Antibacterial activity of Lactobacillus plantarum bacteriocin as a dermal probiotic against Pseudomonas aeruginosa isolated from diabetic foot ulcer

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Abstract:

The current study was considered to select a appropriate isolate of *Lactobacillus* sp. in order to use it as an antibacterial formula for treating diabetic foot ulcer disease in diabetic mellitus patients, over two hundred *Lactobacillus* isolates were collected from different sources for this purpose and subjected to a screening program to evaluate their antagonism activities against multi-drug resistant (*Pseudomonas aeruginosa*) foot ulcer pathogen which were chosen from one hundred and twenty pathogenic isolates. several *Lactobacillus* isolates were selected after primary and secondary screening according to their ability to inhibit the growth of *P. aeruginosa* and ability to produce bacteriocin**.** Results showed the isolate *Lactobacillus* L40 was selected as a highest bacteriocin producing isolate which was further characterized as *Lactobacillus plantarum* through Vitek 2 system. Bacteriocin from *L. plantarum* L40 was partially purified by precipitation with ammonium sulphate with 80% saturation and then separated with sephadex G-150 gel filtration. The specific activity of the resulted partial purified bacteriocin was increased to 864.86 AU/mg with 13-fold purification and 64% yield. The study of bacteriocin characterization revealed that the activity of bacteriocin was stable after 10 min at 20, 30, 40C° whereas, more than 50% of the bacteriocin activity was lost after exposure to 50C° and decreased to approximately 20 AU/ml at 60,70 and 80C°. In addition, bacteriocin activity showed stability at pH 6 and 7 for 30 min while, it was decreased by approximately 50% at pH 5 and 8, and completely inhibited at pH 4 and 9. Antimicrobial activity tests of the partial purified bacteriocin against *P. aeruginosa* clinical isolates showed that bacteriocin was active against Gram negative bacteria. Formula was selected and optimized that can be used in this study, formula containing bacteriocin produced by *L. plantarum* L40 formula showed antibacterial activity by inhibiting (*P. aeruginosa)* growth. The results showed the possibility of using partially purified bacteriocin from *L. plantarum* L40 as an effective probiotic to deal with some skin pathogens, and treat skin diseases such as diabetic foot ulcer disease. The results supported the idea of using probiotic as an alternative method for the treatment with antibiotics.

Keywords: *Lactobacillus*, bacteriocin, antibacterial, probiotic.

الفعالية المضادة لبكتريوسين Lactobacillus plant arum كمعزز حيوي جلدي ضد بكتريا الزائفة الزنجارية المعزولة من قرحة القدم السكري

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الخلاصة:

تم النظر في الدراسة الحالية لاختيار العزلة المناسبة من Lactobacillus sp من أجل استخدامه كتركيبة مضادة للبكتيريا لعلاج مرض قرحة القدم السكرية لدى مرضى السكري ، تم جمع أكثر من مائتي عزلة من Lactobacillus من مصادر مختلفة لهذا الغرض وخضعت لبرنامج فحص لتقييم نشاطها العدائي ضد مقاومة الأدوية المتعددة (الزائفة الزنجارية) المسببة لقرحة القدم السكري والذي تم اختياره من مائة وعشرين عزلة ممرضة. تم اختيار العديد من عزلات Lactobacillus بعد الفحص الأولي والثانوي وفقًا لقدرتها على تثبيط نمو الزائفة الزنجارية والقدرة على إنتاج البكتريوسين. أوضحت النتائج أن العزلة Lactobacillus L40 اختيرت كأعلى عزلة منتجة للبكتيريوسين والتي شخصت كذلك بأنها Lactobacillus plantarum من خلال نظام Vitek 2. تمت تنقية البكتيريوسين من L. plantarum L40 جزئيًا عن طريق الترسيب مع كبريتات الأمونيوم بنسبة تشبع 80٪ ثم فصلها باستخدام sephadex G–150 بالترشيح الهلامي. تمت زيادة النشاط النوعي للبكتريوسين المنقى الجزئي الناتج إلى 864.86 AU / mg مع

