

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

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Abstract

Twenty vaginal swab samples were obtained from healthy married women (with and without children), all samples 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 a slight 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 $37^{\circ}C$ under anaerobic condition for 24-48 h.

Bacteria. The lactic acid bacteria were grown under anaerobic conditions in De Man-Rogosa-Sharpe (MRS) broth, for 24 h at $37^{\circ}C$. Spent culture supernatant was obtained by centrifugation at $10,000 g$ for 30 min at $4^{\circ}C$. 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 $37^{\circ}C$ to give approximately 10^6 cells/ml. 1 ml of bacterial broth (containing approximately 10^6 bacteria) was added to equal volume of UECs. The mixture was incubated at $37^{\circ}C$ 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)

Bateria 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 37C° in 5% CO₂, for 48 h.

The uropathogens were inoculated in brain-heart infusion broth and were incubated at 37C°, 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 37C° 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 (Arepresentative 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 37C° under aerobic atmosphere. *Lactobacillus* strains were grown in MRS broth at 37C° 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 37C° 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 Trimethoprimewhere 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.acidophilus*8,1 against *pseudomonas aeruginosa*,*proteus miriblis*,and *klebsiella pneumoniae* with inhibition zone (2.1mm).while *L.acidophilus*3,4,5 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 ahealthy 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.acidophilus*2 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 is 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 to wards *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(figure7) 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(figure8).these adhesions may partially or completely absent on it.*lactobacilli* can use many mechanism 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-factorial, 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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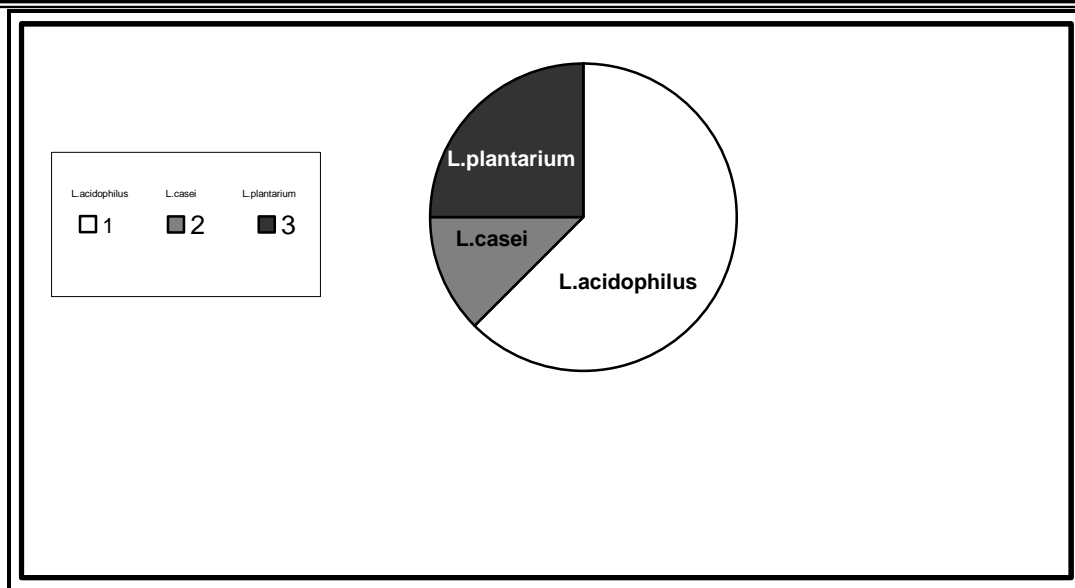
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signalling compounds that modulate immunity.(23). Inhibition of uropathogens adherence are detailed in figure(9). The blocking effect for the same uropathogen varied greatly among the *lactobacilli*.*lactobacillus acidophilus*,*L.plantarum* showed 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 a little 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 biosurfactants (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 a broad range of uropathogens(26).

