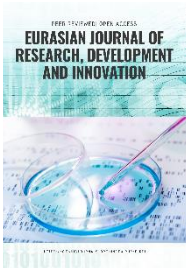

Utilization of *Lactobacillus* SP as prophylactic treatment againstMDR *Staphylococcus aureus* associated with burn wound infection

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ABSTRACT

Infection remains the most common complication after burn injury and can result in sepsis and death, despite the use of topical and systemic antibiotics. Staphylococcus aureus is a frequently implicated pathogen. This study will evaluate the ability of the probiotic organism Lactobacillus sp. to inhibit the pathogenic activity of Pseudomonas aeruginosa, both in vitro and in vivo 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.

Keywords: wound infection, *Staphylococcus aureus,* Lactobacillus,

Introduction

Among the lactic acid bacteria (LAB), Lactobacilli (the species of the genus Lactobacillus) are an essential member of the intestinal microbiota of vertebrates, including humans. Lactobacillus are generally recognized as safe (GRAS) and therefore, can be used as probiotics ((Fuller, 1989). The antibacterial activity of probiotic Lactobacilli act against different pathogenic bacteria through multifunctional ways by secreting antimicrobial substances (organic acids, bacteriocins, H_2O_2, lactic acid and other), counteracting the spread within the colonized body or competing for nutrients and binding sites (Eid, 2016). Lactic acid bacteria produce a wide variety of bacteriocins. Their non-toxic property on eukaryotic cells and inhibitory spectra make gram-positive bacteriocins a unique useful tool for many medicinal and industrial applications (Balciunas et al., 2013).

Aim Of Study

Infection remains the most common complication after burn injury and can result in sepsis and death, despite the use of topical and systemic antibiotics. Staphylococcus aureus is a frequently implicated pathogen. This study will evaluate the ability of the probiotic organism Lactobacillus sp. to inhibit the pathogenic activity of Pseudomonas aeruginosa, both in vitro and in vivo 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.

Objectives

1. Isolation and identification of Lactobacillus from different sources.
2. Select some clinical isolates of Staphylococcus aureus in order to use it as indicators for bacteriocin detection.
3. Screening of isolates in order to select the higher bacteriocin producing isolate using agar plugs diffusion method.

2.1. Probiotics

Probiotics are live microorganisms promoted with claims that they provide health benefits when consumed, generally by improving or restoring the gut microbiota.[1][2] Probiotics are considered generally safe to consume, but may cause bacteria-host interactions and unwanted side effects in rare cases.[3][4][5]

There is some evidence that probiotics are beneficial for some conditions, but there is little evidence for many of the health benefits claimed for them.[1] The first discovered probiotic was a certain strain of bacillus in Bulgarian yoghurt, called Lactobacillus bulgaricus. The discovery was made in 1905 by Bulgarian physician and microbiologist Stamen Grigorov. The modern-day theory is generally attributed to Russian Nobel laureate Élie Metchnikoff, who postulated around 1907 that yoghurt-consuming Bulgarian peasants lived longer.[6]

A growing probiotics market has led to the need for stricter requirements for scientific substantiation of putative benefits conferred by microorganisms claimed to be probiotic.[7] Although numerous claimed benefits are marketed towards using consumer probiotic products, such as reducing gastrointestinal discomfort, improving immune health,[8] relieving constipation, or avoiding the common cold, such claims are not supported by scientific evidence,[7][9][10] and are prohibited as deceptive advertising in the United States by the Federal Trade Commission.[11] As of 2019, numerous applications for approval of health claims by European manufacturers of probiotic dietary supplements have been rejected by the European Food Safety Authority for insufficient evidence of beneficial mechanism or efficacy.[8][12]

2.2. Lactobacillus Sp

Lactobacillus is a genus of Gram-positive, aerotolerant anaerobes or microaerophilic, rod-shaped, non-spore-forming bacteria.[2][3] Until 2020, the genus Lactobacillus comprised over 260 phylogenetically, ecologically, and metabolically diverse species; a taxonomic revision of the genus assigned lactobacilli to 25 genera (see § Taxonomy below).[3]

