International Journal of Design & Nature and Ecodynamics
Vol. 19, No. 6, December, 2024, pp. 1971-1977
Journal homepage: http://iieta.org/journals/ijdne

Antimicrobial Activity of Partially Purified Bacteriocin from *Lactobacillus Reuteri* Against Pathogenic Bacteria and Fungi

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https://doi.org/10.18280/ijdne.190613

Received: 16 October 2024
Revised: 21 November 2024
Accepted: 29 November 2024
Available online: 27 December 2024

Keywords:
antimicrobial, bacteriocin, reuterin, Lactobacillus reuteri, P. aeruginosa, Staph. Aureus, C. albicans, protease

ABSTRACT

Reuterin is an antimicrobial factor produced by *Lactobacillus reuteri,* a lactic acid bacterium involved in food fermentation and found in various ecological niches such as the intestinal gut. This is significant as the consumption of contaminated food leads to large economic losses in the nutrition industry. The aim of this study is to evaluate and estimate the antimicrobial activity of bacteriocin called reuterin produced by *Lactobacillus reuteri* isolated from dairy products against some pathogenic bacteria and fungi in Baghdad hospitals. Twenty-four clinical isolates were collected from patients suffering from urinary tract infections, including 20 (83.3%) urine isolates and 4 (16.6%) blood isolates. All *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* isolates were identified using the Vitek 2E compact system. The sensitivity of all bacterial isolates to 12 antibiotics was tested. On the other hand, 3 isolates of *Lactobacillus reuteri* were isolated from fermented dairy products, all isolates were undergone to the cultural, microscopically, biochemical test and API 50 CHL for the identification of species. Six of these antibiotics belonged to the cephalosporin group. The results showed that *P. aeruginosa* was 100% resistant to cefotaxime, ceftriaxone, and ceftazidime, but exhibited only 12% resistance to imipenem. There was a significant difference in their resistance levels, with S. aureus isolates showing high resistance to cefotaxime (91%) and ceftriaxone (70%), but a lower resistance to imipenem (12%). The results showed that reuterin at a bacteriocin concentration of 30% saturation produced the highest inhibition zones—21.3, 21.4, and 23.2 mm—against *P. aeruginosa*, S. aureus, and C. albicans, respectively. When bacteria were cultured under appropriate conditions on MRS medium, crude reuterin extract (bacteriocin) showed a good inhibitory effect against at levels (stock solution to 10^{-6}) that all pathogenic isolates loss the Protease production, while little and normal activities were observed at concentrations (10^{-7} to 10^{-10}). The result of our research was that *P. aeruginosa* 3 had the highest activity (19 mm) by examining the diameter of the lysis zone on skim milk agar medium, but the P.2 and C.9 were (12 mm in diameter). Bacteriocin (reuterin) demonstrates the greatest inhibition zone against pathogenic bacteria at concentrations (10^{-7} to 10^{-10}).

1. INTRODUCTION

Microorganisms, particularly lactic acid bacteria (LAB), play a key role in food fermentation and have been used in this process for centuries [1, 2].

There is increasing interest in LAB as probiotics due to rising antibiotic resistance [3]. The antibacterial effects of LAB inhibit the growth of potential pathogens in various ways, including reducing pH, secreting bactericidal proteins, and impeding bacterial adhesion to epithelial cells [4]. LAB was a group of gram-positive, non-spore-forming bacteria, including the genera *Streptococcus, Lactobacillus, Lactococcus, Pediococcus, and Leuconostoc*. These cocci or bacilli all produce lactic acid as the end product during carbohydrate fermentation [5]. These organisms create many compounds like organic acids, diacetyl, H_2O_2, acetaldehyde and bacteriocin through lactic fermentation [6]. Many studies have attempted to clarify the mechanisms behind reuterin's antibacterial influence, which has proven difficult to elucidate. This is due to the highly reactive aldehyde group in reuterin, which can convert into various compounds in aqueous solutions [7, 8].

Bacteriocins are peptides manufactured by ribosomes with antimicrobial properties, produced by bacteria such as LAB (*Lactococcus, Lactobacillus*, *Pediococcus* spp., *Enterococcus*, and *Leuconostoc*) that are of particular interest due to their potential use as bio-preservatives in the food industry [9, 10]. Bacteriocin is an antimicrobial factor generated by *Lactobacillus reuteri,* a food fermentative lactic acid bacterium found in different of environmental like intestinal gut [11]. Factors affecting the activity of bacteriocins in various food systems are constitution (proteins and lipids),

hydrolysis of enzymes, synthesis process, physical properties, like pH, antimicrobial activity, and partial description of bacteriocin-like inhibitory material produced by *Lactobacillus* spp. Therefore, the successes of using bacteriocins in the food synthesis to control various pathogens are necessary to prove the antimicrobial activity of bacteriocins through laboratory studies and in food systems [12].

