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Study the antibacterial activity of probiotics against uropathogenic (E. coli) of chronic kidney disease patients

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***Abstract*- Chronic kidney disease (CKD) is a gradual decline of kidneys that results in kidney failure. This disease compromises the body's immune system, elevating susceptibility to infections. One of the serious risk factors for CKD is urinary tract infections (UTIs), which may also be the most common infectious disease associated with CKD patients. Recently, antibiotic resistance has become the biggest problem facing specialists in the field of treating infections; thus, probiotics could be an alternative solution for fighting pathogenic attacks, especially bacterial ones. This study aimed to investigate the antibacterial activity of probiotic effect against CKD-isolateduropathogenic Escherichia coli (*E. coli*) . From October 2023 to April 2024, 300 urine samples were collected from patients aged ≥ 18 years with suspected chronic kidney disease (CKD), and only 44 of the urine samples gave positive results for cultures. Uropathogenic *(E.col)* isolates which were double diagnosed by vitek-2 system showed almost high resistance results to 16 used antibiotics, and it showed that Ampicillin, Cefuroxime, and Cefurxime Axetil had the highest resistance results. The biofilm formation profile for 44 tested isolates showed 0% of non-formation, 0% of weak formation, 45.4% of moderate formation, and 54.6% of strong formation respectively. It was detected that only the bacteriocin concentrations of 125×10^3 µg/ml and 62.500 µg/ml showed the minimum inhibitory concentrations (MICs) for highly resistant bacterial isolates, and the broth microdilution method (BMD) was the best choice for this detection.**

***Keywords*- Probiotic, Uropathogenic E. coli (UPEC), Bacteriocin, Microdilution.**

I. INTRODUCTION

Chronic kidney disease (CKD) is a gradually declining disease that impacts more than 10% of the global population, which is equivalent to about 800 million people. CKD is more common in elderly adults, women, racial minorities, and individuals with diabetes mellitus and hypertension [1, 2].

One of the serious risk factors for Chronic kidney disease (CKD) is urinary tract infections (UTIs) [3]. UTIs can accelerate kidney function decline,, particularly in stages G3-G5 of CKD [1]. UTIs are a common microbial illness affecting people of all ages and sexes, causing inflammation in the urinary system, and they can range from mild cystitis to severe uroseptic shock. Both males and females experience at least one UTI symptomatic infection throughout their lifetime [4].

Pathogenic *E.coli* are divided into two groups: diarrheagenic and extraintestinal pathogenic *E. coli* . The last mentioned group is causing UTIs, termed uropathogenic *E. coli* (UPEC), and is the most common cause of UTIs worldwide [5]. Pathogenic factors such as secreted proteins, hemolysins, capsules, lipopolysaccharides, biofilm, fimbriae adhesions, and iron acquisition systems support the multidrug-resistant(MDR) UPEC's ability to cause severe septic [6].

Antimicrobial resistance (AMR) is a critical worldwide public health concern that impacts healthcare, veterinary, and agricultural industries, endangering the achievement of sustainable development goals, and it is correlated with uropathogenic *E.coli*. Microbes have developed acquired AMR to many drugs due to high selection pressure from the increasing use and misuse of antibiotics over the years [7].

The potential for antibiotics to cause nephrotoxicity, particularly when administered inappropriately, is a significant factor in chronic kidney disease. This can happen in several ways, such as through interstitial nephritis, direct toxicity to the kidney tubules, acute tubular necrosis, crystal deposits in the tubules, immune system dysfunction, and less blood flow to the kidneys [8, 9]. The fact that individuals with chronic kidney disease often interact with healthcare facilities and undergo invasive medical procedures, both of which increase their exposure to multi-drug-resistant bacteria, heightens the complexity. Consequently, they face a higher likelihood of nephrotoxicity [10]. A significant proportion of antibiotics interfere with the normal activity of the favorable microbiome, particularly the one in the gastrointestinal tract. Studies suggest co-administering probiotics like *Lactobacillus rhamnosus* GR-1 or *Lactobacillus reuteri* can minimize antibiotic impact on the microbiome, potentially replacing antibiotics in certain patients with uncomplicated urinary tract infections [11].

Probiotics seem to be a promising approach to prevent and even reduce the symptoms of such clinical states as an

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adjuvant therapy [12]. Probiotics are live microorganisms that are not harmful and, when consumed in adequate quantities (at least 106 viable CFU/g), can have a positive effect on the host by enhancing the balance of microbes in the gut and contributing to metabolism [13].

