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Original Article

Novel isolation of *Enterococcus* spp. from common carp, *Cyprinus carpio* in Iraq: Antibiotic resistance and susceptibility to *Syzygium aromaticum*

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Abstract: The aquaculture industry, particularly in high-density and intensive settings, has suffered significant economic setbacks due to various bacterial diseases. Consequently, it is imperative to investigate the presence of pathogenic species, accurately identify them, and evaluate their resistance to antibiotics or natural extracts to safeguard fish populations. This study aimed to examine 120 specimens of common carp, *Cyprinus carpio*, collected from two cage farms along the Al Hillah River in Babil Governorate, Iraq, during August, September, and October 2023. Swabs were collected from the skin, gills, and intestines for bacterial culture, followed by isolation and identification using the VITEK 2 system. Sensitivity testing was conducted on the isolated bacteria, and the susceptibility of plant cloves, *Syzygium aromaticum*, was assessed as a natural extract. The findings indicated the isolation of two Enterococcus species, namely *E. gallinarum* and *E. casseliflavus*, from the fish's gills, intestines, fins, and skin. Antibiotic susceptibility testing revealed that the bacteria were sensitive to the minimum inhibitory concentrations (MIC) of imipenem, ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole, while exhibiting resistance to cefoxitin and cefazolin. The susceptibility of the plant clove was evaluated using the disc diffusion method on blood agar, measuring the diameter of the inhibition zone to assess the effectiveness of alcohol-extracted clove at four concentrations: 10%, 15%, 20%, and 25%. The results showed that *S. aromaticum* extract effectively inhibited infections caused by *E. gallinarum* and *E. casseliflavus*, particularly at the highest concentration of 25%. The findings of this study provide compelling evidence for the existence of *E. gallinarum* and *E. casseliflavus* in common carp farming in the Hilla River, marking their first isolation in Iraq. Furthermore, the research assessed the susceptibility and resistance of these bacterial strains to various commonly used antibiotics. Additionally, the investigation included an evaluation of clove extract as a botanical agent, which demonstrated antimicrobial activity against intestinal bacteria across a range of concentrations.

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Introduction

Aquaculture is recognized as a highly promising sector for animal food production, with expectations for rapid growth in the forthcoming years. Over recent decades, fish production through aquaculture has consistently increased to meet the global demand for fish consumption. Fish farming has emerged as a lucrative aquaculture practice, effectively addressing the rising demand for fish. Nonetheless, various factors can influence fish production in aquaculture, including water quality, temperature, feed availability, and seasonal variations, rendering this farming approach variable and less predictable (Khalidah et al., 2020).

Focusing on a significant aquaculture species, the common carp (*Cyprinus carpio* L.) is relatively easy to cultivate and can thrive in environments with poor water quality, including polluted waters. It is also recognized for its favorable growth rate and palatability, making it a long-established choice for consumption. *Cyprinus carpio* is the second most cultivated freshwater fish globally, following Nile tilapia (Al-Jubouri et al., 2017). This species is remarkably adaptable to various aquatic environments

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and is often found in brackish or nutrient-rich waters (Ahmed et al., 2020).

Ziarati et al. (2022) reported that the aquaculture and fishing sectors face serious problems related to zoonotic diseases caused by fish and aquatic organisms. Fish should be free of infectious pathogens and have long-term food value, as bacterial infections are believed to be the main cause of high mortality rates and economic losses among fish and fish farms (Tsfaye et al., 2018). Many studies on bacterial diseases affecting *C. carpio* in the Al-Hilla River and fish in Babylon Province have been conducted (Al-Jubouri et al., 2017, 2020; Al-Haider et al., 2019; Al-Niaeem et al., 2020). Bacterial diseases have a negative economic impact on aquaculture and decrease fish production (Mohammed et al., 2023).

These topics have engaged public interest, highlighting the importance of fish diseases. On the other hand, disease progression is a complex process that cannot rely solely on the ability of bacteria to cause health problems; it also depends on the immune status of the fish, environmental factors, and the virulence of the disease. Consequently, changes in freshwater systems seem to play a critical role in developing any disease, including emerging diseases (Johnson and Paull, 2011).

