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Synergistic effect of *Lactobacillus* supernatant and gentamicin against biofilms of uropathogenic *Escherichia coli*

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ABSTRACT

Background: Infection control poses a challenge due to the emergence of antibiotic-resistant bacteria and biofilm formation. Probiotics have been shown to positively affect the host, with certain strains of *Lactobacilli* able to prevent or alleviate inflammation and infections. **Aim:** This work aimed to investigate the synergistic effects of gentamicin and the cell-free supernatant (CFS) of the probiotic *Lactobacillus plantarum* on the biofilm formation of *Escherichia coli* isolated from urinary tract infections (UTIs). **Methods:** We analyzed 422 urine samples from type 2 diabetic patients with UTIs, and identified bacterial isolates using phenotypic methods and the automated system VITEK-2. The antibiotic susceptibility test was conducted using the disc diffusion method, while the inhibitory effects of the CFS were assessed using an agar-well diffusion assay. *E. coli* biofilm formation was detected using a microtiter plate (MTP) method, and the minimum inhibitory concentration (MIC) was estimated via the microdilution method. Finally, the interaction between CFS and gentamicin against *E. coli* was tested using the checkerboard method and fractional inhibitory concentration (FIC). **Results:** The *E. coli* strains isolated from UTIs were found to be multidrug-resistant. The cell-free supernatant (CFS) exhibited a significant bactericidal effect on *E. coli* growth, with MICs of 12.5 U/mL for CFS and 128 µg/mL for gentamicin. The FICI for CFS and gentamicin was 0.37, suggesting a synergistic effect. **Conclusion:** Combining probiotics with antimicrobial agents shows promise for developing new bactericidal formulations for medical and pharmaceutical applications.

Introduction

1. Urinary tract infections (UTIs) are most common in diabetic patients and primarily manifest as cystitis, but infections can also progress to the kidneys, resulting in pyelonephritis [1]. Glycosuria is responsible for the growth of various microbial strains [2]. The primary cause of UTI development is *E. coli* [3]. *Escherichia coli* is the leading cause of

community-acquired urinary tract infections (UTIs) (80%) and a significant portion of nosocomial UTIs (50%), leading to high morbidity and medical costs worldwide [4]. The chronic nature of some UTIs is attributed to the ability of *E. coli* to form biofilms [5].

2. Biofilms are microorganisms that adhere to a surface within a polymeric matrix. These exopolysaccharides are typically produced by

bacteria and their hosts, forming a complex community over time. The water channels that form between exopolysaccharide-enclosed bacteria create stalk- or mushroom-like structures that may contain one or more microbial species [6]. Bacterial adhesion to the urothelium plays a crucial role in UTI development because it encourages bacteria to remain in the urinary tract rather than being flushed out by urine flow [7]. When the biofilm forms and creates a physical barrier to drug penetration, it leads to an increase in uropathogenic antimicrobial resistance [8].

3. Antibiotic resistance has become a significant global concern, resulting in increased mortality rates, morbidity, and healthcare costs [9]. The search for alternative antimicrobial treatments to combat multidrug-resistant microorganisms has become imperative. One such solution gaining traction is the exploration of antibacterial compounds derived from natural sources. To enhance the efficacy of antibiotics against pathogenic strains, experts recommend using probiotics and/or their metabolites in conjunction with antibiotics as antimicrobial agents [10, 11].

4. Probiotics are live microorganisms known to provide health benefits when consumed in appropriate amounts [12]. *Lactobacillus* spp. are a common type of probiotic bacteria that are nonpathogenic and offer essential medicinal benefits. It produces chemicals such as lactic acid, hydrogen peroxide, bacteriocins, and bacteriocin-like compounds. These chemicals also produce various metabolic byproducts, such as biosurfactants, and may compete for nutrients and binding sites [13].

5. Urinary tract infections and biofilm formation caused by *E. coli* are common in diabetic patients. However, there are limited studies on the combined use of antibiotics and probiotics against this pathogen. Consequently, a study was conducted to investigate the potential antimicrobial effect of *Lactobacillus* supernatant on *E. coli* bacterial growth and biofilm formation. This study also aimed to evaluate the synergistic interactions between the supernatant and gentamicin.

