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Inhibition of uropathogenic biofilm growth on silicone rubber in human urine by lactobacilli – a teleologic approach

Abstract The ability of three *Lactobacillus* strains to inhibit the adhesion and growth of naturally occurring uropathogens on silicone rubber was investigated in human urine. The importance of biosurfactant production by *Lactobacillus* in discouraging uropathogen growth was determined in relation to the binding affinities of the lactobacilli for silicone rubber. *L. fermentum* B54 markedly inhibited uropathogen growth on the silicone rubber disks after 8 days for all five men included in the study, albeit to various extents ranging from 77% to 100%. In urine from women, however, this inhibition was less clear, as it was absent for two of the four women participating in this study. *L. casei rhamnosus* 36 completely discouraged uropathogen growth on the disks after 8 days for three of the four women, whereas its effect in urine from men was less pronounced (inhibition ranged from 48% to 100% and was absent for one man). *L. casei rhamnosus* ATCC 7469^T was the least inhibitory *Lactobacillus* strain tested and inhibition was absent for a number of both male and female participants, possibly as a result of the low binding affinity of this strain for silicone rubber and of its inability to release biosurfactants. We conclude that the inhibition of uropathogen growth is dependent on the *Lactobacillus* strain involved, and for *L. fermentum* B54 it was demonstrated to be sex-related. Hence, inhibition must be considered a multifactorial process.

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Upon insertion of a urinary catheter, infection of the device with microorganisms colonizing the periurethral skin readily occurs. The risk of urinary tract infection is proportional to the time over which the catheter remains in the body, and all patients eventually become infected if catheterized long enough [20]. Whereas patients on short-term catheterization usually become bacteriuric with a single species, chronically catheterized patients suffer from polymicrobial bacteriuria involving mainly Gram-negative nosocomial species, each being present in high concentration [$\geq 3 \times 10^5$ colony-forming units (cfu)/ml] [20]. Particularly the persistent polymicrobial infections are characterized by a poor response to antibiotic therapy, recurrence of infection, and, ultimately, the selection of resistant pathogens [20]. These problems in the management of catheter-associated urinary tract infection have prompted research into more preventive measures against the disease, especially because scenarios in modern medicine predict that "...the era of antibiotics will soon come to an end as a result of emerging microbial resistance".

A new approach to the treatment and prevention of infectious diseases is the use of probiotics. Over the years the term probiotic has expanded to include not only live organisms and substances (with the exception of antibiotics) that contribute to intestinal microbial balance but also those beneficial to the host in general [5]. Some clinicians have attempted to treat persistent or recurrent urogenital infections by restoring the healthy microflora of the female urogenital tract through the use of probiotic lactobacilli, the major indigenous organisms, or through the use of estrogen to stimulate the reappearance of these bacteria. Oral estrogen administration or topical application of an intravaginal estriol cream in postmenopausal women suffering from recurrent urinary tract infections, for instance, led to an

increase in the number of women harboring vaginal lactobacilli and a decrease in the number colonized with Enterobacteriaceae, possibly due to a lowering of the vaginal pH [11]. Several studies have shown that intravaginal instillation with viable lactobacilli can be effective in preventing recurrent urinary tract infections in women [15].

To date, clinical trials have not explained the therapeutic action of lactobacilli, but in vitro studies have suggested some possible mechanisms by which these probiotic bacteria may interfere with uropathogen adhesion. Lactobacilli can displace adhering uropathogens from catheter materials such as silicone rubber [9] and can block the adhesion of uropathogenic bacteria to uroepithelial cells [4] and polymer substrata [6]. Furthermore, lactobacilli are capable of coaggregating with uropathogenic bacteria [13], which, in combination with inhibitor production, might lead to elimination of the pathogens [8]. Lactobacilli can produce various metabolic by-products with antimicrobial activity, including lactic acid, hydrogen peroxide, and bacteriocins [1]. Recently, the production of antiadhesive biosurfactants (surface active microbial compounds) by lactobacilli has been described [17]. These biosurfactants were shown to inhibit the initial adhesion of uropathogens to hydrophobic and hydrophilic substrata when suspended in phosphate-buffered saline or urine as studied in a parallel-plate flow chamber [17, 19].