The first record of *Enterococcus gallinarum* was made during a taxonomic survey of *Enterococcus* strains isolated from chickens (Barnes et al., 1978). It was identified as a distinct species called *S. gallinarum* by Bridge and Sneath (Bridge et al., 1982), who described this type as an intestinal *Streptococcus* of domestic birds. *Streptococcus gallinarum*, as well as *S. casseliflavus*, were transferred to the newly accepted genus *Enterococcus* in 1984 (Collins et al., 1984). *Enterococcus gallinarum* and *E. casseliflavus* have been linked to various diseases in both fish and humans, including gastrointestinal disease (Zhang, 2015), urinary tract infections (Chen, 2013), bloodstream infections, skin lesions (Liu et al., 2017), and meningitis, with some cases resulting in severe symptoms and death (Zhang, 2019, 2020).

Identifying water sources with suitable temperatures and quality is essential for successfully farming *C. carpio*. The Al-Hilla River basin has been identified as providing favorable conditions for cultivating this fish. Despite the abundant populations of *C. carpio* in this region, its microbiome remains largely unstudied. The bacteria in the fish's body may benefit the host, but they pose potential health risks to fish and humans. Consequently, this study investigates antimicrobial-resistant and susceptible strains of *E. gallinarum* and *E. casseliflavus* isolated from *C. carpio* cultivated in floating cages.

Materials and Methods

Sample collection and processing: Samples were obtained from two cage farms along the Al Hillah River in the Babil Governorate, Iraq, during August, September, and October 2023. The first farm, designated as Street 1 (44˚24'9.55"E, 32˚26'62.59"N), is positioned before the city center, while the second farm, referred to as Street 2 (44˚40'50.4"E, 32˚23'2.84"N), is located beyond the city center (Fig. 1). A total of 120 common carp were collected, with individual lengths ranging from 30 to 50 cm and weights varying between 400 and 1120 grams. The fish were transported alive to the Pathology Laboratory at the College of Veterinary Medicine, Al-Qasim Green University, within a few hours, where they were subsequently euthanized. Swabs were taken from the skin, gills, intestines, and any lesions observed on the skin and gills for bacterial culture. The total length of each specimen was recorded to the nearest 0.1 cm, and the total weight was measured to the nearest gram using a one-pan scale.

Isolation and identification of *Enterococcus gallinarum* and *E. casseliflavus*: The collected fish specimens were subjected to dissection, during which sterile techniques were employed to obtain bacterial samples from the skin, gills, fins, and intestines using a sterile loop. MacConkey agar medium was utilized to isolate the bacteria. The inoculated medium was incubated at 37°C for 24 hours.

Total Count of Bacteria: Sterile test tubes were prepared in quartets, with each tube containing nine milliliters of sterile distilled water. Subsequently, one

