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Antibiotic Resistance of *Klebsiella pneumonia* Isolated From Inpatients and the Inhibitory Effect of Probiotics Against Metallo-β-lactamases-producing *K. pneumonia*

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ABSTRACT

Background: Increased resistance to various antibiotics has challenged therapeutic options for the treatment of nosocomial infections, including infections caused by *Klebsiella pneumoniae*. Metallo-β-lactamase (MBL) producing *K. pneumoniae* is a serious concern due to its resistance to many antibiotics, including carbapenems, a class of last-resort antibiotics. This resistance is often encoded by genes such as bla_{IMP}, bla_{VIM}, and bla_{NDM}, which are carried on plasmids, making them easily transmissible. The emergence and spread of MBL-producing *K. pneumoniae* strains is a rising threat, particularly in hospital settings, and can increase mortality, morbidity, and healthcare costs. This study investigates the inhibitory effect of commercial and native probiotics on these strains.

Materials and Methods In the present study, first, the resistance of 50 *K. pneumoniae* isolates from hospitalized patients in Isfahan hospitals, Isfahan City, Iran, to 11 antibiotics was determined according to the Clinical & Laboratory Standards Institute (CLSI) protocol. Subsequently, production of MBL enzymes was determined by the combined disks. Then, the bla_{NDM}, bla_{VIM}, and bla_{IMP} genes were evaluated. Next, *K. pneumoniae* strains carrying plasmid-borne *MBL* genes were exposed to commercial FlorMidabil sachets manufactured in Italy. FlorMidabil sachets contained *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus paracasei*, as well as standard native *L. plantarum* (PTCC1058) and *Lactobacillus rhamnosus* (PTCC1637). The goal was to investigate the inhibitory effect of these probiotics on *K. pneumoniae* strains. This study was conducted in three ways: The radial line method, the effects of probiotic supernatants, the effect of live probiotics on Klebsiella strains using the microtiter plate method, and the evaluation of *K. pneumoniae* viability in the presence of probiotics and re-culture on solid medium.

Results: All *K. pneumoniae* strains were resistant to the following antibiotics: meropenem, imipenem, ceftazidime, cefotaxime, cefepime, amikacin, gentamicin, cotrimoxazole, ciprofloxacin, and aztreonam. The results showed that the most effective antibiotic against the studied strains was colistin, a polymyxin. All strains carried plasmid-borne *MBL* genes, and bla_{NDM} and bla_{IMP} were confirmed, but bla_{VIM} was not detected by polymerase chain reaction (PCR). According to the results, 37 isolates (74%) contained the bla_{NDM} gene, and 13 isolates (26%) contained the bla_{IMP}. This study is the first finding of *K. pneumoniae* carrying the bla_{NDM-5} gene in Iran. In the latter part of the study, it

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was identified that commercial probiotic sachets and standard native *L. plantaru*m and *L. rhamnosus* had effects against the *K. pneumoniae* strain studied. The inhibitory effect of *K. pneumoniae* on the radial line method was observed in radial lines with or without slight growth in *K. pneumoniae* strains. In a co-culture method using probiotic bacteria with *K. pneumoniae* isolates, no significant change in optical density (OD) was observed after 24 h of incubation. However, in a liquid culture containing live probiotic bacteria and the *K. pneumoniae* strain, the increase in OD after 24 h was due to the growth of the probiotics. Considering the absorption rate on the second day in this experiment, it was found that native and standard probiotics of *L. rhamnosu*s and *L. plantarum* had a lower inhibitory effect than commercial products. After cultivating wells containing probiotic and *K. pneumoniae* bacteria on solid medium, only small amounts of probiotic bacteria were recovered. As a result, cultivation of wells containing probiotic supernatants inoculated with *K. pneumoniae* did not result in growth.

Conclusion: The present study demonstrates the high frequency of *K. pneumoniae* strains with multiple resistance, high carbapenem resistance, and high bla_{NDM} gene prevalence, which necessitate the identification and use of non-harmful alternative methods for their control and removal. Two *L. plantaru* and *L.* rhamnosus strains, as well as commercial probiotic products, are recommended.

Klebsiella pneumoniae (*K. pneumoniae*) is a gram-negative, encapsulated, and non-motile bacterium. This bacterium is considered an opportunistic pathogen and is resistant to different antibiotic classes. *K. pneumoniae* causes important diseases such as urinary tract infections, sepsis, and pneumonia in hospitalized patients with a high mortality rate [1]. Although this bacterium is part of the normal flora of the mouth, skin, and intestines, it can cause dangerous pneumonia [2]. *K. pneumoniae* is especially common in alcoholics, hospitalized people, and people with immune suppression. Carbapenem-resistant *K. pneumoniae* (CRKP) refers to strains of *K. pneumoniae* that are resistant to carbapenems, a class of last-resort antibiotics used to treat severe infections. This resistance is often mediated by the production of carbapenemases, such as KPC (*K. pneumoniae* carbapenemase), NDM (New Delhi metallo-β-lactamase [MBL]), OXA-48-like enzymes, VIM, and IMP (MBLs) [3]. The global burden of CRKP is a significant and growing public health concern due to its association with high mortality rates, limited treatment options, prolonged hospitalization, and rapid spread across healthcare settings worldwide [4]. Therefore, the major challenge in hospital-acquired infections caused by *K. pneumoniae* is treatment and long-term care [4].

Probiotics are living, specific microorganisms that mostly belong to the large group of bacteria that constitute the main microbial flora of the human intestine. These microorganisms can play a protective role against various diseases [5]. Probiotic products are available in the form of tablets, capsules, powders, and fortified yogurts, milk, and cheese, and no significant negative effects have been reported with their use [6].

