# Comparative Antimicrobial Activity of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on Clinically Isolated *Escherichia coli* : An *In Vitro* Study


## ABSTRACT

**Aim:** The aim of present study is to evaluate the comparative antimicrobial activity of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on clinical isolates of *Escherichia coli.*

**Study design:** Cross-sectional observational study

**Place and Duration of Study:** This study was conducted in Department of Medical Microbiology, School of Medical Education between January 2023 to September 2023.

**Methodology:** A total of 100 *E. coli* isolates collected from various diagnostic laboratories were included in the sample population and the prevalence of XDR, MDR, and non MDR isolates among them were determined by Kirby-Bauer disk diffusion method. The inhibitory activity of untreated and treated (pH adjusted) suspension of standard strains of *L. acidophilus* and *L. rhamnosus* on *E. coli* were determined by agar overlay method and the data was statistically analysed using ANOVA single-factor.

**Result:** The antimicrobial activity was confirmed for untreated and treated suspension of *Lactobacillus spp.* by measuring the zone of inhibition surrounding *E. coli* strains spotted on MRS medium but treated suspension exerted greater inhibitory activity than untreated suspensions of both *Lactobacillus spp*. and among the treated suspension *L. rhamnosus* exhibit greater inhibitory activity. Statistical analysis of this data using ANOVA single-factor was found to be not significant (*P >0.05*), that is treated suspension of both *L. acidophilus* and *L. rhamnosus* has the independent activity against *E. coli.* While untreated suspensions of both *L. acidophilus* and *L. rhamnosus* was significant (*P =0.03*), that is untreated suspension of *L. rhamnosus* (mean inhibition of 12.19 mm) has greater inhibitory activity than untreated cell cultures of *L. acidophilus* (mean inhibition of 11.39 mm).

**Conclusion:** This result, disclosed that even if both *Lactobacillus spp.* exhibit antimicrobial activity against *E. coli*, *L. rhamnosus* showed greater inhibition than *L. acidophilus*. The study suggested the use probiotic *Lactobacillus* as a biotherapeutic in antibiotic resistant *E. coli* infection and should be further studied for their human health benefits.



***Keywords:*** *Probiotics, Antimicrobial resistance, Escherichia coli, XDR, MDR, Lactobacillus rhamnosus, Lactobacillus acidophilus*


## INTRODUCTION

Human gastrointestinal tract makes a complex system with many commensal microbiota, consisting of $10^{10}$ to $10^{12}$ of 100 different species. The most common intestinal flora that maintains a balanced healthy intestine include *Lactobacilli*, *Bacteroides*, *Clostridia*, *Streptococci* and *Coliform* which function as a biological barrier and protect the host from pathogenic microorganisms [1]. Insufficient or depleted intestinal microbiota in human body result in serious infections or disorders such as irritable bowel syndrome, inflammatory bowel disease, antibiotic- induced diarrhoea, etc [2]. Although *Escherichia coli* is an important commensal found in the gastrointestinal tract of human and animals, certain virulent strains of *E. coli* can cause intestinal and extraintestinal infections such as diarrhoea, urinary tract infections, bacteremia, enteritis, biliary tract infections, pneumonia, and neonatal meningitis etc. Pathogenic strains of *E. coli* that cause extra intestinal infection are termed as ExPEC that are responsible for a spectrum of serious infections [3]. Prior to its harmful operations, *E. coli* must escape from host defences, acquire nutrients for survival and compete with other inhabitants of human host. Apart from being pathogenic several strains of *E. coli* have developed resistance to conventionally used antibiotics [4]. Rise in antibiotic resistance increased the necessity of innovative and alternative solution to treat infectious diseases or this has led to the switching of treatment from specific pathogenic elimination to altering bacterial ecology by use of probiotics [5].

