INFECTION AND IMMUNITY, Dec. 2002, p. 7169–7171 Vol. 70, No. 12
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.12.7169–7171.2002

Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* Species That Are Commonly Isolated from the Female Genital Tract

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Received 26 April 2002/Returned for modification 20 June 2002/Accepted 23 August 2002

Epidemiological studies suggest H_2O_2-producing lactobacilli protect women against gonorrhea. Here we demonstrate that *Lactobacillus crispatus* and *Lactobacillus jensenii*, the most common lactobacilli in the female genital tract, inhibit gonococci in both acidic and neutral pH conditions. Inhibition was neutralized by bovine catalase, suggesting that H_2O_2 is the primary mediator of inhibition.

Neisseria gonorrhoeae has a major impact on health worldwide, with the highest morbidity and mortality occurring in females. The most common site of gonococcal infection in females of reproductive age is the endocervix (9). A variety of host factors may contribute to the success or failure of *N. gonorrhoeae* to infect this site, including the types of commensal flora that inhabit the lower genital tract. Lactobacilli, the most common facultatively anaerobic bacteria of the vagina (13) and endocervix (7), play an important role in maintaining a normal vaginal ecosystem through the production of organic acids, bacteriocins, and hydrogen peroxide, all of which may protect against pathogens (13). Among the many microbes that inhibit *N. gonorrhoeae* in vitro (2, 8, 15, 16), lactobacilli are of particular interest due to reported associations between a reduced risk of gonorrhea and colonization by lactobacilli (1, 10, 15).

Lactobacillus crispatus and *Lactobacillus jensenii* are the predominant *Lactobacillus* spp. in the female lower genital tract. *Lactobacillus acidophilus* and *Lactobacillus gasseri* are also frequently isolated (1, 4, 6, 14). Inhibition of *N. gonorrhoeae* in vitro has been reported only for *L. acidophilus* (20), however, and for unidentified H_2O_2-producing clinical isolates of lactobacilli (15). The capacity of the predominant *Lactobacillus* spp. of the genital tract to inhibit *N. gonorrhoeae* is therefore unclear. Here we tested H_2O_2-producing strains of *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. acidophilus* for the capacity to inhibit two gonococcal laboratory strains (MS11 and FA1090) that are infectious in male volunteers (3, 18) and four clinical isolates of *N. gonorrhoeae* (Table 1) using a modified version of the agar overlay technique of Saigh et al. (15). Briefly, saline suspensions containing ca. 10^8 CFU of lactobacilli harvested from lactobacillus-MRS agar plates per ml were prepared. Fifty-microliter samples of the suspensions were inoculated onto heart infusion agar (HIA) that was adjusted to the desired pH (range, 5.8 to 7.6) prior to autoclaving. After 20 to 24 h of incubation, 7.5 ml of GC agar were poured onto the HIA plates and allowed to solidify. Suspensions (100 μl) containing ca. 10^6 CFU of the *Neisseria* species or *Escherichia coli* strains to be tested (target organisms) were spread onto the agar overlay and incubated for 20 to 24 h. The presence of a zone of growth inhibition around the target strain was considered positive for inhibition. For all experiments, the number of CFU in the lactobacillus and target cell suspensions was confirmed by standard serial dilution and culture. Growth of lactobacilli on HIA did not appreciably change the pH of the agar as determined by the use of pH indicators (data not shown). All media were purchased from Difco Laboratories (Detroit, Mich.). All incubations were at 37°C in 5% CO_2.

All four lactobacillus strains inhibited all gonococcal strains tested at low pH; only *L. jensenii* and *L. crispatus* inhibited *N. gonorrhoeae* at neutral pH. None of the lactobacilli inhibited *E. coli*, and only *L. jensenii* inhibited *Neisseria cinerea*, a commensal organism of the respiratory and genital tracts (Table 2). Serial dilution of the lactobacillus suspensions before inoculating the base agar resulted in visibly fewer lactobacilli within the inoculated region. On the basis of this semiquantitative evaluation of the number of lactobacilli present during the assay, *L. jensenii* consistently demonstrated higher levels of inhibition against *N. gonorrhoeae* than the other three lactobacillus strains (Fig. 1). Inoculation of the overlay agar with $>10^6$ CFU of *N. gonorrhoeae* significantly reduced the zones of inhibition and reproducibility of the assay (D. J. Kuch and A. E. Jerse, unpublished observations).