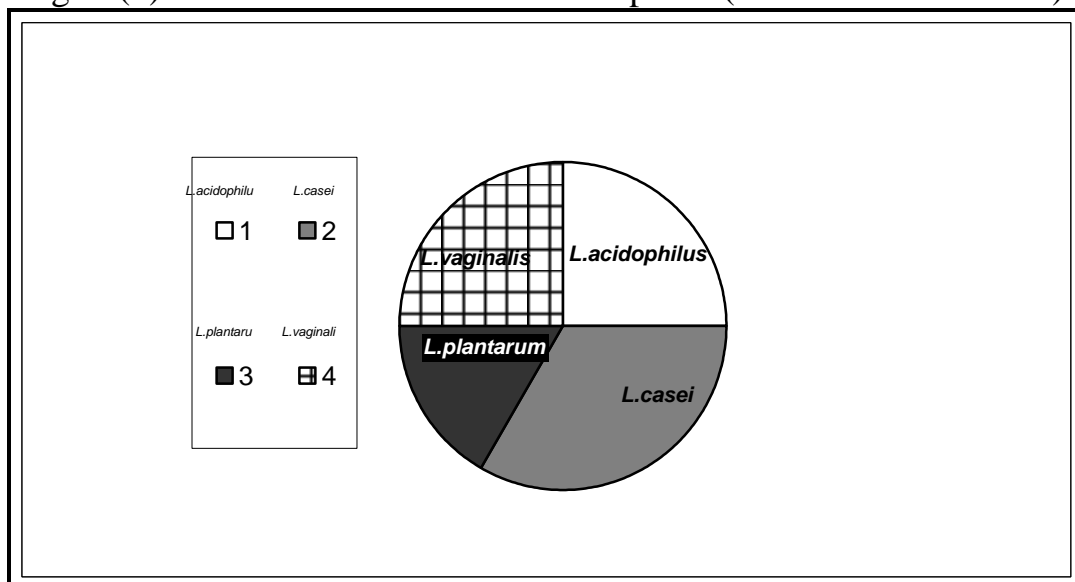
Our results suggest that the adherence of *lactobacilli* to crop epithelium may be important for successful colonization as well as for exclusion of pathogenic bacteria by steric hindrance. Hence, the adhesion of *lactobacilli* to the epithelial surfaces may play a role in the prevention of pathogen colonization and invasion.

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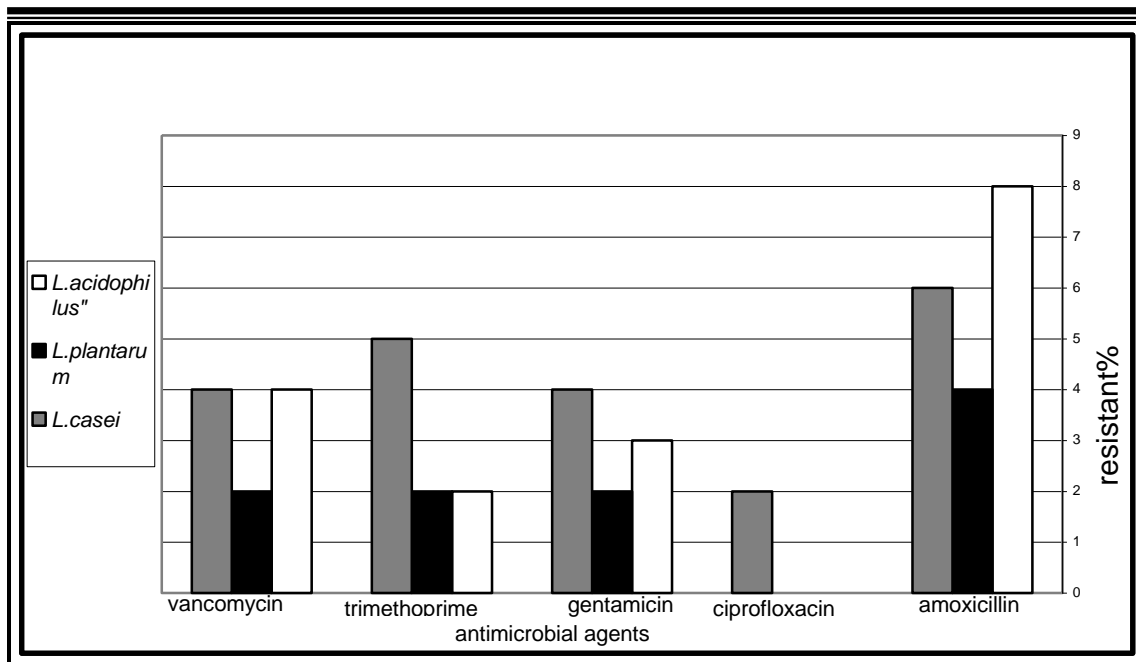
figure(1):Distribution of *Lactobacillus* species(women with children)