Probiotics are live microorganisms with many beneficial properties. They are generally regarded as safe and are considered as one of the strategies to control many disorders [6]. Lactic acid bacteria, especially Lactobacilli are potential probiotic strain which are able to survive and adapt the hostile environment in oral, GI and vagina of human host [7]. Apart from being able to survive, they must be able to adhere and colonise to host cells in gastrointestinal tract. Furthermore, an effective probiotic strain must be able to tolerate acidic gastric juice, basic pancreatic juice, lysozyme and bile salts. Lactobacilli are most commonly used probiotic with many antimicrobial and antagonistic properties. These include the ability to produce acids, hydrogen peroxide and bacteriocin [8]. A number of studies have reported the crucial role of *Lactobacillus* in immune modulation such as local control of immune responses, allergic and inflammatory diseases by increasing the activity of macrophages and Ig A production. Since the antimicrobial properties of lactobacilli are strain specific, they should be correctly identified and their properties must be well investigated before human trials [9]. The probiotic members of *Lactobacillus* include the following species such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus* [10]. In recent years, the use of probiotics in the treatment and prevention of various diseases are increasing globally. As more antibiotics are rendered ineffective, the need of such new treatment is necessary to fight against resistant pathogens [11]. Here we determine the antimicrobial potential of *L. acidophilus* and *L. rhamnosus* against clinical *E. coli* isolates.

## MATERIALS AND METHODS

The present cross-sectional study was conducted in Department of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kerala, India, between January 2023 to September 2023.

## Microorganism and growth conditions

Two standard strains of Lactobacilli; *L. acidophilus* MTCC 10307 and *L. rhamnosus* MTCC 1408 acquired from Institute of Microbial Technology, Chandigarh were included in the study. Both strains of lactobacilli were maintained in MRS agar (HiMedia Laboratories Pvt. Ltd, Mumbai) incubated at 37°C for 48 hours with 5- 10% carbon dioxide and for further study subcultures were done on BHI broth. A total of 100 clinical isolates collected from various diagnostic laboratory were used as the test strain. The indicator strains used in the study were cultured on MacConkey agar, identified using routine biochemical test and its antimicrobial susceptibility test were performed.

## Antimicrobial susceptibility testing of *E. coli*

Antibiotic susceptibility profiles of the *E. coli* were determined by the Kirby-Bauer disk diffusion on MHA at 37°c for 24 hours under aerobic conditions as prescribed by CLSI M02-A13 [12] conditions and analysed using interpretive standards of CLSI M100-S33 [13] and are categorized into MDR, XDR and Non-MDR groups based on CDC/ECDC guidelines [14]. The antibiotic disc used were: Amikacin (30µg), Amoxicillin/Clavulanic Acid (20/10µg), Gentamicin (10µg), Tetracycline (30µg), Ciprofloxacin (5µg), Imipenem (10µg), Cefuroxime (30µg), Cefoxitin (30µg), Aztreonam (30µg), Ampicillin (10µg), Cefixime (5µg), Ceftazidime (30µg), Cefotaxime (30µg).

## Detection of probiotic activity of *L. acidophilus* and *L. rhamnosus* on *E. coli.*

Probiotic activity of *L. acidophilus* and *L. rhamnosus* was detected using Agar overlay method described by Fleming et al, with minor modifications [15]. Both *L. acidophilus* and *L. rhamnosus* were initially cultured on MRS agar for 24 hours at 5-10% $CO_2$. A distinct colony from MRS agar was transferred to BHI broth and incubated at 37°C overnight. Similarly, *E. coli* isolates were incubated in BHI broth at 37°C for 24 hours.

Briefly, a layer of MRS agar was prepared and allowed to solidify. Then, the surface of MRS agar was spot inoculated with 5µL of an overnight culture of *L. acidophilus* and *L. rhamnosus* (untreated), each was done in duplicate and mean was calculated. Also, the overnight culture of *Lactobacillus spp*. was adjusted to 6.5 – 7.0 pH using 1N NaOH (treated) and were spot inoculated on MRS agar. Then the plates were incubated for 24 hr at 37˚C in 5 – 10% $CO_2$. After incubation visible spot appears on the surface of the MRS medium. Then the MRS agar plates with *lactobacillus* spots were thereafter overlaid with 7ml of molten BHI soft agar (0.75%) cooled to 40-45˚C, which was seeded with 100µL of *E. coli* to be tested. After 24 hours of incubation at 37˚C in 5-10% $CO_2$, the inhibition zones around *lactobacillus* spots were diametrically measured and expressed in millimetres and interpreted following Shokryazdan et al. with modifications [16]. The zone of inhibition diameter ≥ 12 mm, 8-11 mm, 4-7 mm and < 4 mm were considered as strong, intermediate, weak and no inhibition respectively.

