# *In Vitro* Antagonistic Activity of Probiotic Strains against Antibiotic-Resistant *Klebsiella pneumoniae* isolated from Preterm Infant Stools

**Angel Yun-Kuan Thye[1,2,7], Yatinesh Kumari[3], Kok-Gan Chan[1,2,4,5], Jimmy Kok-Foo Lee[6], Loh Teng-Hern Tan[2,5], Vengadesh Letchumanan[2,7]\*, Learn-Han Lee[1,2,5]\*, Jodi Woan-Fei Law[1,2,5]\***

[1]Next-Generation Precision Medicine and Therapeutics Research Group (NMeT), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor Darul Ehsan, Malaysia

[2]Novel Bacteria and Drug Discovery Research Group (NBDD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor Darul Ehsan, Malaysia; angel.thye1@monash.edu (AY-KT)

[3]Neurological Disorder and Aging Research Group (NDA), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Subang Jaya 47500, Selangor, Malaysia; yatinesh.kumari@monash.edu (YK)

[4]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; kokgan@um.edu.my (K-GC)

[5]Microbiome Research Group, Research Center for Life Science and Healthcare, Nottingham Ningbo China Beacons of Excellence Research and Innovation Institute (CBI), University of Nottingham Ningbo China, Ningbo 315000, China; loh-teng-hern.tan@nottingham.edu.cn (LT-HT)

[6]Clinical School Johor Bahru, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Johor Bahru 80100, Malaysia; jimmy.lee@monash.edu (JK-FL)

[7]Pathogen Resistome Virulome and Diagnostic Research Group (PathRiD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor Darul Ehsan, Malaysia; vengadesh.letchumanan1@monash.edu (VL)

\*Corresponding authors: Vengadesh Letchumanan; Pathogen Resistome Virulome and Diagnostic Research Group (PathRiD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia; vengadesh.letchumanan1@monash.edu (VL); Jodi Woan-Fei Law and Learn-Han Lee; Microbiome Research Group, Research Centre for Life Science and Healthcare, Nottingham Ningbo China Beacons of Excellence Research and Innovation Institute (CBI), University of Nottingham Ningbo China, Ningbo 315000, China; jodi-woan-fei.law@nottingham.edu.cn (JW-FL); learn-han.lee@nottingham.edu.cn (L-HL)

**Abstract:** *Klebsiella pneumoniae* is one of the most common opportunistic pathogens colonizing the preterm infant gut, and it is associated with neonatal infections. However, the use of antibiotics against infections can disrupt the gut microbiota and lead to emergence of antibiotic-resistant bacteria. Probiotics possess antimicrobial or antagonistic properties that play a key role in inhibiting pathogens. Probiotics (*Bifidobacterium* and *Lactobacillus*) could be a promising prophylactic or alternative therapy in aiding to curtail the spread of antibiotic-resistant bacteria in the gut and to overcome infection in preterm infants. This study aims to present findings on the antagonistic potential of probiotic strains against the isolated antibiotic-resistant *Klebsiella pneumoniae* from preterm infant stool samples. The antagonistic activity of these probiotic strains was assessed using cross-streak assay. Results demonstrated that *Bifidobacterium longum* subsp. *infantis* JCM 1222$^T$, and *Lactiplantibacillus plantarum* JCM11125 exhibited remarkable antagonistic effects on *Klebsiella pneumoniae* isolated from preterm infant stools, while the antagonistic activity exhibited by *Bifidobacterium bifidum* JCM 1255$^T$ was weaker. Overall, our findings showed that selected probiotic strains could be a promising adjunct or preventive strategy for the management of *Klebsiella pneumoniae* infections in preterm infants, especially in settings where antibiotic resistance is prevalent. Nonetheless, future in vivo studies and clinical trials are essential to validate these findings, as well as to determine the optimal combinations, dosages, and safety profiles for their clinical applicability for neonatal use.

**Keywords:** *Klebsiella pneumoniae*; probiotics; preterm baby; stool microbe; multidrug-resistant pathogens; SDG 3 Good health and well-being

---

## 1. Introduction

*Klebsiella pneumoniae* is one of the most common opportunistic pathogens colonizing the preterm infant gut, and it is associated with neonatal infections[1–4]. Importantly, a recent paper published in 2019, identified *Klebsiella pneumoniae* as a pathogen associated with the highest neonatal deaths[5]. That being said, antibiotics are one of the most commonly prescribed medicines in the neonatal intensive care unit (NICU)[6]. However, the use of antibiotics against infections can disrupt the gut microbiota[7,8], increase the risk of adverse outcomes[9,10], and lead to the emergence of antibiotic-resistant bacteria[6,11]. In fact, multi-drug resistant *Klebsiella pneumoniae* has been found in the gut of preterm infants[2,3,12]. These issues highlight the urgent need for viable alternatives or adjunctive strategies to mitigate the risks associated with antibiotic therapy.

In recent years, there has been a growing body of evidence linking the relationship between the gut microbiome and the role of probiotics for health and the prevention and/or

treatment of various diseases [13–22]. Thus, one promising approach is the oral administration of probiotic supplements, which can encourage gut colonization with beneficial members of the early life microbiota. This approach not only has the potential to improve health outcomes in infants but may also contribute to reducing the spread of antimicrobial resistance by limiting the excessive use of antibiotics. Ongoing research is also investigating the use of next-generation probiotics, and interestingly, it is worthy to note that in Malaysia, there have been studies evaluating the antimicrobial potential of probiotic *Streptomyces*[23–27].

