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The Resistance of *Aeromonas* Bacteria to Antibiotics and the Effectiveness of Lactic Acid Bacteria Against Pathogenic Strains in *Cyprinus carpio* L.

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ABSTRACT

This study included 110 fish, and the proportion of isolated bacteria was determined to be 85% in the intestines, 25% in the skin, and 55% in the liver, with *Aeromonas* spp. making up the remaining 25%. Oxytetracycline (OX30), with a concentration of 4.5 ± 0.10mm, proved to be the most effective antibiotic in suppressing *Aeromonas* according to antibiotic sensitivity testing. In contrast, tetracycline (TI30) yielded unimpressive results. Antibiotic efficacy was enhanced by adding cell-free supernatants (CFS) derived from lactic acid bacteria. The data indicated that the optimal concentration of CFS was 50mg/ ml, suggesting that lactic acid bacteria could be a valuable tool in combating antibiotic resistance and improving fish health. In conclusion, this study suggests that lactic acid bacteria may help inhibit the growth of harmful *Aeromonas* strains, highlighting the need to reconsider current approaches to managing drug-resistant bacteria.

INTRODUCTION

The bacteria play a significant role in the well-being and productivity of several fish species, including the common carp (*Cyprinus carpio* L.), and are thus considered a major source of illness in fish farms. Hemorrhagic septicemia and ulcer disease are two of the many health issues caused by *Aeromonas*, which stands out among these bacteria **(Pereira, 2023)**. One challenge in treating bacterial infections is the antibiotic resistance exhibited by *Aeromonas* bacteria, which has been observed due to their widespread use in fish farming **(Semwal *et al*., 2023; Aljoburi *et al*., 2024)**. As a strategy to counteract harmful strains and maintain healthy fish, lactic acid bacteria (LAB) show significant promise. These microorganisms have the potential to improve fish health and protect them against bacterial diseases **(Amador *et al*., 2023; Dewi *et al*., 2023)**. Several therapeutic approaches are being explored to prevent the spread of *Aeromonas* strains that could harm fish health **(Van, 2015; Jumma, 2024)**. One widely accepted method for illness prevention in fish aquaculture is the use of LAB probiotics, such as *Lactobacillus* spp. **(Kuley *et al*., 2021; Al-Shammari, 2024)**. The aim of this study was to assess whether *Lactobacillus* CFS

extract serves as an effective approach for combating antibiotic resistance and enhancing fish health. The combined use of antibiotics and *Lactobacillus* CFS enhanced therapeutic efficacy and contributed to the amplification of antibiotic effects.

MATERIALS AND METHODS

1- Fish bacteria isolation and identification

The current investigation was carried out in a common carp pond located in Basra Governorate, targeting 110 fish, of which 10 were healthy for the isolation of *Lactobacillus* bacteria from their intestines, while the remaining fish were suspected of infection with *Aeromonas hydrophila*. Specimens were obtained from the ocular region, ulcerated dermal sites, kidneys, liver, and spleen. Swabs were used to isolate bacteria, which were then transferred to sterile test tubes with 9ml of the dilution solution (peptone water). First, the tubes were combined well to achieve a decimal dilution, then more were made. L-Shape diffusion was used to transport 0.1ml of the required dilution to plates containing pre-prepared solid nutrient medium Nutrient Agar **(Whittenbury *et al.*, 1970)**. The plates were incubated at 37°C for 24-48 hours. A loop carrier was used to take a swab from each type of colony that differed in shape and color and plant it on solid nutrient medium. The plates were incubated at 37°C for 24-48 hours.

