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Authors

Zuend, Christina Farr
Tobin, Nicole H
Vera, Trisha
et al.

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Pregnancy associates with alterations to the host and microbial proteome in vaginal mucosa

Christina Farr Zuend[1,2] | Nicole H. Tobin[3] | Trisha Vera[1,2] | Lani Kotyrba[1,2] | Laura Noël-Romas[1,2] | Kenzie Birse[1,2] | Sarah Mutch[1,2] | Fan Li[3] | David Lee[3] | Stuart McCorrister[4] | Garrett Westmacott[4] | Grace M. Aldrovandi[3] | Adam D. Burgener[2,5,6,7]

[1]National HIV and Retrovirology Labs, J.C. Wilt Infectious Diseases Research Centre, Public Health Agency of Canada, Winnipeg, MB, Canada

[2]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada

[3]Department of Pediatrics, University of California, Los Angeles, Los Angeles, CA, USA

[4]Mass Spectrometry and Proteomics Core Facility, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada

[5]Department of Obstetrics and Gynecology, University of Manitoba, Winnipeg, MB, Canada

[6]Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA

[7]Unit of Infectious Diseases, Department of Medicine Solna, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden

Correspondence
Adam D. Burgener, Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA.
Email: adam.burgener@case.edu

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Abstract

Problem: Pregnant women are at increased risk of HIV acquisition, but the biological mechanisms contributing to this observation are not well understood.

Method of Study: Here, we assessed host immune and microbiome differences in the vaginal mucosa of healthy pregnant and non-pregnant women using a metaproteomics approach. Cervicovaginal lavage (CVL) samples were collected from 23 pregnant and 25 non-pregnant women.

Results: Mass spectrometry analysis of CVL identified 550 human proteins and 376 bacterial proteins from 11 genera. Host proteome analysis indicated 56 human proteins (10%) were differentially abundant ($P < .05$) between pregnant and non-pregnant women, including proteins involved in angiogenesis ($P = 3.36E-3$), cell movement of phagocytes ($P = 1.34E-6$), and permeability of blood vessels ($P = 1.27E-4$). The major bacterial genera identified were *Lactobacillus*, *Gardnerella*, *Prevotella*, *Megasphaera*, and *Atopobium*. Pregnant women had higher levels of *Lactobacillus* species ($P = .017$) compared with non-pregnant women. Functional pathway analysis indicated that pregnancy associated with changes to bacterial metabolic pathway involved in energy metabolism, which were increased in pregnant women ($P = .035$).

Conclusion: Overall, pregnant women showed differences in the cervicovaginal proteome and microbiome that may be important for HIV infection risk.

KEYWORDS

HIV, microbiome, pregnancy, proteomics

1 | INTRODUCTION

Human immunodeficiency virus (HIV) is the leading cause of death of reproductive age women, with 1.5 million pregnancies affected in 2013, a statistic that has not improved since 2009.[1] Pregnant women are thought to be particularly susceptible to HIV infection, with high HIV incidence during pregnancy reported among women from several populations.[2-5] However, prospective studies exploring HIV acquisition risk during pregnancy have found inconsistent results, with some studies showing up to a threefold increased risk during pregnancy[6-8] and others finding no increased risk,[5-7,9-14] indicating some variability in HIV-risk estimates.

Many behavioral factors could contribute to increased HIV susceptibility during pregnancy.[6] While pregnant women tend to report less risky sexual activity, including being less likely to have multiple partners or to use alcohol and/or drugs during sex,[12,13] pregnant women are more likely to report some unprotected sex.[7,12,13] Furthermore, overall sexual activity of pregnant women decreases during pregnancy and the post-partum period, which may lead to riskier behavior among male partners.[13,15] This suggests that pregnant women are likely being exposed to HIV in a way that is increasing their acquisition risk despite decreased sexual activity, perhaps by decreased condom usage, combined with increased biological susceptibility to infection.

Biological factors could also contribute to increased HIV susceptibility in pregnant women, including immunological, structural, and microbiome changes that have been reported. Endocrine and immunologic processes during gestation induce changes to the cellular, cytokine, and chemokine environments within the female genital tract (FGT), including a shift to a Th2 environment, increased regulatory T cells and production of suppressive factors to promote fetal tolerance.[16-24] Pregnant women have been reported to have anti-inflammatory changes in systemic cytokine profiles[25] which may not be mirrored in the cervicovaginal mucosa.[26] The plasma proteome of pregnant women has previously been investigated and demonstrated enrichment in proteins involved in antimicrobial responses, leukocyte migration, and macrophage differentiation,[27] but the effects in the mucosa are not well studied. While the immune system is not suppressed during pregnancy, differential responses to pathogens occur.[16,28,29] Both systemic and local immune system modulations during pregnancy can make pregnant women particularly susceptible to infections[17,24,30,31] and may increase HIV acquisition risk.[32,33]

