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In Vitro Evaluation of *Limosilactobacillus fermentum* Antagonistic Ability against MDR *Pseudomonas aeruginosa* Associated with Burn Wound Infection

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Abstract: As the interest grows in bacteria that populate the human body rather than merely those that infect it, research is attempting to manage the microbiome in a specific niche to promote human health. Probiotics has experienced significant growth as a result of this utilization of beneficial bacteria. The aim of the study that several isolates of *Lactobacillus* were examined in order to use it as a probiotic for treating *Pseudomonas aeruginosa* associated with burn infections. Thirty-six *Lactobacillus* isolates were collected from different sources and subjected to a screening program to evaluate their antagonism activities against MDR *P. aeruginosa* that collected from patient with burn infection. 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 *L. fermentum* HLB12, in particular its biosafety and suitability to be a successful probiotic. Results showed that *L. fermentum* HLB12 was resistance to β-lactams group such as Ticarcillin/Cavulnate whereas, it was sensitive to Amikacin, Ciprofloxacin, Levofloxacin. Moreover, results confirmed that *L. fermentum* HLB12 was safe as probiotic without any health impacts because it has no haemolytic 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 *P. aeruginosa*. It was concluded showed a highly antagonistic activity against *P. aeruginosa* which supported the idea of using *L. fermentum* as a probiotic that can be used as an alternative for the treatment with antibiotics.

Keywords: *Limosilactobacillus fermentum; Pseudomonas aeruginosa;* burn infection; agar overlay assay.

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Introduction

The emergence of MDR bacteria has led to an urgent need for alternative therapies to treat burn infections (1). *Pseudomonas aeruginosa* is an opportunistic pathogen, therefore, it can be found in patients with immunocompromised. It is most commonly found in hospitals and private care lounges and considered as a dominant colony of burn wounds for its ability to spread rapidly within damaged tissues cause serious diseases and chronic and acute infections. It is one of the most common bacteria that cause hospital infection such as urinary tract infections, skin infections, and pneumonia (2). Acquired injuries to burn patients are caused by two sources, one of which is endogenous infections. It occurs by the presence of an organism that is part of the normal life of the patient. The other source is exogenous infections that occurs by exposure to the hospital staff, medical devices or hospital environment (3). Due to the numerous virulence factors created by

P. aeruginosa, it is considered one of the most important and dangerous organisms in human infections (4). Probiotics are non-pathogenic live microorganisms having the potential to exert health benefits to the host (5), such as reducing inflammation, speeding the wound healing process, and strengthening the immune system(4).

Globally, burn injuries are significant health issues impacting millions of people (6). 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 (7). Burn wound infections are challenging to treat due to the emergence of antibiotic-resistant bacteria, such as *Pseudomonas aeruginosa*.

Several species of *Lactobacillus* have proven to exert a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens, reduction of blood cholesterol levels and others (8). Therefore, lactobacilli were among the first bacteria that described and used as probiotics (8). There are a number of probiotics, including *Lactobacillus*, that 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 (10). 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. Antimicrobial activity can be produced by these bacteria by the secretion of antimicrobial compounds such fatty acids, hydrogen peroxide, and bacteriocins (9). Infection remains the most common complication after burn injury and can result in sepsis and death, despite the use of topical and systemic antibiotics. This study will evaluate the ability of the probiotic organism *Lactobacillus* sp. to inhibit the pathogenic activity of *Pseudomonas aeruginosa in vitro* so that it can be used as prophylactic treatment to prevent the infection. Using probiotics directly to burn wounds is an attractive novel intervention that avoids the pitfalls of standard antibiotic therapies.

Materials and methods

Collection of samples

A total of 151 clinical samples were collected from patients suffering from burns, 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 (10). Next, all isolates of *P. aeruginosa* were 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 can be used as a probiotic in this study. The antibiotics used were (Aztreonam 30µg, Imipenem 10µg, Meropenem 10µg, Piperacillin-tazobactam 100/10µg, Piperacillin 100µg, Ceftazidime 30µg, Tobramycin 10µg, Gentamicin 10µg, Netilmicin 30µg, Levofloxacin 5µg and Ofloxacin 5µg).