تنقية 13 ضعفًا ونسبة 64٪. كشفت دراسة توصيف البكتريوسين أن نشاط البكتريوسين كان مستقرًا بعد 10 دقائق عند 20 ، 30 ، 40 درجة مئوية ، بينما فقد أكثر من 50 ٪ من نشاط البكتريوسين بعد التعرض إلى 50 درجة مئوية وانخفض إلى حوالي 20 وحدة فلكية / مل عند 60 درجة مئوية. ، 70 و 80 درجة مئوية. بالإضافة إلى ذلك ، أظهر نشاط البكتريوسين ثباتًا عند الأس الهيدروجيني 6 و 7 لمدة 30 دقيقة بينما انخفض بنسبة 50٪ تقريبًا عند الأس الهيدروجيني 5 و 8 ، وتم تثبيطه تمامًا عند الأس الهيدروجيني 4 و 9. الفعالية المضادة للبكتريا (للبكتريوسين المنقى جزئياً) ضد الزائفة الزنجارية السريرية أظهرت أن البكتريوسين كان فعالاً ضد البكتيريا سالبة الجرام. تم اختيار التركيبة وتحسينها بحيث يمكن استخدامها في هذه الدراسة ، حيث أظهرت التركيبة المحتوية على البكتريوسين المنتج بواسطة تركيبة L. plantarum L40 نشاطًا مضادًا للبكتيريا عن طريق تثبيط نمو (الزائفة الزنجارية). أظهرت النتائج إمكانية استخدام البكتريوسين المنقى جزئياً من L. plantarum L40 كبروبيوتيك فعال للتعامل مع بعض مسببات الأمراض الجلدية وعلاج الأمراض الجلدية مثل مرض قرحة القدم السكرية. دعمت النتائج فكرة استخدام البر وبيوتيك كطريقة بديلة للعلاج بالمضادات الحيوية.

الكلمات المفتاحية: بكتريوسين، مضاد حيوي، مضاد بكتيري، بكتريا حمض اللاكتك

Introduction:

Nearly a third of the anticipated US$176 billion in direct healthcare expenditures for diabetes care in 2012 are attributable to the treatment of diabetic foot ulcers [1]. Despite the enormous financial investment in healthcare, 20% of patients still aren't well a year after treatment. About 40% of people who have had diabetic foot ulcers heal will experience a recurrence of their condition within a year [2]. Despite the fact that there are defined guidelines for the management of diabetic foot ulcers, therapy of these ulcers can be difficult. A wide range of new interventions to improve wound healing are being studied [3].

High rates of morbidity, death, and healthcare costs have been linked to diabetic foot ulcers (DFU), a common consequence of diabetes. The International Diabetes Federation estimates that between 19% and 34% of patients with diabetes may develop DFU at some point during their lifespan [2]. Despite its long clinical therapeutic attempts, diabetes mellitus still represents a major health concern for millions of people globally and in particular, its most severe complications which is the development of diabetic foot ulcers [2]. Therefore, finding new therapeutic protocols for diabetic foot ulcers confrontation are certainly important and urgent. In this context, several strategies were recently proposed such as antimicrobial peptides, using probiotics and even utilization of some bacteriophages as alternatives to currently available antibiotics, probiotic therapy provides promising results [4].

The purpose of this study is to identify a locally isolated *Lactobacillus* sp. that is able to produce a bacteriocin with antimicrobial activity against *Psedomonas aeruginosa* isolates seen in diabetic foot ulcer samples. The study is particularly, will focus on the multidrug-resistant bacterial isolates that associated with the diabetic foot ulcers. In addition, this work will investigate the possibility of using the *Lactobacillus* bacteriocin as a dermal probiotic for treatment of DFU. In order to achieve these goals, the following objectives were followed:

a) Isolation and identification of diabetic foot ulcer bacterial pathogens and *Lactobacillus* sp. from different sources.
b) Screening of *Lactobacillus* isolates for bacteriocin production and purification and characterization of bacteriocin stability.
c) Preparation of suitable formula containing viable cells of the selected *Lactobacillus* cells or its bacteriocin.
d) Evaluation of antibacterial activity of bacteriocin against *P. aeruginosa in virto*

Materials and Methods

Collection of pathogenic isolates

A total of one hundred twenty specimens taken by swab from individuals who suffer from diabetic foot ulcer, then were cultured in different media and primarily identified through morphological characteristics and regular Biochemical tests, including catalase, oxidase, indole and hemolysis test and further identified by Vitek 2 system.