Probiotics are expected to possess a broad antimicrobial spectrum and exhibit strong antagonism against pathogenic bacteria. This antimicrobial or antagonistic activity is considered a crucial functional attribute of probiotic strains[28]. The antagonistic activity of one microorganism against another can result from various mechanisms such as competitive exclusion of pathogens, immune modulation, stimulation of host defense systems, and the production of signaling molecules that trigger changes in gene expression[28–30]. In addition, the production of organic acids and hydrogen peroxide, which lowers the pH, along with the production of antimicrobials like bacteriocins, contributes to the suppression of pathogenic microorganisms[28,31,32]. This antimicrobial potential is particularly relevant in a clinical context where the gut microbiota of preterm infants is disrupted, making them highly susceptible to colonization of opportunistic pathogens, including *Klebsiella pneumoniae*. Hence, assessing the ability of specific probiotic strains to inhibit such pathogens can provide insights into an alternative approach for infection prevention, especially in preterm infants who are a population at higher risk of antibiotic-associated complications, as mentioned above.

Among the diverse probiotic genera, *Bifidobacterium* and *Lactobacillus* are among the key organisms involved in maintaining the balance of gut microflora, and are natural inhabitants of the healthy human gut. Due to their beneficial roles, these genera are commonly explored for inclusion in probiotic formulations and functional foods; however, not all strains possess the required characteristics. In particular, their ability to exhibit antimicrobial activity against pathogenic, carcinogenic, and opportunistic microorganisms remains one of the key criteria in strain selection[33]. Importantly, given that the probiotic properties of both *Bifidobacterium* and *Lactobacillus* are strain-specific, it is essential to evaluate the antimicrobial properties of individual strains, particularly when targeting vulnerable populations such as preterm infants[34]. Furthermore, *Bifidobacterium* and *Lactobacillus* are the most commonly used probiotic genera in clinical interventions for preterm infants, due to their established roles in gut microbiota development and pathogen inhibition[35–37].

This study aims to evaluate the antagonistic activity of probiotic strains—*Bifidobacterium longum* subsp. *infantis* JCM 1222[T], *Bifidobacterium bifidum* JCM 1255[T], and *Lactiplantibacillus plantarum* JCM11125 (formerly known as *Lactobacillus plantarum*)—against the antibiotic-resistant *Klebsiella pneumoniae* isolated from preterm infant stool samples via *in vitro* cross-streak assay. Findings from this research will provide insights into the antagonistic potential of these three probiotic strains and their future therapeutic applications in neonatal care.

## 2. Materials and Methods

### 2.1. Culturing of Klebsiella pneumoniae

A total of 56 antibiotic-resistant *Klebsiella pneumoniae* were previously isolated from preterm infant stool samples collected from a neonatal intensive care unit (NICU) in Johor Bharu, Malaysia. Antibiotic-resistant *Klebsiella pneumoniae* isolates were revived and cultured in tryptic soya broth (TSB) (HiMedia, India), and incubated overnight in a shaking incubator at 37°C, 200rpm.

### 2.2. Culturing of probiotic strains and their growth conditions

Probiotic type strains were purchased from Riken BioResource Center (Tsukaba, Japan). Probiotic stock strains were anaerobically cultured in Mann–Rogosa–Sharpe (MRS) medium (HiMedia, India). The optimum duration of the incubation period was determined by growing the respective probiotic stock strains in MRS broth, as well as onto MRS agar plates (HiMedia, India) at 37°C, in an anaerobic chamber (0% oxygen, 5% carbon dioxide, 30% humidity). All strains were kept and maintained in MRS broth containing 30 % glycerol at −80 °C. The probiotic type strains used and their respective incubation period are listed in Table 1.

**Table 1:** Probiotic type strains and their incubation period.

| Probiotic type strains | Accession number | Incubation period |
|:---:|:---:|:---:|
| *Bifidobacterium longum* subsp. *infantis* | JCM 1222[T] | 72 hours (3 days) |
| *Bifidobacterium bifidum* | JCM 1255 [T] | 96 hours (4 days) |
| *Lactiplantibacillus plantarum* | JCM 11125 | 48 hours (2 days) |

### 2.3. Cross-streak assay

The cross-streak assay was adapted from Lertcanawanichakul *et al.*[38], and Bhuiyan *et al.*[39], with modifications. The respective probiotic strain (approximately 50ul of seed

culture) was pipetted and streaked onto the center of MRS agar plate and incubated anaerobically at 37°C for the duration as per Table 1. After incubation, the antibiotic-resistant *Klebsiella pneumoniae* isolates (approximately 25ul of seed culture) were cross-streaked perpendicular to the line of probiotic strain growth. Each streak started from near the edge of the plate and streaked towards the growth line of the probiotic strain. The plates were incubated aerobically for 18 hours at 37°C. Antagonistic activity was observed through inhibition zones between the probiotic strain and the clinical isolate. *Klebsiella pneumoniae* controls were included to confirm the validity of the assay. These controls are *Klebsiella pneumoniae* isolates that are streaked onto MRS agar plate without probiotic strains to ensure that any lack of growth or inhibition were due to the probiotics and not the medium or the *Klebsiella pneumoniae* isolates itself. This experiment was performed in duplicates. The results of the cross-streak assay will be categorized as full inhibition, intermediate inhibition, minimal inhibition, and no inhibition. Full inhibition refers to 100% inhibition against *Klebsiella pneumoniae*; intermediate inhibition refers to >30 – 99% inhibition against *Klebsiella pneumoniae*; minimal inhibition refers to ≤ 30% inhibition against *Klebsiella pneumoniae*; and no inhibition refers to 0% inhibition against *Klebsiella pneumoniae*.

## 3. Results

The antimicrobial potential of three probiotic strains—*Bifidobacterium longum* subsp. *infantis* JCM 1222[T], *Bifidobacterium bifidum* JCM 1255[T], and *Lactiplantibacillus plantarum* JCM11125—was evaluated against 56 antibiotic-resistant *Klebsiella pneumoniae* isolates using the *in vitro* cross-streak assay. Following optimization, the ideal incubation period for each probiotic strain was determined (Table 1). The consistency between the cross-streak assay duplicates were similar. All 56 selected *Klebsiella pneumoniae* isolates were completely inhibited by *Bifidobacterium longum* subsp. *infantis* JCM 1222[T], and *Lactiplantibacillus plantarum* JCM11125 in the cross-streak assay. On the contrary, four isolates (MPB 4, MPB 7, MPB 8A, MPB 45) showed no inhibition, 41 isolates showed minimal inhibition and only 1 isolate (MPB 101) showed intermediate inhibition by *Bifidobacterium bifidum* JCM 1255[T] in the cross-streak assay. Results of the cross-streak assay are presented in Table 2.