Structural changes in the female genital tract (FGT) may also be important for HIV susceptibility during pregnancy. In particular, cervical ectopy, which occurs when the columnar epithelium of the endocervical canal extends outwards into the stratified squamous epithelium of the ectocervix, may increase HIV infection.[34] Ectopy has been reported to be increased during pregnancy.[34,35] Ectopy has been associated with a twofold to fivefold increase in HIV acquisition among non-pregnant women in some studies, while others have found no association.[34-36]

The vaginal microbiome is also important for HIV risk, particularly the absence of *Lactobacillus*, which has been associated with an increased risk of acquiring HIV.[37-42] The loss of *Lactobacillus* leads to increased vaginal pH, more HIV target cells, and epithelial barrier disruption, all of which may contribute to HIV infection.[37-45] Pregnant women have been reported to have increased levels of *Lactobacillus* species and decreased microbial diversity, suggesting that the vaginal microbiome may in fact protect from HIV infection during pregnancy.[45-51]

While previous studies have shed light on immunomodulatory and microbiome alterations during pregnancy, they have been limited to examining targeted factors and have primarily been focused on systemic rather than mucosal changes. A better understanding of mucosal differences at the systems level in the vaginal mucosa during pregnancy could provide information on HIV infection susceptibility as well as other adverse outcomes such as preterm birth. In this study, we used a metaproteomics approach to characterize mucosal system differences, including microbial structure and function as well as the host proteome, in pregnant and non-pregnant women.

2 | MATERIALS AND METHODS

2.1 | Study population

Healthy pregnant (n = 23) and non-pregnant (n = 25) women were recruited from an Obstetrics and Gynecology Clinic in Los Angeles, California as described previously.[26] The enrollment criteria included age 17-45 years, no use of hormonal contraceptive in the previous 6 months, no intrauterine device, not actively menstruating, and no reported sexual intercourse in the last 24 hours. Cervicovaginal lavage (CVL), clinical data including cervical photograph, and demographic data were collected. All women provided written consent and the study was approved by the institutional review board at the University of Southern California, Los Angeles, CA and Children's Hospital Los Angeles (CHLA) (Los Angeles, CA) and the research ethics board at the University of Manitoba.

2.2 | Data and sample collection

Methods for data and sample collection have previously been described.[26] Briefly, demographic, obstetric, and gynecological data were collected by structured questionnaire. Cervical ectopy was measured by taking a digital picture of the cervix with an inserted endocervical wick (Tear-Flo™) serving as a length standard. A woman was considered to have ectopy if there was any endocervical epithelium visible. The size of the ectopic area was determined by measuring the total size of the ectopic area compared with the total size of the cervix. CVL samples were collected by bathing the cervical os in phosphate-buffered saline and aspirating fluid from the vaginal vault.

2.3 | Sample preparation for mass spectrometry

Cervicovaginal lavage sample preparation was performed as previously described.[52-54] Briefly, 50μg of protein from each sample was denatured for 20 minutes at room temperature with urea exchange buffer (8M urea; GE HealthCare; 50 mmol/L HEPES pH 8.0; Sigma), reduced with 25 mmol/L dithiothreitol (Sigma), alkylated with 50 mmol/L iodoacetamide (Sigma), and digested with trypsin (Promega). Peptides were eluted and dried via vacuum centrifugation. Reversed-phase liquid chromatography (high pH RP, Agilent 1200 series microflow pump; Water XBridge column) was used for desalting and detergent removal of peptides using a step-function gradient as described previously.[55] Peptides were quantified using the FluoroProfile® quantification kit (Sigma) following the Lava Pep peptide quantification protocol. Samples were randomized and aliquoted with a final peptide concentration of 0.5 μg/μL in LC buffer (2% acetonitrile, 0.1% formic acid) to a volume of 15 μL.

2.4 | Mass spectrometry analysis

Cervicovaginal lavage peptides samples were analyzed by label-free tandem mass spectrometry as described previously.[55] Equal amounts of sample peptides were injected into a nanoflow LC system (Easy nLC; Thermo Fisher) connected inline to a Q Exactive Quadrupole mass spectrometer (Thermo Fisher) and analyzed in a label-free manner. Raw data exported from the mass spectrometer was run through Progenesis QI software using default parameters.

2.5 | Human proteome data analysis

Mascot (Matrix Science, v2.4) was used to search peptide sequences against the SwissProt (2013) human database. A decoy database was included to determine the rate of false discovery. Protein identifications were confirmed using Scaffold software (v4.4.1; Proteome software) with confidence thresholds set at 95% protein identification confidence, requiring at least two unique peptides and 80% peptide identification confidence. Normalized relative abundances of each protein within each sample were obtained from Progenesis QI (v.21.38.1432; Nonlinear Dynamics). Relative protein abundances were calculated by dividing by median intensity across all samples, followed by a log transformation (base 2). Only proteins that had an average covariance of <25% (550 proteins), as determined through measurements of a standard reference sample run at 10 sample intervals (total six times) were used in downstream analysis to exclude proteins with higher technical measurement variability.

