Gardnerella vaginalis growth is eliminated by a novel narrow-spectrum factor secreted by *Lactobacillus jensenii*

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Abstract

Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age. While the vaginal microbiota of health women is dominated by lactobacilli, it shifts to favor other bacteria, specifically *Gardnerella vaginalis* (Gv), in women with BV. Lactobacilli produce antimicrobial factors including hydrogen peroxide, lactic acid, and bacteriocins. We hypothesize that besides these factors, lactobacilli and Gv influence each other's growth within the vaginal environment through additional unidentified factors. To assess the interaction of Gv with *Lactobacillus jensenii* (Lj), one of the vaginal lactobacilli, we grew the organisms in transwell co-culture in medium simulating vaginal fluid. Lj significantly reduced Gv growth at 20 h post inoculation (hpi) and eliminated Gv at 24 hpi. Cell-free culture supernatant (CFS) of Lj harvested at 16 hpi did not affect Gv growth while Lj CFS harvested at 24 hpi eliminated Gv growth entirely. Growth of Lj was unaffected by either 16h- or 24h-CFS. Additionally, 24h-CFS did not affect growth of other vaginal lactobacilli (*L. gasseri* and *L. crispatus*), *Enterococcus faecalis*, *Staphylococcus epidermidis*, or *Escherichia coli*. Time course experiments using 24h-CFS showed that elimination of Gv began at 2 hpi and was complete by 4 hpi. To rule out hydrogen peroxide and D-lactic acid as the responsible agents, we tested concentrations double that produced by Lj; neither inhibited Gv growth. Fractionation of 24h-CFS using 100-, 30-, 10-, and 5-kDa molecular weight cut off columns revealed that the fraction containing ≤ 30-kDa proteins retained the inhibitory effect while the fraction containing less than ≤ 10-kDa proteins had no effect. These results suggest that: 1) a novel 10- to 30-kDa Lj secreted product eliminates Gv and 2) the effect of this factor is unique to Gv.

Introduction

Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age (14-49 years of age). In the U.S., about 21 million women in this range suffer from BV (30%). Of those women, it is estimated that 84% will remain asymptomatic while others exhibit symptoms, including thin greyish vaginal discharge, foul-smelling fishy odor, vaginal itching, and burning sensation during urination. BV is associated with various health complications including increased risk of premature delivery or miscarriage, pelvic inflammatory disease, and increased susceptibility to sexually transmitted diseases.

In healthy women, the vaginal ecosystem is dominated by the *Lactobacillus* spp. *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*. Through their production of antimicrobial compounds, including lactic acid, hydrogen peroxide, and bacteriocins, lactobacilli form a critical line of defense against potential pathogens. Lactobacilli provide additional protection through coaggregation, competitive exclusion, and immune-modulation. Disruption of the population of lactobacilli along with an increase in vaginal pH to 4.5 or higher allows overgrowth of pathogenic microorganisms. Specifically, BV is associated with a decrease in the numbers of lactobacilli and an increase in anaerobic bacteria, especially *Gardnerella vaginalis* (Gv), the most common microorganism identified from the vaginal samples of women with BV. Gv harbors a variety of virulence factors including sialidase and vaginolysin. Sialidase hydrolyzes sialic acid residue from mucus sialoglycans in the vagina and catabolizes free carbohydrate thereby contributing to the degradation of the vaginal mucus barriers. Vaginolysin is a pore gorming toxic compound belonging to the cholesterol-dependent cytolysin family and facilitates the lysis of target cells such as vaginal epithelial cells.

The rod-shaped, anaerobic gram-positive *L. jensenii* (Lj) is second most common species of lactobacilli indigenous to the vaginal tract of healthy woman. Lj accounts for 23% of all vaginal colonies, following *L. crispatus* at 32%. Research has shown that Lj also inhibits *Neisseria gonorrhoeae* adherence to and invasion of epithelial cells. Additional studies suggested that Lj contain at least two constitutively produced inhibitory proteins that target *N. gonorrhoeae.* Considering the complexity of the microbial interaction within the vaginal environment, Lj may target pathogenic bacteria such as GV through additional yet to be identified mechanisms. While previous *in vitro* and clinical studies investigated the complex interaction between lactobacilli and Gv, a detailed and an accurate assessment of such interaction requires growth medium that closely mimics the conditions within the vagina and supports the growth of both lactobacilli and Gv. One such medium is the medium simulating vaginal fluid (MSVF) that contains many of the components of vaginal fluid, including glycogen, mucin, albumin, acetic acid, and lactic acid.

In this study, we examined the possibility that upon growth in MSVF, Lj produces secreted factor(s) that inhibits or eliminates the growth of Gv.

