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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, and Richard B. Pyles^{a,c,*}

^aDepartment of Pediatrics, University of Texas Medical Branch, Galveston, Texas, United States of America

^bDepartment of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Texas, United States of America

^cDepartment of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, United States of America

^dDepartment of Pathology, University of Texas Medical Branch, Galveston, Texas, United States of America

^eOak Crest Institute of Science, Monrovia, California, United States of America

Abstract

The human vagina constitutes a complex ecosystem created through relationships established between host mucosa and bacterial communities. In this ecosystem, classically defined strict bacterial aerobes and anaerobes thrive as communities in the microaerophilic environment. Levels of CO₂ and O₂ 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 intact 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 microbiomes (VMB) collected from healthy and symptomatic donors. Parallel vaginal epithelial cells (VEC)-VMB co-cultures were exposed to increasingly microaerophilic conditions to study the impact of CO₂ concentrations upon the anaerobic bacteria associated with dysbiosis and inflammation. Our data suggest that in the context of intact VMBs, increased CO₂ concentrations favored specific lactobacilli species defined as aerobes or microaerophiles when grown as monocultures. 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

*Corresponding author.

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adversarial relationships that are formed in microbial communities present in the human vaginal ecosystem.

Keywords

vaginal microbiome; *Lactobacillus jensenii*; *Lactobacillus crispatus*; microaerophiles; anaerobes; aerobes

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, the “eco-ome”, 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 CO₂/O₂ 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 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 (IVA) or are dominated by anaerobes associated with BV generally lacking a substantial number of lactobacilli (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 CO₂ 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, douching, etc.) [14, 15]. The limited published clinical data suggest that typical vaginal lumen CO₂ levels are related to the CO₂ present in venous blood and are approximately AA supplemented with 5% CO₂ [14, 16]. It is likely that CO₂ 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 CO₂ may be a predisposing factor for creating dysbiotic VMBs 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 intact VMBs transplanted from samples collected from woman 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 VMB under typical (AA+5% CO₂ and ~20% O₂) and increased CO₂ (AA+10%CO₂ and 18.9% O₂) environments.

VMB shifts created by the increasing CO₂ would be predicted to produce compensatory changes in VEC contributions to the eco-ome 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 CO₂ in the vaginal lumen favors selected lactobacilli that are defined as microaerophiles or aerobes and are considered probiotic organisms. Interestingly, in dysbiotic VMBs that lacked detectable lactobacilli, *G. vaginalis* was enhanced by higher CO₂ 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 vaginal microbiome (VMB) samples from healthy volunteers was approved by the University of Texas Medical Branch’s institutional review board. 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 (V19I) as previously described [18, 20] or established from de-identified, discarded surgical material (BVEC02I) collected during surgical prolapse repair.

2.2 Clinical VMB

Vaginal swabs, collected by clinical staff, were obtained from healthy women 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 2ml of sterile, $\text{Ca}^{++}/\text{Mg}^{++}$ -free Dulbecco's Phosphate Buffered Saline (DPBS; Cellgro, Herndon, VA), and transported at 4°C to the lab. The subsequent procedures were all completed in a sterile class II biosafety cabinet. Each swab was thoroughly vortexed to release the collected bacteria and human cells 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 VMB community aliquots. All aliquots were stored at -80°C until utilized. DNA or RNA was extracted on an automated MagNA Pure 96 (Roche). VMBs were characterized by customized qPCR array as described previously [17]. Any VMB samples molecularly positive for STI, yeast or Y chromosome were excluded from the study.

2.3 Ex vivo VEC multilayer production and VMB colonization

Immortalized human VEC (V19I and BVEC02I) were cultured as described [20] and used to produce stratified squamous vaginal multilayers [17, 18]. Briefly, VEC from one of 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] leading to stratification and differentiation of multilayers.

At maturation (7–9 days) VMB were diluted to 10^3 - 10^4 total genomes and applied to the apical surface of the multilayers. VMB were selected from our cryo-repository based on community profiles established from initial swab evaluations. In supplementation studies we used 1,000 cfu of our previous *L. jensenii* clinical isolate [18]. Parallel triplicate cultures were created and immediately placed in prepared 37°C, humidified incubators containing AA supplemented with either 5% or 10% CO_2 . The cultures were maintained for 48h before harvesting in MagNA Pure 96 External lysis buffer (Roche). Each study was repeated at least once.