**Table 2:** Cross-streak assay results on the antagonistic effect probiotic strains against *Klebsiella pneumoniae* isolates.

| *Klebsiella pneumoniae* Isolates (MPB) | Probiotic Strains | | |
|---|---|---|---|
| | JCM 1222[T] | JCM 1255 [T] | JCM 11125 |
| 1 | FI | MI | FI |
| 2 | FI | MI | FI |

| | **Probiotic Strains** | | |
|:---:|:---:|:---:|:---:|
| *Klebsiella pneumoniae* **Isolates (MPB)** | **JCM 1222[T]** | **JCM 1255 [T]** | **JCM 11125** |
| 3 | FI | MI | FI |
| 4 | FI | NI | FI |
| 7 | FI | NI | FI |
| 8A | FI | NI | FI |
| 9A(i) | FI | MI | FI |
| 9A(ii) | FI | MI | FI |
| 12 | FI | MI | FI |
| 13 | FI | MI | FI |
| 14 | FI | MI | FI |
| 15 | FI | MI | FI |
| 16 | FI | MI | FI |
| 17 | FI | MI | FI |
| 18 | FI | MI | FI |
| 19 | FI | MI | FI |
| 41 | FI | MI | FI |
| 42 | FI | MI | FI |
| 43 | FI | MI | FI |
| 44 | FI | MI | FI |
| 45 | FI | NI | FI |
| 46 | FI | MI | FI |
| 48 | FI | MI | FI |
| 49 | FI | MI | FI |
| 50 | FI | MI | FI |
| 83 | FI | MI | FI |
| 84 | FI | MI | FI |
| 85 | FI | MI | FI |
| 89 | FI | MI | FI |
| 91 | FI | MI | FI |
| 93 | FI | MI | FI |
| 94 | FI | MI | FI |
| 95 | FI | MI | FI |
| 96 | FI | MI | FI |
| 97 | FI | MI | FI |
| 98 | FI | MI | FI |
| 99 | FI | MI | FI |
| 100 | FI | MI | FI |
| 101 | FI | II | FI |

| *Klebsiella pneumoniae* Isolates (MPB) | Probiotic Strains | | |
|---|---|---|---|
| | JCM 1222[T] | JCM 1255 [T] | JCM 11125 |
| 102 | FI | MI | FI |
| 103 | FI | MI | FI |
| 104 | FI | MI | FI |
| 105 | FI | MI | FI |
| 106 | FI | MI | FI |
| 107 | FI | MI | FI |
| 108 | FI | MI | FI |
| 109 | FI | MI | FI |
| 110 | FI | MI | FI |
| 138 | FI | MI | FI |
| 143 | FI | MI | FI |
| 150 | FI | MI | FI |
| 151 | FI | MI | FI |
| 152 | FI | MI | FI |
| 175 | FI | MI | FI |
| 176 | FI | MI | FI |
| 177 | FI | MI | FI |
| FI: Full inhibition; II: Intermediate inhibition; MI: Minimal inhibition; NI: No inhibition | | | |

## 4. Discussion

In order to curtail the spread of antibiotic-resistant bacteria and to overcome infection in preterm infants, probiotics could be a promising prophylactic or alternative therapy[40]. Probiotics possess antimicrobial or antagonistic properties that play a key role in inhibiting pathogens. These effects are mediated through the production of antimicrobial substances such as bacteriocins, enhancement of the intestinal barrier function in resisting pathogens, competitive exclusion of pathogens, and enhancing the host's immune system to combat pathogens[41–43]. These antagonistic properties are fundamental to the therapeutic potential of probiotics, significantly contributing to their ability in preventing and managing infections. Overall, the cross-streak assay findings suggest that probiotics—particularly *Bifidobacterium longum* subsp. *infantis* and *Lactiplantibacillus plantarum*—hold promise as therapeutic agents against infectious diseases caused by *Klebsiella pneumoniae* given their promising antagonistic activity.

In terms of ascertaining the antimicrobial properties of probiotics, a wide range of *in vitro* and *in vivo* methods are employed. *In vitro* methods include modified versions of the spot-on lawn assay, agar-well diffusion assay, co-culturing methods, the use of cell lines, and other related approaches, while *in vivo* method mainly employs the use of animal models[28,41]. In this study, the antimicrobial potential of three probiotic strains—*Bifidobacterium longum* subsp. *infantis* JCM 1222[T], *Bifidobacterium bifidum* JCM 1255[T], and *Lactiplantibacillus plantarum* JCM 11125[T] —was evaluated against antibiotic-resistant *Klebsiella pneumoniae* isolates using the in vitro cross-streak assay. The cross-streak assay was performed in duplicates on the 56 selected *Klebsiella pneumoniae* isolates to assess their interaction with the probiotic strains. This simple, fast, and cost-effective initial screening tool evaluates the direct antagonistic activity of the probiotic strains against the *Klebsiella pneumoniae* isolates on solid media. One of the key advantages of this assay is its simplicity and minimal requirement for specialized equipment and resources, making it an affordable and accessible method for assessing antimicrobial activity[44]. Cross-streak assay allows for direct visual observation of inhibition at the intersection of the streaks, facilitating a straightforward qualitative assessment of antimicrobial effectiveness. However, it does not provide quantitative data on the potency of the antimicrobial agent. Due to its subjective nature, there is also potential for variability or bias in interpreting the absence or presence of growth inhibition[44]. Additionally, the assay allows for comparative evaluation by streaking different target microorganisms perpendicular to the test strain (in this experiment—probiotic strain) in distinct regions of the same plate, allowing assessment of inhibitory effects across multiple strains within a single experiment[44]. However, when using a single plate for multiple target strains, careful technique is required to prevent cross-contamination and ensure reliable interpretation of results.

