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The Effect of Aeromonas Hydrophila Bacteria on Malondialdehyde Levels in The Gills, Liver Enzymes, And Liver Tissues of Cyprinus Carpio L. Fish Fed a Diet Containing Prosopis Farcta and Its Comparison with The Antibiotic Oxytetracycline

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Abstract

The study showed the effect of *Aeromonas hydrophila* on various dietary treatments supplemented with powder, and alcoholic and aqueous extracts of the *Prosopis farcta* plant compared to the control treatment containing the antibiotic oxytetracycline. This was assessed by examining MDA levels, liver enzymes, and liver tissue in *Cyprinus carpio* carp fish injected with a 3×10^{10} CFU/mL dose. The *A. hydrophila* caused the death of approximately 22% of the fish within two weeks after injection. In this study, infection with *A. hydrophila* induced motile Aeromonas septicemia and a higher mortality rate among the fish. The control treatment recorded the highest MDA level of 2.35 mg MDA/g at the $P \leq 0.05$ significance level, while the third treatment had the lowest at 0.679 mg MDA/g in the fish gills. Blood and liver tissue samples were collected on day 14 post-infection. In contrast, treatments 3, 5, 6, and 7 recorded the highest concentrations at 263, 264, 281, and 276 IU/L, respectively at the $P \leq 0.05$ significance level, whereas the uninfected negative control (T0) recorded the lowest at 169 IU/L for the AST enzyme. Liver enzymes served as clear indicators of infection, reflecting the infiltration of inflammatory cells and showed signs of infection and inflammation in various regions of the fish's body, including skin ulcers, necrosis, and degeneration of the soft fin rays. Furthermore, hemorrhaging and necrosis in the internal organs led to increased accumulations of phagocytic cells in the liver. Based on these results, infection with *A. hydrophila* may cause oxidative stress and liver cell damage, directly contributing to the pathophysiology and progression of diseases in infected fish.

Keywords: Prosopis farcta, Challenge, A. hydrophila, Cyprinus carpio.

تأثير بكتيريا Aeromonas hydrophila على مستويات المالون داي- ألديهايد في الغلاصم، وأنزيمات الكبد، وأنسجة الكبد لأسماك .Cyprinus carpio L التي تتغذى عليقة تحتوي على Prosopis farcta ومقارنتها بالمضاد الحيوي أوكسي تتراسيكلين

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الخلاصة

أظهرت الدراسة تأثير بكتيريا *Aeromonas hydrophila* المغذاة على علائق مختلفة من مسحوق والمستخلص الكحولي والمائي لنبات *Prosopis farcta* مقارنة بمعاملة السيطرة الموجبة المحتوية على المضاد الحيوي أوكسي تتراسايكلين على مستوى MDA وأنزيمات الكبد وأعضاء الكبد لأسماك الكارب *Cyprinus carpio* المحقونة بجرعة. (3×10^{10} CFU/mL) تسببت بكتيريا A. hydrophila في موت حوالي 22% من الأسماك خلال أسبوعين بعد حقن الأسماك بالبكتيريا. وفي الدراسة الحالية تسبب عدوى A. hydrophila في حدوث مرض تسمم الدم المتحرك وارتفاع معدل الهلاكات بين الأسماك، كما أظهرت النتائج أن معاملة السيطرة سجلت أعلى مستوى MDA/g 2.35mg عند مستوى معنوية ($P\leq0.05$)، بينما سجلت المعاملة الثالثة أقل مستوى (0.679mgMDA/g) في غلاصم الأسماك. تم أخذ عينات من الدم وأنسجة الكبد في اليوم الرابع عشر من الإصابة، وأظهرت النتائج أنه على الرغم من ارتفاع مستويات إنزيمات ALT، إلا أنه لا توجد فروق معنوية بينها. أما بالنسبة لأنزيم AST فقد سجلت المعاملة الثالثة والخامسة والسادسة والسابعة أعلى تركيز (IU/L 263، 264، 281، 276) على التوالي عند مستوى معنوية ($P\leq0.05$) في حين سجلت معاملة السيطرة السالبة الغير محقونة

T0 أقل تركيز (169 IU/L) لنفس الإنزيم. وكانت إنزيمات الكبد دليلاً واضحاً على الإصابة وتسلل الخلايا الالتهابية مع بعض علامات الإصابة والالتهاب في مناطق مختلفة من جسم السمكة وتقرحات على الجلد ونخر وتنكس الأشعة الناعمة للزعانف. كما أدى النزيف والنخر في الأعضاء الداخلية للأسماك إلى زيادة تراكم الخلايا البلعمية في الكبد. وبناءً على هذه النتائج، فإن الإصابة ببكتريا *Aeromonas hydrophila* قد تسبب الإجهاد التأكسدي وتلف خلايا الكبد، مما يساهم بشكل مباشر في الفسيولوجيا المرضية وتطور الأمراض في الأسماك المصابة.

كلمات مفتاحية: نبات الينبوت، تحدي بكتريا أريموناس هايدروفيلا، الكارب الشائع.