2.4 VMB profiling and VEC molecular transporter gene expression evaluation by custom qPCR array analyses

Half of each lysate was subjected to automated DNA extraction using MagNA Pure 96 DNA and Viral NA Small Volume kit (Roche). Molecular evaluation of VMBs was completed by qPCR array as previously described [17]. RNA extraction was completed using the second half of each cell lysate using the MagNA Pure 96 Cellular RNA large volume kit (Roche) and then converted immediately to cDNA (iScript; Bio-Rad, Hercules, CA). Custom qPCR arrays were built for selected immune response-related genes and molecular transporters (Supplemental Tables 1 and 2) [21–23] and subjected to qPCR with SYBR green detection in CFX amplification systems (Bio-Rad) as described previously [24]. Gene targets were selected following previously identified gene expression in human vaginal mucosa tissues

[19]. Melt temperatures for each amplified target were matched to historic controls and genes with melt curves outside ± 0.6 C were eliminated from further analysis. Ct values for triplicate independent replicates were tabulated and genes present 2 out of 3 times were determined as authentic for detection and further analysis. Expression levels were normalized to 3 housekeeping genes (C1orf4, CHMP2A and PSMB2) [23]. After normalization, the standardized delta delta Ct algorithm was used to compare gene expression levels. Statistical significance was determined using t-tests and P values of 0.05 were concluded as significant. Heat map clustering analyses were performed using QCanvas [25]. Input data were scaled to a maximum of ± 10 -fold change for visualization.

2.5 Statistics

All experiments were repeated at least once. A minimum of 2 representative VMB for each dominant lactobacilli or dysbiotic VMBs were cultivated in triplicate for each environmental condition. Most data are presented as averaged proportions of targeted bacterial species within the community. Replicate data was used to calculate average absolute genomic copies in parallel 5% and 10% CO₂ groups and statistical significance was determined using Student's t test with $P < 0.05$ considered significant. Statistical evaluations and proportional graphing were completed with Prism v7.0a (GraphPad, San Diego, CA).

3. Results

3.1 Increased CO₂ shifts VMB community profiles

The impact of a variety of activities like intercourse, douching, diet, and menstruation alter the vaginal ecosystem producing potential changes in the physiology of both bacteria and VEC that in turn further alter health status [26–28]. To begin the study of these complex interactions we have assessed the impact of the fluctuation of the gaseous composition in the vaginal lumen. The impact of changing O₂/CO₂ ratios on VMB profiles was measured using our *ex vivo* culture system with V19I VEC multilayers colonized with selected VMBs. Initial evaluations of 25 distinct VMB were completed to uncover potential trends in community alteration. These studies included at least 2 representatives for each lactobacilli-dominated and dysbiotic community that were cultivated in the presence of either 5 or 10% CO₂ supplemented AA in triplicate cultures. Surprisingly, these initial studies illustrated that increased luminal CO₂ favored selected lactobacilli and suggested a hierarchy in this genus that was contrary to the aerobic categorization of their cultivation as individual species in the laboratory. Further, this enhancement was associated with reductions in bacteria categorized as strict anaerobes in selected communities (e.g. *G. vaginalis*).

Representative VMB were selected to complete focused confirmatory analyses designed to identify the *Lactobacillus spp* that were most or least favored by increased CO₂. Absolute abundance values for selected organisms within communities cultivated in parallel at 5% or 10% supplemented CO₂ were used to establish average abundance and compared statistically to identify significantly increased titers of lactobacilli in general and *L. crispatus* and *L. jensenii* specifically (Table 1; $P < 0.05$). There were general trends across multiple VMB that did not achieve significance indicating increases in *L. gasseri* were common to

VMB lacking *L. jensenii* or *L. crispatus*. *L. iners* and *G. vaginalis* were suppressed by cultivation at AA+10% CO₂ in VMB that had *L. jensenii* or *L. crispatus* (Table 1).