Lactobacillus reuteri is a heterofermentative lactic acid bacteria that belongs to the autochthonous microbiota of humans and animals [13]. Reuterin is an antimicrobial agent 0utput *Lactobacillus reuteri,* and has been suggested as a method of umpire, in part, the health benefits of probiotics impute to these bacteria. For all 20 years of investigation, the device of work by which reuterin apply its antibacterial effects has carry on shifty. Reuterin is a potent antimicrobial agent active opposed to Gram-positive and Gram-negative bacteria, beside yeasts, molds and protozoa [14].

Reuterin has been postulated that reuterin plays a major role in the probiotic action of *L. reuteri*. Reuterin is effective against enteric pathogens, yeasts, fungi, protozoa, bacteria and viruses [15, 16].

This study aims to isolate and identify *Lactobacillus reuteri* from dairy products and extract bacteriocins (Reuterin), assessing the antimicrobial activity of these bacteriocins against pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1 Collection of samples

2.1.1 Isolates of pathogenic bacteria of all *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

Twenty-four clinical isolates were collected from patients suffering of urinary tract infections from Baghdad teaching laboratories in medical city hospitals in Baghdad city.

2.1.2 Isolates of *Lactobacillus reuteri*

Lactobacillus reuteri isolated from fermented dairy products, all isolates were undergone to the cultural, microscopically, biochemical test and API 50 CHL.

2.2 Culture media

- Nutrient agar and selective medium

Nutrient agar medium was used for the growth and conservation of pathogenic, such as *Staph. aureus* and *P. aeruginosa*, while *Pseudomonas* agar medium used as selective for *P. aeruginosa* and Mannitol salt agar medium was to select for *Staph. aureus* growth [17, 18].

- Potato dextrose agar (PDA) and Sabouraud agar

Selective media was used for growth and conservation of *Candida albicans* [19].

- MRS medium (De Man- Rogosa- Sharp medium)

Lactobacillus reuteri was grown in MRS broth and the medium was used to preserve the bacteria and to specify the growth density of the probiotics under study and their production of bacteriocins.

- Skim milk agar selective medium for protease activity

SMA medium was prepared with a composition of 2% skim milk (Lactona) and 2% agar, then bacteria were inoculated and incubated at 37°C for 24 to 48 h. After the incubation period, it was possible to observe the indication that the growing bacteria were proteobacteria by the good growth of the isolates and the presence of a clear zone around the colony. This activity was tested before and after adding the reuterin extract to the N. agar medium at concentrations (10^{-1} to 10^{-10}) [16, 18].

2.3 Solutions

2.3.1 Standard McFarland solution

McFarland solution was made rendering to (MacFaddin, 2000).

- Prepare a 1% solution of ($BaCl_2$), and a 1% solution of (H_2SO_4).
- Integrate and wholly mix of barium chloride (0.05) ml with H_2SO_4 solution (99.5) ml to form a turbid suspension A approx. cell density (1×10^8 CFU/ml).

2.3.2 Physiological saline solution

It was prepared by dissolving 8.5g of NaCl in 1000 ml of distilled water, then sterilized by autoclave, and then kept at 4°C until use.

2.3.3 KH_2PO_4 buffer 20mM, pH 7.0

It is prepared as following:

1. Liquefied KH_2PO_4 0.34gm / 100 ml distilled water.
2. Liquefy 0.27gm of KH_2PO_4 in 100 ml of distilled water.

Next 61 ml of KH_2PO_4 and 39 ml of KH_2PO_4 solutions were mixed and pH was adjusted to 7.0 and executed to 200 ml.

2.3.4 Ammonium per sulfate solution $(NH_4)_2SO_4$

It is freshly prepared by dissolving 750g of $(NH_4)_2SO_4$ to liter of distilled water in a container or beaker. Simply stir the solution at 25°C - 30°C with a magnetic stirrer for 15min. or until saturation. Pour out the clear supernatant solution. Carefully allow the undissolved solids to settle gently at the bottom of the container [19, 20].

