



ISSN: 0067-2904

Antibacterial Activity of Live Cells of *Lactobacillus Plantarum* L40 as A Probiotic Against Pathogens Associated with Diabetic Foot Infections

Haneen majid abdukkareem alasadi*, Khalid Jaber Kadhum Luti

Department of biotechnology, College of science, Baghdad University, Baghdad, Iraq

Received: 11/11/2022

Accepted: 10/1/2023

Published: 30/11/2023

Abstract

The present study was designed to select a suitable isolate of *Lactobacillus* sp. in order to use it as a probiotic formula for treating diabetic foot ulcer disease in diabetic mellitus patients. In order to determine the inhibitory action, 240 isolates of *Lactobacillus* from different sources, were taken and exposed for screening tests to evaluate the (antagonism-activities) toward 4 multi-drug resistant foot ulcer pathogens which were chosen from 120 pathogenic isolates: *Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus mirabilis*, and *P. aeruginosa*. Twenty *Lactobacillus* isolates were selected from primary screening according to the capacity to restrain its expansion of four markers, that were exposed toward second screenings to detect their ability to produce a high amount of bacteriocin. Based on the results, the isolate *Lactobacillus* L40 was selected as the highest bacteriocin producing isolate which was further characterized as *Lactobacillus plantarum* Next, several tests were performed to study the properties of *L. plantarum* L40, in particular its biosafety and its suitability to be a successful probiotic. Results showed that *L. plantarum* was resistant to cephalosporin groups such as ceftriaxone and cefepime whereas, it was sensitive to amikacin and chloramphenicol. In addition, results revealed that this bacterium was strongly adherent and a good biofilm producer; had high auto-aggregation capability and a high ability to adhere with *S. Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus mirabilis* and *P. aeruginosa*. A gel formula containing live cells of *L. plantarum* L40 was prepared and based on the results, cells were successfully survived in this formula over 18 days. Therefore, it was selected to be utilized as a vector to store and transport viable cells of *L. plantarum* L40. This gel formula showed antibacterial activity by inhibiting *Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus mirabilis*, and *P. aeruginosa* growth. The results showed the possibility of using viable cells of bacteriocin-producing *Lactobacillus plantarum* as an effective probiotic to deal with some skin pathogens, and hence treat skin diseases such as diabetic foot ulcer disease.

Keywords: Diabetic foot ulcer, *lactobacillus plantarum*, probiotic, formula.

الفعالية المضادة للبكتيريا الحية *Lactobacillus plantarum* L40 كمعزز حيوي ضد الممرضات

المرتبطة بعدوى القدم السكرية

حنين ماجد عبد الكريم الأسدي*، خالد جابر كاظم لوتي

التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

*Email: haneenm989@gmail.com

الخلاصة

صممت الدراسة الحالية لاختيار عزلة *Lactobacillus* ملائمة لاستخدامها كمعزز حيوي في تركيبية (مرهم) لمكافحة مرض قرحة القدم السكري عند مرضى السكر. من اجل تحديد التأثير المثبط ولهذا الغرض تم جمع 240 عزلة *Lactobacillus* من مصادر مختلفة ثم أخضعت لبرنامج العزلة لتقييم نشاطها المضاد لأربعة ممرضات مسببة للتقرح والمقاومة للأدوية والتي تم اختيارها من مجموع 120 عزلة مرضية مقاومة من قرحة القدم السكري وبعد اجراء تشخيص كيميائي تم اختيار *Staphylococcus aureus*, *Proteus mirabilis*, *P. aeruginosa* and *Klebsiella pneumonia* كمؤشرات لهذه الدراسة. تم اختيار 20 عزلة *Lactobacillus* من العزلة الأولية اعتمادًا على قابليتها لتثبيط نمو الكواشف الاربعة والتي بدورها اخضعت لعزلة ثانوية للكشف عن قابليتها على انتاج اكير كمية من البكتريوسين. اعتمادًا على النتائج اختيرت عزلة *Lactobacillus* L40 كأفضل عزلة منتجة للبكتريوسين والتي تم تشخيصها بانها *Lactobacillus plantarum*. بعد ذلك تم تنفيذ اختبارات متعددة لدراسة خصائص العزلة، وعلى وجه الخصوص اختبار تحديد ما اذا كانت العزلة امنة وملائمة لتكون معزز حيوي ناجح. أظهرت النتائج أن العزلة مقاومة لمضادات مجموعة سيفالوسبورين مثل مثل سيفترياكسون وسيفيفيم بينما كانت حساسة للأميكاسين وكلورامفينيكول. بالإضافة الى أن النتائج كشفت ان هذه العزلة لها قابلية التصاق عالية ومنتجة جيدة للأغشية الحيوية كما ان لها قابلية التصاق عالية مع بكتريا *Staphylococcus aureus*, *Proteus mirabilis*, *P. aeruginosa* و *Klebsiella pneumoniae*. تم تحضير تركيبية هلامية وتحسينها تحتوي على خلايا حية من *L. plantarum* L40 لها ، بناءً على النتائج ، نجحت خلايا *L. plantarum* L40 في البقاء على قيد الحياة في التركيبية الجل ولمدة 18 يومًا ، وبالتالي ، تم اختيارها للاستخدام كناقل لتخزين ونقل الخلايا القابلة للحياة من *L. plantarum* L40. أظهرت التركيبية فعالية مضادة للبكتريا عن طريق تثبيط نمو *S. aureus* ، *P. aeruginosa* ، *Klebsiella pneumonia* ، *Proteus mirabilis* مما يعزز إمكانية استخدام الخلايا الحية لبكتريا *Lactobacillus plantarum* كمعزز حيوي فعال للتعامل مع بعض مسببات الأمراض الجلدية وبالتالي علاج الامراض الجلدية مثل مرض قرحة القدم السكري.

Introduction

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 [1]. Diabetic foot ulcers have minimal practical significance. The development of diabetic foot ulcers has been linked to about a five% death risk during the first twelve months besides about a 42% mortality rate within 5 years, according to cohort research conducted in the United Kingdom. A study found that patients with diabetic foot ulcers had a 2.5-fold raised risk of death in comparison with those with diabetes but no foot lesions. They have a lot of patients to see every day [2].

With increasing resistance to the most antibiotics in use today, it becomes vital to develop and find new therapeutic strategies to combat microbial infections without affecting the patient. One of the suggested strategies, in this context, is to use bacteria from the resident human microbiome with probiotic characteristics to provide resistance against disease [3].

Treating diabetic foot ulcers accounts for nearly a 3rd of the entire price of diabetes care, which is valued at US\$176 billion in direct healthcare expenses in 2012 [4]. Despite the fact that there are defined guidelines for the organization of diabetic foot sores, therapy for these sores can be difficult. Wide range of new interventions to improve wound healing are being studied [5]. finding new therapeutic protocols for diabetic foot ulcer 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 [6].

The purpose of this study is to identify a locally isolated *Lactobacillus* sp. that is able to produce a bacteriocin with antimicrobial activity against several pathogenic bacterial isolates 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 look into the viability of administering the *Lactobacillus* isolate topically (dermal probiotic) to treat DFU.

