Academic Journal of International University of Erbil

Journal Homepage: https://ojs.cihanrtv.com/index.php/public

PRINT-ISSN: 2519-6928

Online-ISSN: 3080-7174

Evaluation of the Antibacterial and Antibiofilm Activity of Commercial Probiotics Against Drug-Resistant Gram-Negative Bacteria

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DOI: https://doi.org/10.63841/iue3261071

Received 30 Aug 2025; Accepted 13 Oct 2025; Available online 25 Apr 2026.

ABSTRACT:

The increasing prevalence of drug resistant Gram-negative bacteria has become a serious public health concern, these pathogens often form biofilms that protect them from antibiotics, complicating treatment. Probiotics have recently gained attention as potential adjuncts due to their antibacterial and antibiofilm effects. This study evaluated the resistance profiles, biofilm formation, and in vitro antibacterial and antibiofilm effects of two commercial probiotics Vitalactic B (Lactobacillus only) and PROIBS (Lactobacillus and yeast) against multidrug-resistant Gram-negative isolates in Erbil, Iraq. From 120 clinical samples, 62 drug-resistant isolates were identified using the VITEK2 system. Biofilm formation was assessed by Congo Red and microtiter plate method, and antibacterial activity was tested using the agar slab method. The synergistic interaction with meropenem was also analyzed. Results showed that the isolates included *Escherichia coli* (37.1%), *Klebsiella spp.* (30.6%), *Pseudomonas aeruginosa* (12.9%), *Enterobacter spp.* (12.9%), *Acinetobacter baumannii* (3.2%), *Morganella morganii* (1.6%), and *Burkholderia cepacia* (1.6%). The resistance phenotypes were MDR 45.2%, XDR 25.8%, PDR 22.6%, and Drug sensitive 6.5%. Biofilm detection revealed 47 (75.8%) positives by the Congo Red method and 35 (56.4%) by the microtiter assay. In antibacterial testing, PROIBS demonstrated significantly stronger inhibition than Vitalactic B very strong (≥16 mm) in 61.3% vs 19.4%; strong 29.0% vs 40.3%; moderate 6.5% vs 16.1%; weak 1.6% vs 8.1%; and no inhibition 1.6% vs 16.1%, respectively. Overall inhibition occurred in 98.4% of cases with PROIBS and 83.9% with Vitalactic B. In combination assays, probiotics enhanced meropenem inhibition zones in 57.7% of comparisons, indicating a significant synergistic effect. The high frequency of MDR/XDR/PDR pathogens highlights the need for alternative therapies. PROIBS demonstrated superior antibacterial, antibiofilm, and synergistic effects, suggesting its potential as an adjunct against carbapenem-resistant infections.

Keywords: Gram-negative bacteria; MDR/XDR/PDR; Biofilm; In-vitro synergistic effect, drug resistance.

1 INTRODUCTION

Over the last two decades, there has been a dramatic increase in the MDR organisms, particularly carbapenem-resistant Enterobacteriaceae which are generally difficult to treat, particularly among hospitalized patients [1]. Carbapenem antibiotics are used as a last resort for treating high-risk Gram-negative bacteria, which have become a major source of resistance[2]. Antibacterial resistance has reached worrying levels, with important pathogens (including Gram-negative bacteria like *K. pneumoniae, A. baumannii, and P. aeruginosa*) rapidly evolving into pan-drug resistance (PDR) strains. Inadequate response to this medical issue may lead to a significant increase in the burden and mortality associated with infectious diseases, particularly hospital-acquired bacterial infections [3]. The phenomenon of multidrug resistance (MDR) is generally attributed the repeated and indiscriminate utilization of antibiotics, together with the presence of certain antimicrobial resistance (AMR) genes [4] Multidrug resistance is a developing problem among Gram-negative bacteria. Carbapenems are broad-spectrum β-lactam antibiotics, recognized as a safe and highly effective class utilized as a last resort for managing multidrug-resistant Gram-negative bacteria, including Enterobacteriaceae that

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produce extended-spectrum β-lactamases. Increasing resistance to these last-line treatments is being observed, and there is an increased need to swiftly detect and respond to MDR organisms that are also carbapenem-resistant (CR).The swift identification of carbapenem resistance and the elucidation of its mechanisms are crucial for therapeutic treatment, as well as for the establishment of effective infection control programs and the detection and study of outbreaks [5]. Because of their broad-spectrum activity, carbapenems are commonly used to treat infections that can be fatal. CR rates have risen due to the overuse of these drugs. One of the primary causes of the expression of carbapenemase genes in this family is the overuse of these medications [6]. Probiotics are live microorganisms' bacteria or fungi that are consumed directly and have positive health effects on the host. Probiotics production of short-chain fatty acids (SCFAs) from metabolic precursors has downstream effects similar to immune modulation and enhanced mucosal barrier function [7]. *Lactobacillus spp*.(LAB) is a diverse genus of bacteria containing approximately 145 species of non-spore-forming, typically non-motile, Gram-positive rods that are catalase negative, lack cytochrome, can thrive in anaerobic or microaerophilic environments, and produce lactic acid as the primary or only byproduct of fermentative metabolism[8].Furthermore, earlier research revealed that the Lactobacilli bacteria create a number of bactericidal substances. The results revealed that the primary isolates of Lactobacillus strains' antimicrobial activity are their synthesis of organic acids and bacteriocins [9]. In addition to bacterial probiotics, the yeast Saccharomyces cerevisiae, specifically the subspecies boulardii, is acknowledged for its probiotic characteristics. This substance enhances gut health by improving intestinal barrier function, modulating immune responses, and demonstrating direct antagonistic effects against various pathogens, thereby serving as a valuable adjunct in the management of dysbiosis and infections. The utilization of probiotics in the feed may help to improve animal health and slow the emergence of resistance to antibiotics [10]. In Erbil, research has emphasized the ongoing problem of biofilm producing bacteria in healthcare environments, highlighting the regional significance of resistance mechanisms and the necessity for alternate therapeutic approaches [11].Moreover, research into innovative strategies such as phage therapy targeting multidrug-resistant Klebsiella pneumoniae biofilms in regional hospitals illustrates the persistent endeavours to address these issues[12]. The aim of this study to investigate the prevalence and characteristics of carbapenem resistance in Gram-negative bacteria isolated from clinical samples in Erbil, Iraq, and to evaluate the antibacterial, antibiofilm, and synergistic effects of commercial probiotic formulations (*Lactobacillus* and *Saccharomyces* species) alone and in combination with meropenem against these resistant strains.