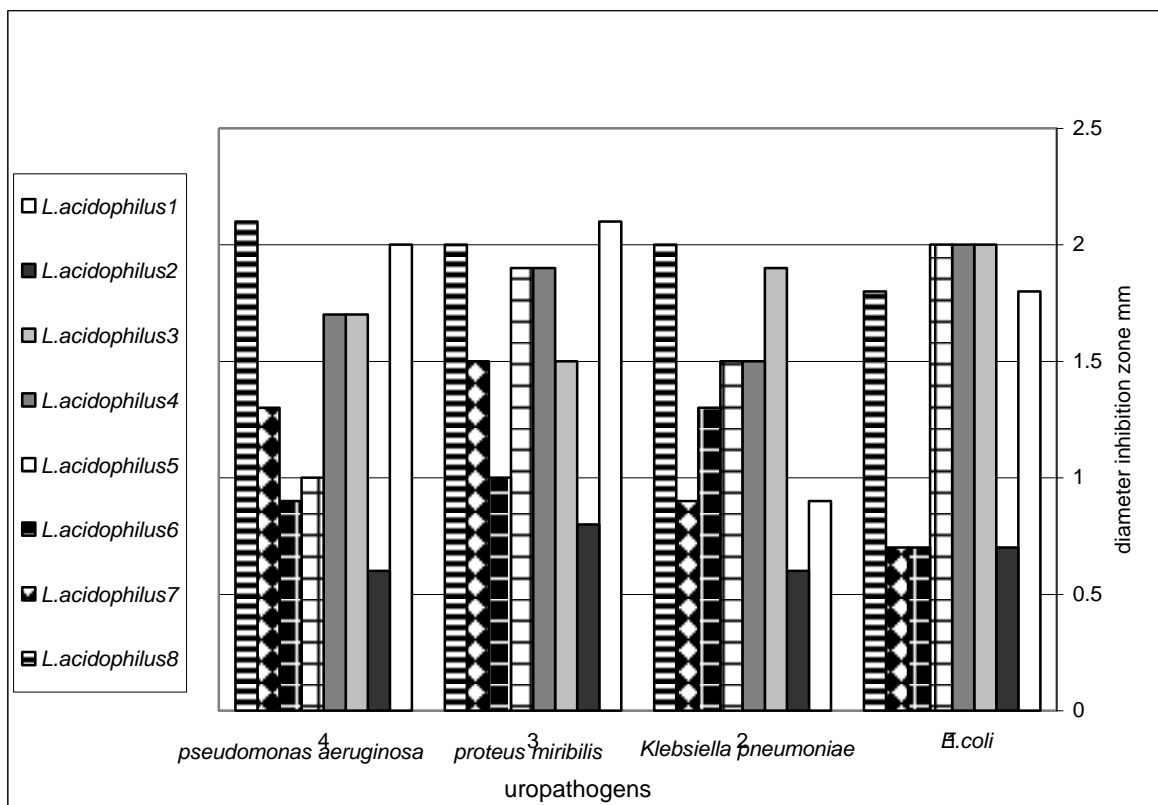
figure(2)Distribution of *Lactobacillus* species(women without children)