Collection of Lactobacillus isolates:

A total of two hundred and forty were collected from Jan 2021 to July 2021 were collected from different sources as follows:

- Human specimens taken from mouth of healthy individuals

- Dairy products : including yogurt, cow milk and cheese.

The samples were primarily grown on de Mann Rogosa Sharpe (MRS) agar, incubate at 37ºC for 48 hours, which in anaerobic environments using candle-jar. To identify the isolates primarily, each colony was investigated for morphology, Gram staining, catalase activity test and oxidase test and further identified by Vitek 2 system. All bacteria isolates were stored for further study. All of particular bacterial isolates then kept at 4 °C in MRS broth supplemented with glycerol (30% v/v) [5].

Partial purification of bacteriocin

Protein concentration assay

The concentration of protein was estimated according to [6) by adding 0.1 ml of the sample into a test tube. Then, 2.5 ml of Coomassie stain and 0.4 Tris-HCl (0.02 M, pH 8.0) buffer were added to sample tube. The contents were mixed well and absorbance was measured at 595 nm after 2 minutes against the blank (2.5 ml of Coomassie stain and 0.5 Tris-HCl buffer). The protein concentration was estimated according to the bovine serum albumin standard curve which is prepared as follows:

A total of six different BSA concentrations (0, 20, 40, 60, 80, and 100 g/ml) were mixed up in a 0.1-milliliter test tube. A 0.4 ml from Tris-HCl buffer was added to each tube. A total of 2.5 ml of Comassie G-250 solution was added, thoroughly mixed, and then permitted to sit for 5 minutes. The spectrophotometric absorbance at 595 nm was determined. Both the Tris-HCl buffer (0.5 ml) and the Coomassie G-250 (2.5 ml) are included in the blank. It was determined by plotting a standard curve of BSA concentration vs absorbance [6].

Separation and purification of bacteriocin

a- Ammonium sulfate precipitation

Broth culture was centrifuged at (8000 rpm) for 30 minutes at 4ºC. bacteriocin activity and protein concentration were estimated. Cell free supernatant (CFS) was transferred to a beaker placed in an ice bath on a magnetic stirrer. An appropriate amount of ammonium sulphate (NH4)2SO4 was added gradually to the CFS in order to achieve 30, 40, 50, 60, 70 and 80 % (w/v) saturations under slow constant stirring at 4ºC. The stirring was continued for an additional 30 min. Each saturation level's precipitate was centrifuged separately for 30 minutes at 10000 rpm. Then, supernatant was decanted and the precipitates were re-dissolved in phosphate buffer pH 8. Next, the dissolved precipitates were dialyzed separately, in phosphate buffer using dialysis membrane tubes (1 kDa MW cutoff). Then, the dialysis bags contained precipitates were placed in 0.5 liter of phosphate buffer overnight at 4ºC. The buffer was replaced four times [7].

b- Sephadex gel filtration Chromatography

Further purification was carried out by gel filtration chromatography using Sephadex G-150 gel. The gel was loaded carefully in the column to obtain (1.6x 43 cm). The column was equilibrated with 0.1M phosphate buffer (pH 7.2). The dialyzed protein sample (4 ml) was then applied to the column and proteins were eluted using 0.1M phosphate buffer at room temperature. By setting the flow rate to 30 ml/hr. Absorption at 280 nm was determined for samples collected using a UV-spectrophotometer. Well agar diffusion assay was used to check the fractions for their ability to kill an indicator strain of bacteria. Protein concentration and bacteriocin activity were measured after combining the antimicrobial active fractions in a single test tube [8].

Preparation of bacterial pathogen as indicator

Pathogenic isolate was used: (*P. aeruginosa*) isolated from diabetic foot ulcer. This indicator was prepared as follow: A chosen colony was transferred via a sterile wire loop into a tube containing 5ml of brain heart infusion broth, and the tube was placed in an incubator at 37°C for 24 hours. After that, we standardized the cell density in the McFarland tube to 1×10^8 cells/ml at 600 nm [9].