2.6 | Microbial proteome data analysis

Protein database searches were initially conducted against all bacterial proteins in the TrEMBL database using Mascot (v2.4.0; Matrix Science). Identity searches for bacterial peptides were then performed a second time using a manually curated database limited to the major genera identified in the initial search. Our curated database included proteins from the following genera (from most to least abundant): *Lactobacillus*, *Gardnerella*, *Acinetobacter*, *Pseudomonas*, *Paenibacillus*, *Chlamydia*, *Megasphaera*, *Delftia*, *Butyrivibrio*, *Bifidobacterium*, *Atopobium*, *Bradyrhizobium*, *Prevotella*, *Clostridium*, and *Roseburia*. The curated database included sequence data from *Homo sapiens* to rule out potential homologies. Search results were then imported into Scaffold (v4.4.1) to validate these protein identifications, using the following criteria: ≤0.1% FDR for peptide identification, ≤1% FDR for protein identification, and at least two unique peptides identified per protein. Microbial abundance was calculated by taking the sum of normalized total spectral counts from Scaffold for all proteins associated with each genus. One woman was removed from the analysis because no bacterial proteins were detected by MS.

2.7 | Functional microbiome analysis

Non-homologous bacterial proteins identified in each patient were mapped against the KEGG ontology database using GhostKOALA (v.2.0; Kyoto University Bioinformatics Center). Wilcoxon rank sum and permutation were used to determine functional differences in the microbiota between pregnant and non-pregnant women. A total of 24 bacterial functions at the ko-level could be assessed with 80% power (COV = 1.45, power = 0.80, FD = 1.5, 20% sample coverage).

2.8 | Statistical and pathway analysis

All statistical analyses were performed using R version 3.6.0 and additional packages "ggplot2" (v.3.2.1), "dplyr" (v0.8.3), "digest" (v0.6.20), "ggrepel" (v.0.8.1), "NMF" (v.0.21.0), "dendextend" (v.1.12.0), "RColorBrewer" (v.1.1-2), "ggfortify" (v.0.4.7), and "vegan" (v.2.5-5) or Prism. Differences in epidemiological characteristics between pregnant and non-pregnant women were assessed using Fisher's exact test and Mann-Whitney *U* tests, where appropriate. Unpaired *t* tests were conducted to compare the host protein expression levels between pregnant and non-pregnant women, while Mann-Whitney *U* tests were used to determine differences in microbial composition and functional pathways. Comparisons were considered statistically significant if they had a $P < .05$. The Benjamini-Hochberg false discovery rate (FDR) method was used to correct for multiple hypotheses. The Pearson's correlation (uncentered) and complete linkage was set as the distance metric. Enrichment of pregnancy and ectopy variables within dendrogram clusters were assessed using two-tailed Fisher's exact tests. Correlations between the host proteome and clinical data including length of gestation and size of ectopic area were assessed using Spearman's rank tests and were adjusted for multiple hypothesis testing correction as above. Proteins significantly associated with pregnancy ($P < .05$) were analyzed using QIAGEN's

Ingenuity Pathway Analysis software (IPA, Qiagen Redwood City), which determined the top enriched biological functions, with corresponding activation z-scores to infer activation/deactivation of biological pathways according to proteome effects. Significant pathways associated with pregnancy passed a critical value of $\alpha < 0.05$ and activation *z* score> |2|.

3 | RESULTS

3.1 | Participant characteristics

Characteristics of this cohort have previously been reported.[26] Twenty-three pregnant (47.9%) and 25 non-pregnant (52.1%) women (n = 48) were enrolled. Pregnant women were significantly younger than non-pregnant women (mean 27.8 years vs 33.3 years, *P* = .02), and the majority of women (93.8%) identified as Hispanic. There was no significant difference in the presence of cervical ectopy (60.9% vs 40%, *P* = .54), gravida (*P* = .4), or parity (*P* = .44) between groups. The mean gestational age among pregnant women was 25 weeks (range 14-37 weeks). Non-pregnant women were not currently menstruating. The date of last menstrual period was not recorded. Upon gynecological examination, vaginal candidiasis was suspected in two pregnant women. While bacterial vaginosis was suspected in one non-pregnant woman based on gynecological observations, no testing for bacterial vaginosis was performed. (Table 1).

3.2 | Mucosal proteome in pregnant women reflects alterations to inflammatory pathways

Mass spectrometry analysis of CVL samples collected from study participants identified 550 unique human proteins. Of these, 56 (10%) were found to be differentially abundant (*P* < .05) between pregnant and non-pregnant women (Figure 1A, Table S1), although AMY1 (involved in carbohydrate metabolic processes) and IGHA1 (involved in humoral immunity) were the only proteins that passed multiple comparison correction at FDR < 0.05. Given that pregnant women were significantly younger, we performed correlation analysis between age and differentially abundant proteins. Seven proteins involved in inflammatory processes significantly correlated with age. Three of these proteins (PAEP, LCN2, S100A7) correlated with age in pregnant women and 5 of them in non-pregnant women (RNASET2, ACE, PZP, S100A7, and APOL1). Four of these proteins (APOL1, ACE, RNASET2, and PAEP) remained significantly associated with pregnancy status after adjustment for age. Hierarchical clustering of differentially abundant proteins clearly distinguished women based on pregnancy status (*P* < .0001, Fischer's exact test) (Figure 1B). While the differentially abundant proteins did not cluster based on presence of ectopy (*P* = .245, Fisher's exact test), cornified envelope proteins were negatively correlated with the size of the ectopic area (TGM3: *r* = −.4034, *P* = .0045; DMKN: *r* = −.373, *P* = .0090).

