

Inhibitory Effect of *Lactobacillus reuteri* on Some Pathogenic Bacteria Isolated From Women With Bacterial Vaginosis

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Background: Considering the high prevalence of bacterial vaginosis and its association with urinary tract infection in women and treatment of gynecologic problems occur when a high recurrence of bacterial vaginosis is often treated with antibiotics.

Objectives: The purpose of this study was to investigate the inhibitory effect of *Lactobacillus reuteri* on pathogenic bacteria isolated from women with bacterial vaginosis.

Materials and Methods: Ninety-six samples were obtained from vaginal discharge of women with bacterial vaginosis by a gynecologist with a Dacron swab and put in sterile tubes containing TSB broth and Thioglycollate broth. Then were immediately sent to the laboratory in cold chain for further assessment. Afterward, culture was transferred on blood agar, EMB, Palcam and differential diagnosis environments. Then cultures were incubated for 24 hours at 37 °C. *Lactobacillus reuteri* strains were cultured in MRS environment and transferred to laboratory. After purification of pathogenic bacteria, *Lactobacillus reuteri* inhibitory effect on pathogenic bacteria was evaluated by minimum inhibitory concentration (MIC) and antibiogram. Statistical analysis was performed using SPSS software v.16.

Results: The results of this study demonstrated the inhibitory effect of *Lactobacillus reuteri* on some pathogenic bacteria that cause bacterial, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Enterococcus*, *Listeria monocytogenes* and *E. coli*. Microscopic examination of stained smears of most *Lactobacillus* and pathogenic bacteria showed reduced. The prevalence of abnormal vaginal discharge, history of drug use, contraceptive methods and douching were 61%, 55%, 42% and 13%, respectively. Significant difference was observed between the use and non-use of IUD in women with bacterial.

Conclusions: Our findings indicated the inhibitory effect of *Lactobacillus reuteri* on pathogenic bacteria that cause bacterial vaginosis. The results of this study confirmed the inhibitory effect of probiotics on pathogens growth that cause bacterial vaginosis, which can be considered in the prevention and treatment of bacterial vaginosis in further investigations.

Keywords: *Lactobacillus reuteri*; probiotics; bacterial

1. Background

Bacterial vaginosis is a vaginal inflammation due to a decrease in *lactobacilli* normal flora accompanied by overgrowth of anaerobic bacteria, Gram-negative, and in some cases Gram-positive cocci pathogens and alteration of vaginal pH to more than 4.5. Anaerobic bacteria which overgrowth during bacterial vaginosis include *Gardnerella vaginalis*, *Mobiluncus*, *Bacteroides*, *Prevotella*, *Mycoplasma* and other bacteria including *Staphylococcus aureus*, *Streptococcus* Group B, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Listeria monocytogenes*, *E. coli* and *Enterococcus* (1-3).

In total, 80% to 95% of vaginal flora consists predominantly of *lactobacilli*, including *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. iners*, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. vaginalis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri*, *L. rhamnosus* (2).

These bacteria colonize the mucosal membrane and

block excessive proliferation of potentially pathogenic microorganisms. Their inhibitory properties include: specific adhesion to epithelial cells surface, and production of substances with antimicrobial activity, such as hydrogen peroxide and bacteriocins (4).

Recurrence rate is very high in bacterial vaginosis (5) and risk of sexually transmitted diseases, such as HIV, as well as urinary tract infections is increased in postmenopausal women (6, 7).

Amsel criteria are generally used in clinical practice to diagnose both symptomatic and asymptomatic BV (Bacterial Vaginosis). At least three of the four criteria should be met: (1) homogeneous, milky vaginal discharge, copious; (2) bad-fish odor, due to the release of volatile amines, on the Whiff test; (3) vaginal pH > 4.5; and (4) identification of bacteria-covered epithelial cells (clue-cells) under light microscopy (8).

Bacterial vaginosis is usually treated with metronidazole or clindamycin (2, 9), but probiotics could be used instead due to antibiotic resistance, side effects and recurrence of infection (7, 10).