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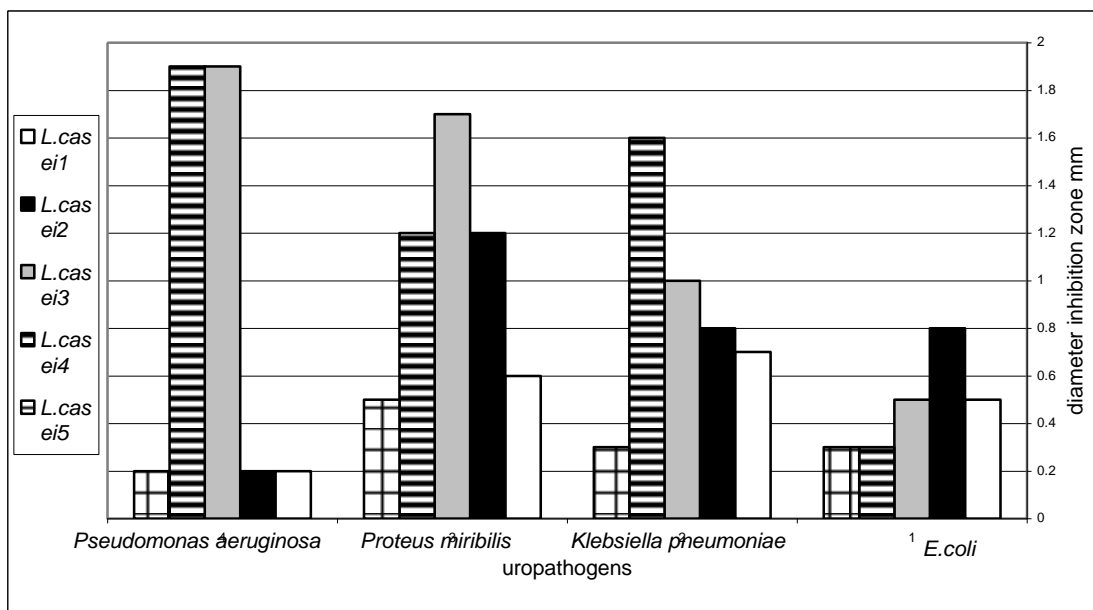
figure(3):sensitivity test of *Lactobacillus* species



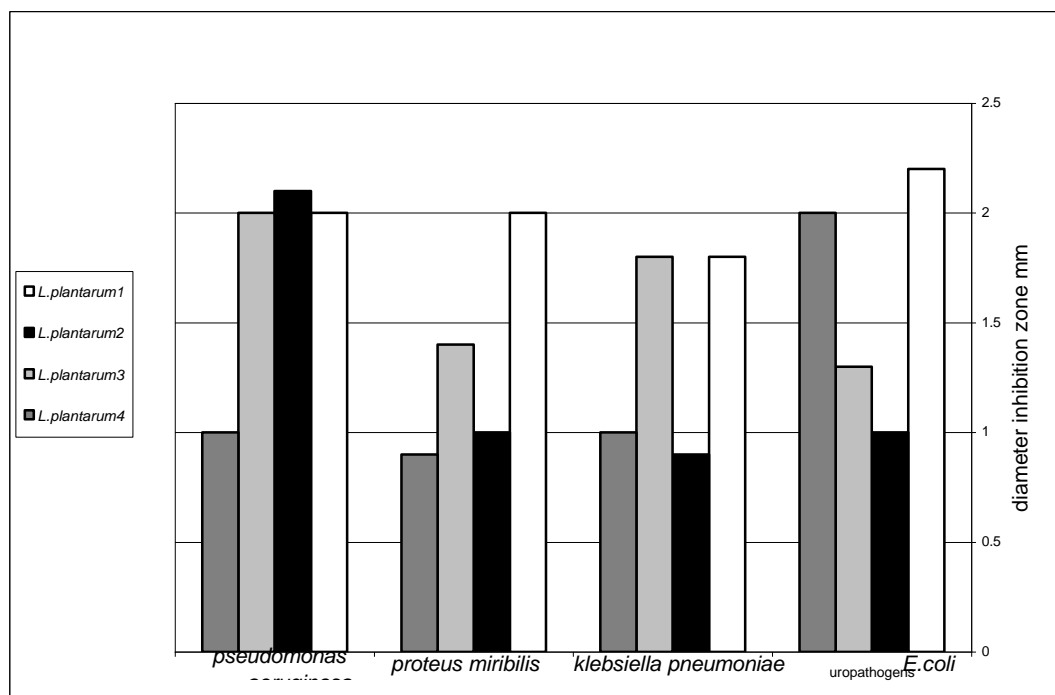
figure(4):inhibitory effect of *Lactobacillus acidophilus* against uropathogens