2 MATERIAL AND METHODS

2.1 SPECIMENS COLLECTION, ISOLATION AND IDENTIFICATION OF BACTERIA

From July to December 2024, we collected 120 Gram-negative bacilli cultures, comprising both non-fermenting bacteria and Enterobacteriaceae, from various clinical specimens. All isolates were obtained from patients in both hospital and community settings across Erbil, Kurdistan Region, Iraq.Upon collection, specimens were cultivated on blood and MacConkey agar plates and incubated at 37 °C for 24 hours. Gram staining confirmed Gram-negative morphology. All isolates were subsequently identified using the VITEK-2 Compact System (bioMérieux, Marcy L'Étoile, France), following the manufacturer's instructions for phenotypic identification via biochemical processes. From these 120 initial cultures, 62 isolates were selected for further comprehensive testing, comprising 35 carbapenem-resistant (CR) and 27 non-carbapenem-resistant strains

2.2 ANTIBIOTIC SENSITIVITY TESTS

The VITEK-2 Compact System (bioMérieux, Marcy L'Étoile, France) was employed in clinical laboratories for the screening and identification of each isolate. Procedures were performed in accordance with the manufacturer's guidelines. This method, known as phenotypic identification, uses biochemical processes to characterize isolates. After overnight incubation at 37 °C, the specimens were inoculated onto MacConkey agar plates. Following incubation, a single colony was selected and suspended in saline. The turbidity of the suspension was adjusted to a McFarland 0.5 standard using a 0.45% VITEK-saline solution and a VITEK DensiCHEK (bioMérieux, France) photometric device. The VITEK-2 Compact System was then loaded with the VITEK 2 ID-GN (Gram-negative) card and the prepared bacterial suspension [13]. Following the identification of Gram-negative isolates, their antimicrobial susceptibility patterns were assessed using the VITEK-2 Compact System for 11 therapeutically relevant antibiotics, adhering to the designated AST-N417 and AST-N419 criteria established by the Clinical and Laboratory Standards Institute (CLSI). The isolates were classified based on their antimicrobial resistance profiles. Classification was based on the criteria defined by the European Centre for Disease Prevention and Control (ECDC). According to ECDC definitions: the MDR isolates exhibit resistance to at least one agent in a minimum of three antimicrobial categories. XDR isolates show resistance to at least one agent in all but two or fewer antimicrobial categories. PDR isolates are resistant to all agents in all tested antimicrobial categories [14].

2.3 PHENOTYPIC DETECTION OF BIOFILM-FORMING ISOLATES

2.3.1 MICROTITER PLATE METHOD

According to Ghellai et al [15], the biofilm formation test was conducted as follows: a 96-well flat-bottom microplate was inoculated with 180 µL of brain heart infusion (BHI) broth supplemented with 1% glucose (BHIG) and 20 µL of an overnight bacterial culture (1.5×10^8 CFU/mL). A volume of 200 µL of BHIG broth alone was used as the negative control. After sealing with Parafilm, each plate was incubated for 24 hours at 37 °C under aerobic conditions. Following incubation, the wells were gently washed three times with phosphate-buffered saline (PBS, pH 7.1) after removing non-adherent bacterial cells. The plate was then oven-dried for 60 minutes at 60 °C. After drying, 100 µL of 0.1% crystal violet solution was added to each well and left for 20 minutes. Excess crystal violet was discarded, and each well was washed three times with PBS to remove unbound stain. The plate was left to air-dry at room temperature for 15 minutes. Subsequent, 200 µL of 95% ethanol was added to each well, and the plate was incubated at 4 °C for 30 minutes. Biofilm formation was quantified by measuring the absorbance at 630 nm for each well, including the negative control, using an ELISA microplate reader.

2.3.2 CONGO RED AGAR METHOD

Congo red agar (CRA) is a specially prepared medium composed of brain heart infusion (BHI) broth (37 g/L) supplemented with sucrose (50 g/L), agar No. 1 (10 g/L), and Congo red (0.8 g/L). A concentrated aqueous solution of Congo red stain was prepared and autoclaved for 15 minutes at 121 °C. It was then added to the BHI agar, which had been previously autoclaved and cooled to 55 °C, along with the sucrose supplement. The isolated pathogens were inoculated onto the prepared CRA plates, which were then incubated aerobically at 37 °C for 24 hours. On the CRA plates, the presence of black, dry, crystalline colonies indicated biofilm production, while the colonies of non-biofilm producers remained colorless [16].

Reference strain Quality control strain Escherichia coli ATCC 25922, a known susceptible strain to carbapenems used as a negative control[17] .

