Glycogen availability and pH variation influence the growth of vaginal *Lactobacillus* species and *Gardnerella vaginalis*

Stephany Navarro[1], Habib Abla[2], Betsaida Delgado[3], Jane A. Colmer-Hamood[1,4], Gary Ventolini[5], Abdul N. Hamood[1]

[1]Dept. of Immunology & Molecular Microbiology, TTUHSC, Lubbock; [2]School of Medicine, TTUHSC, Lubbock; [3]Honors College, TTU, Lubbock; [4]Dept. of Medical Education, TTUHSC, Lubbock; [5]School of Medicine at the Permian Basin, TTUHSC, Odessa

TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER™

Abstract

Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age. Previous studies showed that, in women diagnosed with BV, a shift in the population of *Lactobacillus* and *Gardnerella* species occurs. The exact mechanism for this phenomenon is not clearly defined. *Gardnerella vaginalis* (GV) exists within the vaginal microflora of healthy women, but their numbers are extremely low compared with those of the *Lactobacillus*. Prior to the onset of BV, either host or bacterial factors, or both, increase the vaginal pH to 4.5. We hypothesize that environmental changes within the vagina, specifically the availability of nutrients and pH variation, influence the microbial population. The vaginal pH of healthy women is 3.8- 4.2 favoring the growth of lactobacilli while that of women with BV is 4.5 or above, favoring the growth of GV. Metabolism of free glycogen within the vaginal fluid is likely to be essential for the colonization and growth of vaginal microbiota. In this study, we used the medium simulating vaginal fluid (MSVF) to assess the growth of the *Lactobacillus* strains *L. jensenii, L. gasseri,* and *L crispatus* as well as GV in the presence of different glycogen concentrations and at pH 4, 4.5, and 5. MSVF contains glucose and glycogen as carbon sources. Only *L. gasseri* grew in the absence of glycogen; while only *L. jensenii* survived at all pH conditions. *L. crispatus* was the most restricted, surviving only at pH 5 in 5 and 10 g/L glycogen. Of the lactobacilli, *L. jensenii* was the most versatile. GV showed the highest growth (3 logs over starting CFU).These results suggest that: 1) within the vagina, glycogen is essential for the growth of different *Lactobacillus* spp. as well as for GV; and 2) vaginal pH influences the ability of the strains to utilize glycogen.

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 serious symptoms, including thin greyish vaginal discharge, foul-smelling fishy odor, vaginal itching, and burning sensation during urination. BV is also associated with various health complications such as an increased risk of premature delivery, miscarriage, pelvic inflammatory disease, and increased susceptibility to sexually transmitted diseases. In healthy women, the vaginal microflora is primarily composed of *Lactobacillus* species (dominant flora). BV is associated with a shift in the vaginal microbial population; a decrease in *Lactobacillus* and an increase in anaerobic bacteria, specifically *G. vaginalis* (GV). This shift in bacterial population is associated with an increase in pH of the vaginal environment to 4.5 or higher.

Despite numerous studies, the exact mechanism through which the shift in the vaginal microflora occurs during BV is not defined. GV exists within the vaginal microflora of healthy women, but their numbers are extremely low compared with that of *Lactobacillus*. Prior to the onset of BV, either host or bacterial factors, or both, increase the vaginal pH to 4.5 or above. This pH change significantly increases the GV population while reducing the population of *Lactobacillus*. To address the role of pH in the population shift it is essential to grow both lactobacilli and *G. vaginalis* in one medium and assess the effect of a pH change on their growth. For diagnostic and research purposes, *Lactobacillus* spp. are grown in de Man, Rogosa and Sharpe (MRS) medium while GV is grown in New York City III (NYC) medium. Both organisms are grown at 37°C under 5% CO_2. We recently showed that the previously described medium simulating vaginal fluid (MSVF) supports the growth of lactobacilli and GV. MSVF contains many of the components of vaginal fluid, including glycogen, mucin, albumin, acetic acid, and lactic acid.

Glycogen production and deposition within the vaginal and cervical epithelial cells is influenced by the levels of estrogen and progesterone hormones. Once released in the vaginal environment, glycogen is broken down into smaller metabolites by host as well as microbial enzymes. Amylase, produced by the vaginal epithelial cells, converts a considerable amount of the glycogen to maltose, maltotriose, and α-limit dextrin. *Lactobacillus* extracellular cell-attached debranching enzyme, pullulanase, breaks down glycogen into maltotriose and maltodextrins. While the GV α-glucosidase reduces glycogen to glucose. The activity of pullulanase and α-glucosidase varies in response to the environmental pH. Pullulanase is active at pH 2.5-6.5 with optimal activity at pH 4.5-5, whereas α-glucosidase is active at pH 4-8, optimum pH 6-7.