Methods

Sample collection (DFU-pathogens):

A total of 120 specimens were taken by swab from individuals who suffer from diabetic foot ulcers attending at the (Al-kindy teaching hospital, Special Center for Diabetes and Endocrinology glands and medical city /Baghdad) during the period January 2021 to April 2021. The primary methods used to identify these isolates were morphological features and standard biochemical assays. including: blood hemolysis, coagulase, catalase, oxidase and Gram stain. All isolates were further confirmed using VITEK system after it was primarily identified by culturing on MacConkey agar and Mannitol salt agar.

Lactobacillus collection

240 samples in total were collected; 80 mouths of humans (40 male, 40 female) of healthy individuals and 160 samples from the dairy product (50 samples from cow milk, 60 samples from yogurt, 50 samples from cheese); during the period from (12/January/ 2021 to 30/April/ 2021). After microaerophilic incubation at 37°C for 48 hours in a candle jar, swabs were re-cultured in the agar of MRS like single colonies medium for another 48 hours. The obtained isolates were subjected to different morphological and biochemical tests to be identified presumably [3].

Lactobacillus samples Screening

Primary screen

The following Agar plug diffusing technique was employed for the initial screening [7]: The *Lactobacillus* isolates were placed into the MRS agar media via streaks all across surface of the plate after overnight growth in MRS broth. They were then maintained at 37°C. over roughly 24 hours inside a candle container. Following incubation, plugs of every inoculum measuring 0.5 cm wide have been created using an aseptic cork borer and afterward positioned on Mueller-Hinton Petri dishes containing 200 ml of bacterial isolate of overnight growth culture of the markers (*Staphylococcus aureus*, *Klebsiella*, *P. mirabilis*, *Pseudomonas aeruginosa*) having about 1×10^8 cell by ml. The same procedures then were used for an overnight incubation of dishes. Plates were then incubated under the same conditions. A Zones surrounding the bacterial plugs were measured to determine the antibacterial activity of each isolate.

Secondary screening

Inoculums of *Lactobacillus* isolates were prepared as follows: a few loop-full of pure isolate by culturing about 24 hrs. were taken and placed on MRS solid media placed in 10ml of MRS liquid media after that raised at (37°C) overnight. Furthermore, counted cell by spectrophotometer at 600 nm in order to reach the number of the cell of about 1×10^8 cell/ml. the next step was cultivation method as follow: universal tubes containing 20 ml of MRS broth were inoculated with 2% of an overnight culture of the isolate reaching

approximately 10^8 cells/ml. Then, tubes were incubated for 48 hours under anaerobic conditions at 37°C. Afterward incubation, the broth culture was centrifuged at 10000 rpm for 15 min to collect the cell-free supernatant (CFS) and bacteriocin activity was determined as described in the following section [8].

Bacteriocin activity determination

The significant diluting method, that is comparable to a lowest inhibitory concentration approach (MIC) for antibacterial evaluation, was used to evaluate the action of bacteriocins [8], [9]. Such a procedure required making a serial of a double diluted sample of a cell-free supernatant. of *Lactobacillus* culture to be tested as follows: CFS was filtered with 0.22 µm Millipore filter paper under sterile conditions. To counteract overall impact that organic acids, a mixture of NaOH plus catalase was administered. H₂O₂ activity respectively. A series of Eppendorf tubes were prepared each containing 500 µl of sterile phosphate buffer pH7. A sterile micropipette was used to transfer 500µl of the *Lactobacillus* culture to the first Eppendorf tube and then mixed vigorously using a vortex mixer; this is the first two-fold dilution. Then, 500 µl from the first two-fold dilution was transferred to the second tube to carry out a second two-fold dilution. The series of two-fold dilutions were continued to make a series of two-fold dilutions. 100 µl of each dilution was filtered and added to the wells of plates which were streaked by the selected indicator and after that, dishes had been kept at 37 degrees Celsius for 24 hours [10]. The diameter of the inhibitory area that includes every well was then calculated. The level of bacteriocin activity was measured by the maximum dilution of an inhibitory circle. A bacteriocin efficacy is inversely related to a maximum dilution factor (DF) that results in an apparent inhibitory zone. The below equation was used to determine the number of bacteriocin activity units (AU), that were given as follows Eq.(1):

$$\text{Arbitrary Units AU/MI} = \frac{1}{DF} \times \frac{1000}{\text{volume observed } \mu\text{l}} \quad (1)$$

Characterization of the selected Lactobacillus isolates as a dermal probiotic

The selected *Lactobacillus* isolate was characterized as a prospective probiotic via investigation of some factors related to its growth such as

Antibiotic Susceptibility

This test was examine the ability of the selected isolate to resist some antibiotics including (Cefotaxime, Ceftriaxone and Vancomycin, Clindamycin, and Erythromycin) by using antibiotic disc diffusion method [11].

Detection of the Ability of Biofilm Formation

A *lactobacillus* strain were cultivated over 48 hours at 37°C inside MRS-broth containing 1 percentage glucose anaerobically. Then, A sterile tryptic soya broth was prepared. Inside a microtiter tray, 180 µl of TSB and 20 µl *Lactobacillus* (grown for 48 hours) were poured for every well. After incubating the well's growth at 37 C for 72 hours without shaking, it was mixed 10 times with a pipette. The liquid was taken out post incubation, then phosphate buffered was used to rinse every well three times. Two hundred microliters of about 1% crystal violet were added and left on the counter for 15 minutes. A phosphate buffer solution was used to wash the wells three times before they were allowed to dry in the air for half an hour. After 15 minutes, the supernatant was discarded and 200 ml of 96% ethanol was added The negative control was a sterile medium. The result was read using an ELISA reader at 630nm [12].

Auto-aggregation assay

After an overnight culture of *Lactobacillus* isolates, cells were harvested at 7000 rpm for 15 min, and then the collected cells were washed twice with phosphate buffer saline (pH 6.0) and the concentration was modified to 10⁸ CFU/ml. 4 ml of *Lactobacillus* cells suspension were mixed by vortex for 10 seconds. During 5hr, auto-aggregation of *Lactobacillus* was determined

For ten seconds, a vortex was used to mix the solution at room temp, and an auto-aggregation of *Lactobacillus* was found to be caused by moving 100 µl of the suspension tube's top surface to a 3.9 ml tube containing PBS. At 600 nm, the absorbance was then calculated. The below equation Eq.(2) was used to calculate the auto-aggregation %: [13].

$$\% \text{ Auto-aggregation} = [\text{ODi}-\text{ODf}/ \text{ODi}] \times 100 \tag{2}$$

Where OD_i represents the absorbance of the start time (t = zero) for auto-aggregation and OD_f represents: absorbance in 1,2,3,4 and 5hr.