2.4 Preparation of lactic acid bacteria (LAB) supernatant (Bacteriocin-like substance assay)

The bacteria were cultured in MRS broth at 37°C for 24 hours. Subsequently, 10% of the subculture from 1000 ml of MRS broth was taken and incubated at 37°C for 72 hours. After this, the efficacy of the crude extract of *Lactobacillus reuteri* as an antibacterial agent against pathogens was evaluated for 25 minutes at 4°C. The supernatant was collected, and its pH was adjusted to 6.5 using 5N NaOH, then sterilized through a cellulose acetate filter with a pore size of 0.2 µm [19].

To obtain the crude reuterin extract, the supernatant was placed in a water bath at 80°C for 10 min.

2.5 Partial purification and concentration of a bacteriocin by ammonium sulfate precipitation

1. Sample of bacteriocin solution transferred to beaker containing a stir bar and put in an ice box, then place it on magnetic stirrer. Stir the sample for about 30 minutes, then gradually add $(NH_4)_2SO_4$ to achieve final concentrations ranging from 20% to 90% saturation. Afterwards, centrifuge the tubes at 6000 rpm for 30 minutes.
2. Softly eliminate and discard precipitates into the waste vessel.
3. Resuspend granule in 1 ml of D.W. and Transfer protein solution (bacteriocin) to dialysis tubing and

dialyze versus the distilled water.

4. Protein solution eliminates from the tubing then centrifuged to eliminate any remaining debris.
5. The concentration specifies and saved at -8°C for long dated save [21].

2.6 Bacterial antibiotics sensitivity test

The sensitivity of bacteria to some antibiotics was tested using the (1966) method. A bacterial suspension was prepared from the isolate to be tested by transferring (4-5) single, pure colonies, (24) hours old, growing on the nutrient agar medium using a microbial carrier, to (5) ml of the prepared functional saline solution. The turbidity of the suspension was compared with the turbidity of the prepared standard turbidity constant solution (McFarland (2.2.1)), which gives a number of cells approximately (10^8 × 1.5) CFU/ml. Using a clean, sterile cotton swab, a portion of the bacterial suspension was spread on the surface of plates containing Mueller-Hinton agar medium. Then, antibiotic discs were distributed on the surface of the agar at a rate of (6) discs for each plate by using sterile forceps, then plates incubated at 37°C for 24 hr. Later that, results were observed and the inhibition zones around each disc were measured. By referring to standard tables specific to each type of antibiotic, it was determined whether the isolate was resistant or sensitive to antibiotics.

2.7 Evaluation of the efficacy of (Bacteriocin) reuterin extract of *Lactobacillus reuteri* as an antimicrobial opposite pathogenic bacteria

Three replicates were prepared for each isolate.

- Bacterial suspension was prepared from pathogenic bacteria using physiological saline solution (see section 2.3.2). The turbidity was measured using McFarland's standard solution (see section 2.3.1) and then incubated for 24 hours.
- The agar well diffusion assay (WDA) was performed by transferring 0.1 ml of the pathogenic suspension to Mueller Hinton Agar (MHA) medium, which was then evenly spread using the matting method.
- Wells with a diameter of 8 mm were made in the agar, and the plates were left to dry.
- Put (0.1) ml from bacteriocin in wells.
- The inhibition of growth was assessed by measuring the diameter of the inhibition zone around each well [22].

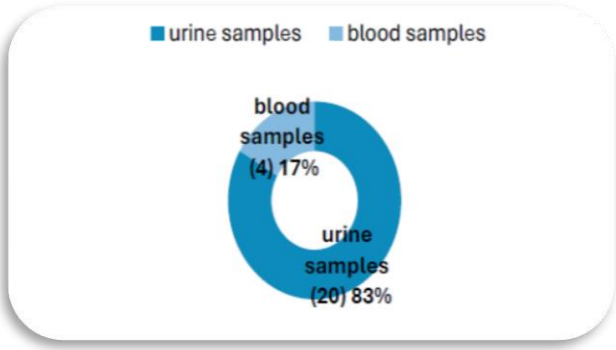

Figure 1. Positive of percentage of urine and blood infections

3. RESULTS AND DISCUSSION

3.1 Identification of all *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

Twenty-four clinical isolates were collected from patients suffering from urinary tract infections; these included 20 isolates (83.3%) from urine and 4 isolates (16.6%) from blood samples. All isolates of *P. aeruginosa*, *S. aureus*, and *C. albicans* were identified using microscopic and biochemical tests and confirmed with the Vitek 2E compact system. These results were illustrated in Table 1 and Figure 1.

