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Research Article

Exploring Anti-*Klebsiella pneumoniae* Activity of Probiotic Lactobacilli of Curd Origin

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Abstract

The current research evaluates the bacterial growth inhibitory activity of indigenous probiotic lactobacilli and standard *Lactobacillus* strains against multidrug resistant (MDR) *Klebsiella pneumoniae*. The probiotic lactobacilli strains (*Lactobacillus animalis* LMEM6, *L. plantarum* LMEM7, *L. acidophilus* LMEM8 and *L. rhamnosus* LMEM9) from curd samples, and *Lactobacillus fermentum* MTCC 9748 standard strain were tested against indicator bacterial pathogens (n = 2): *K. pneumoniae* B535 clinical and *K. pneumoniae* NTCC 703603 standard strains, following agar overlay and agar-well diffusion techniques. The indicator bacterial strains were tested as MDR against 6 or more antibiotics with MAR (multiple antibiotic index) 0.4 - 0.73. The ZDI (zone diameter of inhibition) values from the action of test lactobacilli strains ranged 13.00 ± 1.00 - 33.33 ± 1.53, following agar-overlay method, and 12.00 ± 1.00 - 22.00 ± 1.00 mm, following agar-well method, against MDR *K. pneumoniae* indicator strains. The "R" value of lactobacilli strains ranged 3.00 - 13.17 mm, while the bacteriocin activity, in terms of activity unit (AU/ml), ranged 155.60 - 293.33. The study suggests that the indigenous lactobacilli might play an important role in the protection of host against MDR *K. pneumoniae* infection, and such probiotic strains may beneficially be employed as biotherapeutic agents in partial replacement or adjunct to antibiotic therapy in the treatment of MDR *K. pneumoniae* infection.

Keywords: Probiotic Lactobacilli; MDR Klebsiella pneumoniae; Antagonistic Activity; Bacteriocin Activity; MAR Index

Introduction

The findings of beneficial bacteria, viz., probiotic lactobacilli, as preventive and therapeutic agents to eliminate potential bacterial pathogens are increasing globally [1,2]. The need of such new treatment is important in the treatment of health care-associated infections caused by antibiotic resistant (MDR: multidrug resistant; XDR: extensively drug-resistant; PDR: pandrug resistant) bacteria. The *Klebsiella pneumoniae* is such an opportunistic gram-negative bacterial pathogen, and has been recently recognized as a significant threat to global public health [3]. They are pervasive in nature and generally colonize a diversity of body sites including the human gut, and remain embedded forming biofilm that protects them from the action of antibiotics, and make the situation more difficult to treat the infection [4]. The lactobacilli (genus *Lactobacillus*), which are generally recognize as safe (GRAS), are the prime members of the intestinal microbiota of humans, and had already been reported as alternative to antibiotics as a promising candidate to compete with the harmful bacteria [2].

The combined antibacterial activity of lactobacilli strains (between *L. animalis* and *L. gasseri*) were reported and found effective against *K. pneumoniae* [5]. The lactobacilli have extensively been studied for their remarkable capacity to inhibit the growth of other microorganisms through the inhibitory property [5,6]. The antibiofilm activity of *L. plantarum* strain against *K. pneumoniae* has been reported by Lagrafeuille., *et al* [7]. The culture filtrates of lactobacilli strains were also reported as the inhibitory agent against *K. pneumoniae* [8]. However, no scientific report has been recorded on the prevention of MDR *K. pneumoniae* strain with probiotic therapy from indigenous lactobacilli strains, at least from this part of the globe. Hence, the present study has been aimed to explore the antagonistic activity of probiotic curd lactobacilli strains as alternative therapeutics to prevent MDR *K. pneumoniae* infection.

Materials and Methods

Bacterial strains

Four curd lactobacilli probiotic strains: *Lactobacillus animalis* LMEM6, *L. plantarum* LMEM7, *L. acidophilus* LMEM8, *L. rhamnosus* LMEM9, were utilized in the current study. The methodol-

ogy details for procurement and identification of the isolates were explained earlier [2]. The standard strain used was *Lactobacillus fermentum* MTCC 9748. All the strains were maintained in MRS broth (Hi-Media, India) culture and MRS agar (Hi-Media, India) stabs, in the Laboratory of Microbiology and Experimental Medicine, Department of Zoology, University of Gour Banga, Malda. The indicator strains used in the study included *K. pneumoniae* (n = 2): *K. pneumoniae* B535 clinical and *K. pneumoniae* NTCC 703603 standard strains, which were maintained in the same laboratory in nutrient broth (Hi-Media, India) culture as well as in cystine tryptone agar (Hi-Media, India) stabs.