Two clusters of upregulated and downregulated proteins clearly discriminated pregnant women from non-pregnant women (Figure 1B). Factors decreased in pregnant women were primarily associated with immunity, including neutrophils (ACE, RNASET2), immunoglobulins (IGJ, PIGR, IGHG2, IGHA2), and complement (CF1) (Figure 1C). However, several innate immunity factors were also increased in pregnancy including neutrophil-associated factors (S100A7, LCN2, CTSH, ANXA2) and complement (C1RL), as well as adaptive immunity (UBE2V1, STX7) (Figure 1C,D). Principal component analysis of the 27 immune-related proteins that were differentially abundant between pregnant and non-pregnant women provided clear separation based on pregnancy status (Figure 1C). Proteins increased in pregnant women also included factors previously described to be associated with pregnancy (PZP, KRT19, RAP1A) (Figure 1E) as well as with angiogenesis (S100A7, CTSH, ANXA2) (Figure 1F). Among pregnant women, KRT19 was positively correlated with gestational age (r = 0.4755, *P* = .0218), although this did not pass correction for multiple comparisons.

Upregulated biofunctions significantly associated (*P* < .05, Activation *z* score ≥ 2) with pregnancy included angiogenesis,

TABLE 1 Participant characteristics

Variable	Pregnant (n = 23)	Non-pregnant (n = 25)	*P* value[a]
Socio-demographic			
Mean Age ± SD (range)	27.8 ± 5.8 (17-38)	33.3 ± 7.3 (19-44)	0.02
Hispanic (n, %)	21 (91.3%)	24 (96%)	0.60[b]
Obstetric/Gynecological			
Mean gestational age ± SD (range)	25 ± 7 (14-37)	—	—
Cervical ectopy (n, %)	14 (60.9%)	10 (40%)	0.54
Gravida (Mean, Range)[c]	3 (1-8)	2 (0-7)	0.40
Parity (Mean, Range)[c]	1 (0-5)	2 (0-5)	0.44

[a]Mann-Whitney *U* test unless otherwise indicated.

[b]Fisher's exact test where all non-Hispanic participants are grouped as "other".

[c]Data not available for two non-pregnant participants.