Scientific classification	
Domain.	Bacteria
Phylum.	Bacillota
Class.	Bacilli
Order.	Lactobacillales
Family.	Lactobacillaceae
Genus.	Lactobacillus

Lactobacillus species constitute a significant component of the human and animal microbiota at a number of body sites, such as the digestive system, and the female genital system.[4] In women of European ancestry, Lactobacillus species are normally a major part of the vaginal microbiota.[5][6] Lactobacillus forms biofilms in the vaginal and gut microbiota,[7] allowing them to persist during harsh environmental conditions and maintain ample populations.[8] Lactobacillus exhibits a mutualistic relationship with the human body, as it protects the host against potential invasions by pathogens, and in turn, the host provides a source of nutrients.[9] Lactobacilli are among the most common probiotic found in food such as yogurt, and it is diverse in its application to maintain human well-being, as it can help treat diarrhea, vaginal infections, and skin disorderssuch as eczema.[10]

2.2.1. Metabolism

Lactobacilli are homofermentative, i.e. hexoses are metabolised by glycolysis to lactate as major end product, or heterofermentative, i.e. hexoses are metabolised by the Phosphoketolase pathway to lactate, CO_2 and acetate or ethanol as major end products.[11] Most lactobacilli are aerotolerant and some species respire if heme and menaquinone are present in the growth medium.[11] Aerotolerance of lactobacilli is manganese-dependent and has been explored (and explained) in Lactiplantibacillus plantarum (previously Lactobacillus plantarum).[12] Lactobacilli generally do not require iron for growth.[13]

The Lactobacillaceae are the only family of the lactic acid bacteria (LAB) that includes homofermentative and heterofermentative organisms; in the Lactobacillaceae, homofermentative or heterofermentative metabolism is shared by all strains of a genus.[3][11] Lactobacillus species are all homofermentative, do not express pyruvate formate lyase, and most speciesdo not ferment pentoses.[3][11] In L. crispatus, pentose metabolism is strain specific and acquired by lateral gene transfer.[14]

2.2.2. Genomes

The genomes of lactobacilli are highly variable, ranging in size from 1.2 to 4.9 Mb (megabases).[3] Accordingly, the number of protein-coding genes ranges from 1,267 to about 4,758 genes (in Fructilactobacillus sanfranciscensis and Lentilactobacillus parakefiri, respectively).[19][20] Even within a single species there can be substantial variation. For instance, strains of L. crispatus have genome sizes ranging from 1.83 to 2.7 Mb, or 1,839 to 2,688 open reading frames.[21] Lactobacillus contains a wealth of compound microsatellites in the coding region of the genome, which are imperfect and have variant motifs.[22] Many lactobacilli also contain multiple plasmids. A recent study has revealed that plasmids encode the genes which are required for adaptation of lactobacilli to the given environment.[23]

2.2.3. Species

The genus Lactobacillus comprises the following species:[24][25]

- Lactobacillus acetotolerans Entani et al. 1986
- Lactobacillus acidophilus (Moro 1900) Hansen and Mocquot 1970 (Approved Lists 1980)
- "Lactobacillus alvi" Kim et al. 2011
- Lactobacillus amylolyticus Bohak et al. 1999
- Lactobacillus amylovorus Nakamura 1981
- Lactobacillus apis Killer et al. 2014
- "Lactobacillus backi" Bohak et al. 2006

2.2.4. Taxonomy

The genus Lactobacillus currently contains 44 species which are adapted to vertebrate hosts or to insects.[3] In recent years, other members of the genus Lactobacillus (formerly known as the Leuconostoc branch of Lactobacillus) have been reclassified into the genera Atopobium, Carnobacterium, Weissella, Oenococcus, and Leuconostoc. The Pediococcus species P.dextrinicus has been reclassified as a Lapidilactobacillus dextrinicus

[3][26] and most lactobacilli were assigned to Paralactobacillus or one of the 23 novel genera of the Lactobacillaceae.[3] Two websites inform on the assignment of species to the novel genera or species