At this time, several questions regarding the availability of glycogen and the efficiency of its utilization by either lactobacilli or GV need to be addressed. Based on the observed difference in the pH level for the optimum activity of pullulanase or α-glucosidase, we hypothesized that variations in the vaginal pH contribute to the population shift in the vaginal microflora, specifically among lactobacilli and GV. At a certain pH optimum for the activity of its glycogen-metabolizing enzyme, lactobacilli or GV may grow better, produce more of their extracellular factors, and attach more efficiently to the vaginal epithelial cells. In this study, we examined this hypothesis by assessing the growth of *L. jensenii*, *L. gasseri*, *L. crispatus*, and GV in MSVF with different levels of pH and at different glycogen concentrations.

Funding

This project was funded in part by the Department of Obstetrics and Gynecology, TTUHSC-Permian Basin, Odessa, TX

Hypothesis

Variations in the glycogen level at different starting pH conditions influence the growth of either lactobacilli or *Gardnerella vaginalis*, or both.

Methods

Growth medium: *Lactobacillus* strains and GV were grown in the medium simulating vaginal fluid (MSVF) (**Table 1**).

Table 1. Components in MSVF

Component (g/L)	Function
Glucose (10)	Metabolized by lactobacilli to lactic acid
Glycogen (10)	Broken down by α-amylase to glucose
Lactic acid (2)	Produced by lactobacilli to lower vaginal pH to 3.8-4.2
Acetic acid (1)	Produced by anaerobic bacteria during glucose fermentation
Albumin (2)	Protein found in cervical mucus
Mucin (0.25)	Glycoprotein of cervical mucus; contributes to viscoelastic gel properties of mucus
Urea (0.5)	No defined function
NaCl (3.5)	Electrolyte important for regulation of lubrication and transudation
KCl (1.5)	Same as NaCl
Tween 80 (1.064)	No defined function
Cystein-HCl (0.5)	No defined function
Water (0.89)	Vaginal fluid is 90-95% water

Strains and growth conditions: All strains were obtained from ATCC: *G. vaginalis*, *L. jensenii*, *L. crispatus*, and *L. gasseri*. *Lactobacillus* spp. were grown in MRS broth and GV was grown in NYC broth at 37ºC under 5% CO_2 for 48 h. Between 10^4 colony forming units (CFU)/mL of bacterial cells were inoculated into MSVF in wells of a microtiter plate. The plates were sealed with a gas-permeable membrane and incubated at 37ºC under 5% CO_2 for up to 16 days. At specific time points, the cultures were serially diluted (tenfold) and 10-µL aliquots were plated on MRS agar or chocolate agar plates for lactobacilli and GV, respectively. The plates were incubated for 48 h at 37ºC under 5% CO_2. CFU were calculated by the formula (CFU counted × dilution factor) × 100 = CFU/mL.

Experimental conditions: To determine whether glycogen is essential for growth of the strains, we varied the glycogen content of the MSVF from 10 g/L (normal amount) to 5 g/L to 0 g/L. To determine the effect of pH on the growth and ability of the strains to metabolize glycogen, we adjusted the pH of the MSVF to 4, 4.5, and 5. All strains were tested in MSVF at each pH and each glycogen concentration. Points on graphs represent means of 3 independent experiments ± SEM.

Results

Glycogen is essential for the growth of GV under starting pH conditions of 4.5 or 5.0

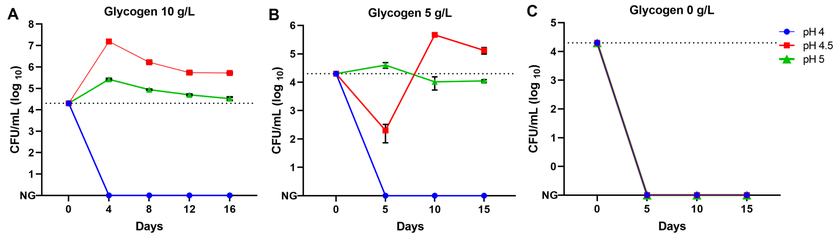

Figure 1. (A) GV grows at pH 4.5 and 5 in the presence of 10 g/L glycogen, but not it does not grow at pH 4. The peak of growth occurred at 4 days-post-inoculation (dpi) with a gradual decline at 8, 12, and 16 dpi. The greatest increase in growth (3 logs) occurred in MSVF pH 4.5 at 4 dpi. **(B)** GV also grows in presence of 5 g/L glycogen at pH 4.5 and 5, although its growth did not reach the level attained in 10 g/L glycogen at pH 4.5. At pH 4.5, there was a sharp drop in CFU/mL at 5 dpi with a steep increase at 10 dpi. There was little increase or decrease in growth at pH 5 (maintenance of starting CFU/mL). As seen in (A), GV did not grow at pH 4. **(C)** GV requires glycogen for its growth in MSVF. No growth occurred without glycogen in the medium at any starting pH. Dotted lines on all graphs, in this figure and the following figures, indicate the starting CFU/mL of the strains.