Antibiotic susceptibility of indicator bacteria

The antibiotic susceptibility of the indicator bacterial strains (*K. pneumoniae* B535 clinical and *K. pneumoniae* NTCC 703603 standard) was determined by disc diffusion method [9], using Mueller-Hinton agar (Hi-Media, India), and 15 antibiotic discs (Hi-Media, India): Ak: amikacin (30-µg/disc); Amc: amoxyclav (30-µg/disc); Amp: ampicillin (10-µg/disc); Cpd: cefpodoxime (10-µg/disc); Cx: cefoxitin (30-µg/disc); Ctx: cefotaxime (30-µg/disc); Gen: gentamicin (10-µg/disc); Ipm: imipenem (10-µg/disc); K: kanamycin (30-µg/disc); Met: methicillin (5-µg/disc); Mrp: meropenem (10-µg/disc); Te: tetracycline (10-µg/disc); Na: Nalidixic acid (10-µg/disc); Tr: trimethoprim (5-µg/disc) and Va: vancomycin (30- µg/disc). The susceptibility test results, in terms of ZDI (zone diameter of inhibition) values, were interpreted as following the criteria of Clinical Laboratory Standards Institute [9], to label the isolates as resistant, sensitive or intermediately susceptible. The MAR (multiple antibiotic resistance) indices were calculated following standard protocol as described before [10].

Antagonistic activity of lactobacilli

The antagonistic activity of four indigenous lactobacilli: *L. animalis* LMEM6, *L. plantarum* LMEM7, *L. acidophilus* LMEM8, *L. rhamnosus* LMEM9, and the standard *L. fermentum* MTCC 9748 strain was determined by agar-well diffusion as well as agar overlay methods, against *K. pneumoniae* strains (n = 2), one clinical and one standard, as mentioned above. The results (ZDI: zone diameter of inhibition) obtained from the earlier method was interpreted according to the criteria mentioned elsewhere [2], while the ZDIs values from the later method were interpreted following Shokryazdan *et al.* [11]: the ZDI > 20 mm, 10 - 20 mm and < 10 mm were considered as strong, intermediate and weak inhibitions, respectively.

Determination of "R" value of lactobacilli

The "R" (width of clear zone) values were also calculated, following agar overlay method, as per the formula stated earlier [5]: $R = \frac{(\text{d Inhib} - \text{d Spot})}{2}$ (where, "d Inhib": the diameter of clear zone around the "d Spot"; and "d Spot": the diameter of spot form of lactobacilli grown on MRS agar plate). The scores of "R" were interpreted following the criteria mentioned earlier [12,13]. All the tests were performed in triplicate, and the data were represented as mean ± SD (standard deviation).

Determination of AU/mL of cell free supernatant of lactobacilli

The antagonistic activity of the test lactobacilli strains in terms of arbitrary unit per mL (AU/mL) was calculated (mean ± SD) as 'a measure of production of bioactive components' using the formula mentioned elsewhere [14]: $\text{AU/mL} = \frac{ZDI \times 100}{volume\ taken\ in\ the\ well\ (\mu L)}$; where ZDI denotes "zone diameter of inhibition", following agar-well diffusion method.

Results and Discussion

The gram-negative enteric bacteria, *K. pneumoniae* possesses the capacity to cause community-acquired as well as healthcare-associated infections, such as pneumonia, bloodstream infection, surgical site infections, urinary tract infection, intra-abdominal infection, skin and soft tissue infection, liver abscess and meningitis [15,16]. In addition, the antibiotic resistance, among *K. pneumoniae*, is an emerging global healthcare crisis [17], and of particular the emergence of MDR *K. pneumoniae* incites impediment in the antibiotic therapy (even with last resort carbapenems: imipenem and meropenem) by escalating the dissemination of resistant pathogens causing protracted infection time among infected individuals [17,18]. In the current study, the indicator bacterial pathogens were MDR (Figure 1 and Table 1); *K. pneumoniae* B535 clinical strain showed resistance to 11 and *K. pneumoniae* NTCC 703603 standard to 6 antibiotics tested, thus having MAR index of 0.73 and 0.4, respectively.