2.3. Burn Wound Infections

The burn wound represents a susceptible site for opportunistic colonization by organisms of endogenous and exogenous origin. Patient factors such as age, extent of injury, and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, and motility determine the likelihood of invasive burn wound infection. Burn wound infections can be classified on the basis of the causative organism, the depth of invasion, and the tissue response. Diagnostic procedures and therapy must be based on an understanding of the pathophysiology of the burn wound and the pathogenesis of the various forms of burn wound infection. The time- related changes in the predominant flora of the burn wound from gram- positive to gram-negative recapitulate the history of burn wound infection. Proper clinical and culture surveillance of the burn wound permits early diagnosis of gram-positive cellulitis, and the stable susceptibility of β- hemolytic streptococci to penicillin has eliminated the threat of this once common burn wound pathogen. Selection and dissemination of intrinsic and acquired resistance mechanisms increase the probability of burn wound colonization by resistant species such as

3- Materials &Methods

3.1. Material

3.1.1 Equipment And Instruments:

Equipment and instruments used in this study and their sources are given inTable (3. 1) Equipment and instruments Company

Equipment and instruments	Company	Origin
1-Autoclave	Hirayama	**Japan**
2-Eppendorf centrifuge	Heraeus	**Germany**
3-Incubator	Dragon Lap	**USA**
4-Micropipette	Dragon Lap	**China**
5-Oven	Memmert	**Germany**
6-pH meter	Hanna	**Italy**
7-Refrigerator	Concord	**Koria**
8-Sensitive balance	Precia	**Swiss**
9-Hood (Safety cabinet)	Sterile card lll	**USA**
10-Hot plate with Magnetic stirrer	Gallenkamp	**UK**
11-Loop	Himedia	**India**
12-Flasks of Different sizes	Assistant(Assipette)	**Germany**
13-Glass Tubes	Himedia	**India**
14-Graduated Cylinder	Assistant(Assipette)	**Germany**
15-Plane Tubes	Himedia	**India**
16-Petri Dishes	Himedia	**India**
17-Anaerobic Jar	**Oxoid**	**Germany**

3.1.2. Chemicals

Chemicals and biological materials used in this study and their sources aregiven in Table (2.2).

Chemicals	Company	Origin
1-HCl	Hi media	**India**
2-NaoH	Hi media	**India**
3-Catalase solution	Oxoid	**Germany**
4-Agar- Agar	Himedia	**India**
5-Formalin	**Certified Company**	**Korea**

3.1.3. Culture Media

Ready to use media

Culture Media and their sources used in this study are given in Table (2-4). All these media were prepared according to company instructions. They were sterilized by autoclaving for 15 min at 121°C and1.5 PSI

Table (2-4): Culture media used in this study.

Media	Company	Origin
1-De Man, Rogosa and Sharpe agar , broth (MRS)	HiMedia	**India**
2-Nutrient agar , broth (NB)	HiMedia	**India**
3-Mullar-Hinton agar , broth (MHA)	**HiMedia**	**India**

They were prepared by suspend suitable quantities (grams) in 1L distilled water (D.W). Heat to boiling to dissolve the medium completely. They weresterilized by autoclaving for 15 min at 121°C and1.5 PSI . Mix well and pourinto sterile petri plates .

3.1.3.Laboratory Prepared Media

The following media were prepared and

used throughout this study which were already sterilized by autoclaving for 15 min at 121°C and1.5 PSI

A- Mrs Broth

Prepared MRS broth by dissolving all components in table (2-5) in 1L of distilled water(D.W). It was sterilized by autoclaving for 15 min at 121°C and1.5 PSI .this medium was used in determination of best production medium (Atlas , 1995).

Components	gm\L
1-Glucose	**20.0**
2-Yeast Extract	**5.0**
3-Meat Extract	**10**
4-Peptone	**10**
5-NH4Cr	**2.0**
6-CH3COONa	**5.0**
7-K2PO4	**2.0**
8-MgSO4	**0.1**
9-MnSO4	**0.05**
Final pH (at 25°C) = 6.5 ± 0.2	

3.1.4-REAGENTS AND SOLUTIONS

3.1.4.1-CHEMICALS SOLUTIONS

A. NORMAL SALINE

This solution was prepared by dissolving 0.85gm of NaCl in 90ml distilled water, adjusted the pH to 7 and then completed the volume to 100ml with distilled water, then sterilized by autoclaving at 121ºC for 15 minutes (Atlas*et al.*, 1995).