In terms of the source of the probiotic strains used in this study, *Bifidobacterium longum* subsp. *infantis* (JCM 1222[T]) was isolated from intestine of infant, *Bifidobacterium bifidum* (JCM 1255[T]) was isolated from feces of a breastfed infant, and *Lactiplantibacillus plantarum* (JCM 11125) was isolated from jojoba meal. *Bifidobacterium longum* subsp. *infantis* has a symbiotic relationship with the human host, protecting neonates by nourishing a healthy gut microbiota prior to weaning. This strain is well-adapted to the infant gut, having evolved alongside the mother-infant relationship and microbiome, partly due to its ability to digest complex carbohydrates present in human milk[45].

A recent study by Yu *et al.*[46], found that *Bifidobacterium longum* subsp. *infantis* carried a number of bacteriocin gene clusters, demonstrating new evidence on the competitive interactions of *Bifidobacterium* in the infant gut. On the other hand, *Bifidobacterium bifidum* are also genetically adapted to utilize host-produced glycans such

as mucins and human milk oligosaccharides[47–49]. Interestingly, *Bifidobacterium bifidum* have been shown to displace and compete with pathogens[50]. This was demonstrated in an *in vitro* study, whereby *Bifidobacterium bifidum* PRL2010 significantly inhibited the adhesion of enteropathogens such as *Escherichia coli*, and *Cronobacter sakazakii*, both commonly associated with severe gastrointestinal diseases in infants[50,51]. With regards to *Lactiplantibacillus plantarum*, it is worth noting that it was previously known as *Lactobacillus plantarum*, which is one of the most significant members of the lactobacilli presenting with good gastrointestinal tolerance, adhesion, antibacterial, and antioxidant properties[43].

*4.1. Clinical Studies of Probiotics Against Klebsiella Species*

There are however, very few studies that have examined the antimicrobial effect of these probiotic strains against *Klebsiella* isolated from stool of preterm infants. Despite our study showing promising results of *Bifidobacterium longum* subsp. *infantis* JCM 1222[T] against *Klebsiella pneumoniae* isolates, Toscano *et al.*[52], found *Bifidobacterium longum* subsp. *infantis* M-63 showed no zone of inhibition against *Klebsiella pneumoniae* via agar-well diffusion assay. With regards to the antimicrobial activity of *Bifidobacterium bifidum*, Srinu *et al.*[53], assessed the antimicrobial activity of *Bifidobacterium bifidum* against clinical isolates of *Klebsiella pneumoniae*, *Esherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* using agar well diffusion assay. Findings showed that *Bifidobacterium bifidum* 229 and *Bifidobacterium bifidum* 232 demonstrated good antimicrobial activity against tested isolates with inhibition zone ranging from 14mm to 16mm and 13mm–15mm, respectively. Specifically, both strains produced inhibition zones of 14mm against *Klebsiella pneumoniae* and 15mm against *Esherichia coli*[53]. Hence, *Bifidobacterium bifidum* exhibits strong antimicrobial activity against these pathogens. However, this contradicts our findings as most of the *Klebsiella pneumoniae* when tested against *Bifidobacterium bifidum* JCM 1255[T] were exhibiting minimal growth inhibition. Nonetheless, the differences in findings observed in both *Bifidobacterium longum* subsp. *infantis* JCM 1222[T] and *Bifidobacterium bifidum* JCM 1255[T] could be due to strain-specificity of the probiotics and pathogens.

*Lactobacillus plantarum* strains have been shown to produce different antimicrobial compounds such as organic acids, hydrogen peroxide, diacetyl, and also bacteriocins and antimicrobial peptides, both denoted by a variable spectrum of action[54]. Several studies used probiotic strain *Lactiplantibacillus plantarum* to test its antagonistic effect on *Klebsiella* species. Zhou *et al.*[55], determined the *in vitro* antibacterial effect of *Lactiplantibacillus plantarum* ZFM518 isolated from feces of healthy newborns against *Klebsiella pneumoniae* ZFM4 using the inhibition zone test and cell assay. The authors used

an in vitro model of the neonatal distal colon and found that *Lactiplantibacillus plantarum* ZFM518 significantly decreased the relative abundance of *Klebsiella* and *Clostridium_sensu_stricto_1* from fecal samples of NEC newborn infants. It also reduced the cytotoxicity and adhesion rate and *Klebsiella pneumoniae* ZFM4 towards Caco-2 cells and increased the prevalence of *Lactiplantibacillus*, *Bifidobacterium*, and *Faecalibacterium* in *Klebsiella pneumoniae*-infected feces[55]. Another study by Savino *et al.* [56], studied the antagonistic activity of twenty-seven *Lactobacillus* strains, in which three strains were identified as *Lactobacillus plantarum* against coliforms isolated from feces of breastfed colicky infants using agar-plates. Although coliforms identified included *Escherichia coli* (55.45%), *Klebsiella oxytoca* (22.15%), *Klebsiella pneumoniae* (12.34%), *Enterococcus faecalis* (6.20%), *Enterobacter aerogenes* (2.70%), and *Enterobacter cloacae* (2.50%) but only one isolate from each species was tested for the antimicrobial activity. Their findings showed *Lactobacillus plantarum* 456 exhibited strong inhibitory activity against all six coliforms with *Klebsiella pneumoniae* CG 23a, *Klebsiella oxytoca* GC Y, and for *Escherichia coli* CG 15b having an inhibition halo of 9.83mm, 7.75mm, and 8.33mm, respectively[56]. Furthermore, Abdel-motaal *et al.*[57], reported that six *Lactobacillus plantarum* strains (isolated from processed cheese, camel manure, sand lake water, and baby stool) exhibited antimicrobial activity, demonstrating high inhibition zone of >15mm against *Klebsiella* spp, highlighting their potential as effective antimicrobial agents. These findings are in-agreement with our findings which demonstrated *Lactiplantibacillus plantarum* JCM11125 exhibited strong inhibitory effect against *Klebsiella pneumoniae* isolated from stools of preterm infants.