At a glycogen concentration of 10 g/L , *L. jensenii* maintained growth in MSVF at all three pH conditions

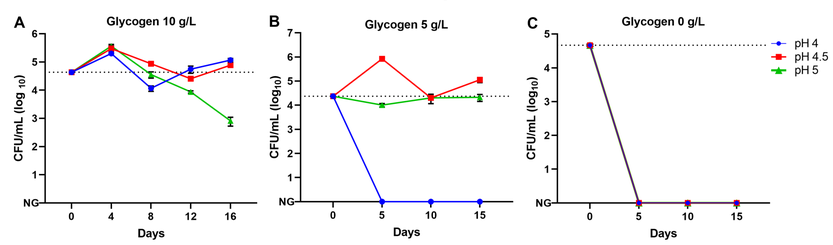

Figure 2. (A) In the presence of 10 g/L glycogen, *L. jensenii* reached its peak growth at 4 dpi under all three starting pH conditions. At pH 4, the lowest amount of growth occurred at 8 dpi, after which its growth increased to the end of the growth cycle. At pH 4.5, the lowest growth amount of growth occurred at 12 dpi with an increase in growth by 16 dpi. In contrast, at pH 5, the growth of *L. jensenii* declined throughout the growth cycle. **(B)** In the presence of 5 g/L glycogen, *L. jensenii* grew under starting pH conditions of 4.5 only. Its pattern of growth was similar that 10 g/L glycogen at pH 4.5 – the peak of growth occurred at 5 dpi, with a drop to the lowest level at 10 dpi followed by an increase in CFU/mL at 15 dpi. Under starting pH condition of 5.0 and in the presence of 5 g/L glycogen, *L. jensenii* maintained its starting CFU/mL across the 15 d growth cycle. **(C)** *L. jensenii* was unable to grow without glycogen regardless of pH.

L. crispatus requires glycogen at 10 g/L and a starting pH of 5 to survive throughout the growth cycle

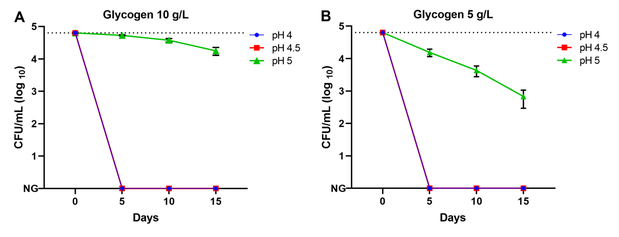

Figure 3. *L. crispatus* did not grow at a starting pH of 4 or 4.5 regardless of the glycogen level. **(A)** At a glycogen concentration of 10 g/L at pH 5, *L. crispatus* maintained a relatively steady concentration across the 15 d growth cycle. **(B)** At a glycogen concentration of 5 g/L at pH 5, the growth of *L. crispatus* steadily decreased, registering a 2-log reduction by 15 dpi. *L. crispatus* was unable to grow without glycogen.

At pH 5, glycogen is not essential for the growth of *L. gasseri*

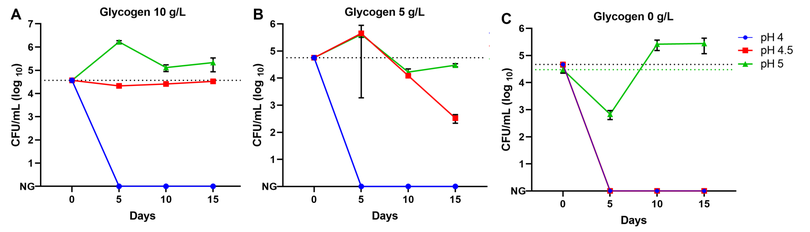

Figure 4. *L. gasseri* was unable to grow at pH 4 in any concentration of glycogen. **(A)** In the presence of 10 g/L glycogen in MSVF at pH 4.5, *L. gasseri* maintained its starting CFU/mL for 15 dpi. At pH 5, *L. gasseri* reached its peak growth at 5 dpi, followed by a 1-log drop at 10 dpi, but above the starting CFU. This level rose slightly by 15 dpi. **(B)** In MSVF containing 5 g/L glycogen, *L. gasseri* again reached its peak of growth at 5 dpi at both pH 4.5 and 5. A drop in CFU/mL occurred at both pH 4.5 and 5, this time to a lower level than the starting CFU/mL. The drop continued to 15 dpi at pH 4.5, but was followed by a slight increase at pH 5. **(C)** Unlike the other strains, *L. gasseri* was able to grow without glycogen at pH 5. Following a drop in CFU/mL at 5 dpi, growth increased by 2 logs at 10 dpi and was maintained through 15 dpi.

Conclusions

- **Vaginal pH influences the ability of lactobacilli and GV to grow in the presence of variable glycogen concentrations.**
- **Under higher pH conditions (5.0), GV competes with lactobacilli in utilizing available glycogen.**
- **Under normal glycogen levels, *L. jensenii* is an essential component of healthy vaginal microflora at pH 4, a condition unfavorable for the growth of GV, *L. crispatus*, and *L. gasseri*.**
- ***L. gasseri* utilizes carbon sources, other than glycogen, in the MSVF at pH 5.**