ISSN: 0067-2904

Utilization of *Limosilactobacillus fermentum* Cells in a Gel Formula as a Dermal Probiotic against MDR *Pseudomonas aeruginosa* Associated with Burn Wound Infection

Ahmad Hameed Khalaf Darweesh, Khalid Jaber Kadhum Luti
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 27/9/2023 Accepted: 9/12/2023 Published: 30/12/2024

Abstract

This work aimed to select a safe and bacteriocin-producing isolate of *Lactobacillus* to use it as a probiotic for treating *Pseudomonas aeruginosa* associated with burn infections. For this purpose, 151 burn wound swabs were collected from patients to isolate the most common multi-drug resistance *Pseudomonas aeruginosa* that could be used as an indicator. In addition, thirty-six *Lactobacillus* isolates were collected from different sources and subjected to a screening program to evaluate their antagonism activities against MDR *P. aeruginosa*. Based on results, the isolate *Lactobacillus* HLB12 showed a highest bacteriocin production which was further characterized as *Limosilactobacillus fermentum* through 16s ribosomal RNA. Several tests were performed to study the properties of *Limosilactobacillus fermentum* HLB12, particularly its biosafety and suitability to be a successful probiotic. Results showed that *L. fermentum* HLB12 was resistant to β-lactams group such as ticarcillin/clavulanate. Whereas it was sensitive to amikacin, ciprofloxacin and levofloxacin. Moreover, the results confirmed that *L. fermentum* HLB12 was safe as a probiotic without any health impacts as it has no hemolytic activity. Furthermore, results revealed that this bacterium was strongly adherent and good biofilm producer, had high auto-aggregation capability and high ability of adhering with *Pseudomonas aeruginosa*. *Limosilactobacillus fermentum* cells were successfully incorporated into a gel base formula. The results revealed that cells kept their viability over 21 days with an ability to produce and release the bacteriocin. The formula was tested *in vitro* and results showed a high antagonistic activity against *Pseudomonas aeruginosa*. Next, the formula was tested *in vivo* to treat rabbit models with full-thickness skin burn. Upon examining the healing process, results showed signs of remodeling stage by day four and scar tissue formation by day nine. The results supported the idea of using probiotic as an alternative method for the treatment with antibiotics.

Keywords: *Limosilactobacillus fermentum; Pseudomonas aeruginosa;* Burn infection; Gel formula.

استخدام خلايا *Limosilactobacillus fermentum* في تركيبة هلامية كبروبيوتيك جلدي ضد بكتريا *Pseudomonas aeruginosa* المتعددة المقاومة المرتبطة بعدوى الحروق

احمد حميد خلف درويش، خالد جابر كاظم لوتي
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

*Email: ahmed.hameed1606a@sc.uobaghdad.edu.iq

الخلاصة

كان الهدف من هذا العمل هو اختيار سلالة آمنة منتجة للبكتريوسين من *Limosilactobacillus fermentum* لاستخدامها كبروبيوتيك لعلاج بكتريا الزائفة الزنجارية المرتبطة بعدوى الحروق *Pseudomonas aeruginosa*. لهذا الغرض ، تم جمع 151 عينة من جروح الحروق من المرضى لعزل سلالة الزائفة الزنجارية الأكثر مقاومة للمضادات المتعددة. بالإضافة إلى ذلك ، تم جمع 36 عينة من *Limosilactobacillus* من مصادر مختلفة وإخضاعها لبرنامج فحص لتقييم أنشطتها التنافسية ضد بكتريا الزائفة الزنجارية المقاومة للمضادات المتعددة. استنادًا إلى النتائج ، أظهرت سلالة *Lactobacillus* HLB12 أعلى إنتاج للبكتريوسين والتي تم تشخيصها *Limosilactobacillus fermentum* من خلال 16S RNA الريبوسومي. تم إجراء العديد من الاختبارات لدراسة خصائص هذا النوع ، لا سيما السلامة البيولوجية وملاءمتها لتكون معزز حيوي ناجح. أظهرت النتائج أن المكورات اللبنية المخمرة HLB12 مقاومة لمجموعة بيتا لاكتام مثل تيكارسيلين / كلافولانات ، في حين أنها حساسة للأميكاسين والسيبروفلوكساسين والليفوفلوكساسين. علاوة على ذلك ، أكدت النتائج أن المكورات اللبنية المخمرة HLB12 آمنة كمعزز حيوي بدون أي آثار صحية لأنها ليس لها نشاط انحلالي. علاوة على ذلك ، كشفت النتائج أن خلايا هذه البكتيريا لها قدرة عالية على التكتل الذاتي وانها منتج جيد للأغشية الحيوية ولديها قدرة عالية على التكتل والالتصاق بالزائفة الزنجارية. تم اضافة خلايا المكورات اللبنية المخمرة بنجاح الى تركيبة هلامية وأظهرت النتائج أن الخلايا حافظت على حيويتها لأكثر من 21 يومًا مع القدرة على إنتاج وإطلاق البكتيريوسين. تم اختبار التركيبة في المختبر وأظهرت النتائج نشاطًا مضادًا للزائفة الزنجارية. بعد ذلك ، تم اختبار التركيبة في الجسم الحي لعلاج عينات الأرانب بالحروق الجلدية الكاملة. تم فحص عملية الشفاء ، وأظهرت النتائج علامات مرحلة إعادة بناء النسيج بحلول اليوم الرابع وتكوين نسيج الندبة بحلول اليوم التاسع. تدعم النتائج الحالية فكرة استخدام خلايا المعزز الحيوي كطريقة بديلة للعلاج بالمضادات الحيوية.

الكلمات المفتاحية: المكورات اللبنية المخمرة ; الزائفة الزنجارية ; التهابات الحروق ; مستحلب الهلام

1. Introduction

Globally, burn injuries are significant health issues impacting millions of people [1]. In addition to the initial trauma, burn patients are at high risk for infections which can result in prolonged hospitalization, extensive scarring and even death [2]. Due to the emergence of antibiotic-resistant bacteria, such as *P. aeruginosa*, burn wound infections are challenging to treat. The emergence of MDR bacteria has led to an urgent need for alternative therapies to treat burn infections [3]. Since *P. aeruginosa* is an opportunistic infection, it can be discovered in immunocompromised patients. It is most frequently discovered in hospitals and private care facilities and is regarded as a dominant colony of burn wounds due to its capacity to spread quickly within damaged tissues and cause catastrophic diseases as well as chronic and acute infections. It is one of the most prevalent bacteria that causes infections in hospitals including pneumonia, skin infections and urinary tract infections [4, 5]. Two factors, including endogenous infections, can result in acquired damage in burn victims. It happens when an organism is present in the patient's daily life. Exogenous infections are another cause that can be acquired by contact with hospital personnel, equipment or surroundings [6, 7]. *P. aeruginosa* is regarded as one of the most significant and hazardous organisms in human infections because of the multiple virulence factors it has produced [8]. Probiotics are live, non-pathogenic microorganisms that have the ability to improve the host's health [9], including lowering inflammation, accelerating wound healing, and boosting the immune system [8]. Numerous *Lactobacillus* species strains have been shown to exert a variety of health-promoting effects including immunomodulation, improved pathogen resistance, lowering blood cholesterol levels and others [10-12]. *Lactobacilli* were among the first bacteria to be identified and utilized as probiotics [13]. Several probiotics, including

