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Growth Inhibition of Metronidazole-Susceptible and Metronidazole-Resistant Strains of *Gardnerella vaginalis* by Lactobacilli In Vitro

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Metronidazole resistance was produced in susceptible *Gardnerella vaginalis* after subculture in the presence of metronidazole. Metronidazole-resistant gardnerellae were less susceptible to growth inhibition by *Lactobacillus* culture filtrates. A low pH (±4) and lactic acid accounted for 60 to 95% of inhibitory activity, and H_2O_2 accounted for only 0 to 30%. However, in the presence of myeloperoxidase, H_2O_2-producing lactobacilli decreased the viability of metronidazole-susceptible gardnerellae 2,000-fold.

Bacterial vaginosis (BV) is one of the most common vaginal infections encountered in medical practice and can represent a quarter of the cases presenting at sexually transmitted disease clinics and up to 50% of all diagnoses made therein (12). BV is characterized by a thin, homogeneous, greyish white discharge, a vaginal pH of greater than 4.5, the presence of clue cells, a positive KOH amine test, and a dramatic shift in vaginal flora from one which is normally dominated by facultative anaerobic lactobacilli to a flora with greatly reduced numbers of lactobacilli (100- to 1,000-fold decrease) and massive increases in the numbers of *Gardnerella vaginalis*, *Bacteroides* species, *Mycoplasma hominis*, and other anaerobic microorganisms. *G. vaginalis* is the predominant microorganism isolated from patients suffering from BV (2).

Lactobacilli are the most prevalent and often the most numerous bacteria found in a healthy human vagina (1). It is widely assumed that lactobacilli serve as a nonspecific form of defense against the overgrowth of potential pathogens in the vagina and thus aid in the maintenance of a healthy state (14). Lactobacilli have been shown to inhibit the growth of a number of microorganisms in vitro, including *G. vaginalis*. This inhibitory effect has been attributed variously to the production of dissociated short-chain fatty acids and a low pH (13) and to the production of hydrogen peroxide (H_2O_2) by lactobacilli (10). This study was carried out to determine whether vaginal *Lactobacillus* species could inhibit the growth of metronidazole (MTZ)-susceptible and -resistant strains of *G. vaginalis* and to define and quantify the nature of the inhibitory substance(s).

A number of strains of *Lactobacillus acidophilus*, *L. rhamnosus*, *L. jensenii*, *L. casei*, and *L. fermentum* were previously isolated from vaginal swabs taken from healthy women. Twelve *G. vaginalis* strains were isolated from vaginal swabs taken from women with suspected BV and identified by the protocol of Taylor and Phillips (20). In addition, type strains of *G. vaginalis*, NCTC 11292 and NCTC 10915, were compared with the clinical isolates. Lactobacilli were cultured in MRS broth or agar (Difco), and *G. vaginalis* was cultured on peptone, dextrose, starch broth, and agar (PDS agar [3]) and incubated under anaerobic conditions in an atmosphere of 65% N_2–30% H_2–5% CO_2 at 37°C for 24 h unless stated otherwise. By disc diffusion assay, seven of the clinical *G. vaginalis* isolates were susceptible to 50-μg MTZ discs (MTZ_{50}) and five strains were completely resistant (Table 1). MTZ-susceptible clinical isolates were significantly less susceptible to MTZ_{50} than were NCTC 11292 and NCTC 10915 ($P < 0.001$; Student t test). Kharsany et al. (9) found that the MICs of MTZ for 93 strains of *G. vaginalis* ranged from 2 to 128 μg/ml, with only one strain showing marked resistance (128 μg/ml). Only 1 year later, we have found that 42% of clinical *G. vaginalis* isolates show complete resistance to MTZ_{50}, albeit in a much smaller study. The resistance of *G. vaginalis* to MTZ may have arisen from widespread use of this drug to treat BV. We found that all the originally MTZ-susceptible *G. vaginalis* isolates became completely resistant to MTZ_{50}, but not to 2- and 10-μg clindamycin discs, after five subcultures in the presence, but not the absence, of the respective antibiotic. A closely related organism, *Helicobacter pylori*, which has been implicated as a major causative factor in chronic gastritis and peptic ulcer disease rapidly develops resistance to MTZ both in vitro (7) and in vivo (6). It has been suggested that this resistance is due to decreased uptake of the drug and/or an alteration in bacterial metabolism of the drug (11). The activity of MTZ is known to be enhanced under acidic conditions (4). Electron microscopic examination of *G. vaginalis* 1 (MTZ resistant), 82141 (MTZ susceptible), and NCTC 11292 (MTZ susceptible) has shown that *G. vaginalis* 1 and 82141 possess a denser surface fibrillar layer consisting of fimbriae than that of NCTC 11292 (unpublished observations). The presence of fimbriae on gardnerellae has been reported previously (18). This outer surface layer may impede the penetration of MTZ into the bacterial cell, rendering these isolates less sensitive to MTZ. The clinical isolates may have been exposed to selective pressures to which the type strains have not, including the presence of MTZ in vivo, and thus developed a degree of resistance to this drug.