Co-aggregation assay

To examine the co-aggregation ability between the selected isolate *Lactobacillus* and the indicator *S. aureus*, *P. aeruginosa*, *Klebsiella* and *Proteus mirabilis*, 2 ml of each of both bacteria were mixed by vortex for 10 seconds. 4 ml of each bacterial suspension was utilized as controls. Finally, the absorbance was measured at 600 nm during 5hr at room temperature [13], The percentage of co-aggregation was counted by using this equation Eq.(3):

$$\% \text{ Co-aggregation} = \frac{[(\text{Ax}+\text{Ay})/2] - \text{A}(\text{x}+\text{y})/[\text{Ax}+\text{Ay}]}{2} \times 100 \tag{3}$$

Where Y represents indicator, Ax represents the absorbance of *Lactobacillus*, Ay represents the absorbance of indicator, A(x+y) is the absorbance of the mixture of both bacteria and X represents *Lactobacillus* isolates.

Preparation of gel formula contained live cells of the selected *Lactobacillus*

Lactobacillus biomass preparation

Lactobacillus Inoculums were prepared as follows: a few loopfuls of *Lactobacillus* growth from an overnight culture on MRS agar was inoculated in 10ml of MRS Broth, incubating it at 37°C, pH 6 for 24 hours. After being incubated, the final cell count was adjusted using a 0.5 McFarland Standard tube to a concentration of about 1×10⁸ cell/ ml per milliliter. The media was then poured into the precipitant after the biomass had been obtained with a centrifuge at twelve thousand rpm for 15 minutes. As useful biomass, this sample solution was saved to be added to the recipe later.

Bacterial pathogens Preparation

Four pathogenic isolates were used: (*S. aureus*, *P. aeruginosa*, *Klebsiella* and *Proteus mirabilis*) isolated from diabetic foot ulcer. These indicators were prepared as follows: 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, the cell density was standardized in the McFarland tube to 1×10⁸ cells/ml at 600 nm.

Preparation of gel formula 100gm [14]:

Carbopol 941 was dissolved in D.W. using an aliquot weighing 4 gm, and the mixture was next vigorously stirred with a magnetic stirrer. Then, 0.1 grams of methyl paraben was applied. The mix was then allowed to remain over 24 hours. After that, a sample (*Lactobacillus* L40) solution in MRS medium was added and well combined. The formula's total volume was then finished to 100g. The ingredients with all components, including biomass, medium, and other formula substances, were blended for a whole hour. Triethanolamine was later added in sufficient quantities to create the desired texture for the

gelling agent, and the pH was adjusted to pH 6. The formula was kept at 4°C in a well closed container.

Results & Discussion

As mentioned earlier, the present study aimed to investigate the antibacterial properties of a potential probiotic *Lactobacillus* sp. against pathogenic bacterial isolates collected from samples of diabetic foot infection. Therefore, to achieve such an aim, it was necessary to select diabetic foot ulcer isolates in order to use them as indicators in the selection of a suitable *Lactobacillus* isolate that can be used as a dermal probiotic in this study. For this purpose, one hundred and twenty subjects were included in this study, 60 (50%) males and 60(50%) females who were pre-diagnosed with diabetic mellitus disease and diabetic foot ulcer. 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%), *E. coli* (7.5%). Whereas, Gram-positive presented 25.82% for both *S. aureus* and *Staph. epidemidis*. These results come in align with some previous studies [15] that reported p, clindamycin, chloramphenicol, tetracycline and amoxicillin with clavulanic acid in order to select the multi-drug resistant isolates that can be used as indicators in this study. Four isolates were used as indicators: *S. aureus*, *Klebsiella pneumonia*, *Proteus mirabilis* and *P. aeruginosa*. [16].

Two hundred and forty samples were collected from different dairy product samples including raw milk, yogurt, cheese as well as from the human mouths of healthy individuals. Based, on results, the homemade yoghurt and raw cow milk were the best source for *Lactobacillus*. All isolates were subjected to a number of biochemical tests. As a total isolation process for the present study the following isolates were collected: 71 (29.58%) isolates were *Lactobacillus* sp., 68 (28.3%) isolates were cocci shape, whereas 56 (23.3%) samples had no growth and 45 (18.7 %) samples were contaminated. 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 on MRS agar were white, rounded in shape and ranged in consistency from creamy white in color to glossy white and moist-mucoid colony appearance on the surface. the growth of *Lactobacillus* isolates appeared as a single colony on MRS agar after anaerobic incubation of 48 h at 37 °C. 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 [17].

To choose the isolate with the highest bacteriocin production that may be employed in the current study's additional investigations, all isolates were put through a screening procedure. The screening procedure was carried out to see if Lacto strains might stop the multi-drug resistance bacteria that cause diabetic foot ulcers from growing. To emphasize the conflict among these isolates and the markers, the agar plugs diffusing technique was applied. It involves making an agar plug from the culture of *Lactobacillus* sp isolates to be tested for antimicrobial production and then deposit on the agar of another plate before inoculating with the indicator bacterium. Ordinarily, microbial cells secrete antimicrobial active compounds which diffuse in the agar medium from the plug. Now, this antimicrobial activity of the *Lactobacillus* is recognized by the appearance of an inhibition area round of agar plug. Results showed that several *Lactobacillus* samples had the ability to give the activity of bacteriocin alongside the four markers used with different inhibition zones. Consequently, the strategy used to select *Lactobacillus* isolates for the secondary screening were based on:

a) The isolate is active against indicators with an inhibition zone of 15 mm and above.

b) The isolate is active against more than two indicators.

According to results, twenty *Lactobacillus* isolates had antagonistic activity against more than two indicators with an inhibition zone of 15 mm and above., The rest of isolates had no or weak (less than 10 mm) or medium (less than 15 mm) inhibitory activity. Therefore, only twenty isolates were selected for further experiments in the secondary screening.

Research has demonstrated that this inhibitory activity of *lactobacilli* can be different in broth compared to solid medium because of better diffusion of the substance secreted by *Lactobacillus* [18]. Therefore, in the second step of screening, the well diffusion method was used to detect the ability of *Lactobacillus* isolates to produce bacteriocin in liquid culture. The highest bacteriocin-producing isolate was detected based on its performance by forming an inhibition zone around the well in an agar plate containing cell-free supernatant. Results demonstrated that the activity was different (320,160,80 40, 20 AU/ml) of bacteriocin produced by *lactobacillus* isolates. According to the results, the isolate *Lactobacillus* L40 from yoghurt showed the best activity isolates to antibacterial activity of 320 AU/ml toward 4 indicators: (*Staphylococcus aureus* *Pseudomonas. aeruginosa*, *Proteus. mirabilis*, and *Klebsiella pneumonia*) as described in (Table and Figure 1). Thus, the isolate numbered L40 was selected to be employed in the work.