Lactobacillus, are safe, immune-modifying host biology and biological treatments that are generally recognized as safe (GRAS). Multiple antimicrobial mechanisms of *Lactobacillus* have recently been identified including competition for food, generation of inhibitory substances, stimulation of the immune system, and competition for binding sites [14, 15]. It is possible that the most crucial mechanism is the ability of *Lactobacillus* to produce lactic acid, acetic acid, formic acid and other acids that lower gut pH. These bacteria produce antimicrobial activity by secreting antimicrobial compounds such fatty acids, hydrogen peroxide and bacteriocins [14]. Despite using topical and systemic antibiotics, this infection is still the most frequent consequence following burn injuries causing sepsis and death. This study evaluated the ability of the probiotic organism *Lactobacillus* sp. to inhibit the pathogenic activity of *P. aeruginosa*, both *in vitro* and *in vivo* so that it could be used as prophylactic treatment to prevent the infection. An alluring experimental strategy that avoids the drawbacks of conventional antibiotic therapy is the direct application of probiotics to burn wounds.

2. Material and Method

2.1 Isolation of P. aeruginosa

A total of 151 clinical samples were collected from patients suffering from burns who were admitted to the Medical City Hospital for the period from September 2022 to January 2023. In order to prevent any potential contamination, all samples were carefully collected using a sterile swab from patients with first-degree burns. All samples were grown on MacConkey agar and incubated for 24 hours at 37ºC. Bacterial samples were subjected to morphological identification, biochemical test, VITEK-2 System as well as the capability of these colonies to produce pyocyanin pigment [16]. All isolates of *P. aeruginosa* were then subjected to antibiotic susceptibility tests in order to select the most multidrug-resistant isolates that can be used as indicator in the choice of a suitable *Lactobacillus* isolate that could be used as a probiotic in this study. The antibiotics used were (aztreonam, imipenem, meropenem, piperacillin-tazobactam, piperacillin, ceftazidime, tobramycin, gentamicin, netilmicin, levofloxacin and ofloxacin)

2.2 Isolation of Lactobacillus spp.

Thirty-six *Lactobacillus* isolates were collected from samples of dairy products including pasteurized milk, cow milk, yoghurt, drinking yogurt, handmade yoghurt, Activia yogurt and from healthy women's vagina. Samples were collected in sterile containers which were kept refrigerated until they were delivered to the lab. Bacterial samples were cultured in MRS agar and incubated at 37°C for 48 hours under microaerophilic conditions before being re-cultured as a single colony in de Man, Rogosa and Sharpe agar under the same circumstances [17]. Bergey's handbook of Systematic Bacteriology culture features and a few biochemical tests were used to identify the bacterial isolates [18].

2.3 Screening of Lactobacillus Isolates for Bacteriocin Production

The MRS agar medium was cultured with the isolates by streaking on the plate surface following an overnight growth in MRS broth. The isolates were then incubated at 37°C for 24 hours under microaerophilic conditions in a candle jar. After incubation, sterile cork borers were used to create plugs of 6mm in diameter for each isolate. These plugs were placed on the Muller-Hinton agar plates previously streaked with 100 µl of an overnight growth culture of the indicator bacterium (MDR *P. aeruginosa*) in BHI broth medium containing 1×10^8 cells/ml. The same conditions were then used for an overnight incubation of plates. The antibacterial activity of each agent was assessed using the zones of inhibition surrounding the agar plugs [19]. In the second step of the screening, the well diffusion method was used to

detect the ability of *Lactobacillus* isolates to produce bacteriocin in liquid culture. A series of Eppendorf tubes were prepared, each of which contained 500 µl of sterile normal saline. A sterile micropipette was used to transfer 500 µl of the cell-free supernatant (CFS) of *Lactobacillus* growth culture to the first Eppendorf tube and then mixed by vortex mixer. This was 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 sequence of dilutions was repeated to make a series of two-fold dilutions. The MDR *P. aeruginosa* was employed as an indicator isolate to evaluate the bacteriocin activity in each dilution using the agar well diffusion assay. To ascertain the bacteriocin activity, the highest dilution factor (DF) offering a discernible inhibitory zone was detected. The bacteriocin activity, commonly known as arbitrary units (AU), was calculated using the equation below [18, 20].

$$\mathbf{AU/ml} = \frac{\mathbf{1}}{\boldsymbol{DF}} \frac{\mathbf{1000}}{\boldsymbol{volums\ spotted\ in\ \mu l}}$$

2.4 Identification of 16S- ribosomal RNA

The chosen bacterial isolate was utilized to extract the nucleic acids using a commercial DNA extraction kit called the Presto Mini-DNA Bacteria Kit from Geneaid Biotech Ltd. in Taiwan. The amount of DNA that was extracted was determined using a Nanodrop and UV-spectrophotometer (ACTGene avans, Taiwan) at two different wave lengths, 260 and 280 nm. The 16S ribosomal RNA gene was used as the basis for the creation of the PCR primers which were provided by Macrogen Company (Korea) using the NCBI Gene sequence data base (MF664480.1).

Amplicon	Reference Locus Sequences (5′ - 3′)	Length
16S rRNA Sequences	GCGGGCGCGTGCTATACTGCAGTCGAACGCGTTGGCC CAATTGATTGATGGTGCTTGCACCTGATTGATTTTGGT CGCCAACGATTGGCGGACGGGTGAGTAACGCGTAGGT AACCTGCCCAGAACCGGGGGACAACGTTTGGAAACA AATGCTAATACCGCATAACAACGTTGTTCGCATGAAC AACGCTAAAAAGATGGCTTCTCGCTATCACTTCTGGA TGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAACG GCCTACCAAGGCGATGATGCATAGCCAAGTTGAGAGA CTGATCGGCCACAATGGGACTGAAACACGGCCCATAC TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATG GGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAA GAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAA GAACACGTATGAGAGTAACTGTTCATACGTTGACGGT ATTT	484 bp

PCR master mix (MaximePCR Premix Kit, iNtRON. Korea) was made using a master mix reagent, and the procedure was then followed exactly as directed by the manufacturer. EZ EZ-10 Spin Column DNA Gel Extraction Kit, available from Biobasic Canada, was used to separate the 16S rRNA PCR product from the *Lactobacillus sp*. isolate from the agarose gel. The PCR products were then examined by electrophoresis (Atta, Korea) with a 1% agarose gel (Promega, USA). Purified 16S rRNA gene PCR product samples were sent to Macrogen Company in Korea to perform DNA sequencing using the 16S rRNA forward primer by the AB DNA sequencing apparatus.

2.5 Determination of Best Production Medium

In order to determine which nutritional medium is better to promote the bacteriocin synthesis by the chosen *Lactobacillus* isolate, a variety of nutritional media were tested including nutrient broth, brain heart infusion broth, tryptic soy broth and Mueller Hinton broth [21].