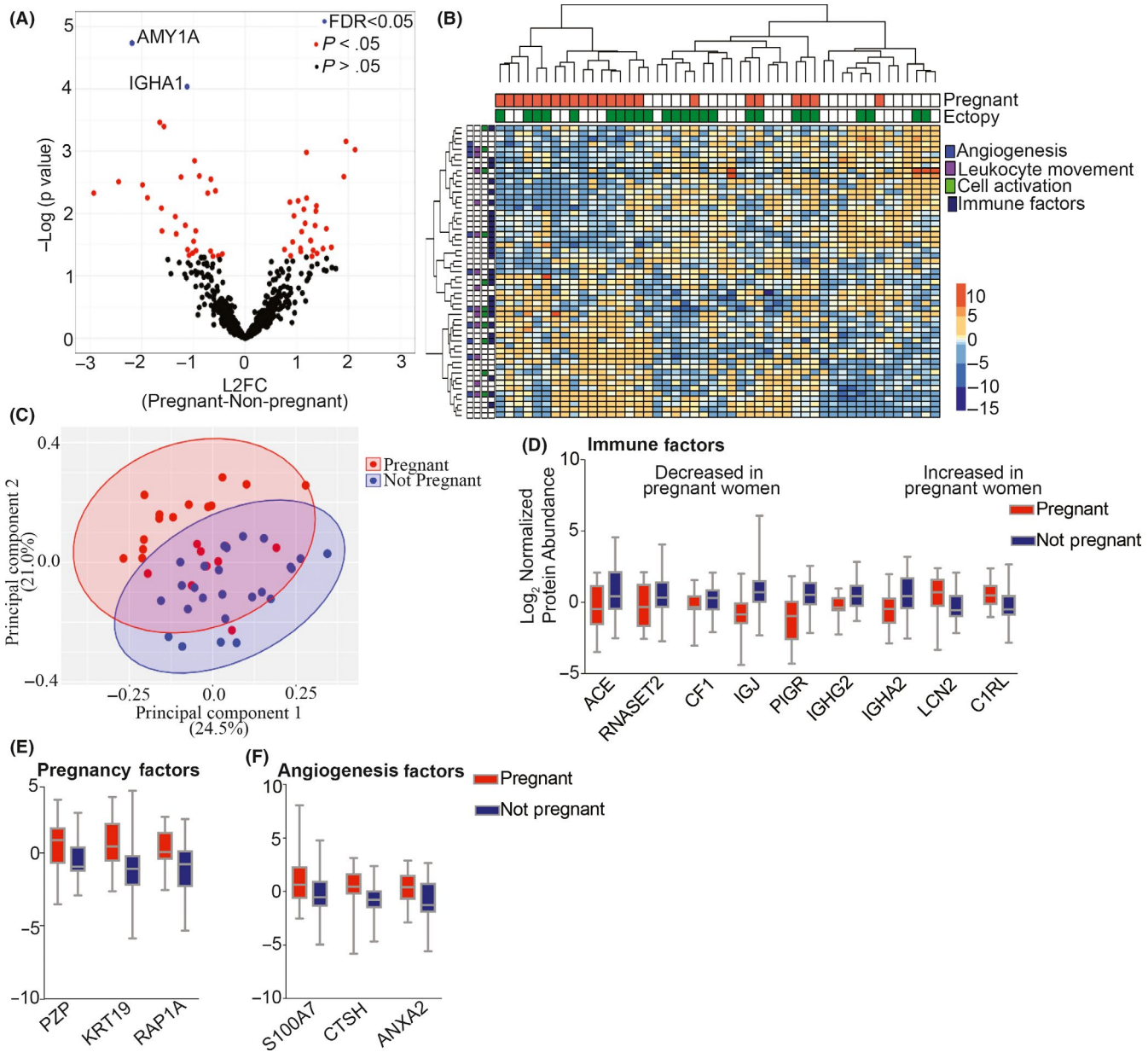

FIGURE 1 Cervicovaginal proteome pathways that are differentially abundant between pregnant and non-pregnant women. A, Volcano plot of all proteins identified comparing pregnant and non-pregnant women using two-tailed independent *t* tests. B, Hierarchical clustering of differentially abundant ($P < .05$) proteins between pregnant and non-pregnant women. Proteins that are overabundant are represented in the heat map in red and those that are underabundant are represented in blue. Pregnancy status and ectopy status are shown. Proteins involved in biofunctions significantly associated with pregnancy (angiogenesis including vasculogenesis and blood vessel permeability; movement of leukocytes; activation of cells; and immune factors) are highlighted. A total of 56 proteins were differentially abundant between pregnant and non-pregnant women, with 27 overabundant in pregnant women and 29 underabundant. C, Principal component analysis of 27 immune-related factors that were significantly different between pregnant and non-pregnant women. Boxplots depicting $\log_2$ normalized protein abundance for immune (D), angiogenesis (E), and pregnancy (F) factors that were differentially abundant ($P < .05$) between pregnant and non-pregnant women

vasculogenesis, permeability of blood vessel, cell movement of phagocytes and leukocytes, and activation of cells (Figure 1B, Table S2). No biofunctions were associated with downregulated proteins in pregnant women. Gene ontology analysis associated differentially abundant proteins with immunoglobulin receptor binding, complement activation, and leukocyte infiltration (Table S3).

3.3 | Pregnant women have a microbiome dominated by *Lactobacillus*

We detected microbial protein expression in pregnant and non-pregnant women using mass spectrometry (MS), which was then used to infer taxa compositions within the metabolically active portion of the vaginal microbiome (Figure 2A). MS detected 376 bacterial