Table 1: *Lactobacillus* isolates Secondary screening for production of bacteriocin

Isolate	Bacteriocin activity (AU/ml)			
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>Klebsiella Pneumoniae</i>	<i>Proteus mirabilis</i>
L1	320	160	40	320
L2	320	20	80	40
L3	20	20	20	20
L4	320	40	20	20
L8	40	20	20	20
L10	40	20	20	160
L11	160	40	80	20
L12	40	40	20	20
L14	320	40	20	20
L40	320	320	320	320
L51	20	20	80	20
L55	40	20	160	20
L65	40	80	20	20
L126	20	80	40	20
L130	320	80	160	80
L160	20	80	20	80
L199	20	20	20	160
L219	40	20	40	20
L239	40	80	20	20
L257	320	40	40	40

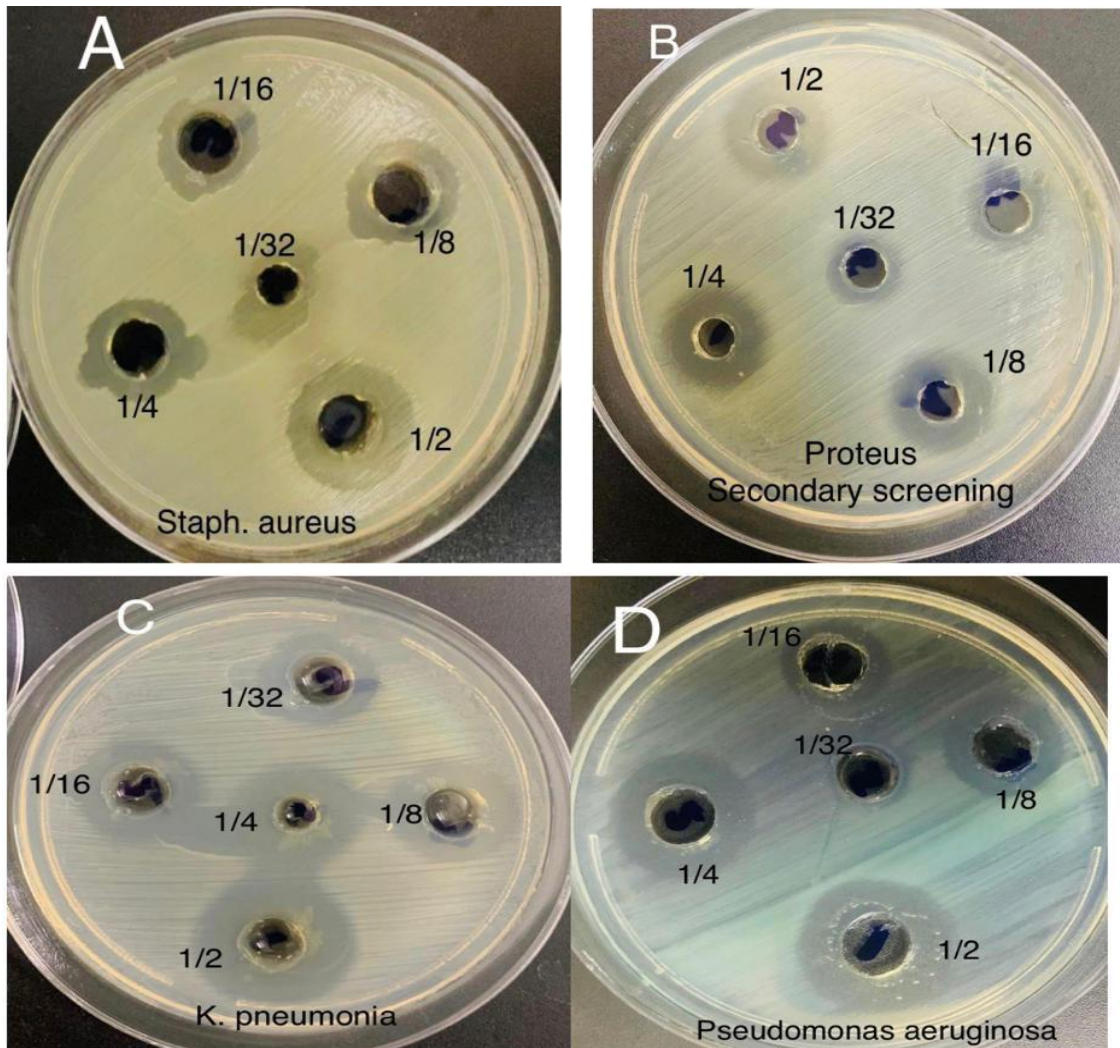


Figure 1: *Lactobacillus* L40 isolate Secondary screening with four indicators (A: *S. aureus*, B: *Proteus mirabilis*, C: *Klebsiella pneumoniae* D: *Ps. aeruginosa*) by using well diffusion method.

Lactobacillus L40 was ten and subjected to a process of identification according to VITEK 2 system in order to determine its species. According to results, the selected *Lactobacillus* isolate showed positive results for 18 biochemical tests and negative results for the rest. These tests were performed to differentiate the species that belong to the genus *Lactobacillus*. The results revealed that the selected isolate L40 was *Lactobacillus plantarum* with a very good probability of 93%. The main target of this work was to use the isolate *Lactobacillus plantarum* L40 as probiotic, therefore, it was useful to take the antibiotic sensitivity profile of the isolate into consideration. For this purpose, nine different antimicrobial drugs were utilized and tested against *Lactobacillus plantarum* L40. Disc diffusion method was utilized in this study to identify the antibiotic susceptibility [19]. Based on results, *Lactobacillus plantarum* was resistant to beta-lactam groups like ampicillin and penicillin whereas, that one showed sensitivity toward Amikacin and chloramphenicol. In addition, *Lactobacillus plantarum* L40 showed resistance to Cefotaxime, Ceftriaxone and Vancomycin, Clindamycin and Erythromycin. The result similarity [20] and [21] reported *Lactobacillus sp* were usually susceptible toward antibacterial that inhibit protein production like chloramphenicol and tetracycline, and strongly resistant to aminoglycoside like streptomycin and gentamicin.

In general, to evaluate the biofilm formation, different methods were used including quantitative methods, for example, tissue culture plate (TCP) [22] which is considered as a standard method as well as qualitative methods using the Congo red agar (CRA) [23]. and tube method (TM) [24]. For the current research, TCP techniques were applied for Detection the Biofilm Formation Ability by *Lactobacillus plantarum* L40. The TCP method provides a numerical value based on OD determination and each value represents a certain phenotype result non-producer, weak, moderate and strong [25]. Therefore, this method is a quantitative assay to determine the ability of an isolate to produce biofilm by calculating the degree of biofilm production. The result showed that OD of sample was 0.37 whereas, OD of control was 0.08, So this isolate is a strongly adherent and good biofilm producer. This result was agreed with [26] who confirmed the ability of *L. plantarum* to produce biofilm. In addition, many studies reported the ability of different species of *Lactobacillus* to produce biofilm [27], [28].