2.6 Assessment of Safety and Virulence Factors of Lactobacillus

Multiple Antibiotic Resistance Index (MAR Index)

According to the Kirby-Bauer disc diffusion method, the susceptibility of a *Lactobacillus* isolate to several antimicrobials was evaluated using amikacin, ciprofloxacin, levofloxacin, meropenem, piperacillin, ticarcillin/cavulnate, and tobramycin [18].

Hemolytic Activity

The purpose of this test was to identify the ability of *Lactobacillus* to make hemolysin. Blood agar plates were inoculated with sampled bacteria and incubating for 48 hours at 37°C in a candle jar. The type of hemolytic generated by colonies was then examined [22].

2.7 Assessment of the Probiotic Potential of Lactobacillus

Auto-aggregation Assay

Cells were taken from an overnight culture of *Lactobacillus* isolate after 15 minutes centrifugation at 7000 rpm. Two washes in phosphate buffered saline (pH 6.0) were made for the cells. The concentration was adjusted to 10^8 CFU/ml. A vortex was used to mix 4 ml of *Lactobacillus* cells culture for 10 seconds. Auto-aggregation of *Lactobacillus* was estimated at room temperature by transferring 100 µl of the suspension tube's top layer to a 3.9 ml tube containing PBS. The absorbance was then measured at 600 nm [23]. The Following equation was used to calculate the auto-aggregation percentage:

[ODi - ODf / ODi] 100, where auto-aggregation (%)

Where ODi is for the absorbance at the start of auto-aggregation (t = 0), and ODf stands for the absorbance at 1, 2, 3, 4, and 5 hours.

2.8 Detection of Biofilm Formation

A *Lactobacillus* isolate was grown in MRS broth containing 1% glucose for 48 hours at 37°C in microaerophilic circumstances. After adding 20 µl of 48 h. cultivated *Lactobacillus*, each well of a microtiter plate was filled with 180 µl of sterile TSB. The well broth was blended with a pipette ten times . The next step was incubating the sample without shaking for 72 hours at 37°C. The supernatant was removed after incubation before washing each well thrice with phosphate buffer. Crystal violet solution at 1% was added in 200 l aliquots for 15 minutes. The wells were cleaned with phosphate buffer three times and then dried in the air for 30 minutes. Two hundred µl of 96% ethanol were added and allowed to sit for 15 minutes. Sterile media was utilized as a bad control. A 630nm ELISA reader (Huma reader HS, Germany) was used to read the outcome [16].

2.9 Pharmaceutical Formula Including Viable Lactobacillus Cells

This formula, which included two phases, was prepared based on the method described by [24] with modification:

Phase A: Using a magnetic stirrer, 1.08 g of span 60 was dissolved in 30 ml of olive oil, and the mixture was well stirred at 45°C before being allowed to cool. The oily mixture was

dissolved with 0.5 gm of carbopol 934, and 0.1 gm of methyl paraben was added while stirring continuously.

Phase B/ The Aqueous Phase: Five gm propylene glycol was added to 59.8 ml D.W.
Phases A and B were then gradually combined, and the mixture was then emulsified for 10 minutes at a low speed using a mixer. The speed was gradually increased until the resulting emulsion was homogenized. Later 1g of hydroxypropyl methylcellulose (a thickening agent) was added and mixed for 45 minutes at low speed.

A few loopfuls of *Lactobacillus* growth were inoculated into 10 ml of MRS broth after an overnight culture on MRS agar. The mixture was then incubated at 37°C for 24 hours. A 0.5 McFarland standard tube was used to regulate the cell density after incubation so that it was approximately10^8 cells/ml. In order to collect the biomass, a centrifuge at 12000 rpm for 15 minutes was used and then the optimum medium was added to the collected biomass. The appropriate amount of biomass and a certain quantity of MRS broth were then added to the mixture. A gelling emulsion was created by adding triethanolamine, drop by drop until the pH was adjusted to 6.5 in the formula which also included biomass and MRS broth with all compounds from phases A and B. The formula was kept at 4ºC in a tightly sealed and sterilized container.

2.10 In vitro Evaluation of Antibacterial Activity of Emul Gel Formula against P. aeruginosa

Antibacterial activity of the *Lactobacillus* cells in the formula was assessed using the agar well diffusion method. In this experiment, 100 µl of an overnight culture of the multidrug-resistant *P. aeruginosa* containing approximately 10^8cells/ml was streaked over a of Mueller-Hinton agar plates by sterilized cotton swap. A sterile cork borer was used to create circular wells each 5 mm in diameter. Low melting agar was utilized to seal the bottom of the wells. Next, 100µl of the formula containing *Lactobacillus* cells was poured into the well. Following incubation, the diameter of the inhibition zone around each well was observed, showing the formula's antibacterial efficacy against *P. aeruginosa* [25].

2.11 In vivo Evaluation of Antibacterial Activity of Emul Gel Formula against P. aeruginosa Burns Infection Model

Six local rabbits, approximately 1kg in weight fed with pellet 3rd stage, were used in the *in vivo* experiments. They were divided into three groups as the following: A test group that contained two rabbits, the second group contained two rabbits as a negative control, the third group, which contained two rabbits, was considered as a positive control [26]. After adaptation period of 3 days, rabbits shoulder region was shaved and injured using a scalpel of about 1×2cm then burned by heated spatula. Next, each rabbit group was infected with the indicator bacterium (MDR *P. aeruginosa)* by spraying 100µl of a BHI broth contained 1×10^8 cells/ml. The test group that had been exposed to the bacterial indicator was given the final formulation twice daily for 10 days which contained active material (*Lactobacillus* as biomass). The *P. aeruginosa*-infected rabbit from the positive group were given 1% Hamazine antibacterial cream twice daily for 10 days. *P. aeruginosa* infection went untreated in the negative group. The length of the experiment relied on when the wound had fully healed.

3. Results and Discussion

A total of 151 clinical samples were collected from patients suffering from burns and based on results. Seventy percent of the total samples were diagnosed as *P. aeruginosa.*

Identification of *P. aeruginosa* was achieved by using macroscopic examination. Results showed a negative gram reaction, very small rods in shape, single or in pairs and non-spore forming bacteria. Biochemical tests revealed positive result to oxidase, catalase and citrate while indole, urease and coagulase gave negative results. Further confirmation was performed by the Vitek 2 system with accuracy reaching up to 99%. Pathogens of specific concern in the burn population included MDR strains of *P. aeruginosa*, *Acinetobacter baumannii* and *S. aureus* [27].