Collection of lactobacillus isolates

Lactobacillus isolates were collected from samples of dairy products including pasteurized milk, cow milk, yoghurt, drinking yogurt, handmade yoghurt, activia yougert and from healthy women's vagina. Samples were collected in sterile containers and kept refrigerated until they were delivered to the lab. Bacterial samples were streaked in MRS agar and incubated for 48 hours at 37°C under microaerophilic conditions, after which they were re-cultured as a single colony in MRS agar under the same circumstances. The Bergey's handbook of Systematic Bacteriology and a few biochemical assays were used to identify the bacterial isolates (11) based on their cultural characters.

Screening of *Lactobacillus* isolates for bacteriocin production

Primary screening

The MRS agar medium was cultured with the isolates by streaking on the plate surface following an overnight growth in MRS broth, and then the isolates were 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 0.5 cm in diameter for each isolate. These plugs were placed on the mullar-hinton agar plates previously streaked with 100 µl of an overnight growth culture of the indicator bacterium (MDR *Pseudomonas 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.

Secondary screening

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 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. To create a series of two-fold dilutions, the sequence of dilutions was repeated. 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 and the bacteriocin activity, commonly known as arbitrary units (AU), was calculated using the equation below (12).

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

Identification of selected isolate by using 16S ribosomal RNA

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

Table (1): The position and length of the 484bp PCR amplicons that used to amplify a portion of the 16S sequences within *Limosilactobacillus fermentum* genomic sequences (GenBank acc. no. MF664480.1).

Amplicon	Reference locus sequences (5′ - 3′)	Length
16S rRNA sequences	GCGGGCGCGTGCTATACTGCAGTCGAACGCGTTGGCCCAATTG ATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGATT GGCGGACGGGTGAGTAACGCGTAGGTAACCTGCCCAGAACCG GGGGACAACGTTTGGAAACAAATGCTAATACCGCATAACAAC GTTGTTCGCATGAACAACGCTAAAAAGATGGCTTCTCGCTATC ACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTA ACGGCCTACCAAGGCGATGATGCATAGCCAAGTTGAGAGACT GATCGGCCACAATGGGACTGAAACACGGCCCATACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTG ATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTA AAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTC ATACGTTGACGGTATTT	484 bp

Table (2): Designed Primers Sanger sequencing used for detection of SNPs.

Gene name and SNP	Primers Sequence 5′→3′		GC %	Product size (bp)	Annealing Temp.
SNP* of selected *gene	**F**	TGTCGTGAGATGTTGGGTTAAG	55%	221	57±5
	R	CCGGTTTTAAGAGATTTGCTTG	50%		

Master mix reagent was used to prepare the PCR master mix (MaximePCR Premix Kit, iNtRON. Korea), which was then carried out in accordance with the manufacturer's instructions. The 16S rRNA PCR product from the Lactobacillus sp. isolate was purified from the agarose gel using the EZ EZ-10 Spin Column DNA Gel Extraction Kit, available from Biobasic Canada, and the PCR products were analyzed by electrophoresis (Atta, Korea) with a 1% agarose gel (Promega, USA). To execute DNA sequencing utilizing the 16S rRNA forward primer by the AB DNA sequencing equipment, the purified 16S rRNA gene PCR product samples were delivered to Macrogen Company in Korea.

In vitro determination of *Lactobacillus* activity as probiotic

Multiple antibiotic resistance index (MAR index)

The Kirby-Bauer disc diffusion method was used to assess the lactobacillus isolate's sensitivity to several antimicrobials (11). Amikacin and Ciprofloxacin antibiotics were employed. Levofloxacin Ticarcillin/ Cavulnate, Meropenem, Piperacillin, and Tobramycin.

Haemolytic 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. Then, the type of hemolytic generated by colonies was examined(13).

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 and the concentration was adjusted to 10^8 CFU/ml. A vortex was used to mix 4 ml of *Lactobacillus* cells culture for 10 seconds. At room temperature, the auto-aggregation of *Lactobacillus* was estimated by transferring 100 µl of the suspension tube's top layer to a 3.9 ml tube containing PBS. At 600 nm, the

absorbance was then measured (14) and 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.

