Studying the ability of lactobacillus bacteria to inhibit the growth of uropathogens and their adhesion to uroepithelial cells

Kawther Hkeem Ibrahem, Sosan Hasan

Abstract

Twenty vaginal swab samples were obtained from healthy married women (with and without children), all sample were subjected to conventional morphological and cultural characteristics. 12 isolates distributed among (Lactobacillus acidophilus (3), Lactobacillus casei (4), Lactobacillus plantarum (2) and Lactobacillus vaginalis (3) isolates. While in figure (2), married women without children the same bacteria but different distributed (Lactobacillus acidophilus (5), Lactobacillus casei (1), Lactobacillus plantarum (2)). After subjecting lactobacillus isolates to the sensitivity test. some isolates all L. acidophilus and L. plantarum were resistance Amoxicillin, most strains of L. acidophilus and L. plantarum were sensitive to ciprofloxacin. L. plantarum was moderate sensitivity to gentamicin, trimethoprim, and vancomycin.

antagonism activity of Lactobacillus species against uropathogens E. coli, Pseudomonas aeruginosa, Proteus mirabilis and Klebsiella. Pneumoniae. result showed highly antagonistic activity by L. acidophilus against all uropathogens isolates, while L. casei showed the lowest antagonistic activity. there was aslight degree of difference in the adhesion properties observed among vaginal lactobacillus. adherence inhibition was tested L. acidophilus, L. plantarum showed higher activity than L. casei in blocking adherence against all uropathogens isolates.

INTRODUCTION:

Urinary tract infection (UTI) is one of the most common bacterial infections encountered in clinical practice in Europe and North America. It is estimated that 150 million cases of Urinary tract infections (UTIs) are common, painful and disruptive. (1) the recurrent nature of urogenital infection, emergence of multidrug resistant bacteria and patient dissatisfaction with side effects of drugs need better ways to diagnose, treat, and prevent infection. Alternative strategies like probiotics would be a beneficial treatment option. (2)
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The vaginal flora was first reported by Albert Döderlein, as early as 1892. Döderlein found that the microflora was homogenously colonized with Gram-positive rods, which were designated the name “Döderlein’s bacilli”. Over the years, these bacilli have been identified as *Lactobacillus* spp. *Lactobacilli*, the predominant micro-organisms of the vaginal microbiota, play a major role in the maintenance of a healthy urogenital tract by preventing the colonization of pathogenic bacteria. In healthy women, the vaginal microflora is dominated by *Lactobacillus* species, at a level of $10^7$-$10^8$ CFU g$^{-1}$ of fluid, which exert a significant influence on the microflora of the ecosystem(3). It has been observed that indigenous *lactobacilli* prevent the overgrowth and invasion of pathogenic bacteria by a combination of competitive exclusion, competition for nutrients, and release of antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants(4). In consequence, a depletion of vaginal lactobacilli has been directly associated with an increase in the incidence of genital and urinary infections (5)the *Lactobacilli* have been shown to produce biosurfactants and collagen binding proteins that inhibit pathogen adhesion and displace the pathogens.(6)

The aim of this study was to block the adherence of uropathogens (*E.coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis*) to uroepithelial cells and to inhibit the growth of such uropathogens by using *lactobacillus species* from women.

**MATERIALS AND METHODS:**

Samples collection: A total of (25) samples of vaginal swabs were collected from healthy women in baghdad hospitals, then cultured in MRS media at 37°C under anaerobic condition for 24-48 h.

Bacteria. The lactic acid bacteria were grown under anaerobic conditions in De Man-Rogosa-Sharp (MRS) broth, for 24 h at 37°C. Spent culture supernatant was obtained by centrifugation at 10,000 g for 30 min at 4°C. Filtered lactic acid bacteria was passed through a sterile 0.22-Millipore. Concentrated filterates were obtained by freeze-drying.

Uroepithelial cells: uroepithelial cells(UECs) were obtained from freshly voided midstream urine samples from healthy women. uroepithelial cells were harvested by centrifugation, washed four times with phosphate buffer saline(PBS) at pH=6, and suspended in it to a concentration $10^5$ cel/ml(7)

Adhesion of *Lactobacillus* species to uro epithelial cells. *Lactobacillus species* were grown in MRS broth for 18 hours at 37°C to give approximately $10^6$ cells/ml. 1 ml of bacterial broth (containing approximately $10^8$ bacteria) was added to equal volume of UECs. The mixture was incubated at 37°C for 60 min. The mixture was washed with PBS and re-suspended by repeated centrifugation for 10 min to eliminate unattached bacteria. A drop of
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final UECs suspension was deposited on glass slide, air dried, heat fixed and Gram stained. Examination of slide was done by light microscope under oil immersion to demonstrate the attachment of bacteria to UECs. (8)

Bacteria isolates: (4) Bacterial isolates of uropathogens (E. coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella Pneumoniae) were obtained from college of science, Al- Mustansiriya University.

Adherence inhibition assay: To study the capacity of lactobacilli and uropathogens to adhere to UECs, Lactobacillus was sequentially subcultured in (MRS) broth at 37°C in 5% CO2, for 48 h.