Normal amount of *Lactobacilli* in the vagina is 10^7 . When the balance of normal vaginal flora is disrupted together with reduced levels of *Lactobacilli*, probiotics can be used to reestablish the balance in the vagina. *Lactobacilli* are bacteria which maintain the host's health status if present in sufficient amounts (11).

Due to the high prevalence of bacterial vaginosis as a major health problem in women visiting midwifery clinics and side effects of medications or recurrence of infections, probiotics including *Lactobacilli*, have been under research in the treatment of vaginosis. Various studies have been conducted to examine the effects of different forms of *Lactobacilli* (douching, vaginal suppositories, tampons, and some others.) in the treatment of vaginosis. Mechanisms by which probiotics exert healthy effects are not completely understood. Some authors declared competitive inhibition with pathogenic bacteria, effects on barrier function, antagonism through the production of antimicrobial substances (hydrogen peroxide and bacteriocins) and modulation of the immune system (12).

2. Objectives

The purpose of this study was to investigate the inhibitory effect of *Lactobacillus reuteri* on pathogenic bacteria isolated from women with bacterial vaginosis.

3. Materials and Methods

Lactobacillus reuteri (DMS 20016) strains were frozen in the liquid Man, Rogosa and Sharpe plus glycerol at -70 fridge, and cultured in MRS solid and incubated in a CO₂ incubator for 48 hours. The *Lactobacilli* grown on solid MRS medium was inoculated in liquid MRS medium, and after 24 hours liquid MRS broth was removed and transferred to another environment, to strengthen *Lactobacilli*.

Ninety-six samples were obtained from vaginal discharge of women with symptoms of bacterial vaginosis by a gynecologist with Dakron swab and inoculated on blood agar medium and kept in incubator. After 24 hours, due to the growth of several types of colonies on blood agar medium, each of the colonies were cultured on blood agar, EMB agar and Palcam.

The next day, catalase test was performed on colonies grown on blood agar, and also slides were prepared and Gram-stain was performed. For Gram-positive cocci and catalase-positive strains, diagnostic tests including those related to family Staphylococcaceae such as coagulase, oxidase and susceptibility to bacitracin discs and Novobiocin were performed and *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* were detected.

For Gram-positive and catalase-negative cocci of Streptococcaceae family including diagnostic tests to check blood agar hemolysis and Bile esculin tests and PYR, growth in the presence of 40% bile, Hippurate hydrolysis test, Camp and sensitivity test discs Optochin and Bacitracin were performed.

Regarding Enterococcus species, *Streptococcus agalactiae* strains were detected in samples. For bacteria grown on Palcam agar medium, catalase test, Gram-slide preparation and camp were performed. *L. monocytogenes* was detected when Gram-positive. catalase test and camp had positive findings.

For bacteria grown on blood agar and EMB, we prepared slides and Gram-stained to differentiate Enterobacteriaceae from other Gram-negative bacteria tested oxidase and Gallery diagnostic testing Enterobacteriaceae family were performed, and bacteria *E. coli* obtained.

First, blank discs were put in MRS broth medium containing *Lactobacillus reuteri* isolated from patients and then put by a half McFarland was created and on Mueller-Hinton medium was cultured according to antibiotic disk diffusion method and treated *Lactobacilli* and ciprofloxacin disks were placed. After 24 hours of incubation, inhibition zone around the discs impregnated with bacteria *Lactobacilli* and antibiotic ciprofloxacin was observed.

In other methods, 250 Lambda of removed bacteria was poured into 750 Lambda of BHI medium containing *Lactobacillus rhamnosus* and BHI medium containing *Lactobacillus reuteri* and incubated for 24 hours. Then cultured on Mueller Hinton agar medium. After 24 hours, Gram staining and molar method were performed on slides.

The final method of *Lactobacillus* cultures in BHI broth was removed and cultured on Muller Hinton medium. We then used a sterile swab of bacteria diluted to 0.5 McFarland standard and draw on Muller Hinton medium. After 24 hours, we reviewed the results.