2.4 PREPARE THE PROBIOTICS

2.4.1 AGAR SLAB METHOD

This study featured two commercial probiotic formulations, Vitalactic B capsule and PROIBS sachet, both obtained directly from a local pharmacy. Vitalactic B, a product of Vitane Pharmaceuticals originating in the USA, comprises a probiotic bacterial culture predominantly consisting of *Lactobacillus plantarum* and *Lactobacillus acidophilus*. The second formulation, PROIBS, manufactured by the Swedish company Probi, has a proprietary combination of aloe leaf extract and, in this specific formulation, also incorporates *Saccharomyces* species.Both formulations were prepared to a density of 1.8 × 10^9 CFU/mL (6.0 per McFarland Standard) and inoculated onto Petri dishes containing MRS agar, then incubated for 24 hours at 37 °C. Disks with a diameter of 10 mm were subsequently cut in triplicate from the solid medium using a sterile cork borer and placed on an appropriate agar medium containing strains of carbapenem-resistant pathogens at a density of 6.0 × 10^8 CFU/mL, corresponding to a McFarland Standard of 2.0. Following a 24/48-hour incubation at 37 °C, the zones of growth inhibition were measured, with the diameter of the disc subtracted from the results.The following criteria were adopted to compare the antagonistic activity of the LAB strains against various microorganisms [18].The following criteria were adopted: the growth inhibition diameter above 16 mm very strong inhibition, 11–15.9 mm strong inhibition, 6–10.9 mm moderate inhibition, 1–5.9 mm weak inhibition, 0 mm no inhibition as shown in Graphical Abstract 1.

2.4.2 PREPARATION OF CFS

The cell-free supernatant (CFS) of the studied probiotic strains was prepared as previously described by Sutyak K et al [19]. These are specific lactobacilli strains. After being added to MRS broth, these bacteria were incubated aerobically for 24 hours at 37 °C. Centrifugation (4480 rpm for 30 min at 4 °C) was used to extract the cells. A Millipore 0.45 µm polytetrafluoroethylene (PTFE) syringe filter was used to filter-sterilize the collected supernatants, which were then stored in sterile tubes at 4 °C until needed. This is regarded as a 100% stock solution of probiotic microorganisms from CFS.

2.5 DETERMINATION OF ANTIBIOFILM ACTIVITY OF LACTIC ACID BACTERIA AGAINST PATHOGENIC BACTERIA

Lactic acid bacteria that have been were sub-cultured in tubes of 15 ml containing 5 ml of MRS broth and then placed in an incubator at 37°C for a period of 16 hours. Isolates of pathogenic were re cultured in brain heart infusion broth, which was then incubated at 37 °C for 18 hours, after determining McFarland standard solution, a total of 100 µL of the culture was dispensed into each well of the 96 well plates. After this step, 100 µL of LAB supernatant was added. Finally, as a negative control, MRS Broth was added to the plates. The plates were kept in an incubator for 16 hours at 37 °C: Crystal violet was used to investigate biofilm formation. The cultures were removed and the plates were submerged in water

three times to clean them. Each well filled with 125 µL of 0.1% crystal violet solution and was incubated at room temperature (RT) for 15 minutes. The staining solution was removed and the plate was rinsed with sterile water three times to remove excess stain. After dilution with 125 µL of 30% acetic acid per well and 15 minutes of incubation at room temperature, the optical density (OD) of the supernatant and pellet was measured at 570nm using an enzyme-linked immunosorbent assay (ELISA) reader [20].

2.6 ANTIBIOTICS AND PROBIOTICS COMBINATION

The effectiveness of antibiotics and probiotics was examined separately. The efficacy of these agents was then assessed in combination during the experiment. Pathogenic strains were initially suspended and spread onto Müller-Hinton agar plates. Subsequently, a meropenem disc was combined with 20 µL of each of two commercial probiotics and placed on the MHA agar. The plates were incubated at 37 °C for 24 hours. Subsequently, the sizes of the inhibitory zones were measured and recorded [21].

2.7 ETHICAL STATEMENT

This study was reviewed and approved by the Institutional Ethics Committee of Hawler Medical University College of Health Sciences, with Approval Code 10B5, ensuring compliance with ethical standards for research involving human subjects.

2.9 STATISTICAL ANALYSIS

Data were analyzed using SPSS version 27.0. Categorical variables were assessed using Chi-square and Fisher's Exact Tests, and one-way ANOVA, primer 7. A p-value < 0.05 was considered statistically significant.

3 RESULT AND DISCUSSION

This study meticulously analyzed 120 Gram-negative bacterial isolates to delineate the prevalence and distribution of carbapenem resistance (CR) across various demographic and clinical parameters. Of the total isolates, 35 (29.17%) exhibited carbapenem resistance. Regarding sample source, urine was the most frequently isolated specimen, accounting for 79 (65.85%) of all isolates and 22 (62.86%) of the CR isolates, Comparative analysis across specimen types revealed a significant association between CR and urine samples ($\chi^2 = 0.007$, $p < 0.05$) underscoring its significant role in the epidemiology of these infections. While stool samples contributed a low proportion of both total (10.00%) and CR isolates (2.86%), blood samples, despite representing only 6.67% of total isolates, yielded a notable 14.29% (5/35) of CR isolates. In terms of hospital affiliation, Bio lab (40.0%) and Dayk private hospital (26.67%) were the primary sources of overall isolates; however, Nanakali hospital contributed the highest percentage of CR isolates (22.86%), followed closely by Bio lab (31.43%). Conversely, Floria lab, comprising 1.67% of total isolates, yielded no CR isolates. Analyzing patient by sex, females constituted a larger proportion of the total isolates (75.0%), but the distribution of CR isolates was more balanced, with females accounting for 54.3% and males for 45.7%. Age-based comparison revealed a strong association between CR and older age groups. Patients aged ≥50 years accounted for 27.5% of total isolates and 40.0% of CR isolates, indicating a statistically significant relationship (Fisher's Exact, $p = 0.03$). The 20–29-year group, despite contributing 15.8% of total isolates, exhibited no carbapenem resistance ($p < 0.05$).Finally, regarding types of bacteria, *Escherichia coli* was the most prevalent species overall (50.83%), but *Klebsiella sp.* emerged as the predominant CR species, accounting for 40.0% of all CR isolates, followed by *E. coli* (25.7%) and *Pseudomonas sp.* (17.1%), Primer 7 statistics underscored several epidemiologic links: *Klebsiella* ↔ urine ($\chi^2 = 0.007$, $p < 0.05$); 42% of *E. coli* cases occurred in ages 40–49 (Fisher's Exact, $p = 0.002$); Pseudomonas in wounds were exclusively PDR ($\chi^2 = 0.03$, $p < 0.05$); Acinetobacter occurred only in sputum (Fisher's Exact, $p < 0.001$); MDR ↔ urine ($\chi^2 = 0.001$, $p < 0.01$); XDR ↔ age ≥50 (Fisher's Exact, $p = 0.03$); and PDR ↔ wounds ($\chi^2 = 0.02$, $p < 0.05$). Collectively, these indicate specimen type and age are strong determinants of resistance distribution. highlighting their significant contribution to carbapenem resistance in the region. The frequency of carbapenem-resistant isolates in this study was 29.17%, which indicates a significant level of carbapenem resistance among the Gram-negative bacilli in Erbil city. This finding is consistent with the increasing prevalence of carbapenem resistance reported in other developing countries, including Pakistan by [22].Since blaNDM-1 was first reported from India in 2009, it is believed that Carbapenem Producing Organisms are highly prevalent in this region and are disseminating globally from there, in this study, *Klebsiella* sp. was the most prevalent carbapenem-resistant strain, accounting for 40.0% of the carbapenem-resistant isolates. This finding is consistent with the results reported by[23],that done in Najaf- Iraq , in the recent years, carbapenem resistance in *K. pneumoniae* is emerging in Iraq [24],followed by *E.coli* 25.7% which is second more resistance strain after *klebsiella sp*. A statistical comparison revealed a significantly higher prevalence of carbapenem resistance in *Klebsiella sp.* isolates (40.0%) compared to *E. coli* isolates (25.7%) p value = <0.005, indicating a notable difference in resistance rates between these two species. In contrast another result was reported in India by[25] that *E.coli* more resistance strain in comparison with *klebsiella sp.* The prevalence and distribution of specific carbapenemase-producing organisms can vary significantly across geographical regions due to differences in antibiotic usage patterns, infection control practices, and circulating bacterial clones. as represented in Table 1.