As mentioned earlier, the purpose of this research was to examine the antibacterial abilities of a possible probiotic *Lactobacillus* sp. against pathogenic *P. aeruginosa* gathered from samples linked to burn illnesses. Therefore, to achieve such an aim, it was necessary to select a pathogenic isolate to use it as indicator in the selection of suitable *Lactobacillus* isolate that could be used as a probiotic in this study. The pathogenic isolates of *P. aeruginosa* were subjected to antibiotic susceptibility tests in order to select the most multidrug-resistant isolates. According to results, the isolate *P. aeruginosa* A23 showed a resistance to all antibiotics examined including aztreonam, imipenem, meropenem, piperacillin-tazobactam, piperacillin, ceftazidime, tobramycin, gentamicin, netilmicin, levofloxacin and ofloxacin. It was, therefore, considered as multi-drug resistant compared to other isolates and was subsequently selected to be used as an indicator in this study. On the other hand, 36 isolates of *Lactobacillus* sp. were collected in this study from different diary product samples including pasteurized milk, cow milk, yoghurt, drinking yogurt, handmade yoghurt, Activia, as well as healthy women's vagina.

All samples were primarily grown on de Man, Rogosa, and Sharpe agar (MRS) and then subjected to a number of biochemical tests and morphological identification. Based on results, the handmade yoghurt and raw cow milk were the best source for *Lactobacillus*. The potential of *Lactobacillus* isolates to inhibit the growth of multi-drug resistant *P. aeruginosa* which causes burn infections, was tested during the screening phase (primary and secondary) by the agar plug diffusion and the well diffusion methods respectively. The primary screening revealed that nine isolates had antagonistic activity against the indicator *P. aeruginosa* with inhibition zone of 15mm and above and, were, hence, considered as strong active isolates. These isolates were selected for the next experiment for secondary screening. The results showed that the isolate *Lactobacillus* HLB12 obtained from Activia yogurt, had the highest antibacterial activity with 80 AU/ml against the indicator bacterium. In order to conduct additional research in this investigation, this isolate was chosen by using agarose gel electrophoresis to analyze the 16S ribosomal RNA gene (PCR) amplification products. It was discovered that the amplified DNA fragment was 221bp in length (Figure 1). Next, the DNA sequences were examined using NCBI- BLASTn for homology sequence identity and BioEdit Sequence Alignment Editor Software Version 7.1. This DNA is 100% equivalent to *L. fermentum* as evidenced by the results shown in Figure 2.

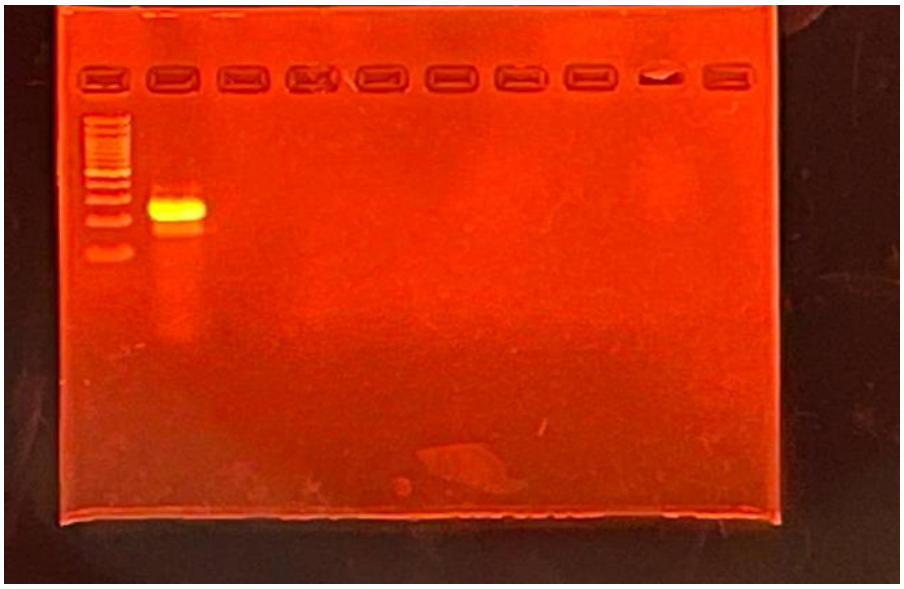

Figure 1: The molecular detection of 16S ribosomal RNA gene for *Lactobacillus* isolate shows the PCR product with a band (221bp) in 2% agarose gel electrophoresis stained with ethidium bromide, 100-1000bp ladder under 75 volt.

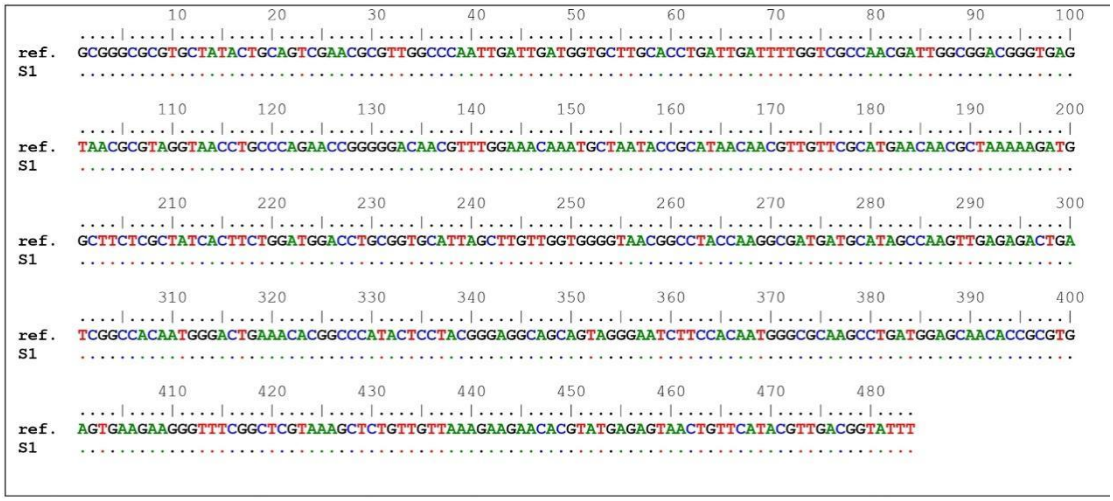

Figure 2: Nucleic acid sequences alignment of one sample with its corresponding reference sequences of the 16S sequences. The symbol "ref" refers to the NCBI referring sequence, letter "S", followed by a number refers to the sample number.

The next step was to determine best production medium to promote the bacteriocin synthesis by the chosen *L. fermentum* HLB12. For this purpose, numerous nutritional media were investigated including nutrient broth, brain heart infusion broth, tryptic soy broth and Mueller-Hinton broth, to ascertain whether nutritional medium was best to encourage the bacteriocin synthesis. Based on results, the best bacteriocin production (80 AU/ml) and the higher biomass (16.7 mg/ml) were obtained in MRS medium (Figure 3). These results agreed with Nouralhuda and Luti [34] who found that MRS broth gave the highest bacteriocin production by *L. parabuchneri* Nu14. Therefore, MRS medium was utilized in the rest of experiments to culture *L. fermentum* HLB12.