2-Diagnostic bacteria tests

The study involved two methods for bacterial isolation. The first involved collecting samples from healthy fish and using MRS Agar culture medium. The samples were incubated under aerobic conditions for 24-48 hours, with small, white colonies monitored. Additional tests were conducted to confirm the identity of *Lactobacillus*. The second method involved collecting samples from various parts of the body, including eyes, ulcerated areas, kidneys, liver and spleen. Bacterial isolation was performed using *Aeromonas* Agar (RYAN), and colonies with a dark green color were confirmed through confirmatory tests. Gram staining was used to diagnose isolated bacteria. After 24 hours, a bacterial culture was prepared and smears were stained with Crystal Violet. The slides were then washed, iodine solution was added; in addition, ethanol decolorizer and neutralizing stain were applied. The slides were then analyzed under a microscope for microbial morphology and type **(Mulaw *et al*., 2019)**.

3-The molecular study

The study involved inoculating *Aeromonas* spp. samples in Brain-Heart Infusion Broth at 28°C for 24 hours. DNA was extracted from the skin, spleen, liver, muscle, and blood samples, which were cultured in Brain Heart Infusion Broth (HIMEDIA, India) at 28°C for 24 hours. DNA extraction was then performed using the DNA extraction kit from BIORON (Germany). Primers used to detect *Aeromonas hydrophila* included:

F: 5’-AACCTGGTTCCGCTCAAGCCGTTG-3’

R: 5’-TTGCCTCGCCTCGGCCCAGCAGCT-3’

React mixture and thermocyclers program: accord to that giving 760bp **(Swaminathan *et al*., 2004)**.

4-Susceptibility testing of *Aeromonas* to antibiotics and combination with CFS (Cell free supernatant) of *Lactobacilli* isolates

The Kirby-Bauer disc diffusion method **(Kyeremeh, 2010)** showed that very few common antibiotics achieved the maximum growth inhibition level when evaluated using the disc diffusion technique on Muller-Hinton Agar, leading to the exclusion of many strains from susceptibility data. We performed an antibiotic susceptibility test using the following antibiotics: Amikacin (AK30), Doxycycline (DO20), Oxacillin (OX30), Ticarcillin (TI30), Cefoxitin (ZOX30), Ceftazidime (KF30), and Chloramphenicol (C30). The inhibitory zones were measured 24 hours after incubation, following the manufacturer's instructions and conducting the tests in duplicate.

Cell-free supernatant (CFS) of *Lactobacilli* isolates was prepared based on the method described by **Grau *et al.* (2017)**. The resulting supernatants were either used for further experiments or stored at -20°C until needed. After preparing the CFSs, 10µl of each CFS was combined with the corresponding antibiotic disc to evaluate the antimicrobial combined action of the CFS and antibiotics against *Aeromonas*. The agar plates were incubated at 37°C for 24 hours under aerobic conditions. After incubation, the diameters of the inhibition zones around the antibiotic discs and CFS were measured. The results were classified as resistant (R) or sensitive (S) based on the size of the inhibition zones.

5- Determining the minimum inhibitory concentration (MIC) of CFE for LAB isolates against test bacteria

The desired bacterial species were cultured in a suitable nutrient medium. The preparation of cell-free supernatant (CFS) involved extracting the liquid from cultured bacterial cells after a specified growth period. Five distinct concentrations of CFS were prepared (50, 25, 10, 5mg/ ml). Equal volumes of each CFS concentration were administered to the bacterial culture plates. To use CFS autonomously, the solution was applied without the addition of bacteria. The plates were then incubated at the specified temperature for 24 to 48 hours, and the results were observed.

Evaluation of growth inhibition

After the incubation period, a scale was used to measure the inhibition zone. Statistical software was then applied to analyze the results and determine the percentage of bacterial growth inhibition for each variant. The results were evaluated by assessing the inhibition effects for each bacterial species at different concentrations of CFS, and the data were presented in a graph depicting the percentage of bacterial growth inhibition. This method evaluates the effectiveness of CFS against various bacterial species and provides valuable insights for potential therapeutic applications.

Statistical analysis

SPSS was used to analyze the results, where values representing the mean and standard error were obtained, then the data were analyzed using one-way ANOVA, and using Duncan's multiple range tests. The difference between groups were determined at a probability level ($P \leq 0.05$).

