alterations may encompass necrosis, inflammatory disorders, hyperplasia, degeneration, and various structural anomalies (Hinton *et al.,* 2001). Histopathological changes frequently indicate the initial impacts of pollutants, including heavy metals, chemical pesticides, and industrial waste water, rendering them significant indicators of the environment's condition and fish health (Martínez-Gómez et al., 2009). *Aeromonas hydrophilia* is one of most prevalent bacterial pathogens in worldwide freshwater habitats. *Aeromonas hydrophilia* is considered an opportunistic infection that affects a variety of fish, encompassing carps in both aquaculture and natural environments The illness often encompasses septicemia, tail rot, fin decay, bleeding from ulcers, dropsy, and abnormal distension, all of which are associated with a wide variety of freshwater fish (Musa *et al.*, 2008).

Antibiotics can alter the microbes in the gut and foster resistant bacterial populations perhaps leading to unforeseen long-term consequences for public health hence generating concerns regarding their use as a preventive strategy. Recent studies indicate that natural and herbal immune stimulating agents positively influence fish disease management, enhance growth and activate both specific and generalized immune responses in finfish and shellfish.

(Harikrishnan *et al.,* 2009). Probiotics are live microorganisms that enhance the host's health when administered in adequate amounts. In common carp by enhancing gut microbial balance and enzyme activity probiotics improve digestion and nutrient absorption. Probiotics such as *Lactobacillus plantarum* and *Saccharomyces cerevisiae* induces weight gain in common carp, enhances (FCR) and elevates their specific growth rate. *Lactobacillus plantarum* food supplementation enhanced common carp development and feed utilization (Hoseinifer *et al.*, 2017).

Probiotics boost both innate and adaptive immune responses in common carp. They boost the synthesis of cytokines and immunoglobulins and raise the activity of immune-related enzymes (such lysozyme). Common carp supplementation with Bacillus subtilis enhanced immunological measures like respiratory burst and phagocytic activity (Beck *et al.*, 2015) Probiotics lower the risk of infections by competing with harmful bacteria for resources and adhesion sites. They generate antimicrobial substances (such bacteriocins) that prevent the growth of infections such as *Aeromonas hydrophilia* (Nayak., 2010).

Materials & Methodology:

Samples of *Cyprinus carpio* were captured with the help of netting method from Fisheries Complex Bahawalpur (Location 1). Samples were transferred in polythene bags filled with oxygenated water for better respiration. Samples were transported to the General zoology lab of University of Education Lahore Vehari campus (Location 2). Samples were acclimatized for 3 to 5 days**.** Specimens were acclimatized in an aquarium using typical settings. In order to get rid of the related bacteria and fungi, the 50 L (24 units) aquarium utilize in this study were first cleaned with fresh water and then disinfected with 10 PPM chlorine. After that aquariums were cleaned with water until they were cleaned and allowed to dry in the sun for about 24 hours. Each vessel was filled up with 100 L of water and fitted with an aerator (Hamka *et al.*, 2020). During acclimation 25 °C temperature was measured in aquarium. (pH) of aquarium was 7 to 8. (DO) levels of 5 were the acclimatization parameters to 8 mg/L

(Abedi *et al.,* 2012). Samples were divided into 3 groups each group had 10 samples. Aquarium G_o as a controlled group. Similiarly, aquarium G1 and G2 were used as experimental group 1 & 2.

A virulent strain of *Aeromonas hydrophila* was isolated from the kidney and liver tissues of naturally infected fish showing typical clinical signs such as hemorrhages and ulcerations. Tissue samples were aseptically collected and streaked onto Rimler–Shotts agar and incubated at 28 °C for 24-48 hours. Colonies with typical morphology yellow with a black center were subcultured for purification. Preliminary identification was carried out using Gram staining, oxidase, catalase, and motility tests. Gram-negative, oxidase-positive, motile rods were considered presumptive *Aeromonas* spp.

