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

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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 aimed 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 Bahru, 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, 51 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.

	Probiotic Strains		
***Klebsiella pneumoniae* Isolates (MPB)**	**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

	Probiotic Strains		
***Klebsiella pneumoniae* Isolates (MPB)**	**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—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 (*Lantiplantibacillus 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 *Esherichia 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 *Salmonella 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.

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