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The composition of the vaginal microbiome in first trimester pregnant women influences the level of autophagy and stress in vaginal epithelial cells

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ABSTRACT

Epithelial cells lining the vagina are major components of genital tract immunity. The influence of the vaginal microbiome on properties of host epithelial cells is largely unexplored. We evaluated whether differences in the most abundant lactobacilli species or bacterial genera in the vagina of first trimester pregnant women were associated with variations in the extent of stress and autophagy in vaginal epithelial cells. Vaginal swabs from 154 first trimester pregnant women were analyzed for bacterial composition by amplification and sequencing of the V1–V3 region of bacterial 16S rRNA genes. Vaginal epithelial cells were lysed and autophagy quantitated by measurement of p62. Intracellular levels of the inducible 70 kDa heat shock protein (hsp70), an indicator of cell stress and an autophagy inhibitor, were determined. When *Lactobacillus crispatus* was the most abundant member of the vaginal microbiota, epithelial p62 and hsp70 levels were lowest as compared to when other bacterial taxa were most abundant. The highest concentrations of p62 and hsp70 were associated with *Streptococcus* and *Bifidobacterium* abundance. The p62 level associated with *Gardnerella* abundance was lower than that observed when lactobacilli other than *L. crispatus* were most abundant. In conclusion, in the first trimester of pregnancy the abundance of different bacterial taxa is associated with variations in autophagy and magnitude of the stress response in vaginal epithelial cells.

1. Introduction

The presence and persistence of lactobacilli as the most abundant component of the vaginal microbiota in the majority of reproductive age women is maximal during pregnancy (MacIntyre et al., 2015; Romero et al., 2014). These bacteria likely prevent colonization by other microorganisms that can potentially interfere with gestation. Their production of lactic acid acidifies the vagina leading to the direct killing or inhibition of other bacteria (Alakomi et al., 2000; O'Hanlon et al., 2001). Lactic acid also primes anti-microbial immune responses (Aldunate et al., 2015; Linhares et al., 2011; Witkin et al., 2013).Lactobacilli produce bacteriocins to kill other bacteria and their adherence to vaginal epithelial cells prevents other microorganisms from binding to the cell surface (Abramov et al., 2014; Ojala et al., 2014).

In the majority of women the abundance of only one lactobacilli species or a bacterium of another genera greatly exceeds the level of any other microorganism in the vagina (MacIntyre et al., 2015; Romero et al., 2014). The influence of individual members of the vaginal microbiota on properties of host vaginal epithelial cells has received very limited attention and is the focus of this investigation.

Autophagy is a basic process present in almost all eukaryotic cells to maintain intracellular homeostasis. Aged or defective organelles such as mitochondria, aggregated and dysfunctional proteins as well as bacteria and viruses that have invaded the cell and their precursor components are sequestered in a double membraned structure called an autophagosome. The autophagosome then fuses with a lysosome and the contents are degraded by lysosomal enzymes (Wang and Klionsky 2003). The components of the degraded macromolecules are returned to the cytoplasm for reutilization by the cell.

In the present study we detail the relationship between the most abundant bacterial species and genera in the vaginal microbiota of first trimester pregnant women, the extent of autophagy in vaginal epithelial

Abbreviations: hsp70, 70 kDa heat shock protein; L. crispatus, Lactobacillus crispatus; L. iners, Lactobacillus iners; L. gasseri, Lactobacillus gasseri; L. jensenii, Lactobacillus jensenii; L. helveticus, Lactobacillus helveticus; L. acidophilus, Lactobacillus acidophilus; L. johnsonii, Lactobacillus johnsonii

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cells and the intracellular epithelial cell expression of the inducible 70 kDa heat shock protein (hsp70), a sensitive indicator of stress (Craig 1985). The present investigation is an expansion of an earlier report detailing epithelial cell stress and autophagy in relation to the relative vaginal levels only of *L. crispatus* and *L. iners* in this population (Leizer et al., 2017).

2. Material and methods

2.1. Subjects

The study population consisted of 154 women at 8–12 weeks of gestation seen as outpatients for a routine examination between September 2013 and November 2014 at Weill Cornell Medicine. Exclusion criteria from this prospective observational study included the use of antibiotics for any reason in the previous four weeks, a multifetal gestation, the presence of a genital tract infection, vaginal bleeding and the inability to give informed written consent. Only after completion of the microbiome and biochemical analyses was the outcome of the index pregnancy as well as pregnancy history and demographic medical data obtained from each woman's chart. The study was approved by the Institutional Review Board at Weill Cornell Medicine and all participants provided informed written consent and the study performed in accordance with the ethical standards of the Helsinki Declaration.

