PROBIOTIC EFFECT OF LACTOBACILLI ON MICE INCISIONAL WOUND INFECTIONS

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Abstract

Three lactobacilli species, namely; *L. bulgaricus*, *L. plantarum*, and *L. acidophilus* were isolated from yoghurt, vinegar, and vagina, respectively, showed inhibition activity on *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from several wound infections specimens. However, *S. aureus* was more affected than *P. aeruginosa* by the all lactobacilli isolates, on the other hand, *L. plantarum* showed the highest inhibition activity on both pathogenic isolates. *Lactobacillus plantarum* cells or supernatant succeeded in preventing *S. aureus* and *P. aeruginosa* from establishing wound infection. Since the inflammation and histopathological signs which developed by either *S. aureus* or *P. aeruginosa* were disappeared when the wounds were treated by lactobacilli cells or supernatant.

Introduction

Lactobacilli are non-pathogenic, gram-positive lactic acid bacteria found in the normal intestinal microflora of animals and humans (1) and are classified as probiotic agents. *Lactobacillus* derived products, including culture supernatants have been used for their wound healing and antiviral properties as they are believed to boost energy and to be effective remedies for allergies, common cold, lactose intolerance, and have also been shown to reduce cholesterol levels and the risk of colon cancer (2-4). Supernatants of *Lactobacillus acidophilus* were also proved to be effective against *Helicobacter pylori in vitro* and *in vivo* in people and were shown to possess antimicrobial activities against *Bacillus anthracis* and *E. coli* (3). *Lactobacillus* supernatants (LS) promote inflammatory response during tissue repair in rodents (5), stimulate proliferation of embryonic cells (6), and that subcutaneous injections of *Lactobacillus* supernatants into the ears of rats lead to angiogenesis (5). Using a cytokine antibody array, leptin and several other cytokines (e.g., IL-6, IL-8 and TGFβ) were detected in medium conditioned by bovine endothelial cells exposed to LS

Fermented dairy products containing *Lactobacillus* have traditionally been used to modulate the microbial ecology (7) to prevent infection of pathogenic bacteria (8) to stimulate the immune system (9) and to normalize gastrointestinal disorders (10). Also a significant probiotic strain with proven health benefits and therapeutic applications in the treatment of diarrhea (11) irritable bowel syndrome (12), atopic eczema (13) and the prevention of urinary tract infections (14).

Staphylococcus aureus is a major opportunistic pathogen that can cause a variety of local and systemic infections ranging from skin abscesses, bone and soft tissue surgical infections, sepsis, invasive endocarditis, and toxic shock syndrome (TSS) (15). *Pseudomonas aeruginosa* produces infection of wounds and burns, giving rise to blue-green pus; meningitis, and urinary tract infection, necrotizing pneumonia, otitis externa in swimmers. It may cause invasive (malignant) otitis externa in diabetic patients. Infection of the eye, occurs most commonly after injury or surgical procedures. In infants or debilitated persons, *P aeruginosa* may invade the bloodstream and result in fatal sepsis; in patients with severe burns (15).

The aim of the present study was to determine, in a mouse animal model, the capability of *Lactobacillus* to inhibit the wound infection caused by pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Materials and methods

Isolation and identification

Pseudomonas aeruginosa and *S.aureus* were isolated from several wound infections specimens which obtained by sterile cotton swabs, streaked on blood agar, MacConkey agar plates and mannitol salt agar (all media were purchased from Himedia, India), incubated at 37° C for 24 h., thereafter, the grown colonies were identified according to (16).

To isolate lactobacilli, three different samples: vinegar, yogurt, and vagina, were streaked onto De Mann-Rogosa-Sharpe (MRS) agar (Himedia, India) agar plates (pH 5.5) and incubated at 37°C for 48 h under anaerobic conditions. The Lactobacilli were initially identified by their ability to grow on the selective MRSA, gram-positive staining, rod shape, and catalase-negative phenotype. Biochemical analyses, including sugar fermentation profile and gas production in MRS broth, were conducted as described in (16).