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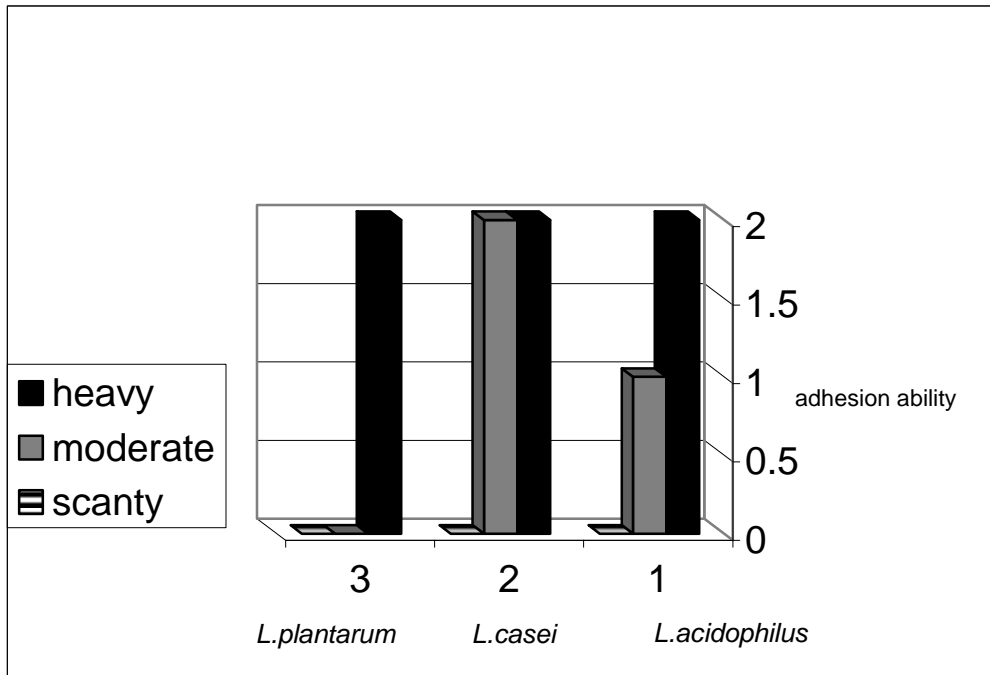
figure(5):inhibitory effect of *Lactobacillus casei* against uropathogens



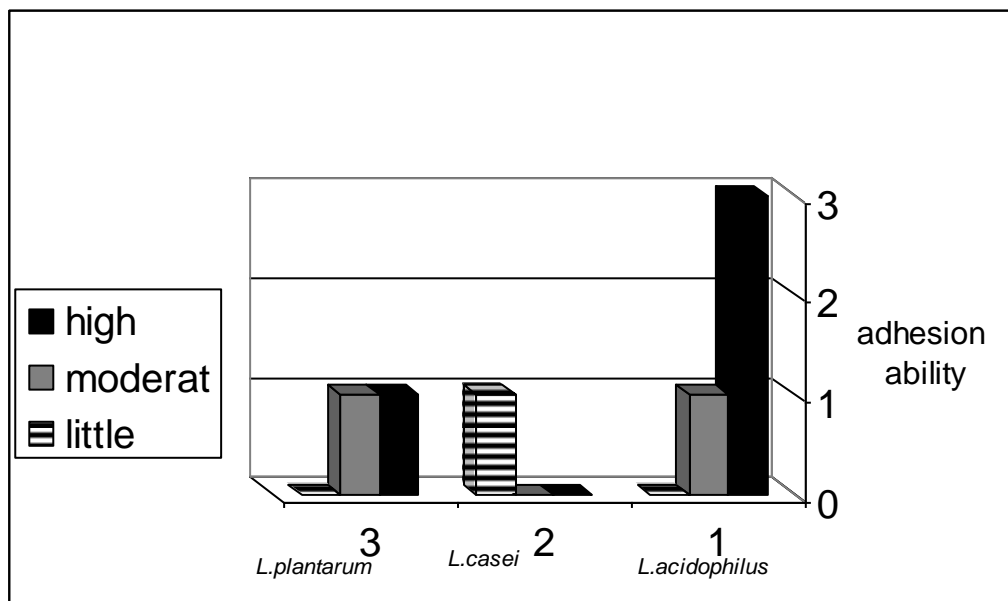
figure(6):inhibitory effect of *Lactobacillus plantarum* against uropathogens