Materials and Methods

Study design and inclusion criteria

This study was conducted at the Ramadi Teaching Hospital in Anbar, Iraq, from December 2022 to July 2023. The study included patients who had been clinically diagnosed with one or more of the following symptoms: dysuria, frequency, urgency, and suprapubic syndrome. Patients also experienced discomfort or pain in the flank. The study involved 422 individuals over the age of 30 with type 2 diabetes.

The Ethical Approval Committee of the University of Anbar in Ramadi, Iraq, approved all research methods used in the study (Approval No. 65, June 21, 2022). All patients participating in the study provided written informed consent.

Isolation and identification of *E. coli*

The urine sample was collected in the morning and placed in a sterile container. The samples were then inoculated onto two agar mediums: MacConkey's agar and Eosin methylene blue media. They were then incubated at 37 °C overnight. Positive culture plates were used to identify *E. coli* using standard microbiology techniques, including colony morphology, microscopic examination (Gram stain), and biochemical tests [14]. The VITEK2 Compact System (BioMerieux, France) was used to confirm *E. coli* identification.

Antibiotic susceptibility

In accordance with the recommendations of the Clinical and Laboratory Standards Institute [15], the sensitivity of the samples was assessed using the Mueller-Hinton agar and disk diffusion method. Eleven antibiotic disks (supplied by Mast Group, UK) were used: Piperacillin (100 µg), Gentamicin (10 µg), Imipenem (10 µg), Amikacin (30 µg), Ciprofloxacin (30 µg), Nitrofurantoin (100 µg), Tetracycline (30 µg), Ceftazidime (30 µg), Ceftriaxone (10 µg), Trimethoprim-sulfamethoxazole (1.25/23.75 µg), and Chloramphenicol (30 µg). The reference strain of *E. coli* ATCC 25922 was used in the current study as a quality control measure. A bacterial suspension (0.5 McFarland) was cultivated for this purpose on Mueller-Hinton agar plates, after which antibiotic discs were added to the medium and incubated at 37°C for 18 to 24 hours.

Cultivation of probiotic bacteria

Lactobacillus plantarum was obtained from the Department of Biology at the College of

Science, University of Baghdad, Iraq. The sample enriched with *Lactobacillus* was placed on De Man–Rogosa–Sharpe (MRS) agar Petri plates (Himedia, India), which were then incubated anaerobically at 37 °C for 48 hours. The bacterial growth was monitored during the incubation period [16].

Preparation of *Lactobacillus* supernatant

200 ml of sterile MRS medium was inoculated with 0.5 McFarland of *L. plantarum* (1.5×10^8 CFU/mL) and incubated anaerobically for 48 hours at 37°C. The culture broth was then centrifuged at 5000 rpm for 15 minutes, and the supernatant was collected. The supernatant was filtered using a Millipore filter (0.22 nm). After filtration, a loop full of the filtrate was streaked onto an MRS agar plate and incubated for 48 hours at 37 °C under anaerobic conditions to confirm the sterilization of the filtrate. The filtrate was stored at 4 °C until further use [17].

Antibacterial activity of CFS

The agar-well diffusion method was used to determine the antibacterial activity of *Lactobacillus* supernatant according to the method described by Kiousi, *et al* [18]. Initially, a 100-μl sample of 24-hour bacterial culture of *E. coli* in BHI broth was spread on the surface of Muller-Hinton agar. Then, two wells with a diameter of 6 mm were made using a sterile cork borer. One well was filled with crude *Lactobacillus* supernatant, while the other was filled with sterile distilled water as a negative control. The plates were then incubated at 37 °C for 24 hours before measuring the diameter (in mm) of the zone of inhibition.