## Statistical analysis

The data was analysed using Microsoft Excel 2019. Descriptive statistics such as mean and standard error and inferential statistics such as ANOVA single-factor were employed in the present study. $P = 0.05$ was considered statistically significant.

## RESULTS

### Antibiotic susceptibility test

Zone of inhibition around antibiotic disc was interpreted and results of antibiotic susceptibility test *of E. coli* are shown in Fig 1. The result shows that out of 100 *E. coli* isolates, 68% were XDR, 11% MDR and 21% were Non MDR (Fig 2).

Fig1: Antibiotic susceptibility test of *E. coli* (n = 100)

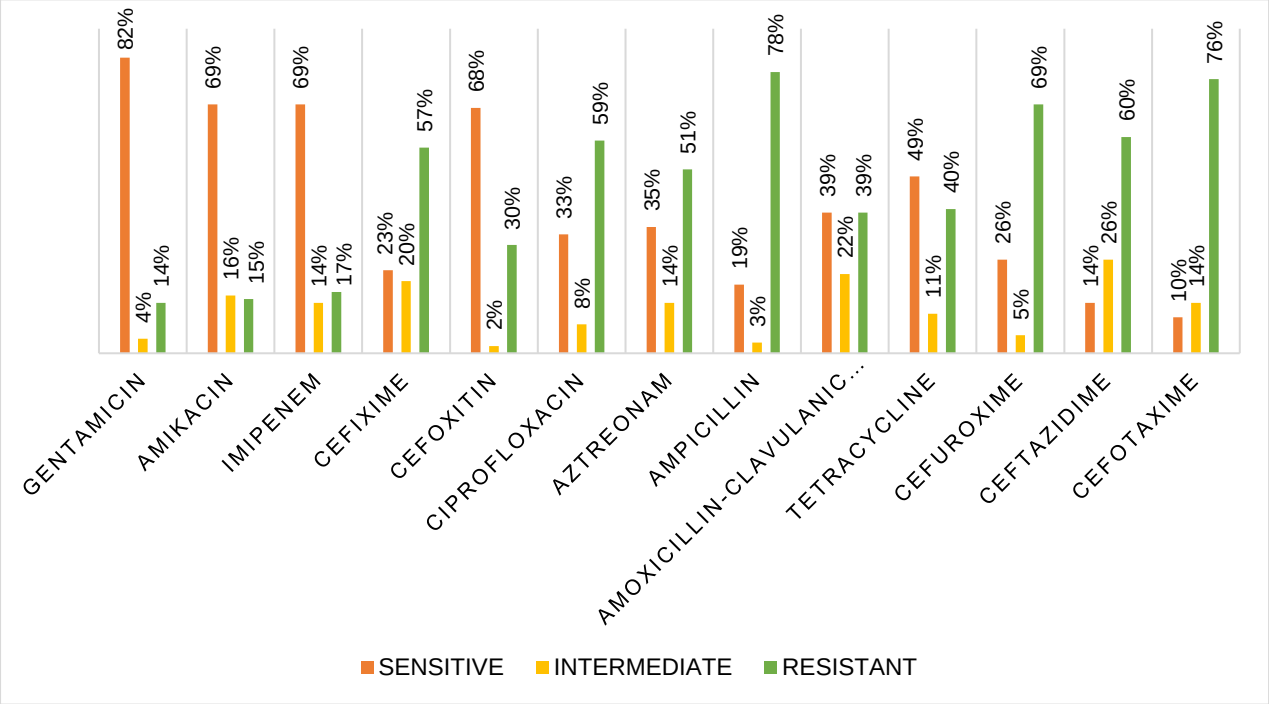
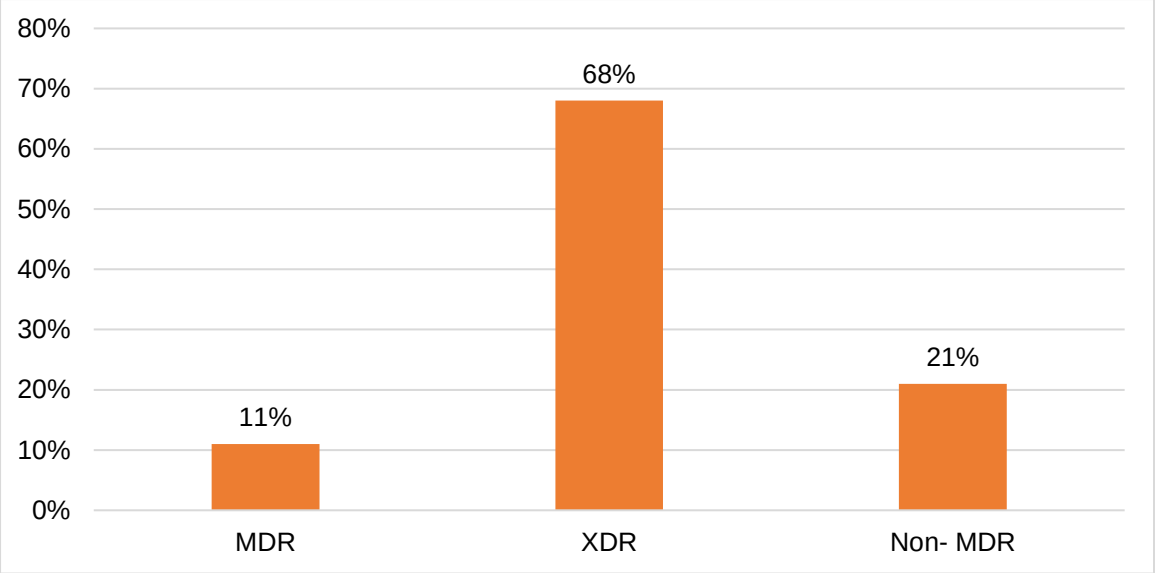