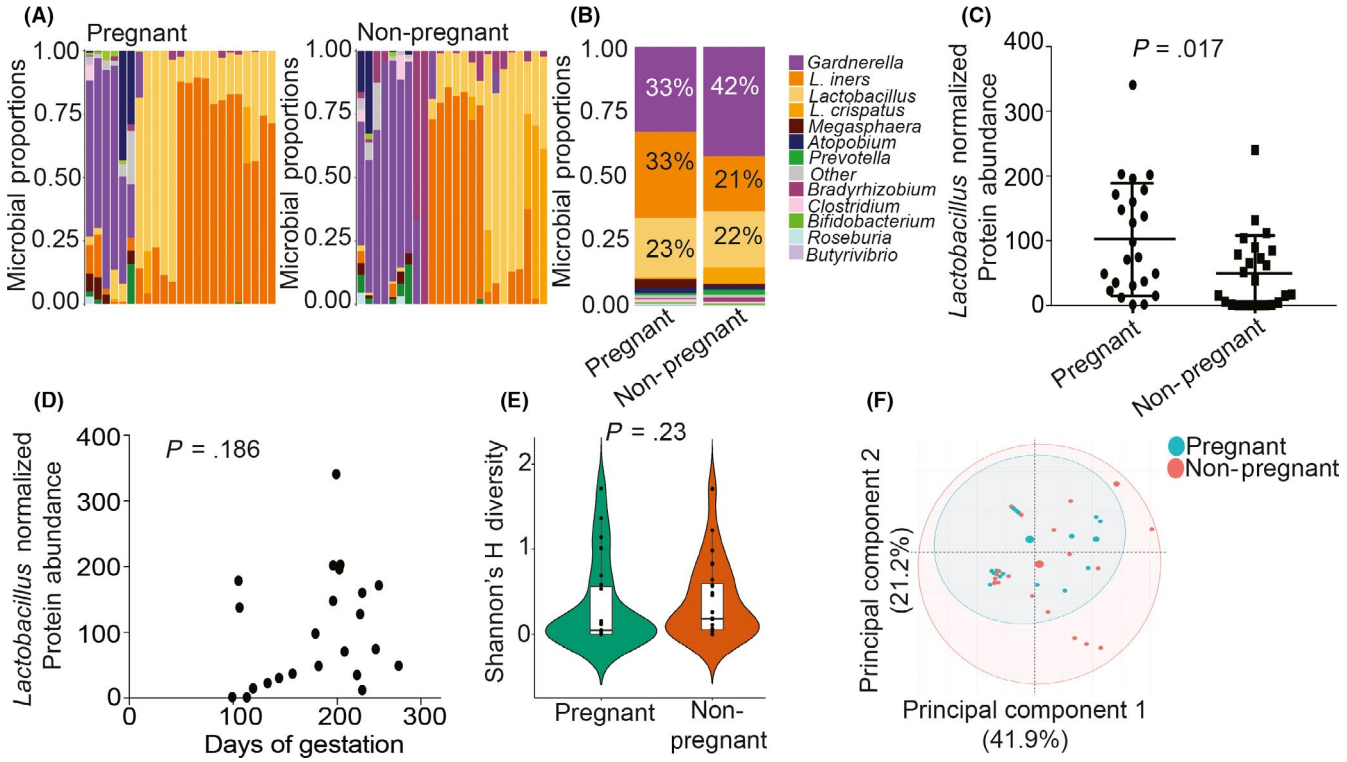

FIGURE 2 The microbiome in pregnant women is dominated by *Lactobacillus*. A, Taxa proportion plots of each individual based on pregnancy status detected by MS *Lactobacillus* is displayed to the species level for the two most abundant species detected, *L iners* and *L crispatus*. B, Summary of distribution of bacterial taxa by pregnancy status. The average percentages for the top three bacterial taxa are shown. C, Normalized protein abundance for all *Lactobacillus* species in pregnant and non-pregnant women. *P* value was calculated using the Mann-Whitney *U* test. D, Normalized protein abundance for all *Lactobacillus* species in pregnant women compared with gestational age. Spearman's $r = .2862$, $P = .1855$. E, Shannon's Diversity Index by pregnancy status. Wilcoxon *P* value is shown. F, Principal component analysis of all bacterial proteins detected in pregnant and non-pregnant women

proteins from 11 genera. The top bacterial genera were *Lactobacillus*, *Gardnerella*, *Prevotella*, *Megasphaera*, and *Atopobium*. In agreement with previous studies, *Lactobacillus* species were significantly increased in pregnant women compared with non-pregnant women ($P = .016$) (Figure 2B,C). *L iners* was the predominant species detected and was significantly increased in pregnant women ($P = .0081$), composing 33% of their microbiome compared with 21% in non-pregnant women (Figure 2B,C). While *Lactobacillus* tended to increase with gestational age, this was not statistically significant ($r = .286$, $P = .186$) (Figure 2D). There was no difference in bacterial diversity (Shannon's H $P = .23$) between pregnant and non-pregnant women (Figure 2E). Principal component analysis of all bacterial proteins detected indicated that there was no difference between pregnant and non-pregnant women, suggesting that women with *Lactobacillus* dominant proteins are more similar to each other regardless of pregnancy status (Figure 2F).

3.4 | Pregnancy associates with increases in carbon fixation pathways in *Lactobacillus* species

Functional shifts in the microbiome may be important for proper mucosal system functioning, including inflammation status and barrier function,[54] but have never been explored in the context of pregnancy. We matched 61.5% of the bacterial proteins to KEGG gene ontology with one or more functions. Overall, 24 bacterial functions were identified at the ko-level, primarily related to metabolism (Figure 3A). Carbon fixation pathways in prokaryotes (ko00720) were significantly increased in pregnant women (Log2 fold change = 1.5, $P = .035$) (Figure 3B). This functional shift is primarily derived from *Lactobacillus* proteins (54.7%), although proteins from *Megasphaera* (14%), *Gardnerella* (10.4%), *Atopobium* (9.1%), *Clostridium* (6.9%), and *Prevotella* (5%) also contributed (Figure 3C). Principal component analysis of ko-level protein groups demonstrated clustering by microbiome status (*Lactobacillus* dominant (LD) where > 50% of bacterial proteins are from *Lactobacillus* vs non-*Lactobacillus* dominant (nLD)) but not pregnancy status (Figure 3D). In support of this, 18 ko-level functions were significantly different ($P < .05$) based on *Lactobacillus* dominance. However, the carbon fixation pathway (ko00720) was not ($P > .999$), suggesting that this bacterial function is uniquely associated with pregnancy. When both pregnancy status and microbiome composition were taken into consideration, women with LD microbiomes clustered together regardless of pregnancy status (Figure 3E). L-lactate dehydrogenase, Glyceraldehyde-3-phophospate dehydrogenase type I, pyruvate kinase, and phosphoglycerate kinase were primarily driving the variance within the LD