To illustrate the impact of elevated CO₂ on representative VMB, proportional bar charts are presented in Figure 1. The first VMB that was dominated by *L. crispatus* but also contained a substantial proportion of *G. vaginalis* illustrates the impact of 10% CO₂ that led to *L. crispatus* enhancement while *G. vaginalis* loads were reduced (Fig. 1A VMB1 bright blue v bright red color blocks). A second VMB dominated by *L. gasseri* also with a large portion of *G. vaginalis*, again showed reduction of *G. vaginalis* and an expansion of *L. gasseri* dominance in high CO₂ relative to 5% supplementation (Fig. 1A; VMB2 turquoise v bright red). VMB3 was selected to illustrate a community dominated by *L. iners* at 5% supplemented CO₂ (Fig. 1A; orchid) that lacked other lactobacilli. Remarkably, *L. iners*, categorized as an anaerobic vaginal lactobacillus when grown as a single species [29, 30], was not enhanced by increasing the CO₂ levels showing a marked reduction in proportion (Fig. 1A VMB3). In this community, *G. vaginalis* became the dominant organism suggesting this VMB transitioned towards dysbiosis in the higher CO₂ environment. A second VMB dominated by *L. iners* but containing small proportions of both *L. crispatus* and *L. jensenii* was tested (VMB4). At 10% CO₂, this community also showed marked reduction in *L. iners* and a coincident expansion of both *L. crispatus* and *L. jensenii* (Fig. 1A VMB4). In fact, VMBs in our panel of 25 that were dominated by or contained measurable *L. iners* (n=7) consistently experienced a reduction in this species during cultivation in V19I cultures at 10% CO₂.

To confirm the trends observed with the microaerophilic lactobacilli and begin to develop a hierarchy for CO₂ enhancement, VMB5 was selected as a representative dysbiotic community that was dominated by *G. vaginalis* but contained small proportions of *L. crispatus* (2%), *L. iners* (10%) and *L. jensenii* (14%) at 5% CO₂. When cultivated with 10% CO₂ supplementation, VMB5 showed increases in the proportion of *L. jensenii* and a non-significant increase in *L. crispatus*. Coincident contractions of *G. vaginalis* and *L. iners* were observed as well (Fig. 1A). Expansion of *L. jensenii* was also illustrated in the VMB2 community where a significant 13-fold increase was detected (P=0.029) indicating this species was the most affected by increased CO₂ regardless of community profile. VMB6, dominated by *L. jensenii*, showed proportions of 97.8% at 5% CO₂ and 99.2% in the higher CO₂ illustrating the consistent dominance of this species in multiple community types (Fig. 1A). Across all tested VMB including those that were already dominated by *L. jensenii*, an average 8-fold increase in absolute abundance was observed (Table 1, P=0.022).

As a final confirmation of the dominance of *L. jensenii* under higher CO₂ conditions, healthy (VMB7) and dysbiotic (VMB1) communities that lacked detectable *L. jensenii* were selected for exogenous supplementation with a clinical *L. jensenii* isolate (1,000 cfu) [18]. In both VMB, the exogenous *L. jensenii* became the dominant organism after 48 hours in 5% CO₂ conditions (Fig. 1B). At 10% CO₂, the non-native, exogenous *L. jensenii* expanded from 74.1% to 86.5 % in the healthy and from 70% to 92% in the dysbiotic VMBs tested (Fig. 1B). Both native VMB contained proportions of *L. crispatus* that also expanded after cultivation in higher CO₂ levels as seen in previous studies but failed to surpass the growth of the exogenous *L. jensenii* (Fig. 1B).

3.2 Increased CO₂ enhanced *L. jensenii* in African American VEC but other vaginal bacteria were differently impacted

Current clinical data associate African lineages with VMBs that have reduced *Lactobacillus* content and higher proportions of anaerobes [7, 31]. Utilizing VEC from genetically distinct donors, the multilayer culture system offers the opportunity to establish distinct eco-omes to directly compare VMB community formation. For these studies, we compared the data from VMB created in either V19I VEC (Caucasian; haplogroup T2b) or BVEC02I cells from a distinct donor (African American; haplogroup L1b1). The same clinical VMBs were used to colonize BVEC02I VEC with the exception of one community (VMB1) that was no longer available and was substituted with a similar community (VMB8).