Simultaneous and deferred antagonism assays were performed to assess the ability of lactobacilli to inhibit the growth of *G. vaginalis* isolates. A 1-ml aliquot of 10^9 washed *Lactobacillus* cells or 1 ml of culture filtrate was added to 10 ml of molten MRS agar, poured into a petri dish, and allowed to solidify. Molten PDS agar was overlaid, and a range of *G. vaginalis* concentrations was spread on solidified agar. Plates were examined for the growth of *G. vaginalis* after 24, 48, and

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TABLE 1. Susceptibilities of clinical isolates of *G. vaginalis* to MTZ

G. vaginalis strain	Source of isolate	Radius of inhibition zone (mm)[a]
NCTC 10915	Type strain	15.5
NCTC 11292	Type strain	16
49506	Vaginal swab	12.5
49453	Vaginal swab	12.5
50726	Vaginal swab	16
82141	Vaginal swab	6.5
82141 H	Vaginal swab	6.5
54203	Vaginal swab	1.5
2	Vaginal swab	4
1	Vaginal swab	0
6	Vaginal swab	0
53111	Vaginal swab	0
53100	Vaginal swab	0
54204	Vaginal swab	0

[a] Surrounding MTZ_{50} disc.

72 h of incubation. The results after 48 h were determined, as no further growth of *G. vaginalis* was observed after this time. Deferred antagonism by agar well diffusion assay was then used to measure the inhibition due to all 23 *Lactobacillus* isolates tested and to partially characterize the inhibition due to the following lactobacilli selected for further study: *L. fermentum* 1 and *L. acidophilus* L44l, L44s, L8, and J1l. Briefly, 400 μl of *Lactobacillus* culture filtrates was added to 16-mm-diameter wells cut in freshly poured PDS agar containing 3×10^9 *G. vaginalis* cells per ml. Plates were incubated for 48 h, and then the inhibition zone radii were measured from the outer edges of wells to the edges of bacterial growth.

All 23 *Lactobacillus* isolates completely inhibited the growth of *G. vaginalis* NCTC 11292 in the sandwich plate assay in a bacteriostatic manner. The well diffusion assay indicated that the degree of inhibition of *G. vaginalis* growth by *Lactobacillus* spp. varied (Table 2). There was a highly significant negative correlation between the pH of *Lactobacillus* culture filtrates and inhibition zone size ($P < 0.01$; Kendall rank correlation coefficient). The lower the pH produced by lactobacilli, the larger the inhibition zone of gardnerellae. Of the five *Lactobacillus* strains chosen for further study, the *L. fermentum* 1 culture filtrate produced significantly less inhibition of *G. vaginalis* growth than did the *L. acidophilus* strains tested ($P < 0.05$; Student *t* test). In addition, the MTZ-susceptible *G. vaginalis* isolates (NCTC 11292 and 82141) were significantly more susceptible to inhibition by *Lactobacillus* supernatants than was MTZ-resistant *G. vaginalis* 1, with NCTC 11292 being the most susceptible of all ($P < 0.05$; Student *t* test).

