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The Vaginal Microbiome of Transgender Men

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BACKGROUND: Hormonal changes influence the composition of vaginal flora, which is directly related to the health of an individual. Transgender men prescribed testosterone experience a vaginal hormone composition that differs from cisgender women. To the author's knowledge, there are no clinical studies evaluating the influence that testosterone administration has on the vaginal microbiome.

METHODS: Vaginal swabs were self-collected by a cohort of self-identified healthy transgender men prescribed testosterone for at least 1 year (n = 28) and from cisgender women who were used as the comparator (n = 8). Participants completed a questionnaire to indicate the mode and dose of testosterone administration, sexual history, and vaginal health. Serum was collected for hormone analysis. Bacterial community profiles were assessed with broad-range PCR primers targeting the V3–V4 hypervariable region of the 16S bacterial rRNA, next-generation sequencing, and analysis by phylogenetic placement.

RESULTS: Compared to cisgender women, the vaginal floras of transgender men were less likely to have *Lactobacillus* as their primary genus. Intravaginal estrogen administration was positively associated with the presence of *Lactobacillus* in transgender men ($P = 0.045$). Transgender men had a significantly increased relative abundance of >30 species and a significantly higher α diversity ($P = 0.0003$). The presence of *Lactobacillus* was significantly associated with a lower α diversity index ($P = 0.017$).

CONCLUSIONS: The vaginal microbiome of transgender men who were assigned a female sex at birth and use testosterone may differ from that of cisgender women. Intravaginal estrogen administration may reduce these differences by promoting colonization with *Lactobacillus* species and decreasing α diversity.

Socialized dogma that engrains sex and gender as a synonymous binary has led to the lack of transgender representation in the medical literature *(1)*. This lack is particularly amplified when addressing areas of sexual health for transgender men *(2)*. Transgender men were assigned a female sex at birth on the basis of the presence of a vulva and/or labia, which implies that the additional reproductive organs include a uterus and ovaries, thus defining the sex of the newborn. However, the development of gender is more complicated than the visual appearance of sex organs, and as such gender incongruence consequently occurs when some individuals assigned a specific sex at birth do not identify with the equivalent gender *(3)*. Transgender men fall into this category and will often use testosterone hormone therapy, which allows them to develop physical characteristics associated with masculinity (i.e., facial hair, increased muscle mass) and to affirm their gender identity. Other physiological changes can include cessation of menses, increased clitoral size, atrophic vaginitis, decreased vaginal epithelium thickness, and ovarian hyperplasia *(4)*. Some transgender men will pursue gender-affirming surgeries, such as chest masculinization (so-called top surgery) and/or partial or complete hysterectomy, but many will still retain their natal reproductive organs, particularly their genitalia *(5, 6)*.

To the best of our knowledge, there have been no clinical studies evaluating the changes in vaginal flora that occur when transgender men are exposed to testosterone. Data collected on cisgender women indicate that hormonal fluxes play a significant role in the development and maintenance of vaginal microbes *(7)*. Throughout childhood, the vaginal flora resembles that of the gut and skin. The onset of puberty, with its rapid increases in estrogen and progesterone, promotes anatomical and biochemical changes that support the growth of lactobacilli, which remain a dominant member of the healthy vaginal microbiome throughout the reproductive years. With the onset of menopause, the vaginal architecture restructures to an environment that is less supportive of lactobacilli. This reproductive transition is

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often accompanied with irritability, vaginal atrophy and dryness, and an overall decrease in sexual health *(8)*. Research indicates that oral or intravaginal estrogen administration relieves these symptoms *(9, 10)*.

Developing a basic understanding of the vaginal microbiome for the transgender male population has important applications to their general health, the practice of preventative medicine, and decision support during the course of primary care. The objective of this study was to catalog and explore differences between the vaginal microbiota of transgender men receiving testosterone and cisgender women by use of next-generation 16sr-RNA gene sequencing *(11, 12)*.