The uropathogens were inoculated in brain-heart infusion broth and were incubated at 37°C, twice for 24 h. 0.5 ml of bacterial broth (containing approximately 10⁸ bacteria) was added to equal volume of UECs and lactobacillus bacteria. The mixture was incubated at 37°C for 60 min. The mixture was washed with PBS and re-suspended by repeated centrifugation for 10 min to eliminate unattached bacteria. A drop of final UECs suspension was deposited on glass slide, air dried, heat fixed and Gram stained. Examination of slide was done by light microscope under oil immersion to demonstrate the attachment of bacteria to UECs. The number of microorganisms attached to 50 consecutive VECs was counted. (8).

Antibiotic Susceptibility test:
Antimicrobial agents susceptibility test by disk diffusion was performed by the procedure recommended by the National Committee for Clinical Laboratory Standards (9). Interpretative chart for disk diffusion tests were these described in the current National Committee for Clinical Laboratory Standards documents (10). The following disks were used: Amoxicillin, ciprofloxacin, gentamicin, trimethoprim and vancomycin (A representative disks from oxoid/England).

Inhibition of growth. The capacity of Lactobacillus to inhibit the growth of uropathogens was studied in solid media. Uropathogens were inoculated in brain-heart infusion broth and were incubated for 24 h at 37°C under aerobic atmosphere. Lactobacillus strains were grown in MRS broth at 37°C with 5% CO2 for 48 h. Assays in solid media were carried out with the 17 Lactobacillus against the following 4 uropathogens: E. coli, Pseudomonas aeruginosa, Proteus mirabilis, K. Pneumoniae. from women suffering from recurrent UTI, thin layer of Muller Hinton agar was displaced in to sterile petridish. 0.1 ml (containing approximately 10⁸ bacteria) of an 18 hours culture of E.coli broth was streaked on the surface of Muller Hinton agar. A cork borer was use to make pore (5x5mm in diameter) on the surface of streaked agar. Plates were incubated at 37°C for 18-24 hours. The inhibitory activity of
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Lactobacillus species against E.coli, p aeruginosa, P. mirabilis, K. Pneumoniae isolates was determined by measuring the inhibition zones around each pore(11).

RESULTS AND DISCUSSION

Lactobacilli were selected from 20 human vaginal isolates by collected 25 swabs from vagina of healthy married women.these isolates were identified at the lab.by classical microbiological tests(cultural,morphological and biochemical tests) as shown in figure(1),12 isolates were selected from married women with children distributed among (Lactobacillus acidophilus(3),Lactobacillus casei(4),Lactobacillus plantarum(2) and Lactobacillus vaginalis(3) isolates. While in figure(2), married women without children the same bacteria but different distribute(Lactobacillus acidophilus(5), Lactobacillus casei(1),Lactobacillus plantarum(2). The urogenital microflora of a healthy woman comprises ~50 species of organisms, which differ in composition according to reproductive stages and exposure to several factors, including antibiotics and spermicides. Infections are very common with > 300 million cases of urinary tract infections(12).

Figure(3) represents the effect of five antimicrobial agents strains of L.acidophilus,L.plantarum and L.casei. All L.acidophilus and L.plantarum were resistance to Amoxicillin,L.casei to Amoxillin and Trimethoprim.where as most strains of L.acidophilus and L.plantarum were sensitive to ciprofloxacin.L.plantarum was moderate sensitivity to gentamicin, trimethoprim, and vancomycin.

Zarazaga et al.(1999) observed that antibiotic resistance among species could be attributed to many factors including enzymatic inactivation,decrease intracellular drug accumulation or presence of gene that confer antibiotic resistance(13).the antagonism properties of lactobacillus against urinary pathogens were investigated using well diffusion method figure(4).result showed highly antagonistic activity by L.acidophilus8,1 against pseudomonas aeruginosa,proteus mirbilis,and klebsiella pneumoniae with inhibition zone (2.1mm).while L.acidophilus3,4,5 gave the same inhibition zone (2mm) against E.coli.A number of metabolic products of lactobacilli are believed to contribute to their ability to aid in the maintenance of a healthy urogenital tract. Lactic acid and hydrogen peroxide are toxic to a number of bacterial species and have been demonstrated to inhibit potential pathogens(14).little inhibitory activity was observed among L.acidophilus2 to ward E.coli and klebsiella with inhibition zone(0.7,0.6)mm respectively In addition, a number of bacteriocins produced by lactobacilli have been described that are active against a wide range of bacteria(15).coconnier et al.(1997)The spent culture supernatant of the human Lactobacillus acidophilus strain produces an antibacterial activity against a
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wide range of gram-negative and gram-positive pathogens. It decreased the in vitro viability of Staphylococcus aureus, Listeria monocytogenes, Salmonella typhimurium, Shigella flexneri, Escherichia coli, Klebsiella pneumoniae, Bacillus cereus, Pseudomonas aeruginosa, and Enterobacter(16). McGroarty and Reid described that its bacteriocin derived from lactobacilli showed activity in vitro against uropathogenic E. coli and Enterococcus species. Bacteriocins are proteinaceous bacterial products that have bactericidal activity (17). They are produced by various lactic acid bacteria, including lactococci, lactobacilli, and pediococci. Barefoot and Klaenhammer found that 63% of the L. acidophilus strains they surveyed produced bacteriocin(18). L. plantarum 1 produced a greater inhibitory effect towards E. coli, Proteus, Pseudomonas L. plantarum 2 produced inhibition zone (2.1) mm against pseudomonas, (1) mm E. coli and Proteus. In the other hand L. plantarum 4 produced lower antagonism (0.9, 1) mm against proteus, klebsiella, pseudomonas. The lowest inhibitory activity produced by L. casei against all uropathogens isolates.