Biofilm-producing microorganisms have an advantage that is not found in planktonic ones such as protection and resistance to drugs (antibiotics), adhesion capability, mechanical properties, avoiding immune system (antibodies and phagocytes), protection from environmental stresses and cellular communication [29]. Biofilm formation is a property that provides the ability to colonize probiotic cells and avoid the colonization of pathogens [30]. Auto-aggregation is an important property of probiotics, Some *Lactobacillus* strains have the ability to inhibit adherence of pathogens either by forming a barrier via auto aggregation or by direct co-aggregation with the pathogens [31], [32]. Therefore, this test is important to determine the possibility of using *L. plantarum* L40 free-cells equally prospective probiotics. As the results presented in Figure (2), *Lactobacillus plantarum* L40 had a high auto-aggregation capability of approximately 87.2% after 5 hrs. This test clearly proves that this isolate has a good ability to adhere and inhibit adherence to pathogens. The results agreed with [28] who reported that *Lactobacillus* Nu14 cells had a high auto-aggregation capability of more than 86% after 5 hrs. Furthermore, *Lactobacillus plantarum* L40 had a high Co-aggregation capability of approximately 76.1% after 5 hrs. Figure (3). This test clearly proves that this isolate has a good ability to adhere to inhibit adherence to pathogens. The results agreed with [28] who reported the high ability of *L. crispatus* and *L. parabuchneri* to adhere with *S. mutans* which reached 71.66% after 5 hrs. The obtained result was also in agreement with study that reported the ability of *Lactobacillus* to co-aggregate with pathogens [33].

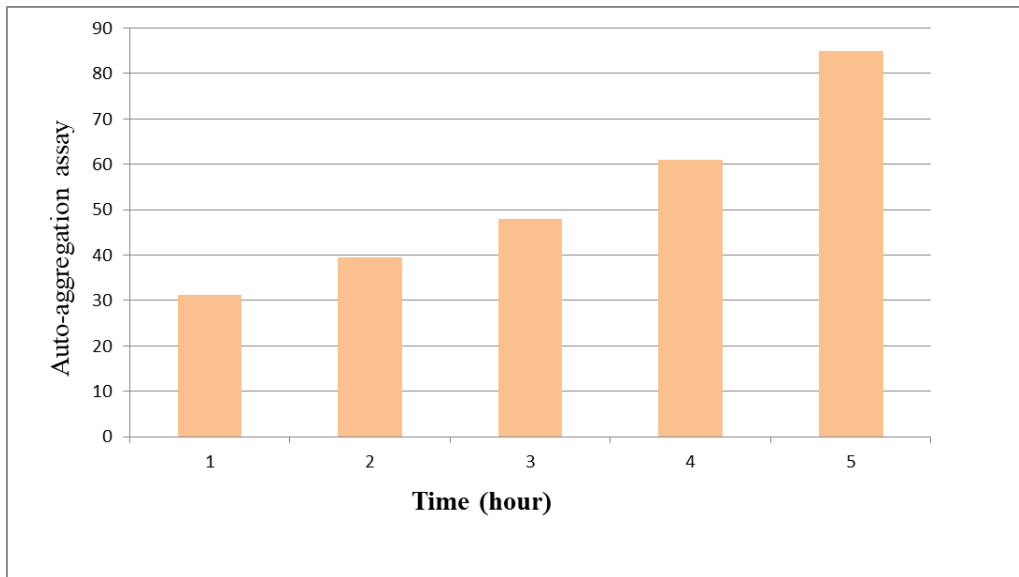


Figure 2: Auto-aggregation assay after 5 hours of incubation

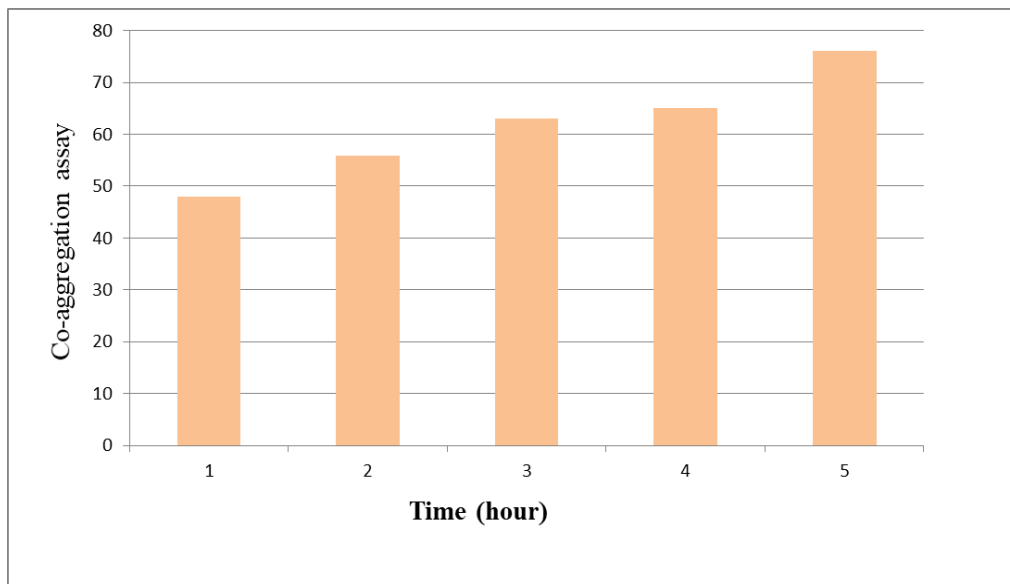


Figure 3: Co-aggregation assay after 5 hours of incubation

According to the definition by the World Health Organization (WHO) and the American Food and Agricultural Organization (FAO), a probiotic is “a live organism which has a beneficial effect on human health when they take in enough amounts” [34]. Antagonistic activity is a very important criterion for the selection of probiotics because they are natural antimicrobials for potentially harmful bacteria. Therefore, CFSs from *Lactobacillus* spp. isolated from dairy products and the mouth of healthy individuals were screened for their antagonistic activity against Gram-positive and Gram-negative diabetic foot ulcer-causing pathogens. In this context, different bacterial probiotic cells, in particular *Lactobacillus* sp., were utilized in formula to treat several diseases such as *Lactobacillus plantarum* cells in an emul formula that is used against some skin pathogens [35]. In addition, [27] used *Lactobacillus crispatus* in an emul gel formula against some vaginal pathogens. This study was performed to select an acceptable formula which can be used to provide a suitable medium for *Lactobacillus plantarum* L40 to survive.

In fact, the challenge in this study was to select a useful formula which can act as a vector to store and transport viable cells of *Lactobacillus plantarum* L40 into the skin with an ulcer in diabetic patients. After several tests, formula containing *lactobacillus plantarum* L40 (live cells) was selected and optimized to choose the most useful one that can be used in this study.

Lactobacillus plantarum L40 was used in this study against four MDR pathogenic bacteria that isolated from diabetic foot ulcer. The physiological characteristics of the created emul gel formula include a silky finish, a white appearance that is clear and homogenous, and no odor. *Lactobacillus plantarum* L40 biomass was successfully included in the formulation of the emul gel bases in this investigation. By daily culture on MRS agar following manufacture and homogeneity of the formula ingredients, the vitality of *Lactobacillus plantarum* L40 cells was monitored for three weeks. According to the findings, cells maintained their vitality for 18 days. A number of factors, including pH, homogeneity, spreadability, and skin irritancy, are often used to assess the manufactured formula emul gels [36]. In vitro monitoring of pH and homogeneity made the formula's stability clear. Through the course of 18 days, the produced formula demonstrated homogeneity and stability at pH (5.9-6.5).