*4.2. Studies Testing Probiotics Against Non-Klebsiella Species*

With regards to antimicrobial activity against *Enterobacteriaceae* that are not from preterm stool, some studies found that *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* have antimicrobial activity against enteropathogenic *Enterobactericeae*. A Malaysian-based study by Yusof *et al.*[58], isolated *Bifidobacterium* strains from stools of breastfed infants, whereby three *Bifidobacterium infantis* (Bifi-11, Bifi-19 and Bifi-20) showed strong antagonistic activity against enteropathogenic *Escherichia coli* 0157 and *Salmonella typhimurium*. They found that *Bifidobacterium* inhibited *Esherichia coli* better than *Salmonella typhimurium* as a result of low pH[58]. After 24 hours of incubation, *Bifidobacterium infantis* inhibited around 98% of *Esherichia coli*[58]. The authors also suggested that the inhibitory effect of *Bifidobacterium* strains in weaning food against the growth of *Esherichia coli* and *Salmonella typhimurium* was attributed primarily to the lower pH and production of volatile acid components by the bacteria[58]. This somewhat is in-agreement with Duar *et al.*[59], who proposed that low pH is a key factor in preventing the

invasion and overgrowth of pathogenic bacteria in the infant gut—a mechanism referred to as colonization resistance. Additionally, Cai *et al.*[60] isolated *Bifidobacterium* strains, including *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* from the stools of healthy, breastfed full-term infants to evaluate their antimicrobial activity against seven enteropathogenic bacteria, consisting of *Salmonella typhimurium* CICC 10420, *Listeria monocytogenes* CGMCC 1.9136, *Salmonella enterica* subsp. *enterica* CGMCC 1.1754, *Staphylococcus aureus* CICC 21600 and three different *Escherichia coli* strains: *Escherichia coli* EPEC O127: K63 (CICC 10411), *Escherichia coli* ETEC O78: K80 (CICC10421), and *Escherichia coli* EHEC O157: H7 (CICC 21530) which were selected due to their varying pathogenic effects and their representation of diarrheagenic types of *Escherichia coli*. Results demonstrated that all strains demonstrated bacteriostatic ability against *Escherichia coli* EPEC O127: K63 (CICC 10411), *Escherichia coli* ETEC O78: K80 (CICC 10421), and *S. typhimurium* CICC 10420 [60]. Interestingly, *Bifidobacterium longum* subsp. *infantis* (BF48-2, BF17-4, BF67-13) and *Bifodobacterium bifidum* (BF87-11, BF52-1) inhibited all broad-spectrum pathogenic bacteria tested in the experiment, highlighting their antimicrobial properties[60]. Overall, the authors reported that the fourteen representative *Bifidobacterium* strains in their experiment exhibited strong inhibitory activity against *Esherichia coli*, *Salmonella typhimurium*, and *Salmonella enterica,* potentially due to the production of organic acids or antimicrobial substances (ablastin), during their metabolic processes[60].

Some studies also found that *Bifidobacterium bifidum* and *Lactiplantibacillus plantarum* have antimicrobial activity against non-*Enterobacteriaceae*. Yildirim *et al.*[61], found that a *Bifidobacterium bifidum* excreted bacteriocin called Bifido B that was active against several gram-positive food-borne pathogens and food-spoilage bacteria, including *Bacillus*, *Leuconostoc*, *Lactobacillus*, *Listeria*, *Enterococcus*, and *Pediococcus*. In addition, Campana *et al.*[62], evaluated the antimicrobial activity of various lactic acid bacteria—*Bifidobacterium bifidum* W23 (DSM 26331), *Lactobacillus salivarius* W24 (DSM 26403), *Lactobacillus acidophilus* W37 (DSM 26412), *Lactobacillus casei* W56 (DSM 26388), *Lactococcus lactis* W58 (DSM 26390), *Lactobacillus plantarum* W21 (DSM 26401) and *Lactobacillus rhamnosus* W71 (DSM 26396) against five human intestinal pathogens—*Salmonella enteritidis* ATCC 13076, *L. monocytogenes* ATCC 7644, *E. coli* O157: H7 ATCC 35150, *Cronobacter sakazakii* ATCC 29544 and *Campylobacter jejuni* ATCC 33291 using agar well diffusion assay. Focusing on *Bifidobacterium bifidum* W23 (DSM 26331) and *Lactobacillus plantarum* W21 (DSM 26401), results showed that their individual inhibitory effects against *Salmonella enteritidis*, *Listeria monocytogenes*, *Esherichia coli*, *Cronobacter sakazakii*, and *Camphylobacter jejuni* ranged from 10.1 to 12.1mm for

*Bifidobacterium bifidum* W23, and 10.1 to 14.1mm for *Lactobacillus plantarum* W21[62]. Notably, *Bifidobacterium bifidum* W23 showed no visible inhibitory effect against *Salmonella enteritidis*[62]. Furthermore, it is worthy to note that the individual lactic acid bacteria strains in their experiment showed strain-specific abilities to reduce the invasion of intestinal pathogens in an interference model with Caco-2 cells[62]. Besides that, Bibalan *et al.*[63] isolated seventy-two *Lactobacillus* species from the stools of healthy volunteers and evaluated their antimicrobial activity using agar spot test and well-diffusion assay. Findings showed that approximately 40% of all *Lactobacillus* isolates had antimicrobial activity against one or more microorganisms. Among these strains, 17.4% were active against all four indicator bacteria—Enteropathogenic *Esherichia coli*, Enteroaggregative *Esherichia coli*, *Salmonella typhi*, and *Shigella dysenteriae*[63]. Additionally, another study tested ten lactic acid bacteria from calf-gut origin (6 *Lactobacillus reuteri*, and 2 *Pediococcus pentasaceus*, 1 *Lactobacillus johnsoni*, 1 *Lactobacillus ingluviei*) and against enteric pathogen *Esherichia coli* ATCC strain, found varying antagonism against *Esherichia coli* ATCC strain, with the minimum zone of inhibition being 13.5mm (isolate RM151- *Lactobacillus ingluviei* LC 383825.1) while the maximum zone of inhibition reaching 19mm (RM 122- *Pediococcus pentasaceus* LC274609.1) via well-diffusion assay[64].