The aim of this study was to determine the significance of the binding affinity of lactobacilli for silicone rubber in relation to the production of the antiadhesive biosurfactant in discouraging uropathogen growth on a catheter material. To achieve this goal we selected three *Lactobacillus* strains with an appropriate combination of biosurfactant-releasing properties and binding affinities for silicone rubber. The influence of each of these strains on the adhesion and growth of naturally occurring uropathogens was investigated on silicone-rubber disks in nonpooled specimens of human urine.

Materials and methods

Strains and culture conditions

The *Lactobacillus* strains used in this study comprised *L. fermentum* B54, a poultry isolate [14]; *L. casei rhamnosus* 36, an isolate from a woman with a history of urogenital infection [14]; and *L. casei rhamnosus* ATCC 7469^T (type strain), obtained from the American Type Culture Collection. *L. fermentum* B54 is a strong producer of an antiadhesive, proteinaceous biosurfactant, which has been shown to inhibit the initial adhesion of *Enterococcus faecalis* and various other uropathogens to silicone rubber and glass substrata [17, 19]. *L. casei rhamnosus* ATCC 7469^T and 36, on the other hand, do not release this antiadhesive biosurfactant [17, 18].

All strains were stored at −60 °C in MRS broth (*Lactobacillus* broth described by De Man, Rogosa, and Sharpe; number 1.10661, Merck, Darmstadt, Germany) containing 7% (v/v) dimethylsulfoxide. Precultures were prepared by inoculation of 10 ml of MRS broth with 10 µl of frozen stock followed by incubation at 37 °C in an atmosphere containing 5% CO_2 for 24 h. Stationary cultures were obtained by incubation of 250 ml of MRS broth with 1 ml of a preculture under the same conditions for 18 h.

For the *Lactobacillus* adhesion assay, stationary cells were harvested by centrifugation and washed twice in demineralized water (10,000 g, 5 min, 10 °C). The cells were sonicated three times on ice for 10 s at 30 W with a Vibra Cell 375 device (Sonics and Materials, Danbury, Conn.) to break the aggregates and chains. The lactobacilli were counted in a Bürker-Türk counting chamber and then diluted in filter-sterilized, pooled samples of human urine (see Velraeds et al. [19] for details) to a final density of 3×10^8 cells/ml.

For the biofilm experiments, stationary *Lactobacillus* cells were harvested by centrifugation (10,000 g, 5 min, 10 °C), and the cell pellet was resuspended in 4 ml of sterilized phosphate-buffered saline (10 m*M* KH_2PO_4/K_2HPO_4 and 150 m*M* NaCl, with the pH adjusted to 7.0). After the counting process the lactobacilli were diluted in human urine to a final density of 10^{10} cells/ml. The collection of the urine is described below.

Lactobacillus adhesion assay

For determination of the affinity of the *Lactobacillus* strains for silicone rubber, adhesion assays were performed in a parallel-plate flow-chamber system. This system allows in situ observation of microbial adhesion to a substratum surface by automated image analysis over a field of view covering 0.011 mm^2 [17].

The bottom plate of the flow chamber consisted of a silicone-rubber substratum measuring 5.5 × 3.8 cm (Silastic medical-grade silicone rubber Q7-4750, Nusil, Belgium), which was fixed to a polymethyl methacrylate plate to make its surface more rigid. The silicone-rubber surface was separated from a glass upper plate of equal dimensions by two Teflon spacers measuring 0.06 cm in thickness. Bottom and upper plates were cleaned as described elsewhere [17].

A *Lactobacillus* suspension of 3×10^8 cells/ml in filter-sterilized, pooled specimens of human urine was flowed through the flow chamber at room temperature. A pulse-free flow (0.034 ml/s) was created by hydrostatic pressure, and the suspension was recirculated by a Multiperpex 2115 peristaltic pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) that maintained a constant shear rate of 15/s. Based on estimated daily urine production and internal catheter diameter, this shear rate is similar to that found at the luminal surface of a urinary catheter and corresponds to a Reynolds number of 1, well within the laminar flow regimen. Images were grabbed during the experiment and stored in the computer.