In addition, Chi-square and Fisher's Exact tests from Primer 7 highlighted further associations: *Klebsiella* isolates were significantly associated with urine specimens ($\chi^2 = 0.007$), while 42% of *E. coli* isolates occurred in the 40–49 age group (Fisher's Exact, $p = 0.002$). *Pseudomonas* from wound samples were exclusively PDR ($\chi^2 = 0.03$), and *Acinetobacter* was found only in sputum specimens (Fisher's Exact, $p < 0.001$). MDR isolates were strongly associated with urine samples ($\chi^2 = 0.001$), XDR isolates were most frequent in patients ≥50 years (Fisher's Exact, $p = 0.03$), and PDR isolates clustered in wound samples ($\chi^2 = 0.02$). these comparative findings demonstrate that carbapenem resistance is significantly associated with older age, urinary specimens, and Klebsiella spp. prevalence, highlighting their critical role in the regional dissemination of resistant Gram-negative pathogens.

The distribution of antimicrobial resistance patterns among the 62 Gram-negative isolates showed that most of them were MDR, XDR or PDR. Only a small number of isolates were fully drug sensitive.

Klebsiella spp. made up a large number of resistant isolates, with 42.1% being MDR, 21.1% being XDR, and 31.6% being PDR. Only 5.3% were fully susceptible. Resistance among *E. coli* was also high: 56.5% MDR, 26.1% XDR, and 13.0% PDR, with only 4.3% drug sensitive isolates. Statistical comparison showed no significant difference in overall MDR frequency between *E. coli* and *Klebsiella spp.* ($p > 0.05$), but the proportion of PDR isolates was slightly higher in *Klebsiella spp.* than in *E. coli* ($p < 0.05$) This shows that it is becoming harder to treat this common pathogen. The prevalence of XDR and PDR in *Klebsiella spp*., *E. coli*, *P.aeruginosa*, and *A.baumannii* signifies that these pathogens impose the most significant burden of antimicrobial resistance in the dataset. In contrast another study in Ramadi- Iraq by [26], show that phenotypic classes of resistances on *A. baumanni* all finding was MDR, XDR respectively .The Iraqi studies in different provinces of Iraq reveals different rates of resistance bacteria , these differences in the mean prevalence rates among various studies could be related to differences in methods that used in bacterial detection ,geographical locations and hygienic practices of the populations[27]. as displayed in table 2.

In this study, most resistance was in urine specimen 65.71% similar result was reported by [28]. that done in Thiqar-Iraq followed by blood 14.29%. Most studies found that Gram-negative bacteria are the most common cause of UTIs rather than Gram-positive bacteria [29], This phenomenon is attributed to the elevated concentration of Gram-negative bacteria in the intestine, alongside various virulence factors inherent to these bacteria, including their unique structural composition and specific adhesion proteins that facilitate adherence to urinary epithelial cells, thereby accelerating the proliferation of urinary tract infections [30]. The statistical analysis indicated a notable difference in the prevalence of various bacterial pathogens and carbapenemase-resistant isolates obtained from diverse sample types ($p < 0.05$)

The analysis of antimicrobial resistance by age and sex revealed distinct patterns. Drug-resistant infections were most common in patients aged 50 years and older, who accounted for 42.86% of all PDR cases and also had the highest numbers of MDR and XDR infections, particularly with *E. coli* and *Klebsiella spp.* This age group showed a significantly higher prevalence of resistance compared with all younger age groups (Fisher's Exact, $p = 0.03$). The 40–49-year age group also contributed significantly to the burden of XDR and PDR. In contrast, resistant infections were least frequent among younger groups, especially those aged 10–19 years and 20–29 years, each representing only 7.14% of PDR cases. The 0–10-year age group showed a moderate proportion of resistance, contributing 14.29% of PDR cases, primarily due to *E.* coli and *Klebsiella spp.* infections.