Fig 2: Distribution of XDR, MDR, NON-MDR *E. coli* isolates.




**Probiotic activity of treated and untreated suspensions of *L. acidophilus* and *L. rhamnosus* against *E. coli***

The antimicrobial activity assay showed that both untreated and treated suspension of *L. acidophilus* and *L. rhamnosus* has inhibitory activity against test strains of *E. coli* (Fig 3 and 4).

Fig 3: visible zone of inhibition around (A) *L. acidophilus* 1(untreated), (B) *L. acidophilus* 2 (untreated), (C) *L. rhamnosus* 1 (untreated) & (D) *L. rhamnosus* 2 (untreated) spots on MRS agar

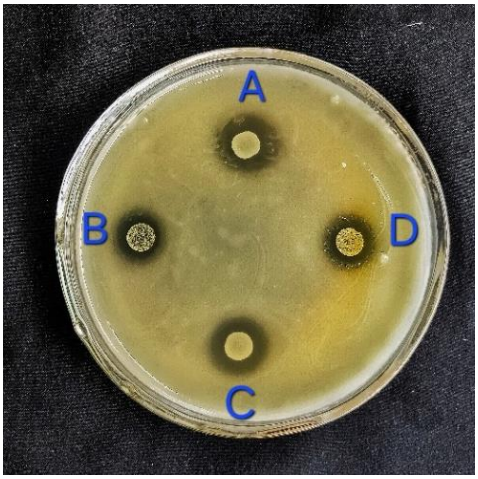
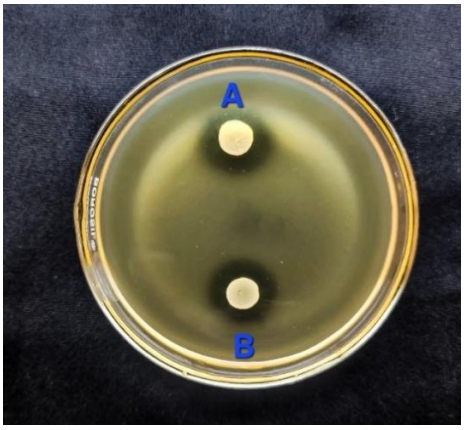



Fig 4: visible zone of inhibition around (A*) L. acidophilus* (treated) and (B) *L. rhamnosus* (treated) spots on MRS agar




The mean zone of inhibition of untreated and treated suspension of *L. acidophilus* against *E. coli* was 11.39 and 11.98. Mean zone of inhibition of *L. rhamnosus* untreated and treated was 12.19 and 12.66 respectively (Fig 5). Statistical analysis of this data was found to be not significant that is both untreated and treated suspension of *L. acidophilus* and *L. rhamnosus* has independent activity against *E. coli* (Table 1 & 2).