From the initial, linear increase in the number of adhering bacteria per unit area with time, the initial deposition rate was calculated by a linear least-squares fitting procedure. The number of lactobacilli adhering to the silicone rubber after 3 h was also determined.

Urine

Fresh urine containing urethral bacteria acted as the test system for a better simulation of the in vivo situation in men and women. Healthy volunteers of both sexes (age 20–48 years) were requested to clean the periurethral area with water and to collect their midstream morning urine in a sterile container. Fresh urine samples were brought into the laboratory daily over the course of the experiment except for Saturday and Sunday, which were days 5 and 6, respectively. The urine was left untreated and used directly in the biofilm experiments.

Setup of biofilm experiments

The influence of each of the three *Lactobacillus* strains on uropathogenic biofilm formation was investigated in urine specimens from nine test subjects, including five men and four women. On day 0 of the experiment, two sterile screw-cap containers, each holding two autoclaved silicone-rubber disks of 0.6-cm diameter

and 0.1-cm thickness (Silastic medical-grade silicone rubber Q7-4750, Nusil, Belgium), were prepared for each test subject. One container was filled with 25 ml of urine and designated as the control. To the second container, 25 ml of urine supplemented with a *Lactobacillus* strain (10^{10} cells/ml) was added. Subsequently, the containers were incubated with the caps slightly opened at 37 °C in an atmosphere containing 5% CO_2 to stimulate *Lactobacillus* growth. On every day except days 5 and 6 the old urine was removed and replaced with fresh midstream morning urine and freshly cultured lactobacilli. On days 4 and 8, one disk was aseptically isolated from each system: the control urine and the *Lactobacillus*-supplemented urine. Each disk was taken twice through an air-liquid interface at low speed in a test tube containing 1 ml of phosphate-buffered saline, was transferred to a second test tube with 1 ml of phosphate-buffered saline, and was then sonicated for 1 min to remove the adhering microorganisms. The sonicated samples were dilution-plated on blood-agar plates (aerobic, 37 °C) and MRS-agar plates (5% CO_2, 37 °C) for the culture of uropathogenic microorganisms and lactobacilli, respectively. After incubation for 1 or 2 days, the numbers of colony-forming units per square centimeter of silicone rubber were calculated for the control and *Lactobacillus*-treated disks. The types of microorganisms were determined by microscopic evaluation.

This teleologic approach [16], which utilizes naturally occurring uropathogens instead of working with single isolated, pure cultures of uropathogens (a reductionistic approach), was chosen because it more closely resembles the clinical situation, where it is not known a priori which naturally occurring bacteria will cause infection. Although both reductionistic and teleologic approaches have their drawbacks in microbiology, a teleologic approach circumvents the need for decisions as to which uropathogens should be used and how many isolates would be required for a conclusion to be drawn, among others.

Results

Table 1 presents the binding affinities of the three *Lactobacillus* strains for silicone rubber in terms of the initial deposition and the numbers of adhering bacteria as measured in a parallel-plate flow chamber in the presence of urine. *L. casei rhamnosus* 36 and *L. fermentum* B54 showed comparably high initial deposition rates and adhesion numbers after 3 h, whereas *L. casei rhamnosus* ATCC 7469^T did not adhere to the silicone-rubber substratum at all.

In the biofilm experiments the naturally occurring pathogens in the urine specimens from the test subjects grew to an average density of 5×10^9 cells/ml on days 4 and 8. Of these, many organisms adhered to the silicone-rubber disks placed in the containers, resulting in uropathogen counts ranging from 10^3 to 10^6 cfu/cm^2 on the control disks and from 0 to 10^5 cfu/cm^2 on the *Lactobacillus*-treated disks on day 8. The types of uropathogenic microorganisms that were cultured from the disks were similar for urine samples from men and women and comprised cocci, streptococci, staphylococci, and rod-shaped bacteria. Occasionally a few yeast cells were spotted among the bacteria, but their proportion was negligible as compared with the bacterial counts.

Lactobacilli were solely found on silicone-rubber disks incubated in *Lactobacillus*-supplemented urine, and they grew as well in urine from men as in that from women. On day 8 the average *Lactobacillus* counts on the disks amounted to 1.1×10^6 and 1.4×10^5 cfu/cm^2 for *L. casei rhamnosus* 36 and 7469^T, respectively. For some reason the average numbers of *L. fermentum* B54 on day 8 were higher on disks incubated in urine from men (1.3×10^6 cfu/cm^2) as opposed to that from women (2.6×10^4 cfu/cm^2).