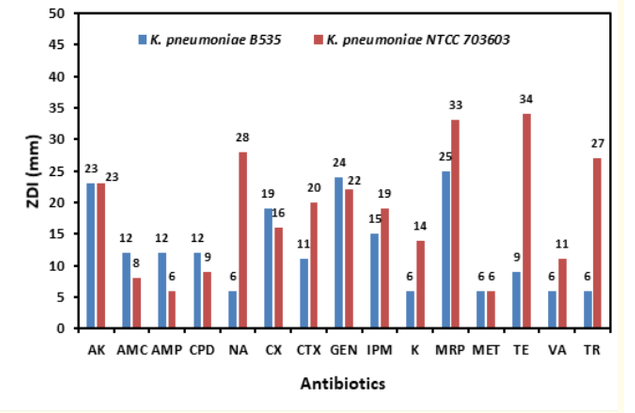

Figure 1: The ZDI (zone diameter of inhibition) values from antibiotic action against the indicator bacteria.

Ak: Amikacin; Amc: Amoxyclav; Amp: Ampicillin; Cpd: Cefpodoxime; Cx: Cefoxitin; Ctx: Cefotaxime; Gen: Gentamicin; Ipm: Imipenem; K: Kanamycin; Met: Methicillin; Mrp: Meropenem; Te: Tetracycline; Na: Nalidixic Acid; Tr: Trimethoprim; Va: Vancomycin.

Strain	Resistant	Intermediately susceptible	Sensitive
K. pneumoniae B535	Amc, Amp, Ctx, Cpd, Ipm, K, Met, Na, Te, Tr, Va	-	Ak, Cx, Gen, Mrp,
K. pneumoniae NTCC 703603	Amc, Amp, Ctx, Cpd, Met, Va	Cx, Ipm, K	Ak, Gen, Mrp, Na, Te, Tr

Table 1: Antibiotic susceptibility test results for indicator bacteria.

Ak: Amikacin; Amc: Amoxyclav; Amp: Ampicillin; Cpd: Cefpodoxime; Cx: Cefoxitin; Ctx: Cefotaxime; Gen: Gentamicin; Ipm: Imipenem; K: Kanamycin; Met: Methicillin; Mrp: Meropenem; Te: Tetracycline; Na: Nalidixic Acid; Tr: Trimethoprim; Va: Vancomycin.

Considering the situation mentioned, the antagonistic property of four indigenous lactobacilli procured from locally available niches against *K. pneumoniae* has been investigated in order to prepare anti- *K. pneumoniae* biotherapeutics. The antibacterial activity of non-haemolytic probiotic lactobacilli strains, following agar-well method as well as agar-overlay method, against *K. pneumoniae* has been shown in figure 2. Among the fermented food products curd belongs to excellent source of probiotic microorganisms, viz., *Lactobacillus* and being used worldwide dates back to thousands years. Recently, curd has been considered as a novel source of probiotic strains to be used against pathogenic bacteria [2].