Detection of biofilm formation

A *Lactobacillus* isolate was cultivated for 48 hours at 37°C in microaerophilic conditions in MRS broth with 1% glucose. Each well of a microtiter plate received 180 µl of sterile TSB before 20 µl of 48 h cultivated Lactobacillus was added. By using a pipette, the broth in the well was mixed ten times. It was then incubated for 72 hours at 37°C without being shaken. After incubation, the supernatant was taken out, and phosphate buffer was used to wash each well three times. 200 µl aliquots of 1% crystal violet solution were added for 15 minutes. With phosphate buffer, the wells were rinsed three times before being dried in the air for 30 minutes. For 15 minutes, 200 µl of ethanol (96%) were added. As a negative control, sterile media was utilized. A 630nm ELISA reader (Huma reader HS, Germany) was used to read the outcome(15).

Antagonistic activity of *Lactobacillus* by agar overlay assay: -

a. Spot-on lawn antimicrobial assay

According to Pilasombut (16): Preparation of Mueller-Hinton agar plates preparation of the chosen indicator microorganism *P. aeruginosa* in BHI broth and kept at the concentration 1×10^8 cell/ml. The investigated probiotic bacterium was spotted onto the Muller-Hinton agar plate by a drop of 75µl of *Lactobacillus* grown in MRS broth on the center of the plate. Then the plate was sprayed with *P. aeruginosa* broth with by sterilized spray bottle. After incubation for 24h, the antimicrobial activity is expressed as inhibition zone around the spot.

b. Agar spot antimicrobial assay

MRS agar plates were prepared and inoculated with *Lactobacillus* by spreading. Then, plates were incubated at 37°C for 48h in anaerobic condition to develop a heavy growth culture. Muller-Hinton agar plates were prepared and a loopful culture of the *Lactobacillus* biomass was transferred on the center of the plate. *P. aeruginosa* growth was prepared in BHI broth and kept at the concentration 1×10^8 cell/ml. Then, the plate was sprayed with *P. aeruginosa* broth with by sterilized spray bottle. The plates were then incubated aerobically and after 24h incubation, the inhibition zone was detected. A clear zone of more than 1 mm around the spot is considered as positive (17).

Results and discussion

There are a number of microorganisms that colonize the surfaces of skin burn very quickly. If the patient is not treated with antibiotics, after 5-7 days the skin becomes an environment for the colonization of different microbes including Gram positive and Gram-negative bacteria (18).

In this study, a total of 151 clinical samples were collected from patients suffering from burns and based on results, 70% of the total samples were diagnosed as *P. aeruginosa.* Identification of *P. aeruginosa* was done by using macroscopic and microscopic examination as well as number of biochemical tests such as MacConky agar, Cetrimide agar as in (Figure 1).and further confirmed by the Vitek 2 system with accuracy reaching up to 99%. The results agreed with (19).

Pathogens of specific concern in the burn population include MDR strains of *P. aeruginosa*, *Acinetobacter baumannii*, and *S. aureus* (20).

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 in order to use it as indicator in the selection of suitable *Lactobacillus* isolate that can 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, therefore it was considered as multi-drug resistant compare with other isolates and hence it was selected to be used as an indicator in this study.

Figure (1): *P. aeruginosa* grown at 37°C for 24 hr on (A): MacConky agar (B): Cetrimide agar.

In addition, thirty-six 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 screening process (primary and secondary) by agar pluge diffusion method and well diffusion method respectively was used to testing the ability of *Lactobacillus* isolates to stop the growth of multi-drug resistant *P. aeruginosa* that causes burn infections. The primary screening revealed that nine isolates had antagonistic activity against the indicator *P. aeruginosa* with inhibition zone of 15mm and above and hence were considered as strong active isolates (Figure 2).

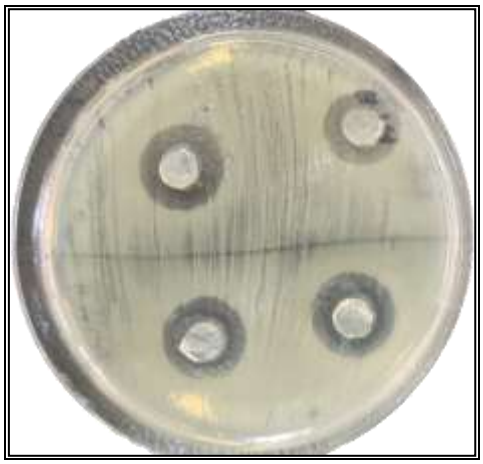

Figure (2): *L. fermentum HLB12* agar plug on Mueller-Hinton agar streaked with *P. aeruginosa* at 37°C for 24 hr.