Generally, *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species are the main probiotic species used in commercial products. They inhibit pathogen growth through various mechanisms, including competitive inhibition of bacterial binding sites on intestinal epithelial surfaces, elimination of toxin receptors, food competition, and boosting the immune system. As well as [7]. According to the crisis of antibiotic resistance and valuable probiotic features, probiotics synthesize various compounds like short-chain fatty acids (SCFAs), organic acids, hydrogen peroxide and bacteriocins. Organic acids can keep lower the pH of the gut and inhibit the growth of certain pathogens. Hydrogen peroxide damages pathogen cell membranes and DNA. Bacteriocins can inhibit or kill other bacteria that may be potential candidates for infectious disease treatment. Hence, this study has been conducted to determine the effects of commercial probiotics, including FlorMidabil sachets manufactured in Italy. FlorMidabil sachets contained *Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus plantarum*, and *Lactobacillus paracasei*, as well as standard native *L. plantarum* (PTCC1058) and *Lactobacillus rhamnosus* (PTCC1637). They were chosen because no research experiment had yet been conducted to investigate their inhibitory effect against MBL-producing *K. pneumoniae*.

Materials and Methods

Bacterial samples collection and identification

In a cross-sectional descriptive study, samples were collected from 4 hospitals in Isfahan City, Iran. They were cultured on blood agar and eosin methylene blue (EMB) agar, and then, using standard microbiological phenotypic methods and biochemical tests, *K. pneumoniae* isolates were confirmed [7]. There were large, mucoid, dome-shaped colonies due to capsule production on blood agar and mucoid, pink/purple colonies on EMB. After gram staining, gram-negative, short, plump rods (bacilli) were observed.

Phenotypic study of antibiotic susceptibility

Antibiotic susceptibility testing of isolates was performed using the Kirby-Bauer disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines. A panel of 11 antibiotic disks, including ceftazidime, cefepime, cefotaxime, ciprofloxacin, amikacin, gentamicin, imipenem, meropenem, cotrimoxazole, aztreonam, and colistin, was used. Results diameters were interpreted as susceptible, intermediate, or resistant according to the company guideline tables.

Phenotypic validation of MBL enzyme production

Since MBLs are inhibited by Ethylenediaminetetraacetic acid (EDTA), a combination test of EDTA with meropenem was performed. In this experiment, the zone diameters of the meropenem and EDTA hybrid disks were compared with the zone diameters of the meropenem disk alone. Increasing the diameter of the non-growth space, in combination with a disk, to more than 5 mm, compared with meropenem alone, indicates the presence of MBLs [8].

Molecular detection of antibiotic resistance genes

The DNA of *K. pneumoniae* isolates was extracted by the boiling method. The DNA samples were then stored at −20 ˚C until used. Three antibiotic-resistance genes were selected for antimicrobial agents belonging to the carbapenem and β-lactam classes. Finally, their primers and DNA samples were used for polymerase chain reaction (PCR), as shown in Tables 1 and 2.

Sequencing of bla_{NDM} genes

After detection of bla_{NDM} genes, the PCR products were sent to Bionier (South Korea) for sequencing. Then, by analyzing and editing the determined sequence, the nucleotides were identified using the Basic Local Alignment Search Tool (BLAST) at the NCBI site. Finally, the approved sequences were submitted to the The DNA Data Bank of Japan (DDBJ).

Evaluation of the antibacterial activity of probiotics

To evaluate the antibacterial effects of probiotics (Tables 2 and 3) on *K. pneumoniae* isolates and *Staphylococcus aureus* (ATCC25923), we used 3 different methods.

Radial streak method

First, a 0.5 McFarland concentration of commercial and native probiotic bacteria was cultured in the centre of the MRS agar plate (a 1 cm diameter circle) and incubated at 35 ˚C for 48 h. Then, using a swab of the 0.5 McFarland concentration of *S. aureus* and *K. pneumoniae* containing the *MBL* gene, a radial line was drawn from the bottom of the plate to the centre, where the probiotics had grown to the edge of the probiotic culture. If probiotics inhibit these bacteria, the radial growth line of pathogenic bacteria will not be observed around, and the stronger the antibacterial effect of probiotics, the shorter the radial line of pathogenic bacteria will be [9].

Plate microtiter method

At first, commercial and native probiotics were cultured separately in 100 mL of MRS broth and incubated at 35 ˚C for 48 h (Tables 4 and 5). Then, to prepare pure supernatant from probiotics, the culture medium was centrifuged at 3000 g and passed through a 0.4-µ filter. Then, to evaluate the effect of the supernatant on *K. pneumoniae* growth, the isolates were cultured in native and commercial probiotic supernatants. As a negative control, 1000 µL of MRS broth culture medium was added in triplicate, and 1000 µL of nutrient broth was added in triplicate. As a positive control, 1000 µL of a 0.5 McFarland concentration of probiotics (prepared in MRS broth medium) and 1000 µL of 0.5 McFarland concentration of *K. pneumoniae* were added in triplicate. To investigate the inhibitory effect of probiotics, 500 µL of a 0.5 McFarland concentration of probiotics grown in MRS broth medium was added along with 500 µL of a 0.5 McFarland concentration of *K. pneumoniae*, as well as 500 µL of the pure supernatant solution of probiotics

and 500 µL of a half McFarland concentration of *K. pneumoniae*. After filling all the wells, the initial absorbance (optical density 1 [OD1]) was measured at 610 nm using a spectrophotometer to compare changes in absorbance after incubation. The plates were then incubated for 24 h at 35 ˚C. After this time, the secondary optical absorption (OD2) was measured again with a spectrophotometer and compared with the primary optical absorption and the optical absorption of the negative and positive controls, and the level of inhibitory effect was calculated. The absence of an increase in secondary light absorption relative to primary light absorption in wells containing co-cultures of probiotic bacteria or their supernatant and *K. pneumoniae*, or in wells with absorption similar to or close to the negative control, indicates the inhibitory effect of probiotics [10]. OD 610 was selected because it is appropriate for checking the accuracy of adjusting the bacterial concentration to 0.5 McFarland before incubation and for measuring bacterial growth and comparing the proliferation rate after incubation. This wavelength is used to measure light scattering by live and dead cells [10].