Methods

PATIENT RECRUITMENT, QUESTIONNAIRES, AND SAMPLE COLLECTION

Self-identifying transgender men who were assigned a female sex at birth were prospectively recruited from a lesbian, gay, bisexual, transgender, queer (LGBTQ)[3]–oriented primary care and internal medicine clinic in Seattle, Washington. Individuals who identified as nonbinary and who met our inclusion criteria (assigned the female sex at birth and use testosterone as gender-affirming hormone therapy) were grouped with the transgender men. The gender identity of nonbinary people is distinct from transgender men *(3)*, but under these criteria, exposure of the reproductive organs to testosterone would be identical. Control participants were recruited either from the same clinic or from the author's institution. Those recruited from the clinic all identified as LGBQ, while only 2 of the cisgender controls recruited from outside the clinic identified as LGBQ. The number of control specimens analyzed (n = 8) was selected for practicality and limited resources. Primarily, we wanted to make sure that our experiments recovered bacterial species in their relative abundance similarly to what has been previously published. Secondarily, the reagents and time needed to perform this testing is resource-intensive and the addition of this number of control samples was manageable within our experimental runs. All participants self-identified as healthy and were receiving primary care appointments for a variety of wellness reasons, such as gender-affirming hormone use follow-up, annual physical, contraception, or mental health follow-up.

Informed consent was obtained from all participants, and participants were asked to provide 2 vaginal swabs (Puritan® 6-inch sterile standard foam swabs) on a voluntary basis by following self-collection instructions (see material in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue1) *(13)*. Additionally, venipuncture was used to obtain a gold-top serum sample (5 mL) for serum estrogen and testosterone measurements. Vaginal flora can be affected by age and sexual practices. For that reason, we collected data on these factors. Basic demographic information and sexual history including type of intercourse, history of sexually transmitted infection, number of partners, overall genital concerns, contraception and hormonal therapy with dosage were collected in a standardized questionnaire (see material in the online Data Supplement). Study numbers were used in place of participant names; no patient identifiers were retained.

All participants were at least 18 years old. Self-identified transgender participants had been prescribed gender-affirming testosterone hormone therapy for at least 1 year and consented to collection of relevant samples and information. Recruitment was consecutive, as inclusion criteria, consent, and clinic work flow allowed. Cisgender controls were selected to reflect a range of ages, sexual orientations, contraception use, and fertility status. Participants were excluded if they had any preexisting conditions that are known to influence general chemistry or hematology laboratory measurements. More specifically, patients were excluded if they had a past history of diabetes, severe cardiovascular event (e.g., myocardial infarction or stroke), HIV infection, obstructive sleep apnea, or clotting disease. Patients were also excluded if they were currently pregnant, actively breast feeding, were a current smoker or had a body mass index higher than 30. Participants were not included or excluded on the basis of previous sexual activity, history of herpes simplex virus (HSV), human papilloma virus, gonorrhea, *Chlamydia*, bacterial vaginosis (BV), or other vaginal concerns. Exclusion criteria were selected because the participants in this study were also being recruited for a study designed to establish hematology, chemistry, and special chemistry reference intervals in transgender men and women (unpublished observations). The Western Institutional Review Board approved the protocol (study number 1179338).

SERUM HORMONE CONCENTRATIONS

Estradiol and total testosterone were measured within 8 h of serum collection by use of the DxI 800 competitive immunoassays (Beckman Coulter), which are linear from 30 to 4800 pg/mL and 0.3 to 16 ng/mL, respectively. Adult reference intervals are sex, age, and/or reproductive phase specific, as follows. Testosterone: women, ≤0.8 ng/mL; men ages 17–19 years, 2.5–10 ng/mL; men ages 20–29 years, 2.2–7.8 ng/mL; men ages 30–39 years, 2.0–7.3 ng/mL; men ages 40–49 years, 1.8–6.8 ng/mL; men ages 50–59 years, 1.7–6.3 ng/mL. Estrogen: women, midfollicular, 27–122 pg/mL; women, midlu-

[3] Nonstandard abbreviations: LGBTQ, lesbian, gay, bisexual, transgender, queer; HSV, herpes simplex virus; BV, bacterial vaginosis.

Table 1. Demographics of study cohorts.

	Transgender men (n = 28)	Cisgender women (n = 8)
Average age years (range)	30.6 (21–55)	35.5 (27–50)
Mode of testosterone administration	Injection (n = 24) Topical (n = 3)	NA
Average injected testosterone dose (range)	74.2 mg/wk (45–100 mg/wk)	NA
Topical testosterone dose	50 mg/d	NA
Other hormones	Estrogen ring (n = 3) Vaginal estrogen cream (n = 1)	Oral contraceptives (n = 2) IUD (n = 3) Estrogen ring (n = 1)

teal, 49–291 pg/mL; women, periovulatory, 95–433 pg/mL; women, postmenopausal, <41 pg/mL; men, <48 pg/mL.