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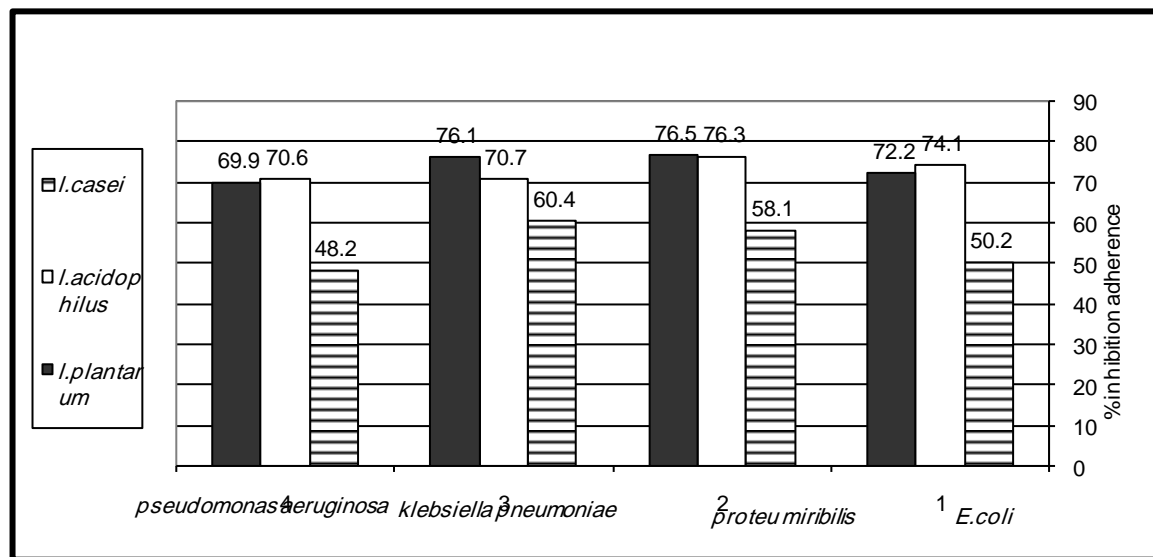
figure(7):adhesion ability of *Lactobacillus* species to uroepithelial cells in married women with children



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

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figure(9):inhibition of uropathogens adherence by *Lactobacillus* species

REFERENCES

- 1.Kucheria,R.;Dasgupta,P.;Sacks,S.;Khan,M. ;and Sheerin ,N.S .2005.Urinary tract infections: new insights into a common problem.J.Postgraduate Med.,81:83-86(2007).
2. Jassawala,M.J.Probiotics and Women's Health. J. Obstet Gynecol India ,57(1):19-21(2007).
- 3.Boris S, Barbés C. Role played by *lactobacilli* in controlling the population of vaginal pathogens. Microb. and Infect;2(5):543–546(2000).
4. Aroutcheva A; Dominique G; Melissa S; Susan S..Defence factors of vaginal *lactobacilli*. American Journal of Obstet. and Gynecol., 185(2):375-379(2001).
- 5.Pavlova SI, Kilic AO, Kilic SS,Genetic diversity of vaginal *lactobacilli* from women in different countries based on 16S rRNA gene sequences. J. Appl. Microb.,92(3):451–459(2002).
- 6.Jassawala,M.J.Probiotics and Womens heath.J.Obstet Gynecol. India, 2005, 54(1):19-21
- 7.Lomberg,H;Cedegen,B;Loffler,H.;Nilsson,B.;Carlstrom,A.S.;and Eden,C.S.Influence of blood group on the a vailability of receptor for attachment of uropathogenic *Escherichiacoli*. Infect.Immun.,51 :919.(1986).
- 8.Bartolomer,R.;Osset,J.and Garcia,E.Assessment of the capacity of *Lactobacilus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells.J.Infect.Dis.,183.(2001)
- 9-National Committee for Clinical Laboratory Standards.Performance Standard for Antimicrobial Susceptibility Testing.Approved Standard M2-A7,7th Ed.,National Committee for Clinical laboratory Standard.(2000), Pennsylvania.
- 10-NationalCommittee for Clinical Laboratory Standards.Performance Standard for Antimicrobial Susceptibility Testing.12th Ed., National Committee for Clinical laboratory Standard.(2002), Pennsylvania.