Survival rate of *K. pneumoniae*

To evaluate the survival rate of *K. pneumoniae*, we re-cultured the contents of each well on nutrient agar for 24 h, then counted the colonies.

Results

Isolation and identification of *K. pneumoniae*

Fifty samples of *K. pneumoniae* were isolated from various clinical samples collected at four hospitals in Isfahan. The majority of patients are from the intensive care units (ICUs). Most of the samples were collected in the ICU. Surgery and Gynecology wards also appear a few times. The number of male patients was slightly higher than that of females. Patient ages range from 23 to 87 years. Most patients fell within the 40-75 age range. Elderly female patients (over 60) were mostly in the ICU and commonly had respiratory or urinary symptoms. Older male patients had samples from respiratory tubes or blood. Respiratory tubes were the most common sample type (28 samples). Urine was the second most frequent (12 samples). Other types included sterile fluids, wounds, blood, and trachea (10 samples).

Antibiotic resistance phenotypes

In this study, *K. pneumoniae* isolates were completely resistant to the following antibiotics: ceftazidime, cefotaxime, cefepime, aztreonam, cotrimoxazole, gentamicin, ciprofloxacin, amikacin, imipenem, and meropenem. However, they were sensitive to colistin. The phenotypic frequency of MBLs was evaluated by the combined disk and EDTA methods. All 50 *K. pneumoniae* isolates showed zone inhibition of more than 5 mm and were positive for MBL.

Antibiotic resistance genotyping

According to the results, 37 isolates (74%) contained the bla_{NDM} gene, and 13(26%) contained the bla_{IMP} gene. Two *K. pneumoniae* isolates carried bla_{NDM-5}. However, no bla_{VIM} gene was detected. This study is the first finding of bla_{NDM-5} in Iran.

Antimicrobial effect of commercial and native probiotics by the radial streak method

Radial streak results indicated that both commercial and native probiotics inhibited the growth of *K. pneumoniae* isolates. However, there was no similar effect on *S. aureus* (Figure 1).

Co-culture and survival rate of *K. pneumoniae* with probiotics

In liquid culture containing live probiotics (FlorMidabil) and *K. pneumoniae* isolates, light absorption in-

Table 1. Standard biochemical tests

Test	Result	Test	Result
Oxidase	Negative	Methyl red (MR)	Negative
Catalase	Positive	Voges–proskauer (VP)	Positive
Indole	Negative	Urease	Positive (slow)
Citrate utilization	Positive	TSI agar	A/A, gas (+), H_2S (–)
Lysine decarboxylase	Positive	ONPG test	Positive

Table 2. Antibiotic susceptibility pattern according to 2018 CLSI

Antibiotic	Abbreviation	Disk Content (µg)	Susceptible Zone Diameter (mm)	Intermediate Zone Diameter (mm)	Resistant Zone Diameter (mm)
Ceftazidime	CAZ	30	21≤	20-18	17≥
Cefotaxime	CTX	30	26≤	25-23	22≥
Cefepime	CPM	30	25≤	24-19	18≥
Amikacin	AN	30	17≤	16-15	14≥
Gentamicin	GM	10	15≤	14-13	12≥
Ciprofloxacin	CIP	5	21≤	16-20	15≥
Imipenem	IMP	10	23≤	20-22	19≥
Meropenem	MEN	10	23≤	20-22	19≥
Trimethoprim/Sulfamethoxazole	COT	1.25/23.75	16≤	11-15	10≥
Aztreonam	AZT	30	21≤	18-20	17≥
Colistin	COL	10	15≤	-	14≥

creased significantly after 24 hours (P<0.0001) (Table 6). Also, in co-culture *K. pneumoniae* with FlorMidabil supernatant, the same effect was detected (P<0.0001) (Table 7) (Figures 2A and 2B). Additionally, it was found that native probiotics of *L. rhamnosus* and *L. plantarum* had a significant inhibitory effect in liquid culture containing live probiotics and co-culture with probiotic supernatant (P<0.0001) (Table 8) (Figures 2C and 2D). Totally, the results of the plate microtiter method confirmed the results obtained in the radial streak method.

Evaluation of the survival rate of *K. pneumoniae* in the presence of probiotics and recultivation on solid medium

The results of this method, as with the previous two, showed that probiotic bacteria inhibited Klebsiella isolates. After culturing wells containing probiotic bacteria and *K. pneumoniae* on solid nutrient agar, only small amounts of probiotic bacteria grew, and no growth was observed when wells containing probiotic supernatants

Table 3. Thermocycler programming and primer sequences for detecting *MBL* genes

Primer Name	Initial Denaturation		Denaturation		Annealing		Extension		Final Extension	
bla_{NDM} (621 bp)	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp
	4 min	94˚C	40 s	94˚C	50 s	57 °C	50 s	72˚C	4 min	72 °C
Sequence (5´-3´)	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC									
bla_{IMP} (239 bp)	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp
	4 min	94 °C	40 s	94 °C	50 s	54 °C	50 s	72 °C	4 min	72 °C
Sequence (5´-3´)	F-GGAATAGAGTGGCTTAAYTC R-TCGGTTTAAYAAAACAACCACC									
bla_{VIM} (390 bp)	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp
	4 min	94 °C	40 s	94 °C	50 s	54 °C	50 s	72 °C	4 min	72 °C
Sequence (5´-3´)	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG									

Table 4. Commercial probiotics list

Commercial Probiotics	Commercial Name, Country
B. lactis	Sachet, FlorMidabil, Italy
L. acidophilus	Sachet, FlorMidabil, Italy
L. plantarum	Sachet, FlorMidabil, Italy
L. paracasei	Sachet, FlorMidabil, Italy

Table 5. Native probiotics list

Native Probiotics	PTCC
L. plantarum	PTCC1058
L. rhamnosus	PTCC1637

PTCC: Persian type culture collection.

were cultured with *K. pneumoniae*. After preparing direct slides and staining, culturing the contents of the wells on MRS and nutrient agar media, and counting colonies (CFU/mL), it was found that the increase in absorption was due to the growth of probiotic bacteria.