Quantification of bacterial suspensions was adjusted to approximately 1.5×10^8 CFU/ml by comparison to McFarland turbidity standards confirmed by enumeration using the spread plate technique.

In vitro study

Antibacterial activity of *Lactobacillus* cells coculture

Lactobacilli were cultured anaerobically on MRS agar for 48 hrs. at 37°C. Thereafter, 5 mm agar discs (triplicates) were cut out by a sterile Pasteur pipette and placed on a Muller Hinton agar (Himedia, India) plates seeded with *S. aureus* or *P. aeruginosa.* Then the plates were cultivated for 24 h. at 37° C. Inhibition zones were measured in millimeters by an ordinary ruler (17).

Preparation of Lactobacillus supernatants

Briefly, cultures of lactobacilli were grown in MRS broth (Himedia, India) at 37°C for 24 hr under anaerobic conditions. Overnight bacterial cultures contained 1.5×10^8 CFU/ml, and these cultures were centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatants were filtered through a 0.2 μm membrane filter to remove the remaining bacteria and debris (18). Plating on MRS agar plates showed no evidence of lactobacilli growth.

Antibacterial activity of *Lactobacillus* supernatants

The bacterial suspension of *S. aureus* or *P. aeruginosa* was transferred evenly on three Mueller Hinton agar plates. Four wells were made in each agar plate with a sterile Pasteur pipette; 50 μl of culture supernatant from the three different lactic acid bacteria were added to three wells. While, in the fourth well MRS broth was added to determine possible inhibitory activity of the medium (as control). After aerobic incubation for 24 h 37 ^{o}C Inhibition zones were measured in millimeters by an ordinary ruler (19).

In vivo study

Animals

Six-week-old female white mice (*Mus musculus)* from the inbreed colony of department of biology, college of Science, university of Baghdad, each weighing from 25 to 30 g, were used throughout the investigation. Animals were housed in plastic cages and fed *ad libitum* with a conventional diet. All the animals were randomly assigned to the following experimental groups (as triplicates): (1) sterile saline solution treated group, (2) *Lactobacillus plantarum* treated group, (3) *L. plantarum* supernatant treated group, (4) *S. aureus* treated group (5) *P. aeruginosa* treated group. (6) *L. plantarum* and *S. aureus* treated group, (7) *L. plantarum* supernatant and *S. aureus* treated group (8) *L. plantarum* with *P. aeruginosa* treated group, (9) *L. plantarum* supernatant with *P. aeruginosa* treated group.

Microorganisms inoculation procedure

Overnight cultures of lactobacilli grown in MRS broth, *P. aeruginosa* or *S. aureus* grown on brain hearth infusion broth (24 hours, 37°C) were centrifuged at 10 000 g for 10 minutes at 4°C, washed twice with sterile saline solution.

Transcutaneous 6 mm in diameter wounds were performed on the backs of the mice as it described by (5)**,** bacterial suspension or supernatant were applied to the wound using cotton swab. Mice were sacrificed after two days; the injured skin specimens were

aseptically removed, fixed with 10% formalin for 24 hours at room temperature, and then embedded in paraffin according to standard histological methods (20).

Statistical analysis

The results were analysed statistically in the Statistical program with the rest of the Least Significant Difference (LSD).

Results and discussion

Three lactobacilli species *L. bulgaricus, L. plantarum,* and *L. acidophilus* were isolated from yoghurt, vinegar, and vagina, respectively.

Staphylococcus aureus and *P. aeruginosa* were inhibited, variably, by the supernatant as well as the coculture of the three isolates of *Lactobacillus.* However, *L. plantarum* showed the highest inhibition activity among the three lactobacilli Table (1). Depending on these results *L. plantarum* was elected for the in vivo study. However, these results strongly indicated that the source of lactobacilli is of great importance in the selection of probiotic microorganism.

Table (1)
Antibacterial activity of lactobacilli isolates.