RESULTS AND DISCUSSION

1-Molecular diagnosis

Aeromonas hydrophila was confirmed using the polymerase chain reaction (PCR) technique, specifically targeting the amplification of the 16S rRNA gene, followed by sequencing, which is one of the most common methods used. Standard bacteriological methods were often insufficient to identify fish pathogens **(Abdelsalam *et al.*, 2023)**. The work was carried out in the central laboratory at the College of Education - Qurna, using the PCR-Bonier-USA device. A total of 110 fish were analyzed, and various organs were examined to determine the percentage of bacteria isolated. The results of the PCR analysis are shown in Fig. (1), which presents the electrophoresis analysis of DNA molecules from *Aeromonas hydrophila* using agarose gel.

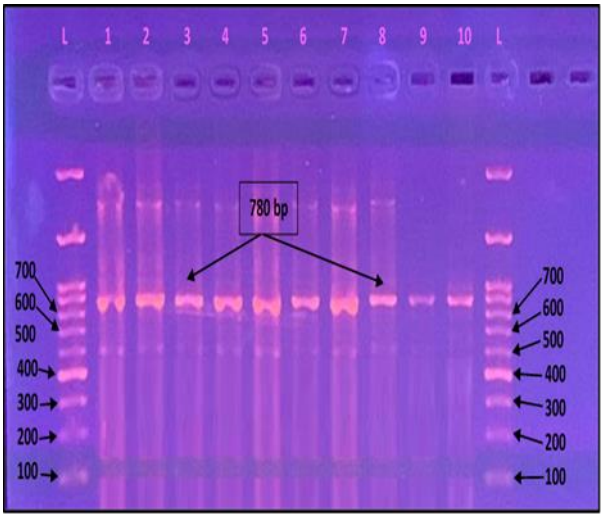

Fig. 1. PCR products using Gel Documentation System of 16s RNA (780bp). Region electrophoresis conducted on 1.6% agarose gel. L: ladder *Aeromonas hydrophila* (1,2,3,4,5,6,7,8,9,10)

2- Fish bacteria isolation and identification

A number of fishes were examined to determine the percentage of *Aeromonas* spp. and *Lactobacillus* spp. bacteria in different organs. The results showed that *Aeromonas* spp. were more prevalent in the liver (55%), while the highest percentage of *Lactobacillus* spp. was found in the intestines (85%). All the studied organs showed a varied total of isolated bacteria, with the intestines having the highest percentage (100%). Overall, the results

indicated a diverse presence of bacteria in different organs, highlighting the importance of studying microbial interactions in fish health. The intestines of fish had the largest percentage of total bacteria isolated (100%), mostly from *Lactobacillus* spp. (85%) **(Miyazaki *et al*., 2010; Techo, 2017)**. This finding is in line with the results shown in Table (1), which shows the bacteria recovered from different organs of the fish. *Lactobacillus* spp. are known to be part of the normal gut flora, and they help keep hazardous organisms from multiplying by competing for resources and secreting chemicals that slow their growth **(Colautti *et al*., 2022; Saha *et al*., 2022)**. *Aeromonas* spp. made up the vast majority of the bacteria found in the skin and liver, which together accounted for 25% of the total bacteria isolated. According to **Rahayu *et al.* (2022)**, certain organs such the kidney, spleen, and eye had high levels of *Aeromonas* spp., but no *Lactobacillus* spp. were found in these same organs. Consistent with studies **(Okon *et al*., 2023; Lagadec *et al*., 2024)** linking *Aeromonas* spp. to a variety of diseases, including infections **(Elbialy *et al*., 2024)**, the increased concentrations of *Aeromonas* spp. in the liver and skin suggest a possible association with health issues in fish.