Discussion

Antibiotic resistance profile

The phenomenon of antibiotic resistance among pathogenic bacteria, such as MBLs, is further expanded through horizontal gene transfer between bacteria. Patients admitted to the intensive care unit are at higher risk of nosocomial infections, with approximately 25% of such infections occurring in hospital ICUs [8]. Given that antibiotic resistance in *K. pneumoniae* strains is increasing due to the presence of MBL genes, preventing and controlling *K. pneumoniae* infections has been recognized as an important challenge in hospitals, especially in ICUs. Due to limitations in antibiotic therapy and the increasing prevalence of antibiotic resistance, many efforts have been made to find suitable alternatives for controlling *K. pneumoniae* infection. Probiotics, such as *Lactobacillus* species, have shown potential to combat drug-resistant bacteria through several biological mechanisms. *L. rhamnosus* and *L. plantarum* synthesize various compounds like SCFAs. These organic acids can keep the pH and create conditions unfavorable for the growth of MBL-positive bacteria. MBL-producing bacteria, such as *K. pneumoniae*, often rely on biofilms for protection and survival. Probiotics can prevent biofilm formation and disturb existing biofilms. Some probiotics may reduce the horizontal transfer of resistance genes (such as MBL genes on plasmids). They change the local environment which inhibit bacterial conjugation and limit the spread of resistance, with help hydrogen peroxide damage pathogen cell membranes and DNA, and with bacteriocins can inhibit or kill other bacteria that may be a good candidate as an alternative method for controlling bacterial infections that are resistant to antibiotics due to their effectiveness in suppressing pathogenic bacteria and their potential preventive and therapeutic roles in some types of infections [11].

The cell-free supernatant of probiotic suspensions contains various antimicrobial metabolites secreted by probiotics, such as *L. plantarum* and *L. rhamnosus*. These compounds exert multiple inhibitory effects against *K. pneumoniae*, including multidrug-resistant and MBL-producing strains [6].

The cell-free supernatant contains organic acids, such as lactic acid and acetic acid, which significantly reduce the pH, disturb the membrane integrity of *K. pneumoniae*, and prevent optimal growth and replication of bacteria [6].

There are antimicrobial peptides, such as bacteriocins, that form pores in the bacterial membrane and cause cell lysis. Also, hydrogen peroxide (H_2O_2) can cause oxidative damage to DNA, proteins, and lipids. In addition to probiotic supernatant, it interferes with biofilm maturation and limits horizontal gene transfer by reducing bacterial population density [6].

Table 6. The inhibitory effect of 0.5 Mcfarland solution, a commercial product of flormidabil, on *K. pneumoniae* using the microplate method

Sample ID	OD1	OD2	Sample ID	OD1	OD2	Sample ID	OD1	OD2
K.p+FlorMidabil37	0.32	0.84	K.p+FlorMidabil19	0.31	0.9	K.p+FlorMidabil1	0.38	0.85
K.p+FlorMidabil38	0.31	0.81	K.p+FlorMidabil20	0.31	0.8	K.p+FlorMidabil2	0.42	0.76
K.p+FlorMidabil39	0.33	0.88	K.p+FlorMidabil21	0.31	0.77	K.p+FlorMidabil3	0.31	0.88
K.p+FlorMidabil40	0.31	0.8	K.p+FlorMidabil22	0.35	0.8	K.p+FlorMidabil4	0.31	0.83
K.p+FlorMidabil41	0.35	0.78	K.p+FlorMidabil23	0.34	0.81	K.p+FlorMidabil5	0.32	0.81
K.p+FlorMidabil42	0.32	0.81	K.p+FlorMidabil24	0.3	0.75	K.p+FlorMidabil6	0.33	0.88
K.p+FlorMidabil43	0.32	0.82	K.p+FlorMidabil25	0.3	0.8	K.p+FlorMidabil7	0.36	0.84
K.p+FlorMidabil44	0.34	0.82	K.p+FlorMidabil26	0.32	0.81	K.p+FlorMidabil8	0.34	0.82
K.p+FlorMidabil45	0.37	0.85	K.p+FlorMidabil27	0.32	0.85	K.p+FlorMidabil9	0.33	0.8
K.p+FlorMidabil46	0.37	0.87	K.p+FlorMidabil28	0.31	0.85	K.p+FlorMidabil10	0.31	0.82
K.p+FlorMidabil47	0.39	0.82	K.p+FlorMidabil29	0.31	0.81	K.p+FlorMidabil11	0.34	0.86
K.p+FlorMidabil48	0.3	0.84	K.p+FlorMidabil30	0.3	0.81	K.p+FlorMidabil12	0.3	0.8
K.p+FlorMidabil49	0.34	0.82	K.p+FlorMidabil31	0.32	0.82	K.p+FlorMidabil13	0.3	0.89
K.p+FlorMidabil50	0.37	0.81	K.p+FlorMidabil32	0.36	0.84	K.p+FlorMidabil14	0.33	0.84
			K.p+FlorMidabil33	0.37	0.88	K.p+FlorMidabil15	0.36	0.81
Negative control	0.033	0.034	K.p+FlorMidabil34	0.33	0.87	K.p+FlorMidabil16	0.3	0.85
Positive control	0.22	0.98	K.p+FlorMidabil35	0.35	0.85	K.p+FlorMidabil17	0.32	0.79
Positive control	0.35	1.31	K.p+FlorMidabil36	0.33	0.84	K.p+FlorMidabil18	0.3	0.80

K.p: *K. pneumoniae*.