Fig:5 Mean zone of inhibition of *L. acidophilus* and *L. rhamnosus* (untreated and treated)

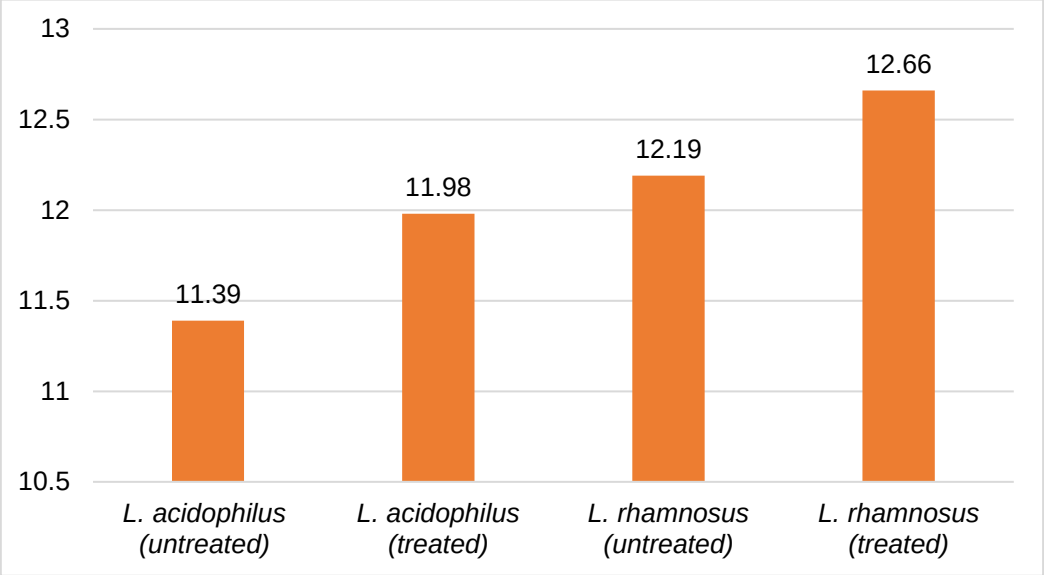



Table 1: Comparative activity of untreated and treated *L. acidophilus*

| *Groups* | *Count* | *Sum* | *Average* | *Variance* | *Standard error* |
|---|---|---|---|---|---|
| *L. acidophilus* (treated) | 100 | 1198 | 11.98 | 4.504646 | 0.212241524 |
| *L. acidophilus* (untreated) | 100 | 1139.5 | 11.395 | 5.597449 | 0.236589296 |
| Source of Variation | *SS* | *df* | *MS* | *F* | *P-value* |
| Between Groups | 17.11125 | 1 | 17.11125 | 3.387663 | 0.067182578 |

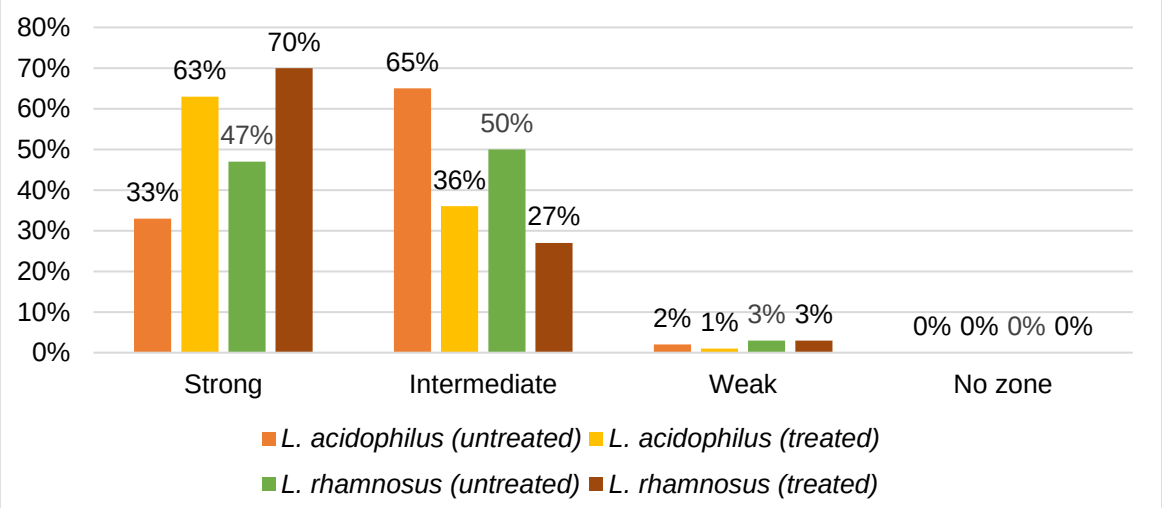
| | | | | | |
|---|---|---|---|---|---|
| Within Groups | 1000.108 | 198 | 5.051048 | | |
| Total | 1017.219 | 199 | | | |
| | | | | | *P*- value significant if <0.05 |