2.2. Sample collection

Vaginal secretions as well as a sampling of the outer layers of vaginal epithelial cells were obtained by vigorously scraping the side walls of the posterior vagina with a cotton swab followed by shaking the swab into a tube containing one ml sterile phosphate-buffered saline. The tube was centrifuged to obtain cell pellet and supernatant fractions. The epithelial cell pellet was immediately resuspended in 130 µl of a cell lysate buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mg/ml deoxyribonuclease, 100 mM protease inhibitor cocktail (Sigma, ST. Louis, MO) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM EGTA, incubated on ice for 30 min and then centrifuged at 11,000 rpm for 10 min. The lysate supernatant was frozen at −80 °C until tested. A separate aliquot from the vaginal wall of each subject was obtained using a Copan Diagnostics ESwab sample collection tube (Fisher Healthcare, Houston, TX). All samples were immediately frozen at −80 °C until shipped on dry ice to the University of Idaho for microbiome analysis.

2.3. Characterization of vaginal bacterial communities

The kinds and relative abundances of bacteria in vaginal communities were determined using cultivation-independent methods based on the classification of partial 16S rRNA gene sequences as previously described (Shen et al., 2016). In brief, a validated method (Yuan et al., 2012) was used to extract and purify total genomic DNA (gDNA) from vaginal swabs. The V1-V3 region of 16S rRNA genes in each gDNA sample was amplified in two rounds of a polymerase chain reaction that introduced barcodes and DNA sequencing adaptors as previously described (Shen et al., 2016). The resulting amplicons were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) and the RDP Bayesian classifier was used to assign sequences to phylotypes (RDP 2.5; http://rdp.cme.msu.edu). For constructing phylotype abundance tables we used a simple heuristic rule: to be included in the table a phylotype had to either (a) present in more than one sample at an abundance of 1% or more, or (b) constitute more than 5% of a single community.

2.4. Autophagy and cell stress

The extent of autophagy in the epithelial cells was determined by measuring the intracellular concentration of p62, otherwise known as sequestosome-1, in the cell lysates by ELISA (Enzo Life Sciences, Farmingdale, NY). p62 is an intracellular adaptor protein that binds to components destined for autophagy removal. Its concentration in the cytoplasm decreases as autophagy is induced and, therefore, p62 levels are inversely proportional to the degree of autophagy induction (Levine et al., 2011). The lower the cytoplasmic concentration of p62 the higher is the extent of autophagy. The concentration of hsp70 in the cell lysates was also measured by ELISA (R & D Systems, Minneapolis, MN). The lower level of detection was 100 pg/ml for p62 and 156 pg/ml for hsp70.

2.5. Statistics

Statistical analysis was performed with the GraphPad (Graphpad Software Inc, San Diego, CA) and SPSS v24 (IBM Corp, Armonk, NY) statistical packages. Distribution of categorical variables was compared with the Fisher's exact test. Normality of distribution of continuous variables was examined with the Shapiro-Wilk test. Given that all variables were non-normally distributed, Kruskall-Wallis and paired Mann-Whitney *U* tests were used. Correlations between p62 and hsp70 levels were analyzed by the Spearman rank correlation test. All p values were 2-sided and the alpha level of statistical significance was set at 0.05.

3. Results

The study subjects' characteristics are shown in Table 1. Almost all women had a spontaneous conception (90.9%) and delivered a healthy baby at term (85.7%). Six women had a spontaneous abortion (3.9%), four had a termination of pregnancy (2.6%) and three delivered preterm (1.9%). The majority of subjects (56.4%) were of White race.

Results of the microbiome analysis revealed that *Lactobacillus crispatus* was the most abundant bacterial species in 70 (45.5%) women, followed by L. iners in 23 women (14.9%), *L. gasseri* and the genus *Gardnerella* in 17 women each (10.4%), *L. jensenii* in 12 women (7.8%), the genus *Streptococcus* in six women (3.9%), the genus Bifidobacterium in five women (3.2%), *L. helveticus* in two women (1.3%) and *L. acidophilus* and L. johnsonii in one woman each (0.6%). The three women with a preterm birth had a vaginal microbiota in which *L. crispatus, L.*

Table 1
Pregnancy-related characteristics of the study population.

Characteristic	Value
Mean age (SD)	33.8 (4.0) years
Conception	
Spontaneous	140 (90.9%)
In vitro fertilization	14 (9.1%)
Outcome	
Term delivery	132 (85.7%)
Preterm delivery[a]	3 (1.9%)
Spontaneous abortion	6 (3.9%)
Induced abortion[b]	4 (2.6%)
Unknown (lost to follow-up)	10 (6.5%)
Race/ethnicity	
White	56.4%
Mixed	25.5%
Asian	12.8%
Hispanic	4.7%
Black	0.7%

The study population consisted of 154 women seen at 8–12 weeks gestation.
[a] 33.3, 35.4, 36.0 weeks gestation.
[b] 2 due to fetal anomalies, 1 due to trisomy 21, 1 unknown.