Hypothesis

In addition to H_2O_2 and lactic acid, lactobacilli and Gv influence each other's growth within the vaginal environment through unidentified factors

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Methods

Co-culture experiments were done using MSVF in Transwell® plates to physically separate Gv from Lj, while allowing the medium and factors produced by the bacteria to flow across a permeable membrane. The upper chamber was inoculated with 10^4 colony forming units (CFU) of Gv and the lower chamber with 10^4 CFU of Lj. Plates were sealed with a gas permeable membrane to prevent desiccation and incubated for the designated time at 37°C under 5% CO_2.

For quantification, bacteria from each chamber were individually collected and serially diluted (tenfold). Ten-µL of aliquots of each dilution on de Man Rogosa Sharp (MRS) for Lj or chocolate agar for Gv. Statistical significance was determined by unpaired, two-tailed *t*-test using GraphPad Prism 9.3.1(471). For each experiment, values represent the average of three independent experiments ± SEM; *P* values are indicated in the legend.

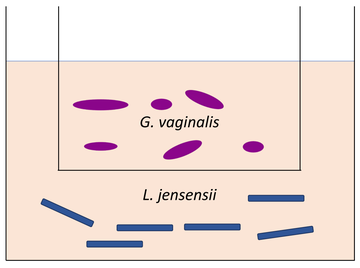

Figure 1. Diagram depicting Gv and Lj co-culture

Results

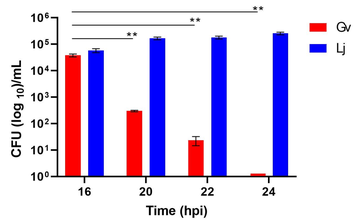

Figure 2. Lj significantly reduced the growth of Gv in co-culture. Co-culture experiments were conducted as described in Methods. Growth (CFU/ml) was assessed at 16, 20, 22, and 24 h post inoculation (hpi). **Growth of Gv was significantly reduced at 20 and 22 hpi (2 and 3 log respectively) and was eliminated at 24 hpi** (**, *P* <0.01).

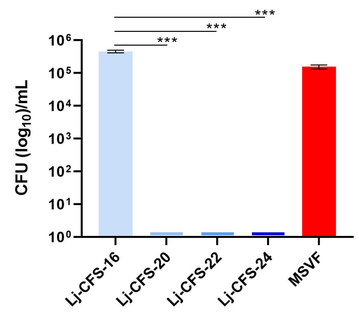

Figure 3. Growth in Lj cell-free culture supernatant eliminated Gv when produced from Lj cultures grown for more than 16 h. Lj was grown in MSVF at 37°C using microtiter well plates under 5% CO_2 for 16, 20, 22, or 24 h. Cell-free supernatant (Lj-CFS) from each time point was harvested by centrifugation followed by filtration to remove any residual bacteria. Gv (10^4 CFU/mL) was inoculated in 1 mL of Lj-CFS from each time point in a 24-well microtiter plate. After 24 h at 37°C under 5% CO_2, Gv growth was quantitated. **Lj-CFS harvested after 16 hpi and used as a growth medium eliminated Gv** (***, *P* <0.001).

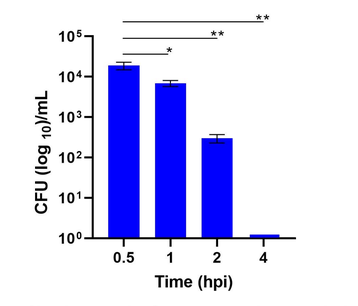

Figure 4. Lj-CFS-24 eliminated Gv growth at 4 hpi when used as a growth medium. Lj-CFS-24 was prepared and used as a culture medium for Gv as described in Fig. 3. Samples were obtained at the indicated time points and the CFU/mL were determined. **Gv growth was significantly reduced by 1 hpi and eliminated at 4 hpi** (*, *P* <0.05; **, *P* <0.01).