Synergistic effects of CFS and gentamicin

The MIC of the cell-free supernatant of *L. plantarum* and gentamicin was tested by the Resazurin Microtiter Plate Assay (REMA) according to Obaid, *et al* [19]. The synergistic effect of the combination of *L. plantarum* cell-free supernatant and gentamicin against *E. coli* was investigated using a checkerboard microdilution technique described by Abdul-Jabbar, *et al* [20]. Synergy was defined as $FICI \leq 0.5$, partial synergy as $0.5 < FICI < 1$, additive as $FICI = 1$, no effect as $FICI < 4$, and antagonism as $FICI > 4$. The FICI values were determined by using the following formula [21]:

$$FICI = \frac{\frac{MIC \text{ of CFS alone}}{MIC \text{ of CFS in combination}}}{\frac{MIC \text{ of gentamicin}}{MIC \text{ of gentamicin in combination}}} \quad (1)$$

Biofilm biomass assay

The microtiter plate (MtP) method was employed to determine if *E. coli* was capable of forming biofilms using a microplate reader and 96-well sterile flat-bottomed polystyrene microtiter plates (Figure 1). The biofilm formation of *E. coli* isolates was evaluated by the modified crystal violet method outlined by Saleh and Hassan [22]. CFS of lactobacilli and gentamicin at concentrations (0.5 MIC, 1 MIC, 2 MIC, and 4 MIC) were used to evaluate their anti-biofilm activity. After the incubation period, the culture medium was gently discarded from each well, and the plate was washed 2–4 times with sterile distilled water to wash away nonadherent bacterial cells. The plate was allowed to air dry at 55 °C for 40 minutes. A volume of 200 μl of a 1% crystal violet solution was introduced into the wells and allowed to incubate at room temperature for a duration of 15 minutes. The wells were subsequently rinsed three times with distilled water to eliminate excess stains. 225 μl of methanol was added to the wells to remove stains. 200 μl of destaining solution was then transferred from each well to another plate. The percentage inhibition of biomass growth for each concentration of the test chemical was calculated using the average absorbance at 595 nm and the following equation:

$$\% \text{ inhibition} = 100 - \left(\frac{OD \text{ sample}}{OD \text{ control}} \times 100 \right)$$

Biofilm metabolic activity assay

The metabolic activity of the biofilms developed by *E. coli* was assessed using a thiazolyl blue tetrazolium bromide reduction (MTT) assay (Figure 2), according to Al-Obaidy and Abdulkareem [23]. The MTT salt was dissolved in phosphate-buffered saline (PBS) to achieve a final concentration of 5 mg/mL. Following the cell attachment assay for 24 hours and the preformed biofilm assay at various time intervals (1 hour, 3 hours, 6 hours, 12 hours, and 24 hours), the culture media was carefully removed and the plates were left to air dry. 200 μl of PBS and 10 μl of MTT solution (5 mg/ml) were carefully pipetted into each well and incubated for 3 hours at 37 °C in a controlled and sterile environment. After the enzymatic hydrolysis of MTT by the dehydrogenase enzyme found in living cells, the insoluble purple

formazan was successfully dissolved in dimethyl sulfoxide (DMSO). Using a microplate reader, the absorbance at 570 nm was measured.

Statistical analysis

The data was analyzed using SPSS version 28. An analysis of variance (ANOVA) was conducted. The group means were compared using the least significant difference at a 5% level of significance ($P \leq 0.05$) [24].

Results

Bacterial Isolates

A total of 422 urine samples were collected from individuals with type 2 diabetes mellitus. Out of these samples, 168 were found to be positive based on culturing and biochemical analysis, representing 39.81% of the total samples. The remaining 254 samples (60.19%) tested negative for culture. Among the positive samples, 125 (74.41%) were from women and 43 (25.59%) were from men. The 168 isolated bacterial cultures were classified into 107 (63.69%) Gram-negative bacteria and 61 (36.31%) Gram-positive bacteria. Out of the 168 diabetic patients with UTIs, 44 had *E. coli*.

Antibiotics Susceptibility of *E. coli*.

The effectiveness of eleven antibiotics from various groups was assessed against 44 strains of *E. coli* isolated from patients with urinary tract infections (UTIs). The study utilized the disk diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines. The results indicated that the isolates exhibited varying levels of antibiotic resistance, as outlined in Table 1. The uropathogenic isolates showed significant resistance to various types of antibiotics. According to the definition of multidrug resistance (MDR), which requires resistance to at least three antimicrobial drugs, 97.72% of the isolates exhibited MDR.

