International Journal of Endorsing
HEALTH SCIENCE RESEARCH

IJEHSR

Original Article

Antibiotic susceptibility and antibacterial activity of neutralized cell-free supernatant of *Lactobacillus rhamnosus* MT539286 against Foodborne and Clinical pathogens.

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Doi: 10.29052/IJEHSR.v9.i1.2021.04-09
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Received 03/07/2020
Accepted 20/10/2020
First Published 17/12/2020

OPEN ACCESS

Abstract

Background: The emergence of horizontally transferable antibiotic resistance (AR) in probiotic strains of lactic acid bacteria (LAB) has incited a need to establish stringent safety measures for the assessment of commercial probiotics. *Lactobacillus rhamnosus* is not only a prevalent constituent of commercially available probiotics but also has an abundant presence in fermented foods.

Methodology: The present study tested antimicrobial activity and antibiotic susceptibility of *L. rhamnosus* isolated from fermented cabbage. Agar well diffusion assay was used to determine the antibacterial activity against a number of Gram-positive and Gram-negative bacteria, and antibiotic susceptibility testing was used to determine the antibiogram of several antibiotics. For this, the neutralized Cell-Free Supernatant (nCFS) of *L. rhamnosus* was used, which exhibited antibacterial activity against several bacteria.

Results: Antagonistic activity of nCFS of *L. rhamnosus* was found against *Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Salmonella typhimurium, Klebsiella pneumoniae, Salmonella enterica*. Maximum inhibitory activity was observed against *Listeria monocytogenes*. Whereas, no activity was found against *Staphylococcus epidermidis, Streptococcus pyogenes, Bacillus subtilis, Vibrio alginolyticus, Proteus mirabilis* and *Proteus vulgaris*. The intensity of inhibition among the isolates was in the sequence of *Listeria monocytogenes* > *Escherichia coli* > *Bacillus cereus* > *Vibrio parahaemolyticus* > *Salmonella typhimurium*. *L. rhamnosus* was found susceptible against Chloramphenicol and Novobiocin. Moreover, it exhibited resistance to Amoxicillin, Levofloxacin, Oxacillin, Streptomycin and Tobramycin.

Conclusion: In conclusion, this study suggests that the use of *L. rhamnosus* as a probiotic requires extensive examination of AR genes present in this strain.

Keywords

Antibacterial Activity, Lactic Acid Bacteria, Antibiotic Susceptibility, *Lactobacillus Rhamnosus.*

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Introduction

The ever-increasing prevalence of microbial resistance to antibiotics is becoming a public health crisis. Deaths as a direct result of antibiotic resistance are estimated to be raised by 300 million by 2050[1]. Antibiotics are also known for drastically altering gut microflora, resulting in reduced diversity and overall presence in the Gastrointestinal (GI) tract. Consequently, the ability of inhabitant GI bacteria to withstand the invasion of pathogenic microorganisms is significantly reduced. Moreover, the effects of antibiotics prevent the restoration of balance in GI microbiota long even after the bacterial count has been restored, making the patients more susceptible to infections and other diseases[2]. This bleak landscape has garnered much attention towards the use of probiotics as prophylaxis, especially during antibiotics therapy. Several clinical studies have evaluated the positive effects of probiotics on GI health[2]. LAB has historically been used as probiotics, and a large number of LAB strains have a beneficial influence over human health and have been assigned generally recognized as safe (GRAS) status by FDA[3]. Research has revealed the LAB's efficacy as a probiotic; its effects range from growth inhibition of pathogenic bacteria to strengthening the immune response and more[4]. This perception has led to the overwhelming growth of probiotic usage from being limited to merely dairy and fermented products to large-scale incorporation of probiotics in fortified foods, dairy products and health supplements[5].

L. rhamnosus is a commonly available commercial probiotic. It is recommended for a variety of health benefits and for treating antibiotic-associated diarrhea[2]. The presence of AR in bacteria can be either intrinsic or acquired. Intrinsic resistance is generally considered risk free due to its horizontal transfer[5]. Characterization of AR in LAB has revealed that many species of LAB, including *L. rhamnosus*, have intrinsic resistance to several antibiotics. However, researchers have also reported its role as a reservoir for transmission of AR genes to pathogenic bacteria[6]. Therefore, this study aims to evaluate the antibiotic susceptibility and antimicrobial activity of *L. rhamnosus* isolated from fermented cabbage.

