Original Article

Some Probiotic Properties of Vaginal Lactobacilli
Isolated from Healthy Women

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(Received February 7, 2006. Accepted June 15, 2006)

SUMMARY: Lactobacilli have long been considered to be protective flora in the vagina. In this study, samples were taken from the lateral vaginal wall of 19 healthy women, and 58 strains of Lactobacillus spp. were isolated from 10 of these women. The identification results revealed that Lactobacillus gasseri was the predominant lactobacilli in healthy women. The amounts of lactic acid and hydrogen peroxide (H$_2$O$_2$) produced by Lactobacillus spp. were determined. The vaginal pH levels induced by Lactobacillus spp. strains ranged from 1.00 to 2.15. The titratable acidity of the strains ranged from 0.27 to 1.33%. The H$_2$O$_2$ concentration produced in the lactobacilli strains ranged between 1.01 µg/ml and 15.50 µg/ml. The antimicrobial effects of the lactobacilli against the pathogenic bacteria Escherichia coli ATCC 11230, Staphylococcus aureus ATCC 2392, Pseudomonas aeruginosa ATCC 29212 and Salmonella enterica serovar Enteritidis RSKK 171 were also determined by the agar diffusion methods.

INTRODUCTION

Lactobacilli are the dominant bacteria of a healthy human vagina, and their presence and number are influenced by estrogen production, which undergoes age- and menstrual cycle-dependent changes (1). A healthy vaginal ecosystem is dominated by certain species of Lactobacillus which exert a significant influence on the microbiology of the vagina (2,3). However, lactobacilli are used in the fermented food industry as probiotics for human and animal nutrition (4). Lately, they have also been suggested as candidate microorganisms to be included in probiotics for vaginal use (5). There is growing interest in the use of lactobacilli of human origin as probiotics against urogenital tract infections. Lactobacilli have long been considered to constitute the primary microbiological barrier against infection by urogenital pathogens (6).

Lactobacilli, particularly those strains that produce H$_2$O$_2$, may have a protective effect against vaginal colonization by pathogenic species such as those that cause bacterial vaginosis (BV) and possibly human immunodeficiency virus (HIV) and gonorrhea (7).

They exert a protective role against pathogen colonization by steric exclusion and production of inhibitory substances. Among these substances, lactic acid produced from carbohydrates helps to maintain a low vaginal pH (8). The healthy vagina is generally found to have a pH of 4 ± 0.5, and this acidity has been shown to be microbicidal for many sexually transmitted disease pathogens, including HIV (9). In addition, different Lactobacillus spp. produce H$_2$O$_2$ and bacteriocin-like substances that may affect undesirable or pathogenic strains even on lactic acid bacteria themselves (8). The hydrogen peroxide-producing microorganisms that are present in the vagina of healthy women have been suggested as the bacterial group that is responsible for the maintenance of the ecological balance, mainly in pregnant women (10,11). Absence of these microorganisms is related to increase in BV, recurrent urinary tract infections by Escherichia coli and acquisition of HIV-1 (12). Hydrogen peroxide-producing lactobacilli also might exert control over vaginal cancer through specific interaction with reactive oxygen species. In addition to lactic acid, the combination of H$_2$O$_2$ further suppresses the endogenous pathogenic bacteria to maintain a healthy vaginal ecosystem (13).

The determinative characteristics of lactobacilli for probiotic use in the human female urogenital tract have been suggested (8,14). The selection of Lactobacillus spp. for probiotic use in the human vagina as a barrier to infections and alternative to long-term use of antibiotics.

The aim of this study was to determine the ability of Lactobacillus spp. strains isolated from the vaginas of healthy women to produce acid and hydrogen peroxide. The inhibitory effects of these lactobacilli on various pathogenic bacteria were also studied. Several of the newly isolated strains that were determined to have certain desirable properties may be available for use as probiotics.

MATERIALS AND METHODS

Bacterial strains and identification of lactobacilli: Vaginal swabs were taken from the lateral vaginal wall of 19 healthy women and were inoculated on lactic agar as a selective medium. Colonies forming a yellow zone were isolated from the lactic agar (15). To identify the strains, each colony was subjected to a gram stain test as well as tests for catalase activity, cell shape, motility, growth at various temperatures (15, 45, and 50°C), and tolerance of different salt levels (MRS broth with 2, 4, and 6.5% NaCl). Production of gas from glucose and production of catalase were also tested. Finally, the carbohydrate fermentation characteristics of all strains were determined by using an API 50 CHL system (BioMerieux, Marcy l’Etoile, France) (16,17). The isolates were stored at ~80°C in MRS broth (de Man, Rogosa and Sharpe Medium; Oxoid, Hampshire, England) containing 30% glycerol and regenerated twice before use in the manipulation. According to the results of the identification tests, 58 strains were identified as Lactobacillus spp. (Figure 1). In preparation for the experiments, Lactobacillus spp. strains were...
cultured under anaerobic conditions for 16-18 h (the exponential growth phase) at 37°C in MRS broth in isolates. The test bacteria were obtained from the Department of Biology Culture Collection of Gazi University.