Using a technique previously described by Mostafa et al. [37], we investigated the antibacterial efficacy of *Lactobacillus plantarum* L40 biomass within emulgel formulations against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Proteus mirabilis*.

(the control is a formula without *lactobacillus*).

The formula's efficiency was demonstrated by its ability to suppress the growth of four indicator bacteria when tested at doses of 10^8 CFU/ml and according to the following:

1. Viability of *Lactobacillus plantarum* L40 cells.
2. Its ability to produce bacteriocin.
3. There is no way for the bacteriocin to become trapped in the formula, and no way for any of the components of the formula to interfere with the bacteriocin's ability to inhibit the tested bacteria as it diffuses out into the surrounding environment (the tested media).

The results showed inhibition of bacterial growth to: *Staph. aureus* with 19mm in diameter with control formula of no inhibition zone Figure (4). While in *Pseudomonas aeruginosa*, *L. plantarum* L40 cells formula showed an inhibition zone of 16mm. Furthermore, *L. plantarum* L40 cells formula showed inhibition toward *Klebsiella pneumonia* with 18mm in diameter and 17mm in *Proteus mirabilis*).

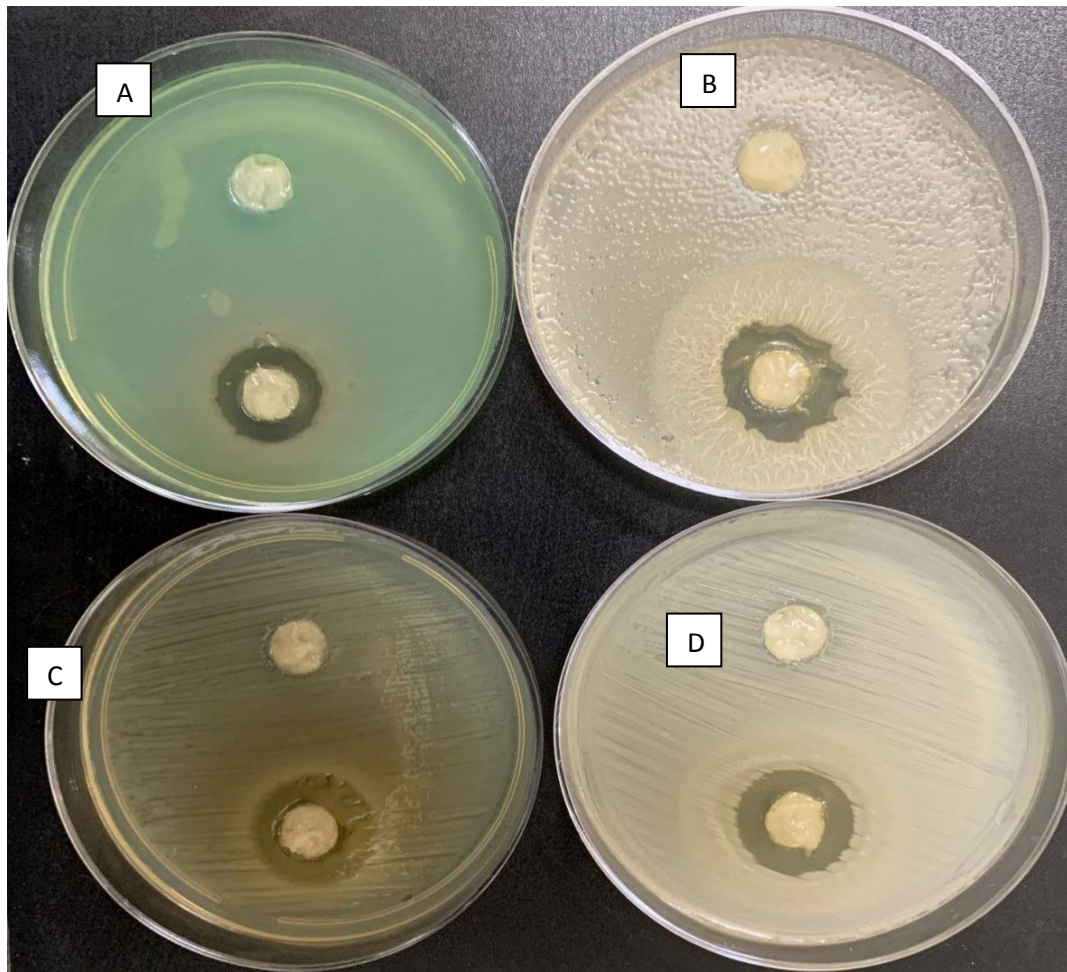


Figure 4: The activity of live cells of *lactobacillus plantarum* L40 formula against diabetic foot infections-causing pathogens compared with control (lack of *lactobacillus* bacteria). **A:** *P. aeruginosa*, **B:** *Klebsiella*, **C:** *Proteus mirabilis*, **D:** *S. aureus*.

According to the results obtained in this study, *Pseudomonas aeruginosa* was the most causing pathogen for foot ulcer therefore, it was used as an indicator to test the formula. Moreover, the formula was tested for eighteen days in order to measure the availability of *lactobacillus plantarum* L40 to inhibit bacterial growth. The L40 cells formula showed increases in activity with 22mm in diameter against *Pseudomonas aeruginosa* and lack of its green color altering into yellow improving the *Lactobacillus plantarum* L40 availability and ability to produce compounds that affect or inhibit bacterial growth Figure(5>Data confirmed that the recipe distributed extremely well, which is important since gel or emul gel's capacity to treat ailments often hinges on how well they spread. Emul gels also have perfect quality in dermal administration due to their exceptional spreading capacity. In contrast, is thus thought to be a crucial component of patient adherence to therapy [38].