Introduction

The genus *Prosopis* comprises approximately 44 species of trees and shrubs, with four native to Asia and Africa and 40 to North and South America. These species thrive in harsh conditions, particularly in arid and semi-arid regions (37), due to their ability to store carbohydrates in lower regions, such as stem bases, stems, tubers, and rhizomes (3 and 40). The species have various applications, including medicinal uses for treating asthma, rheumatism, and poison ivy (20). Feed additives can increase production or reduce costs (8). Antibiotics such as Florfenicol, Sulfadimethoxine/Ormetoprim, Oxytetracycline (OTC), and Sulfamerazine are widely used and approved for aquaculture practices. Oxytetracycline, a broad-spectrum tetracycline antibiotic, is commonly used to treat bacterial infections such as Motile Aeromonas Septicemia (MAS), vibriosis, flavobacteriosis, furunculosis, and columnaris in aquaculture farms (21). *A. hydrophila* is an opportunistic pathogen that causes MAS in various fish species, including *C. carpio*. Due to its genetic diversity, this gram-negative bacterium remains a challenge in aquaculture (44).

Water pollution caused by microbial or toxic pollutants is a global issue that negatively impacts fish health, leading to physiological, biological, and histological alterations over the past decades. Agricultural activities and rapid industrialization have significantly contributed to microbial contamination in aquatic environments, leading to bioaccumulation in fish tissues and posing potential risks to fish and consumers (2). Bacterial pathogens pose a significant challenge for the rapidly expanding aquaculture industry worldwide. These pathogens utilize virulence factors, including toxins, adhesins, effectors, and enzymes, to facilitate infection and colonization (24). Aquaculture remains one of the fastest-growing industries, playing a crucial role in global food security. *C. carpio* is the most widely consumed and cultivated freshwater fish worldwide (23). In fish, *A. hydrophila* infection may elevate the production of reactive oxygen species (ROS) and free radicals within cells, potentially altering antioxidant defense mechanisms and leading to oxidative stress and cellular damage (25). Any elevation in serum aminotransferase levels may be attributed to physiological, pathological, or nutritional factors, or these changes may result from stress or overfeeding, leading to liver dysfunction and impaired function (11). Similar to other vertebrates, fish attempt to mitigate oxidative damage through antioxidant defense systems (17).

Histopathological indicators serve as biomarkers for pathogen-induced tissue damage. Hepatocyte cytoplasmic degeneration, necrosis, atrophy, vacuolar formation, and an increased presence of immune macrophages are pathological changes commonly observed in the livers of freshwater fish (16). The liver and kidneys are the most affected organs during *A. hydrophila* infection, with histopathological pathways contributing to tissue damage (1). These organs play a crucial role and are critical in detoxification, antioxidant immune defense, and cellular protection (18). This study aims to investigate the effects of different treatments on *C. carpio* infected with *A. hydrophila*, specifically examining pathological and histopathological changes in the liver and evaluating the impact of treatments on oxidative stress levels (MDA) following bacterial injection.

Materials and Methods

Experimental design: The experimental fish were transported from a private fish farm in Ramadi in a vehicle equipped with a water pump to the Fish Laboratory, Animal Production Department, College of Agriculture, Anbar University, Ramadi, Iraq. Altogether 270 fish were used and distributed into nine treatments with three replicates each of 10 fish per replicate with an average weight of 25 ± 2 g for each fish in the second experiment. They were transferred to the system tanks at a rate of 10 fish for each of the 27-system aquarium tank with a capacity of 75 L. The experiment was conducted from August 25 to September 10, 2024.

Challenge from *Aeromonas hydrophila*: The experimental fish were treated and infected with *A. hydrophila* bacteria. The experimental fish were injected intramuscularly with a bacterial suspension solution at a concentration of 3×10^{10} CFU/ml and a 0.1 ml/fish dose. The - effects of each powder and the alcoholic and aqueous extract of *P. farcta* and the antibiotic used in the experimental treatments were compared for two weeks from August 8 to September 5, 2024.

Determination of LD_{50}: The LD50 value was calculated according to the method described in (34) and adopted by (30), where the inoculated *A. hydrophila* bacteria were re-isolated from the kidneys of dead fish using trypsin soy agar and identified as *A. hydrophila* by characterization tests. Thus:

Critical mitigations that fall between 50% of deaths = [deaths above 50%-50/deaths above 50% - deaths below 50%].

and, LD_{50}% = mitigation above 50% of deaths + critical mitigation falling between 50% of deaths.

Measurement of malondialdehyde levels in the gills of fishes: The concentration of malondialdehyde (MDA) was detected following (41). The absorbance of the organic layer was measured at 520 and 535 nm using a spectrometer (Lambda 2S, Perkin-Elmer Co., USA).

Biochemical blood tests: Anticoagulant gel tubes were used to collect blood samples from fish, and the blood plasma drawn from them was separated. The absorbance of the standard and sample solutions was measured at 505 nm when analyzing AST, ALT, and ALP using a spectrophotometer model SP-3000 plus, made in Germany, according to (27).

Histopathology examination: Histological examination of the samples was conducted after they were prepared and placed in buffered formalin at a concentration of 10% for 24 hours, as mentioned by (14 and 22).