Increasing the pH of *Lactobacillus* culture filtrates to pH 6.5 with NaOH removed approximately 60% of the inhibitory activities associated with *L. acidophilus* culture filtrates and approximately 95% of the activity of *L. fermentum* 1 culture filtrates (Fig. 1). The predominant acid produced by lactobacilli, as determined by high-performance liquid chromatography (HPLC) with a 5 octyldecyl silane column (Anachem), was lactic acid (Table 3). The addition of lactic acid to MRS broth alone and to neutralized *Lactobacillus* culture supernatants restored inhibitory activity (unpublished observations), confirming previous reports (13, 19) that lactic acid and a low pH are inhibitory to *G. vaginalis*. Organic acids act as protonophores and at high concentrations increase the inward leakage of hydrogen ions into bacterial cells so that the efflux is not rapid enough to maintain an alkaline cytoplasm. The cytoplasm therefore becomes acidic, which disables energy yielding and transport processes, resulting in growth inhibition of bacteria (8). Further experimentation is required to elucidate the mechanism of resistance of gardnerellae to organic acids and a low pH.

TABLE 2. Inhibition of *G. vaginalis* NCTC 11292 by *Lactobacillus* culture supernatants in a well diffusion assay

Species and strain	Radius of inhibition zone surrounding well (mm)	pH of MRS culture supernatant
L. acidophilus L13cl	14.5	3.95
L. acidophilus L13wh	16.0	3.98
L. acidophilus J1s	15.5	3.84
L. acidophilus J11	11.5	3.95
L. acidophilus L44l	14.5	3.90
L. acidophilus L44s	14.5	3.92
L. acidophilus L8	11.0	4.05
L. acidophilus 4504	13.0	4.07
L. acidophilus L18A	14.0	3.97
L. acidophilus L17	14.0	3.96
L. acidophilus J55	12.0	3.91
L. casei subsp. *alactosus* J102A	14.0	4.05
L. casei L40	13.0	3.97
L. fermentum 1	6.5	4.16
L. jensenii J4	11.0	4.45
L. jensenii J24	13.0	4.13
L. jensenii J92	14.0	4.33
L. jensenii L42	14.0	3.97
L. jensenii L14	12.5	3.98
L. jensenii L48	14.5	3.98
L. rhamnosus J3s	12.5	4.07
L. rhamnosus J32	11.5	3.93
L. rhamnosus lcr	15.0	3.91

All 23 *Lactobacillus* isolates tested, with the exception of *L. fermentum* 1, produced H_2O_2 on MRS agar plates supplemented with tetramethylbenzidine and horseradish peroxidase after 48 h of incubation under anaerobic conditions followed by 10 min of exposure to air as described previously (15). Catalase treatment (30 μg/ml) to denature H_2O_2 in the culture supernatants tested in well diffusion assays removed approxi-

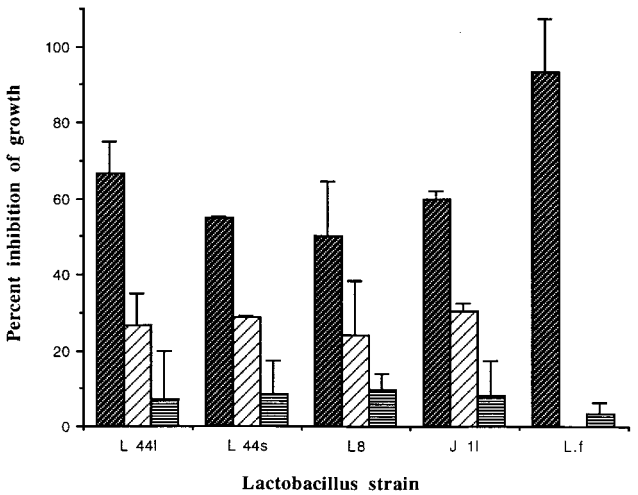

FIG. 1. Effects of catalase treatment and pH neutralization on growth inhibition of *G. vaginalis* NCTC 11292 by *Lactobacillus* culture filtrates. Data are the means of three separate determinations, with error bars showing standard deviations. ▨, supernatant adjusted to pH 6.5; ▨, supernatant treated with catalase; ▤, supernatant treated with catalase and adjusted to pH 6.5. L.f, *L. fermentum.*

TABLE 3. Organic acid production by *Lactobacillus* isolates in MRS broth[a]

Species and strain	Concn (mM) under incubation conditions			
	Aerobic and shaken		Anaerobic and static	
	Lactic acid	Succinic acid	Lactic acid	Succinic acid
L. acidophilus L44l	30	15	63	12
L. acidophilus L44s	30	27	55	8
L. acidophilus L8	37	2	43	2
L. acidophilus J11	56	2	58	1
L. fermentum 1	33	15	43	1