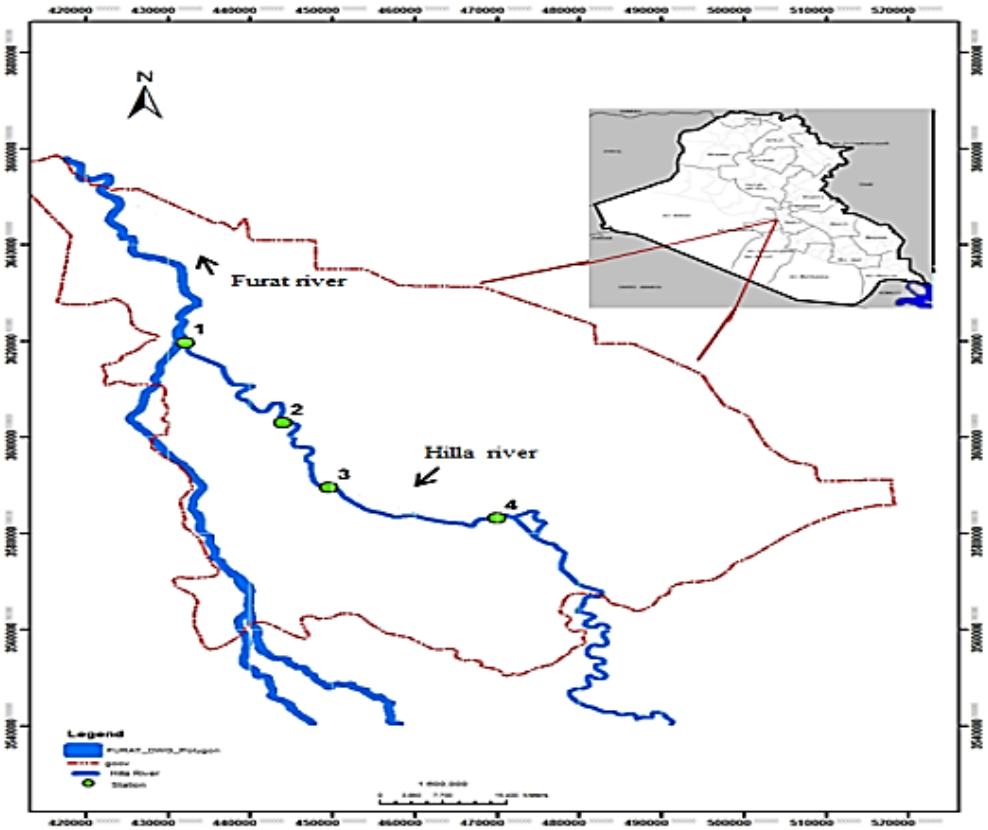

Figure 1. Map of field sampling sites in Al-Hilla River.

milliliter of river water was introduced into the first tube, which was then agitated thoroughly to dilute $1x10^{-1}$. From this first tube, one milliliter was transferred to the second tube, resulting in a dilution of $1x10^{-2}$. This process was continued, transferring one milliliter from the second tube to the third, and so forth, until the eighth tube was reached, which resulted in a dilution of $1x10^{-8}$.

A solid nutrient agar medium was prepared and sterilized using an autoclave. After cooling, the medium was dispensed into two Petri dishes, each containing one milliliter of the $1x10^{-6}$ and $1x10^{-8}$ dilutions, respectively, allowing the river water to interact with the nutrient medium. The Petri dishes were then incubated at 37°C for 48 hours. Following incubation, bacterial colonies were enumerated using a bacterial counting device. The total bacterial count was calculated based on the number of colonies recorded, considering the dilution factor, which ultimately provided the total number of bacteria present in one milliliter of river water (Andrews, 1992).

Diagnosis of bacteria using VITEK 2: The identification of Gram-negative and Gram-positive bacteria was conducted utilizing the VITEK 2 system at the Public Health Laboratory of the Babylon Health Department. This investigation employed three distinct cards for the diagnosis of various microorganisms:

GN: This card is designated to identify Gram-negative aerobic bacteria, including lactose-fermenting and non-fermenting types.

GP: This card is intended to diagnose spherical aerobic bacteria and non-spore-forming Gram-positive bacilli.

AST-GN82: This card is utilized to assess the susceptibility of Gram-negative bacteria to various antibacterial agents.

Antibiotic and plant clove susceptibility testing: Antibiotic and plant clove susceptibility testing are essential in determining the effectiveness of antibiotics and plant-derived compounds against bacterial infections. The results of these tests can help guide treatment decisions and identify potential

alternatives to traditional antibiotics. Table 1 presents the biochemical characteristics of the isolated *Enterococcus* species.