Characterization of bacteriocin

a- Thermal stability of bacteriocin

In order to test the thermo stability of bacteriocin, samples were exposed to different temperatures (20, 30, 40, 50, 60, 70, 80, 90) C for 10 min followed by cooling on an ice-bath. The residual activity was then determined by agar-well diffusion technique against indicator bacteria [10].

b- pH stability of bacteriocin

Partially purified bacteriocin preparation was treated with either 0.1N HCl or 0.1 N NaOH to achieve the desired pH values between 4 and 9. The pH adjusted crude extracts were incubated for 30 min. After incubation, aliquots were neutralized and activity was

measured by agar-well diffusion technique against indicator bacteria [10].

Pharmaceutical formula including partially purified bacteriocin

A cream contained the partial purified bacteriocin was prepared as follow :

•Amount of 0.1 gm of methyl paraben was dissolved in 1ml of ethanol 70%.

•Forty-nine ml of olive oil was gradually added with continues mixing.

•5 ml of partially purified bacteriocin was added with mixing until homogenization. An amount of 50 gm of white petroleum (Vaseline) was added gradually with continuous mixing to homogenization. The prepared cream was stored in close container at 4ºC [11].

In vitro evaluation experiment of pharmaceutical formula

After preparing the suitable formula containing active material (partially purified bacteriocin), and to test the antibacterial activity within the formula, agar well diffusion method was used. An amount of 100µl of an overnight culture of the P. aeruginosa (as indicator) containing approximately 1×10^8 cells/ml was added into 100 ml of a sterile Muller Hinton agar kept at 45-50°C in a water bath. The mixtures were kept at the same temperature until poured into sterile plastic petri dishes and allowed to solidify. Circular wells of 5mm in diameter were made using a sterile cork borer and then low melting temperature of Muller Hinton agar was used to seal the bottom of the wells. Then, aliquots of 100µl of the formula containing partially purified bacteriocin were dispensed in the wells and then plates were incubated for 24 hr. at 37°C. Following the incubation, the diameter of inhibition zone around each well indicating to the antibacterial activity result [12].

Result & Discussions:

Morphological identification of Lactobacillus isolates was mainly achieved by investigating the appearance of colonies on the solid medium (MRS) as well as the microscopic examination. All colonies of isolation MRS agar were white, rounded in shape and range in consistency from creamy white in color to glossy white and moist-mucoid colony appearance on the surface. The results of microscopic examination showed that the isolates were Gram positive with long or short, opaque and smooth without pigment chains. The microscopic field examination for all isolates was investigated. Under light microscope, Lactobacillus is a Gram positive, rod-shaped (bacilli) and occurred singly, in pairs or even in chains [13] were performed to detect the genus Lactobacillus. Results exhibited that all Lactobacillus sp. isolates were oxidase and catalase negative. In conclusion, results of chemical and morphological tests revealed that 71 isolates from 240 samples isolated from cow's milk, sheep's milk, cheese, yoghurt and human mouth were Lactobacillus sp. confirmed by the biochemical tests. All of seventy-one Lactobacillus isolates and its source were employed in this study as stated in the following table (1).

Table (1) Morphological and biochemical results of *Lactobacillus* isolates and isolate source

Source	No. of isolates	Shape	Gram stain	Catalase test	Oxidase test	Hemolysis
Sheep milk	9	Rod in shape	+	-	-	α-hemolysis
Caw milk	12	Rod in shape	+	-	-	α-hemolysis
Yogurt	23	Rod in shape	+	-	-	α-hemolysis
Cheese	8	Rod in shape	+	-	-	α-hemolysis
Human mouth	19	Rod in shape	+	-	-	α-hemolysis
Total	71	Rod in shape	+	-	-	α-hemolysis

One hundred twenty subjects were included in this study, 60 male and 60females who were pre-diagnosed with diabetic mellitus disease and diabetic foot ulcer, the majority of microorganisms isolated were Gram negative bacteria. Most common were S. aureus, E.