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11. Chan, R.C.Y., Reid G, Irvin RT, Bruce AW, Costerton J.W. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infect. Immun.*, 47:84-9 (1985).
12. Reid G; Bruce.. Urogenital infection in women: can probiotics help? *Post graduate med. j.*, 79:428-432(2003).
13. Zarazaga M; Saenz Y. and Portillo, A. In vitro activities of Ketolide HMR3647 Macolides and other antibiotic against *Lactobacillus*, *Leuconostoc* and *Pediococcus* isolates. *Antimicrob. agents and chemother.*, 43(12):3034-3041(1999).
14. Zarate, G.; and Nader-Macias, M.E. Influence of Probiotic vaginal *Lactobacilli* on invitro adhesion of urogenital pathogens to vaginal epithelial cells.
15. Reid, G; Andrew, W.; Bruce, W.; Jacqueline, A.; McGroarty, K. and Cheng, J. Is there a role for *Lactobacilli* in prevention of urogenital and intestinal infections?. *Clin. Microb. Re.*, 3(4):335-344(1990).
16. Coconnier, M.; Lievin, V.; Camard, M.B.; Hudault, S. and Ervin, A. Antibacterial effect of adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob. Agent Chemother.*, 41(5):10-46(1997).
17. McGroarty, J.; and Reid, G. Detection of a *Lactobacillus* substance that inhibits *Escherichia coli*. *American j. clin. nutrition*, 73(2):437-443(2001).
18. Murianat, P.M. and Klaenhammer, T.B. Purification and partial characterization of lactacin F, bacteriocin produced by *Lactobacillus acidophilus* 11088. *APPL. and Environ. Microbiol.*, 79:4281(2003).
19. Ocana, V.S. and Elena, N.M. Adhesion ability of *Lactobacillus* to vaginal epithelial cells: study by microbiological methods. *Method Mol. Biol.*; 268:441-5(2004).
20. Reid, G. Probiotic agents to protect the urogenital tract against infect. *American J. Clin. Nutr.* 73(2):345-44(2001).
21. Chan, R. C. Y., Bruce, A.W. and G. Reid. Adherence of cervical, vaginal and distal urethral normal microbial flora to human uroepithelial cells and the inhibition of adherence of Gram-negative uropathogens by competitive exclusion. *J. Urol.*, 131:596-601(1984)
22. Reid, G. and Bruce, A.W. Urogenital infections in women: can probiotics help?. *postgraduate Med. J.*, 79:428-432(2003).
23. Ocana, V. and Nader, M. Adhesion of *Lactobacillus* vaginal strains with probiotic properties to vaginal epithelial cells. *Bio. Cell.*, 25(3):265-77(2001).
24. Kaewsrirachan J, Peeyanjarassri K, Kongprasertkit J. Selection and identification of anaerobic *Lactobacilli* producing inhibitory compounds against vaginal pathogens. *FEMS Immunol. Med. Microbiol.*; 48(1):75-79(2006).
25. Juarez, T.M.; Ocana, V.S., Wiese, B. Nader, M. Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259, and inhibition of uropathogenic *Escherichia coli*. *J. Med. Microbiol.*; 52:1117-24(2003).
26. Howard, J., C. Heinemann, B. J. Thatcher, B. Martin, B. S. Gan, and G. Reid. Identification of collagen-binding proteins in *Lactobacillus* spp. with surface-enhanced laser desorption/ionization-time of flight Protein Chip technology. *Appl. Environ. Microbiol.* 66: 4396-4400(2000).