With regard to sex, females were disproportionately affected, making up 71.43% of PDR cases and most of the MDR infections, with *E. coli* accounting for 9 of the 13 MDR isolates in women. In contrast, males represented only 28.57% of PDR cases and had fewer MDR or XDR infections overall. These findings highlight a clear difference in resistance burden between males and females, suggesting the need for further investigation into factors such as patterns of antibiotic exposure, healthcare access, and possible biological susceptibility, similarly the South African study [31]. reported a median age of 33 years among patients with CRE bacteremia, with a significant proportion of infections occurring in older adults. The study indicated that patients aged 60 and older exhibited higher risks of in hospital mortality due to various comorbidities, increased rates of hospitalization and antibiotic exposure, and the presence of indwelling catheters. Reports indicate that the rising prevalence of resistant microorganisms leads to ineffective antibiotic therapy in more than half of elderly patients with infections [32]. previous research on CRKP infection in older patients has primarily focused on hospital acquired infections. However, it has been observed that between 0.04 and 29.5% of carbapenem-resistant bacteria are of community origin, with this percentage showing an ongoing rise [33].

Primer 7 statistical analysis also confirmed a strong relationship between specimen type and patient age ($\chi^2 = 68.3$, $p < 0.001$). For example, 54% of urine isolates came from patients aged 40–49 years, and 63% of blood isolates came from children aged 0–10 years. These correlations suggest demographic clustering of resistant infections.

Throughout all age groups, the incidence of urinary tract infections in females exceeds that of males, although the underlying causes and specific pathogens may vary. The structural differences between sexes result in women's urethras being shorter than those of men. Bacteria can travel a shorter distance to reach the bladder in women [34]. Furthermore, a woman's heightened susceptibility to urinary tract infections is primarily attributable to hormonal fluctuations that take place during her menstrual cycle [35]. as displayed in table 3.

The Congo Red method found 47 biofilm-positive cases (75.81%) and 15 biofilm-negative cases (24.19%) out of 62 bacterial isolates. The Microtiter plate method, on the other hand, found 35 biofilm-positive isolates (56.45%) and 27 negative cases (43.55%). The Congo Red method found positive samples 19.36% more often than the other method. The Microtiter plate method found 19.36% more negatives than the Congo Red method for negative samples. This symmetrical difference shows that the two methods always give different results when it comes to detection as shown in table 4, the Congo Red method yielded a higher percentage of positive results than the Microtiter plate method.

Comparative statistical testing (ANOVA, Primer 7) revealed that antibiofilm activity varied significantly with biofilm strength. Vitalactic B demonstrated significant group differences ($F = 4.32$, $p = 0.009$), while PROIBS also showed significant variation ($F = 3.97$, $p = 0.013$). Moreover, Chi-square confirmed a strong association between biofilm formation and probiotic inhibition: Vitalactic B ($\chi^2 = 27.8$, $p = 0.0001$) and PROIBS ($\chi^2 = 19.4$, $p = 0.003$). These results validate that probiotic effects were not random but strongly dependent on the biofilm phenotype of the isolates.

The formation of biofilm by bacteria is a significant virulence factor, contributing to increased antibiotic resistance and persistent infections [36]. Many Gram-negative bacteria can form biofilms, providing a protective environment that enhances bacterial survival and resistance to both environmental stresses and antimicrobials [37]. Within a biofilm, sessile bacteria exhibit a dormant or stationary growth phase with distinct phenotypic characteristics compared to planktonic bacteria [38] .

As shown in table 5 This study investigated the antibiofilm activity of 100% cell-free supernatant (CFS) of *Lactobacillus* alone and in combination with *Saccharomyces* against biofilms. Treatment of weak biofilm forming strains with *Lactobacillus* alone and in combination with *Saccharomyces* CFS resulted in a significant reduction in biofilm structural integrity ($p < 0.05$). In moderate and strong biofilm-forming strains, the combination of *Lactobacillus* and *Saccharomyces* CFS promoted a markedly greater decrease in biofilm integrity compared with Lactobacillus CFS alone. This enhanced effect suggests a synergistic interaction between the metabolites produced by these two probiotic genera. Research by Isayenko et al. [39], has specifically demonstrated the synergistic antibiofilm activity of metabolite complexes derived from *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii* against polyresistant Gram-negative microorganisms, including *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Similarly, studies on polymicrobial biofilms have shown that probiotic combinations, including *Saccharomyces boulardii* and *Lactobacillus* species, are effective in disrupting complex biofilm structures [40].This synergy likely results from the various antimicrobial compounds and biofilm-modulating factors produced by each probiotic, which, when combined, can target multiple pathways involved in biofilm formation and maintenance, leading to more effective disruption.

Similar study reported that lactobacillus has reduced the biofilm forming category [41].another study show similar results that done in France by [42] ,show that significantly reduced biofilm biomass. Khiralla et al. reported that three Lactobacillus strains isolated from traditional products are recommended as biocontrol agents due to their ability to inhibit pathogen biofilm formation [43]. The present work combined with these previous reports, suggest that probiotic-derived CFS, especially the *Lactobacillus* and *Saccharomyces* combination, represents a promising strategy for combating biofilm associated infections caused by drug-resistant bacteria. This approach could potentially reduce the reliance on conventional antibiotics and mitigate the spread of antibiotic resistance. Further research is needed to identify the specific bioactive components responsible for the antibiofilm activity and to evaluate the efficacy of this approach in clinical settings.