Data was analyzed using SPSS software version 16 for statistical analysis. We used relative frequency (%) for qualitative variables and mean and median measures to report quantitative variables.

4. Results

In this study, 96 women with vaginal discharge were examined to study the inhibitory effect of *Lactobacillus reuteri* on pathogenic bacteria causing bacterial vaginosis., Risk factors including age, history of drug use, abnormal discharge, vaginal douching, and contraceptive methods were assessed.

From 96 patients examined, aged between 18-58 years, the maximum age group was 49 to 58 years old. From 96 patients, 69 patients (72%) had bacterial vaginosis. From 69 patients examined in this study, 37 (54%) had a history of using drugs, 41 (59%) had abnormal vaginal discharge, 27 (39%) had a history of contraceptive methods and 9 (13%) had a history of douching (Figures 1- 8 Tables, 1 and 2)

Table 1. Distribution of Bacteria Isolated From Patients With Bacterial Vaginosis

The Age Range	Positive Culture No, (%)	Negative Culture No, (%)	Total (No = 96)
28-18	8 (66)	4 (33)	12
38-29	19 (68)	9 (32)	28
4-39	14 (56)	11 (44)	25
58-49	28 (90)	3 (10)	31
P Value	0.033	0.033	0.033

Table 2. Odds Ratios of Risk Factors

Risk factors	OR (95% CI)	P Value
History of Drug Use	0.79	0.786
Abnormal Vaginal Discharge	0.86	0.93
Means of Preventing Pregnancy	3.42	0.012
Douching	3.3	0.142

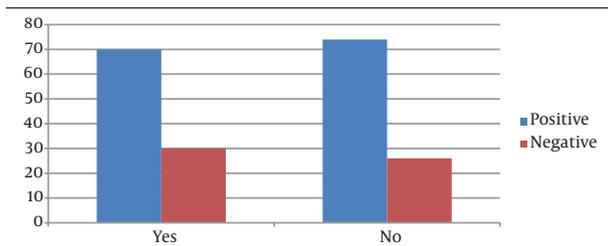


Figure 1. Distribution of Positive and Negative Results for Bacterial Vaginosis Regarding Previous Drug Use

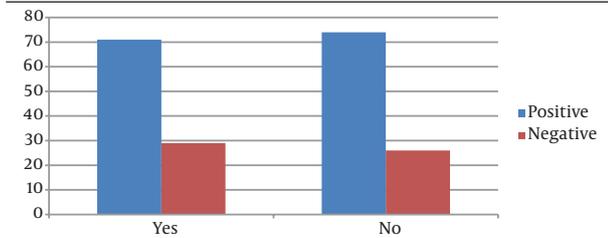


Figure 2. Distribution of Positive and Negative Abnormal Vaginal Discharge

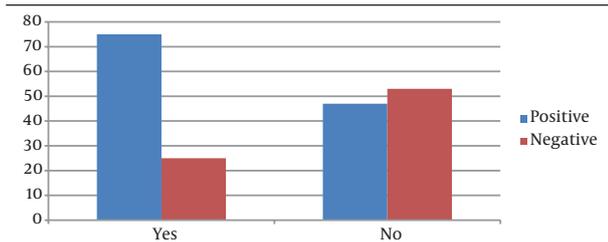


Figure 3. Distribution of Positive and Negative Results For Bacterial Vaginosis Regarding Pregnancy Prevention

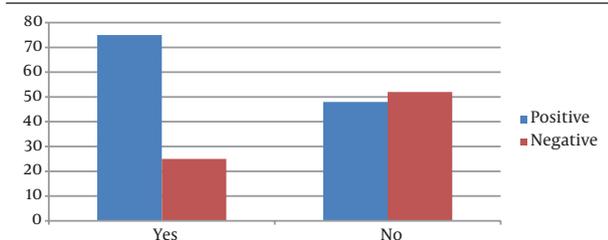


Figure 4. Distribution of Positive and Negative Results Regarding Douching

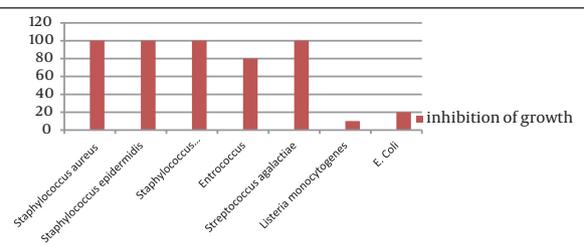


Figure 5. Inhibitory Effect of *Lactobacillus reuteri* on Pathogenic Bacteria Isolated From Patients With Bacterial Vaginosis