Preparation of the Bacterial Inoculum The confirmed *A. hydrophila* strain was cultured in Tryptic Soy Broth (TSB) and incubated at 28 °C with shaking (150 rpm) for 18–24 hours until the culture reached an optical density (OD_{600}). The bacterial suspension was then centrifuged at 4,000 rpm for 10 minutes, washed twice with sterile phosphate-buffered saline (PBS), and resuspended to the desired concentration. The bacterial concentration was adjusted to 10^7 colony-forming units per milliliter (CFU/mL) using serial dilution and confirmed by plate counting on Tryptic Soy Agar (TSA).

The studied samples were intraperitoneally injected with 0.1 mL of the prepared *A. hydrophila* suspension (10^7 CFU/mL). A control group was injected with 0.1 mL of sterile PBS. Each group consisted of 10 fish, and the experiment was conducted in triplicate. Post-injection, fish were monitored daily for 14 days for clinical signs, behavioral changes, morbidity, and mortality. Dead or moribund fish were collected for re-isolation of the bacteria to confirm Koch's postulates. Re-isolation was done using the same bacteriological procedures as mentioned above. Sampls of Gills, Heart and Kidneys were taken at 14^{th} day.

Tissue Collection

Anesthesia:

Fish samples were anesthetized with chloroform

Organ Sampling:

Gills: Gills were carefully excise without damage.

Heart: Heart was carefully excise without damage.

Kidneys: Kidneys were carefully excise without damage

Fixation: Tissues were preserved in 10% buffered formalin solution. Ensure the tissue-to-fixative ratio is at least 1:10.

Tissue Processing

Tissues were passed from following concentrations of ethanol (70%, 95%, 100%) for one hour each for dehydration purpose. xylene was used as an appropriate clearing agent for one hour. Tissues were immersed in molten paraffin for one hour at 55°C. Tissues were embedded in paraffin wax molt and allow to set at room temperature. Extra amount of paraffin wax was removed during trimming and tissues were oriented in blocks. Microtome was used to cut 3-5 µm thick sections. Thick sections of tissues were float on warm water at 45°C for mounting then transfer to glass slides. Paraffin was removed by procced slides from xylene for Deparaffinization and rehydrate the slides through ethanol concentration.

Hematoxylin and Eosin (H&E) Staining:

- **Hematoxylin**: Stained about 5–10 minutes.
- **Rinse**: Tap water used for washing.
- **Eosin**: 1–2 minutes for water staining.
- **Rinse**: 95% ethanol used for washing.

Dehydration: slides were passed through different ethanol concentrations. slides were clear in xylene. **Mounting**: coverslip was used in suitable mounting medium.

RESULTS:

Microscopic Examination

Hematological analysis of fish heart, kidneys and gills:

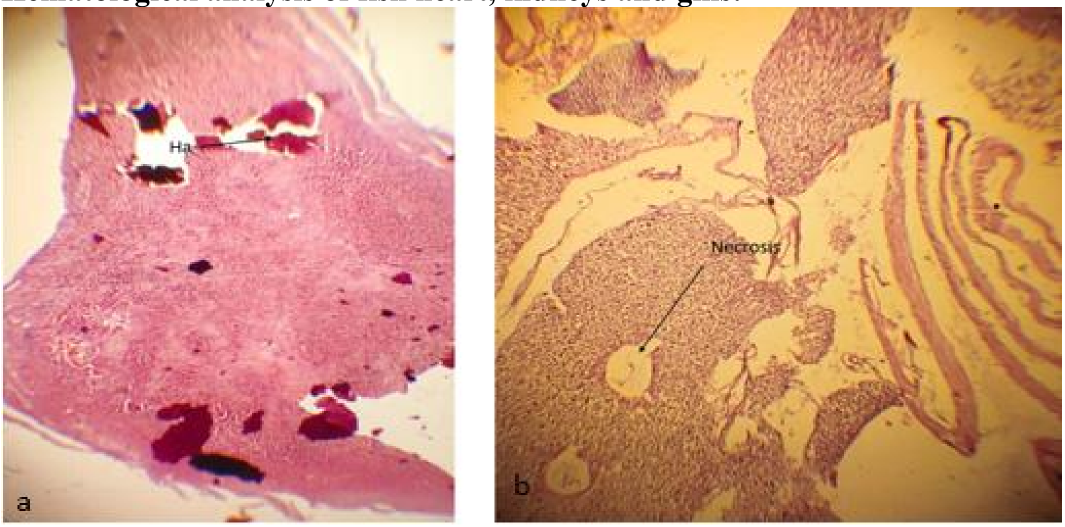
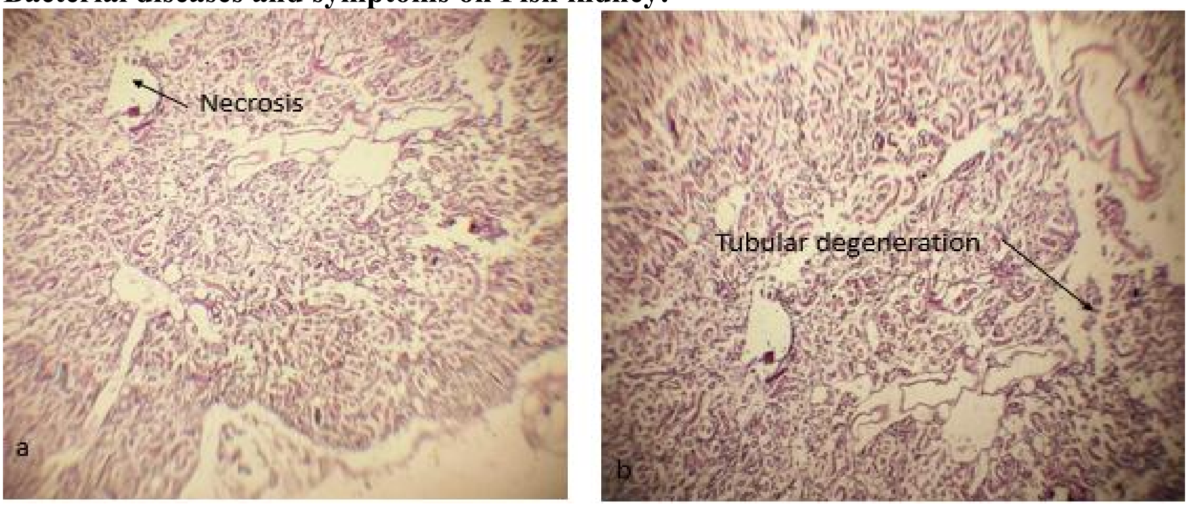


Fig.1a) shows the hemorrhage area of fish heart. The reasons of hemorrhagic area are the damage of cardiac tissues and blood vessels due to *Aeromonas hydrophilia*.Fig.1b) shows the necrosis of fish heart. Necrosis refers to the death of cardiac tissues through inflammation and tissue degradation due to *Aeromonas hydrophilia*.

Bacterial diseases and symptoms on Fish kidney:



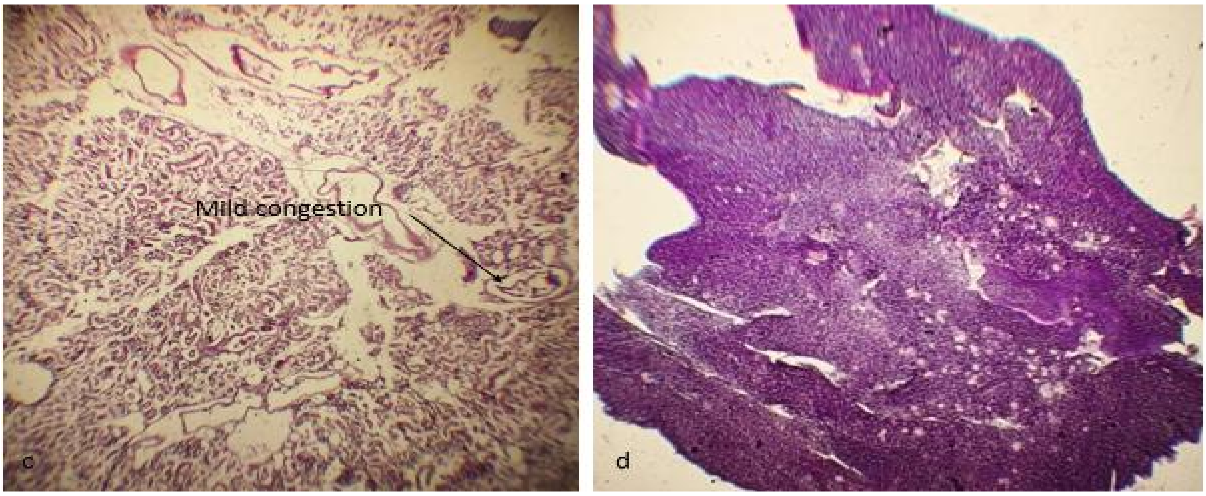


Fig. 2a) shows the necrosis of fish kidneys. Necrosis is the death of renal tisues due to *Aeromonas hydrophilia*. Fig. 2b) sows the tubular degeneration of fish kidneys. Tubular degeneration reffers to the deterioration or demage of renal tubules due to *Aeromonas hydrophilia*. Fig. 2c) shows the mild congestion in the fish kidneys. Mild congestion reffers to the accumulation of excess blood within the blood vessels of renal tissues due to *Aeromonas hydrophilia*. Fig. 2d) shows normal tissues of fish kidney.

Bacterial disease and symptoms on fish gills:

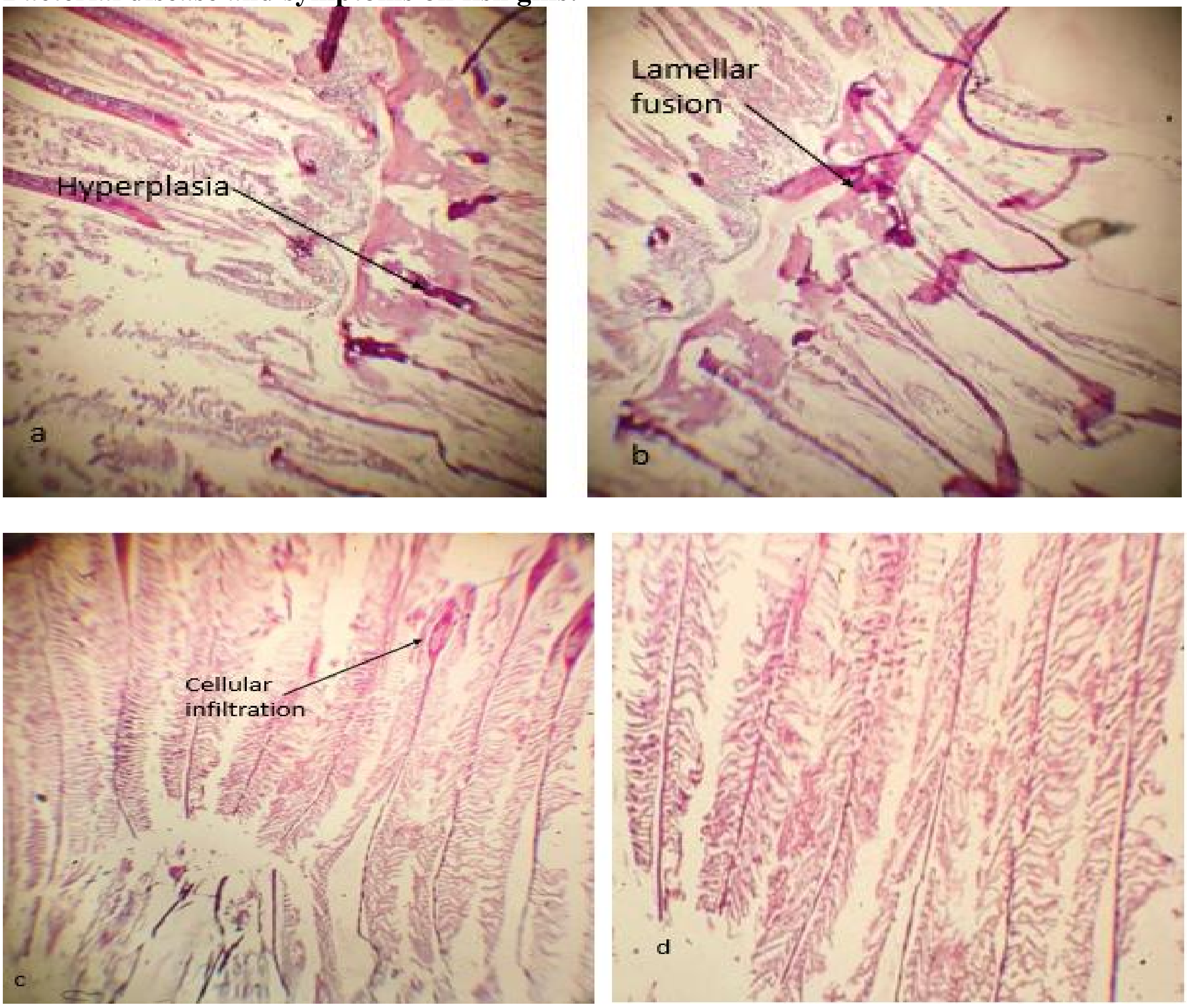


Fig.3a) shows hyperplasia in the fish gills.It is the incease in the size of the tissue leading in tissue enlargemet due to *Aeromonas hydophilia.* Fig.3b) shows the lammelar fusion . It reffers to the abnormal joining of secondary lammelaedue to *Aeromonas hydrophilia. Fig.3c)* shows cellular infiltration. It reffers to accumolation of inflammatory cells due to *Aeromonas hydrophillia.Fig.3d)* shows normal tissues.

Bacterial disease and symptoms on fish Intestine:

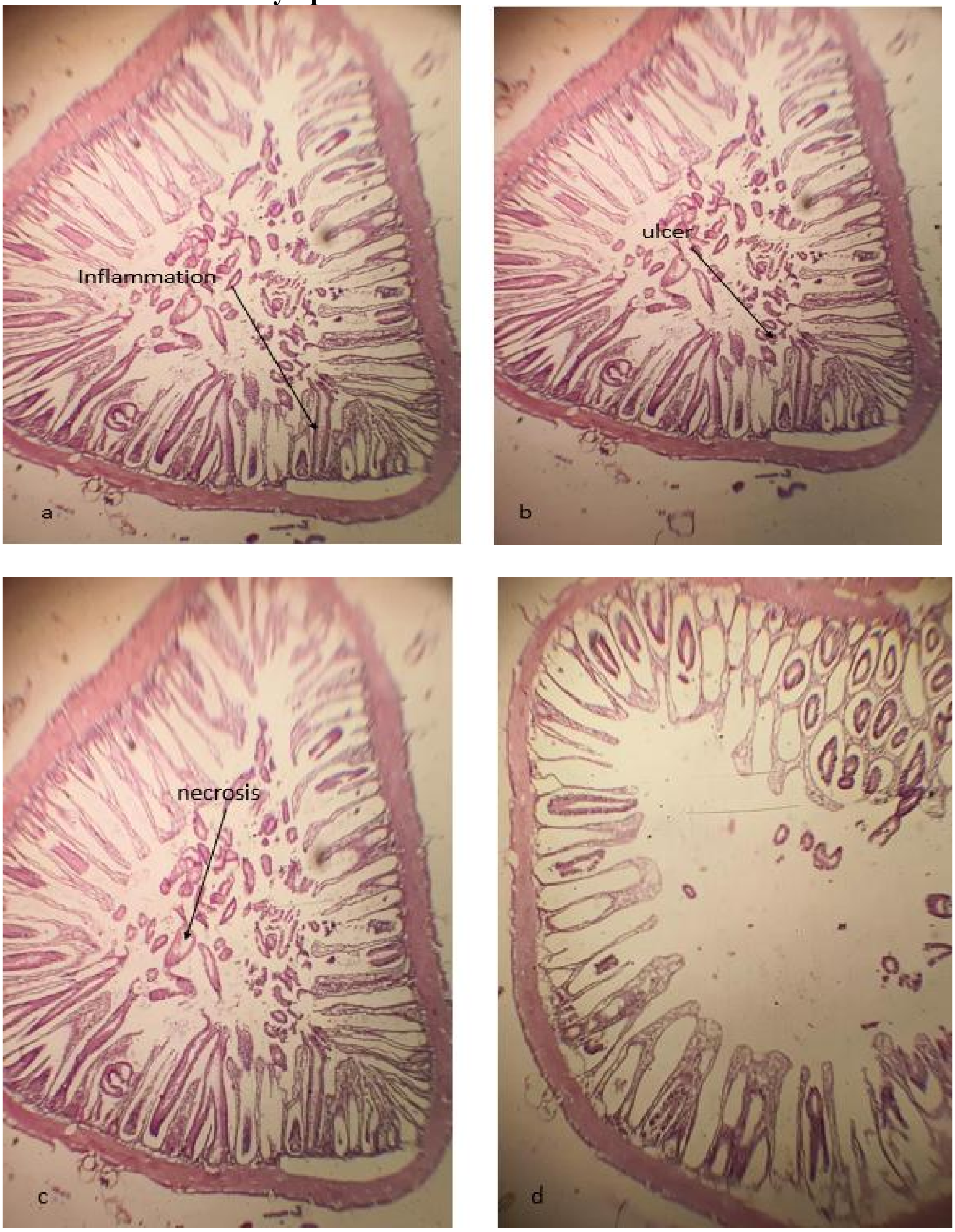


Fig.4a) shows the inflammation in the fish intustine due to *Aeromonas hydrophilia*. Fig.4b) shows ulcer in the fish intestine due to *Aeromonas hydrophilia*.Fig.4c) shows necrosis in the fish intestine .It happened due to death of intestinal tissues. Fig.4d) shows normal tissues.

Discussion:

This study aims to evaluate the preventive and treatment effects of *Lactobacillus*-derived a probiotic supplement on the histopathology integrity of key organs in the common carp (*Cyprinus carpio*) exposed with Aeromonas hydrophila. Our findings underscore the possibility of probiotic administration in mitigating tissue damage often linked to infections with bacteria in aquaculture species. The escalating risk of infections by bacteria in aquaculture demands the investigation of environmentally friendly and efficient replacements to antibiotics. Probiotics, especially those originating from Bacillus species, have demonstrated beneficial health effects in aquatic organisms (Balcázar at el., 2006).

In the present study, we determined the effectiveness of Lactobacillus-based probiotic supplements in alleviating the histopathological damage induced by *Aeromonas hydrophila* in the cardiac system, kidneys, gut, and gills in common carp (*Cyprinus carpio*). The microscopic examination demonstrated substantial defense in probiotic-treated fish, underscoring the potential of beneficial bacteria as a functional supplement in aquaculture.

Histological examinations indicated substantial heart injury in untreated infected fish, characterized by bleeding, necrosis, and post-mortem artifacts .Figures 1a–1c demonstrate significant tissue damage and inflammatory reactions induced by A. hydrophila. Conversely, fish that received probiotic supplementation had diminished pathogenic indicators with cardiac tissues closely approximating healthy histoarchitecture (Fig. 1d). This indicates that Bacterium strains may have cardioprotective benefits may be by modulating immune responses and diminishing pathogen-induced inflammation Histological analysis of the cardiac tissue with *A. hydrophila*-infected fish demonstrated notable structural anomalies including hemorrhage, necrosis and artifacts associated with blood clotting (Fig. 1a–1c). These findings align with the established toxicity of *A. hydrophila* which generates cytotoxins and hemolysins that can harm the cardiac muscles and vasculature. Hemorrhaging signifies the rupture of blood vessels, whereas necrosis denotes cellular death resulting from continual inflammation and oxygen depletion (Nayak *at el*., 2010).