Biofilm formation

In the microtiter plate assay, it was observed that 6 out of 44 *E. coli* isolates (13.63%) had weak biofilm formation capacity, 25 isolates (56.81%) had moderate capacity, and 13 isolates (29.54%) had strong capacity. Out of the 44 isolates, three multidrug-resistant (MDR) *E. coli* isolates were chosen to test the *Lactobacillus* cell-free supernatant's antibacterial and antibiofilm potentials. These isolates exhibited resistance to six or more antibiotics from various antibiotic groups and showed strong biofilm-forming abilities.

Antibacterial activity of *Lactobacillus* supernatant

To assess the effectiveness of *Lactobacillus* supernatant in combating bacterial activity, a well diffusion assay was performed. After 24 hours of incubation with crude *Lactobacillus* supernatant, the inhibition zones were measured. The results showed that the crude supernatant was able to inhibit *E. coli* growth by a range of (16 ± 0.34) mm, indicating potent antibacterial activity.

Synergistic effects

The study found that combining *Lactobacillus* cell-free supernatant (CFS) with gentamicin has a synergistic effect against *E. coli*. The study used a checkerboard assay to determine the effect of the combination, and the results showed a decrease in the minimum inhibitory concentration (MIC) for both substances when used together. The MIC for the CFS alone was 12.5 $\mu\text{L/ml}$, but when combined with the antibiotic, the concentration decreased to 3.12 $\mu\text{L/ml}$. Similarly, the MIC for gentamicin alone was 128 $\mu\text{g/mL}$, but it decreased to 16 $\mu\text{g/mL}$ when combined with CFS. The combination of antibiotics and CFS of the *E. coli* isolate resulted in a synergistic effect, as indicated by the FICI values. This means that using a combination of antibiotics and CFS allows for a lower dose of the antibiotic to be used compared to using the antibiotic alone.

Antibiofilm activity of CFS

In order to evaluate the effectiveness of cell-free supernatant (CFS) and gentamicin in preventing biofilm formation, their effects were tested at different concentrations (0.5 MIC, 1 MIC, 2 MIC, and 4 MIC) on initial cell adhesion development. On two separate plates, a modified crystal violet assay was used to measure the percentage inhibition of biomass adhesion. The results showed that both CFS solution and gentamicin had an inhibitory effect of more than 90% at concentrations of 4MIC and 2MIC on biomass adhesion to both plates. At 0.5 MIC of CFS, the initial cell adherence rate was significantly decreased by 68.3% for clinical *E. coli* ($P < 0.01$), as shown in Figure 3.

The study examined the effectiveness of CFS and gentamicin in treating *E. coli* biofilms by determining their two-fold minimum inhibitory concentrations (MICs). Pre-formed biofilms were tested at different time intervals (1, 3, 6, 12, and 24 hours) to check their inhibition rates. The crystal

violet assay results revealed that after 1 hour of exposure to *L. plantarum* CFS and gentamicin, the inhibition rates were only 18.94% and 19.85%, respectively, for *E. coli* at 2MIC levels. However, after 3 hours of exposure, the inhibition rates significantly increased to 88.69% and 90.8% for *L. plantarum* CFS and gentamicin, respectively. When the tested materials were combined, the inhibition of biofilm formation showed a significant increase compared to their separate exposure under the same conditions, as indicated in Figure 4. According to the study, mature biofilms initially displayed resistance to antibiotics and *L. plantarum* CFS, which reduced inhibitory effects on biofilm development during the first hour of exposure to the test materials (either separately or combined). However, after three hours of exposure, the percentage of inhibition of *E. coli* biofilm formation significantly increased. The investigation indicated that higher concentrations of antibiotics or natural substances were required to inhibit the growth of the *E. coli* biofilm in comparison to the planktonic phase. Prolonged exposure to antibiotics or *L. plantarum* CFS also played a significant role in the anti-biofilm activity.

Anti-metabolic activity of CFS

MTT test results revealed that gentamicin exhibits the highest antiadhesion activity at 4MIC,

2MIC, and 1MIC, with biofilm inhibition rates of 97.9, 92.2, and 87.8%, respectively, with a significant decrease in activity observed at 0.5 MIC where the biofilm inhibition rate was 68.4%. Moreover, the *Lactobacillus* supernatant significantly inhibited cell attachment, with biofilm inhibition rates ranging from 85.2 - 96.5%. However, a decrease in this inhibition was recorded at a concentration of 0.5 MIC to reach 61.8%, as shown in Figure 5. Based on these results, 2MIC was adopted as the lowest concentration which led to a significant rate of inhibition in the subsequent experiment.