In this study, all *K. pneumoniae* isolates are resistant to antibiotics such as cefepime, ceftazidime, cefotaxime, aztreonam, ciprofloxacin, amikacin, gentamicin, and cotrimoxazole, as well as to imipenem and meropenem. Still, the most effective antibiotic against *K. pneumoniae* isolates is colistin. In a study by Japoni-Nejad et al. similar to our findings, *K. pneumoniae* isolates were found to be highly resistant to various antibiotics, including cefotaxime, aztreonam, ciprofloxacin, gentamicin, and meropenem [12]. However, in another study by Ahangarzadeh Rezaee et al. chloramphenicol, amikacin, ciprofloxacin, and imipenem showed significant activity against *K. pneumoniae* isolates from a hospital in Tabriz City, Iran [13]. The study by Hashemizadeh et al. showed that more than 55% of *K. pneumoniae* isolates from Shahrekord city, Iran, were resistant to multiple drugs. In addition, a study by Fazeli et al. in Isfahan on 142 isolates showed that 84% were multidrug-resistant [14]. The study by Sanchez et al. in the United States shows increased resistance of *K. pneumoniae* to the antibiotics cefepime, ceftazidime, cefotaxime, aztreonam, ciprofloxacin, amikacin, gentamicin, and cotrimoxazole, as well as penicillin. Still, the percentage of resistance to all these antibiotics was lower than that in the present study [15].

Molecular resistance genes

Dong et al. (2018) reported that 31% of *K. pneumoniae* isolates from patients' blood were carbapenem-resistant and harbored bla_{NDM} and bla_{IMP} genes. All of these strains were resistant to imipenem, meropenem, ceftriaxone, ceftazidime, cefotaxime, cefepime, aztreonam, and ciprofloxacin, but were sensitive only to colistin and amikacin [16]. The study by Shokri et al. in Isfahan, Iran, showed that among 75 *K. pneumoniae* isolates, 95% and 94% were resistant to imipenem and meropenem, respectively [17], which is close to the results of the pres-

Table 7. The inhibitory effect of the supernatant solution of the commercial product FlorMidabil on *K. pneumoniae* using the microplate method

Sample ID	OD1	OD2	Sample ID	OD1	OD2	Sample ID	OD1	OD2
K.p+F. supernatant37	0.2	0.33	K.p+F. supernatant19	0.25	0.25	K.p+F. supernatant1	0.24	0.3
K.p+F. supernatant38	0.23	0.3	K.p+F. supernatant20	0.2	0.22	K.p+F. supernatant2	0.22	0.29
K.p+F. supernatant39	0.21	0.28	K.p+F. supernatant21	0.21	0.25	K.p+F. supernatant3	0.26	0.33
K.p+F. supernatant40	0.25	0.34	K.p+F. supernatant22	0.21	0.24	K.p+F. supernatant4	0.24	0.31
K.p+F. supernatant41	0.22	0.31	K.p+F. supernatant23	0.2	0.32	K.p+F. supernatant5	0.23	0.34
K.p+F. supernatant42	0.22	0.31	K.p+F. supernatant24	0.22	0.26	K.p+F. supernatant6	0.2	0.35
K.p+F. supernatant43	0.23	0.36	K.p+F. supernatant25	0.23	0.31	K.p+F. supernatant7	0.22	0.31
K.p+F. supernatant44	0.2	0.33	K.p+F. supernatant26	0.22	0.24	K.p+F. supernatant8	0.21	0.3
K.p+F. supernatant45	0.21	0.31	K.p+F. supernatant27	0.24	0.32	K.p+F. supernatant9	0.21	0.29
K.p+F. supernatant46	0.22	0.32	K.p+F. supernatant28	0.21	0.33	K.p+F. supernatant10	0.24	0.2
K.p+F. supernatant47	0.22	0.34	K.p+F. supernatant29	0.2	0.31	K.p+F. supernatant11	0.26	0.28
K.p+F. supernatant48	0.23	0.31	K.p+F. supernatant30	0.26	0.31	K.p+F. supernatant12	0.26	0.3
K.p+F. supernatant49	0.25	0.35	K.p+F. supernatant31	0.24	0.31	K.p+F. supernatant13	0.26	0.31
K.p+F. supernatant50	0.24	0.31	K.p+F. supernatant32	0.25	0.23	K.p+F. supernatant14	0.24	0.27
			K.p+F. supernatant33	0.21	0.35	K.p+F. supernatant15	0.22	0.26
Negative control	0.031	0.034	K.p+F. supernatant34	0.2	0.3	K.p+F. supernatant16	0.22	0.3
Positive control	0.23	0.99	K.p+F. supernatant35	0.22	0.3	K.p+F. supernatant17	0.2	0.24
Positive control	0.34	1.35	K.p+F. supernatant36	0.22	0.31	K.p+F. supernatant18	0.21	0.27

K.p: *K. pneumoniae;* F.: FlorMidabil.

ent study. The study of Hashemizadeh et al. showed that *K. pneumoniae* isolated from Shahrekord has more than 40% resistance to imipenem and meropenem [14].