Adhesion of lactobacilli to the epithelium has been described as the first step in the formation of a barrier to prevent undesirable microbial colonization(19). The adhesion of lactobacillus to uroepithelial cells varied among species, it was high among L. plantarum and L. casei in married women with children (figure 7) while in women without children L. acidophilus showed high adhesion ability to uroepithelial cells, in the other hand L. vaginalis did not produce adhesion at all (figure 8). These adhesions may partially or completely absente on it. Lactobacilli can use many mechanisms to adhere to surfaces, such as electrostatic, hydrophobic, hydrophilic, capsular and fimbrial mechanism in urogenital tract(20). Chan et al. (1985) demonstrated the attachment of lactobacillus cells to uroepithelial cells appeared to be mediated by the bacterial glycocalyx or cell wall (21). Lactobacilli strains have the ability to interfere with the adherence and growth of uropathogenic bacteria. This interaction is believed to be important in the maintenance of a normal urogenital flora and in the prevention of infection in females(22). Adherence of bacteria to epithelial cells has been shown to be an important factor in the colonization of mucous membranes. However, little is known about the mechanisms by which lactobacilli from the vaginas of healthy young women adhere to vaginal epithelial cells, although the variety of surface structures in these bacteria implies that a spectrum of adherence mechanisms may exist. Furthermore, self-aggregation may substantially increase the colonization potential of lactobacilli in environments with short residence times. The mechanisms appear to be multi-fac torial, and include production of: (i) anti-microbial factors such as lactic acid, bacteriocins, hydrogen peroxide, (ii) biosurfactants or other components that affect pathogen colonization and biofilm formation, (iii) signalling compounds that influence pathogen virulence expression, and (iv)
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signalling compounds that modulate immunity.(23). Inhibition of uropathogens adhesion are detailed in figure(9 ). The blocking effect for the same uropathogen varied greatly among the lactobacilli. lactobacillus acidophilus, L.plantarum showed higher activity than lactobacilli casei in blocking adherence against all uropathogens isolates. L.acidophilus displayed greatest efficacy being able to block adherence of (76.5%, 76.1%) against proteus mirabilis, klebsiella pneumoniae and (72.2%, 69.9%) of E.coli, pseudomonas aeruginosa respectively. L.casei showed alittle activity (60.4%, 58.1%, 50.2%, 48.2%) of block klebsiella, proteus, E.coli, Pseudomonas, although L.plantarum showed better activity represented by 76.3% proteus mirabilis ,74.1% E.coli, 70.7% klebsiella pneumoniae, and 70.6% pseudomonas aeruginosa . Kaewsrichan et al.(2006) believed that the competitive exclusion mode of action is caused by a stronger affinity of Lactobacillus than pathogens to VEC receptors, others suggest that exclusion occurs due to mechanical hindrance by Lactobacillus fragments (24) Lactobacilli are believed to interfere with pathogens by different mechanisms. The first is competitive exclusion of genitourinary pathogens from receptors present on the surface of the genitourinary epithelium. Second, lactobacilli coaggregate with some uropathogenic bacteria a process that, when linked to the production of antimicrobial compounds, such as lactic acid, hydrogen peroxide, bacteriocin-like substances, and possibly biosurfactants, would result in inhibition of the growth of the pathogen(25). Howard et al.,(2000) showed that biosurfacants (compounds released by microbes with a distinct tendency to accumulate at interfaces) produced by certain lactobacilli, not only aid binding of the organisms to collagen on cells, but they inhibit adhesion to surfaces of abroad range of uropathogens(26).

Our results suggest that the adherence of lactobacilli to crop epithelium may be important for successful colonization as well as for exclusion of pathogenic bacteria by steric hindrance. Hence, the adhesion of lactobacilli to the epithelial surfaces may play a role in the prevention of pathogen colonization and invasion.
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figure(1): Distribution of Lactobacillus species (women with children)

figure(2): Distribution of Lactobacillus species (women without children)
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Figure (3): Sensitivity test of Lactobacillus species

Figure (4): Inhibitory effect of Lactobacillus acidophilus against uropathogens
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figure (5): inhibitory effect of *Lactobacillus casei* against uropathogens

figure (6): inhibitory effect of *Lactobacillus plantarum* against uropathogens
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Figure (7): Adhesion ability of Lactobacillus species to uroepithelial cells in married women with children.

Figure (8): Adhesion ability of Lactobacillus species to uroepithelial cells in married women without children.
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