Probiotics are a combination of live beneficial bacteria and/or yeast that naturally live in our bodies. Enteric probiotics have long been used in an attempt at a body-wide transformation, eradicating uropathogens throughout the gut and vagina. Lactobacilli seem to play a protective role in the preservation of bladder well-being beyond their direct bactericidal activity. Intravesical probiotics may have a restorative effect on the internal environment of the bladder. They may modify the urobiome and induce protective changes within the bladder mucosa [14].

One crucial attribute of probiotics is their ability to generate molecules like bacteriocins, anti-carcinogens, and several other compounds that possess health-enhancing or pharmacological qualities [15, 16].

Researchers first discovered bacteriocins as positively charged antimicrobial peptides, a century ago. Lactic acid bacteria synthesise diverse bacteriocins that exhibit wide-ranging antibacterial properties, tolerance to changes in pH and temperature, and are non-toxic. Lactic acid bacteria are susceptible to digesting proteases, which guarantees that they do not have a detrimental impact on the gut microbiota. This property makes them ideal for inactivating infections [17, 18].

Most bacteriocins possess two primary attributes that differentiate them from conventional antibiotics that they are produced by ribosomes and have a limited range of antibacterial effects [19]. Several studies have been published in recent years, suggesting that bacteriocins could be used as an alternative to antibacterial agents in the prevention or even treatment of bacterial infections [20]. The current study aimed: to study the antibacterial activity of probiotics against chronic kidney disease-isolated uropathogenic *E.coli*, to check the antimicrobial susceptibility, and to detect the biofilm formation profile.

II. MATERIALS AND METHODS

A. Sample collection

Urine sample collection was done at the nephrology departments of Al-Imam Ali and Al-Kindi hospitals in Baghdad during a period starting from October 2023 to April 2024. Out of 300 patients aged ≥ 18 years with suspected CKD, only 44 urine samples were gave the positive results for cultures and gave the criteria.

B. Bacterial isolation, identification, and susceptibility tests

Urine samples were cultured on MacConky agar, incubated at 37◦ for 24 hours. Out of 300 samples, only 44 of *E.coli* were isolated. Bacterial isolates were frozen in brain heart infusion broth containing 15% glycerol for preservation [21].

We specifically double-diagnosed the 44 selected Uropathogenic *E.coli* (UPEC) isolates , initially diagnosed as *E. coli* according to the morphological diagnosis, using the Vitek-2 system (BioMrieux, France), and also used it to detect the antibiotic resistance profile of these isolates. Sixteen used antibiotics have been used to determine the minimal inhibiting concentration (MIC) [22].

C. Biofilm formation detection

Quantitative determination of biofilm formation was determined by a colorimetric microtiter plate assay [23]. The study involved cultured isolates of bacteria in brain-heart infusion broth for 24 hours, then transferred to tubes containing normal saline and adjusted turbidity to McFarland 0.5. The bacteria were then added to 96-well polystyrene microtiter plates and incubated under aerobic conditions for 24 hours. After incubation, the plates were washed three times with phosphate buffer solution (PBS) and dried. The biofilms were fixed with methanol and air dried. The plates were then stained with a crystal violet solution and dried at 37°C. The optical density (OD) of each well was read using a microtiter plate reader. The cut-off OD (ODc) to classify the isolates into four groups: non-producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer [23].

D. Probiotic antibacterial activity: It was evaluated by two methods:

1) Agar well diffusion method

The current study used bacteriocin of *Lactobacillus*, pre-extracted and purified as a powder (Sigma Aldrich, USA), as the probiotic. The antibacterial susceptibility assay was done according to the agar well diffusion method. One gram of the bacteriocin was suspended with 2 ml of distilled water. Two concentrations were prepared. 400 µl of the bacteriocin suspension were suspended with 1.6 ml of distilled water, resulting in 0.1 (100%) as the first concentration, and then 0.5 µl of the first concentration were also re-suspended with 1.6 ml of distilled water, resulting in 0.05 µl as the second concentration. According to the agar well diffusion method, the Muller Hinton agar plates were inoculated with 10^8 CFU/ml of tested bacterial suspension. 8-mm-diameter wells were punched and filled with 100 µl of each concentration of bacteriocin, and then the plates were incubated at 37°C. No zones were detected around the wells after 24 hours, and this method [24] was repeated three times, but it showed same result.

2) Broth microdilution method (BMD)

This is a process of antimicrobial susceptibility testing, it involves creating a liquid broth medium with different antimicrobial agent concentrations, inoculating a specific inoculum of microorganisms into it, incubating it, and then monitoring its growth. In, the resazurin-based 96-well plate microdilution method was used to determine the minimum inhibitory concentration (MIC) of the bacteriocin for each testing isolates [25].