In the current study, bla_{NDM} and bla_{IMP} genes were detected, consistent with previous reports. However, in a study conducted in Italy by Esposito, bla_{VIM} was found [18]. Yoon from South Korea reported bla_{NDM} in *K. pneumoniae* isolates [19]. In the study by Esposito et al. [18] *K. pneumoniae* isolates carried the bla_{VIM} gene. Moreover, in the present study, two strains of *K. pneumoniae* harbored the bla_{NDM-5} gene, representing the first report of its presence in Iran. The gene was first isolated from *Escherichia coli* in the UK in 2011 [20]. NDM-5, like NDM-1, is a variant of the New Delhi metallo-beta-lactamase (NDM) enzyme that confers resistance to carbapenems and other beta-lactam antibiotics in bacteria. The NDM-5 differed from NDM-1 in two amino acid replacements at positions 88 (Val→Leu) and 54 (Met→Leu) [21].

It is a concerning resistance profile because the bla_{NDM-5} gene, which encodes the NDM-5 enzyme, is frequently located on plasmids, making it easily transferable between bacteria, and it is often found alongside other resistance genes, contributing to a broader multidrug-resistant phenotype. Also, NDM-5 has been identified in various countries and in different bacterial species, highlighting its potential for widespread dissemination. NDM-5-producing bacteria can result in higher morbidity and mortality, particularly in vulnerable populations like children [21].

The detection of bla_{NDM-5} is not only a laboratory finding but a strong warning signal. It calls for urgent strengthening of molecular-genomic surveillance, stricter infection control, antimicrobial stewardship, and cross-border collaboration, since NDM-5–harboring bacteria can spread rapidly and silently across hospitals, communities, the environment, and international boundaries.

Table 8. The inhibitory effect of *L. plantarum* supernatant on *K. pneumoniae* using the microplate method

Sample ID	OD1	OD2	Sample ID	OD1	OD2	Sample ID	OD1	OD2
K.p + SupernatantL.P37	0.22	0.44	K.p + SupernatantL.P19	0.24	0.42	K.p+supernatantL.P1	0.25	0.45
K.p + SupernatantL.P38	0.21	0.46	K.p + SupernatantL.P20	0.26	0.43	K.p +supernatantL.P2	0.23	0.44
K.p + SupernatantL.P39	0.23	0.48	K.p + SupernatantL.P21	0.23	0.41	K.p + supernatantL.P3	0.25	0.47
K.p + SupernatantL.P40	0.21	0.41	K.p + SupernatantL.P22	0.21	0.43	K.p + supernatantL.P4	0.22	0.47
K.p + SupernatantL.P41	0.21	0.41	K.p + SupernatantL.P23	0.21	0.45	K.p +supernatantL.P5	0.2	0.49
K.p + SupernatantL.P42	0.24	0.4	K.p + SupernatantL.P24	0.23	0.5	K.p + supernatantL.P6	0.21	0.44
K.p + SupernatantL.P43	0.23	0.43	K.p + SupernatantL.P25	0.21	0.4	K.p +supernatantL.P7	0.21	0.44
K.p + SupernatantL.P44	0.2	0.45	K.p + SupernatantL.P26	0.21	0.39	K.p + supernatantL.P8	0.28	0.46
K.p + SupernatantL.P45	0.2	0.44	K.p + SupernatantL.P27	0.24	0.42	K.p + SupernatantL.P9	0.22	0.47
K.p + SupernatantL.P46	0.21	0.47	K.p + SupernatantL.P28	0.22	0.41	K.p + SupernatantL.P10	0.2	0.42
K.p + SupernatantL.P47	0.21	0.5	K.p + SupernatantL.P29	0.29	0.51	K.p + SupernatantL.P11	0.2	0.41
K.p + SupernatantL.P48	0.22	0.41	K.p + SupernatantL.P30	0.22	0.48	K.p + SupernatantL.P12	0.21	0.43
K.p + SupernatantL.P49	0.23	0.42	K.p + SupernatantL.P31	0.26	0.44	K.p + SupernatantL.P13	0.21	0.4
K.p + SupernatantL.P50	0.23	0.41	K.p + SupernatantL.P32	0.23	0.42	K.p + SupernatantL.P14	0.23	0.48
			K.p + SupernatantL.P33	0.23	0.45	K.p + SupernatantL.P15	0.2	0.49
Negative control	0.032	0.033	K.p + SupernatantL.P34	0.22	0.42	K.p + SupernatantL.P16	0.23	0.45
Positive control	0.23	0.99	K.p + SupernatantL.P35	0.2	0.43	K.p + SupernatantL.P17	0.21	0.43
Positive control	0.34	1.22	K.p + SupernatantL.P36	0.2	0.44	K.p + SupernatantL.P18	0.22	0.46

K.p: *K. pneumoniae*.

High transmissibility warning

NDM-5 is typically located on transferable plasmids, enabling horizontal gene transfer across multiple species and high-risk clones (e.g. *E. coli* ST167/ST410). This capability raises concern about silent dissemination in both hospital and community settings, even in the absence of clear epidemiological links.

Need to strengthen surveillance systems

In this regard, we should integrate rapid MBL detection into routine labs: phenotypic screening (mCIM/eCIM, inhibitor-based disk tests with imipenem/meropenem) and molecular confirmation (PCR/multiplex for bla_{NDM} and its variants).

We could also expand genomic surveillance, such as WGS/NGS, to identify subtypes (e.g. NDM-5), plasmid types, track transmission chains, and detect co-carried genes (e.g. ESBLs, 16S RMTases, etc.).

The national data should be centralized, and cross-border data exchange established; alert thresholds for clusters and sudden increases should be defined.

Reinforce infection prevention and control (IPC) in healthcare settings

We should establish targeted screening of high-risk patients (recent hospitalization abroad, medical tourism, transfer from crowded ICUs, extensive antibiotic use).