Slab diffusion method to test the antimicrobial activity of two agents, Vitalactic B and Pro IBS, against a wide range of bacteria that had been isolated in the clinical specimen. We used well-known criteria to figure out what the inhibition zones meant. Zones larger than (16 mm) meant very strong inhibition, (11–15.9 mm) meant strong inhibition, (6–10.9 mm) meant moderate inhibition, (1–5.9 mm) meant weak inhibition, and (0 mm) meant no inhibition. Comparative analysis demonstrated that ProIBS always had more antimicrobial activity than Vitalactic B. Pro IBS caused a lot of strong inhibition (≥16 mm) in both PDR and XDR strains, which is a lot for all of the isolates. For example, *Klebsiella sp*.7 PDR (urine) had a (29 mm) inhibition zone, E. coli 10 PDR (blood) had a (22 mm) zone, *E. coli* 1 XDR (blood) had an (18 mm) zone, and *Pseudomonas sp.*4 PDR (urine) had a (21 mm) zone all of which were very strong inhibition zones. Even strains that were very resistant, like PDR *A.baumannii* and *Burkholderia cepacia,* were strongly to very strongly inhibit by ProIBS. On the other hand, Vitalactic B had a less strong inhibitory effect. When exposed to Vitalactic B, some MDR and XDR strains, like *Klebsiella sp.*1 MDR, *Klebsiella sp*.2 MDR, *Klebsiella sp.*5 MDR, and *E. coli* 19 MDR, showed no inhibition (0 mm). Most of the inhibition zones that did respond were in the moderate to strong range, but there were a few exceptions. For example, *E. coli* 10 PDR and *Pseudomonas sp.*5 XDR showed very strong activity (20 mm and 17 mm, respectively). The distribution of susceptibility responses also changed depending on the type of specimen. The most common source of isolates was urine, and many of them were MDR or XDR. Pro IBS worked very well against these strains. Blood isolates also reacted better to Pro IBS than to Vitalactic B. Younger age groups (0 to 10 years) also had a lot of resistant isolates, which shows that people can be exposed to or carry resistant bacteria at a young age. For instance, *E. coli* 19 MDR and *Klebsiella sp.*3 XDR came from this age group, and in most cases, Pro IBS showed

at least strong inhibition. this study demonstrates that probiotics can inhibit the growth of drug-resistant bacteria in vitro, with a mixed bacteria and yeast probiotic exhibiting greater inhibitory activity than a bacteria only probiotic. This finding aligns with previous research highlighting the potential of probiotics to combat carbapenem resistance. Another study reported by [44]. Research conducted in Turkey indicates that all tested CFSs (*Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus acidophilus, and Lactobacillus rhamnosus*) at various concentrations effectively inhibited the growth of CRK strains. A further study conducted in Korea by [45]. show that *Saccharomyces boulardii* and *Lactobacillus rhamnosus* were selected as probiotics because they have been shown to decolonize multi drug resistance organism including carbapenem resistance. A combination of *Lactobacillus* and *Saccharomyces* may exhibit stronger antibacterial activity than *Lactobacillus* alone due to several potential synergistic mechanisms Lactobacillus primarily exerts antibacterial effects through the production of lactic acid, other organic acids, bacteriocins, and competition for nutrients, *Saccharomyces* (a yeast) may employ different mechanisms, such as the production of mycocins (antimicrobial peptides produced by yeast), competition for binding sites, and modulation of the host's immune response.These diverse mechanisms can work together to inhibit a broader spectrum of bacteria or enhance the inhibitory effect on specific pathogens [46, 47]. These diverse mechanisms can work together to inhibit a broader spectrum of bacteria or enhance the inhibitory effect on specific pathogens. For instance, the combined action of *Lactobacillus* and *Saccharomyces* has been shown to improve gut barrier function and reduce inflammation, indirectly contributing to pathogen control [48]. Furthermore, the unique metabolic byproducts from the co-fermentation of these two distinct microbial types can create an environment less favorable for pathogen growth and survival [49]. Such combinations offer a promising avenue for developing novel strategies to overcome the increasing challenge of carbapenem-resistant bacterial infections. as displayed in table 6.

The present study demonstrates that the combination of probiotics and meropenem displayed in Graphical Abstract 2 in many cases, enhance the inhibitory effect on meropenem resistant bacteria. This observation suggests a potential synergistic interaction between probiotics and this antibiotic. The fact that 7.69% of isolates showed no effect from the combination indicates that this synergy is not universal and may depend on specific bacterial strains or resistance mechanisms. Similarly, the 34.62% of isolates where probiotics had no effect on the zone size suggests that in a subset of cases, the probiotics did not alter the activity of meropenem. The observation that PROIBS enhanced the zone diameter to a greater extent than Vitalactic B in 57.69% of cases suggests that the specific composition of the probiotic influences its interaction with antibiotics. This difference in efficacy may be attributed to variations in the bacterial or yeast strains present in each probiotic formulation, or to differences in the concentration or types of bioactive compounds produced Similar study reported in Taiwan by [50].that probiotic enhance zone diameter of carbapenem resistance Enterobacteriaceae. Similarly another study in India reported by [51]. This study evaluated the antibacterial effects of six *Lactobacillus* strains on *B. cereus*, *S. aureus*, and *E. coli* using a cut well assay. *L. rhamnosus* showed the strongest inhibition against all tested pathogens. Conversely, *L. acidophilus* and *L. bulgaricus* did not inhibit *S. aureus*. The study concluded that while most *Lactobacillus* strains possess significant antibacterial activity, the effectiveness varies among strains, potentially due to acid production and other microbial components, aligning with prior research. The synergistic enhancement of meropenem activity by probiotics against carbapenem-resistant Gram-negative bacteria can be explained by various underlying mechanisms. Probiotics, specifically Lactobacillus and Saccharomyces species, generate a variety of metabolites that can directly or indirectly affect bacterial resistance mechanisms. A critical mechanism involves the permeabilization of the outer membrane in Gram-negative bacteria. The outer membrane serves as a significant barrier, restricting the penetration of various antibiotics, such as meropenem. Compounds derived from probiotics, including specific peptides or organic acids, can compromise the barrier, increasing the permeability of bacterial cells and rendering them more vulnerable to antibiotics that would typically be excluded [52, 53]. Additionally, certain probiotic metabolites have demonstrated the ability to inhibit bacterial efflux pumps. Efflux pumps represent a significant mechanism of multidrug resistance in Gram-negative bacteria, facilitating the active expulsion of antibiotics from the bacterial cell. Reducing the activity of these pumps allows probiotics to increase the intracellular concentration of meropenem, thus restoring or enhancing its antibacterial efficacy [54].This is especially pertinent to carbapenem resistance, as efflux systems frequently play a substantial role in the resistant phenotype. In addition to direct interactions, probiotics can modulate the host immune response, create an unfavorable microenvironment through the production of short-chain fatty acids that lower pH, and compete with pathogens for nutrients and adhesion sites. These mechanisms can indirectly contribute to a reduction in bacterial burden and enhance the effectiveness of antibiotics [49]. The differing efficacy of PROIBS and Vitalactic B likely indicates variations in their specific strains and the distinct combinations of bioactive compounds they generate, resulting in varied effects on complex resistance mechanisms. This comprehensive strategy, which integrates direct antimicrobial actions, biofilm disruption, and interference with resistance mechanisms such as efflux pumps and membrane permeability, highlights the considerable potential of probiotics as effective adjuncts to standard antibiotic therapy in addressing the increasing challenge of carbapenem resistance [55].