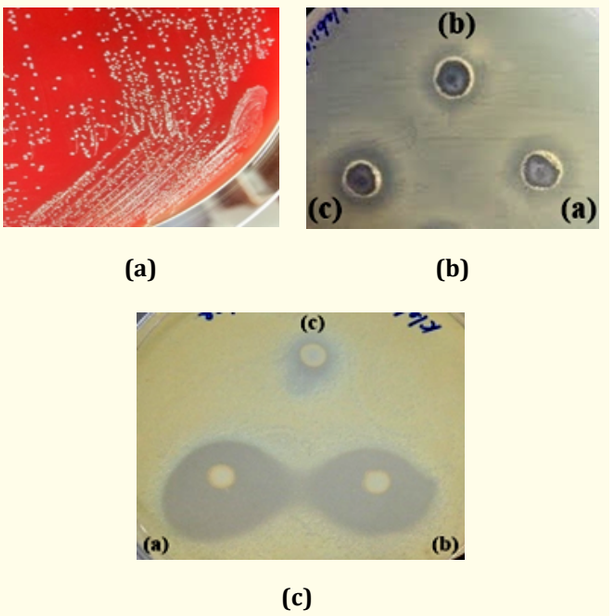

Figure 2: Lactobacilli strains: (A) γ-haemolytic colonies of *Lactobacillus plantarum* LMEM7 strain on sheep blood agar (Hi_Media) plate; (B) Antibacterial activity of lactobacilli (a: *L. animalis* LMEM6; b: *L. fermentum* MTCC 9748; c: *L. acidophilus* LMEM8) against *K. pneumoniae* in agar-well method; (C) Antibacterial activity of lactobacilli (a: *L. plantarum* LMEM 7; b: *L. acidophilus* LMEM8; c: *L. animalis* LMEM6) against *K. pneumoniae* in agar-overlay method.

The antibacterial activity of the test lactobacilli strains against *K. pneumoniae*, in terms of ZDIs following agar-overlay method, is represented in figure 3; for *K. pneumoniae* B535 clinical strain the ZDIs ranged from 13.00 ± 1.00 mm to 33.33 ± 1.53 mm (due to the action of *L. acidophilus* LMEM8 and *L. rhamnosus* LMEM9, respectively), while the ZDIs ranged from 14.67 ± 1.53 mm (from the action of *L. acidophilus* LMEM8) to 25.33 ± 1.53 mm (from the action of *L. fermentum* MTCC 9748). Following agar-well diffusion method, the ZDIs obtained around the test lactobacilli strains against *K. pneumoniae* ranged from 12.00 ± 1.00 to 22.00 ± 1.00 (Figure 4). The probiotic bacteria can impede the growth of microbial pathogens within the human gut, utilizing various metabolic pathways, through the synthesis of distinct metabolites, such as hydrogen peroxide, bacteriocin and lactic acid [19-21]. Harith., *et al*. [8] reported, unlike treatment with aminoglycosides: amikacin and gentamicin, which left live bacterial cells, Lactobacillus supernatant left no live cells in the *K. pneumoniae* biofilms. Also, the findings, as represented by Lagrafeuille., *et al.* [7], greatly suggested the lactobacilli culture supernatant as a potent antimicrobial agent against *K. pneumoniae*. The lactobacilli strains, such as, *L. plantarum* CIRM653, *L. delbrueckii* CIRM267 and *L. delbrueckii* CIRM268, were found effective against *K. pneumoniae* [22]. The *L. fermentum, L. plantarum, L. casei* and *L. brevis* isolated from fermented dairy foods had growth inhibitory activity against *K. pneumoniae* having ZDIs 7 - 14 mm [23].

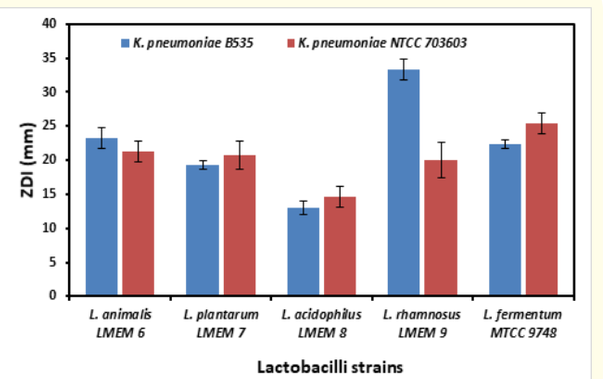

Figure 3: Antibacterial activity of lactobacilli strains in agar-overlay method.

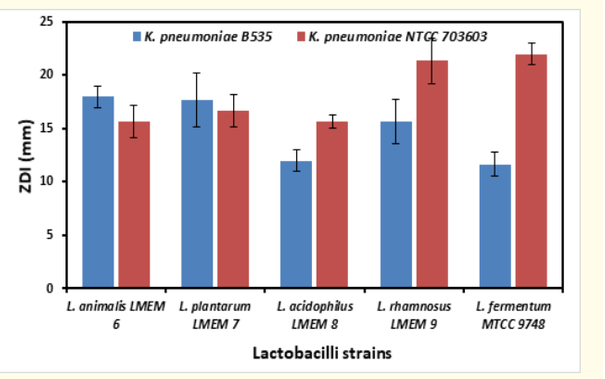

Figure 4: Antibacterial activity of lactobacilli strains in agar-well diffusion method.