In contrast to the consistent contraction of *L. iners* proportions observed in V19I VEC, BVEC02I VEC produced VMB communities with enhanced *L. iners* proportions at higher CO₂ levels (Fig. 2A VMB8, VMB3 and VMB4). The trend was consistent but did not achieve statistical significance across the tested VMB (Table 1, P=0.09). The marked increase in *L. iners* in these three VMB included both eubiotic (VMB8 and 4) and dysbiotic (VMB 3) communities. Consistent with the results in V19I, BVEC02I VEC also supported a dominant expansion of *L. jensenii* at the 10% CO₂ supplementation. Across the tested VMB, an average 3.5-fold increase in *L. jensenii* was observed (Table 1; P=0.0001). VMB2, 5 and 6 communities cultivated in BVEC02I cultures produced similar *L. jensenii* proportions to those observed in V19I at AA+10% CO₂ (Fig. 1A vs. 2B). In BVEC02I cultures, VMB5 also illustrated an increase in *L. crispatus* consistent with the outcomes in V19I during cultivation in 10% CO₂. However, the average increase of 2-fold across the tested VMB was not significant (Table 1, P=0.14).

3.3 Luminal CO₂ VMB shifts produced VEC transcription changes in immune response genes and molecular transporters

VMB shifts are known to cause changes in the inflammatory state and overall health of the vaginal mucosa [8]. An inflammatory state can cause tissue damage and create disruptions in the vaginal mucosa leaving women more susceptible to STIs like HIV [32–34]. These same conditions also appear to impact the efficacy of topically applied antimicrobials [17, 34, 35]. To begin to elucidate the molecular mechanisms associated with compensatory changes in the ecosystem, we exploited the shifted VMB outcomes to study associated differences in expression of selected genes related to immune responses and molecular transport. RNA from triplicate cultures was harvested from sterile V19I VEC or multilayers colonized with VMB1 or VMB6 with or without *L. jensenii* supplementation that were cultivated in 5% or 10% CO₂. The RNA was converted to cDNA and subjected to custom RT-PCR arrays to quantify changes in expression of selected immune response genes and molecular transporters (Supplemental Tables 1 and 2). After normalization the fold change expression patterns were statistically compared and selected significant changes are described below and in Figures 3 and 4. Genes that were impacted by CO₂ in sterile cultures were first identified and then eliminated from further consideration. Remaining targets were used to create baseline gene expression values for sterile cultures at 5% and 10% supplemented CO₂ and used as comparators for parallel cultures created with VMB with or without *L. jensenii*

supplementation as shown in Fig. 1B. Up-regulated expression is depicted on the heat map in red, down regulation by green and average fold change is shown in the table.

Expression patterns for the immune response and signaling genes impacted by shifted VMB (Fig. 3A) clustered the samples based on CO₂ supplementation (5% CO₂ outcomes clustered on the left). Genes that were generally up-regulated by any VMB community, regardless of CO₂ levels, included MMPs 1, 9, 14 and 28 as well as ICAM-1 (Fig. 3). Interestingly, these genes are associated with epithelial barrier integrity. Immune modifiers, including CCL20, IL33 and TGFBI also were generally up-regulated when bacterial communities were added to the V19I VEC (Fig. 3). With regard to shifted VMB impacts, Toll-like receptors (TLR) 4, 5 and 6, responsible for recognition of bacterial products, were significantly down-regulated (4- to 242-fold reduced, $p < 0.05$; Fig. 3) following cultivation in 10% CO₂ while no change or slight increases were observed in 5% CO₂ equivalents. These changes may be attributed to the significant expansion of *L. jensenii* in these communities and a more probiotic community profile. In contrast, TLR1, 2, 3, 7 and 9 expression levels were either unchanged or significantly increased (24- to 275-fold; $p < 0.05$) in AA+10% CO₂ conditions. TLR1 and 3 were most impacted by the VMB6 *L. jensenii*-dominated community that showed up-regulation at both CO₂ concentrations. TLR3, 7 and 9 recognize modified nucleotides and CpG motifs generally related to viral challenge.