B. PHOSPHATE BUFFER SALINE (PBS)

It was prepared according to (Atlas et al., 1995) method from the following components:

Salts	Weight
1-NaCl	**8 gm**
2-KH_2Po_4 (Anhydrous)	**0.2 gm**
3-K_2HPO_4(Anhydrous)	**12 gm**
4-Distilled water	**1000 ml**

The components were dissolved in 950ml of distilled water and the pH was adjusted to 7.2, then the volume was completed to 1 liter of D.W. and autoclaved at 121 °C for 15 minutes, then stored at 4 °C until use.

3.1. Isolation And Identification Of Staphylococcus Aureus Involved In Burn Infections

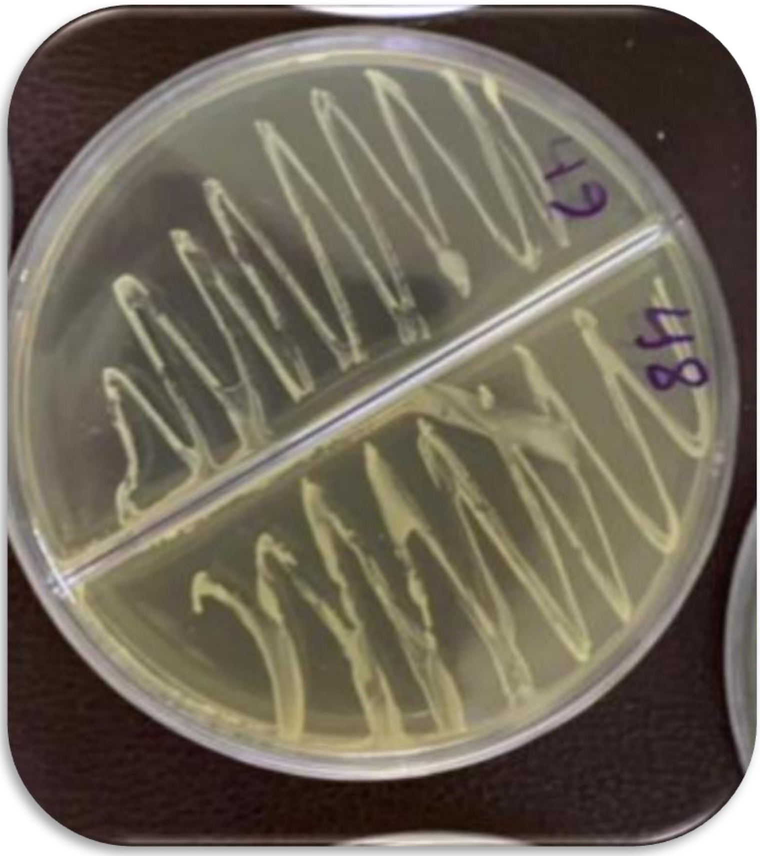
Staphylococcus aureus were isolated and identified using blood agar and Mannitol salt agar (MSA) were used. Mannitol salt agar (MSA) is a differential and selective medium due to it allow the growth of some Gram- positive bacteria such as *Staphylococcus* that tolerate high salt concentrations (about 7.5–10%) of NaCl and inhibits growth of Gram-positive bacteria. In addition, MSA was considered as a differential medium of mannitol- fermenting staphylococci (Bachoon *et al.*, 2008). On MSA, *S. aureus* cells appeared as yellow colonies with yellow zones as result of fermenting mannitol into acid that causes the phenol red (component in MSA) to turn to yellow. On blood agar, *S. aureus* cells appeared as colonies surrounded by clear zones as a result to complete hemolysis (beta - hemolysis). Under microscopic field, Gram positive *S. aureus* was appeared as a round-shaped cells of single, paired or aggregated as a grape cluster. *S. aureus* showed as positive result for catalase.