Previously reported that the lactobacilli from curd samples had brilliant antibacterial activity in agar-well diffusion (ZDI: 13.67 ± 0.58 to 29.50 ± 2.10 mm) as well as agar-overlay (ZDI: 11.33 ± 0.58 to 35.67 ± 2.52 mm) methods, with "R" values 3.17 ± 0.29 - 15.33 ± 1.26 mm [2]. The "R" values of lactobacilli, in the current study, ranged from 3.00 to 13.17 (Table 2), while the AU/mL of the lactobacilli tested against *K. pneumoniae* ranged from 155.60 to 293.33 (Table 3). The bacteriocin activity has been expressed as 200 AU/ml against *Enterococcus faecalis* by Kormin., *et al* [24]. The *Lactococcus lactis* had strong bacteriocin activity (1600 AU/mL) against *K. pneumoniae* ATCC 12296, while some other lactic acid bacteria had inhibitory activity of 800 AU/mL against *Escherichia coli* [25]. In our earlier investigation, the average growth inhibitory activity of lactobacilli strains ranged from 233.34 ± 45.54 to 280.56 ± 83.67 AU/mL, against the test bacterial pathogens [2]. Herein, the current study depicts that among the indigenous lactobacilli, *L. animalis* LMEM6, *L. acidophilus* LMEM8, *L. rhamnosus* LMEM9 had high growth inhibition activity, against *K. pneumoniae*, with R values > 6 mm (range: 6.5 - 13. 17 mm), while, for MTCC strain the R value ranged 7.67 - 9.17 mm; low inhibition capacity was noted for L. acidophilus LMEM8 with "R" values of 3 - 3.84 mm (Table 2). Following agar-overlay method, *L. animalis* LMEM6, *L. rhamnosus* LMEM9 and *L. fermentum* MTCC 9748 strains had strong antagonistic activity against both *K. pneumoniae* B535 and *K. pneumoniae* NTCC 703603 indicators strains (Figure 3). The all test lactobacilli including the standard one (*L. fermentum* MTCC 9748) had high antibacterial activity against *K. pneumoniae* NTCC 703603 standard strain (ZDI: ≥ 15 mm), while 3 indigenous lactobacilli strains (*L. animalis* LMEM6, *L. plantarum* LMEM7 and *L. rhamnosus* LMEM9) were found highly active against *K. pneumoniae* B535 clinical strain (ZDI: ≥ 15 mm); *L. acidophilus* LMEM8 and *L. fermentum* MTCC 9748 strains had moderate activity (ZDI: 11.67 ± 1.15 - 12 ± 1.00 mm), following agar-well diffusion method (Figure 4). Thus, this is first to report with scientific validation of anti-*K. pneumoniae* activity of indigenous curd lactobacilli from this part of the globe.

Indicator bacteria	"R" value for lactobacilli strain				
	LMEM6	LMEM7	LMEM8	LMEM9	MTCC 9748
K. pneumoniae B535	8.17	6.17	3	13.17	7.67
K. pneumoniae NTCC 703603	7.17	6.84	3.84	6.5	9.17

Table 2: "R" value of lactobacilli isolates against *K. pneumonia*

Indicator bacteria	Lactobacilli strain showing growth inhibitory activity (AU/ml)				
	LMEM6	LMEM7	LMEM8	LMEM9	MTCC 9748
K. pneumoniae B535	240	235.60	160	208.93	155.60
K. pneumoniae NTCC 703603	208.93	222.27	208.93	284.40	293.33

Table 3: Growth inhibitory activity of lactobacilli expressed in "AU/ml" for bacterial pathogens

Conclusions

The study suggests that the indigenous lactobacilli might play an important role in the protection of host against MDR *K. pneumoniae* infection, and such probiotic strains may beneficially be employed as biotherapeutic agents in partial replacement or adjunct to antibiotic therapy in the treatment of MDR *K. pneumoniae* infection. Accordingly, the test lactobacilli might be useful in the field of food as well as pharmaceutical industries because of their (lactobacilli) therapeutic potentiality; however, further studies are warranted to extend and authenticate the current indications.

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