Statistical analysis: Statistical analysis was conducted using a completely randomized design (CRD). It included the effect of the experimental coefficients on the studied traits, following the general linear model and using the ready-made SAS statistical program version 9.1 (SAS, 2002). Significant differences between the averages were tested using Duncan's multiple-nominal test (16) at the $P \leq 0.05$ significance level, according to the model:

$$Yij = \mu + Ti + Eij$$

Table 1: Composition of the experimental diet.

No.	Ingredient	Percentage in Diet
1	*Soybean meal 48%*	40
2	Fish meal	20
3	Wheat bran	15
4	Yellow corn	15
5	Barley	5
6	Wheat flour	3
7	Premix	1
8	Sunflower oil	1

* The soybean meal is of Argentine origin, containing 44% crude protein (CP) and 2230 kcal/kg metabolizable energy (ME).
*Fish meal contains 65% protein.

Results and Discussion

Malondialdehyde level in gills after treating fish with bacteria: Figure 1 illustrates the statistical analysis results of MDA levels in the gills, showing significant variations among treatments after injecting the fish with *A. hydrophila*. This indicates the apparent effects of *P. farcta* powder and its alcoholic and aqueous extracts on fish health and their response to stress caused by bacterial infection. The negative control group (T1) recorded the highest MDA concentration at the $P<0.05$ significance level reaching 2.35 mg MDA/g gill, surpassing all other treatments. This reflects the natural breakdown of peroxides, as MDA is a secondary byproduct, suggesting that adding *P. farcta* powder and its alcoholic and aqueous extracts reduces the increase in MDA levels. MDA is considered the most stable byproduct of oxidation caused by free radicals in unsaturated fatty acids and serves as a key indicator for measuring oxidative stress (30). The role of free radical reactions and reactive oxygen species (ROS) in the physiological processes of living organisms, particularly in response to environmental pollutants, contributes to increased oxidative stress associated with bacterial infections.

The *P. farcta* powder treatment (T3) at 150 mg/kg feed recorded the lowest level at 0.679 mg MDA/g, followed by the T7 and T8 aqueous extract treatments at 150 mg/kg and 300 mg/kg feed, respectively. These findings align with (23), which demonstrated the effect of *P. farcta* extract in reducing elevated MDA levels against ethanol-induced oxidative stress in rats. The results of this study are also consistent with (12), who reported increased MDA levels in common carp infected with *A. hydrophila*. As the disease progresses, these processes intensify, with lipid peroxide metabolism playing a crucial role in compensatory mechanisms, which become disrupted during disease progression. The sequential increase in lipid peroxide products, from the onset of infection to the chronic phase, indicates an active process of free radical oxidation (3). Preliminary phytochemical studies of *P. farcta* extracts have shown the presence of alkaloids, tannins, flavonoids, saponins, glycosides, phenols, resins, and sterols (35).

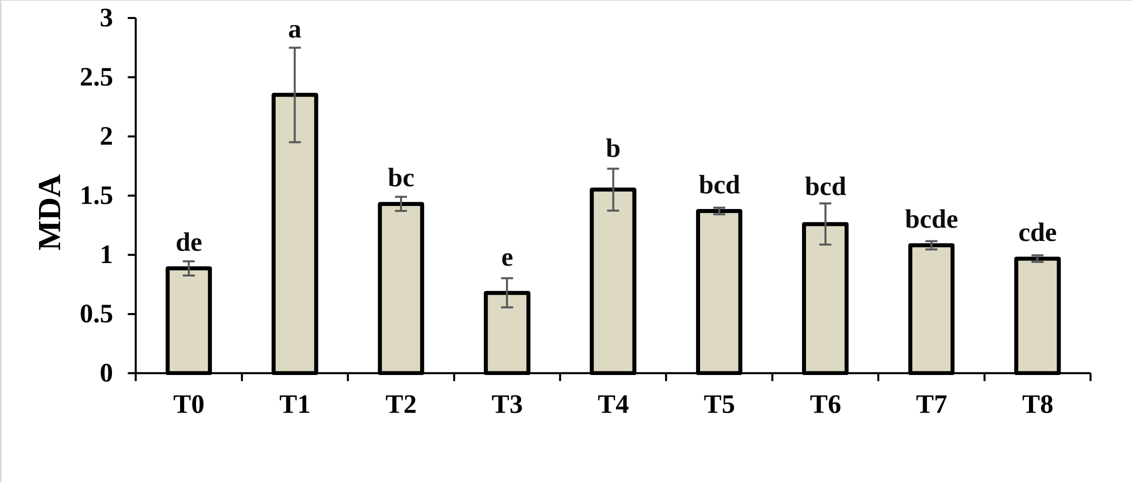

Figure 1: The effect of experimental treatments on the level of MDA in fish gills after injecting them with bacteria.

a, b, c: different letters within the same row indicate significant differences between treatments at the P≤0.05 significance level.