Conversely, fish fed with Lactobacillus-derived probiotics exhibited nearly normal cardiac shape (Fig. 1d) indicating less pathogen-induced damage. The shielding effect may be ascribed to the immunomodulatory properties of *Bacillus spp* which are recognized for stimulating immune responses that are innate as well as adaptive thus augmenting the host's capacity for neutralizing bacterial toxins. Furthermore, probiotics may attenuate pro-inflammatory cytokines hence diminishing tissue damage. The findings correspond with prior research indicating that probiotic supplementation in fish enhances heart histology and fortifies resistance to pathogenic pathogens (Ringø *at el*.,2010).

Untreated fish kidneys exhibited necrosis, tubular degeneration and moderate congestion (Fig. 2a–2c), signifying systemic bacterial infection and impaired renal function. The degenerative alterations were significantly mitigated in the probiotic-supplemented groups where kidneys slices exhibited maintained tissue architecture and negligible indications of degeneration (Fig. 2d). The protective function of Lactobacillus may be ascribed to its capacity to bolster systemic immunity and preserve mucosal barrier integrity thus restricting the dissemination of pathogens to renal organs. The kidney a vital organ responsible for osmoregulation and elimination in fish is particularly vulnerable to systemic infections. In our investigation kidneys from diseased and untreated carp demonstrated necrosis, epithelial breakdown and blood vessel obstruction (Fig. 2a–2c). The clinical characteristics signify significant renal dysfunction possibly resulting from the systemic dissemination of *A. hydrophilia* which compromises blood circulation and tubular integrity. Fish treated with probiotics exhibited markedly smaller histological abnormalities with kidney tissues closely approximating those of healthy controls (Fig. 2d). This indicates that *Lactobacillus*-based probiotics contribute to the maintenance of renal anatomy during bacterial challenges. The method may entail the enhancement of immune defenses at mucosal barriers hence restricting the migration of bacteria and systemic dissemination. *Lactobacillus* species also generate antimicrobial agents, including antibiotics and organic acids that impede pathogen growth. These variables collectively facilitate the preservation of renal tissue integrity and functionality (Newaj at el.,2014).

Gills due to their direct exposure to the outside world, are especially susceptible to infections. In our investigation, infected unmanaged fish gills displayed excessive growth, lamellar fusion and infiltration of cells (Fig. 3a–3c) indicative of a standard immune response to an infection and resultant tissue damage. Probiotic-treated fish had markedly diminished inflammation with gill morphology resembling that of the members of the control group (Fig. 3d). The findings corroborate the notion that probiotic bacteria can maintain gill epithelial cell surfaces and diminish inflammatory infiltration potentially via competitive eradication of pathogens or enhancement of local immunity. Gills serve as the principal location for exchange of gases and a significant entrance point for pathogens in fish. Consequently, gill injury frequently signifies initial indications of infection. In the infected but non-supplemented cohort, gills exhibited hyperplasia, lamellar fusion while and cellular invasion (Fig. 3a–3c). Hyperplasia along with lamellar fusing are adaptive mechanisms to irritants and infection intended to minimize additional exposure, albeit at the expense of respiratory efficiency. Cellular infiltration signifies a confined immune response endeavoring to eliminate invading microorganisms. In sharp contrast, gill tissue from fish fed with probiotics displayed normal laminated structures with negligible indications of inflammation or structural deformation (Fig. 3d). This indicates that probiotics may aid in preserving gill health by diminishing pathogen colonization and enhancing epithelial cell turnover. *Lactobacillus spp* may function by excluding competitors, accumulating mucus binding sites and so obstructing the attachment and penetration of pathogens such as *A. hydrophila*. Furthermore, by stabilizing the mucus layer and augmenting antioxidant activity, probiotics bolster the overall resistance of gill tissues under viral stress (Pandiyan *at el*.,2013).

The intestine a principal location for nutritional absorption and immunological activity exhibited significant pathological alterations in infected fish, including inflammatory processes, the development of ulcers and mortality (Fig. 4a–4c). These lesions can significantly compromise fish health by interfering with digestive and absorptive activities. Probiotic-treated fish exhibited negligible intestinal damage with the structure of tissue reverting to normal (Fig. 4d). This highlights the essential function of *Lactobacillus* in preserving intestinal homeostasis, potentially via strengthening epithelial tight junctions, secreting antimicrobial peptides and modulating gut microbiota. The intestine serves as both an organ of digestion and a significant element of the fish defenses. The gastrointestinal tract serves as the principal target and site of action for orally delivered probiotics. This investigation revealed significant histological alterations in infected carp intestines, including inflammation, the development of ulcers and necrotic (Fig. 4a–4c). These findings highlight the severity of damage inflicted by A. hydrophila, particularly compromises epithelial integrity and initiates inflammatory responses, potentially resulting in diminished nutrition absorption and systemic disease.