- Inocula preparation:

Following the CSLI guideline, the OD600 value was altered to reach an equivalent of 108 CFU ml-1 for the inocula [26].

- Cultural medium, bacteriocin, and reagent preparations:

A double concentration of Muller Hinton Broth (MHB) was prepared [27]. Including diluted 0.1 g of the previously used bacteriocin powder with 0.4 ml of

distilled water and vortexed the mixture to produce 250000 g/ml. Resazurin was prepared at 0.015 % by dissolving 0.015 g, vortexed, and filter sterilised (0.22 lm filter), and the preparation should be kept at 4 °C for a maximum of two weeks [26].

- Steps of the assay:

Six (96-well) plates were prepared to hold the 44 E.coli isolates, each plate containing 8 isolates A-H and 10 columns numbered to reflect the serial dilution plus positive and negative control columns. 100µl of the double MHB was added to each well. Each well, with the exception of column 11 (as a negative control), received 10 µl of each inocula (activated isolates). We added 250000 µg/ml of previously prepared bacteriocin to the wells of column 1, resulting in a final concentration of 125000 µg/ml, which marked the beginning of the serial dilution . Using a multichannel pipette, mix and transfer 100 µl of the initial concentration, and continue this process until the dilution concludes. Incubation at 37°C for 24 hours. 0.015 % of resazurin was added. Results were observed. Blue colored wells indicate the minimum inhibitory concentrations(MICs), while pink ones indicate the bacterial growth [28].

E. Statistical analysis

The percentage and chi-square were calculated to study the significance level and P-value between the different factors included in the study. The T-test was used to compare various groups with each other. Results were expressed as mean ± standard deviation (SD). Pearson's correlation test (r) was used to detect the association of the factors under study with each other. The values of $p>0.05$ were considered statistically non-significant while $p\leq0.05$ in the analysis of contingency tables. The statistical analysis was carried out by SPSS (V 20).

III. RESULTS

Antibiotic susceptibility tests could be clearly explained by Fig.1 and Table I**.** Table I determines the resistance and sensitive percentage for each used antibiotic. It shows that: Ampicillin, Cefuroxime, and Cefurxime Axetil had the highest resistance results, whereas Ertapenem, Meropenem, and Nitrofurantoin had the highest sensitivity results.

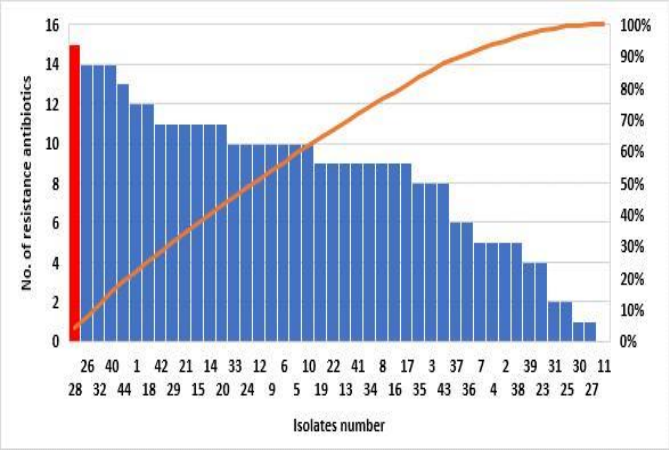

Fig.1: Antibiotic susceptibility pattern of tested isolates; isolates are arranged in descending order from the highest antibiotic resistance to the lowest resistance, thereby determining the percentage for each category.

Table I: Antibiotics profile of uropathogenic *E.coli* isolates

Resistance Profile	AMP	TZP	CXM	CXM Axetil	FOX	CFM	CAZ	CRO	CPM	ETP	MEM	AK	GEN	CIP	NIT	SXT
R No	42	14	38	38	21	37	30	34	23	3	4	9	19	29	4	27
R%	95.45	31.82	86.36	86.36	47.73	84.09	68.18	77.27	52.27	6.82	9.09	20.45	43.18	65.91	9.09	61.36
S No	2	31	6	6	23	7	14	10	21	41	40	35	25	15	40	17
S%	4.55	70.45	13.64	13.64	52.27	15.91	31.82	22.73	47.73	93.18	90.91	79.55	56.82	34.09	90.91	38.64

Ampicillin *AMP*, Piperaciliin/Tazobactam *TZP*, Cefuroxime *CXM*, Cefurxime Axetil *CXM*, Cefoxitin *FOX*, Cefixime *CFM*, Ceftazidime *CFM*, Ceftriaxone *CRO*, Cefepime *CPM*, Ertapenem *ETP*, Meropenem *MEM*, Amikacin *AK*, Gentamicin *GEN*, Ciprofloxacin *CIP*, Nitrofurantoin *NIT*, Trimethoprim/Sulfamethoxazole *SXT*