In an experiment on preformed biofilms, the effects of Gentamicin and *Lactobacillus* supernatant on *E. coli* biofilm formation were observed on 2 MIC. It was found that both Gentamicin and *Lactobacillus* supernatant hindered the activity of *E. coli* biofilm formation when used individually. The repression of metabolic activity increased over time, with the maximum impact observed at 24 hours (Figure 6). When the test materials were combined, an even greater repression of metabolic activity was observed compared to when they were used separately. Additionally, the combined state demonstrated an inhibitory effect, with the most significant impact at 24 hours as shown in Figure

Table 1. Percentages of antibiotics susceptibility testing for *E. coli* isolates.

Antibiotics	Concentration	Sensitive	Intermediate	Resistant
Amikacin	30 µg	42 (95.46%)	0 (0 %)	2 (4.54%)
Ceftazidime	30 µg	3 (6.81%)	2 (4.55%)	39 (88.64%)
Ceftriaxone	10 µg	6 (13.63%)	2 (4.55%)	36 (81.81%)
Chloramphenicol	30 µg	39 (88.64%)	3 (6.81%)	2 (4.54%)
Ciprofloxacin	5 µg	14 (31.81%)	1 (2.27%)	29 (65.91%)
Gentamicin	10 µg	12 (27.28%)	2 (4.54%)	30 (68.18%)
Imipenem	10 µg	42 (95.46%)	0 (0%)	2 (4.54%)
Nitrofurantoin	100 µg	35 (79.54%)	0 (0%)	9 (20.45%)
Piperacillin	100 µg	5 (11.37%)	3 (6.81%)	36 (81.82%)
Tetracycline	30 µg	24 (54.54%)	2 (4.55%)	18 (40.90%)
Trimethoprim-sulfamethoxazol	1.25/23.75 µg	6 (13.63%)	0 (0%)	38 (86.37%)

Table 2. Checkerboard assay result of CFS and gentamicin against *E. coli* isolate

Test materials	Minimum inhibitory concentration (MIC)				FICI value	Type of interaction
	CFS (alone)	CFS (combined)	gentamicin (alone)	gentamicin (combined)		
CFS + Gentamicin	12.5 µL/ml	3.12 µL/ml	128 µg/ml	16 µg/ml	0.37	Synergistic

Figure 1. Biofilm biomass assay of lactobacillus CFS against uropathogenic *E. coli* by the microtiter plate (MtP) method.

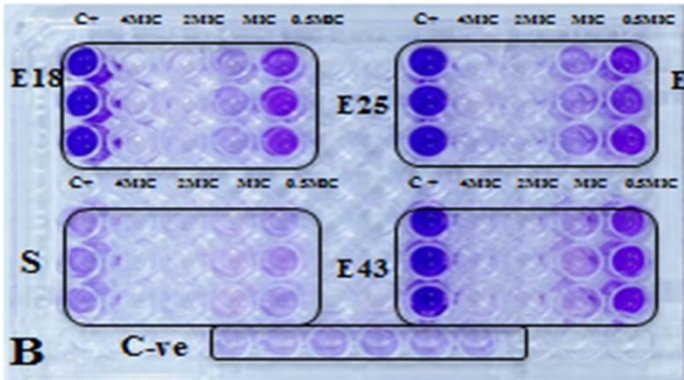


Figure 2. Biofilm metabolic activity assay of lactobacillus CFS against uropathogenic *E. coli* using a thiazolyl blue tetrazolium bromide reduction (MTT) method.

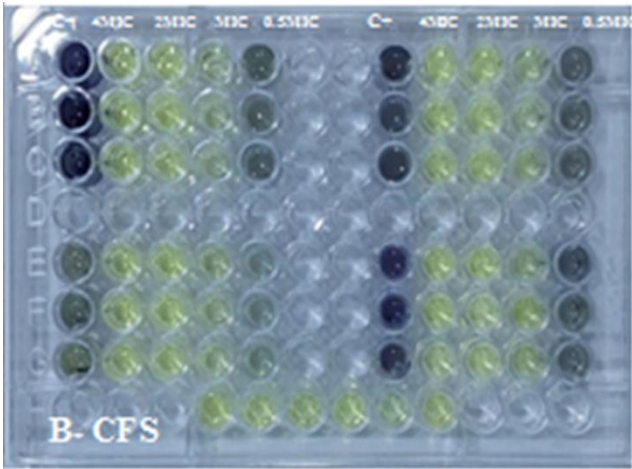


Figure 3. Inhibition percentage of *E. coli* biofilm formation at various concentrations by CFS and Gentamicin.