Table 1. Number of positive bacterial cultures of samples and the percentage of urine and blood infections

Total Number of Bacterial Samples	Positive Culture 24(100)%	
	Number of Urine Samples	Number of Blood Samples
24	20 (83.3%)	4 (16.6%)
Pseudomonas aeruginosa	6	1
Staphylococcus aureus	6	2
Candida albicans	8	1

3.2 Isolation and identification of all *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

- **Isolated and identification *Pseudomonas aeruginosa***

The bacterial isolates were cultured on nutrient agar and Pseudomonas agar, as shown in Figure 2, where they exhibited pyocyanin pigment colonies. This medium was generally used in the laboratory for the selective cultivation of *P. aeruginosa*, a Gram-negative bacterium.

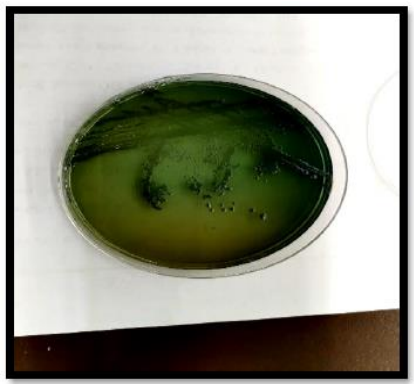

Figure 2. *P. aeruginosa* produced of pyocyanin pigment on nutrient agar

- **Isolate *Staphylococcus aureus***

Staphylococcus aureus isolates were cultured on mannitol salt agar medium which selective growth of *Staphylococcus* spp. Because of its constituents of sodium chloride (75.0 gm/L) is regarded as selective and differential medium for Staphylococci as shown in Figure 3 [18].

Few isolates could the ferment mannitol and composed a large golden colony surrounded by yellow zones and turned the medium color from pink to yellow, while others were non-mannitol-fermenter with white color was manifested on the mannitol agar agree with study [20].

Figure 3. *S. aureus* colonies on mannitol salt agar medium

- **Isolated and identification *Candida albicans***

Clinical Candida was cultured in aerobic and anaerobic blood culture bottles and grown in the automated blood culture system at 35℃. The aliquot was then removed and CHROM agar Candida was plated as a control and cultured on Sabouraud dextrose agar at the same time. No significant difference in colony form or color was observed between blood and control isolates in this agrees with study [20].

3.3 Antibiotic sensitivity tests

In the present study, standard disc diffusion test has been used for detection of susceptibility of pathogenic bacteria to antibiotics, as shown in Figures 4, 5 and 6.

Figure 4. *C. albicans* isolates growth on Muller Hinton agar

Figure 5. Sensitivity test using antimicrobial discs against *P. aeruginosa* cultured on Mueller Hinton agar

P. aeruginosa isolates appears a various resistance levels to antimicrobial as following: the isolates were resistant to Cefotaxime was (100%); Ceftriaxone (100%); Ceftazidime (100%); Cefepime (85%); Cefoxitin (63%); Ciprofloxacin (61%), while revealed lower resistance to Aztreonam (43%); Ampicillin (32%); Vancomycin (35%); Levofloxacin (47%); Rifampin (24%); imipenem was (12%) as shown in Figure 7.

S. aureus isolates revealed a various were relatively of resistant for than *P. aeruginosa* to Antimicrobial Cefotaxime was (91%); Ceftriaxone (70%); Ceftazidime (42%); Cefepime (26%); Cefoxitin (25%); Ciprofloxacin (41%), while Aztreonam (70%); Ampicillin (54%); Vancomycin (74%); Levofloxacin (22%); Rifampin (20%)); Imipenem was (15%) as shown in Figure 8, this does not agree with studies [6, 18].