DNA EXTRACTION AND BACTERIAL COMMUNITY PROFILING

Following collection, swabs were immediately placed in −20 °C storage for up to 72 h before transferring to −80 °C storage. DNA was extracted within 6 months of collection by use of a Qiagen® QIAamp BiOstic Bacteremia DNA Kit, with swabs incubated in 450 μL of lysis buffer for 10 min followed by expressing lysate from the foam applicator tip. Unused sham swab controls and reagent-only controls were extracted in parallel with each round of samples to characterize background bacterial DNA contamination.

Extracted DNA was amplified with broad-range primers targeting the V3–V4 hypervariable region of the 16S bacterial rRNA, as described elsewhere *(11)*. Sequencing was performed by an Illumina MiSeq (Illumina) with 600 cycle V3 chemistries.

READ PROCESSING, PHYLOGENETIC REFERENCE SET CREATION, AND TAXONOMIC ASSIGNMENT

Barcoded reads were demultiplexed by MiSeq Reporter Software (Illumina). DADA2 *(14)* was used to infer error-corrected sequence variants from fastq files downsampled to no more than 100K reads. Sequence variants were compared to a 16S rRNA alignment model with cmsearch *(15)*, and those with an e-value <0.01 were discarded to exclude non-16S rRNA sequences (e.g., those resulting from off-target amplification). To reduce the effects of low-level DNA carryover and barcode misassignment, counts for sequence variants represented by 100 or fewer reads in a given sample were discarded.

The remaining sequence variants were compared to a curated collection of 16S rRNA records downloaded from NCBI to identify related species; representative reference sequences were aligned with cmalign *(15)*, and a phylogenetic tree was calculated with RAxML *(16)*. Sequence annotation, multiple alignment, and tree were packaged into a phylogenetic reference set with taxtastic acquired from the GitHub repository.

To perform taxonomic assignment, sequence variants were aligned with cmalign, placed onto the reference tree with pplacer, and classified with "guppy classify" *(17)*. Details of these pipelines, software versions, and parameters are provided in the methods in the online Data Supplement. The phylogenetic reference set used for classification is available as material in the online Data Supplement.

STATISTICAL ANALYSES OF MICROBIOME DATA

Phyloseq v1.22.3 *(18)* was used to calculate and plot the Shannon index for all specimens; Shannon index is an α-diversity measure accounting for both evenness and abundance of bacterial organisms within a given specimen *(19)*. A higher Shannon index represents increased α diversity. Phyloseq was also used to visualize bacterial population characteristics (heat map). DESeq2 v1.18.1 *(20)* was used to determine bacterial organisms with differing abundance in transgender men compared to cisgender women. The Fisher exact test was used to determine statistical significance of intravaginal estrogen administration; Mann–Whitney *U*-tests were used to determine significance of all other comparisons.

Results

STUDY COHORTS AND SERUM HORMONE CONCENTRATIONS

Vaginal microbiome composition was determined on a total of 28 transgender men and 8 cisgender women (Table 1). The serum testosterone concentration in transgender men were all within or slightly above the corresponding adult cisgender male reference interval, while the serum estrogen concentrations were within the adult cisgender male reference interval for the majority of transgender men (n = 20) and above the cisgender reference interval for a subset (n = 8) (Fig. 1). Compared to the cisgender female reference intervals, 17 of the transgender men were within the postmenopausal reference in-