The assessment of antibiotic susceptibility, involving seven different antimicrobials, was performed utilizing the VITEK 2 system. The susceptibility testing for the plant species was carried out through the disc diffusion method on blood agar, which was incubated at 37°C for 24 hours. This procedure adhered to the standardized disc agar diffusion protocol established by the National Committee for Clinical Laboratory Standards for antimicrobial susceptibility testing (Finegold and Martin, 1982). Following the incubation period, the diameter of the inhibition zone was measured to evaluate the sensitivity of the plant clove (*Syzygium aromaticum*), which was extracted using alcohol at varying concentrations of 10, 15, 20, and 25%. The clove, *S. aromaticum*, used in this research was sourced from a local market in Al-Hilla, Iraq. It was classified by the National Center for Herbal Medicine and the Al-Razi Center for Medical Herbs. The clove buds were cleaned with a 5% sodium hypochlorite solution (NaOCl), rinsed three times with distilled water, and subsequently allowed to dry. The dried plant material was ground into a fine powder.

Statistical analysis: The results were analyzed using SPSS version 20, employing one-way ANOVA to determine group differences.

Results and Discussions

Bacterial fish diseases are a significant threat to the health and well-being of fish populations worldwide. These diseases are caused by various species of bacteria, such as *Aeromonas hydrophila*, *A. salmonicida*, and *Edwardsiela ictaluri*, which are examples of common carp pathogens (Austen and Austen, 2012; Aisah et al., 2019). Therefore, it is imperative to periodically investigate pathogenic bacteria, accurately identify them, and evaluate their resistance to antibiotics or natural extracts to safeguard fish populations.

Bacterial isolation and identification: *Enterococcus*

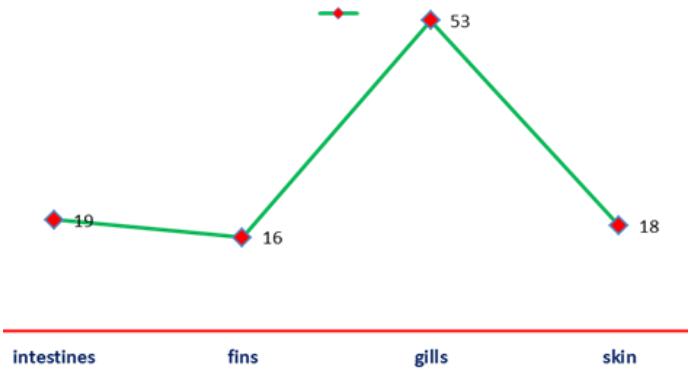

Figure 2. Percentage of isolated *Enterococcus gallinarum* from *Cyprinus carpio* in Al-Hilla River.

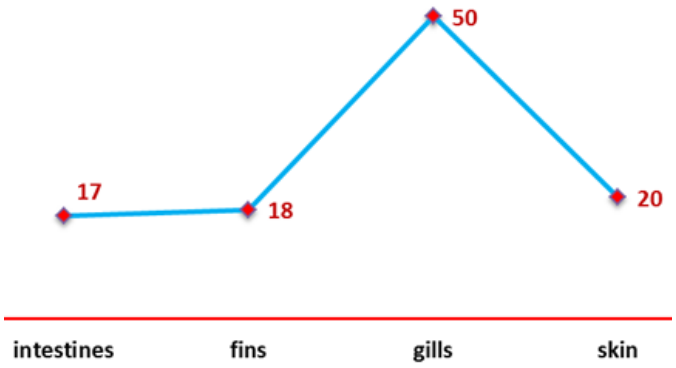

Figure 3. Percentage of isolated *Enterococcus casseliflavus* from *Cyprinus carpio* in Al-Hilla River.

gallinarum and *E. casseliflavus* were first identified in Iraq from the gills, skin, intestines, and fins of common carp across all sampling locations. Infected fish exhibited clinical symptoms characterized by hemorrhages on the body, particularly red spots above the gill cover and on the lower abdomen. Figures 2 and 3 illustrate the varying percentages of bacterial presence in the different anatomical regions of common carp, with the gills showing the highest prevalence at 53%, while the fins exhibited the lowest at 16%. This disparity suggests that the gills, being in direct contact with water, are more susceptible to bacterial colonization.