Several studies used *Lactiplantibacillus plantarum* to evaluate the antimicrobial activity against other bacteria, other than *Klebsiella* spp. In a study by Mulaw *et al.*[65], three probiotic strains—*Lactococcus lactis* E124, *Lactobacillus paracasei* K114, and *Lactobacillus plantarum* K132—and their combination successfully inhibited the growth of *Salmonella typhimurium* DT104 under *in vitro* conditions of the co-culturing assay, in which *Lactobacillus plantarum* K132 specifically showed inhibition of 96.50%. Furthermore, they concluded that a combination of probiotic strains *Lactococcus lactis* E124, *Lactobacillus paracasei* K114, and *Lactobacillus plantarum* K132 was significantly more effective than individual strains in reducing fecal *Salmonella* counts in mice infected with *Salmonella typhimurium* DT104, compared to the control group (monoculture of *Salmonella typhimurium*) DT104. Additionally, Arena *et al.*[54] evaluated the antimicrobial activity of *Lactobacillus plantarum* isolated from wine and must against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella Enteritidis* using agar spot test, well-diffusion method, and broth microdilution method. They found that all *Lactobacillus* strains inhibited the growth of pathogens in a lactobacillus strain- and pathogen strain- depending manner. Via the agar spot method, seventeen *Lactobacillus plantarum* were classified as very strong inhibitors, with halos exceeding 5mm against most of the food-pathogen tested[54]. Kumar *et al.*[66] investigated the antimicrobial activity of *Lactobacillus plantarum* against three multidrug-resistant enteroaggregative *Escherichia coli* (MDR-

EAEC) isolated from diarrhoeal cases of human infants. Findings from their in vitro assay showed that *Lactobacillus plantarum,* when co-cultured with MDR-EAEC isolates showed a reduction in MDR-EAEC counts (eosin–methylene blue agar) in a dose- and time-dependent manner: probiotics at a dose rate of $10^{10}$ CFU inhibited MDR-EAEC isolates at 72 h post-inoculation (PI), whereas at lower concentrations ($10^8$ and $10^9$ CFU) MDR-EAEC isolates were inhibited at 96 h PI, suggesting that *Lactobacillus plantarum* has potential to mitigate MDR-EAEC-associated diarrhoea[66]. Overall, these studies demonstrated that *Bifidobacterium infantis*, *Bifidobacterium bifidum*, and *Lactiplantibacillus plantarum* have antimicrobial effects.

## 5. Conclusions

In conclusion, this study demonstrated that the probiotic strains *Bifidobacterium longum* subsp. *infantis* JCM 1222$^T$, and *Lactiplantibacillus plantarum* JCM11125 exhibit remarkable *in vitro* antagonistic activity against antibiotic-resistant *Klebsiella pneumoniae* isolated from preterm infant stool samples, while the antagonistic activity exhibited by *Bifidobacterium bifidum* JCM 1255$^T$ was weaker. Our findings showed that selected probiotic strains could be a promising adjunct or preventive strategy for the management of *Klebsiella pneumoniae* infections in preterm infants, especially in settings where antibiotic resistance is prevalent. Nonetheless, future *in vivo* studies and clinical trials are essential to validate these findings, as well as to determine the optimal combinations, dosages, and safety profiles for their clinical applicability for neonatal use.

## References

1. Wei X, Liang J, Zhang H, *et al*. Clinical features and risk factors of Klebsiella pneumoniae infection in premature infants: a retrospective cohort study. BMC Infect Dis 2024; 24(1): 1311.

2. da Silveira Ferreira IC, de Paula Menezes R, Lopes MSM, *et al*. Multidrug-resistant Klebsiella pneumoniae in critically ill neonates: evidence from a Brazilian cohort. Aust Crit Care 2025; 38(6): 101444.

3.  Milic M, Siljic M, Cirkovic V, *et al*. Colonization with multidrug-resistant bacteria in the first week of life among hospitalized preterm neonates in serbia: Risk factors and outcomes. Microorganisms 2021; 9(12): 2613.

4.  Benboubker M, Oumokhtar B, Oukachou D, *et al*. ESBL-producing Klebsiella pneumoniae gut colonisation and subsequent health-care associated bacteraemia in preterm newborns: a descriptive cohort with nested case–control study. Epidemiol Infect 2025; 153: e121.

5.  Ikuta KS, Swetschinski LR, Aguilar GR, *et al*. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet 2022; 400(10369): 2221-2248.

6.  Zou Z-H, Liu D, Li H-D, *et al*. Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. Ann Clin Microbiol 2018; 17: 1–11.

7.  Arboleya S, Sánchez B, Milani C, *et al*. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. J Pediatr 2015; 166(3): 538–544.

8.  Fouhy F, Guinane CM, Hussey S, *et al*. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob Agents Chemother 2012; 56(11): 5811–5820.

9.  Ting JY, Synnes A, Roberts A, *et al*. Association between antibiotic use and neonatal mortality and morbidities in very low-birth-weight infants without culture-proven sepsis or necrotizing enterocolitis. JAMA Pediatr 2016; 170(12): 1181–1187.

10.  Kuppala VS, Meinzen-Derr J, Morrow AL, *et al*. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. J Pediatr 2011; 159(5): 720–725.

11.  Yuan H, Xu J, Wang Y, *et al*. The longitudinal trend and influential factors exploring of global antimicrobial resistance in Klebsiella pneumoniae. Sci Total Environ 2024: 175357.