Biochemical blood characteristics after treating fish with Aeromonas hydrophila: The results in Figures 2, 3, and 4 on the ALT, AST, and ALP blood biochemical parameters showed no significant differences in ALT enzyme levels among the treatments despite an increase in their values after bacterial exposure. Elevated liver enzyme levels indicate oxidative stress, often caused by disease, infection, poor management, or inadequate nutrition. This finding is further supported by the increase in MDA levels following bacterial exposure, whereas its levels were normal before treatment. Regarding AST, there was a significant increase in the T3, T5, and T6 treatments compared to the others at the $P<0.05$ significance level while no significant differences were observed among the remaining treatments. As expected, the uninfected negative control (T0) recorded the lowest MDA level, since it was not exposed to infection. The elevated MDA levels in the aforementioned treatments represent a natural response and an indicator of fish infection, reflecting the body's defense mechanism against disease. As for ALP, treatment T3 (150 mg *P. farcta* powder/kg feed) significantly outperformed the other treatments at $P<0.05$, recording the highest level at 435 IU/L.

Meanwhile, the T7 treatment recorded the lowest MDA concentration among all treatments, which may be attributed to the effect of the aqueous extract of *P. farcta*. Liver enzymes have a crucial role in assessing liver tissue health (13). Liver damage leads to the release of liver enzymes into the bloodstream, resulting in increased enzyme activity in blood serum (38). ALT and AST are two of the most important enzymes involved in amino acid catabolism in the liver. Elevated ALT and AST serum levels are associated with liver cell damage. Some herbs have been reported to possess hepatoprotective properties and enhance liver function (37). ALT and AST enzyme levels in *Labeo rohita* fish infected with *A. hydrophila* increased significantly but returned to normal with *Achyranthes* treatment (33). The findings of this study match (11) who reported that yeast beta-glucan reduced ALT and AST activities in *C. auratus gibelio*. In their study, ALT and AST activities declined in fish fed appropriate doses of 0.5 or 1.0 g/kg and infected with *A. hydrophila*, but increased at higher doses of 2.0 and 4.0 g/kg.

ALP is one of the most important phosphatases in phagocytic cells. Its levels have been reported to decrease significantly in *Labeo rohita* infected with *A. hydrophila*, and treatment with *Achyranthes* restored these levels to normal compared to uninfected fish (33). Moreover, a significant increase in ALP levels was observed in crucian carp fed moderate doses of 0.5–1.0 g/kg of CVP (*Coriolus versicolor* polysaccharides). However, higher CVP doses of 2.0 and 4.0 g/kg lowered ALP levels on days 42 and 56 compared to the control group (41). Furthermore, the results of this study agree with (43) on the effect of probiotics on the blood biochemical parameters in Atlantic salmon infected with *A. hydrophila*. Although the liver enzymes were elevated in this study, they remained within the normal range, indicating the stress experienced by the fish during infection.

The fact that the enzyme levels stayed within the normal range reflects the effect of the powder and extracts of *P. farcta* in reducing stress levels. High concentrations of copper in the form of copper sulfate and copper nanoparticles negatively affect specific and relative growth rates. It acts as an oxidant. The challenge here is whether vitamins E and C can mitigate the effect of concentrations on oxidative stress resulting from exposure to large doses of copper sulfate and nanoparticle copper (19).

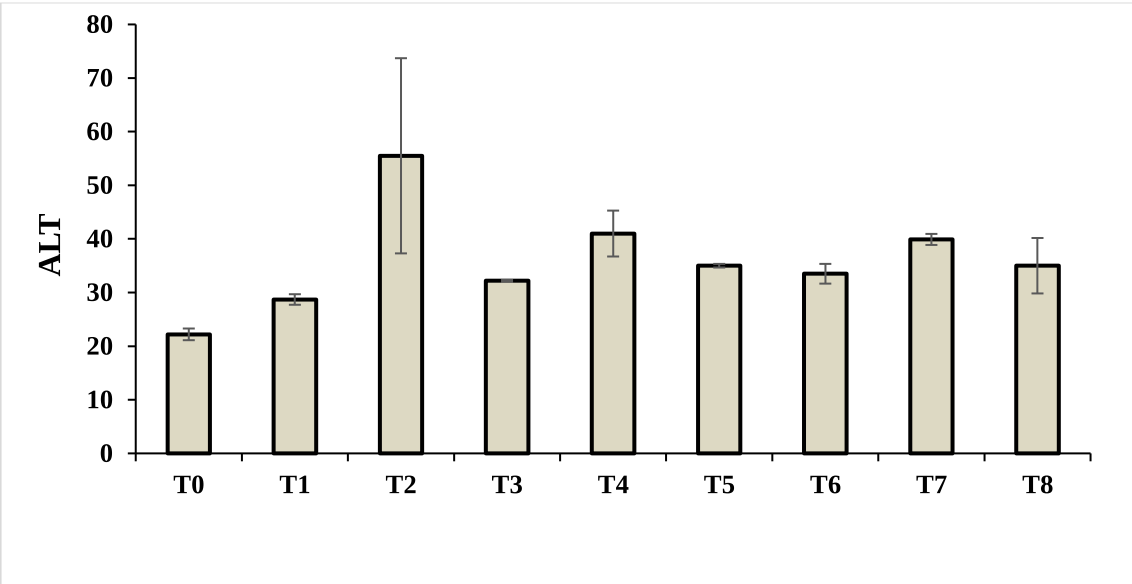

Figure 2: The effect of treatments on the ALT enzyme after injecting the fishes with bacteria.

a, b, c: different letters within the same row indicate significant differences between treatments at the P≤0.05 significance level.