The Al-Hilla River, along with other freshwater sources, is notably contaminated with sewage, which serves as a significant source of bacterial pathogens, particularly in proximity to populated areas where untreated sewage is discharged. Consequently, freshwater environments are often reservoirs for

various pathogenic bacteria (Amal et al., 2015). The findings of this study align with those of Al-Jubouri et al. (2023) and Alwan et al. (2023), who reported similar results in tilapia from the Diwaniya River. They posited that the quality of water, along with anthropogenic activities, influenced the vulnerability of fish to infections and emphasized the importance of total bacterial counts as indicators of microbial quality and water health, as well as the potential presence of diseases. Variations in bacterial types among different farms may be attributed to the pollution of aquatic ecosystems resulting from the excessive discharge of agricultural runoff and sewage (Koshy and Nayar, 1999). According to Monticelli et al. (2018).

The decline in water quality, particularly in proximity to breeding habitats, is characterized by factors such as ammonia levels, temperature fluctuations, and dissolved oxygen concentrations, which adversely impact aquatic ecosystems. This degradation can increase fish's susceptibility to bacterial infections and diseases due to compromised immune responses. Furthermore, Monticelli et al. (2018b) indicated that *E. gallinarum* and *E. casseliflavus* exhibit significant resistance to vancomycin, a critical concern as these *Enterococci* are often implicated in healthcare-associated infections, notably bloodstream, and urinary tract infections, as well as surgical site infections. The prevalence of these pathogenic bacteria is rising globally, particularly among patients with hepatobiliary disorders or concurrent hematological conditions. Their distinct resistance to vancomycin, attributed to the chromosomally encoded VanC factor and intrinsic resistance to various antibiotics beyond glycopeptides, severely restricts available treatment options.

Antibiotic susceptibility test: The antibiotic susceptibility test serves as a crucial instrument in diagnosing and managing bacterial infections, as it aids in identifying antibiotic-resistant bacteria, thereby contributing to the prevention of the dissemination of antibiotic-resistant infections. Table 1 illustrates that the most effective antibiotics against *E. gallinarum* and *E. casseliflavus* were ciprofloxacin (≤0.11 mcg/ml), imipenem (≤0.25 μg/ml), and levofloxacin (≤0.22 μg/ml). In laboratory tests, levofloxacin demonstrated efficacy at ≤0.25 μg/ml concentrations for *E. gallinarum* and *E. casseliflavus*. These strains resisted cefoxitin at ≤66 μg/ml and ≤70 μg/ml, respectively. The findings of this study align with previous research conducted by Al-Hider (2019), Hade (2022), Al-Jabouri (2023), and Alwan (2023), which also noted the antibiotic-producing capabilities of *E. gallinarum*, as reported by Jennes et al. (2000). Conversely, Kumada et al. (2009) and Suzuki et al. (2011) identified *E. gallinarum* strain 012, isolated from the duodenum of an ostrich, as a producer of enterocin 012, which exhibits activity against various pathogens, including *E. faecalis*, *Lactobacillus acidophilus*, *Listeria innocua*, *Propionibacterium acidipropionici*, *P.* spp., *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Additionally, certain studies have reported inhibitory effects from Enterobacter species, with *E. faecium* noted for its ability to impede biofilm formation by cariogenic *Streptococci*. The prolonged use of antibiotics has been linked to the emergence of bacterial resistance, the presence of drug residues in fish, and concerns regarding environmental safety. Langaoen et al. (2018) further emphasized that antibiotics remain significant commercially exploited secondary metabolites produced by bacteria and fungi, commonly employed to prevent or manage disease outbreaks in aquaculture. However, the excessive application of antibiotics in fish farming poses a risk of developing antibiotic-resistant pathogens that can affect both farmed animals and humans.

Susceptibility of plant cloves (*Syzygium aromaticum*): *Syzygium aromaticum*, widely recognized as cloves, has been employed in various capacities, such as a spice, medicinal agent, and food preservative, owing to its extensively documented antimicrobial properties. The current investigation demonstrated that extracts from clove plants can inhibit infections caused by *E. gallinarum* and *E. casseliflavus*. Specifically, the extracts significantly suppressed the growth of *E. gallinarum* and *E. casseliflavus* (P<0.01) at the highest

Table 1. Antibiotic susceptibility *Enterococcus gallinarum* and *E. casseliflavus*. *MIC: Minimum Inhibitory Concentration (µg/ ml), S: Sensitive, R: Resistant.