Fish that received probiotic supplementation displayed intestinal cells with normal histological characteristics and preserved mucosal layers (Fig. 4d). This enhancement presumably arises from many probiotic-mediated pathways. Lactobacillus strains can fortify the intestinal wall by increasing the expression of restrictive junction proteins, therefore inhibiting the movement of microorganisms and endotoxins. Secondly, probiotics regulate the gut microbiome by enhancing beneficial bacteria that compete with diseases for resources and habitat. Probiotics affect host immunity by enhancing mucosal immunity (e.g., promoting secretory IgM production) and diminishing inflammatory cytokine expression, therefore mitigating tissue damage (Ghosh at el.,2008).

Probiotic therapy consistently yielded enhanced histology results across all analyzed organs compared to controls without treatment. These results correspond with earlier research illustrating the immunological, anti-inflammatory, and barrier-enhancing properties of Lactobacillus in aquatic species. The decrease in tissue damage in several organs indicates that Lactobacillus-derived probiotics may function as an effective preventive and therapeutic approach against bacterial infections such as A. hydrophila, diminishing the reliance on antibiotics and promoting sustainable aquaculture methods (Kumar at el.,2006).

The organ-specific histology data collectively indicate a similar trend: Lactobacillus-derived probiotic intake greatly alleviates the deleterious effects of A. hydrophila infections across various critical systems. This indicates a systemic preventative effect, perhaps based on both immediate and systemic immunological regulation. Probiotics diminish bacterial burden and avert significant tissue harm by fortifying the intestinal barrier and systemic immunity. These findings have significant implications for the management of health in aquaculture. The excessive application of medicines in aquaculture jeopardizes the emergence of antimicrobial resistance and disturbs the natural microbial equilibrium in aquatic ecosystems. Probiotics provide a natural, sustainable solution that promotes fish health and preservation of the environment. Moreover, augmenting fish resistance via dietary supplements may diminish deaths, enhance the performance of growth, and optimize feed conversion efficiency—essential elements for the economic sustainability of aquaculture enterprises (Shelby *at el*.,2006).

The histopathological findings robustly endorse the advantageous effects of probiotics; nevertheless, the study is constrained by the absence of molecular data, including cytokines profiling, microbe study and gene expression investigations pertinent to immune signaling pathways. Subsequent research should integrate these methodologies to further comprehension of the processes via which probiotics provide organ-specific protection. Furthermore, prolonged experiments assessing lifespan, development and reproductive efficacy in natural environments would yield practical information into the feasibility of beneficial bacteria as a sustainable solution in aquaculture (Sugita et al.,1991).

This study's findings indicate that Lactobacillus-derived probiotics have substantial protective effects against damage generated by Aeromonas hydrophila in the chambers of the heart, kidneys, gills, and gut of common carp. The diminished histopathological damages in probiotic-treated fish highlights their contribution to bolstering immunological defense, preserving tissue integrity, and promoting overall fish health. The findings endorse the incorporation of probiotics as a fundamental element in comprehensive aquaculture health management approaches, potentially diminishing dependence on antibiotics and fostering sustainable fish farming practices.

Conclusion:

Aeromonas hydrophila is the major etiological agent of illnesses affecting essential fish organs, demonstrating significant virulence and pathogenicity. The histological evidence substantiates that *Aeromonas hydrophila* contamination induces multi-organ damage, impacting essential systems including circulatory, renal, breathing, and gastrointestinal systems in fish. These alterations can lead to elevated mortality and morbidity rates in aquaculture, underscoring the necessity for prompt identification, efficient treatment, and appropriate biosecurity protocols to mitigate the dissemination of this virus.

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