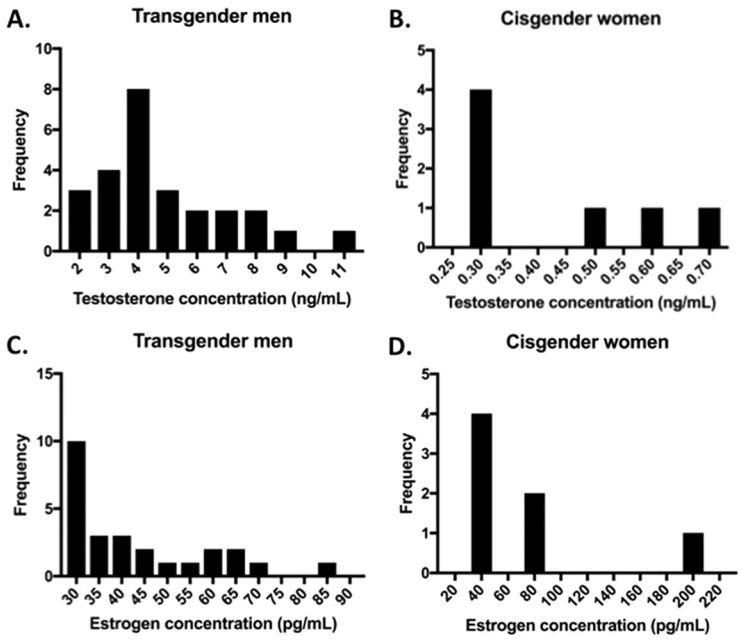

Fig. 1. Frequency distribution indicating the serum testosterone and estrogen concentrations in the transgender men (A and C) and cisgender women (B and D) cohorts.

Adult reference intervals are sex, age, and/or reproductive phase specific, as follows. Testosterone: women, ≤0.8 ng/mL; men ages 17–19 years, 2.5–10 ng/mL; men ages 20–29 years, 2.2–7.8 ng/mL; men ages 30–39 years, 2.0–7.3 ng/mL; men ages 40–49 years, 1.8–6.8; men ages 50–59 years, 1.7–6.3 ng/mL. Estrogen: women, midfollicular, 27–122 pg/mL; women, midluteal, 49–291 pg/mL; women, periovulatory, 95–433 pg/mL; women, postmenopausal, <41 pg/mL; men, <48 pg/mL.

terval; the remaining 11 were within the midfollicular reference interval. All cisgender women had serum testosterone concentrations within the adult female reference interval, and serum estrogen concentrations were within the expected reproductive cycle–specific interval. There was a statistically significant difference between the testosterone concentrations of transgender men and cisgender women ($P < 0.0001$), but there was no difference found in estrogen concentrations ($P = 0.84$).

In the previous year, the transgender men had a median of 1 sexual partner [range, 0–30; interquartile range (IQR), 1–3 partners], as did the cisgender women (range, 0–12; IQR, 1–4.75 partners). The difference in the distribution of sex partners was not found to be significant ($P = 0.63$). In the previous 5 years, 17 transgender male and 3 cisgender female participants had intercourse with partners who had a penis and partners who had a vagina; 6 transgender male and 0 cisgender female participants had intercourse only with partners who have a vagina; 5 transgender male and 5 cisgender female participants had intercourse only with partners who have a penis.

A history of HSV, gonorrhea, HSV and gonorrhea, or BV was documented in 4, 2, 1, and 1 transgender male participants and 1, 0, 0, and 1 of the cisgender female participants, respectively. There were no current infections with gonorrhea and to the best of our knowledge, no active flairs of HSV or BV in the transgender cohort; symptomatic BV was present in 1 of the cisgender women.

Vaginal estrogen was prescribed to 4 of the transgender men who had a history of either vaginal atrophy or BV. Symptoms had resolved in 3 of these individuals who were prescribed estrogen as a ring; 1 individual remained symptomatic and administered estrogen with a vaginal tablet. Additional hormones were not prescribed to this cohort.

Contraception was prescribed to 6 of the cisgender women orally (n = 2), as an intrauterine device (n = 3), or as an estrogen ring (n = 1).

BACTERIAL COMPOSITION AND DIFFERENTIAL ABUNDANCE

Lactobacillus was identified as the primary genus (>90%) inhabiting the vagina of all cisgender women evaluated, even for the participant (C11) with symptomatic BV (Fig. 2). In contrast, most of the transgender men (n = 20) had very little relative abundance (<2%) of *Lactobacillus* species. Five transgender men had minor (<25%) and 3 had major (>90%) relative abundance of *Lactobacillus* in the vagina. Closer analysis of the participants