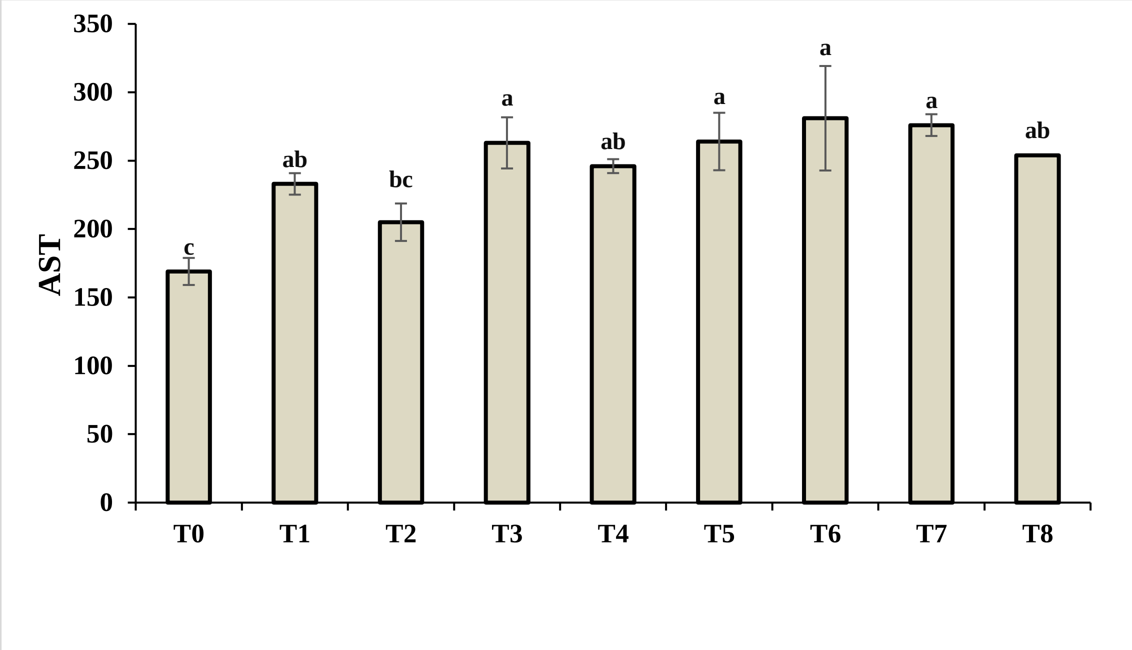

Figure 3: The effect of treatments on the AST enzyme after injecting the fishes with bacteria.

a, b, c: different letters within the same row indicate significant differences between treatments at the P≤0.05 significance level.

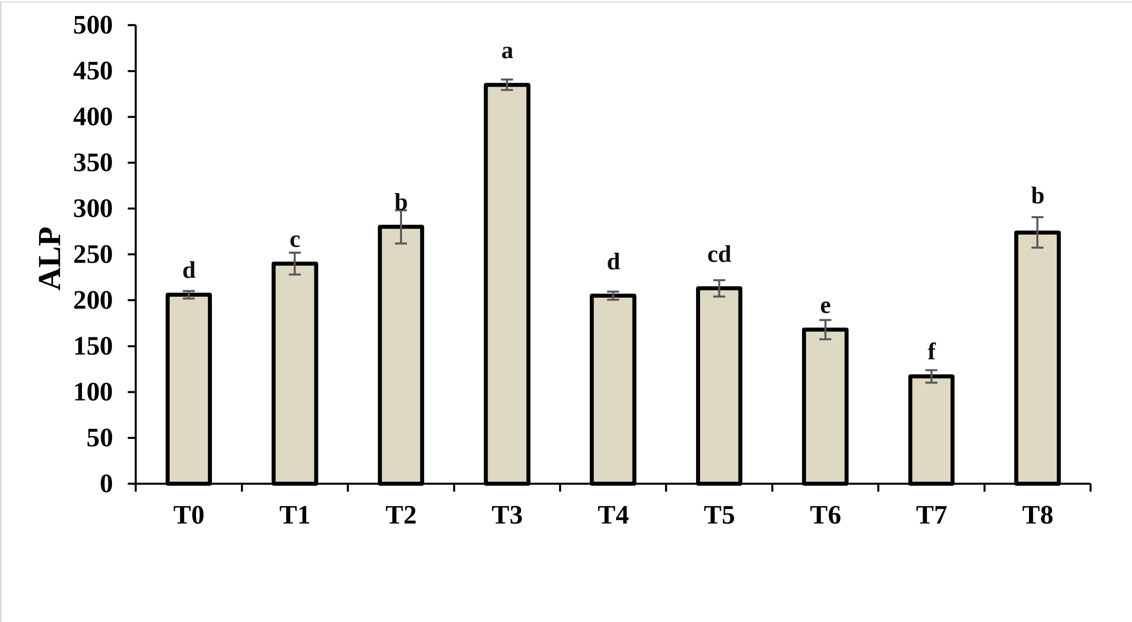

Figure 4: The effect of treatments on the ALP enzyme after injecting the fishes with bacteria.

a, b, c: different letters within the same row indicate significant differences between treatments at the P≤0.05 significance level.