**Determination of lactic acid production ability:** The acid production of the lactobacilli strains was determined in 10 ml of sterilized MRS broth. One percent inoculum from an overnight culture was used and the incubation proceeded for 16-18 h at 37°C. After incubation, the pH of samples was measured by an electronic digital type pH meter. In addition, the samples were titrated with 0.1 N NaOH using phenolphthalein as an indicator. The titratable acidity was expressed as a percentage of that of lactic acid (17).

**Determination of hydrogen peroxide (H₂O₂) production ability:** Lactobacilli were added (2% v/v) to 10 ml MRS broth and inoculated at 37°C for 16-18 h. Samples were adjusted to room temperature before adding the desired amount of H₂O₂. Ten milliliters of samples were immediately adjusted to pH 4.5 with 0.1 N HCl. Two milliliters of 0.1 M acetate buffer (pH 4.5) were added and the mixture was diluted to 20 ml with distilled water. The samples were then filtered through Whatman no. 42 filter paper to remove the curd. Centrifugation of the samples at 3,400 × g for 10 min may also be used to remove curd. To have the filtrate for each sample serve as the blank, 5 ml of the filtrate for each sample was added to a tube containing 1 ml distilled water and 0.1 ml o-dianisidine solution, and then another 5 ml of the filtrate was added to a tube containing 1 ml peroxidase solution and 0.1 ml o-dianisidine. The assay tubes were then incubated for 10 min at room temperature (23-26°C), 0.2 ml of 4 N HCl was added to each tube, and the optical density was determined after an additional 5 min of incubation (18). The H₂O₂ (30%) was obtained from Merck and was diluted to the desired concentration with distilled water just before each assay. H₂O₂ was quantified by using a H₂O₂ standard curve, established for peroxide (H₂O₂) and lactic acid production abilities were determined (Table 1). The level of pH induced by lactobacilli strains ranged from 1.00 to 2.15. The titratable acidities (%) of the strains were between 0.27 and 1.33%. L. gasseri R4, R5 (1.33, 1.18 %) and L. acidophilus R1, R6 (1.17, 1.12 %) strains induced a higher level of acidity than all the other lactobacilli strains. There were striking differences between the amounts of H₂O₂ production (range, 1.01 to 15.50 μg/ml H₂O₂), and 23 strains of lactobacilli did not produce any detectable amount of H₂O₂. The H₂O₂ production of L. vaginalis H5 was the highest.

The antimicrobial effect of Lactobacillus spp. was studied by using the agar diffusion technique on the test bacteria (P. aeruginosa ATCC 29212, S. aureus ATTC 2392, S. enterica serovar Enteritidis RSKK 171 and E. coli ATTC 11230) which caused infections in the genital and urogenital system. The strains of Lactobacillus spp. had an 85% inhibitory effect on S. aureus ATTC 29212, a 71% inhibitory effect on S. enterica serovar Enteritidis RSKK 171, and an 43% inhibitory effect on E. coli ATTC 11230. Although most of the strains were found to produce inhibition zones against some pathogenic bacteria, the strains L. gasseri O2, L. vaginis S1, L. salivarius S2, and L. plantarum T1 did not show antimicrobial activity against any of the test bacteria. P. aeruginosa ATCC 29212 was the most sensitive, while E. coli ATTC 11230 was the most resistant to the supernatants of Lactobacillus strains (Table 2).

**RESULTS**

In this study, the samples were taken from the lateral vaginal wall of 19 healthy women. Fifty-eight strains of Lactobacillus spp. were isolated from 10 of these women. Lactic agar was used as a selective medium. Only isolates that formed a yellow colony on lactic agar, indicating a high level of acid production, were selected. The 58 Lactobacillus isolates were classified as 21% L. gasseri, 16% L. vaginalis, 16% L. acidophilus, 14% L. delbrueckii spp. lactis, 14% L. crispatus, 5% L. plantarum, 3% L. cebllobius, 3% L. jenseni, 3% L. salivarius, 2% L. curvatus, 2% L. brevis and 2% L. oris (Figure 1). L. gasseri was the predominant Lactobacillus.

The Lactobacillus spp. were isolated and their hydrogen peroxide (H₂O₂) and lactic acid production abilities were determined (Table 1). The level of pH induced by lactobacilli strains ranged from 1.00 to 2.15. The titratable acidities (%) of the strains were between 0.27 and 1.33%. L. gasseri R4, R5 (1.33, 1.18 %) and L. acidophilus R1, R6 (1.17, 1.12 %) strains induced a higher level of acidity than all the other lactobacilli strains.