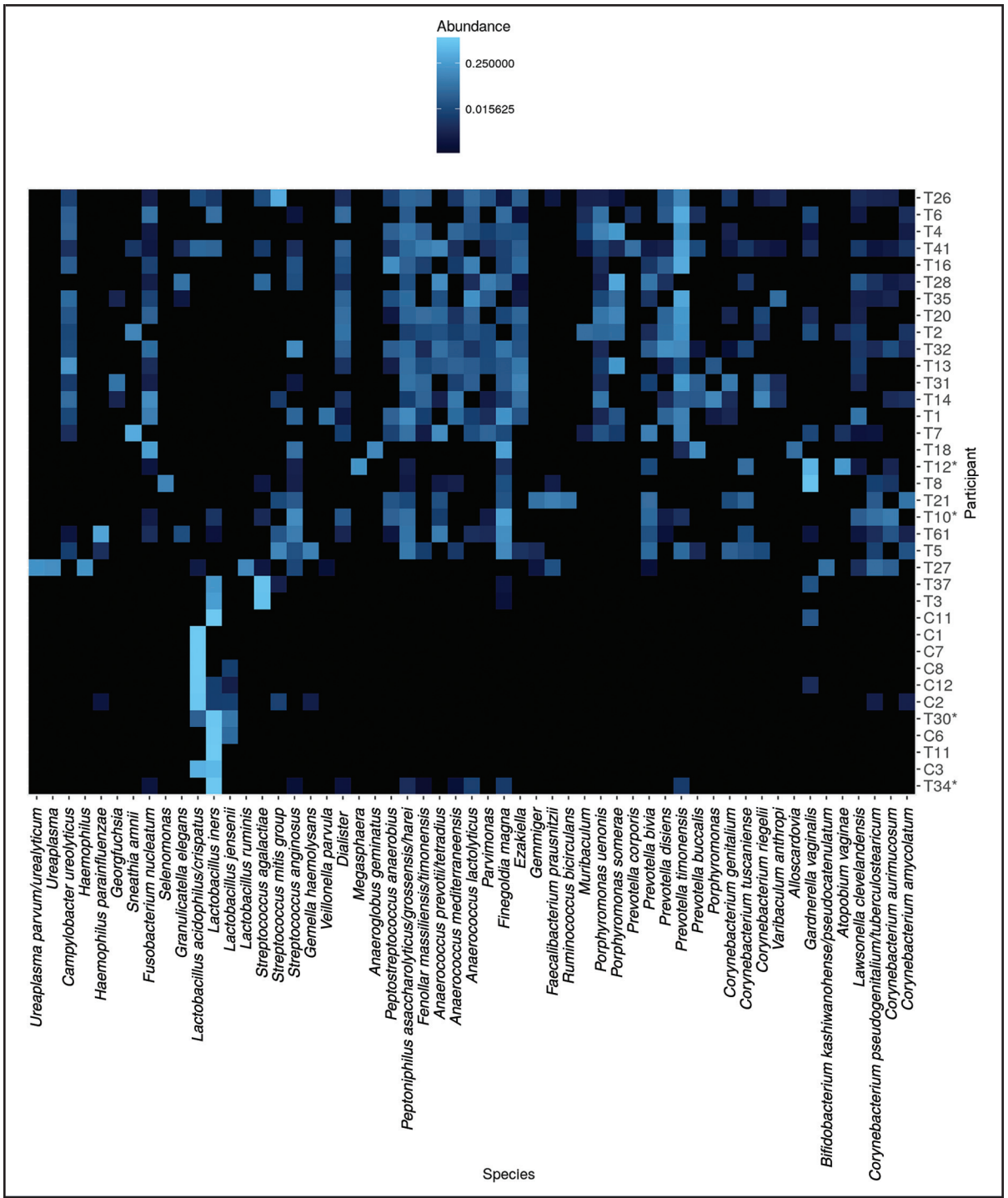

Fig. 2. Heat map illustrating the most specific classifications available for the set of organisms present in at least 5% relative abundance in at least 1 specimen.

Specimens are labeled on the *x* axis and grouped on the basis of species similarity. "C" indicates cisgender woman; "T" indicates transgender man. Transgender men administering intravaginal estrogen are labeled with an asterisk (*).