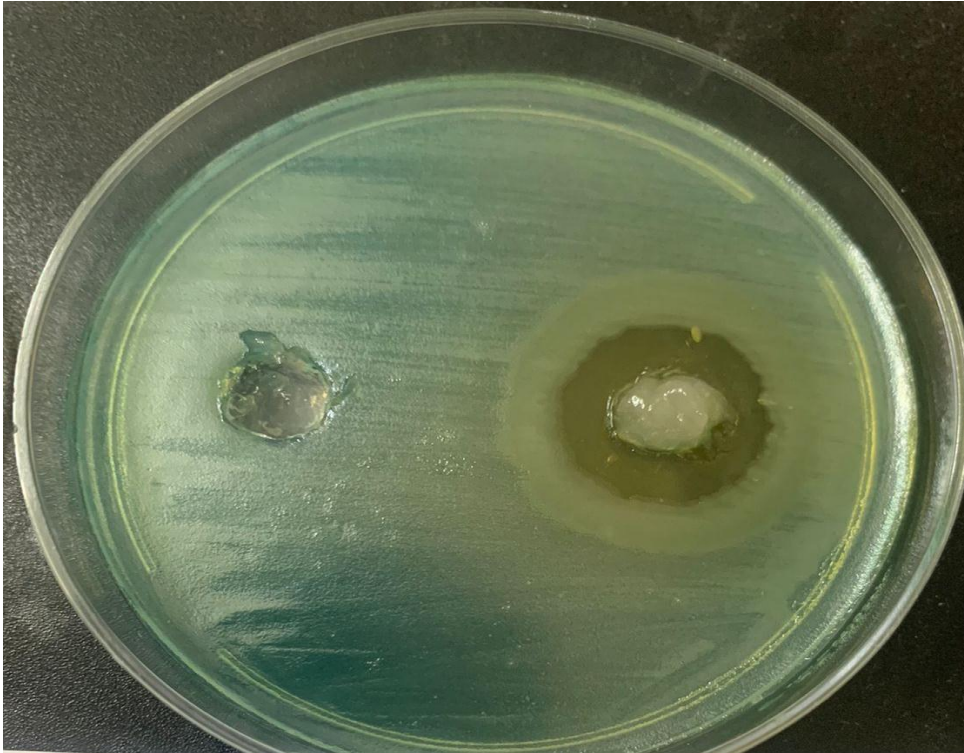


Figure 5: Antibacterial activity of live *Lactobacillus plantarum* L40 cells formula against *P. aeruginosa* after 18 days

Based on results of the viable cell count method, *Lactobacillus plantarum* L40 successfully survived in this formula over 18 days as shown in Figure (6). The percentage of viable cells decreased after three weeks. Therefore, this formula was selected to use in this study which can be utilized as a vector to store and transport viable cells of *Lactobacillus plantarum* L40. the presence of glycerol in the formula may play an important role to store bacteria for long term at 4°C, and hence, may participate in maintaining *Lactobacillus parabuchnei* Nu14 cells viable for long time [28].

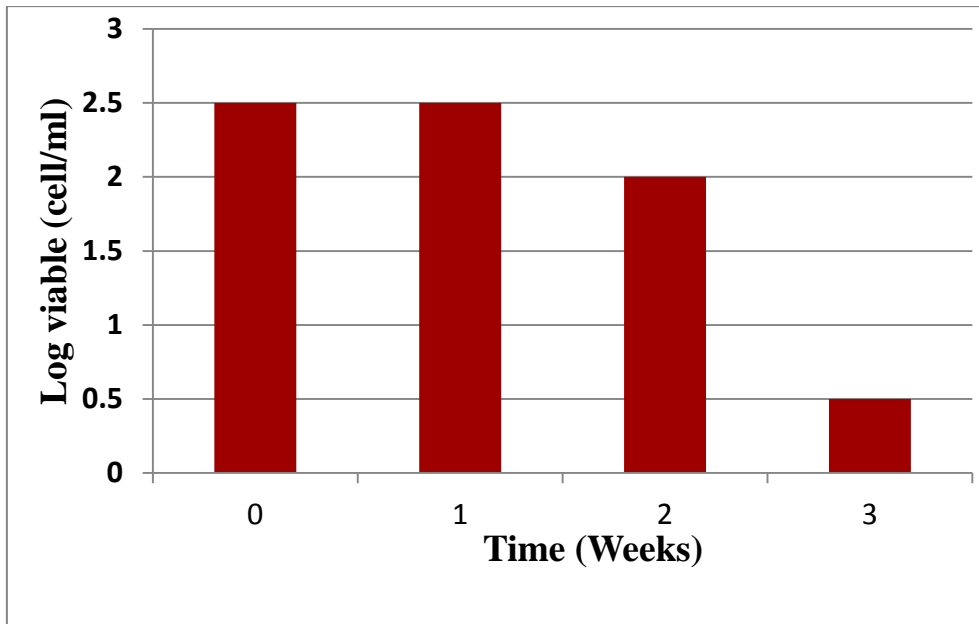


Figure 6: Monitoring of viability of *Lactobacillus plantarum* L40 in formula for three weeks

Conclusions

In conclusion, the present study supports the idea of using vital cells as a dermal probiotic for the treatment of skin infections and as an alternative method to face the widespread multi-drug resistance. The results showed the possibility of using viable cells of bacteriocin-producing *Lactobacillus plantarum* as an effective probiotic to deal with some skin pathogens, and hence treat some skin diseases related to diabetic foot ulcer patients. The results of this study validated the survivability of the *Lactobacillus plantarum* L40 cells within the formulas and their capacity to generate and secrete bacteriocin without interacting with formula ingredients, demonstrating the effectiveness of the cells-containing formula.

References

- [1] G. Armstrong, J. M. Andrew. M. D. Boulton and A. Busicco, "Diabetic Foot Ulcers and Their Recurrence," *new Engl J of Med*, pp. 376: 2367–75, 2017.
- [2] J. Walsh, O. Hoffstad and M. Sullivan, "Association of diabetic foot ulcer and death in a population-based cohort from the United Kingdom," *new Engl J of Med*, p. 33(11):1493–1498, 2016.
- [3] N. Dyaee and K. Luti, "Classical and Statistical Optimization by Response Surface Methodology For Enhancing Biomass and Bacteriocin Production by *Lactobacillus Plantarum*," *Iraqi Journal of Science*, no. vol. 60, no. 3, pp. 494–508, pp. vol. 60, no. 3, pp. 494–508, Mar. 2019.
- [4] G. deSmet, L. Kroese and A. Menon, "Oxygen therapies and their effects on wound healing," *Wound Repair Regen*, 2017.
- [5] L. Prompers, N. Schaper and et al, "Prediction of outcome in individuals with diabetic foot ulcers: focus on the differences between individuals with and without peripheral arterial disease.," *The EURODIALE Study. Diabetologia*, p. 51:747–755., 2008.
- [6] O. Taha, P. Connerton, P. L. Connerton, I. A. Connerton and El-Shibiny, "Potential Treatment for *Klebsiella pneumoniae* Isolated From Diabetic Foot Patients " *Front. Microbiol.*, p. 9:2127., 2018.
- [7] F. N Abd, and K. J. Luti, "An exploitation of interspecies interaction for promoting bacteriocin production by local isolate of *Bacillus* sp.," *J Baghdad for Science*, pp. 14(3): 524-538, 2017.
- [8] A. Mayr-Harting, A. J. Hedges and C. W. Berkeley, "Methods for studying bacteriocins," *Methods Microbiol*, p. 7: 315–412., 1972.