In Tables 2–4 the abilities of the lactobacilli to reduce uropathogen growth on silicone-rubber disks incubated in urine are presented as percentages of inhibition. According to Table 2, *L. fermentum* B54 inhibited the numbers of uropathogens isolated from the disks after 8 days of incubation in urine from all five male test subjects, albeit to various extents. A complete inhibition of uropathogen growth was seen in two cases (M1 and M2), whereas in the other three cases this inhibition was partial. For the women participating in the study the effect of *L. fermentum* B54 on the development of a uropathogenic biofilm on silicone rubber in urine was less clear. Whereas a decrease in the numbers of uropathogens on the *Lactobacillus*-treated disks was seen in cases F2 and F3 on day 8, no inhibition was observed for cases F1 and F4.

From Table 3 it can be concluded that *L. casei rhamnosus* 36 was capable of completely discouraging uropathogenic biofilm growth on the silicone-rubber disks after 8 days of incubation in urine from three of the four women. For the remaining woman (F3) the number of uropathogens was not reduced on the *Lactobacillus*-treated disk on day 8. *L. casei rhamnosus* 36 also suppressed the growth of uropathogens on silicone

Table 1 Initial deposition rates and numbers of adhering lactobacilli after 3 h of incubation on silicone rubber in urine as studied in a parallel-plate flow chamber

Lactobacillus strain	Initial deposition rate	Adhesion after 3 h
L. fermentum B54[a]	466 cm^{-2} · s	1.0×10^6 cm^{-2}
L. casei rhamnosus 36[b]	306 cm^{-2} · s	1.1×10^6 cm^{-2}
L. casei rhamnosus ATCC 7469^T[a]	0	0

[a] Data from Millsap et al. [10]
[b] Experiments were performed in duplicate with separately grown cultures at a shear rate of 15/s and the results coincided within 20%

Table 2 Percentage of inhibition of uropathogenic biofilm growth on silicone-rubber disks after 4 and 8 days of incubation in urine from healthy men (*M*) and women (*F*) as induced by supplemented *Lactobacillus fermentum* B54 (10^{10} cells/ml)

Test person M/F (age, years)	Inhibition of uropathogen growth (%)	
	Day 4	Day 8
M1 (22)	0	100
M2 (33)	100	100
M3 (37)	100	97
M4 (38)	88	91
M5 (23)	0	77
F1 (20)	99	0
F2 (48)	89	78
F3 (21)	100	71
F4 (22)	0	0

Table 3 Percentage of inhibition of uropathogenic biofilm growth on silicone-rubber disks after 4 and 8 days of incubation in urine from healthy men (*M*) and women (*F*) as induced by supplemented *L. casei rhamnosus* 36 (10^{10} cells/ml)

Test person M/F (age, years)	Inhibition of uropathogen growth (%)	
	Day 4	Day 8
M1 (27)	93	48
M2 (25)	0	87
M3 (39)	63	56
M4 (24)	100	100
M5 (27)	59	0
F1 (30)	93	100
F2 (39)	100	100
F3 (27)	77	0
F4 (44)	84	100

Table 4 Percentage of inhibition of uropathogenic biofilm growth on silicone-rubber disks after 4 and 8 days of incubation in urine from healthy men (*M*) and women (*F*) as induced by supplemented *L. casei rhamnosus* ATCC 7469^T (10^{10} cells/ml)

Test person M/F (age, years)	Inhibition of uropathogen growth (%)	
	Day 4	Day 8
M1 (31)	96	0
M2 (25)	53	0
M3 (39)	88	92
M4 (41)	96	99
M5 (24)	0	99
F1 (20)	24	53
F2 (41)	100	100
F3 (27)	0	0
F4 (44)	90	0

rubber after 8 days for four of the five men, but this inhibition was generally weaker than that observed for the female test subjects. Additionally, a similar experiment with this *Lactobacillus* strain was carried out for test subject M4, whereby an *Escherichia coli* strain was added daily to the control urine and the *Lactobacillus*-supplemented urine to a final density of 10^5 cells/ml. On day 8 the number of *E. coli* on the *Lactobacillus*-treated disk was 50 times (or 98%) lower than that on the control disk.