Histopathological analysis: The histological section of the liver of the fish (T0) not injected with bacteria showed regular hepatocyte cords with normal appearance of cells (black arrow) and pancreatic vesicles with ducts (red arrow) stained with hematoxylin and eosin (Image 1). The histological sections of the fish liver in the first treatment (negative control) after injecting the *A. hydrophila* bacteria showed the occurrence of moderate hepatitis. This was characterized by irregularity of the hepatocyte cords with apparent atrophy of the cells and the formation of necrotic foci of the hepatocytes and accumulation of mononuclear inflammatory cells. Other sections showed degeneration and slight necrosis of the hepatocytes with a slight infiltration of mononuclear inflammatory cells in the sinusoids with clear congestion of the bile canaliculi with bile (Image 2). The histological section of the liver in T2-positive control fish (oxytetracycline) in (Image 3) shows regular hepatocyte cords with a typical appearance of most cells, slight congestion, and formation of a few necrotic foci (black arrow) and pancreatic alveolitis (red arrow).

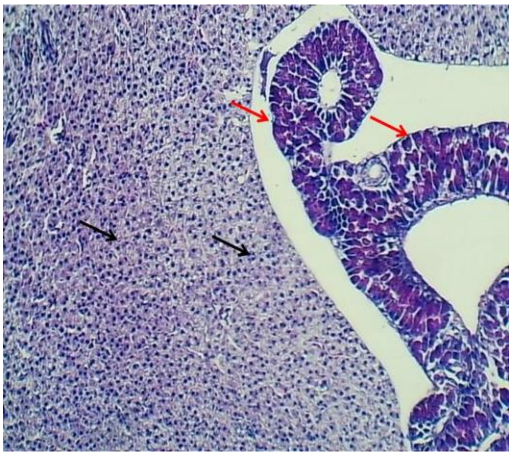

Image 1: T0 section of fish liver after bacterial injection.

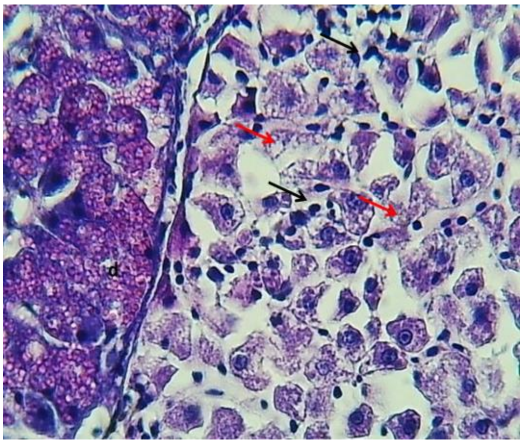

Image 2: T1 section of fish liver after bacterial injection.

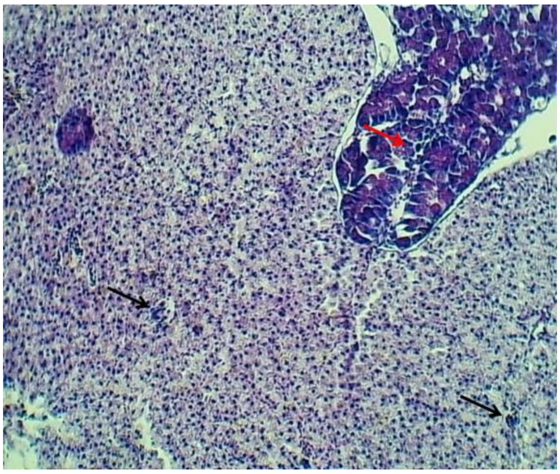

Image 3: T2 section of fish liver after bacterial injection.

The histological results of the liver section in fish from the third treatment group (150 mg/g feed of *Prosopis* powder) after injection with *A. hydrophila* showed a regular arrangement of hepatic cell cords, with normal morphology of hepatocytes and sinusoids, as shown in the image 4. The histological results of fish from the fourth treatment group (300 mg/kg feed of *Prosopis* powder) after bacterial injection revealed a regular arrangement of hepatic cell cords, with a generally normal appearance of most hepatocytes and sinusoids. However, small foci of fatty degeneration and congestion of the central vein were observed, as shown in the image 5.

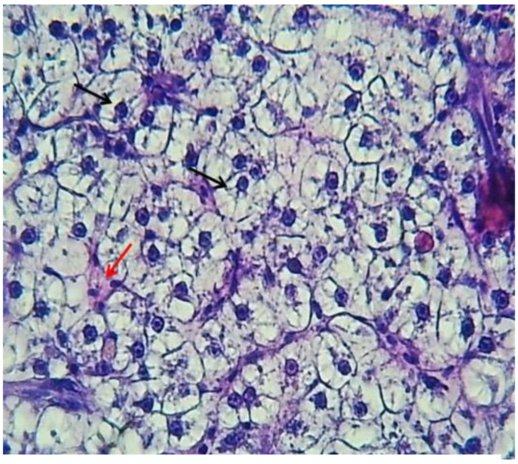

Image 4: T3 section of fish liver after bacterial injection.