Lactobacillus species	Zone of inhibition (mm)±SD			
	P. aeruginosa		*S. aureus*	
	supernatant	coculture	supernatant	coculture
L. bulgaricus	13.5 ± 1.5 a	13.3 ± 2 a	17 ± 1.7 b	17.3 ± 1.1b
L. plantarum	20.3 ± 0.5 c	19.3 ± 0.5c	25.6 ± 2.3 c	24.6 ± 1.1c
L. acidophilus	8.6 ± 0.5 d	9 ± 1.7 d	12.6 ± 2 a	14.3 ± 1.5a

Each value is the mean of three replicate (mean ± SD)
Values with different letter have significant differences (P>0.05)

Obviously, Table (1) demonstrates that *S. aureus* was more affected than *P. aeruginosa* by the all lactobacilli isolates, a result agreed with Gilliland and Speck (1977) (21) who reported that Lactobacilli showed stronger antibacterial properties against gram-positive bacteria (*S. aureus* and *Clostridium perfringens*) than gram-negative bacteria (*Escherichia* coli and *Salmonella typhimurium*). While others (22) reported that strains of *Lactobacillus* had an 85% inhibitory effect on *P. aeruginosa* and 48 % *S. aureus*. Moreover, wild strains of *Lactobacillus* species were isolated from Kunun zaki (fermented millet drink) and Fresh Cow milk. The results showed that the isolates were found to be the most effective against *S. aureus*. Also observed to be next in effectiveness is against *Escherichia coli*. However, the least level of inhibition was recorded against *Klebsiella pneumoniae*. The inhibition recorded in the case of the isolates that have antagonistic effect may be due to the production of organic acids, bacteriocins and hydrogen peroxide (23).

Characterisation of the lactobacilli metabolic product for antimicrobial agents reveals that lactic acid may be responsible for the inhibition of the pathogenic bacteria (24).

In regard to the control group Fig. (1), *S. aureus* and *P. aeruginosa* were able to cause an infection in the experimentally wounds, represented by infiltration of inflammatory cells Fig.(2) and the dilatation along with increase in number of the blood capillaries, as it can be seen from Fig.(3). Such activity was observed in transcutaneous wounds in mice treated with *Lactobacillus* supernatant resulted in prolonged inflammatory phase of wound healing and delayed wound closure, including reepithelialization (5).

Lactobacillus plantarum cells or supernatant succeeded in preventing *S. aureus* and *P. aeruginosa* from establishing wound infection. Since the inflammation and histopathological signs which developed by either *S. aureus* or *P. aeruginosa* were disappeared when the wounds were treated by lactobacilli cells

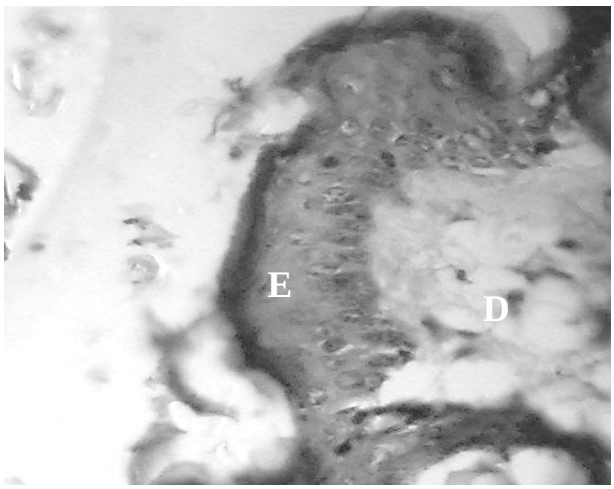

Fig.(1) : Normal mouse skin structure. E= epidermis, D= Dermis. X400, H&E.