Table 2: Comparative activity of untreated and treated *L. rhamnosus*

| *Groups* | *Count* | *Sum* | *Average* | *Variance* | *Standard error* |
|---|---|---|---|---|---|
| *L. rhamnosus* (treated) | 100 | 1266 | 12.66 | 9.52 | 0.31 |
| *L. rhamnosus* (untreated) | 100 | 1219.5 | 12.19 | 8.84 | 0.29 |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* |
| Between Groups | 10.81 | 1 | 10.81 | 1.18 | 0.279177692 |
| Within Groups | 1817.88 | 198 | 9.18 | | |
| Total | 1828.69 | 199 | | | |
| | | | | | *P*- value significant if <0.05 |

Grading of probiotic activity of untreated and treated suspension of *L. acidophilus* and *L. rhamnosus* are shown in Fig: 6. It was found that treated suspension of *L. acidophilus* and *L. rhamnosus* exhibit strong activity against *E. coli* strains. While untreated suspension showed intermediate action against *E. coli*. Isolates with weak inhibitory activity were comparatively lower.

Fig 6: Grading of probiotic activity of untreated and untreated suspension of *L. acidophilus* and *L. rhamnosus.*




On analysing the data obtained from the study, untreated and treated suspensions of *L. acidophilus* and *L. rhamnosus* exhibited inhibitory activity against XDR, MDR and Non MDR *E. coli* strains. This interpretation was based on the result obtained from agar spot test against *E. coli*. Inhibitory activity of *L. acidophilus* and *L. rhamnosus* was summarized in Table 3 & 4.

Table 3: Inhibitory activity of *L. acidophilus* on *E. coli*

| XDR/MDR/NON-MDR | Inhibitory activity of *L. acidophilus* treated and untreated | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Strong | | Intermediate | | Weak | | No inhibition | |
| | *L. acidophilus* (untreated) | *L. acidophilus* (treated) | *L. acidophilus* (untreated) | *L. acidophilus* (treated) | *L. acidophilus* (untreated) | *L. acidophilus* (treated) | *L. acidophilus* (untreated) | *L. acidophilus* (treated) |
| XDR | 47.05% (n=32) | 75% (n=51) | 50% (n=34) | 23.52% (n=16) | 2.94% (n=2) | 1.47% (n=1) | 0% | 0% |
| MDR | 45.45% (n=5) | 54.54 % (n=6) | 54.54% (n=6) | 36.36% (n=4) | 0% | 9.09% (n=1) | 0% | 0% |
| NON-MDR | 47.61% (n=10) | 66.66 % (n=14) | 47.61% (n=10) | 33.33% (n=7) | 4.76% (n=1) | 0% | 0% | 0% |

Table 4: Inhibitory activity of *L. rhamnosus* on *E. coli*

| XDR/ MDR/NON-MDR | Interpretation of *L. rhamnosus* treated and untreated | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Strong | | Intermediate | | Weak | | No inhibition | |
| | *L. rhamnosus* (untreated) | *L. rhamnosus* (treated) | *L. rhamnosus* (untreated) | *L. rhamnosus* (treated) | *L. rhamnosus* (untreated) | *L. rhamnosus* (treated) | *L. rhamnosus (untreated)* | *L. rhamnosus (treated)* |
| XDR | 33.82% (n=23) | 63.23% (n=43) | 63.23% (n=43) | 35.29% (n=24) | 2.94% (n=2) | 1.47% (n=1) | 0% | 0% |
| MDR | 45.45% (n=5) | 81.81% (n=9) | 54.54% (n=6) | 18.18% (n=2) | 0% | 0% | 0% | 0% |
| NON-MDR | 23.80% (n=5) | 52.38% (n=11) | 76.19% (n=16) | 47.61% (n=10) | 0% | 0% | 0% | 0% |