Table II: Biofilm formation profile for *E.coli* isolates in current study

Isolation sources N(%)	Non N(%)	Weak N(%)	Moderate N(%)	Strong N(%)
Female 22	0	0	18(40.9)	4(9.2)
Male 22	0	0	2(4.5)	20(45.4)
Total 44	**0**	**0**	**20(45.4)**	**24(54.6)**
P value	**The chi-square statistic is 23.4667. The *p*-value is < 0.00001. The result is significant at *p* <0 .05**			

The biofilm formation profile revealed the ability of the 44 bacterial isolates isolated from 22 males and 22 females of chronic kidney disease patients to form only moderate and strong, 45.5% and 54.6%, respectively (table II).

The broth microdilution method revealed the MIC results for all 44 *E.coli* isolates of the first two concentrations (125000µl/m &62.500µl/ml) fig.2, and fig.3 represent the results in one of the six plates used in this method.

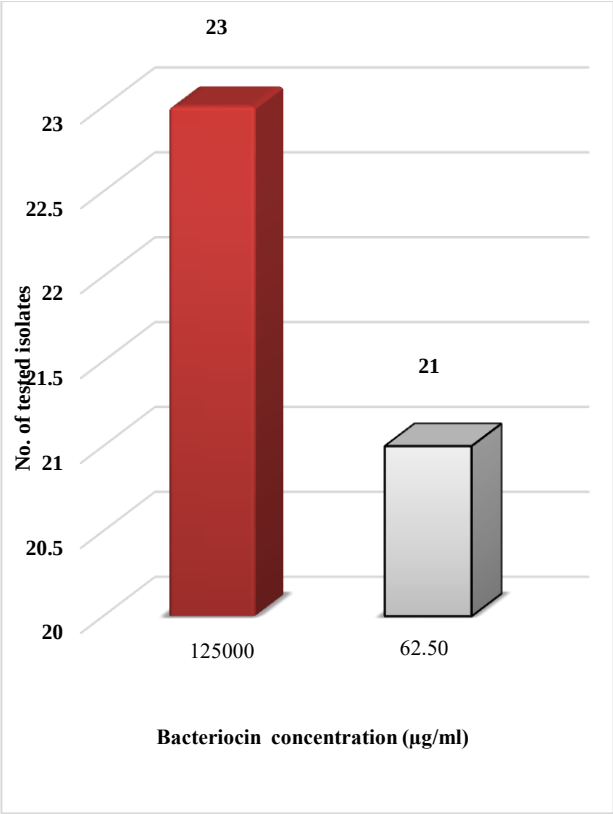

Fig.2: The two antibacterial concentrations among all the tested isolates show 23 isolates were affected by 125000 µg/ml , and 21 isolates affected by 62.5 µg/ml of bacteriocin.

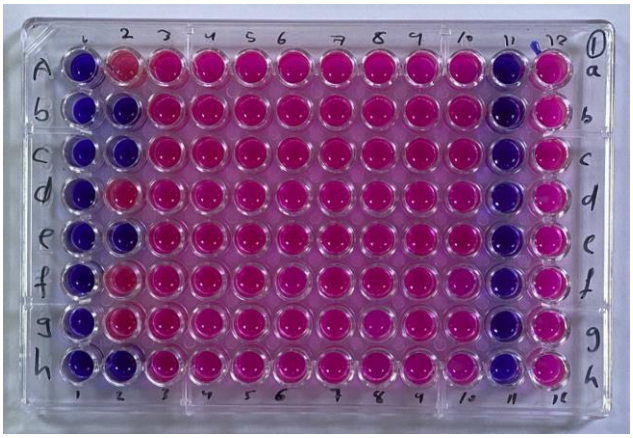

Fig.3: MIC results for eight of the tested isolates; Columns 1 and 2 show the bacteriocin's antibacterial effect (blue coloured), while the serial dilution from Column 3 to Column 10 shows no effect, bacterial growth(pink coloured) is obvious.

Table III displays a relationship between the two concentrations, the antibiotic resistance, and the biofilm formation profile of the tested isolates. The first concentration affected isolates had a mean antibiotic resistance of 9.33, matching 12 isolates with moderate biofilm formation and 11 isolates with strong biofilm formation, while the second concentration's mean (8.04) matched 8 isolates with moderate biofilm formation and 13 isolates with strong biofilm formation.