Table 1. The analysis reflecting the diversity of bacteria found in different parts of the examination fish

No. of examined fish	Organs	Percentage of *Aeromonas* spp.	Percentage of *Lactobacillus* spp.	%Total isolated bacteria
20	Skin	10	15	25
10	Eye	20	-	20
20	Liver	55	-	55
10	Spleen	20	-	20
20	Kidney	40	-	40
30	Intestines	15	85	100

3- Susceptibility testing of *Aeromonas* to antibiotics and its combination with the cell-free supernatant (CFS) of *Lactobacilli* isolates

The zone of inhibition (in mm) was measured when the antibiotic was used alone and in combination with cell-free supernatants (CFS) from *Lactobacillus* spp. against *Aeromonas* spp. The antimicrobial activity of CFS was determined by measuring the inhibition zone using the agar well diffusion assay. The results, presented in Table (2), show the inhibition zone measurements (in millimeters) for three treatment modalities: antibiotics alone, CFS alone, and the combination of antibiotics with CFS.

Among the antibiotics tested, oxacillin (OX30) exhibited the strongest effectiveness against *Aeromonas* bacteria, as evidenced by the highest inhibition zone of 4.5 ± 0.10mm. In contrast, tetracycline (TI30) showed a minimal inhibition zone of 0.5 ± 0.55mm, suggesting limited effectiveness. Inhibition zones measuring 0.33 to 1.5mm

indicated that CFS alone was ineffective. While CFS demonstrated some antibacterial properties, it was not sufficient to be used as a standalone treatment.

However, when combined with CFS, the inhibitory efficacy of all antibiotics was significantly enhanced. For example, tetracycline (TI30), when combined with CFS, produced the highest inhibition zone (9.5 ± 0.97mm), indicating that the antibiotic's effect was greatly amplified. Chloramphenicol (C30) achieved an inhibition zone of 8.5 ± 0.56mm, suggesting strong efficacy when combined with CFS.

Table 2. Zone of inhibition antibiotics and combination with (Cell free supernatant) of *Lactobacilli* against *Aeromonas* spp.

Type of antibiotic	Zone of inhibition (mm)		
	Antibiotic only	**CFS only**	**Antibiotic + CFS**
Amikacin AK30	3.5 ± 0.33	1.1 ± 0.33	8.5± 0.76
Doxycycline DO20	2.5 ± 0.13	0.52 ± 0.13	5.5 ± 0.82
Oxacillin OX30	4.5 ± 0.10	1.5 ± 0.10	6.5 ± 0.61
Ticarcillin TI30	.5 ± 0.552	0.5 ± 0.55	9.5 ± 0.97
Cefoxitin ZOX30	1.5 ± 0.78	0.5 ± 0.78	4.5 ± 0.61
Ceftazidime KF30	3.5 ± 0.56	0.33 ± 0.56	7.5 ± 0.97
Chloramphenicol C30	1.02± 0.56	0.5 ± 0.56	8.5± 0.56

The findings show that CFSs and antibiotics had synergistic interactions, which is in line with a study that showed that lactic acid bacteria can produce a variety of metabolic substances, including bacteriocins, peroxide, hydrogen, diacetyl, and lactic acid **(Albano *et al.*, 2007; Alrudainy & Jumaa, 2016)**. Some chemicals found in probiotic cell-free supernatants (CFS), such as antimicrobial peptides and bacteriocins, have the ability to inhibit the growth of other bacteria, as stated by **Houssni *et al.* (2023)**. According to **Do *et al.* (2024)**, CFS exhibit inhibitory effects on a variety of harmful bacteria, and all antibiotics show enhanced inhibitory efficacy when administered with CFS. This combined effect can improve therapeutic efficacy, as noted by **Zanetta *et al.* (2022)** and **Al-Shammari *et al.* (2024)**. For example, the antibiotic tetracycline (TI30) had the largest inhibition zone (9.5 ± 0.97mm) when paired with CFS, indicating that CFS significantly enhances the antibiotic's effectiveness. Chloramphenicol (C30) also showed a strong inhibition zone of 8.5 ± 0.56mm when combined with CFS, further suggesting its potent effect. According to **Wang *et al.* (2024)**, antibiotics work more effectively in the presence of free cellular CFS.