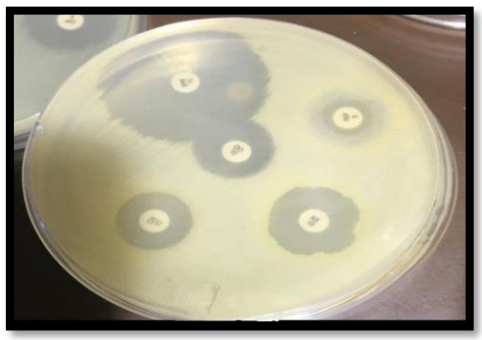

Figure 6. Sensitivity test using antimicrobial discs against *S. aureus* cultured on Mueller Hinton agar

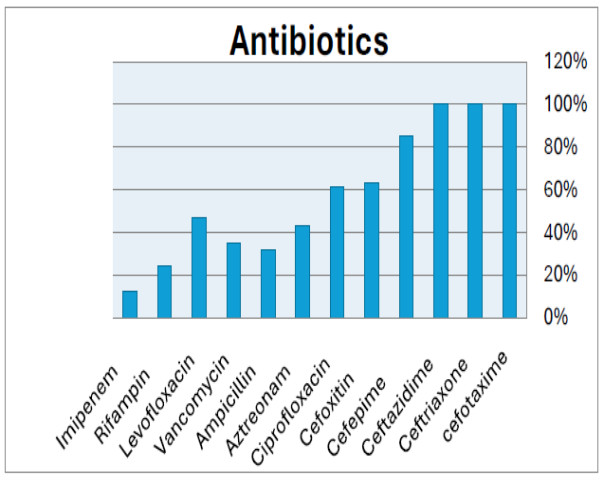

Figure 7. Results of susceptibility tests of *P. aeruginosa* against 12 antimicrobial agents according to (CLSI 2015)

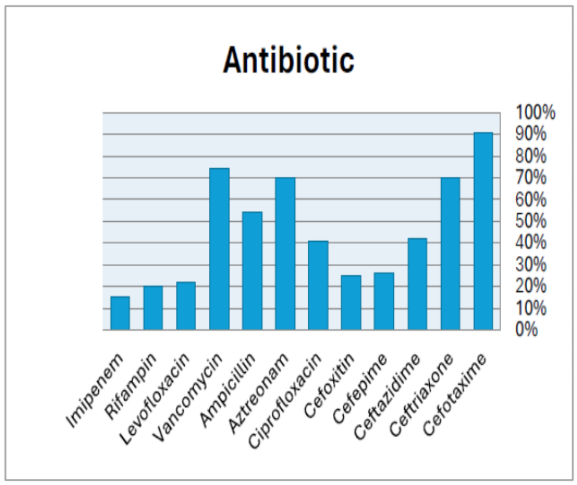

Figure 8. Results of susceptibility tests of *S. aureus* against 12 antimicrobial agents according to (CLSI 2015)

3.4 Antibacterial activity of crud and partially purified reuterin (bacteriocin) from *Lactobacillus reuteri* on growth of some pathogenic bacteria

P. aeruginosa, Staph. aureus and C. albicans isolates were possessing high resistance to antibiotics and able to produce virulence factors.

The protease extracted from some pathogenic bacteria important a virulence factor that was especially for study. The results of this study showed that *P. aeruginosa* (3) were the most active (19 mm diameter) through measuring of the inhibition zone on skim milk medium (or by the well procedure), at the same time as the *P*. (2) and *C*. (9) were the lowest down protease activity (12 mm) in diameter as illustrated in Table 2.

Table 2. Protease activity produced of *P. aeruginosa*, *S. aureus* and *C. albicans* isolates detected measuring the diameter of lysis position later 18-24 hours at 37℃ of incubation

ACTIVITY Isolate No.	PROTEASE Activity Diameter of Lysis Area in (mm)
P. 1	14
P. 2	12
P. 3	19
P. 4	16
S. 5	14
S. 6	14
S. 7	16
S. 8	14
C. 9	12
C. 10	15
C. 11	14
C. 12	14

The reuterin extract, a bacteriocin, showed a good inhibitory effect on *P. aeruginosa*, *Staph. aureus* and *C. albicans* at concentrations ranging from the stock solution to 10^{-6}. All isolates of these pathogens lacked protease enzyme activity, while few or normal activities were observed at dilutions ranging from 10^{-7} to 10^{-10}, as shown in Table 3. This suggests that contact with other microbes in urine increases the production of reuterin, which inhibits bacterial growth by modifying thiol groups. This indicates that reuterin negatively affects multiple cell compounds, a finding that was consistent with study [7].