Contact precautions, including strict hand hygiene, isolation, and environmental monitoring, must be in place.

Cohort culture screening during outbreaks, such as decontaminating high-risk reservoirs (sinks, drains), must be promoted.

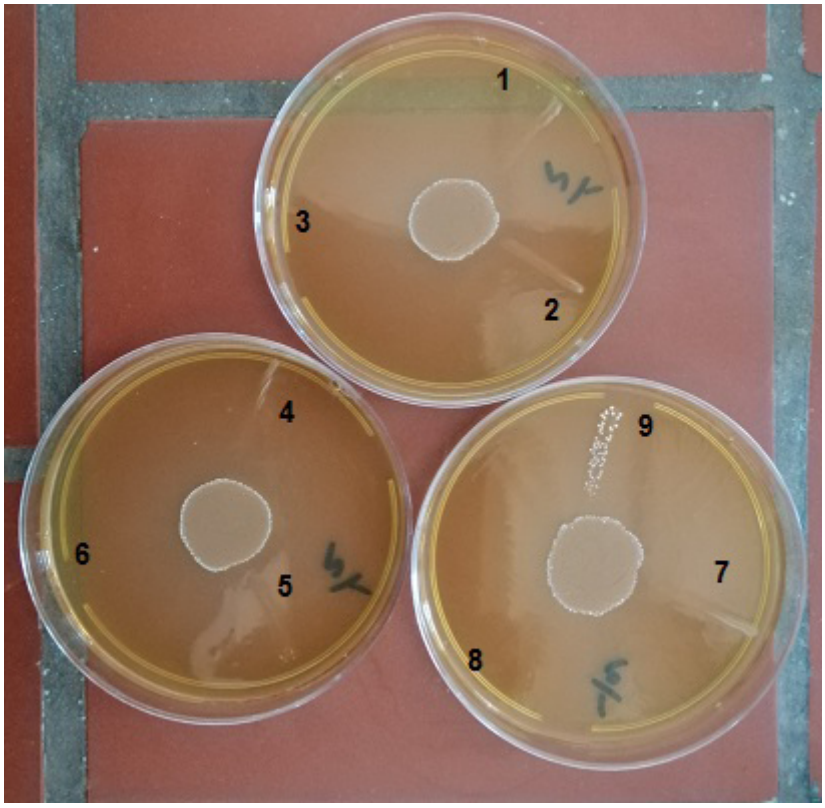

Figure 1. The radial streak method

Note: *L. rhamnosus* was located in the center of the plate, and radial numbers 3 to 8 contained *K. pneumoniae* isolates. Radial streak number 9 was *S. aureus*. Inhibitory effect against *K. pneumoniae* in the form of radial lines without bacterial growth or with very little visible growth.

Antimicrobial stewardship and therapeutic policy

We must update empirical treatment guidelines for high-risk infections, given reduced carbapenem effectiveness against NDM producers.

We advise enhancing stewardship, restricting carbapenem use, rationally applying active combinations (e.g. aztreonam with β-lactamase inhibitors) or reserving drugs based on AST results and availability, and monitoring and reporting consumption and resistance trends.

One health approach and non-clinical surveillance

Non-human reservoirs (hospital/municipal wastewater, livestock, food products, surface water) should be monitored to trace plasmid circulation and hotspots. Hygiene in food supply chains and wastewater management must be strengthened, particularly in high-density cities.

Cross-border collaboration and mobility management

Given regional travel, pilgrimage, and medical tourism, "cross-country notification" of NDM cases and the adoption of common patient screening protocols are essential. We should harmonize laboratory standards and share reference strains across neighboring countries to trace sources and transmission routes.

Laboratory capacity building and data governance

The national reference labs for molecular/genomic confirmation must be empowered, and training for regional laboratories must be provided. We should mandate reporting of carbapenemase-producing organisms with gene/subtype identification (including NDM-5), and publish regular bulletins for healthcare providers.

Outbreak preparedness and response

Establish rapid response teams with predefined protocols (screening, isolation, environmental sampling, se-