4- The analysis of the sensitivity test results of *Aeromonas* bacteria to antibiotics and their combination with CFS originated from *Lactobacillus* bacteria

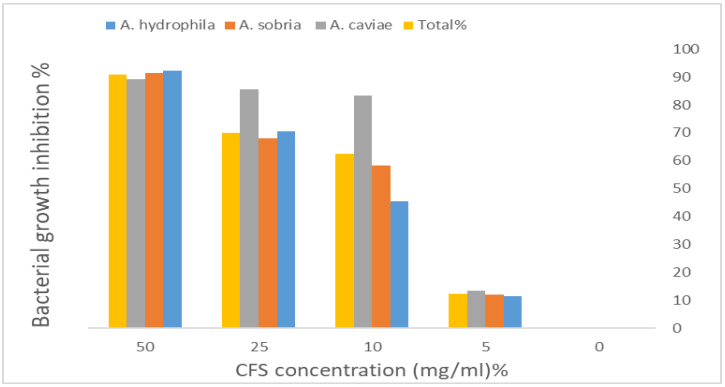

Chart 1. The detection of a significant difference in bacterial growth inhibition upon using CFS of *L. acidophilus* at all concentrations (5, 10, 25 and 50%) compared to the control (0) (*P*-value < 0.05)

The chart illustrates the impact of cell-free supernatant (CFS) concentration on the suppression of bacterial growth for the three strains: *A. hydrophila*, *A. sobria*, and *A. caviae*, at varying concentrations (50, 25, 10, and 5mg/ ml). Evaluation of the impact of the maximum concentration of 50mg/ ml results in the greatest bacterial growth inhibition, approaching 100% for most species, demonstrating the liquid's robust efficacy at this level. *A. hydrophila* exhibits a significant inhibition rate, signifying its heightened sensitivity to this treatment. At reduced concentrations, specifically 25mg/ ml, there is a notable decline in growth inhibition, which persists at even lower concentrations of 10 and 5mg/ ml. The total line (Total%) indicates the overall performance of various concentrations, demonstrating that the efficacy of CFS persists at lower concentrations, albeit to a diminished extent. These findings show that CFS can effectively inhibit the growth of specific bacterial types at suitable concentrations; however, the sensitivity of various species and the effects of low concentrations still require further investigation

The findings show that CFSs and antibiotics had synergistic interactions, which is in line with a study that showed that lactic acid bacteria can produce a variety of metabolic substances, including bacteriocins, diacetyl, hydrogen peroxide and lactic acid **(Albano *et al.*, 2007; Hussein & Jumma, 2024)**. Some chemicals found in probiotics cell free supernatants (CFS), such as antimicrobial peptides and bacteriocins, have the ability to prevent the grow of other bacterial, as stated by **Houssni *et al.* (2023)** and **Alshumary *et al*. (2024)**. According to **Do *et al.* (2024)**, CFS have inhibitory effects on a variety of harmful bacteria. All antibiotics show enhanced inhibitory efficacy when administered with CFS. An example of this is the concept that combined effects can improve therapeutic efficacy, as stated by **Zanetta *et al.* (2022)** and **Al-Juhaishi *et al.* (2024)**. The antibiotic tetracycline (TI30) exhibited the largest inhibition zone (9.5 ± 0.97mm) when paired with CFS, indicating that CFS significantly enhances the antibiotic's effectiveness. An inhibition zone of 8.5 ± 0.56mm was also observed with chloramphenicol (C30), suggesting strong

potency. According to **Wang *et al.* (2024)**, antibiotics are more effective in the presence of free cellular CFS.

CONCLUSION

According to the findings of this study, the interaction between antibiotics and free cell-free supernatant (CFS) from *Lactobacillus* fermentative secretions enhanced treatment efficacy, suggesting that CFS plays a role in amplifying the effects of antibiotics. The highest effective concentration of *L. acidophilus* for bacterial inhibition was 50mg/ ml, highlighting the need to explore its potential medicinal uses. These findings underscore the importance of incorporating probiotics into integrated therapy regimens to improve fish health and infection management.

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