Table 3. Explain the activity of (Bacteriocin) levels on protease product by pathogenic bacteria

BACTERIOCIN Activity Levels	PROTEASE Producing Activity Pathogenic
Stock solution	No-producing
Con.10^{-1}	No-producing
Con. 10^{-2}	No-producing
Con. 10^{-3}	No-producing
Con. 10^{-4}	No-producing
Con. 10^{-5}	No-producing
Con. 10^{-6}	No-producing
Con. 10^{-7}	Little (10mm)
Con. 10^{-8}	Normal (12mm)
Con. 10^{-9}	Normal (14mm)
Con. 10^{-10}	Normal (14mm)

3.5 Evaluating the antibacterial activity of (Bacteriocin) reuterin extract of *Lactobacillus reuteri* against pathogenic bacteria

Lactic acid bacteria were used as antimicrobial agent. This comes down to its capability to secrete antibacterial materials that prevent the growth of pathogenics. LAB secretes anti-microbial composites (bacteriocins). This study shed light on a bacterium called reuterin, which is produced by *Lactobacillus reuteri*, and assesses its antibacterial activity against various bacterial and fungal pathogens found in hospitals. Roterin production (bacteriocin concentration (30%) saturation) showed the highest inhibition zone (21.3, 21.4 and 23.2 mm) opposed *P. aeruginosa, Staph. aureus* and *C. albicans*, respectively, at it's grown anaerobically on MRS agar, pH 6.5 at 37℃ for one day. This study has permissible us to utilizes reuteri as an antimicrobial into reduce pathogenics. This agrees with study [7] that appears the reuterin has inhibitory activity against Gram (+) and Gram (-) bacteria. The inhibition zones were up measuring 23mm and 21mm in diameter contra *Staph. aureus* and *E. coli*, respectively.

Using the agar well diffusion assay (WDA), 0.1 ml of the suspension, at concentrations ranging from the stock solution to 10^{-10}, was transferred to Mueller-Hinton agar and spread using the matting method. Wells with a diameter of 8 mm were created, and the plates were then left to dry.

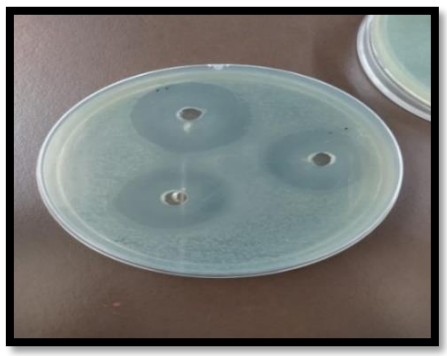

Figure 9. Antibacterial activity of crude and partially purified reuterin (Bacteriocin) against *P. aeruginosa*

Figure 10. Antibacterial activity of crude and partially purified reuterin (Bacteriocin) against *Candida albicans*

Subsequently, 0.1 ml of reuterin extract (bacteriocin) was added to each well. After four hours, the plates were incubated under different conditions: anaerobically at 4℃ for lactic acid bacteria (LAB), and aerobically at 37℃ for other pathogens,

for 18 to 24 hours. Three replicates were conducted for each isolate. The inhibition of growth was assessed by measuring the zone of inhibition surrounding each well, as illustrated in Figures 9 and 10.

There are supports our results through study [11] that reuterin showed in yogurt, a (fungistatic) effect at 1.4 mM of level but a (fungicidal) effect was geted at concentration of 7 mM. so, reuterin has a high possible as a nutrient protector, a specialty having to its biochemical characterizes and antimicrobial effectiveness es. in addition, reuterin illustrated a fungicidal effectiveness (killed 99.9% of all tested M.Os) at levels equal or less than 15.6 mM as indicated by MFC.

4. CONCLUSIONS

1. *Lactobacilli* can display antipathogenic bacterial activity through numerous mechanisms, as production of antimicrobial bioactive agents that can inhibit the growth of the pathogens.

2. Bacteriocins are antimicrobial compounds named reuterin which are produced by *Lactobacillus reuteri* have antimicrobial effectives against some hospitalized pathogens.

3. Bacteriocin (reuterin) demonstrates the greatest inhibition zone against pathogenic bacteria at concentrations (10^{-7} to 10^{-10}). The results of our research were that *P. aeruginosa* 3 had the elevate effective (19 mm) by examining of the inhibition zone on skim milk agar medium, but the P. 2 and C. 9 were (12 mm in diameter).

4. The reuterin (Bacteriocin) is used as therapeutic agents.

ACKNOWLEDGMENT

The authors express their sincere gratitude to Mustansiriyah University, College of Science, Departments of Microbiology and Biology, for continued support of our research program.

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