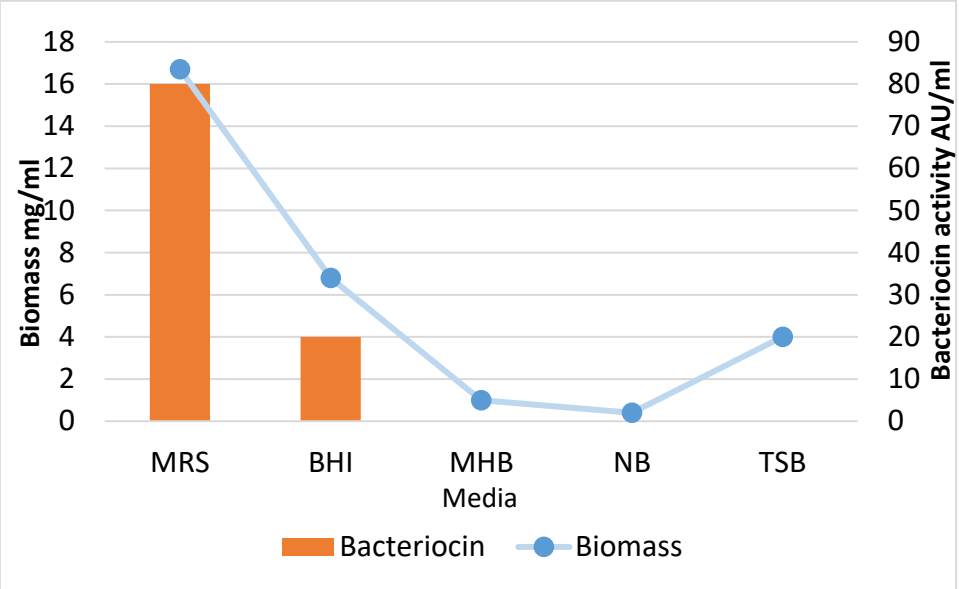

Figure 3: The effect of different media on bacteriocin and biomass production by *L. fermentum HLB12* after incubation for 24 hr. at 37ºC at pH6.

The main goal of this research was to use the isolated *Lactobacillus* as a probiotic. It was therefore important to assess the safety of the selected isolate *L. fermentum HLB12*. The antibiotic sensitivity and hemolytic activity showed the safety of *L. fermentum HLB12* isolate [28]**.** The results presented in Table 2 revealed that *Lactobacillus* was resistance to β-lactams group such as ticarcillin/clavulanate. Whereas it was sensitive to amikacin, ciprofloxacin, levofloxacin and intermediate to meropenem. In addition, *Lactobacillus* showed resistance to tobramycin and piperacillin. The hemolytic activity of *L. fermentum* HLB12 was also tested. The results revealed that the type of hemolytic activity was α-hemolysis indicating non-hemolytic activity of *L. fermentum* HLB12 which was thought to be a secure prerequisite for the chosen probiotic strain according to [29].

In accordance with similar findings, *Lactobacillus* species isolated from dairy products have been demonstrated to be non-hemolytic [30]. In addition, 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] and [32]. It was found that after 5 hrs. *Lactobacillus* HLB12 had high auto-aggregation capability of approximately 76.3%. This test clearly proved that this isolate has a good ability to adhere and inhibit adherence of pathogens (Figure 4).

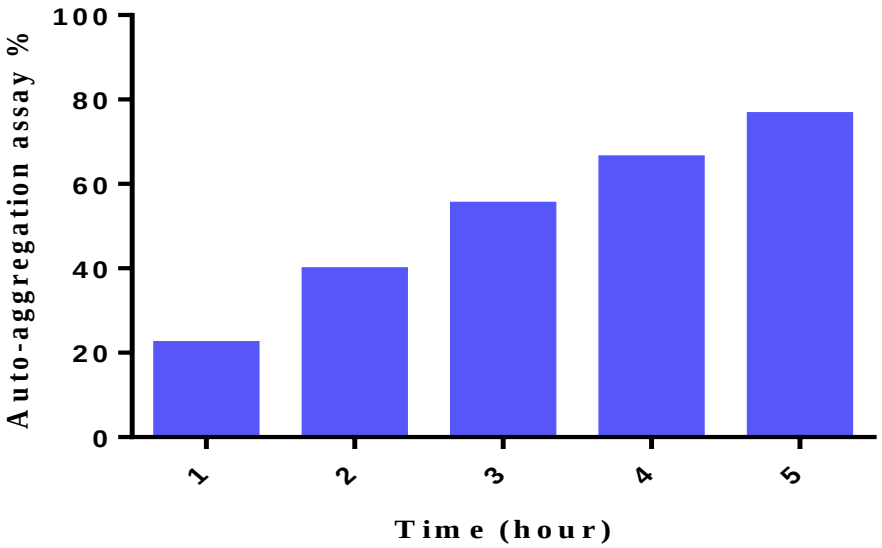

Figure 4: Auto-aggregation assay by *Lactobacillus* after 5 hours of incubation at room temperature using PBS at pH 6.

Another important property of probiotics is biofilm formation. TCP method, which is preferred and more useful, was used because of its superiority in comparison with other biofilm detection methods. The TCP method provides a numerical value based on OD determination and each value represents a certain phenotype result presenting as non-producer, weak, moderate and strong [33]. Biofilm formation is a property that provides the ability to colonize probiotic cells and avoid colonization of pathogens [34].

Based on result, the selected isolate *L. fermentum* HLB12 was a strongly adherent and a good biofilm producer. As can be noticed from the results presented in Figure 5, *L. fermentum* HLB12 had high co-aggregation capability of approximately 92.2% after 5 hrs. This test clearly proved that this isolate has an excellent ability to inhibit adherence of pathogens. These results agree with Nouralhuda and Luti [35] 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 also agrees with that by Younas *et al.* [36] who reported the ability of *Lactobacillus* to co-aggregate with pathogens, as well as Tareq and Luti [37] reported that *L. crispatus* IS30 showed 58% of *Pseudomonas* co-aggregation percentage after 5 hrs. of incubation.

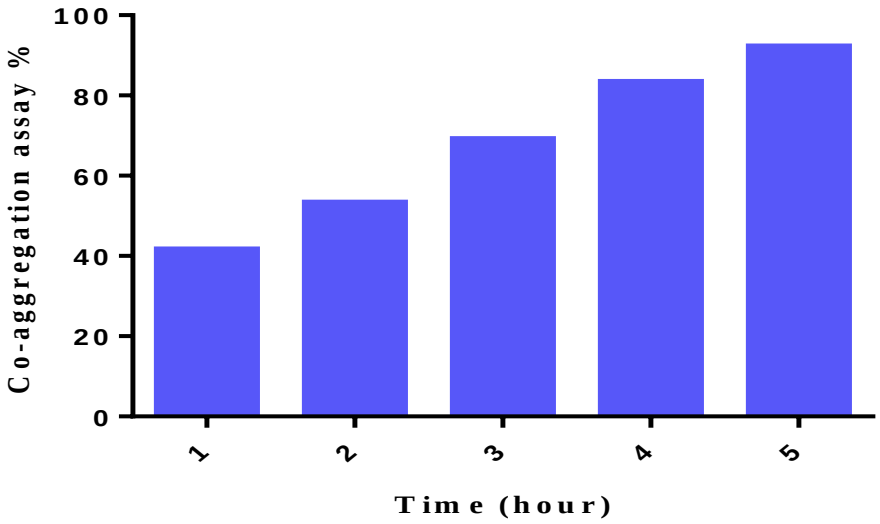

Figure 5: Co-aggregation assay of *L. fermentum* HLB12 after 5 hours of incubation at room temperature using PBS at pH 6.