L. casei rhamnosus ATCC 7469^T strongly inhibited uropathogen growth on silicone rubber after 8 days of incubation in urine from three of the five male test subjects (Table 4). Its effect in urine from women, however, was less pronounced.

No net increase was observed in the inhibition of uropathogenic biofilm formation with time for any of the three *Lactobacillus* strains, and the inhibition generally did not correlate with the *Lactobacillus* counts on the silicone-rubber disks.

Discussion

The abilities of three *Lactobacillus* strains to inhibit the adhesion and growth of naturally occurring uropathogens were investigated in a teleologic approach mimicking the presence of a silicone-rubber urinary catheter in the urethra. A teleologic approach has proven merits for oral [16] and oropharyngeal [7] biofilm formation. The lactobacilli were capable of discouraging, in some instances completely, the formation of a uropathogenic biofilm, but the degree of inhibition depended on the *Lactobacillus* strain involved. Furthermore, inhibition appeared to occur more often, but not always more strongly, in men than in women. *L. fermentum* B54 showed the strongest inhibition of uropathogen growth on disks incubated in urine from men, whereas *L. casei rhamnosus* 36 was the most inhibitory strain in urine from women. The observed differences between "male" and "female" urine specimens to support uropathogen interference were found to be statistically significant only for *L. fermentum* B54 ($P < 0.025$, Student's *t*-test).

Altogether, both *L. fermentum* B54 and *L. casei rhamnosus* 36 induced inhibition in seven of nine test subjects and, on average, this inhibition was evenly strong (see Tables 2, 3). The two strains had relatively high binding affinities, in contrast to *L. casei rhamnosus* ATCC 7469^T. However, *L. casei rhamnosus* 36 does not release the antiadhesive biosurfactant inhibitory for uropathogen adhesion, whereas *L. fermentum* B54 does. *L. casei rhamnosus* ATCC 7469^T was capable of inhibiting uropathogenic biofilm growth in five of nine urine specimens. This *Lactobacillus* did not adhere to silicone rubber in the parallel-plate flow chamber after 3 h, yet it was isolated from the disks in the urine experiments. This discrepancy might be explained by the presence of other bacteria in the urine, which could influence the adhesion of *L. casei rhamnosus* ATCC 7469^T to the disks. In addition, *L. casei rhamnosus* ATCC 7469^T also does not produce the antiadhesive biosurfactant. This means that besides biosurfactant production and binding affinity for the catheter material, other factors probably also play a role in the interference with uropathogenic biofilm growth, such as the ability of a *Lactobacillus* to survive and to multiply in urine or to coadhere with adhering uropathogens. Furthermore, these findings indicate that strains for possible clinical application should be carefully selected.

Higher binding affinities were not always associated with higher *Lactobacillus* counts on the silicone-rubber disks. The numbers of lactobacilli on the disks incubated in urine from female subjects that had been supplemented with *L. fermentum* B54, for instance, were relatively low. This observation might be attributable to accidental variations in the consistency of the urine. Due to the occurrence of hormonal changes during the various stages of a woman's life and to variations over the course of the menstrual cycle, the hormonal consistency of women's urine is not as constant as that of men's urine. Estrogen levels in urine differ over the menstrual cycle in a way that can alter *Lactobacillus* and uropathogen adhesion [2, 3, 12].

In summary, these data show that *L. fermentum* B54, *L. casei rhamnosus* 36, and *L. casei rhamnosus* ATCC

7469^T have the ability to inhibit the growth of a uropathogenic biofilm on silicone rubber for at least 8 days. The degree of inhibition depended on the *Lactobacillus* strain involved, and for *L. fermentum* B54 the inhibition was demonstrated to be sex-related. It appeared that several factors were involved in the discouragement of uropathogen growth, such as binding affinity and production of the antiadhesive biosurfactant, but the survival and growth of lactobacilli in urine and their ability to coadhere with adhering uropathogens may have played a role as well.

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