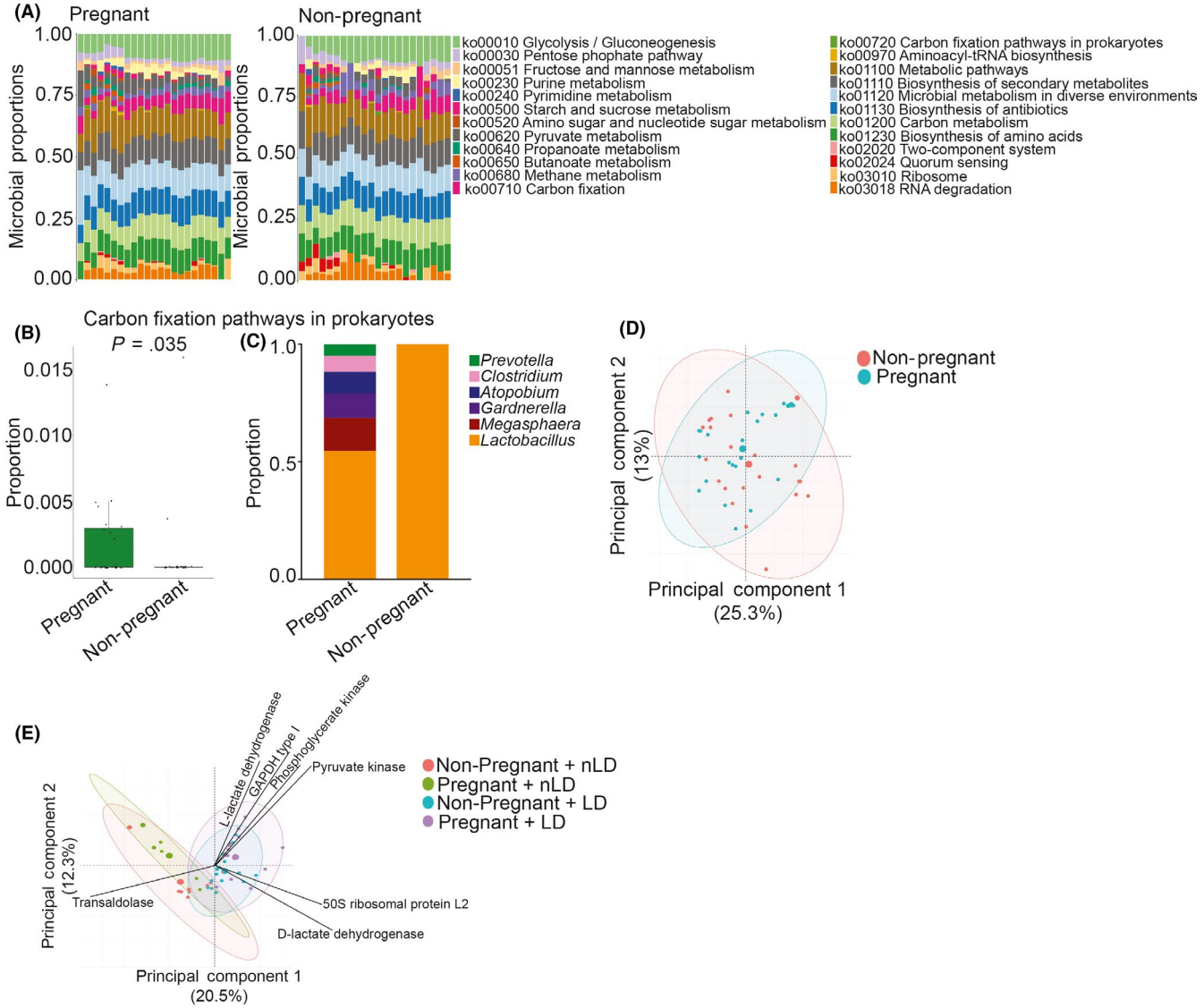

FIGURE 3 Functional microbiome pathway analysis. A, ko-level bacterial functions in pregnant and non-pregnant women. B, Carbon fixation pathways in prokaryotes are increased in pregnant women. C, Bacterial genera that contribute proteins to carbon fixation pathways in prokaryotes in pregnant and non-pregnant women. D, Principal component analysis of ko-level bacterial functional data with pregnancy status. E, Principal component analysis of ko-level bacterial function data with both pregnancy and microbiome (LD vs nLD) status. Lines indicate bacterial proteins that are driving the variances

group, while transaldolase was responsible for the variation in the nLD group.

4 | DISCUSSION

This study is the first comprehensive proteomic investigation of mucosal factors in healthy pregnant women and several key findings were identified. The first was that pregnant women had increased proteomic signatures of blood vessel formation and immune cell recruitment. Secondly, there were key differences in the vaginal microbiome, including increased *Lactobacillus* levels and bacterial pathways important for energy metabolism. Finally, pregnant women had high rates of cervical ectopy. This demonstrates that the vaginal mucosa of pregnant women differs from non-pregnant women at the structural, microbial, and immunological levels.

