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Vaginal ecosystem modeling of growth patterns of anaerobic bacteria in microaerophilic conditions

Audrie A. Medina-Colorado [a], Kathleen L. Vincent [b], Aaron L. Miller [a], Carrie A. Maxwell [c], Lauren N. Dawson [b], Trevelyn Olive [b], Elena V. Kozlova [d], Marc M. Baum [e], Richard B. Pyles [a, c, *]

[a] Department of Pediatrics, University of Texas Medical Branch, Galveston, TX, USA
[b] Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX, USA
[c] Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA
[d] Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA
[e] Oak Crest Institute of Science, Monrovia, CA, USA

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ABSTRACT

The human vagina constitutes a complex ecosystem created through relationships established between host mucosa and bacterial communities. In this ecosystem, classically defined bacterial aerobes and anaerobes thrive as communities in the microaerophilic environment. Levels of CO_2 and O_2 present in the vaginal lumen are impacted by both the ecosystem's physiology and the behavior and health of the human host. Study of such complex relationships requires controlled and reproducible causational approaches that are not possible in the human host that, until recently, was the only place these bacterial communities thrived. To address this need we have utilized our *ex vivo* human vaginal mucosa culture system to support controlled, reproducible colonization by vaginal bacterial communities (VBC) collected from healthy, asymptomatic donors. Parallel vaginal epithelial cells (VEC)-VBC co-cultures were exposed to two different atmospheric conditions to study the impact of CO_2 concentrations upon the anaerobic bacteria associated with dysbiosis and inflammation. Our data suggest that in the context of transplanted VBC, increased CO_2 favored specific lactobacilli species defined as microaerophiles when grown as monocultures. In preliminary studies, the observed community changes also led to shifts in host VEC phenotypes with significant changes in the host transcriptome, including altered expression of select molecular transporter genes. These findings support the need for additional study of the environmental changes associated with behavior and health upon the symbiotic and adversarial relationships that are formed in microbial communities present in the human vaginal ecosystem.

1. Introduction

The vaginal microbiome (VMB) plays critical roles in maintaining women's reproductive and general health [1]. VMB communities are part of a complex ecosystem that contribute to profound changes of the host mucosa created by the stratified squamous vaginal epithelial cell (VEC) multilayer as well as individual bacterial phenotypes. The bacterial phenotypes are altered by their ongoing survival efforts that can be favored or limited by host changes including the alteration of CO_2/O_2 levels in the vaginal lumen. During periods of eubiosis, the symbiotic relationship of the VMB and VEC creates optimal carbon supplies for the bacteria and protective chemical and physical barriers for the host to exclude pathogens including bacterial and viral sexually-transmitted infections (STIs) [1]. Conversely, dysbiotic VMB, reported to be dominated by expanded populations of anaerobic bacteria, cause inflammation and increased susceptibility to many pathogenic outcomes [2].

VMB dysbiosis can lead to the symptomatic syndrome, bacterial vaginosis (BV), the most common bacterial infection of reproductive age women (14–49 years), afflicting 29.2% women overall with a higher prevalence in non-Caucasian women [2–4]. It is becoming increasingly clear that VMB differ among women of different age

* Corresponding author. Department of Pediatrics, University of Texas Medical Branch, Galveston, TX, USA.
E-mail address: rbpyles@utmb.edu (R.B. Pyles).

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and racial background but the contributing factors are still being elucidated [5–7]. Categorical schemes have been described to aid identification of associations between specific VMBs with clinical outcomes. Ravel and co-workers used next generation sequencing (NGS) to classify 6 VMB community state types (CSTs) based on the most abundant *Lactobacillus* species present in the community. Specifically, CST I communities are dominated by *L. crispatus,* CST II by *L. gasseri,* CST III by *L. iners,* and CST V by *L. jensenii* [7]. CST IV communities carry a mixed population of lactobacilli (CST IVA) or are dominated by anaerobes associated with BV generally lacking a substantial number of lactobacilli (CST IVB). A more recent schema linked VMB profile with host health state leading to 4 categorical "cervicotypes" (CTs) [8]. For this schema, CT1 showed *L. crispatus* domination, CT2 was dominated by *L. iners*, CT3 by *Gardnerella* spp, and CT4 containing mixed VMB communities that included *Prevotella* spp [8].

Additional NGS and other 'omic approaches correlated to clinical datasets have provided associative outcomes that implicate growing lists of environmental, behavioral and genetic confounders influencing the VMB profile [9–13]. Such associations have linked inflammatory profiles and VMBs associated with symptomatic BV dominated by traditionally defined anaerobes, including *G. vaginalis* and *Atopobium vaginae,* suggesting that conditions related to increased CO_2 levels in the vaginal lumen may support VMB shifts to inflammatory states and increased susceptibility to STI pathogens. The vaginal lumen is considered a microaerophilic to slightly hypoxic environment that can be impacted by introduction of ambient air (AA) by sexual activity as well as vaginal hygiene methods (e.g. tampon usage, etc.) [14,15]. The limited published clinical data suggest that typical vaginal lumen CO_2 levels are related to the CO_2 present in venous blood and are approximately AA supplemented with 5% CO_2 [14,16]. It is likely that CO_2 levels are impacted also by both the carbon metabolism of the VEC multilayer as well as the composition of VMB community.