CONCLUSION

The research revealed a significant incidence of multidrug-, extensively drug-, and pan-drug-resistant Gram-negative bacteria in clinical samples from Erbil, Iraq, with *E. coli* and *Klebsiella spp.* as the predominant pathogens. Comparative analyses demonstrated that the probiotic PROIBS (comprising *Lactobacillus* and *Saccharomyces*) displayed markedly

superior antibacterial and antibiofilm properties compared to Vitalactic B *(Lactobacillus* alone). Both probiotics had a synergistic effect with meropenem, with PROIBS yielding a more pronounced augmentation of inhibition zones. These results validate those probiotic formulations, especially those integrating bacterial and yeast species, are capable of fighting drug-resistant Gram-negative bacteria and may operate as beneficial complements to antibiotic treatment.

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Table 1. Distribution of Carbapenem-Resistant Gram-Negative Bacterial Isolates by Source, Hospital, Sex, Age, and Species

Parameter	Category	Total Isolates (%)	CR Isolates (%)
Sample Source	Urine	79 (65.9%)	22 (62.9%)
	Blood	8 (6.7%)	5 (14.3%)
	Stool	12 (10.0%)	1 (2.9%)
	Other (swab, sputum, etc.)	21 (17.4%)	7 (20.0%)
Hospital	Bio lab	48 (40.0%)	11 (31.4%)
	Nanakali Hospital	10 (8.3%)	8 (22.9%)
	Others (combined)	62 (51.7%)	16 (45.7%)
Sex	Female	90 (75.0%)	19 (54.3%)
	Male	30 (25.0%)	16 (45.7%)
Age Group	≥50 years	33 (27.5%)	14 (40.0%)
	40–49 years	21 (17.5%)	10 (28.6%)
	0–10 years	15 (12.5%)	7 (20.0%)
	Other ages	51 (42.5%)	4 (11.4%)
Bacterial Species	*E. coli*	61 (50.8%)	9 (25.7%)
	Klebsiella sp.	32 (26.7%)	14 (40.0%)
	Pseudomonas sp.	13 (10.8%)	6 (17.1%)
	Others (combined)	14 (11.7%)	6 (17.2%)

Table 2. Indicates a significant occurrence of MDR, XDR, and PDR phenotypes in gram negative bacteria.

Organism	N [%]	MDR [%]	XDR [%]	PDR [%]	Drug sensitive [%]
Klebsiella sp.	19[30.6]	8 [42.11]	4[21.05]	6[31.58]	1[5.26]
Enterobacter Spp	4[6.5]	2[50.0]	1[25.0]	0	1[25.0]
Pseudomonas aeruginosa	8[12.9]	2[25.0]	3[37.5	2[25.0]	1[12.5]
Acinetobacter baumanii	2[3.2]	0	1[50.0]	1[50.0]	0
E. coli	23[37.1]	13[56.52]	6[26.09]	3[13.04]	1[4.35]
Morganella morganii	1[1.6]	1[100.0]	0	0	0
Bulkhorderia cepacia	1[1.6]	0	0	1[100.0]	0
Proteus sp.	4[6.5]	2[50.0]	1[25.0]	1[25.0]	0
Total	62	28[45.2]	16[25.8]	14[22.6]	4[6.5]

Table 3. Sex and Age Profile of Patients with Multidrug-Resistant Gram-Negative Bacterial Infections.

Bacteria	Total isolates n (%)	MDR n (%)	XDR n (%)	PDR n (%)	Drug sensitive (%)	0–10	10–19	20–29	30–39	40–49	50+	Male	Female
E. coli	23 (37.1%)	13 (56.5%)	6 (26.1%)	3 (13.0%)	1 (4.3%)	1	1	0	0	0	1	0	3
Klebsiella spp.	19 (30.6%)	8 (42.1%)	4 (21.1%)	6 (31.6%)	1 (5.3%)	0	0	0	2	2	2	2	4
Pseudomonas aeruginosa	8 (12.9%)	2 (25.0%)	3 (37.5%)	2 (25.0%)	1 (12.5%)	0	0	0	0	0	2	1	1
Acinetobacter baumannii	2 (3.2%)	0 (0%)	1 (50.0%)	1 (50.0%)	0 (0%)	0	0	0	0	1	1	0	1
Proteus spp.	2 (3.2%)	2 (100%)	1 (50.0%)	1 (50.0%)	0 (0%)	0	0	1	1	1	0	1	0
Enterobacter spp.	2 (3.2%)	2 (100%)	1 (50.0%)	0 (0%)	1 (50.0%)	1	0	0	1	0	0	1	0
Morganella morganii	1 (1.6%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0	0	1	0	0	0	0	1
Burkholderia cepacia	1 (1.6%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1	0	0	0	0	0	1	0
Total	62	28	16	14	4	11	3	5	15	14	18	26	36

Table 4. Comparison of Biofilm Detection Results Using Congo Red Agar and Microtiter Plate Methods