coli, Proteus mirabilis, Klebsiella pneumoniae and Pseudomonas aeruginosa. The results demonstrated that Gram negative bacteria were the most common bacteria isolated from diabetic foot, including Proteus mirabilis (8.3%), Klebsiella pneumoniae (10.8%), Pseudomonas aeruginosa (12.5%) and E. coli (7.5%). Whereas, Gram positive presented 25.82% for both S. aureus and Staph. epidermidis. The present analysis showed that the most common Gram-negative bacilli in patients with foot syndrome were P. mirabilis, Ps. aeruginosa and Klebsiella spp., which made up more than 39% of the total. The study results were in agreement with a study conducted by [14]. However, subjects in this study have higher ratio of MRSA which is regularly hospital-acquired infection that is hard to exterminate, the study consequences agreed with [15] and[16]. Beside these bacterial isolates, 36 isolates (mixed bacteria and molds), identified morphologically and under microscope showed filaments proved their type. Mixed infections (bacteria with fungi) are uncommon but similarly found in DFU patients, for example Candida species and Aspergillus sp. were seen collective fungal isolates from DFU by [17]. only P. aeruginosa was included in this work.

The studied bacteriocin produced by L. plantarum L40 was precipitated from the culture supernatant by saturation with different concentrations of ammonium sulfate from (30, 40, 50, 60, 70 and 80%,) followed by dialysis to remove salts and impurities. Based on the results maximum bacteriocin precipitation was obtained at 80% saturation level. The bacteriocin activity was 320 AU/ml with specific activity of 336.8 AU/mg as shown in figure(1).

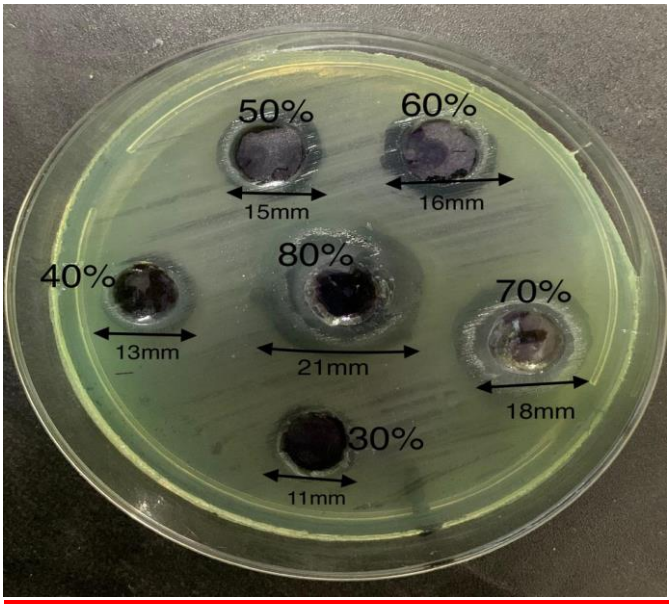

Figure (1) Bacteriocin activity precipitated with different concentrations of ammonium sulfate (clinical *p. aeruginosa*)

The precipitated bacteriocin was loaded in sephadex G-150. Sixty-five fractions of 4 ml were collected at a flow rate of 30 ml/hr. which then monitored at 280nm. Antimicrobial assay was performed for each fraction using *P. aeruginosa* as indicator isolate by agar well diffusion assay. two separated peaks were obtained in the separation profile. The bacteriocin activity was detected in eleven fractions corresponding to the second peak in fractions No. 38 to 49. The active fractions were collected and the specific activity of the partial purified bacteriocin was increased to 864.8 AU/mg resulting in 13-fold purification with 64% yield table (2) and Figure (2). In a similar study, bacteriocin produced by *L.* plantarum was purified using gel chromatography of Sephadex G150 column [18].