[a] The organic acid concentrations present in *Lactobacillus* supernatants from cultures cultivated under aerobic and anaerobic conditions were determined by HPLC with reference to a set of standard curves.

mately 0 (*L. fermentum* 1) to 30% (*L. acidophilus* L44l) of *Gardnerella* growth inhibition (Fig. 1). The amounts of H_2O_2 produced by the five *Lactobacillus* strains chosen for further study were determined by scopoletin oxidation, as described by Root et al. (17). *L. fermentum* 1 did not produce any H_2O_2, whereas *L. acidophilus* L44l, L44s, L8, and J1l produced 8, 8, 6, and 4 μM H_2O_2, respectively, under agitated, aerobic conditions. No detectable H_2O_2 ($<10^{-7}$ M) was produced under anaerobic conditions or static, aerobic conditions. This may partly explain the lower toxicity of the *L. fermentum* 1 culture filtrate for *G. vaginalis* isolates (Fig. 2).

Lactobacilli inhibited the growth of *G. vaginalis* to a greater extent when cultured under static, anaerobic conditions rather than under agitated, aerobic conditions. Alternative metabolic pathways come into action when lactobacilli are grown in an aerobic environment. NADH and pyruvate oxidases compete with lactate dehydrogenase for NADH formed in glycolysis, which favors the production of H_2O_2, carbon dioxide, and acetylphosphate rather than the production of lactate (21). Our findings suggest that lactic acid and a low pH are more

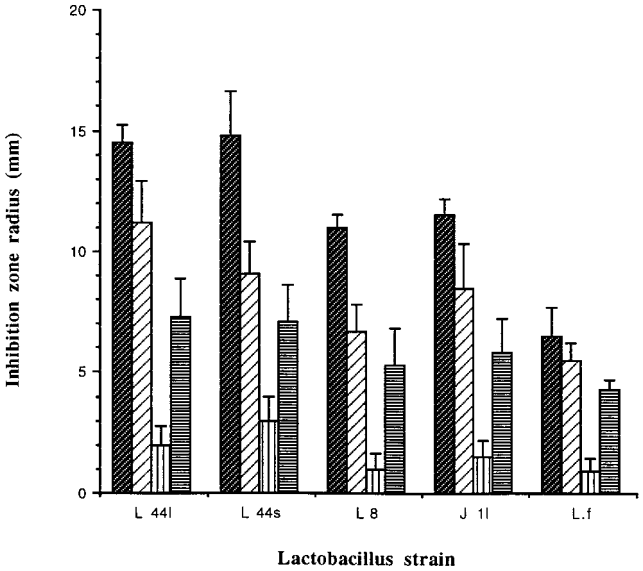

FIG. 2. Growth inhibition of MTZ-sensitive and -resistant *G. vaginalis* isolates by *Lactobacillus* culture filtrates. Data are the means of three separate determinations, with error bars showing standard deviations. ▨, *G. vaginalis* NCTC 11292 (MTZ sensitive); ▨, *G. vaginalis* 82141 (MTZ sensitive); ▥, *G. vaginalis* 82141 (MTZ resistant); ▤, *G. vaginalis* 1 (MTZ resistant). L.f, *L. fermentum.*