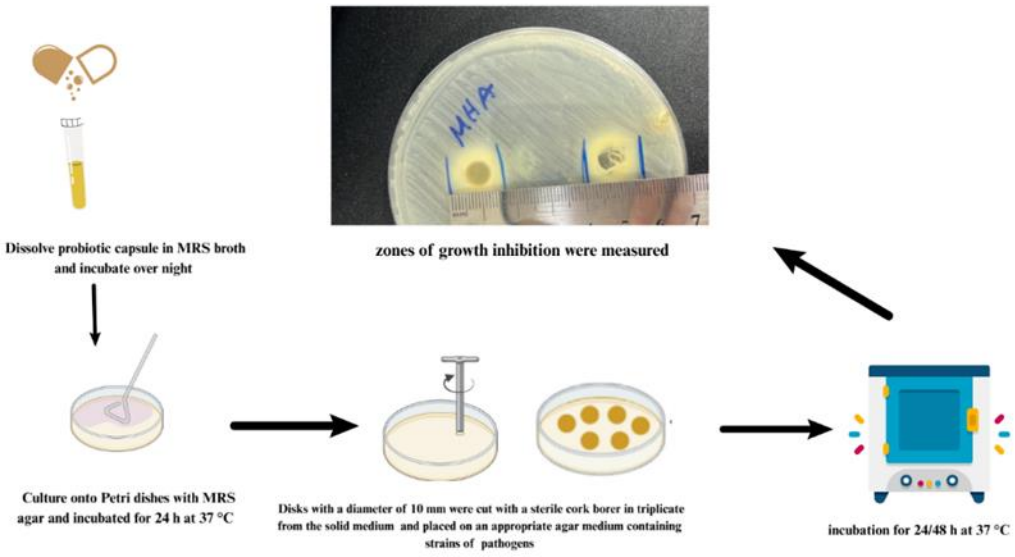
Biofilm detection outcome	Congo red agar	Microtiter plate	p-value
Biofilm-positive isolates	47 (75.81%)	35 (56.45%)	
Biofilm-negative isolates	15 (24.19%)	27 (43.55%)	
Total	62 (100%)	62 (100%)	$\chi^2 = 4.36$, $p = 0.037$

Table 4. Commercial Probiotic Antibiofilm Activity Against Gram-Negative Isolates

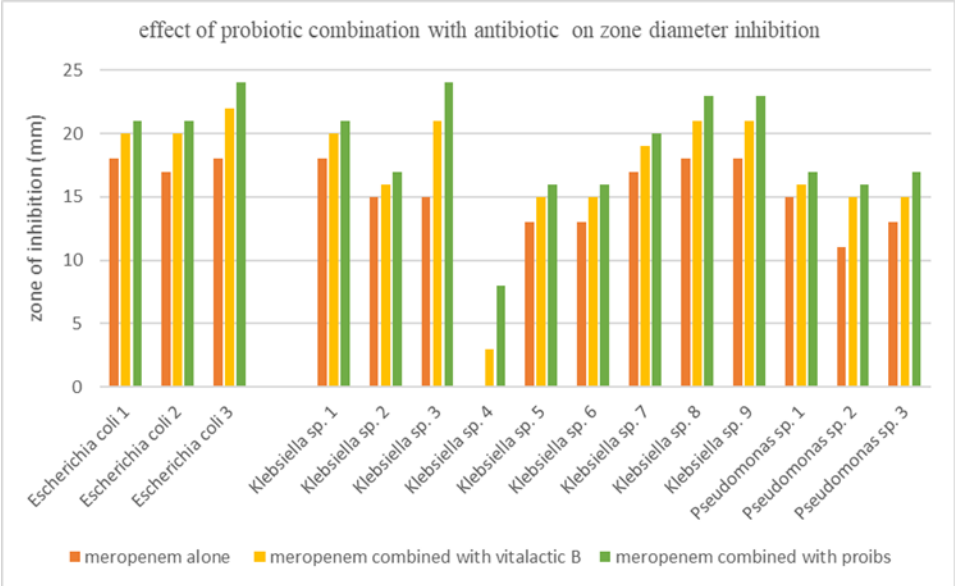
Bacteria	No. of isolates	Predominant biofilm phenotype	Probiotic antibiofilm activity (Vitalactic B)	Probiotic antibiofilm activity (PROIBS)
E. coli	23	Mostly Moderate–Strong	Moderate inhibition in some isolates	Weak to Negative inhibition
Klebsiella spp.	19	Mostly Weak–Moderate	Mostly Weak/Negative inhibition	Mostly Weak/Negative inhibition
Pseudomonas spp.	8	Variable (Weak to Strong)	Some Moderate activity	Mostly Weak/Negative
Morganella morganii	1	Moderate	Moderate	Weak
Burkholderia cepacia	1	Negative (no biofilm)	Negative	Negative
Enterobacter spp.	4	Mixed (Weak to Strong)	Moderate	Weak
Acinetobacter spp.	2	Negative	Negative	Negative
Proteus spp.	4	Mostly Weak–Moderate	Moderate in some isolates	Weak
Total	62	Majority Weak–Moderate	Moderate antibiofilm effect in selected strains	Weak to Negative overall

Table 6. Inhibitory Effects of Two Commercial Probiotics on drug-Resistant Gram-Negative Bacteria

Inhibition Zone Category	Vitalactic B (n, %)	Pro IBS (n, %)
Very Strong (≥16 mm)	12 (19.4%)	38 (61.3%)
Strong (11–15.9 mm)	25 (40.3%)	18 (29.0%)
Moderate (6–10.9 mm)	10 (16.1%)	4 (6.5%)
Weak (1–5.9 mm)	5 (8.1%)	1 (1.6%)
No Inhibition (0 mm)	10 (16.1%)	1 (1.6%)
Total	62 (100.0%)	62 (100.0%)



Graphical Abstract 1. Procedure of the slab method.



Graphical Abstract 2. Enhanced antibacterial activity of meropenem in combination with probiotics against carbapenem-resistant gram-negative bacteria.