### Comparison between probiotic activity of untreated *suspension of L. acidophilus* and *L. rhamnosus*

The mean zone of inhibition against *E. coli* by *L. acidophilus* (untreated) was 11.39 and the mean zone of inhibition of *L. rhamnosus* (untreated) was 12.19. A statistically significant result was found between mean zone of inhibition of *L. acidophilus* and *L. rhamnosus* (P=0.03) (Table 5). Grading of inhibitory

activity of untreated suspension of *Lactobacillus spp*. against *E. coli* showed that both *L. acidophilus* and *L. rhamnosus* exhibit intermediate activity against *E. coli.*

Table 5: Comparative activity of untreated *L. acidophilus* and *L. rhamnosus*

| *Groups* | *Count* | *Sum* | *Average* | *Variance* | *Standard error* |
|---|---|---|---|---|---|
| *L. acidophilus* (untreated) | 100 | 1139.5 | 11.39 | 5.59 | 0.24 |
| *L. rhamnosus* (untreated) | 100 | 1219.5 | 12.19 | 8.84 | 0.29 |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* |
| Between Groups | 32 | 1 | 32 | 4.43 | 0.036499201 |
| Within Groups | 1429.09 | 198 | 7.22 | | |
| Total | 1461.09 | 199 | | | |
| | | | | | *P*- value significant if <0.05 |

## Comparison between probiotic activity of treated *suspension of L. acidophilus* and *L. rhamnosus*

The mean zone of inhibition against *E. coli* by *L. acidophilus* (treated) was 11.98 and the mean zone of inhibition of *L. rhamnosus* (treated) was 12.66. Analysing this data using ANOVA single factor was found to be not significant (*P* >0.07) (Table 6). Grading of inhibitory activity of treated suspension of *Lactobacillus spp*. against *E. coli* showed that both *L. acidophilus* and *L. rhamnosus* exhibit strong activity against majority of *E. coli* strains.

Table 6: Comparative activity of treated *L. acidophilus* and *L. rhamnosus*

| *Groups* | *Count* | *Sum* | *Average* | *Variance* | *Standard error* |
|---|---|---|---|---|---|
| *L. acidophilus* (treated) | 100 | 1198 | 11.98 | 4.50 | 0.21 |
| *L. rhamnosus* (treated) | 100 | 1266 | 12.66 | 9.52 | 0.31 |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* |
| Between Groups | 23.12 | 1 | 23.12 | 3.29 | 0.070963331 |
| With in Groups | 1388.9 | 198 | 7.01 | | |
| Total | 1412.02 | 199 | | | |
| | | | | | *P*- value significant if <0.05 |

## DISCUSSION

*Lactobacillus* species are commensal and non -pathogenic inhabitants of human gastro intestinal, oral, and vaginal microbiota which have been considered as valuable probiotic microorganisms possessing innovative functional characteristics and formulations. They are also an attractive goal in therapeutic strategies to restore the natural microbiota and gaining great interest for their health-promoting effects in the host both on direct interactions between cells and indirectly through their released metabolites, thus making them suitable to be used as probiotic strains. Probiotics are also known to have immune-modulatory roles, anticancer effects, and promote the lowering of cholesterol [17,18]. The commensal intestinal flora, *E. coli* lacks virulence in this setting but can also be the cause of intestinal and extraintestinal illness including urinary tract infections (UTI), pneumonia, bacteremia, peritonitis and many more [19]. In addition, increasing antibiotic resistance among *E. coli* is a serious public health problem worldwide and emergence of MDR, XDR *E. coli* strains were a growing concern due to the unavailability of appropriate treatment options [20]. The application of probiotics and their antimicrobial metabolites for the treatment and prevention of infection is gaining importance in the era of developing antibiotic resistant pathogen.

Antibiotic resistance in *E. coli* is increasing day by day, making it an emerging global healthcare crisis. The present study highlights higher prevalence of XDR (68%) with a considerable number of MDR *E. coli* (11%) which is contrast to the recent findings of Pattnaik et al. which exhibited that from a total of 267 *E. coli* isolates 70.04% were classified as Multidrug-resistant (MDR) [21]. However, the present study investigation revealed a far lower percentage. Also, Pattnaik et al. demonstrate that 19.10% of test strains of *E. coli* were MDR. The variations in population, geographic location, duration between investigations, and clinical specimen types and sizes can all be used to explain why the results of different studies have different findings [22].