Expression of molecular transporter genes, divided into ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies [36, 37], were evaluated using a similar paradigm. In previous work, we employed microarray, qRT-PCR and immunolabeling to evaluate human vaginal tissues and in VEC cultures to identify the subset of ABC and SLC transporters that were expressed [1, 19]. Selected vaginally-expressed transporter genes were evaluated in cultures with shifted VMB profiles created by CO₂ supplementation in the VEC model. Genes impacted by elevated CO₂ in sterile cultures were eliminated from additional evaluation and then clustered creating a clean separation of samples based on CO₂ levels (Fig. 4).

Relative to sterile cultures, three transporters, ABCG1, SLC16A3 and SLC35E4 were consistently upregulated by the presence of VMB bacteria regardless of community or CO₂ supplementation (Fig. 4). Eight transporters were consistently down-regulated in the presence of VMB communities at 10% CO₂ in contrast to 5% CO₂ where they were up-regulated or unchanged relative to sterile baselines (top half of the heat map, Fig. 4). These included ABCC2, C6 and G2 that encode proteins (MRP2, MRP6 and a “White family xenobiotic transporter”, respectively) involved in multidrug resistance and movement of antimicrobials and xenobiotics [37–40]. SLC6A16, 22A20, 25A15, 29A2 and 45A3 were down regulated in AA+10% CO₂ and generally up regulated in 5% CO₂ relative to sterile baselines (Fig. 4). These genes encode proteins associated with transport of organic anions. Of interest to the vaginal mucosa, SLC22A20 encodes a protein (OAT6) that transports estrone sulfate [41]. SLC29A2 encodes HNP36 involved in nucleotide transport including nucleoside analogs employed as antivirals [42, 43]. VMB shifts to *L. jensenii* dominated communities also led to the increased expression on SLC16A5, a gene that encodes a transporter of monocarboxylates including lactate and pyruvate [44]. Collectively these data

illustrate the potential of the system to elucidate the molecular mechanisms in the changing eco-ome associated with VMB communities shifted by controlled luminal CO₂.

4. Discussion

Human vaginal bacterial communities function as part of a complex ecosystem important to maintaining reproductive health and serving as a first line defense against STI pathogens [31]. Several studies have defined the bacterial profile of common VMB in women [6, 7, 45] producing data identifying factors that influence typical community composition including age, race and health status [7, 46]. Additional clinical data illustrate VMB communities are disrupted through sexual intercourse, menstruation, douching, diet, infection, antibiotic therapy, hormonal changes and other yet to be defined factors [26–28] emphasizing the complex interplay of the bacterial community, the mucosa status and vaginal lumen changes. In this regard, clinical data from 1984 suggested the vaginal environment is disrupted by tampon insertion that may introduce AA into the vaginal cavity enriching O₂ levels [15]. However, a more recent report described increased CO₂ levels with coincident decreased O₂ as the tampon resides in the vaginal cavity [14]. These data led to a hypothetical association between altered CO₂/O₂ levels and pathogenic bacterial growth [15] but causal studies were hindered by limited modeling capabilities and clinical research approaches confounded by human genetics and behavior. Our *ex vivo* vaginal model system allowed the controlled study of increasing CO₂ in the simulated vaginal lumen measuring impacts on both VMB community profiles and the phenotype of cultured vaginal mucosa.

Parallel cultivation of transplanted VMB representing eubiotic and dysbiotic communities in 2 genetic backgrounds provided unexpected results that were contrary to predictions based on the categorization of individual species of bacteria. Specifically, in the context of the vaginal eco-ome, elevated CO₂ conditions favored aerobes and microaerophiles considered to be probiotic rather than strict anaerobes associated with BV and inflammation [1]. Such definitions are challenged generally when species are considered in the context of complex communities that thrive in microaerophilic environments such as the vaginal vault. Recent work from Srinivasan and colleagues has begun to enhance lab based cultivation systems without human cells that will help to clarify the nature of the bacterial community members as individual species [30].

The data generated in our *ex vivo* system also support the conclusion that community-based relationships alter the behavior of individual species relative to lab cultivation. Such physiologic changes allow strict anaerobes and aerobes to thrive in microaerophilic environments including the vagina. Some *in vitro* studies support this finding given that culture conditions affect the production of metabolites and antimicrobials associated with vaginal health such as H₂O₂ and lactic acid [47–49]. Understanding the impact of species relationships in the VMB will be a crucial step forward in the development of optimized probiotic approaches that have had mixed results to date [47].