12.  Lee YQ, Kamar AA, Velayuthan RD, *et al*. Clonal relatedness in the acquisition of intestinal carriage and transmission of multidrug resistant (MDR) Klebsiella pneumoniae and Escherichia coli and its risk factors among preterm infants admitted to the neonatal intensive care unit (NICU). Pediatr Neonatol 2021; 62(2): 129–137.

13.  Johnson D, Chua K-O, Selvadurai J, *et al*. Pilot Trial of Probiotics in Major Depressive Disorder: A Randomized, Double-Blind, Placebo-Controlled Approach. Prog Microbes Mol Biol 2025; 8(1).

14.  Kong GY-E, Letchumanan V, Tan LT-H, *et al*. Gut microbiome in obsessive compulsive disorder: potential of probiotics as an adjuvant therapy. Prog Microbes Mol Biol 2022; 5(1): a0000272.

15.  Loo K-Y, Thong JYH, Tan LT-H, *et al*. A Current Overview of Next-Generation Probiotics and Their Prospects in Health and Disease Management. Prog Microbes Mol Biol 2024; 7(1).

16.  Sim AAXH, Cheam JY, Law JWF, *et al*. The ameliorative role of probiotics in 5-fluorouracil induced intestinal mucositis. Prog Microbes Mol Biol 2023; 6(1): a0000339.

17.  Lim WQ, Cheam JY, Law JW-F, *et al*. Role of garlic in chronic diseases: focusing on gut microbiota modulation. Prog Microbes Mol Biol 2022; 5(1): a0000271.

18.  Thye AY-K, Tan LT-H, Law JW-F, *et al*. Long COVID-19: Psychological symptoms in COVID-19 and probiotics as an adjunct therapy. Prog Microbes Mol Biol 2022; 5(1).

19.  Lee L-H. Probiotics in Depression Management: Efficacy, Mechanisms and Future Directions. Prog Microbes Mol Biol 2025; 8(1).

20. Jazuli I, Jazeel A, Selvaratnam L, *et al*. Navigating the Role and Approach of Gut Microbiota in Addressing Alzheimer's Disease Pathogenesis. Prog Microbes Mol Biol 2024; 7(1).

21. Tang EK, Loo KY, Thye AYK, *et al*. The impact of antidepressants on gut microbiome and depression management. Prog Microbes Mol Biol 2024; 7(1): a0000446.

22. El Mazouri S, Aanniz T, Bouyahya A, *et al*. Gut Microbiota in Autism Spectrum Disorder: A Systematic Review. Prog Microbes Mol Biol 2024; 7(1).

23. Goh JXH, Tan LT-H, Law JW-F, *et al*. Streptomyces sp. MUM 195J: A Promising Probiotic for Controlling Vibrio parahaemolyticus Infection in Aquaculture. Prog Microbes Mol Biol 2024; 7(1).

24. Loo K-Y, Tan LT-H, Law JW-F, *et al*. Detection of multidrug resistant Vibrio parahaemolyticus and anti-Vibrio Streptomyces sp. MUM 178J. Prog Microbes Mol Biol 2023; 6(1).

25. Loo K-Y, Tan LT-H, Chua K-O, *et al*. Complete Whole-Genome Sequence of Streptomyces sp. MUM 178J, a Potential Anti-Vibrio Agent. Prog Microbes Mol Biol 2024; 7(1).

26. Loo K-Y, Tan LT-H, Law JW-F, *et al*. Vibrio parahaemolyticus: Exploring its Incidence in Malaysia and the Potential of Streptomyces sp. as an Anti-Vibrio Agent. Prog Microbes Mol Biol 2023; 6(1).

27. Elsalam RM, Goh KW, Mahadi M, *et al*. The antibacterial activities of secondary metabolites derived from Streptomyces sp. Prog Microbes Mol Biol 2022; 5(1).

28. Fijan S. Antimicrobial effect of probiotics against common pathogens. Probiotics and prebiotics in human nutrition and health 2016; 10: 5772.

29. Saxelin M, Tynkkynen S, Mattila-Sandholm T, *et al*. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 2005; 16(2): 204–211.

30. Ratsep M, Naaber P, Koljalg S, *et al*. Effect of Lactobacillus plantarum strains on clinical isolates of Clostridium difficile in vitro. J Probiotics Health 2014; 2(1): 1–5.

31. Makras L and De Vuyst L. The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. Int Dairy J 2006; 16(9): 1049–1057.

32. Rastogi S, Mittal V, and Singh A. Selection of potential probiotic bacteria from exclusively breastfed infant faeces with antagonistic activity against multidrug-resistant ESKAPE pathogens. Probiotics Antimicrob Proteins 2021; 13(3): 739–750.

33. Denkova R, Goranov B, Teneva D, *et al*. Antimicrobial activity of probiotic microorganisms: Mechanisms of interaction and methods of examination. Antimicrob Res Nov Bioknowl Educ Programs 2017; 1: 201–212.

34. Beck LC, Masi AC, Young GR, *et al*. Strain-specific impacts of probiotics are a significant driver of gut microbiome development in very preterm infants. Nat Microbiol 2022; 7(10): 1525–1535.

35. Yang S, He J, Shi J, *et al*. Characteristics of intestinal microbiota in preterm infants and the effects of probiotic supplementation on the microbiota. Front Microbiol 2024; 15: 1339422.

36. Alcon-Giner C, Dalby MJ, Caim S, *et al*. Microbiota supplementation with Bifidobacterium and Lactobacillus modifies the preterm infant gut microbiota and metabolome: an observational study. Cell Rep Med 2020; 1(5).

37. Robertson C, Savva GM, Clapuci R, *et al*. Incidence of necrotising enterocolitis before and after introducing routine prophylactic Lactobacillus and Bifidobacterium probiotics. Arch Dis CHILD-FETAL 2020; 105(4): 380–386.