TABARK HASSN MOHAMMED

3.2. Isolation And Screening Of Lactobacillus Isolates.

Isolates of *Lactobacillus* sp were collected from different diary product samples including raw milk, yogurt and drinking yogurt. All samples were subjected to an isolation method to obtain *Lactobacillus* bacteria. Samples from those products were collected carefully by unsealing the different products and pipetting them into sterilized polypropylene tubes in a clean area in order to limit the possible contamination with other possible pollutant. The samples were cooled-preserved at -4˚C for further investigation. Hyper thick (semi-solid) yogurt products in texture were retextured by vertical mechanical-manual shaking in order to liquidise them before unsealing. Also, the expiration dates for the industrial products and the freshness for thehomemade ones were considered. Based on results, the homemadeyoghurt and raw cow milk were the best source for *Lactobacillus*.All isolates were then subjected to identification process in order to confirm their genus.

Morphological identification of *Lactobacillus* isolates was mainly achieved by investigating the appearance of colonies on the solid medium as well as the microscopic examination. All colonies of isolates on MRS agar were white, rounded in shape and range in consistency from creamy white in colour to glossy white and moist-mucoid colony appearance on the surface. Figure (3-1) shows the growth of *Lactobacillus* isolates as a separated single colony on MRS agar after anaerobic incubation of 48 h at 37 ˚C.



الشكل (3-1): ***Lactobacillus* on MRS agar after 24 h of incubation**

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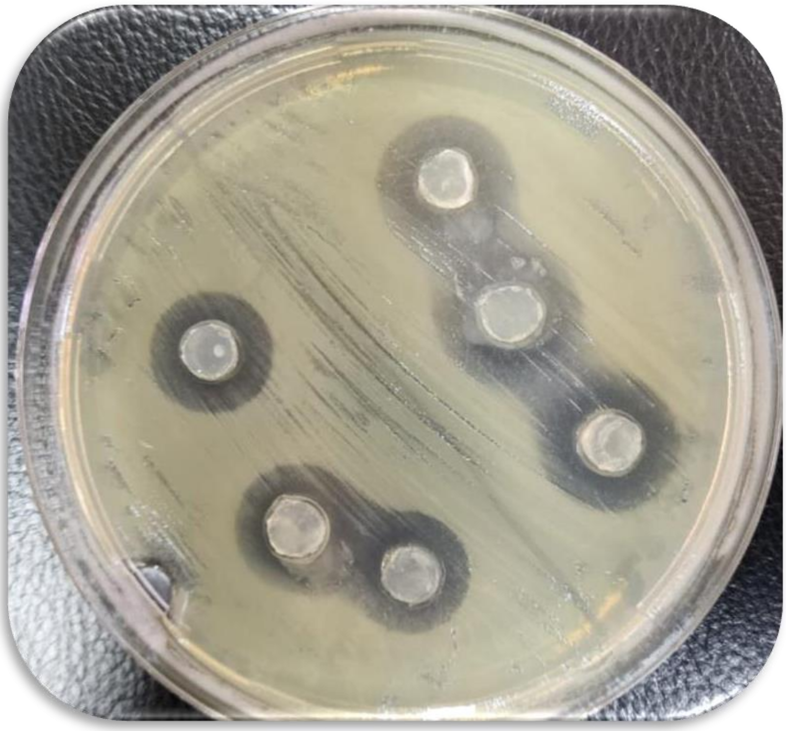
3.3. Assessment Of The Probiotic Potential Of *Lactobacillus*

Agar plug diffusion method used to highlight the antagonism between the isolates. It involves making an agar plug from the culture of the isolate to be tested for antimicrobial molecules production and then deposit on the agar surface of another plate previously inoculated with the indicator bacterium (Valgaset,al. 2007; Balouiri, et, al. 2016). During their growth, microbial cells secrete antimicrobial molecules which diffuse in the agar medium from the plug. Then, the antimicrobial activity of the microbial secreted moleculesis detected by the appearance of an inhibition zone around the agar plug. In this context, (Karadağlıoğlu et al., 2019) reported that agar plug diffusion method is often used to highlight the antagonism between microorganism. As can be noticed from the results presented in Figure (3-2) isolates were able to produce bacteriocin with different size of inhibition zones against *Staphylococcus aureus*.



screening of *Lactobacillus* isolate against *Staphylococcus aureus* by الشكل (3-2)using Agar plug diffusion method.

Conclusions

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. 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 wide spread of multi drug resistance.

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