Our data show the VMB can be shifted in response to potential vaginal environmental changes such as fluctuations in O₂ and CO₂ and that these responses vary between our two VEC genetic backgrounds. In both donor cultures *L. jensenii* was expanded consistently and

significantly during cultivation with increased luminal CO₂ in diverse eubiotic and dysbiotic VMB communities. This organism, defined as a microaerophile, has been studied extensively for probiotic contributions to vaginal health [50] and was tested as a probiotic supplement in a number of published studies [50–53]. *L. crispatus* also showed enhancement in elevated CO₂ conditions but was surpassed by native or exogenous *L. jensenii*. We previously established that these organisms, as single additives, tempered inflammatory responses in the VEC culture system [18]. *L. gasseri* trended towards enhanced growth in selected VMBs but was not increased significantly if either of the other lactobacilli were present in the community.

Although both VEC types favored lactobacilli in general, there were distinct outcomes consistent with clinical data suggesting African genetic lineages support VMB that are dominated by *L. iners* and bacterial anaerobes [6, 7, 26, 31, 45]. Our limited data support this clinical finding and add validity to the *ex vivo* model's predictions and utility. In the VEC-VMB culture system, while there were similarities in the responses of the two tested haplogroups there also were differences in the favored microbiota. Specifically, BVEC02I eco-omes favored *L. iners* and *G. vaginalis* in general and at higher luminal CO₂; these same bacteria were generally inhibited in the V19I cultures. The trends in these initial experiments support the need for additional studies of VMBs representing distinct communities to better predict optimal probiotic approaches that may need to be tailored to genetic differences. The data suggest increased luminal CO₂ and the associated molecular changes might enhance supplementation by probiotic lactobacilli that often are excluded by established communities [47, 49, 54].

Bacterial products and metabolites have been the focus of research efforts to identify biomarkers and molecular mechanisms associated with health and inflammation [50]. Metabolomic and proteomic approaches have identified a number of odorants and enzymatic activities that support clinical diagnostics of BV (e.g. BV Blue® sialidase detection and the Whiff test in Amsel's criteria). These molecules also serve to alter the ecosystem in undefined ways associated with VMB shifts and vaginal mucosal changes. Utilizing the *ex vivo* model system, we completed gene expression studies to establish if transcription changes in the VEC could be specifically attributed to shifted VMB profiles caused by the elevated luminal CO₂. Using customized PCR arrays developed during our previous transcriptomic studies of vaginal mucosa [19] we identified a set of immune response genes including targets related to epithelial barrier integrity and pathogen recognition. Consistent with our findings that *L. jensenii* tempered inflammation [18], VMB shifts that led to expansion of this probiotic organism significantly decreased expression of TLRs 4, 5 and 6 that recognize pathogenic bacterial products [20].

The underlying molecular changes that affect efficacy of vaginally applied drugs remain unclear but likely involve altered movement of drug from the lumen to deeper cell layers and intracellular locations targeted by pathogens. Molecular transporters collectively mobilize metabolites, signaling molecules and carbon sources to and from the mucosa and, until recently, had not been characterized in the vaginal tract [19, 36, 37, 55]. In fact, molecular transporter expression patterns produced from vaginal ecosystems may help to explain differences in clinical results for vaginally-delivered antiretroviral therapies [1, 19, 36, 37,

56–58]. Consistent with previous data suggesting VMB impacted the efficacy of tenofovir against HIV-1 [17] we found ABC and SLC transporters were impacted by the VMB shifts associated with elevated CO₂. Collectively the results for the selected molecular transporters suggested biomarkers for physiological changes will be identified through such studies and can be used to predict the physiological and metabolic changes that would favor probiotic therapies. Such approaches also could enhance vaginally-applied drugs and vaccines.

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Highlights

- The human vagina is a complex ecosystem easily influenced by external stimuli.
- Environmental and behavioral factors shift vaginal bacterial communities.
- Increased luminal CO₂ in the vagina favors lactobacilli over anaerobic bacteria.
- Microbiome shifts result in differential transporter and immune gene expression.