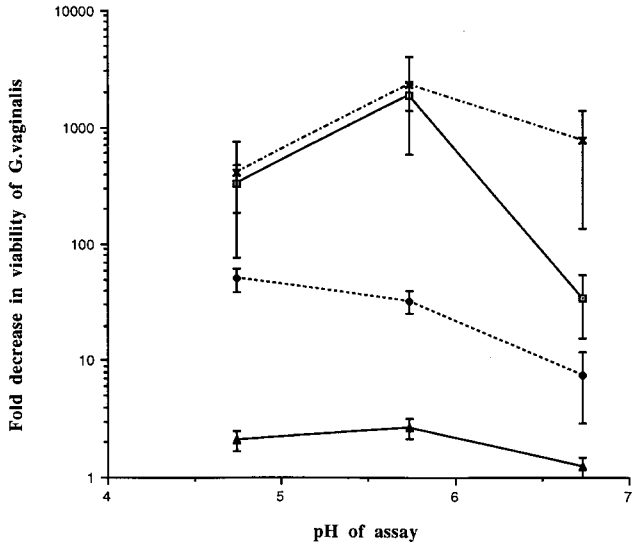

FIG. 3. Bactericidal effect of *L. acidophilus* L44l in combination with myeloperoxidase and chloride on *G. vaginalis* NCTC 11292, 82141, and 1. *L. acidophilus* L44l cultured under anaerobic conditions was significantly less bactericidal than was L44l cultured under aerobic conditions ($P < 0.05$; Student *t* test). *G. vaginalis* 1 was significantly less susceptible to the bactericidal effect than were strains NCTC 11292 and 82141 ($P < 0.05$; Student *t* test). Data are the means of three separate determinations, with error bars showing standard deviations. ◆, *G. vaginalis* NCTC 11292 (MTZ sensitive), cultured under static and anaerobic conditions; ×, *G. vaginalis* NCTC 11292 (MTZ sensitive), cultured under aerobic conditions with agitation; ⊡, *G. vaginalis* 82141 (MTZ sensitive), cultured under aerobic conditions with agitation; ▲, *G. vaginalis* 1 (MTZ resistant), cultured under aerobic conditions with agitation.

important for *G. vaginalis* growth inhibition than is H_2O_2 in vitro. Since the vagina tends toward low-level oxygen tension, the lactobacilli present would be expected to favor lactate rather than H_2O_2 as the major metabolic end product in vivo.

The majority of *G. vaginalis* growth inhibition was abolished by the combination of catalase treatment and pH neutralization. Heat treatment (121°C, 15 min) of supernatants had no effect on the sizes of inhibition zones, discounting the possibility of heat-labile substance(s) playing a role in the inhibition of *G. vaginalis* growth. All MTZ-susceptible *G. vaginalis* isolates became resistant to MTZ_{50} when grown in the presence of this drug for five subcultures. When they were tested in well diffusion assays, these resistant variants showed markedly decreased sensitivities to *Lactobacillus* culture filtrates (Fig. 2).

Nagy et al. (16) and Eschenbach et al. (5) reported the isolation of H_2O_2-producing lactobacilli from the vaginas of a majority of healthy subjects but from the vaginas of only 6 and 23%, respectively, of women with BV. Klebanoff et al. (10) postulated that H_2O_2 production by lactobacilli may contribute to the control of vaginal flora, particularly in the presence of peroxidase and a halide. The toxicities of H_2O_2-producing lactobacilli in the presence of myeloperoxidase and chloride ions, both of which are found in the vagina, toward our *G. vaginalis* isolates were tested by the method of Klebanoff et al. (10). Briefly, washed suspensions of logarithmic-phase lactobacilli containing 10^9 cells per ml were incubated with *G. vaginalis* at a concentration of 10^7 cells per ml at 37°C for 1 h in the presence of myeloperoxidase (75 mU/ml) and NaCl (0.01 M) under either anaerobic or aerobic conditions with or without agitation. The viable counts of *G. vaginalis* and lactobacilli were determined by culturing aliquots of the mixed suspensions on PDS agar and MRS agar. The viability of *G. vaginalis* was reduced by up to 2,000-fold at an optimum pH

between 5 and 6 (Fig. 3), compared with the 3,800-fold reduction reported by Klebanoff et al. (10). The inhibition observed with this system was bactericidal rather than bacteriostatic, as in the well diffusion assay. As in the Klebanoff study, H_2O_2 was implicated as the toxic factor, as the addition of catalase or the use of non-H_2O_2-producing lactobacilli resulted in virtually no bactericidal activity. *G. vaginalis* 1 (MTZ resistant) was significantly less susceptible to killing than was *G. vaginalis* 82141 (MTZ sensitive) or NCTC 11292 (MTZ sensitive) ($P < 0.05$; Student *t* test). This system was effective against *G. vaginalis* only under incubation conditions which favored the production of H_2O_2, i.e., aerobic and agitated. Under conditions more closely resembling those of the vagina, static and anaerobic (22), *G. vaginalis* was not killed. These findings raise the question of how important this system would be in the control of *G. vaginalis* growth in vivo, given the low-level oxygen tension of a healthy vagina, and suggest that the production of short-chain fatty acids, such as lactic acid, and a low pH are of more significance in the vaginal ecosystem.

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