Table (2) Summary of bacteriocin purification of Lactobacillus plantarum L40

Purification Step	Volume (ml)	bacteriocin activity (AU/ml)	Protein Conc. (mg/ml)	Specific activity (AU/mg)	Total activity (AU)	Purification (folds)	Yield %
CrudeExtract	50	80	1.2	66.66	4000	1	100
Ammonium sulfate	10	320	0.95	336.84	3200	5.05	80
Sephadex G-150 after Concentration with Sucrose	4	640	0.74	864.86	2560	13	64

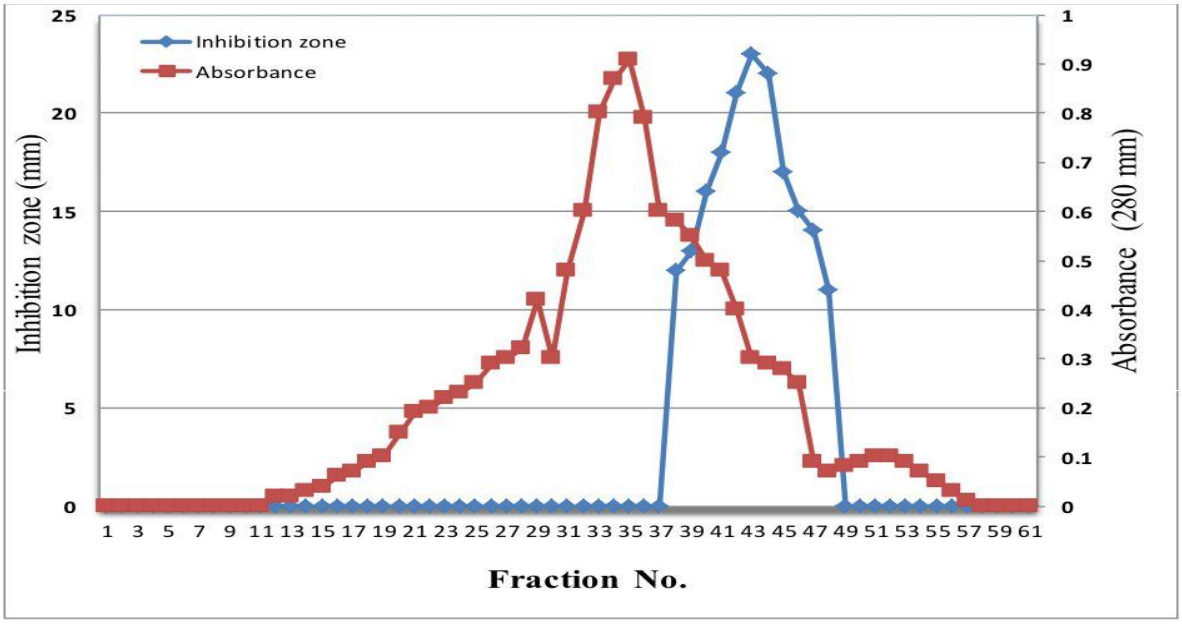

Figure (2) Purification of bacteriocin by Sephadex G-150 column equilibrated and eluted with sodium phosphate buffer, pH 7.2 with flow rate of 30ml/hr.

Thermal stability of bacteriocin is an important criterion that can help to determine whether the bacteriocin is belong to the class of heat-labile or heat –stable protein [19]. In order to examine its thermo stability, the activity of bacteriocin produced by *L. plantarum* L40 was tested in different temperatures ranging from 20 to 90◦C , the activity of bacteriocin was remained stable after 10 min at 20, 30 and 40 C◦ as no effect was observed on its antimicrobial activity. However, bacteriocin activity was significantly decreased after exposure to 50◦C for 10 min. In addition, the activity of bacteriocin was obviously decreased upon exposure to 60, 70 and 80 Co for 10 min, reaching to 20 AU/ml. Bacteriocin activity was disappeared in at 90Co for 10 min. From these results, it can be concluded that the bacteriocin is heat –labile. Some studies reported that no reduction was observed in the inhibitory activity of the CFS on heating to 40˚C for 10 or 30 min, but a slight reduction was observed when the heating was continued for 60 minutes. However, a seven percent % reduction in the activity was observed on heating at 60˚C, while heating to 100˚C or 121˚C resulted in complete loss of the activity regardless of the length of the heating period [8], reported a loss of activity after heat treatment at 121˚C for 15 min.