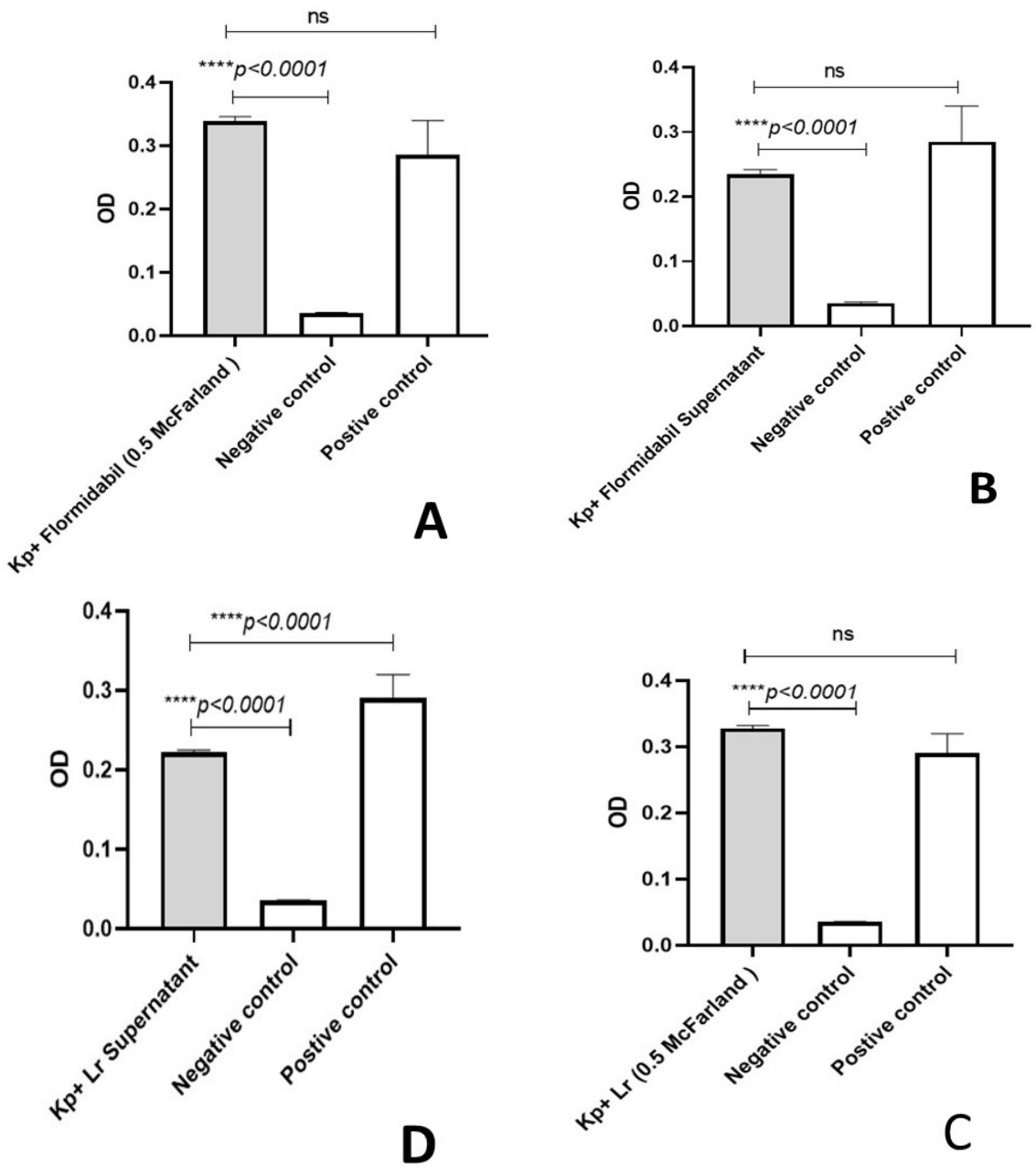

Figure 2. Co-culture probiotic bacteria with *K. pneumoniae* isolates

A) Liquid culture live probiotic (FlorMidabil) with *K. pneumoniae* isolates, B) Supernatant culture from live probiotic (FlorMidabil) with *K. pneumoniae* isolates, C) Liquid culture live native probiotics (*L. rhamnosus*) with *K. pneumoniae* isolates, D) Supernatant culture from live probiotic (*L. rhamnosus*) with *K. pneumoniae* isolates

quencing, and immediate feedback to clinicians and IPC teams).

Probiotic inhibitory effects

There are a few studies on the inhibitory effect of probiotics on *K. pneumoniae*. In one study by Piatek et al. [22], the inhibitory effect of synbiotics (similar to commercial products) against *K. pneumonia*e strains carrying *blaNDM* genes has been reported. The results are completely consistent with the present study [23]. The effects of commercial probiotic products against *K. pneumoniae* isolates carrying bla_{NDM} and bla_{IMP} genes are the first report in this field. Naderi et al. investigated the effect of *Lactobacillus casei* and *L. rhamnosus* against *E. coli* and *K. pneumoniae*. According to this study, the studied lactobacilli had an inhibitory effect only against *E. coli*

[24]. Heydari et al. Investigated the effect of *L. casei*, *L. rhamnosus*, and *L. plantarum* on *K. pneumoniae* ESBL and found probiotics inhibit ESBL bacteria [25]. Mercado also showed the inhibitory effect of *L. casei* on *K. pneumoniae* ESBL [26]. Coman et al. examined the inhibitory effects of *L. rhamnosus*, *L. paracasei*, and synbiotics on a group of gram-positive bacteria, such as *Listeria monocytogenes* and *S. aureus*, and gram-negative bacteria, such as *K. pneumoniae*, as well as fungi. They reported that probiotics and synbiotics have an inhibitory effect on bacteria [9].

Conclusion

The results of this study demonstrated that the *K. pneumoniae* isolate from different clinical samples was highly resistant to antibiotics, especially carbapenems. Considering that these isolates harbored bla_{IMP} and bla_{NDM} genes and that resistance genes can rapidly spread via horizontal gene transfer within the Enterobacteriaceae family, this study suggested using probiotics to control *K. pneumoniae* infection.

Study limitations

The study's conclusions are drawn solely from in vitro experiments, which may not fully capture the complexities of a living organism's environment. Future work should incorporate animal models or clinical samples to confirm these findings. While in vitro assays provide controlled insights, they cannot replicate systemic interactions, immune responses, or pharmacokinetics observed in vivo. Expanding the experimental scope to include multi-organ or organ-on-a-chip platforms could mitigate this limitation.

OD readings can be skewed by differing growth rates, cell morphology, and media opacity when multiple cell types coexist, risking inaccurate quantification of biomass or viability. Employing complementary methods (e.g. flow cytometry, fluorescent tagging, or cell-specific metabolic assays) is advisable to validate OD-derived estimates.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Authors contribution's

Conceptualization: Mohammad Rabbani Khorasgani and Dariush Shokri; Methodology and investigation: Saba Pourrahim; Data curation: Mohammad Rabbani Khorasgani, Dariush Shokri, and Babak Beikzadeh; Writing the original draft: Saba Pourrahim; Review and editing: Saba Pourrahim, Mohammad Rabbani Khorasgani, and Dariush Shokri; Supervision: Mohammad Rabbani Khorasgani.

Conflict of interest

The authors declared no conflict of interest.

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