Figure 6. Growth Inhibition of *Streptococcus agalactiae* by Disk Impregnated With *Lactobacilli*

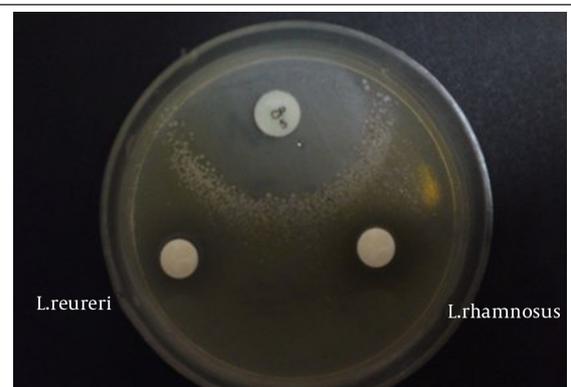


Figure 7. Growth Inhibition of *Staphylococcus epidermidis* by Discs Impregnated With *Lactobacilli*



Figure 8. Growth Inhibition of Pathogenic Bacteria by *Lactobacillus reuteri*

5. Discussion

Treatment with probiotics is based on the assumption of normal microbial flora. Data from various studies indicated the efficacy of probiotics in the treatment of human disease (13). Therefore, it was decided to investigate the inhibitory effects of probiotic *lactobacilli* on bacterial pathogens that cause bacterial vaginosis. The results of this study demonstrated the inhibitory effect of *Lactobacillus reuteri* on some pathogenic bacteria that cause bacterial vaginosis, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes*. Microscopic examination of stained smears of most *Lactobacillus* and pathogenic bacteria showed reduced. In a research performed in 2008 in Europe, 72 postmenopausal women aged 55 to 65 years with asymptomatic bacterial vaginosis were examined. Oral capsules containing *Lactobacillus* CFU *Lactobacillus rhamnosus* GR1 and RC14 *reuteri* were administered for 14 days. The results showed that most women were tested by probiotic treatment (14).

In 2006, the effect of *lactobacilli* probiotic was assessed on in vitro binding of urogenital pathogens to vaginal epithelial cells. The results showed that vaginal *lactobacilli* inhibit the binding of *Staphylococcus aureus* and *Streptococcus* Group B genitourtopathogenic to vaginal epithelial cells. Results indicated the efficacy of probiotic strains of *lactobacilli* in the vagina which suggests further studies on their capacity to prevent and treat infections in women urogenital tract (15). Brotman et al. showed that the prevalence of bacterial vaginosis was 40.2, which was associated with douching (16). In the present study, the prevalence of douching was 13%, a significant difference was observed between using and not using douching in women with bacterial vaginosis. In a study conducted on 357 patients to assess bacterial vaginosis risk factors, use of IUD was found in 47.2% of subjects (17). In the present study, the prevalence of contraceptive methods including IUD was 39%. A significant difference was observed be-

tween using and not using IUD in women with bacterial vaginosis in a study in Sweden in 2000 on 956 women. In addition, 52% of 131 women with bacterial vaginosis were smoking, indicating the association between smoking and the risk of BV (18). In our study, smoking was not assessed due to the small number of smoking women.

According to the results of this study, the prevalence of abnormal vaginal discharge, history of contraceptive drug use, contraceptive methods and douching were 61%, 55%, 42% and 13%, respectively. No significant difference was observed between using douching. Mechanism of action of probiotics should be more clearly explained to select best species for probiotic against a particular pathogen (13).

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