pH stability was used to test the activity of the bacteriocin from L. plantarum L40, bacteriocin activity showed stability at pH 6 and 7 in which the bacteriocin kept its stability of 640 AU/ml for 30 min. However, bacteriocin

activity was decreased to (80 AU/ml) at pH 8, whereas, no activity was observed at pH 4 and 9. The range of pH stability for bacteriocin activity is differing from one to another. Some bacteriocins have a wide range active of pH while others are active at a narrow range, it can be noticed from other studies that, the bacteriocins of Lactobacillus strains were active over a wide range of pH (2-6) and it reduced to alkaline pH, indicating strong probiotic potential, because most of the bacteriocins are resistant to acidic pH more than alkaline pH. The purified bacteriocin of Lactobacillus fermentum showed a maximum activity at an initial pH of 2 and 4 with an activity unit of 1600 AU/ml against S. aureus [20]. The results agreed with [9] who reported that the activity of bacteriocin produced by L. acidophilus HT1 was stable in pH 6, 7 while decreased in pH 8. In this study, P. aeruginosa was the most causing pathogen for foot ulcer, the formula was tested for eighteen days in order to measure the availability of Lactobacillus plantarum L40 bacteriocin to inhibit bacterial growth, the L40 cells formula showed increasing in activity with 22mm in dimeter against P. aeruginosa and lack of its green color altering into yellow improving the L. plantarum L40 availability and ability of producing compounds that affect or inhibit bacterial growth (Figure 3). Results reflected the ability of formula to spread excellently, because the therapeutic efficacy of gels is usually depends on their spread. Emul gels have excellent spreading ability which leading to a perfect quality in topical application. Furthermore, this is considered an important factor in patient compliance with treatment.

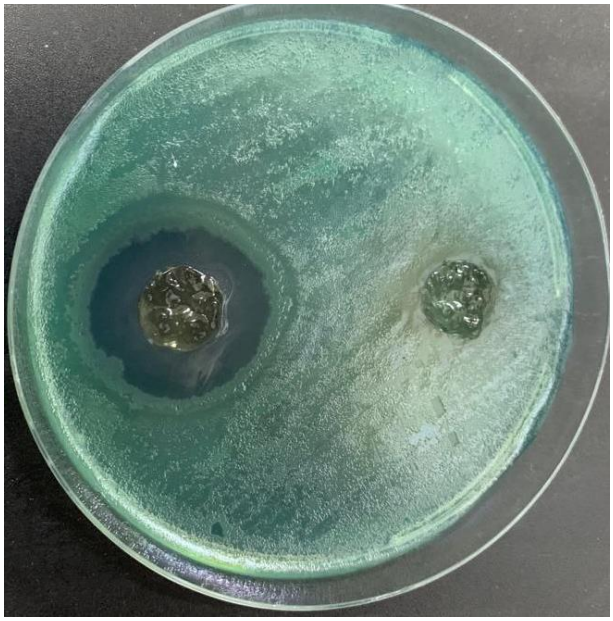

Figure (3) The antibacterial activity of purified bacteriocin formula against the bacterial indicator Pseudomonas aeruginosa

The antibacterial activity of bacteriocin produced from L. plantarum L40 applied in a gel formula preparation was investigated against P. aeruginosa. According to results presented in the study a significant inhibition zones were observed around wells contained the bacteriocin-containing cream. Inhibition of P. aeruginosa growth was certainly demonstrated the efficiency of the formula through several points included:

1- activity of bacteriocin.

2- its releasing throughout the formula to the tested media.

The study results confirmed no interaction between bacteriocin and any component of the formula that may counteract bacteriocin ability to inhibit tested bacteria.

The results showed an inhibition of bacterial growth to: P. aeruginosa, L40 bacteriocin formula showed an inhibition zone of (24 mm). The results of this study were similar with [8] who reported that bacteriocin has a significant antimicrobial activity against Gram positive and negative bacteria with bacteriocin concentration (400,500 and 640) AU/ml. Moreover, [9] reported that bacteriocin from L. acidophilus HT1 have inhibitory activity against P. aeruginosa.

Conclusions

The results showed the possibility of using bacteriocin-producing Lactobacillus plantarum as an effective probiotic to deal with some skin pathogens, and hence treat some skin diseases. L. plantarum showed an ability to produce an effective bacteriocin against some common diabetic foot ulcer pathogens and, therefore this species can be selected as a prospective dermal probiotic that can be applied in a gel formula

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