Pregnant women have been reported to be at an increased risk for HIV acquisition, although the mechanism behind this is not well understood.[2-5] Pregnant women were thought to have a suppressed immune system, although more recent studies have suggested that the immune system of pregnant women is active but tightly regulated, leading to differential responses to pathogens.[16,21-24,28,29] There may be local immune responses within the female reproductive tract that change over the course of pregnancy.[16] Our data demonstrated changes to factors involved in innate and adaptive immunity among pregnant women. A previous study has found a systemic increase in factors related to leukocyte migration during pregnancy,[27] which agreed with our mucosal

findings which show increased proteins associated with leukocyte motility. This may be an important component of blood vessel permeability and formation, processes of which are important for the development and maintenance of pregnancy. These overlapping and sometimes conflicting immune changes during pregnancy indicate a complex balancing act to protect from pathogens while maintaining a semi-allogenic fetus.

In support of previous studies, the microbiome of pregnant women in this study was primarily dominated by *Lactobacillus*. There was also a trend toward decreased bacterial diversity in pregnant women compared with non-pregnant women. During pregnancy, it is thought that the vaginal microbiome shifts to one dominated by *Lactobacillus* to protect against infections, including HIV.[45-50] *Lactobacilli* produce several antimicrobial compounds, lower the environmental pH by production of lactic acid, and provide competitive exclusion for other bacterial species.[43-45] Low vaginal pH in women with *Lactobacillus* can inactivate cell-free and cell-associated HIV,[38] and the absence of *Lactobacillus* is associated with an increased risk of acquiring HIV.[37-42] Furthermore, bacterial vaginosis during pregnancy, which occurs when *Lactobacillus* are replaced by anaerobic bacteria, increases the risk pregnancy complications,[42] suggesting that increases in *Lactobacillus* are important for decreasing adverse pregnancy outcomes. Interestingly, *L iners* was the most commonly identified species of *Lactobacillus* in this study. Previous studies generally found pregnant women to be dominated by *L crispatus*, but many also identified *L iners* within the microbiome.[46-48,51,56] A microbial profile dominated by *L iners* is more likely to shift to dysbiosis[57] and has been associated with elevated levels of proinflammatory mediators including IL-8 and IP-10.[58] As these women were not followed longitudinally, we are not able to determine if there were shifts in the microbiome or if there was any relationship between *L iners* dominance and adverse pregnancy outcomes.

While it has previously been reported that pregnant women are more likely to have a vaginal microbiome dominated by *Lactobacillus*, the functional microbiome differences have not been explored. Here, we determined that there were significant increases in energy metabolism in the microbiome of pregnant women compared with non-pregnant women. This suggests that the microbiome of pregnant women may be more metabolically active than that of non-pregnant women. Principal component analysis of ko-level bacterial functions indicated that women clustered based on their microbiome composition regardless of pregnancy status. However, as this study is small, larger studies would be needed better understand these functional microbiome differences and potential relevance to HIV susceptibility.

The epithelial lining of the FGT provides a structural barrier against pathogen invasion and damage. Changes to this barrier, such as the generation of breaches from pathogen-related damage, may facilitate entry for HIV-1. Proteins associated with epithelial barrier damage are also modified during pregnancy. Cervical ectopy, which was present in 60.9% of the pregnant women, is one structural change that could increase HIV-1 susceptibility.[34,35]

Taken together this data suggests that immune system complexities and epithelial barrier dysfunction that occur during pregnancy may be contributing to increased HIV acquisition in pregnant women, while the microbiome may be protective. While the hormonal changes that occur during pregnancy could be responsible for all of these observed changes, it is difficult to determine if there are additional interactions between the microbiome, epithelial barrier, and immune system that could drive these changes.

Our study has various limitations including the small study size and cross-sectional study design. In addition, the majority of the participants identified as Hispanic, which could impact the representativeness of this data. Furthermore, menstrual cycle phase at time of sample collection was not recorded for the non-pregnant women and, therefore, may represent a source of proteome variation within our control group that we could not account for in our study. However, since we were comparing pregnant women to non-pregnant women, we believe that the dramatic increase in ovarian hormones that occurs during pregnancy (eg, progesterone is 10x higher during pregnancy than the luteal phase of the menstrual cycle) will have a greater influence on the proteome differences observed here than the cyclical shifts observed over the course of the menstrual cycle.[59] Our data do provide several indications of pathways that could contribute to HIV susceptibility during pregnancy; however, HIV acquisition was not measured. Larger, longitudinal studies of pregnant women would be required to better evaluate HIV acquisition and its' relationship to the risk factors measured in our study including cervical ectopy, microbiome, and immune cell recruitment signatures.

Overall, this study demonstrates that the vaginal mucosa of pregnant women differs from non-pregnant women at the structural, microbial, and immunological levels. These findings suggest that immunological and structural changes that occur during pregnancy may increase the risk for HIV acquisition and suggests that condom use should continue during pregnancy to provide protection from HIV.

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AUTHOR CONTRIBUTIONS

GMA and AB designed the study. CFZ, NT, TV, LK, KB, LNR, SH, FL, and DL analyzed the data. TV, LK, SM, and GW performed the experiments. CFZ and AB drafted the manuscript. All authors reviewed the manuscript.

ORCID

Adam D. Burgener https://orcid.org/0000-0002-2303-5738

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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