Antagonistic activity is a very important criterion for selection of probiotics because they are natural antimicrobials to potentially harmful bacteria. Therefore, cells and CFSs from *Lactobacillus* spp. isolated from dairy products and vagina of healthy women were screened for their antagonistic activity against different pathogens associated with burns infection. In this context, different bacterial probiotic cells, in particular *Lactobacillus* sp., were utilized in formula to treat several diseases such as *Lactobacillus* cells in an emulgel formula that is used against some skin pathogens [24]. In addition, Tareq and Luti, [37] used *Lactobacillus crispatus* in an emulgel formula against some vaginal pathogens. This study was performed to select an acceptable formula which could be used to provide a suitable medium for *Lactobacillus* survival. In fact, the challenge in this study was to select a useful formula which could act as a vector to store and transport viable cells of *Lactobacillus* into the skin of burns infection. This study used cells of *L. fermentum* HLB12 to combat MDR *P. aeruginosa* bacteria that were isolated from burn infections. Basically, the created emulgel composition should have an odorless, white color, transparent and smooth texture as its physical characteristics. Based on results, the formula of the emulgel utilized in this investigation was successfully incorporated with *Lactobacillus* biomass. The health of the *Lactobacillus* cells was observed for three weeks by daily cultivation on MRS agar after preparation and making sure that the formula's components were uniform. The results showed that cells had sustained their viability for eighteen days. According to Loveleen and Tarun [38], the created formula emulgels are generally assessed based on a number of factors including pH, homogeneity, spread ability, and skin irritation. *In vitro* monitoring of pH and homogeneity made the stability of formula clear. Through the course of 18 days, the produced formula demonstrated homogeneity and stability at pH 5.9-6.5. The antibacterial activity of *L. fermentum* HLB12 biomass contained within the emulgel formulation was examined *in vitro* against *P. aeruginosa* using the technique described by Mostafa et al. [39]. The concentration of *L. fermentum* HLB12 cells was always kept at 10^8CFU/ml. The results of the effectiveness of formula were demonstrated by the results of the inhibition of pathogenic bacterial growth which contained the following points: the ability of the *Lactobacillus* cells to live, produce bacteriocin, and release that bacteriocin into the environment (tested medium) without becoming trapped or interacting with any other formula ingredient that would reduce bacteriocin's ability to inhibit tested bacteria. The results showed *P. aeruginosa* growth

inhibition with 19mm in diameter, whereas no inhibition zone was observed with control formula (Figure 6b). On the other hand, the formula was tested for twenty-one days by measuring the viability of *Lactobacillus* to inhibit *P. aeruginosa* growth. The *L. fermentum* HLB12 cells formula kept its activity with 20mm in dimeter against *P. aeruginosa* with no green color (Figure 6a). Moreover, results showed that the mixture had good spreading properties which is important because gel or emulgels' capacity to treat illnesses typically hinges on how well they spread. Emulgels have great spreading properties, making them ideal for topical use.

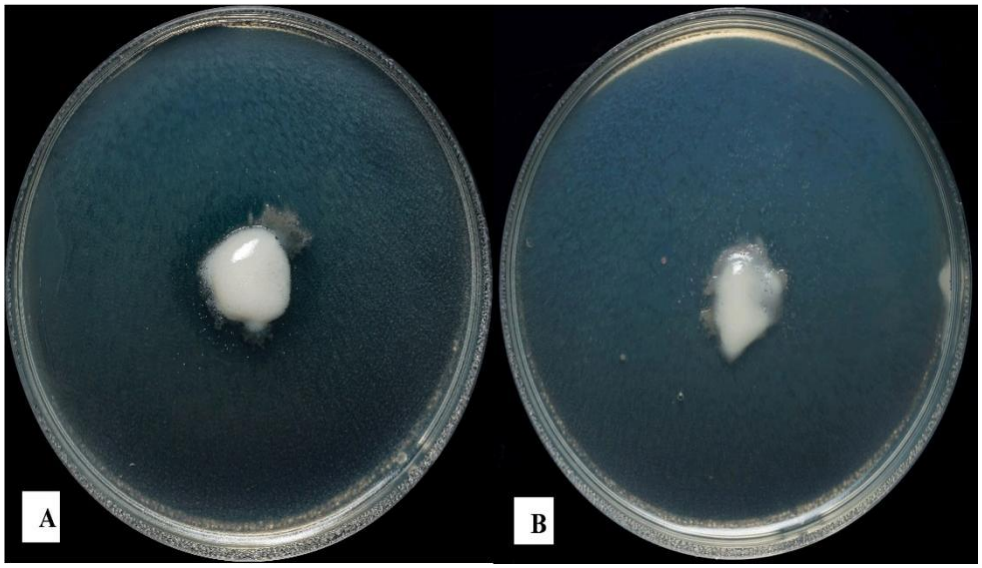

Figure 6: Antimicrobial activity of emulgel on Muller Hinton agar sprayed with *P. aeruginosa* (A) emulgel formula with *L. fermentum* HLB12 biomass (B) emulgel formula without *L. fermentum* HLB12 biomass.

The healing process after a burn can leave scars which is a serious health concern. In this study, the emulgel formula was tested for its efficiency as a probiotic promoting wound healing. In order to evaluate the advantages of using *Lactobacillus* biomass as a treatment, this formula was applied to a rabbit model with full-thickness skin burns. The experiment involved six burned rabbits divided into three groups, as described in the material and methods section. Burn injury involves several healing stages including the inflammatory, proliferative, and remodeling phases. Laboratory experiments on rabbits help researchers understand the mechanisms of burn injury and develop new treatments for burn victims.

Three days after burning and infecting rabbit with the pathogenic isolates, the first stage was attained for the three groups of tested animals with positive and negative control groups. The healing process was examined day by day for all groups. Results showed that the emulgel probiotic group that was applied twice daily and the 1% Hamazine-treated positive group that was also applied twice daily, showed signs of remodeling stage by day four. It was found that the inflammation signs were reduced as the swelling and redness of the skin got better. In the untreated negative group, these signs still existed as the burn ulceration continued with swelling and redness. By day nine, the emulgel formula and 1% Hamazine groups started to show scar tissue formation as they entered the remodeling stage compared with the negative group of untreated animals that showed slow healing and was still in the process of the proliferative stage (Figure 7). Wounds are grouped as chronic and acute due to a chemical, physical, or thermal injury. The healing of wounds shows 4 phases: hemostasis phase, inflammations phase, proliferation or granulation phase, remodeling or maturation phase [40].