The primary mediator of inhibition in all four strains appeared to be H_2O_2 based on the ability to neutralize inhibition by incorporating bovine catalase (Worthington Biochemicals, Lakewood, N.J.) into the overlay medium. Inhibition of *N. gonorrhoeae* by *L. crispatus* and *L. jensenii* when cultured at pH 7.0 was neutralized by 5 U of bovine catalase per ml. At an acidic pH, a 10-fold-higher concentration of catalase was required to neutralize inhibition by *L. jensenii*, and 100-fold-more catalase was required to neutralize inhibition by *L. acidophilus* and *L. crispatus* (Table 3). This result was reproducible, although it appears to be inconsistent with the large inhibitory zones produced by *L. jensenii* compared to those produced by *L. crispatus* (Fig. 1). In general, more catalase was required to neutralize the inhibition by all lactobacilli as the

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Description	Source
N. gonorrhoeae		
FA1090	Laboratory strain	Our laboratory
MS11	Laboratory strain	H. S. Seifert[a]
400	Clinical isolate	H. S. Seifent
644	Clinical isolate	H. S. Seifert
229	Clinical isolate	H. S. Seifert
555	Clinical isolate	H. S. Seifert
N. cinerea 14685	Commensal species	ATCC[b]
Lactobacillus spp.		
L. acidophilus 4356	Human isolate	ATCC
L. crispatus 33197	Human urine isolate	ATCC
L. jensenii 25258	Human vaginal isolate	ATCC
L. gasseri 33323		ATCC
E. coli		
EFC-9	Fecal isolate	H. L. T. Mobley[c]
EFC-10	Fecal isolate	H. L. T. Mobley
J96	Uropathogenic strain	A. D. O'Brien[d]

[a] Northwestern University, Evanston, Ill.
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] University of Maryland, Baltimore, Md.
[d] Uniformed Services University of Health Sciences, Bethesda, Md.

pH of the base agar decreased (pH 7.6, 7.0, 6.6, 6.3, and 5.8) (data not shown). This observation may be explained by increased production of H_2O_2 by lactobacilli at low pH or increased stability of H_2O_2 at low pH (5).

These data support the hypothesis that commensal lactobacilli in the lower genital tract reduce the risk of gonococcal infection in women through the production of H_2O_2. It is not known, however, if lactobacilli produce sufficient amounts of H_2O_2 in the low-oxygen-tension environment of the lower genital tract (12, 19) for inhibition of *N. gonorrhoeae* to occur. Also, theoretically, gonococcal catalase should defend against

TABLE 2. Inhibition of *N. gonorrhoeae* by H_2O_2-producing *Lactobacillus* spp.[a] of the female genital tract under conditions of acidic versus neutral pH

Organism	Inhibition of organism by lactobacilli[b]							
	pH 5.8				pH 7.2			
	LA	LC	LJ	LG	LA	LC	LJ	LG
N. gonorrhoeae								
FA1090	+	+	+	+	−	+	+	−
MS11	+	+	+	+/−	−	+	+	−
229	+	+	+	+	−	+	+	−
397	+	+	+	+	−	+	+	−
400	+	+	+	+	−	+	+	−
644	+	+	+	+	−	+	+	−
N. cinerea	−	−	+	−	−	−	+	−
E. coli								
EFC-9	−	−	−	−	−	−	−	−
EFC-15	−	−	−	−	−	−	−	−
J96	−	−	−	−	−	−	−	−

[a] All *Lactobacillus* spp. produced H_2O_2 as detected in a qualitative H_2O_2 assay by McGroarty et al. (11).
[b] Abbreviations: LA, *L. acidophilus;* LC, *L. crispatus;* LJ, *L. jensenii;* LG, *L. gasseri.* Symbols: +, inhibition; −, absence of inhibition; +/−, weak inhibition.