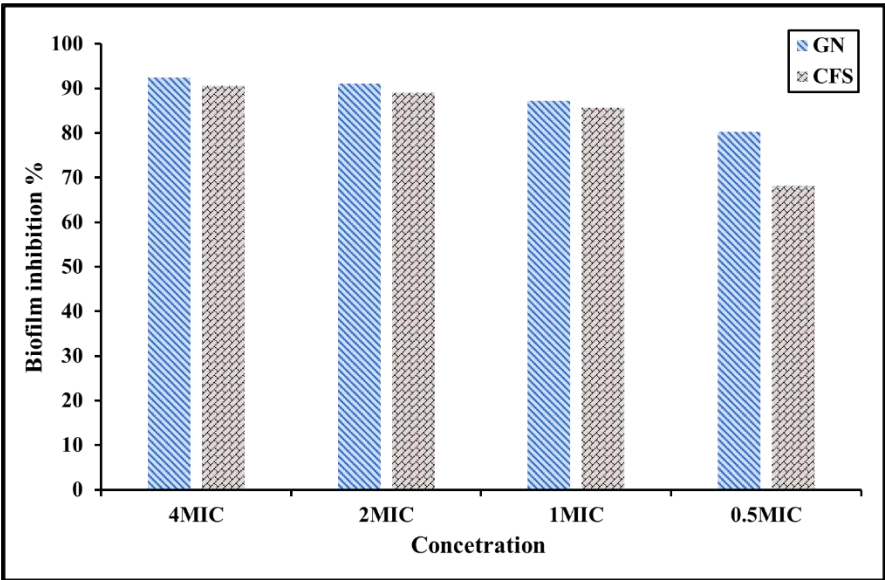
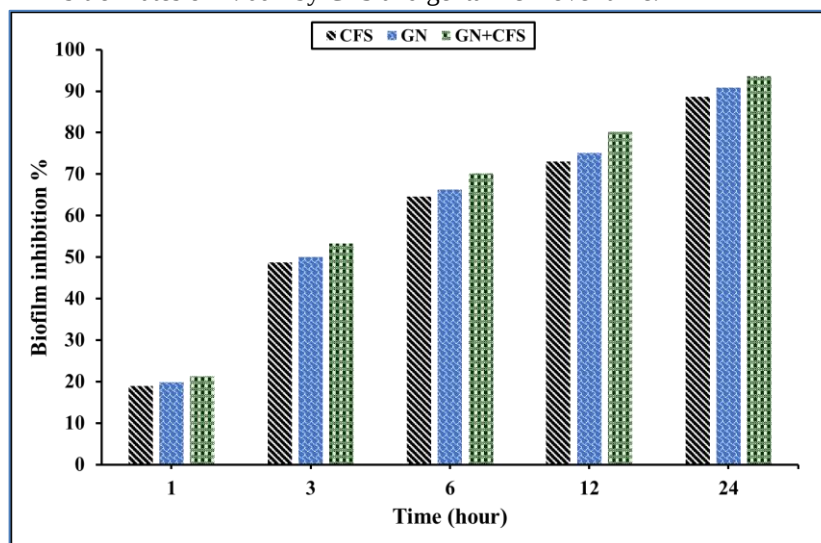
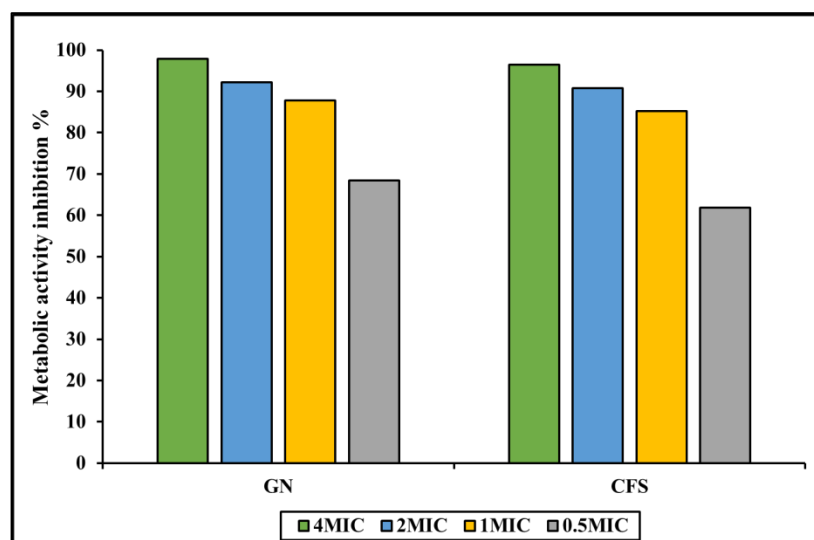
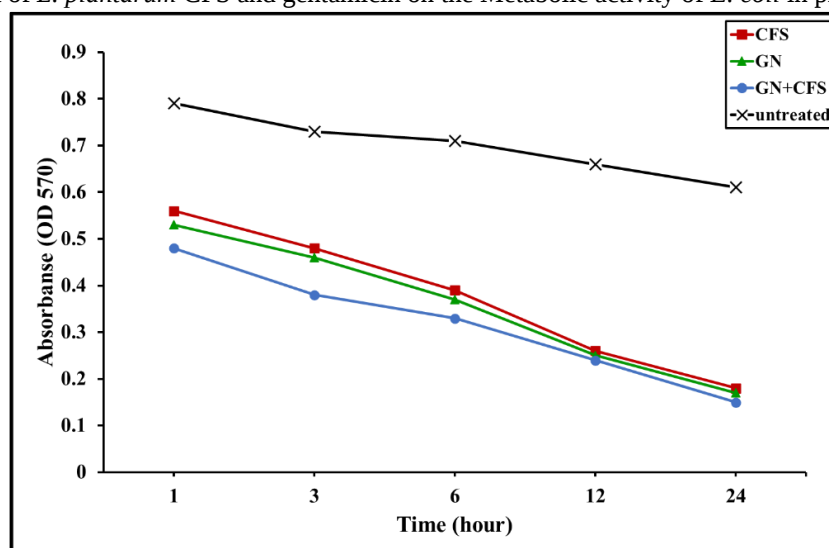