The healing periods for chronic and acute wounds are twelve and eight days respectively [41]. On the other hand, wound burns show. a susceptible location for colonization of opportunistic pathogens of exogenous and endogenous original sites. Patient factors such as burn depth, injury extent, age, and microbial factors such as number and type of pathogen, production of toxin and enzyme, and motility identify the probability of invasive wound burn infection [42].

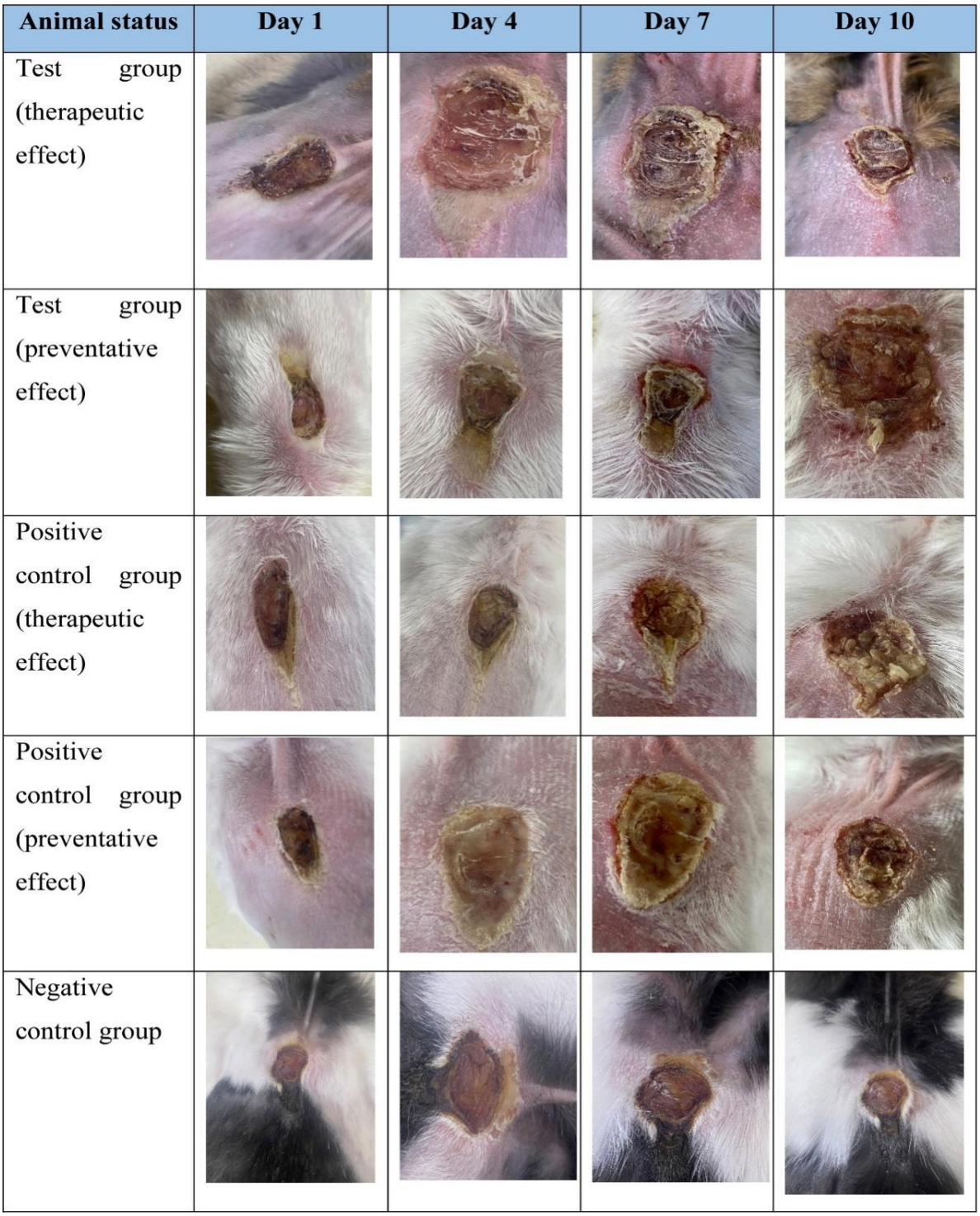

Figure 7: *In vivo* experiment for applying emulgel formula to treat a group of rabbits infected with *P. aeruginosa.*

Conclusion

The present study confirms previous findings as well as contributes additional evidence on using viable cells of bacteriocin-producing *Lactobacillus* as an effective dermal probiotic to deal with multi drug resistance bacteria such as *P. aeruginosa.* Moreover, this study provides evidence with respect to the efficacy of the cells-containing formula in terms of the viability of *Lactobacillus* cells within the formula and its ability to produce and release bacteriocin without interaction with formula components.

Ethical Clearance

This research was ethically approved according to the reference number CSEC/0623/0051 by the ethical committee of university of Baghdad College of Science accepted this work.

Conflict of Interest:

The authors declare that they have no conflict of interest.

References

[1] W. H. Organization, "World Health Organization model list of essential medicines: 22nd list (2021)," World Health Organization, 2021.

[2] R. Kumar *et al.*, "Burn Injury Management, Pathophysiology and Its Future Prospectives," *Journal for Research in Applied Sciences and Biotechnology*, vol. 1, no. 4, pp. 78–89, 2022.

[3] F. D. Halstead *et al.*, "Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens," *Burns*, vol. 41, no. 8, pp. 1683–1694, 2015.

[4] P. Pachori, R. Gothalwal, and P. Gandhi, "Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review," *Genes Dis*, vol. 6, no. 2, pp. 109–119, 2019.

[5] S. N. Arumugam *et al.*, "Antibacterial efficacy of lytic phages against multidrug-resistant Pseudomonas aeruginosa infections in bacteraemia mice models," *BMC Microbiol*, vol. 22, no. 1, p. 187, 2022.

[6] E. J. Kelly, M. A. Oliver, B. C. Carney, and J. W. Shupp, "Infection and burn injury," *European Burn Journal*, vol. 3, no. 1, pp. 165–179, 2022.

[7] K. M. Perkins, S. C. Reddy, R. Fagan, M. J. Arduino, and J. F. Perz, "Investigation of healthcare infection risks from water-related organisms: Summary of CDC consultations, 2014—2017," *Infect Control Hosp Epidemiol*, vol. 40, no. 6, pp. 621–626, 2019.

[8] W. A. J. Al-Kaaby, "Molecular detection of virulence factors genes in Pseudomonas aeruginosa isolated from different infections cases in Al-Diwaniya Hospital," *Al-Qadisiyah Journal of pure science*, vol. 20, no. 2, pp. 53–58, 2015.

[9] A. Latif *et al.*, "Probiotics: mechanism of action, health benefits and their application in food industries," *Front Microbiol*, vol. 14, 2023.

[10] N. Garcia-Gonzalez, N. Battista, R. Prete, and A. Corsetti, "Health-promoting role of Lactiplantibacillus plantarum isolated from fermented foods," *Microorganisms*, vol. 9, no. 2, p. 349, 2021.