*Enterococcus **gallinarum***					
Antimicrobial	**MIC***	**Interpretation**	**Antimicrobial**	**MIC***	**Interpretation**
Ampicillin	5	S	Imipenem	≤0.21	S
Piperacillin/ Tazobactam	≤5	S	Amekacin	≤3	S
Cefazoline	>=42	R	Gentamicin	≤4	S
Cefoxetin	≤66	S	Nitrofurantoin	66	R
Ceftazidime	≤16	S	Ciprofloxacin	≤0.11	S
Cftriaxone	≤7	S	Levofloxacin	≤0.25	S
Cefepime	≤7	S	Tigecycline	4	R
Ertapenem	≤0.62	S	Trimethoprim/Sulfamethoxazole	≤25	S
*Enterococcus **casseliflavus***					
Antimicrobials	**MIC***	**Interpretation**	**Antimicrobial**	**MIC***	**Interpretation**
Ampicillin	2	R	Imipenem	≤0. 25	R
Piperacillin/ Tazobactam	5	S	Amekacin	≤3	S
Cefazoline	>= 33	R	Gentamicin	≤1	S
Cefoxetin	≤70	S	Nitrofurantoin	123	R
Ceftazidime	≤0	S	Ciprofloxacin	≤0.22	S
Ceftriaxone	≤2	S	Levofloxacin	0.25	S
Cefepime	≤1	S	Tigecycline	2	R
Ertapenem	≤1	S	Trimethoprim/Sulfamethoxazole	≤21	S

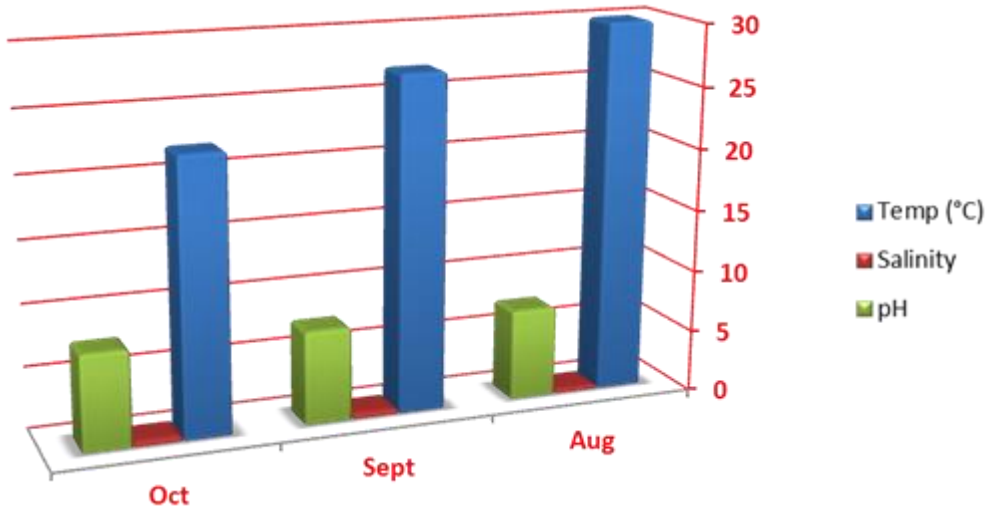

Figure 4. The water parameters in station 1 of the study area in Al-Hilla River.

concentration of 25%. This finding aligns closely with previous studies indicating that clove essential oil exhibits antimicrobial effects against a range of intestinal bacteria, including *E. coli* and *S. aureus* (Kumar., 2017); *B. subtilis* and *P. aeruginosa* (Singh, 2018); and *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Zhang, 2019). The oil's minimum inhibitory concentrations (MICs) varied from 0.5 to 2.5 mg/mL, suggesting its efficacy at concentrations typically utilized in food and pharmaceutical contexts. Further investigation is warranted to comprehensively elucidate the mechanisms of action and potential interactions with other compounds. This study indicates that readily accessible and cost-effective plant extracts may be effective natural agents for preventing *E. gallinarum* and *E. casseliflavus* infections. Additional research is essential to evaluate these plant extracts' efficacy *in vivo* and establish appropriate practices for their application in aquaculture.