Antimicrobial activity is a key feature of probiotic. However, an exact methodology is crucial to rule out its sensible outcome. Most research has focused on either *in -vitro* agar spot test or the well diffusion assay. In our study, Agar overlay inhibition assay was employed with certain modifications to demonstrate the antimicrobial activity of *L. acidophilus* and *L. rhamnosus* against *E. coli* isolates [4]. Cadirici and Citak compared the two method (agar overlay and agar well diffusion) and found the agar overlay method as the effective one in the evaluation of inhibitory activity of Lactobacilli against gram negative bacteria [23]. Several studies have been performed with various modifications of agar overlay method to confirm the inhibitory activity of probiotics against different *E. coli* strains [4]. Study by Jacobsen et al. investigated 47 lactobacilli strains, 30 exhibited antimicrobial activity (including *L. acidophilus* and *L. rhamnosus*) against *E. coli* strains [24]. In a similar study by Davoodabadi et al. reported the inhibitory activity of *Lactobacillu*s strains of human origin and suggested their usefulness as probiotic in controlling intestinal infection with *E. coli [25].*

In the present study, the two standard strains of Lactobacilli exhibit good antimicrobial activity against clinical isolates of *E. coli*. In a similar study by Michèle Delley et al. *Lactobacillus spp*. including *L. acidophilus* and *L. rhamnosus* exhibit moderate activity against *E. coli* [26]. In our study, among the

two strains of *Lactobacillus spp*. *L. rhamnosus* showed greater inhibitory activity against *E. coli*. Hutt et al. studied the probiotic activity of *L. rhamnosus GG* and found that the strain highly suppressed *E. coli* ATCC 700336 [27]. Up on comparing the inhibitory effect of untreated suspensions of *L. acidophilus* and *L. rhamnosus* against *E. coli,* a statistically significant result was obtained; which showed that untreated suspension of *L. rhamnosus* has greater inhibitory activity against *E. coli.* In the study performed by Halder and Mandal, inhibition values of commercial probiotic *Lactobacillus* strains (*L. acidophilus* and *L. rhamnosus)* against *E. coli* are similar [28]. Such slight difference in inhibitory activity of lactobacilli is due to variation in strain and source, and also due to difference in target pathogen, which needs further study to explain the difference. In our study we used standard strains of *L. acidophilus* and *L. rhamnosus* but in the later study *L. acidophilus* and *L. rhamnosus* of commercial origin were used [28]. However, treated suspension of both *L. acidophilus* and *L. rhamnosus* exhibit greater zone of inhibition than untreated suspensions. The findings by Georgieva et al. demonstrate that neutralised supernatant of *L. acidophilus* and *L. rhamnosus* showed inhibitory activity against *E. coli* [29]. Conversely, the findings of Tejero-Sariñena S et al. demonstrate that lower the pH, higher the inhibition against pathogenic microorganisms [30]. Thus, the inhibitory action of treated suspension of both *Lactobacillus spp.* was may be due to some other factors like bacteriocin, hydrogen peroxide etc. Those findings agree with the Zhao et al. who demonstrated that bacteriocin produced by *L. acidophilus* and *L. rhamnosus* exhibit antibacterial mechanism against *E. coli*, the suggested mechanism is via cell membrane damage and intracellular material leakage [31].

## CONCLUSION

The result of our study clearly states that both *L. acidophilus* and *L. rhamnosus* exhibited antimicrobial activity against clinical *E. coli* isolates, but the level of inhibitory action varied between *Lactobacillus* species which may be due to variation in strain and also dependent up on chosen agar overlay method. Our findings emphasized on the use of probiotic *L. acidophilus* and *L. rhamnosus* on clinical isolates of *E. coli. Lactobacillus spp*. as a probiotic may play a promising role in this era of rapidly growing burden of drug resistant organisms. However, further *in vivo* and largescale studies are warranted to clarify this before it can be safely applied in health field.

## ACKNOWLEDGEMENTS

Authors would like to thank Mrs. Rajumol B. Zacharia for her technical assistance

## AUTHORS' CONTRIBUTIONS

All authors equally contributed to the present study.

## ETHICAL APPROVAL

The study was approved by Institutional Ethical Committee, Ref No: IEC/26/MICRO/SME-GNR/2022

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