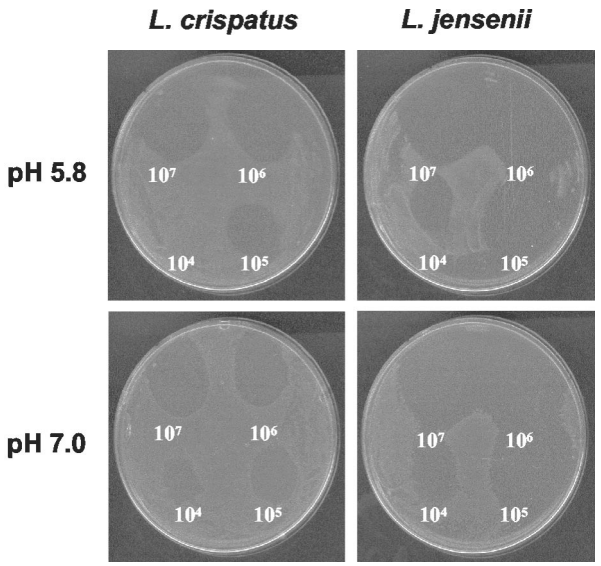

FIG. 1. Inhibition of *N. gonorrhoeae* strain FA1090 by *L. crispatus* and *L. jensenii* when cultured at acidic or neutral pH as detected in an agar overlay assay. An undiluted suspension containing 10^7 CFU of lactobacilli was inoculated onto the upper right quadrant, and 10-fold serial dilutions of this suspension were inoculated in a clockwise direction onto the base agar the day before inoculating with *N. gonorrhoeae*.

H_2O_2-producing lactobacilli by breaking H_2O_2 down to water and molecular oxygen. The inability of gonococcal catalase to neutralize H_2O_2-mediated lactobacillus inhibition in vitro could be due to the production of overwhelming amounts of H_2O_2 or to a lactobacillus-encoded factor that interferes with the ability of gonococcal catalase to cleave H_2O_2.

The relationship between culture pH and the relative inhibitory potential of each *Lactobacillus* species tested is intriguing in light of the cyclical change in pH of the female lower genital tract. The average pH values of vaginal and cervical mucus

TABLE 3. Effect of bovine catalase on inhibition of *N. gonorrhoeae* strain FA1090 by lactobacilli or increasing concentrations of H_2O_2

pH, lactobacillus, and/or H_2O_2 concn	Effect[a] of bovine catalase concn[b] in overlay agar on inhibition				
	0 U	0.5 U	5 U	50 U	500 U
Base agar pH 7.4					
L. crispatus	+	+	−	−	−
L. jensenii	+	+	−	−	−
18 mM H_2O_2	+	−	−	−	−
50 mM H_2O_2	+	+	−	−	−
100 mM H_2O_2	+	+	−	−	−
Base agar pH 5.8					
L. acidophilus	+	+	+	+	−
L. crispatus	+	+	+	+	−
L. jensenii	+	+	+	−	−
L. gasseri	+	+	+	−	−
18 mM H_2O_2	+	−	−	−	−
50 mM H_2O_2	+	+	−	−	−
100 mM H_2O_2	+	+	−	−	−

[a] Symbols: +, inhibition; −, absence of inhibition.
[b] Catalase concentration is expressed in units per mg of protein (1 U decomposes 1 μmol of H_2O_2 per min at 25°C and pH 7.0).

during the proliferative stage of the menstrual cycle are 4.6 (range, 3.3 to 7.4) and 6.8 (range, 5.5 to 8), respectively. A lower pH occurs in the luteal stage, with an average vaginal pH of 4.4 (range, 3.6 to 6.0) and endocervical pH of 6.1 (range, 5.1 to 8.4) (17). On the basis of these data, one might hypothesize that the capacity of commensal lactobacilli to protect women against gonorrhea may depend on both the species and stage of the menstrual cycle. The capacity of *L. jensenii* or *L. crispatus* to inhibit *N. gonorrhoeae* at both low and neutral pHs suggests that these strains may protect against gonorrhea more effectively than the other species tested.

We thank Afrin Begum for technical assistance and David Kuch for helpful reading of the manuscript.

This work was supported in part by USUHS intramural grant G173-HM.

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Editor: D. L. Burns