[11] B. N. Abdul Hakim, N. J. Xuan, and S. N. H. Oslan, "A Comprehensive Review of Bioactive Compounds from Lactic Acid Bacteria: Potential Functions as Functional Food in Dietetics and the Food Industry," *Foods*, vol. 12, no. 15, p. 2850, 2023.

[12] D. Jurášková, S. C. Ribeiro, and C. C. G. Silva, "Exopolysaccharides produced by lactic acid bacteria: From biosynthesis to health-promoting properties," *Foods*, vol. 11, no. 2, p. 156, 2022.

[13] I. De Boeck, I. Spacova, O. M. Vanderveken, and S. Lebeer, "Lactic acid bacteria as probiotics for the nose?" *Microb Biotechnol*, vol. 14, no. 3, pp. 859–869, 2021.

[14] R. Georgieva *et al.*, "Antimicrobial activity and antibiotic susceptibility of Lactobacillus and Bifidobacterium spp. intended for use as starter and probiotic cultures," *Biotechnology & Biotechnological Equipment*, vol. 29, no. 1, pp. 84–91, 2015.

[15] R. H. Perez and A. E. Ancuelo, "Diverse Bioactive Molecules from the Genus Lactobacillus," in *Lactobacillus-A Multifunctional Genus*, IntechOpen, 2022.

[16] 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*, vol. 15, pp. 305–311, 2011.

[17] Ş. A. KARAOĞLU, F. Aydin, S. S. Kilic, and A. O. Kilic, "Antimicrobial activity and characteristics of bacteriocins produced by vaginal lactobacilli," *Turk J Med Sci*, vol. 33, no. 1, pp. 7–13, 2003.

[18] J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams, "Bergey's manual of determinative bacteriology. 9th," *Baltimor: William & Wilkins*, 1994.

[19] F. N. Abd and K. J. K. Luti, "purification and characterization of a bacteriocin produced by Bacillus subtilis NK16 exhibits a significant antimicrobial activity against clinical *Staphylococcus spp*.", *Baghdad Sci. J*, vol. 14, no. 3, p. 0524, Sep. 2017.

[20] M. Balouiri, M. Sadiki, and S. K. Ibnsouda, "Methods for in vitro evaluating antimicrobial activity: A review," *J Pharm Anal*, vol. 6, no. 2, pp. 71–79, 2016.

[21] A. M. Jetten, G. D. Vogels, and F. De Windt, "Production and purification of a Staphylococcus epidermidis bacteriocin," *J Bacteriol*, vol. 112, no. 1, pp. 235–242, 1972.

[22] J. G. Collee, R. S. Miles, and B. Watt, "Tests for identification of bacteria," *Mackie and McCartney practical medical microbiology*, vol. 14, pp. 131–149, 1996.

[23] B. Kos, J. Šušković, S. Vuković, M. Šimpraga, J. Frece, and S. Matošić, "Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92," *J Appl Microbiol*, vol. 94, no. 6, pp. 981–987, 2003.

[24] H. T. Rasheed, K. J. K. Luti, and M. A. Alaubydi, "A PROBIOTIC APPLICATION OF Lactobacillus acidophilus HT1 FOR THE TREATMENT OF SOME SKIN PATHOGENS.," *Iraqi Journal of Agricultural Sciences*, vol. 51, no. 6, 2020.

[25] A. Mayr-Harting, A. J. Hedges, and R. C. W. Berkeley, "Chapter VII methods for studying bacteriocins," in *Methods in microbiology*, vol. 7, Elsevier, 1972, pp. 315–422.

[26] Y. A. Hussein and K. J. K. Luti, "Probiotic Application of Bacteriocin-Producing S. Epidermidis in A Cellulosic Pad to Treat Some Skin Infections", *Iraqi Journal of Science*, vol. 61, no. 8, pp. 1932–1943, Aug. 2020.

[27] H. Kanamori *et al.*, "A prolonged outbreak of KPC-3-producing Enterobacter cloacae and Klebsiella pneumoniae driven by multiple mechanisms of resistance transmission at a large academic burn center," *Antimicrob Agents Chemother*, vol. 61, no. 2, pp. 10–1128, 2017.

[28] D. C. Donohue and S. Salminen, "Safety of probiotic bacteria," *Asia Pac J Clin Nutr*, vol. 5, pp. 25–28, 1996.

[29] M. Araya, P. Gopal, and S. E. Lindgren, "Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food, London, Ontario, Canada, 30 April-1 May 2002." 2002.

[30] P. A. Maragkoudakis, G. Zoumpopoulou, C. Miaris, G. Kalantzopoulos, B. Pot, and E. Tsakalidou, "Probiotic potential of Lactobacillus strains isolated from dairy products," *Int Dairy J*, vol. 16, no. 3, pp. 189–199, 2006.

[31] T. Tatsaporn and K. Kornkanok, "Using potential lactic acid bacteria biofilms and their compounds to control biofilms of foodborne pathogens," *Biotechnology Reports*, vol. 26, p. e00477, 2020.

[32] R. C. Reuben, P. C. Roy, S. L. Sarkar, R.-U. Alam, and I. K. Jahid, "Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics," *BMC Microbiol*, vol. 19, pp. 1–20, 2019.

[33] T. Mathur, S. Singhal, S. Khan, D. J. Upadhyay, T. Fatma, and A. Rattan, "Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods," *Indian J Med Microbiol*, vol. 24, no. 1, pp. 25–29, 2006.

[34] J. C. Post, "Direct evidence of bacterial biofilms in otitis media. 2001," *Laryngoscope*, vol. 125, no. 9, pp. 2003–2014, 2015.

[35] 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*, vol. 60, no. 3, pp. 494–508, Mar. 2019.

[36] S. Younas, B. Mazhar, I. Liaqat, S. Ali, H. M. Tahir, and N. M. Ali, "Bacteriocin Production by Lactobacilli and Their Role as Antibacterial Tool against Common Pathogens," *J Oleo Sci*, vol. 71, no. 4, pp. 541–550, 2022.

[37] 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. 491–507, 2022.

[38] L. P. Kaur, "Topical gel: a recent approach for novel drug delivery," *Asian journal of biomedical and Pharmaceutical Sciences*, vol. 3, no. 17, p. 1, 2013.

[39] M. Khodadadi, M. H. Fotokian, and M. Miransari, "Genetic diversity of wheat (Triticum aestivum L.) genotypes based on cluster and principal component analyses for breeding strategies," *Aust J Crop Sci*, vol. 5, no. 1, pp. 17–24, 2011.

[40] T. W. Fossum, *Small Animal Surgery E-Book*. Elsevier Health Sciences, 2018.

[41] J. Koehler, F. P. Brandl, and A. M. Goepferich, "Hydrogel wound dressings for bioactive treatment of acute and chronic wounds," *Eur Polym J*, vol. 100, pp. 1–11, 2018.

[42] J. Pruitt Basil A, A. T. McManus, S. H. Kim, and C. W. Goodwin, "Burn wound infections: current status," *World J Surg*, vol. 22, pp. 135–145, 1998.