Table III: Correlation of bacteriocin and antibiotic resistance alongside biofilm profile

* No/cons.	Mean of resist. Antibiotic	Biofilm formation profile	
		Moderate	*Strong*
23/125000	9.33	12	11
21/62.500	8.04	8	13
*Isolates number/ Bacteriocin concentration (µm/ml).			

IV. DISCUSSION

Chronic kidney disease is characterized by metabolic and immunological changes, including uremic toxins, pro-inflammatory molecules and immune alterations. This may lead to the development of UTIs, increasing the risk of progression towards end-stage kidney disease (ESKD)[1]. Although long-term oral antibiotic therapy has been successful as a therapeutic alternative, the emergence of bacterial resistance has made this method unreliable. An encouraging option involves utilising living microorganisms, known as probiotics, for the prevention and treatment of UTIs [29].

The prevalence of UTI in patients with chronic kidney disease (CKD) caused by *E.coli* that are resistant to several drugs has been on the rise [30]. In the current study, according to susceptibility tests, most bacterial isolates were resistant to most antibiotics. The development of antibiotic resistance in bacteria can occur through several methods, including as active elimination, enzymatic alterations, modifications of cell components, overexpression of enzymes, changes in membrane permeability, alternative metabolic pathways, and modifications of regulatory systems [31]. Amoxicillin and Cefuroxime had the highest percentage of resistance, results are nearly similar to an Iraqi study included UTI in CKD, conducted by Majeed and Aljanaby [30], while Ertapeneme, Meropeneme, and Nitrofurantoin had the highest percentage of sensitivity, that is in agreement with the results reported by Wijaya *et al* [32] and Kot *et al*. [33].

In this study, biofilm formation profile detection was involved. Generally, biofilm formation is categorised into non-, weak, moderate, and strong formations. the results revealed only moderate and strong formations. Those results show the different behavior of uropathogenic *E. coli* found in CKD patients, which is in agreement with Naziri *et al*. Furthermore, biofilms exhibit resistance to the effects of antimicrobial medications, as the majority of the existing antimicrobial treatments are only effective against the planktonic bacterial growth forms. Hence, probiotic strains that possess both anti-biofilm and antibacterial properties against UPEC might have significant therapeutic value [34]. Lactic acid bacteria can exert antibiofilm action through various methods, such as releasing antimicrobial peptides or lactic acid [35], which can impede bacterial growth. These bacteria synthesise biosurfactants that alter the cell surface characteristics or

attach to solid surfaces, reducing bacterial attachment [36]. They can also modify the structural integrity of biofilms by disrupting cell-to-cell aggregation and surface attachment processes, potentially mediated by exopolysaccharides or the physicochemical characteristics of their cell surface [37].

Last but not least, researchers have been studying the antibacterial activity of probiotics for several years, and this field seems to be crucial for both preventing and treating bacterial infections, especially when antimicrobial resistance to different antibiotics increases over time. As previously mentioned, the first method, the agar diffusion method, showed confusing results when we used *Lactobacillus* bacteriocin against *E. coli* isolates. Several studies such as a study by Younas *et al* [38], concluded that bacteriocin exhibits effective antibacterial activity against *E. coli*. However, in the current study, this method showed no antibacterial activity, this result may be due to inability of bacteriocin to diffuse through the agar media or the insufficient concentration of the bacteriocin, which creates inhibition zones around the bacterial growth.

The broth microdilution method was the second choice. This method has been applied in many other studies to detect the potential of different antimicrobial agents, such as chitosan and different plant extracts, against different pathogens [28, 39, 40]. This method showed the antibacterial activity of the used bacteriocin. The minimum inhibitory concentration (MIC) was determined and observed in the first two serial concentrations as shown in Fig3. Out of the 44 tested isolates, 23 were affected by the MIC of 125000µg/ml, and 21 were affected by the MIC of 62.500µg/ml as it shown in Fg.2. Those results indicate that the uropathogenic *E. coli* isolates are highly resistant. They were affected by the only first two of 10 gradual concentrations that started at 125000 µg/ml and ended at 244 µg/ml.

V. CONCLUSIONS

The bacteriocin's probiotic properties provide another ideal tool for treating infections, particularly in patients with chronic kidney disease. Despite the challenge of managing the bacterial isolates in this study, the probiotic could still have an impact, while a comparative study for evaluation of the efficacy of commercial bacteriocin is recommended.

ETHICAL CLEARANCE

This study has been checked and approved by the ethical scientific committee of Al-Iraqia University /College of Medicine/Department of Microbiology, with the approval number of 261 at 2024/8/11.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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