**DISCUSSION**

Lactobacilli are used in the fermented food industry and as
probiotics for human and animal nutrition; lately, however, they have also been suggested as candidate microorganisms to be included in probiotics for vaginal use (22). There is growing interest in the use of lactobacilli of human origin as probiotics against urogenital tract infections (6). Recently, however, the use of lactobacilli isolated from dairy products as probiotics have been investigated. The potential use of vaginal lactobacilli isolated from healthy women as probiotics has not been examined in detail.

In this study, vaginal swabs were collected from the lateral vaginal wall of 19 healthy Turkish women, and lactobacilli were isolated from only 10 of these women. Lactobacilli are dominant in this habitat, at 10^10 to 10^16 cfu/g of vaginal fluid of healthy premenopausal women (23). As a result of the identification tests, 58 Lactobacillus spp. isolates were identified as 12 L. gasseri, 9 L. vaginalis, 9 L. acidophilus, 8 L. delbrueckii spp. lactis, 8 L. crispatus, 3 L. plantarum, 2 L. cellobiosus, 2 L. jensenii, 2 L. salivarius, 1 L. curvatus, 1 L. brevis, and 1 L. oris (Figure 1). L. gasseri was predominant among the other lactobacilli; these bacteria represented 21% of the isolates, followed by L. acidophilus (16%) and L. vaginalis (16%) in Turkish women. Similar results have been reported by other authors. Benno et al. (24) reported that L. gasseri was the dominant species among lactobacilli isolated from the intestinal tracts of Japanese people. Also, Song et al. (25) reported that L. crispatus and L. gasseri were the predominant lactobacilli in the vaginas of Japanese women. However, Reid and Bruce emphasized that L. iners were the most common organisms, at least in one mainly white population (26). Among them, those belonging to the L. acidophilus group and L. fermentum are most frequently isolated, although others, such as L. plantarum, L. brevis, L. jensenii, L. casei, L. delbrueckii, and L. salivarius are isolated as well (27). A new species, L. vaginalis, was recently isolated; this species resembles L. fermentum and L. reuteri, but is phylogenetically most closely related to L. oris and L. reuteri (14). The inhibitory effect on growth of the pathogen thus involved a combination of both hydrogen peroxide and acidity. That was produced during the lactobacilli growth. One of the mechanisms suggested to control the vaginal ecosystem is the production of these antagonistic substances. Tomas et al. (28) reported that when the vaginal pH is <4.5, colonization of the introitus by E. coli is not frequent. It was found that the titratable acidity of L. gasseri R4 (1.33%) was highest. New strains isolated from healthy women, which are able to high acid production, may be used as probiotics.

In this study, only 10 strains from the 58 human vaginal isolates inhibited all the test bacteria. In their study, Tomas et al. (28) reported that only 6 of 134 isolated strains of vaginal lactobacilli were able to inhibit the growth: all the pathogens such as E. coli, S. aureus, Enterococcus faecalis, and Klebsiella sp.

Lactic acid bacteria have been shown to inhibit the in vitro growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (29). L. gasseri R4 and L. cellobiosus L2 have been shown to strongly inhibit the enteropathogenic bacteria E. coli ATCC 11230 and S. enterica serovar Enteritidis RS KK 171 in vitro. Similar results have been reported (30,31) for other strains of Lactobacillus isolated from chickens that inhibited Salmonella and E. coli growth. In the present study, L. salivarius isolated from the cloaca and vagina produced significant inhibitory activity against the S. enteritidis. P. aeruginosa ATCC 29212 was more sensitive against lactobacilli strains than other test bacteria. Collins and Aramaki (32) have shown that some L. acidophilus strains were able to inhibit the growth of certain Pseudomonas spp. by producing levels of 40-55 μg/ml H₂O₂. L. gasseri O2, L. vaginalis S1, L. plantarum T1, and L. salivarius S2 exhibited no antibacterial activity against any of the test bacteria. Also, these bacteria showed weak H₂O₂ and acid production. However, Aroutheva et al. (13) and Annuk et al. (33) revealed that there was no correlations among bacteriocin activity, lactic
acid and hydrogen peroxide production. They also found that some *Lactobacillus* strains produced H$_2$O$_2$, but did not demonstrate any inhibitory effect. We obtained similar results in the present study: *L. gasseri* R4, R5, R7 and *L. acidophilus* R1 strains produced a high level of lactic acid, but did not produce H$_2$O$_2$. Also, no correlation was found between antimicrobial activity with lactic acid and H$_2$O$_2$ production of strains. Our results indicated that new strains of *Lactobacillus* spp. isolated from the vaginas of healthy women could be promising candidates for use in the preparation of probiotic products and for use as health-promoting bacteria.

**ACKNOWLEDGMENTS**

This study was supported by Gazi University BAB project no. FEF 05/2002-54 and TR Prime Ministry State Planning Organization project no. 2003 K 12470-06.
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