Figure 4. Biofilm inhibition rates of *E. coli* by CFS and gentamicin over time.**Figure 5.** Effect of Lactobacillus CFS and Gentamicin on the metabolic activity of *E. coli* at different concentration**Figure 6.** Effect of *L. plantarum* CFS and gentamicin on the Metabolic activity of *E. coli* in preformed biofilms.

Discussion

Prevalences of MDR isolates have been noted in this study of 97.72%. It's possible that the indiscriminate use of numerous courses of antibiotic therapy for asymptomatic or mildly symptomatic UTIs is to blame for the evolution of multidrug resistant bacterial isolates [25]. The results of this study reveal a relatively higher prevalence of resistance in uropathogenic *E. coli* compared to other previous local studies. A previous study by Abed and Mutter [26] reported a resistance prevalence of 87.8% in UPEC *E. coli*, which is lower than our current study, confirming that *E. coli* resistance increases over time and poses a real threat to human health. Patients with chronic diseases such as diabetics are most potential to harbor multidrug-resistant bacteria during UTI, including fluoroquinolone-resistant uropathogens and extended-spectrum β -lactamase Enterobacteriaceae [27].

In the present study, synergistic interactions were observed between the CFS of *L. plantarum* and the antibiotic gentamicin, as the MIC value of the cell supernatant reached 12.5 μ L/ml and decreased to 3.12 μ L/ml when combined with gentamicin due to the synergistic effect. The checkerboard test confirmed this synergistic effect, which showed an FIC index of less than 0.5, indicating a synergistic interaction between CFS and the antibiotic. The results of our study are superior to those of Aminnezhad *et al* [28], who found that the MIC value of the cell supernatant of *L. casei* and *L. rhamnosus* reached 62.5 μ L/mL and 125 μ L/mL, respectively. This may be due to the difference in the types of probiotic bacteria used in each study. The antibacterial mechanisms of CFS and gentamicin may have worked together to produce more reactive oxygen species (ROS), leading to a higher bactericidal effect of the combination to support our results.