To test the hypothesis that increased CO_2 may be a predisposing factor for creating dysbiotic communities dominated by anaerobic bacteria, we utilized our *ex vivo* human vaginal mucosal culture system [17,18]. The air-interfaced, apical surface of the VEC cultures support colonization with vaginal bacterial communities (VBC) transplanted from vaginal swab samples collected from women during routine gynecological exams. The system allows controlled manipulation of environmental, genetic and behavioral confounders not easily controlled in clinical research to study traditionally defined aerobic and anaerobic bacteria in the context of a stabilized community exposed to controlled gas mixtures in the created "vaginal lumen". For our studies, VEC cultures were created with cells from a Caucasian (V19I) or an African American (BVEC02I) donor and colonized with representative VBC under typical (AA+5% CO_2 and ~20% O_2) and increased (AA+10%CO_2 and 18.9% O_2) CO_2 environments.

VBC shifts created by the increasing CO_2 would be predicted to produce compensatory changes in VEC contributions to the ecosystem including differently regulated gene expression. As an initial study of the molecular signals associated with alterations of the vaginal environment, we quantified changes in immune response and molecular transporter gene expression with custom RT-PCR arrays as an extension of our previous work [19]. Collectively, the results from two genetically distinct VEC donors suggest that increasing CO_2 in the vaginal lumen favors selected lactobacilli that are defined as microaerophiles or anaerobes and are considered probiotic organisms. Interestingly, in VBCs that lacked detectable lactobacilli, *G. vaginalis* was enhanced by higher CO_2 levels. The data suggest that current bacterial species categorization based solely upon single species lab cultivation incubated under aerobic or anaerobic conditions may prove insufficient for predicting behavior in complex communities cultivated on human mucosae.

2. Materials and methods

2.1. Ethics statement

Collection of VBC samples from healthy volunteers was approved by the University of Texas Medical Branch's institutional review board under protocol numbers 12–238, 12–239 and 14–0479. Adult subjects provided written informed consent during enrollment prior to collection of vaginal swabs. Each sample was provided a unique study number by the clinical team and provided as de-identified material to the research team. VEC cultures were either purchased commercially (V19; haplogroup T2b) as previously described [18,20] (MatTek Corp; Ashland, MA, USA) or established from a single African American donor's de-identified, discarded surgical material (BVEC02I; haplogroup L1b1) collected during surgical prolapse repair.

2.2. Clinical vaginal bacterial communities (VBC)

Vaginal swabs, collected by clinical staff, were obtained from healthy, asymptomatic women (Amsel's criteria negative) during routine gynecological examinations using a sterile calcium alginate swab (Fisherbrand, Pittsburgh, PA) passed over the mid-vaginal wall avoiding external sites via the placement of a standard speculum. The swabs were immediately placed into 2 ml of sterile, Ca^{++}/Mg^{++}-free Dulbecco's Phosphate Buffered Saline (DPBS; Cellgro, Herndon, VA), and transported at 4 °C to the lab. All subsequent procedures were completed in a sterile class II biosafety cabinet. Each swab was thoroughly vortexed to release the collected bacteria and then aliquoted for DNA and RNA separately into MagNA Pure 96 External lysis buffer (Roche, Basel, Switzerland) for molecular evaluations or mixed with sterile glycerol (10% w/v) as a cryo-protectant for viable VBC aliquots. Optimized methods were reported previously for use with our VEC culture system to optimally preserve viability of the bacteria collected [17,18]. All aliquots were stored at −80 °C until utilized. DNA or RNA was extracted on an automated MagNA Pure 96 (Roche). VBCs were characterized by customized qPCR array as described previously [17]. Any samples molecularly positive for STI, yeast or Y chromosome were excluded from the study.

2.3. Ex vivo VEC multilayer production and VBC colonization

Immortalized human VEC (V19I and BVEC02I) were cultured as described [20] and used to produce stratified squamous vaginal multilayers [17,18]. Briefly, primary V19 cells (MatTek Corp, Ashland, MA) were immortalized and fully characterized as previously reported [21]. BVEC02I cells were grown from trypsinized discarded vaginal tissue collected during a surgical prolapse repair, immortalized and similarly characterized to V19I. VEC were routinely cultured in a broad-spectrum antimicrobial, Primocin (Invivogen, San Diego, CA). Cells were tested for mycoplasma contamination monthly (MycoSensor, Stratagene, LaJolla, CA). VEC from one of the 2 donors were plated in 24 (10^6 cells/transwell) or 96 (10^5 cells/transwell) well transwell format and cultured at 37 °C with 5% CO_2. After monolayer formation, each culture was subjected to air-interfacing by removal of the apical growth medium. The multilayers were refed basally every other day with antibiotic- and serum-free medium as described [17,18] leading to stratification and differentiation of multilayers.

At maturation (7–9 days) VBC were diluted to 10^3-10^4 total genomes and applied to the apical surface of the multilayers. VBCs