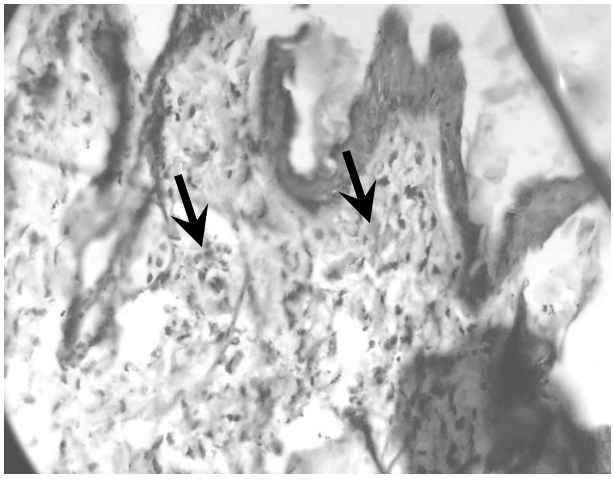

Fig.(2) : Mouse skin (wound) injected with 1.5×10^8 CFU/ml of S. aureus shows the infiltration of inflammatory cells (arrows). X400, H&E.

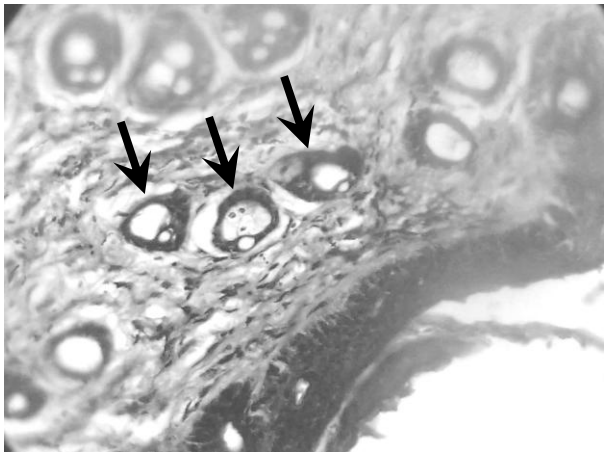

Fig.(3): Mouse skin (wound) injected with 1.5×10^8 CFU/ml of S. aureus shows numerous blood vessels (arrows) with open lumina. X400, H&E.

or supernatant. This result is confirmed with an another study revealed that *L. plantarum, L. delbruekii, L. acidophilus,*and *L. brevis* were shown to produce a bacteriocin-like substance. Their sensitivity varied greatly. *L. plantarum* produced a more heat stable bacteriocin than the other isolated strains, which exhibited a broad spectrum of inhibitory activity. The antibacterial activity of plantaricin was more potent than the other isolated strains (25).

Results of Valdez and others (26) indicated that *L. plantarum* and/or its by-products are potential therapeutic agents for the local treatment of *P. aeruginosa* burn infections due to the inhibition of *P. aeruginosa* colonization and there was an improvement in tissue repair, enhanced phagocytosis of *P. aeruginosa* by tissue phagocytes, and a decrease in apoptosis at 10 days.

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الخلاصة

اظهرت ثلاثة انواع من بكتريا *Lactobacillus* وهي *L. bulgaricus* و *L. plantarum*, و *L. plantarum* عزلت من اللبن و الخل و المهبل على التوالي فعالية تثبيطية على *Pseudomonas aeruginosa* و *Staphylococcus aureus* عزلتا من اخماج الجروح و تبين ان العزلة *S. aureus* كانت الاكثر تاثرا من العزلة *P. aeruginosa* بوساطة عزلات بكتريا *Lactobacillus* في حين اظهرت العزلة *L. plantarum* اعلى فعالية تثبيطية في كلا العزلتين المرضيتين.

نجحت خلايا او طافي بكتريا *L. plantarum* في منع كل من *S. aureus* و *P. aeruginosa* من احداث خمج جروح في الفئران فقد اختفى الالتهاب و العلامات المرضية النسيجية التي تكونت بوساطة العزلة *P. aeruginosa* او العزلة *S. aureus* عندما عومل الجرح بخلايا او طافي بكتريا *Lactobacillus*.