38. Lertcanawanichakul M and Sawangnop S. A comparison of two methods used for measuring the antagonistic activity of Bacillus species. Walailak J Sci & Tech 2008; 5(2): 161–171.

39.     Bhuiyan R, Shill S, Islam A, *et al*. Screening of Lactobacillus spp. from raw goat milk showing probiotic activities against pathogenic bacteria. Afr J Microbiol Res 2017; 11(15): 620–625.

40.     Pradhan D, Mallappa RH, and Grover S. Comprehensive approaches for assessing the safety of probiotic bacteria. Food Control 2020; 108: 106872.

41.     Fijan S. Probiotics and their antimicrobial effect. Microorganisms 2023, 11(2): 528

42.     Van Zyl WF, Deane SM, and Dicks LM. Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria. Gut microbes 2020; 12(1): 1831339.

43.     Echegaray N, Yilmaz B, Sharma H, *et al*. A novel approach to Lactiplantibacillus plantarum: From probiotic properties to the omics insights. Microbiol Res 2023; 268: 127289.

44.     Hossain TJ. Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. Eur J Microbiol Immunol 2024; 14(2): 97–115.

45.     Chichlowski M, Shah N, Wampler JL, *et al*. Bifidobacterium longum subspecies infantis (B. infantis) in pediatric nutrition: current state of knowledge. Nutrients 2020; 12(6): 1581.

46.     Yu D, Pei Z, Chen Y, *et al*. Bifidobacterium longum subsp. infantis as widespread bacteriocin gene clusters carrier stands out among the Bifidobacterium. Appl Environ Microbiol 2023; 89(9): e00979–23.

47.     Turroni F, Peano C, Pass DA, *et al*. Diversity of bifidobacteria within the infant gut microbiota. PloS one 2012; 7(5): e36957.

48.     Turroni F, Bottacini F, Foroni E, *et al*. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. PNAS 2010; 107(45): 19514–19519.

49.     Turroni F, Milani C, van Sinderen D, *et al*. Genetic strategies for mucin metabolism in Bifidobacterium bifidum PRL2010: an example of possible human-microbe co-evolution. Gut microbes 2011; 2(3): 183–189.

50.     Turroni F, Duranti S, Milani C, *et al*. Bifidobacterium bifidum: a key member of the early human gut microbiota. Microorganisms 2019; 7(11): 544.

51.     Serafini F, Strati F, Ruas-Madiedo P, *et al*. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal Bifidobacterium bifidum PRL2010. Anaerobe 2013; 21: 9–17.

52.     Toscano M, De Vecchi E, Gabrieli A, *et al*. Probiotic characteristics and in vitro compatibility of a combination of Bifidobacterium breve M-16 V, Bifidobacterium longum subsp. infantis M-63 and Bifidobacterium longum subsp. longum BB536. Ann Microbiol 2015; 65: 1079–1086.

53.     Srinu B, Devi KK, and Rao TM. Study on synergistic and antimicrobial activity of certain Bifidobacterium bifidum strains and plant extracts against clinical isolates. J Phytol 2024, 13(4): 294–297.

54.     Arena MP, Silvain A, Normanno G, *et al*. Use of Lactobacillus plantarum strains as a bio-control strategy against food-borne pathogenic microorganisms. Front Microbiol 2016; 7: 464.

55.     Zhou Q, Zhang Y, Liu L, *et al*. Effect of Lactiplantibacillus plantarum on the multidrug-resistant Klebsiella pneumoniae in the neonatal distal colon in vitro. J Funct Foods 2025; 129: 106823.

56.     Savino F, Cordisco L, Tarasco V, *et al*. Antagonistic effect of Lactobacillus strains against gas-producing coliforms isolated from colicky infants. BMC Microbiol 2011; 11(1): 157.

57.     Abdel-motaal H, Abdelazez A, Wang P, *et al*. Exploring Phenotype, Genotype, and the Production of Promising GABA Postbiotics by Lactiplantibacillus plantarum: A Comprehensive Investigation. Ferment 2024; 10(6): 309.

58.  Yusof RM, Haque F, Ismail M, *et al*. Isolation of Bifidobacteria infantis and its antagonistic activity against ETEC 0157 and Salmonella typhimurium S-285 in weaning foods. Asia Pac J Clini Nutr 2000; 9(2): 130–135.

59.  Duar RM, Kyle D, and Casaburi G. Colonization resistance in the infant gut: the role of B. infantis in reducing pH and preventing pathogen growth. High-throughput 2020; 9(2): 7.

60.  Cai J, Bai J, Luo B, *et al*. In vitro evaluation of probiotic properties and antioxidant activities of Bifidobacterium strains from infant feces in the Uyghur population of northwestern China. Ann Microbiol 2022; 72(1): 14.

61.  Yildirim Z and Johnson MG. Characterization and antimicrobial spectrum of bifidocin B, a bacteriocin produced by Bifidobacterium bifidum NCFB 1454. J Food Prot 1998; 61(1): 47–51.

62.  Campana R, Van Hemert S, and Baffone W. Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. Gut Pathog 2017; 9(1): 12.

63.  Bibalan MH, Eshaghi M, Rohani M, *et al*. Determination of bacteriocin genes and antibacterial activity of lactobacillus strains isolated from fecal of healthy individuals. Int J Mol Cell Med 2017; 6(1): 50.

64.  Waghe SB *Efficacy of combinations of probiotics in augmenting gut health in rats* 2021, IVRI.

65.  Mulaw G, Muleta D, Tesfaye A, *et al*. Protective effect of potential probiotic strains from fermented ethiopian food against Salmonella Typhimurium DT104 in mice. Int J Microbiol 2020; 2020(1): 7523629.

66.  Kumar M, Dhaka P, Vijay D, *et al*. Antimicrobial effects of Lactobacillus plantarum and Lactobacillus acidophilus against multidrug-resistant enteroaggregative Escherichia coli. Int J Antimicrob Agents 2016; 48(3): 265–270.