These isolates were selected to the next experiment of secondary screening and results showed that the isolate *Lactobacillus* HLB12 which obtained from Activia yogurt had the highest antibacterial activity with 80 AU/ml against the indicator bacterium (Figure 3). Therefore, this isolate was chosen to be the subject of additional research in this investigation. The 16S ribosomal RNA gene (PCR) amplification products were evaluated by agarose gel electrophoresis, and the results showed that the amplified DNA fragment was 221bp in length. BioEdit Sequence Alignment Editor Software Version 7.1 and NCBI- BLASTn for homology sequence identity were used to analyze the DNA sequences and the findings showed that DNA was 100% comparable to *Limosilactobacillus fermentum.*

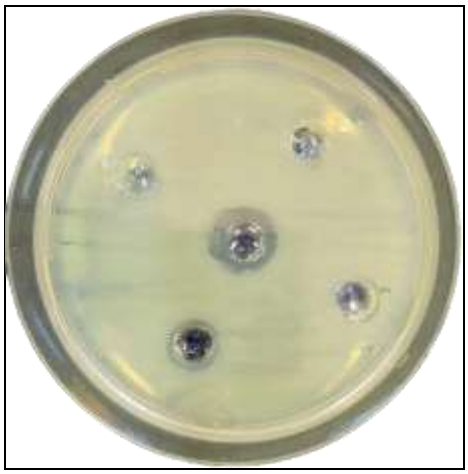

Figure (3): Well diffusion *L. fermentum HLB12* bacteriocin on Mueller-Hinton agar streaked with *P. aeruginosa* at 37°C for 24 hr.

Limosilactobacillus is one of the *Lactobacilli* species that colonize the gastrointestinal system, vagina, and mouth in humans (21). The species *Limosilactobacillus fermentum* is a member of this genus. In addition to being employed for a wide range of purposes, including food and feed fermentation, species in this genus are suited to the digestive system of vertebrates (22). Numerous studies have demonstrated the potential of *L.*

fermentum for usage as probiotics and in the medical area due to their health-promoting benefits, which include lowering gastrointestinal and upper respiratory tract infections and preventing alcoholic illness. Its antioxidant, anti-inflammatory, immunomodulatory, and anti-proliferative properties were also noted (22). A fascinating finding is that, *L. fermentum* has antibacterial action against *Helicobacter pylori*, *Clostridium perfrigens* , *Streptococcus mutans Micrococcus luteus,* frequent food pathogens, and *P. aeruginosa* as well as *E. coli* (24). It has considerable potential for food preservation applications because its ability to synthesis both bacteriocin and lactic acid (25).

For a product to be considered as a probiotic, it must conform to three essential characteristics in particular its biosafety and suitability to be a successful probiotic. As the main goal of this research was to use the isolated *Lactobacillus* as a probiotic; hence it was important to assess firstly, the safety of the selected isolate, *L. fermentum HLB12*. The antibiotic sensitivity and haemolytic activity showed the safety of *L. fermentum HLB12* isolate (26)**.** The results revealed that *Lactobacillus* was resistance to β-lactams group such as Ticarcillin/Cavulnate whereas, it was sensitive to Amikacin, Ciprofloxacin, Levofloxacin and intermediate to Meropenem. In addition, *Lactobacillus* showed resistance to Tobramycin and Piperacillin. The Haemolytic activity of *L. fermentum* HLB12 was tested and 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 (27). In accordance with similar findings, *Lactobacillus* species isolated from dairy products have been demonstrated to be non-haemolytic (28).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 (29). It was found that *Lactobacillus* HLB12 had high auto-aggregation capability of approximately 76.3% after 5 hrs. This test clearly prove that this isolate has a good ability to adhere and inhibit adherence of pathogens (Figure 4). Another important property of probiotics is biofilm formation, the method TCP was used which is preferred and more useful, because of its superiority in comparison with other biofilm detection methods. The TCP method provide a numerical value based on OD determination and each value represent a certain phenotype result presenting as non-producer, weak, moderate and strong (30). Biofilm formation is a property that provide the ability to colonize probiotic cells and avoid colonization of pathogens (30). Based on result, the selected isolate *L. fermentum* HLB12 was a strongly adherent and strong biofilm producer. As can be noticed from the results presented in (Figure 4), *L. fermentum* HLB12 had high co-aggregation capability of approximately 92.2% after 5 hrs. This test clearly prove that this isolate has an excellent ability to adhere to inhibit adherence of pathogens.