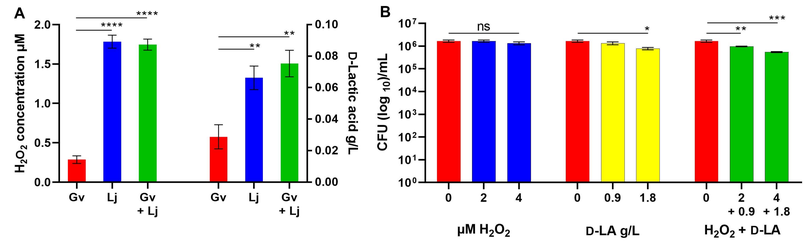

Figure 5. Hydrogen peroxide (H_2O_2) and D-lactic acid at levels produced by Lj modestly affect Gv growth. *(A)* Lj and Gv (individually or in co-culture) were grown in MSVF for 24 h as described above. Cells were pelleted and the supernatants collected to determine the levels of H_2O_2 and D-lactic acid produced using the Pierce™ Quantitative Peroxide Assay Kit and the R-Biopharm Inc. E Lactic Acid Test Kit, respectively. **Lj produces significantly greater amounts of H_2O_2 and D-lactic acid whether grown alone or with Gv** (**, *P* <0.01; ****, *P* <0.0001)**.** *(B)* Gv was grown in the presence of exogenous H_2O_2 and/or D-lactic acid at physiological levels produced by Lj (PL) and two times the PL (2X PL) – 2 µM and 4 µM and 0.9 g/dL and 1.8 g/dL, respectively – or both in MSVF for 24 h. Samples were obtained at 24 hpi and the CFU/mL were determined (ns, no significance; *, *P* <0.5, **, *P* <0.01; ***, *P* <0.001). **H_2O_2 did not affect Gv growth at PL or 2X PL, while D-lactic acid alone at 2X PL decreased Gv growth by 0.3 logs. PL of H_2O_2 and D-lactic acid in combination decreased Gv growth by 0.2 logs and at 2X PL, by 0.5 logs. These decreases are slight compared to the >5-log reduction produced by Lj in the co-culture (Fig. 2).**

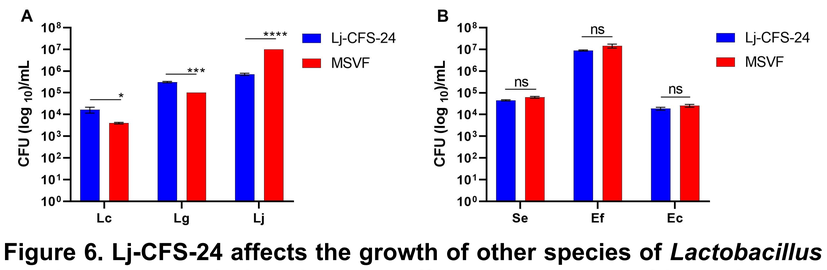

Figure 6. Lj-CFS-24 affects the growth of other species of *Lactobacillus* and its own growth, but does not affect other genera of bacteria. Each species was grown in Lj-CFS-24 or MSVF for 24 h. samples were obtained at 24 hpi and the CFU/mL were determined (ns, no significance; *, *P* <0.5, ***, *P* <0.001; ****, *P* <0.0001). *(A)* Species of vaginal lactobacilli: *L. crispatus* (Lc), *L. gasseri* (Lg), and Lj. **In both media, Lj growth was higher than that of Lg and Lc. Growth of Lc and Lg was incrementally enhanced (~ 0.5 log) by growth in Lj-CFS-24 compared to MSVF. Lj inhibited its own growth by ~1 log.** *(B)* Other genera and species of vaginal bacteria: *Staphylococcus epidermidis* (Se), *Enterococcus faecalis* (Ef), and *Escherichia coli* (Ec). **Growth in Lj-CFS-24 did not affect any of the other bacteria.**

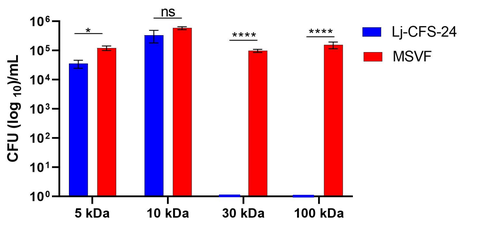

Figure 7. The factor produced by Lj that eliminates Gv resides within the 10- to 29-kDa range. Lj-CFS-24 and MSVF (as a control) were fractionated using 100-, 30-, 10-, and 5-kDa molecular weight cut off columns according to the manufacturers' instructions. Filtrates from each fractionation column were mixed with MSVF at a 1:1 ratio to provide sufficient nutrients for bacterial growth. Gv was inoculated into each mixture at 10^4 CFU/mL in 24-well microtiter plates and incubated as described in Fig. 3. Samples were obtained at 24 hpi and the CFU/mL were determined (ns, no significance; *, *P* <0.5; ****, *P* <0.0001). **While the Lj-CFS-24/5 and Lj-CFS-24/10 fractions had no effect on Gv growth, the Lj-CFS-24/30 and Lj-CFS-24/100 fractions eliminated Gv. Therefore, the molecule that eliminates Gv, which is most likely a protein, resides in the 10- to 29-kDa range.**

Conclusions

- **When grown in a medium and environment simulating the vaginal environment, Lj eliminates Gv growth through a mechanism not directly related to lactic acid or H_2O_2.**
- **This bactericidal effect is unique to Gv, producing minimal effect on the growth of other *Lactobacillus* spp., and no effect on several other bacterial genera found in the vaginal microflora.**
- **The effect, which occurs within 4 h of Gv growth in Lj-CFS -24, is mediated by a potential secreted protein with a molecular weight between 10- to 29-kDa.**