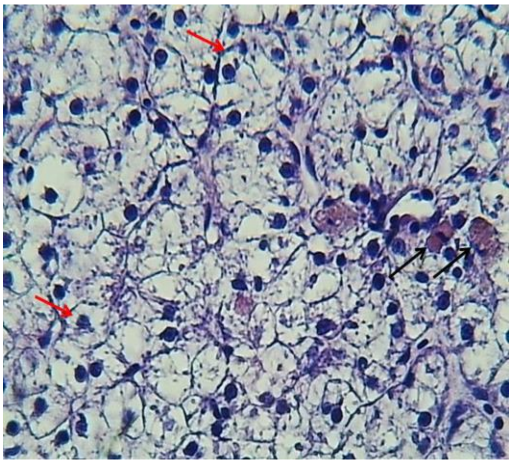

Image 5: T4 section of fish liver after bacterial injection.

The histological results of the liver showed regularity of hepatocyte cords with the formation of small foci of fatty degeneration and severe necrosis of the alveolar cells of the pancreatic glands of the T5 fish after injection with *A. hydrophila* Image 6. There was also the formation of small foci of hepatocyte degeneration inflammation. Additionally, it revealed clear congestion of the alveolar cells in the pancreas, accompanied by infiltration of inflammatory cells and severe congestion in the central vein of the T6 fish after inducing bacterial infection Image 7.

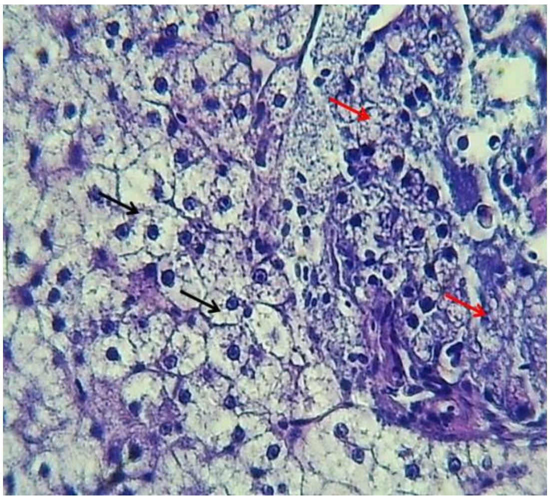

Image 6: T5 section of fish liver after bacterial injection.

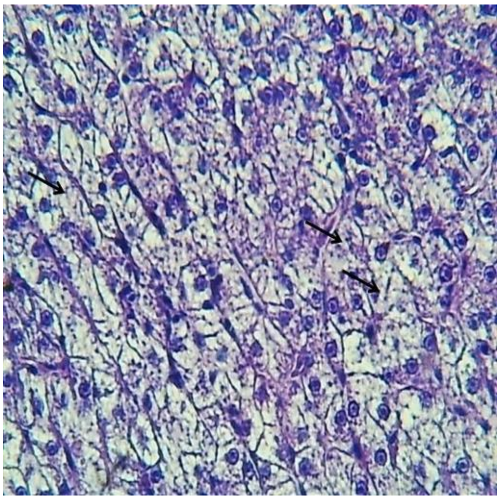

Image 7: T6 section of fish liver after bacterial injection.

After the injection, the histological results of the T7 liver showed irregularity of the hepatocyte cords, accompanied by severe and generalized inflammation of the liver tissue, including the formation of large and multiple necrotic foci of hepatocytes with infiltration of mononuclear inflammatory cells. Additionally, it revealed general degeneration of the hepatocytes, characterized by clear congestion, inflammation, necrosis, and atrophy of the alveolar cells in the pancreas. Image 8 shows the histological changes in the fish infiltration of mononuclear inflammatory cells (black arrow) and general degeneration of hepatocytes (red arrow).

The results of the histological sections of the liver in the T8 fish after injection with *A. hydrophila* showed irregularity of the hepatocyte tendons with general fatty degeneration. Additionally, it revealed severe necrosis of liver tissue and hepatocytes, accompanied by infiltration of mononuclear inflammatory cells, as well as necrosis and atrophy of alveolar cells in the pancreatic glands Image 9.

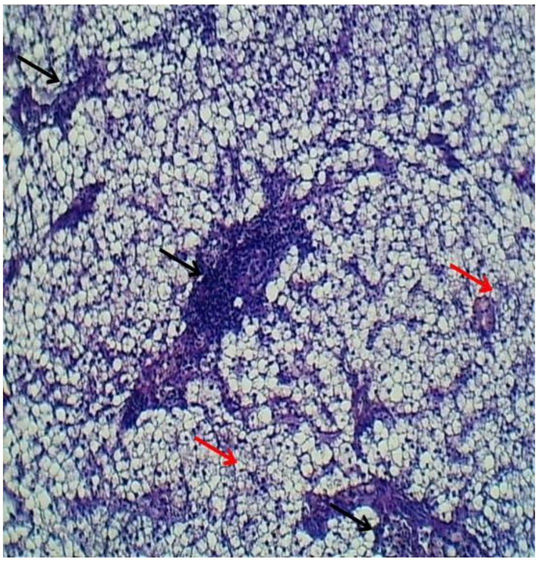

Image 8: T7 section of fish liver after bacterial injection.