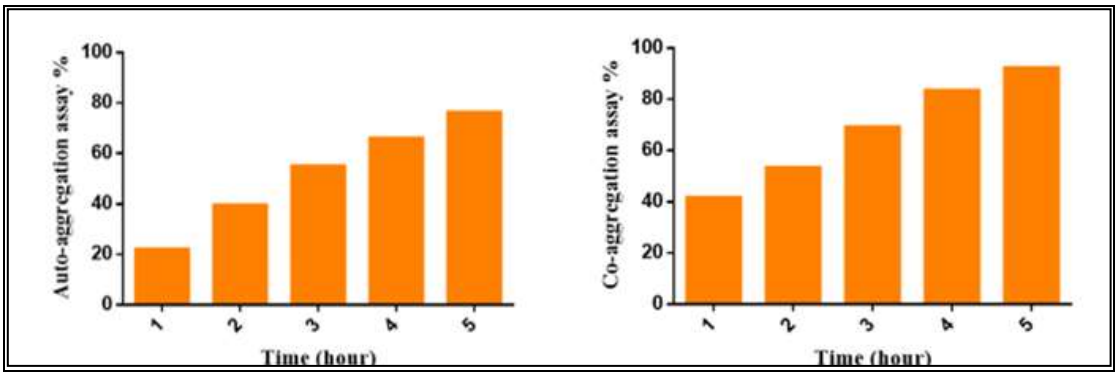

Figure (4): Co-aggregation assay of *L. fermentum* HLB12 after 5 hours of incubation at room temperature using PBS at pH 6.

For probiotics, having an antagonistic ability against pathogenic bacteria with significant antimicrobial activity is particularly important because it is one of their functional requirements. Several factors, such as immune modulation, stimulation of the host defense mechanisms, production of organic acids or hydrogen peroxide that lower pH, production of antioxidants and antimicrobials like bacteriocins, as well as production of signaling molecules that change gene expression, can cause antagonistic activity by competitively excluding one microorganism from another (31). The inhibitory zone surrounding the *Lactobacillus* spot in the MRS broth has a high antagonistic activity against *P. aeruginosa*, as shown in (Figure 5), demonstrating the isolate *Limosilactobacillus fermentum's* good probiotic potential.

Figure (5): De Man, Rogosa and Sharpe (MRS) broth of *Limosilactobacillus fermentum* HLB12 spotted on Mueller-Hinton agar sprayed with *P. aeruginosa.*

The activity of the chosen *Lactobacillus* against *P. aeruginosa* by Agar spot antimicrobial assay was further validated, as seen in (Figure 6). The inhibitory zone surrounding the spot was clearly visible after an overnight incubation at 37°C for 24h, demonstrating that the *Lactobacillus* cells have strong antagonistic activity against *P. aeruginosa*. These findings demonstrate that the chosen isolate of *L. fermentum* HLB12 has outstanding probiotic potential. A crucial characteristic of probiotics is their antibacterial capacity, which includes the generation of antimicrobial

substances, competitive exclusion of pathogens, and improvement of intestinal barrier integrity, among other things. Probiotic strains typically produce many antimicrobial compounds, some of which may work in concert to increase the range of activity against the desired pathogens. This characteristic might be advantageous if the antimicrobial spectrum is only used to treat pathogenic germs (32).

In conclusion the current study supports earlier findings and adds to the body of knowledge about the use of *Lactobacillus* cells capable of producing bacteriocin as a dermal probiotic for the treatment of bacteria with multidrug resistance, such as *Pseudomonas aeruginosa*. Additionally, this study offers a high recommendation for the use of *L. fermentum* as a safe and good dermal probiotic.

Figure (6): Biomass of *L. fermentum* HLB12 spotted on Mueller-Hinton agar sprayed with *P. aeruginosa.*

Conclusion

It was concluded showed a highly antagonistic activity against *P. aeruginosa* which supported the idea of using *L. fermentum* as a probiotic that can be used as an alternative for the treatment with antibiotics.

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