Methodology

Bacterial cultures and growth medium

The isolated strain was identified by polymerase chain reaction (PCR) and 16 s rRNA sequencing. The sequence was deposited in GenBank under the accession number MT539286. The culture was propagated in de Man Rogosa Sharpe (MRS) broth (Oxoid) at 37°C for 24-48 h. The antibacterial assay was performed using Mueller Hinton Agar (MHA) (Oxoid). All the cultures were propagated in their respective medium and were maintained by sub culturing after every two weeks and stored at -20°C with 20% v/v glycerol. Experiments were performed in triplicate. A total of 17 cultures were tested against the *L. rhamnosus,* of which five were American type culture collection (ATCC) food borne pathogens and twelve were clinical pathogens depicted in Table 1. All cultures were obtained from Food Safety Research Group Laboratory, Department of Microbiology, University of Karachi.

Preparation of Cell-Free Supernatant

The strain was sub-cultured with the MRS broth with an overnight incubation at 37°C. The culture was centrifuged at 10,000 rpm for 10 min. and bacterial cells were removed before obtaining CFS. CFS was filtered by 0.2 µm syringe filter corning (Corning, USA) and neutralized by 2M NaOH; pH was adjusted to 6.5.

Standardization of the culture

The culture was standardized by using the McFarland index. 4-5 colonies of pure isolated test cultures were transferred to 0.85% sterile saline. The turbidity was adjusted to 106 CFU/ml, which is equivalent to the 0.5 Mcfarland standard index; the final inoculum was obtained.

Antibacterial Assessment

The antibacterial activity of *L. rhamnosus* was assessed against the indicator organisms by agar well diffusion assay. 100 µL of nCFS was dispensed into each pre-labelled well, and MRS broth was

used as a negative control. The plates were allowed to refrigerate at 4°C for an hour and then incubated at 37°C for 24 hours. Results were recorded by the appearance of the zone of inhibition (ZOI), and diameter was measured.

Antibiotic susceptibility Testing

Antibiotic discs were used to determine the effect of antibiotics against the strain of *L. rhamnosus* by Kirby Bauer disc diffusion assay. Nine antibiotic discs (Sigma-Aldrich, United States) listed in Table 2 were used. Culture suspension of the selected isolate was prepared by adjusting the turbidity with the 0.5 McFarland index and was swabbed on the pre-incubated MRS agar plates. Discs were placed aseptically and incubated at 37°C for 24 hours. ZOI was measured.

All experimental analyses were done in triplicates, and results were presented as mean ± SD by SPSS version 23.0.

Results

The antibacterial activity of nCFS against gram-positive and gram-negative organisms of food borne pathogens and clinical isolates are mentioned in Table 1. Total 17 indicator organisms were used in agar well diffusion assay, out of which 5 were ATCC food isolates while the remaining 12 were clinical isolates. The isolated strain has shown inhibitory activity against four of the five foodborne pathogens, including *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Vibrio parahaemolyticus* and no activity against *Vibrio alginolyticus.* The clinical isolates *Staphylococcus epidermidis, Streptococcus pyogenes, Bacillus subtilis, Proteus vulgaris* and *Proteus mirabilis* showed no activity. Whereas, *Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica* has shown antibacterial activity.

Table 1: Antibacterial activity of *L. rhamnosus* against foodborne and clinical pathogens by agar well diffusion assay

Indicator Organisms	Isolation source	Zone of inhibition
Gram-Positive Organisms		
Staphylococcus aureus	Clinical	5.5±1.00
Staphylococcus epidermidis	Clinical	-
Streptococcus pyogenes	Clinical	-
Enterococcus faecalis	Clinical	4.0 ± 0.50
Bacillus subtilis	Clinical	-
Bacillus cereus ATCC 11778	Food	7.00 ±0.00
Listeria monocytogenes ATCC 13932	Food	10.00±0.50
Micrococcus luteus	Clinical	6.00±1.00
Gram-Negative Organisms		
Vibrio parahaemolyticus ATCC 17802	Food	6.50±0.50
Vibrio alginolyticus ATCC 17749	Food	-
Escherichia coli ATCC 8739	Food	7.66±2.08
Salmonella typhimurium	Clinical	6.33±1.04
Klebsiella pneumoniae	Clinical	4.16±0.76
Pseudomonas aeruginosa	Clinical	6.00±0.50
Proteus vulgaris	Clinical	-
Proteus mirabilis	Clinical	-
Salmonella enterica	Clinical	4.33±0.28

*ZOI (zone of inhibition) diameter in mm (Mean ± Standard deviation), No zone of inhibition = -

Analysis of the antibiogram of *L. rhamnosus* is depicted in Table 2. The majority of the antibiotics used in this study were found resistant to the tested strain of LAB. Furthermore, the Chloramphenicol and Novobiocin were observed susceptible towards the *L. rhamnosus* with a zone diameter of around 28 mm and 30 mm, respectively. Aminoglycosides used in this study were found resistant, but Gentamicin and Tetracycline showed intermediate results. Whereas the beta-lactam antibiotics, Amoxicillin and Oxacillin, showed no susceptibility.