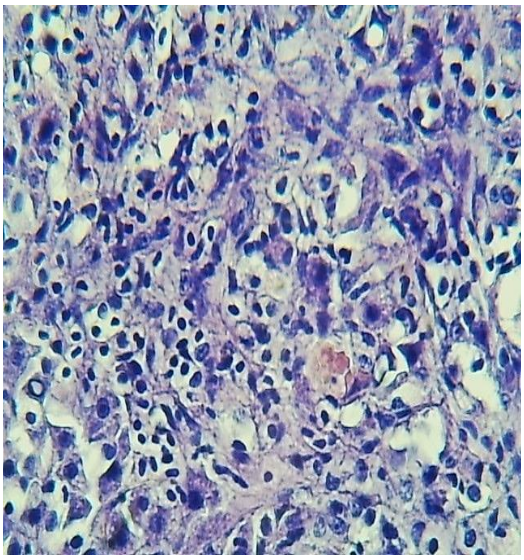

Image 9: T8 section of fish liver after bacterial injection.

The results of the histological sectioning of the liver organ revealed varying degrees of damage in the fish between treatments, with the second (50 mg oxytetracycline/kg feed) being more effective. There were significant differences in bacterial resistance between the treatments for fish infected with the present bacteria, as they were histologically close to the normal state. However, the histological sections taken from the fish organs showed a difference in the extent of damage to their tissues between the treatments, with T7 and T8 causing more injury.

Necrosis can be defined as a pathological process of cell death that can be caused by infection, trauma, hypoxia, or toxins. It becomes uncontrolled, releasing numerous chemicals from the dying cell that cause damage to the surrounding cells. Inflammation is often initiated by necrosis. Coagulative necrosis is generally caused by infarction (a lack of blood flow due to a blockage, resulting in ischemia) and can occur in all body cells except the brain. The heart, kidneys, and spleen are good examples of cells that undergo coagulative necrosis. Necrotic cells can become dry and hard. Interestingly, a gelatinous appearance occurs in dead tissue, but the cell structure is preserved for at least a few days. Coagulation occurs when proteins are degraded and denatured (27 and 32).

The results of this study are consistent with (8), which showed histopathological changes in the liver of *C. carpio* infected with *A. hydrophila* and revealed significant changes following infection. Samples collected over seven days indicated significant damage, highlighting the harmful effects of pathogens on liver structure and function as an extreme reaction appeared in the liver and in the hepatic pancreas. The liver structure showed massive necrosis of hepatocytes by distributed melanoma cells, vacuolar dissolution of most hepatocytes, venule dilation and infiltration of inflammatory cells, vacuolar dissolution of most hepatocytes, venule dilation and infiltration of inflammatory cells, liver tissue fluctuation, and vacuolar dissolution of most hepatocytes.

The results of this study are consistent with a second study by the same researcher (7) on *A. hydrophila* infection in *C. carpio* causing significant histopathological changes, including inflammation, necrosis, and degeneration in liver tissue, in addition to increased accumulation of melanocyte macrophages. This indicates oxidative stress and damage that contribute to the pathophysiology of infected fish. Pathological changes in the gills, liver, and kidney can serve as suitable biomarkers for assessing the pathogenesis of *A. hydrophila* in the common carp. Additionally, the Aeromonas species can pose a threat to public health, particularly individuals in contact with diseased fish. Therefore, proper management of water quality, food hygiene, and quarantine of new fish can control such infections. Microbial contamination also leads to significant tissue damage, including the destruction of gill filaments, enlargement of the nucleus in the liver, and infiltration of inflammatory cells into the heads of all fish (5).

The results of this study are consistent with (29), which showed the susceptibility of red hybrid tilapia to *A. hydrophila* infection. Apart from the threat to farmed fish, the contamination rate of *A. hydrophila* isolates may also pose a health threat to humans. Regarding the potential ability of *A. hydrophila* to infect tilapia, more attention should be paid to diagnosing the disease in farms and effectively controlling this pathogen by using appropriate and environmentally friendly therapeutic measures. The liver is most closely associated with detoxification, metabolism, and serum protein synthesis. However, due to its function, location, and blood supply, it is also one of the organs most affected by pollutants and bacterial toxins that infect fish (10).

Conclusions

A. hydrophila causes severe disease and is associated with high morbidity and mortality rates in *C. carpio* in water. This finding, along with other bacterial pathogens recently described in fish farming, highlights the importance of designing disease control programs based on accurate pathogen identification. This will facilitate timely and important decisions at the fish production level and help identify the pathological tissue on internal organs using specific doses that determine the degree of pathogenicity.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Author 1; methodology, writing—original draft preparation, Author 2 writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:

The study was conducted following the protocol authorized by the Head of the Ethics Committee, University of Anbar.

Informed Consent Statement:

Not applicable.

Data Availability Statement:

Data available upon request.

Conflicts of Interest:

The authors declare no conflict of interest.

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