According to the synergy effect, combining antibiotics with Lactobacillus' cell-free supernatant produces significantly better results than using either component separately. The need for alternative strategies to treat infections caused by drug-resistant bacteria has arisen due to the increase in bacterial resistance to antibiotics and the lack of new treatments [28]. Combinations that restore the effectiveness of traditional antibiotics could expedite their clinical use at a lower cost of development, as the bioactive component of the

combination has already undergone thorough clinical trials. Other advantages of synergistic interactions include increased efficiency, stability, reduced side effects, improved bioavailability, and the need for lower doses compared to synthetic alternatives [29, 30]. This is attributed to the presence of bacteriocin, acetic acid, lactic acid, and H_2O_2 in *L. plantarum* CFS. Organic acids disrupt substrate transport mechanisms by altering the permeability of the cell membrane [31].

The supernatant, which contains lactic acid, hydrogen peroxide, bacteriocins, and bacteriocin-like compounds, is used to prevent bacterial adhesion and biofilm formation. Additionally, it has antimicrobial properties that make it advantageous for biomedicine [32]. The extracellular matrix (EPS) in mature biofilms is thought to be responsible for their increased susceptibility to several antimicrobial agents. EPS has also been linked to antibiotic resistance in *E. coli* biofilms [33]. *L. plantarum* produces many digestive enzymes in CFS that have a strong inhibitory effect on biofilm formation and are associated with disruption of preformed biofilms [34].

Although the crystal violet assay measures biomass levels in biofilms, it does not assess the metabolic activity of the live cells because it stains both viable and non-viable cells that may be present in the developed biofilms [35]. Living cells exclusively possess the capacity to convert thiazolyl blue tetrazolium bromide (MTT) salt into a measurable, photometrically detectable colored compound. Consequently, the MTT assay selectively identifies living cells based on their metabolic functionality [36, 37].

Bacteria generally possess the genetic capacity to evolve and disseminate antibiotic resistance [38]. The potential of antimicrobial combinations to prevent the emergence of resistance mutants and have synergistic effects *in vivo* is investigated. Current antimicrobials may have a longer effective life if they were mixed with natural substances. These combinations may offer therapeutic alternatives for the treatment of infections [39, 40]. Lactic acid bacteria, including species from the genus Lactobacillus, have been associated with the prevention or spread of pathogenic microorganism-induced biofilms. This conclusion is usually associated with the formation of antimicrobial substances such as organic acids,

bacteriocins, hydrogen peroxide, and biosurfactants [41, 42]. The synergistic effect occurs when the natural compounds of the probiotic strains damage the cell wall and create holes in it [43], allowing antibiotics to penetrate and influence the bacterial cell membrane [44].

Conclusions

In our study, *Escherichia coli* was the most common uropathogen, with a 39.8% prevalence of UTIs among diabetes patients. According to the results, the majority of *E. coli* strains were antibiotic-resistant. Antibiotics such as amikacin, imipenem, and chloramphenicol are still among the best options for preventing and treating early-onset *E. coli* infections. Lactobacillus' cell-free supernatant has significant activity against the growth of *E. coli* and biofilm population. When combined with gentamicin, the lactobacillus cell-free supernatant was found to be more effective against MDR *E. coli*, the biofilm formation, and initial cell attachment. The results of the MTT assay show that the lactobacillus cell-free supernatant of Lactobacillus has a greater ability to reduce the metabolic activity of the *E. coli* biofilm, either separately or in combination with gentamicin.

Conflict of interest

The authors declare no conflict of interest.

Financial disclosure

No financial support was received.

Data availability

No data are available for this study.

Authors' contribution

All authors contributed to the conception and design of the study. **Mohammed Koodi:** Investigation, Methodology, Writing – original draft, **Laith Najeeb:** Data curation, Writing – review & editing, **Asra'a Abdul-Jalil:** Supervision, Formal analysis, Visualization. All authors reviewed and approved the final manuscript.

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