Characteristics of water: Figures 4 and 5 illustrate the monthly variations in water temperature, which fluctuated from a low of 22.1°C in October to a high of 30°C in August. Salinity levels ranged from a

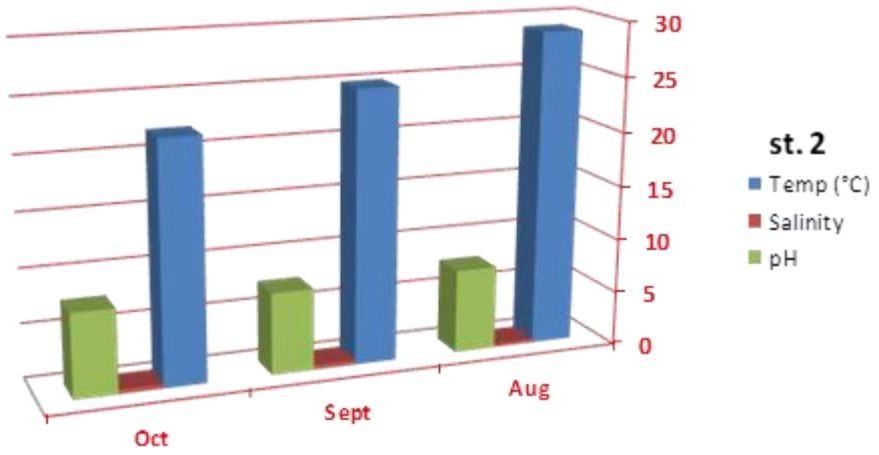

Figure 4. The water parameters in station 2 of the study area in Al-Hilla River.

minimum of 0.3 in August to a maximum of 0.4 in October, while pH values varied between 7.3 in August and 7.7 in October across the two study locations. The results revealed no significant differences between the two sites (t = 0.005, $P>0.05$). These findings align with previous research conducted by Hade (2022), Al-Jabouri (2023), Alwan (2023), and Al-Jabouri (2024) on the Diwaniya River. The present study identified several environmental factors influencing bacterial proliferation, including temperature, salinity, and pH, as noted by Ismail et al. (2016). The bacterium *Enterococcus* spp. thrives in environments with salinity levels up to 6.5%, pH values ranging from 4.0 to 9.6, and temperatures between 10 and 45°C (Ogier and Serror, 2008; Hammad et al., 2014). Its optimal growth temperature is 37°C, with a pH range of 4.4 to 10.6 (Fisher and Phillips, 2009; Ogier and Serror, 2008).

Conclusion

The study findings indicated that *E. gallinarum* and *E. casseliflavus* represent the first recorded *C. carpio* cultivated within floating cages at the Al-Hilla River in Babylon Province, Iraq. The investigation confirmed the existence of antibiotic-resistant *Enterococcus* species in common carp raised in these cages, underscoring the necessity for careful antibiotic application in aquaculture. The excessive use of antibiotics contributes to the development of resistant bacterial strains. Furthermore, the water of the Al-Hilla River is contaminated, with bacteria capable of infecting humans found in the tissues of fish, a situation exacerbated by the activities of the surrounding population. The extract of clove (*S. aromaticum*) demonstrated antimicrobial properties against *E. gallinarum* and *E. casseliflavus* at various concentrations. Additional studies are essential to evaluate plant extracts' efficacy in vivo and establish appropriate practices for aquaculture.

Ethical Considerations Approval—Ethical Clearance: On April 12, 2023, the local ethical committee at the College of Veterinary Medicine, University of Al-Qasim Green University, approved the project under code number 681.

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