Table 2: Antibiotic susceptibility pattern by *L. rhamnosus* using Kirby-Bauer disc diffusion assay

Antibiotics discs	Concentration (µg)	Group of antibiotic	Zone diameter (mm)	Interpretation
Amoxicillin	10	β-lactam	-	R
Chloramphenicol	30	Amphenicols	28.66±0.94	S
Gentamicin	10	Aminoglycosides	16±0.00	I
Levofloxacin	5	Fluoroquinolones	11±1.41	R
Novobiocin	30	Aminocoumarin	30.66±1.88	S
Oxacillin	1	β-lactam	-	R
Streptomycin	10	Aminoglycosides	-	R
Tetracycline	20	Tetracyclines	16±0.00	I
Tobramycin	10	Aminoglycosides	-	R

*I= Intermediate (16-20 mm), R=Resistant (≤ 15 mm) S= Sensitive (≥ 21 mm), No zone = -

Discussion

The antimicrobial properties of LAB have upraised its scope of application in the food industry for controlling the bacteria as an alternative to chemical preservatives[4]. Similar results were recorded in our study that shows the antagonistic potential of *L. rhamnosus* towards foodborne and clinical pathogens. Antibacterial effects of organic acids were prevented from influencing the ZOI by neutralizing pH to 6.5. Therefore, the antibacterial activity exhibited by this strain is due to the inhibitory substances such as hydrogen peroxide, bacteriocin or bacteriocin-like inhibitory substance. This study also signifies the antagonistic behavior of the isolate against major clinical pathogens, including *L. monocytogenes, B. cereus* and *P.aeruginosa*, all of which are known to induce serious infections. The maximum inhibition of 10mm was observed in gram-positive *L. monocytogenes.* Previous studies also reported higher antimicrobial activity against gram-positive bacteria[4,7], which is due to the bactericidal effect of protease, as it was elaborated by De Vuyst[8] whereas, the lack of activity against gram-negative bacteria is due to the presence of hydrogen peroxide[9].

The isolated strain was tested against nine antibiotics (Table 2). Our strain is resistant against the β-lactam group, amoxicillin and oxacillin. These results are in accordance with Halami et al[10]. However, previous studies have also reported susceptibility against these cell wall inhibitors[11, 12]. Similarly, the aminoglycosides group, which included Gentamicin, streptomycin and tobramycin, showed no susceptibility and the results ranged from intermediate to resistant. Resistance to aminoglycosides has been described as intrinsic in some LAB sp. including *L. rhamnosus*[13]. The only inhibitor of protein synthesis in the tested group to demonstrate susceptibility was Chloramphenicol; similar results have been reported by Liasi, 2009[11]. Novobiocin also demonstrated sensitivity, while Tetracycline recorded intermediate ZOI.

Antibiotic susceptibility of LAB has not been explored to a significant extent[11], yet the scope of their prevalence in unregulated probiotics and fermented or fortified foods has caused great concern over the implications of this pattern, the reason being LAB's ability to transfer AR genes to commensal bacteria and pathogens in the GI tract. The presence of LAB with acquired resistance in the

human food chain represents a great threat to human health[3]. On the other hand, the antimicrobial activity of *L. rhamnosus* against foodborne and clinical pathogens have shown promising results by inhibiting the growth of 11 out of 17 pathogens listed in Table 1. The results are in accordance with previous studies by Sharma et al and Gharib and demonstrate the probiotic characteristics of *L. rhamnosus* that signify its efficacy against pathogenic microorganisms[14,15]. However, a systematic review by the Agency for Healthcare Research and Quality (AHRQ) has raised concerns over the lack of information available to draw a conclusive case for safely recommending probiotic-based intervention[16].

Conclusion

In conclusion, the major finding of this study indicates that *L. rhamnosus* has exhibited promising antibacterial activity against a broad spectrum of microorganisms. Even though antibiotic susceptibility was observed against Chloramphenicol and Novobiocin, yet the strain was also observed to be resistant against Amoxicillin, Oxacillin, Streptomycin and Tobramycin. These findings enable us to conclude that while effective as a deterrent against foodborne and clinical pathogens, its antibiotic resistance makes it important to examine the AR-pattern in *L. rhamnosus* before considering it as a viable probiotic in health supplements and a variety of fermented and dairy food products to ensure that it is not acting as a reservoir for transmission of AR genes to pathogenic bacteria.

Conflicts of Interest

None.

Acknowledgement

The authors show high gratitude to Ms. Aribah Naz from the Department of Genetics, the University of Karachi, for providing technical assistance in completing this research.

Funding

None.

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