- [9] M. Santagati, S. Marina, P. Francesco, A. Caterina and S. Stefania, "Bacteriocin-producing oral Streptococci and inhibition of respiratory pathogens," *Immunol. and Medical Microbiol*, pp. J., 22:167-171., 2012.
- [10] N. Dyaee and K. J. K. Luti, "'Classical and Statistical Optimization by Response Surface Methodology For Enhancing Biomass and Bacteriocin Production by Lactobacillus Plantarum," *Iraqi Journal of Science*, pp. vol. 60, no. 3, pp. 494–508, Mar 2019.
- [11] E. Vlková, V. Rada, P. Popelářová, I. Trojanová and J. Killer, "Antimicrobial susceptibility of bifidobacteria isolated from gastrointestinal tract of calves," *Livest Sci.*, p. 105:253–259., 2006.
- [12] A. Hassan, J. Usman, F. Kaleem, M. Omair, A. Khalid and M. Iqbal, "Evaluation of different detection methods of biofilm formation in the clinical isolates," *Brazilian Journal of Infectious Diseases*, pp. 15(4), 305-311, 2011., 2011.
- [13] B. V. Kos, J. Šušković, S. Vuković, M. Šimpraga, J. Frece and S. Matošić, "Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. *Journal of applied microbiology*," *Journal of applied microbiology*, pp. 94(6),981-987, 2003.
- [14] S. Santoyo, A. Arellano, P. Ygartua and C. Martin, "Penetration enhancer effects on the in vitro percutaneous absorption of piroxicam through rat skin.," *Int. J. Pharm*, pp. 117, 219–224, 1995.
- [15] M. S. Bader, "Diabetic foot infection," *Fam. Phys*, p. 78(1): 81, 2008.
- [16] N. Tentolouris, G. Petrikkos and N. Vallianou, "Prevalence of methicillin- resistant Staphylococcus aureus in infected and uninfected diabetic foot ulcers.," *Clin. Microbiol. Infect.*, pp. 12(2):186-189, 2006.
- [17] J. Zheng, S. Wittouck, E. Salvetti, C. M. A. B. Franz, H. M. B. Harris, P. Mattarelli, P. O'Toole, B. Pot, P. Vandamme, J. Walter, K. W. Watanabe, S. Wuyts, G. E. Felis, M. G. Gänzle and S. Lebeer, "A taxonomic note on the genus description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae," *Int. J. Syst. Evol. Microbiol*, pp. 70(4):2782-2858, 2020.
- [18] J. Osset, R. Bartolomé, E. García and A. Andreu, "Assessment of the capacity of Lactobacillus to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells.," *J Infect Dis.*, p. 183:485–491, 2001.
- [19] N. Fating and et al, "Detection of Bacterial Flora in Orofacial Space Infections and Their Antibiotic Sensitivity Profile," *Journal of maxillofacial and oral surgery*. Springer, pp. 13(4), pp. 525–532, 2014.
- [20] M. Ammor, A. Florez and B. Mayo, "Antibiotic resistance in non-enterococcal lactic acid bacteria and Bifidobacteria," *Food. Microbiol*, p. 24:559–570., 2007.
- [21] S. Borrás, " "System of innovation theory and the European Union", " *Science and Public Policy*, p. 31(6) pages 000–000, december 2004.
- [22] G. D. Christensen, W. A. Simpson, J. A. Younger and et al, "Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices," *J Clin Microbiol*, pp. 22, pp. 996-1006, 1995.
- [23] D. Freeman, F. R. Falkiner and C. T. Keane, "New method for detecting slime production by coagulase negative staphylococci.," *J. Clin. Pathol*, pp. 42: 872-874, 1989.
- [24] G. D. Christensen, W. A. Simpson, A. L. Bisno and E. H. Beachey, "Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces.," pp. 37(1):318-26, july 1982.
- [25] M. Diaz, B. del Rio, V. Ladero , B. Redruello, E. Sanchez-Llana, M. Fernandez and et al, "Histamine-producing Lactobacillus parabuchneri strains isolated from grated cheese can form biofilms on stainless steel," *Food Microbiol.*, 2016 b.
- [26] G. Pannella, S. J. Lombardi, F. Coppola, M. Iorizzo, M. Succi, P. Tremonte, C. Iannini, E. Sorrentino and R. Coppola, "Effect of Biofilm Formation by Lactobacillus plantarum on the Malolactic Fermentation in Model Wine," *National library of medicine*, no. (Basel, Switzerland), p. 17;9(6):797, Jun 2020.
- [27] I. Tareq and K. J. K. . Luti, "An application of Bacteriocin-Producing Vaginal Lactobacillus Crispatus IS30 in A Gel Formula Against Some Vaginal Pathogens," *Iraqi Journal of Science*, pp. vol. 63, no. 2, pp. 491–507, Feb. 2022.

- [28] A. H. Nouralhuda and K. J. Luti, "Evaluation of in vitro antimicrobial activity of *Lactobacillus parabuchneri* as a potential probiotic in toothpaste against some oral pathogens," MS.c Thesis, Department of biotechnology, college of science, University of Baghdad., 2022.
- [29] A. L. Santos, A. C. Galdino, T. P. Mello, L. S. Ramos, M. H. Branquinha and A. M. Bolognese, "What are the advantages of living in a community? A microbial biofilm perspective! *Memorias do Instituto Oswaldo Cruz*," National library of medicine, p. Vol. 113(9): e180212, 2018.
- [30] J. Post, "Direct evidence of bacterial biofilms in otitis media," pp. 125:2003-14, 2015.
- [31] M. C. Collado, J. Meriluoto and S. Salminen, "Measurement of aggregation properties between probiotics and pathogens: In vitro evaluation of different methods," *J. Microbiol. Methods*, pp. 71-74, 2007.
- [32] C. L. Ferreira, L. Grzeskowiak, M. C. Collado and S. Salminen, "In vitro evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens," *J. Food Prot.*, pp. 74 pp. 1482-1487, 2011.
- [33] S. Younas, B. Mazhar, I. Liaqat, S. Ali, H. Tahir and N. Ali, "Bacteriocin Production by *Lactobacilli* and Their Role as Antibacterial Tool against Common Pathogens," *J Oleo Sci.*, pp. 1;71(4):541-550, April 2022.
- [34] J. Meurman and V. Iva Stamatova, "Probiotics: Evidence of Oral Health Implications," National Library of Medicine, pp. 1;60(1):21-29, Mar 2018.
- [35] H. T. Rasheed and K. J. K. Luti, "a probiotic application of *Lactobacillus acidophilus* HT1 for the treatment of some skin pathogens," *the Iraqi journal of Agriculture science*, 2020.
- [36] L. Preet Kaur and T. K. Kumar Guleri, "Topical gel: A Recent Approach for novel drug delivery," *Asian journal of Biomedical and pharmaceutical science*, pp. 3(17): 1-5, 2013.
- [37] K. Mostafa, H. F. Mohammad and M. Mohammad, "Genetic diversity of wheat (*Triticum aestivum* L.) genotypes based on cluster and principal component analyses for breeding strategies," *Australian Journal of Crop Science*, pp. 5(1), 17-24, 2011.
- [38] A. R. Silvio, M. D. Camila, A. Larissa, O. C. Ferdinndo, J. L. R. Jackson and R. D. Jackson, "Development and evaluation of stability of a gel formulation containing the monoterpene borneol," *The Scientific World Journal*, 2016.