Figure 1. VMB community profiles changed in response to increased luminal CO₂ in the VEC19I vaginal mucosa model

To determine the effects higher CO₂ conditions have on the VMB profile, selected clinical VMBs were transplanted to matured V19I multilayer cultures and cultivated in either 5% or 10% CO₂ supplemented AA. (A) Paired proportional bar charts, established by qPCR array, revealed the impact of increased CO₂ (5, left bar or 10%, right bar). Native *Lactobacillus* spp dominated over anaerobic BV-associated bacterial species growth in all VMB (VMB1, VMB2, VMB5, VMB6) with the exception of *L. iners* (VMB3, VMB4). (B) Eubiotic (VMB7) or dysbiotic (VMB1) bacterial communities that lacked *L. jensenii* were supplemented with 10³ *L. jensenii* CFU prior to application to sterile V19I multilayers. The resulting community profiles confirmed *L. jensenii* was favored over native *Lactobacillus* spp in AA+10% CO₂.

Figure 2. VMB community profiles established in BVEC02I cultures

VMB communities were cultivated in BVEC02I ecosystems with AA+5 or 10% luminal CO₂ as shown in Figure 1. The same VMB were tested with the exception of VMB8 that was substituted for VMB1 because of its similar profile. (A) VMB with native *L. iners* with or without *G. vaginalis* illustrated the observed expansion of *L. iners* in 10% CO₂. (B) VMB with native *L. jensenii* confirmed that results in V19I cultures supporting the conclusion that this lactobacilli was most enhanced by elevated luminal CO₂. The color legend is depicted in Fig. 1.

Figure 3. Clustering and fold change evaluations of selected immune response genes related to the shifted VMB community profiles produced by increased luminal CO₂

Custom RT-PCR arrays were used to analyze gene expression using delta delta Ct comparisons of sterile multilayers and parallel cultures colonized by the indicated VMB with or without *L. jensenii* (L jen) supplementation. (A) Heat map relational clustering of genes that had significantly altered average expression (triplicate cultures) for at least one condition are indicated as up-regulated (red) or down-regulated (green) relative to the sterile control baseline expression levels. (B) Average fold change values for the clustered data are provided for those conditions that led to altered gene expression. Values that were significantly different ($p < 0.05$) are bolded. At least 3 values are considered for each condition.

Figure 4. Clustering and fold change evaluations of selected molecular transporter genes related to the shifted VMB community profiles produced by increased luminal CO₂

Data for selected molecular transporter genes are presented as described in Fig. 3. (A) Heat map relational clustering illustrated grouping of 5% CO₂ conditions regardless of VMB or *L. jensenii* supplementation. (B) Average fold change values for the clustered data again are provided for those conditions that led to altered gene expression. Values that were significantly different ($p < 0.05$) are bolded. At least 3 values are considered for each condition.

Table 1

Absolute abundance comparisons of selected bacteria cultivated in VMB communities at AA+5 or 10% CO₂

	AA+5% CO ₂	AA+10% CO ₂	p value*
V191 VEC			
	Ava. abundance**	SDEV	Avg. abundance
<i>Lactobacillus spp</i>	4.88	5.22	5.45
<i>L. crispatus</i>	3.45	3.41	4.19
<i>L. jensenii</i>	3.98	4.18	4.91
<i>L. gasseri</i>	4.16	4.56	4.65
<i>L. iners</i>	4.35	4.97	4.10
<i>G. vaginalis</i>	4.13	4.05	4.24
BVEC021 VEC			
	Ava. abundance	SDEV	Avg. abundance
<i>Lactobacillus spp</i>	4.87	4.67	5.16
<i>L. crispatus</i>	4.23	3.72	4.51
<i>L. jensenii</i>	4.58	4.47	5.13
<i>L. gasseri</i>	3.84	3.79	4.38
<i>L. iners</i>	3.3	3.26	3.68
<i>G. vaginalis</i>	3.96	3.42	4.12

Absolute abundance values for genomic titers of each indicated bacteria were established by qPCR and averaged from the 25 tested VMB. Only positive values were considered.

* p values were established by Student's T test. P<0.05 are indicated in bold

** Absolute abundance averages and standard deviations are provided in log10