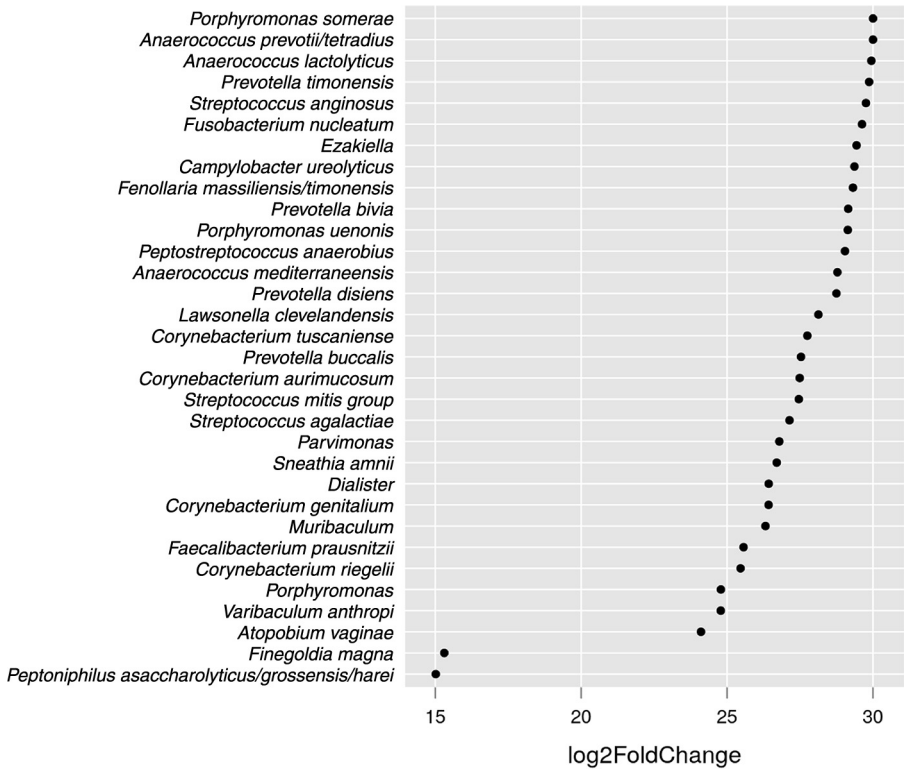

Fig. 3. Comparison of relative abundance for species between cisgender women and transgender men.

Plot only includes organisms with a difference in abundance at $P < 0.01$ and present in ≥5% abundance in at least 1 specimen. Numeric values greater than 0 indicate a higher relative abundance in transgender men. Values are expressed as $\log_2$-fold difference.

with >90% *Lactobacillus* indicated that 2 of these participants were prescribed vaginal estrogen. A Fischer exact test indicated a significant correlation between vaginal estrogen therapy and presence of a predominately *Lactobacillus* vaginal milieu (i.e., relative abundance exceeding 90%) in transgender men ($P = 0.045$). There was no association observed between sexually transmitted infection history and the presence of *Lactobacillus* ($P = 0.94$) or receptive vaginal sex and the presence of *Lactobacillus* ($P = 0.65$). Multiple organisms (n = 32 classified at the species level) were found to be in greater relative abundance in transgender men relative to cisgender women (Fig. 3).

INTRAINDIVIDUAL MICROBIOTA DIVERSITY

Median α diversity was greater in transgender men than cisgender women, with a median Shannon diversity index of 3.14 (range, 0.53–3.75; IQR, 1.93–3.56) and 0.75 (range, 0.12–1.75; IQR, 0.59–1.29), respectively, and the observed increase was statistically significant ($P = 0.0003$; Fig. 4). Shannon indices within the cohort of transgender men followed a bimodal distribution. Evaluation of the relative abundance of *Lactobacillus* species between these 2 modes (Shannon index <2 vs Shannon index >2) indicated that the presence of *Lactobacillus* in transgender men was significantly correlated with a lower Shannon index ($P = 0.017$), or in other words, less overall microbial diversity.

Discussion

Clinical observations have documented that testosterone administration can lead to vaginal atrophy and downstream symptoms in transgender men *(4)*, but no previously published study has evaluated the effect of testosterone on the vaginal microbiome. In postmenopausal women, decreased estrogen has been shown to affect the availability of glycogen, which makes conditions less favorable for *Lactobacillus* species and consequently alters the vaginal microbiota from the premenopausal state *(21)*. The data derived in this study suggest that a similar relationship between estrogen and vaginal *Lactobacillus* abundance may occur in transgender men.

The presence of *Lactobacillus* is the hallmark of a healthy vaginal microbiome *(22)*. The replacement of *Lactobacillus* with an alternative microbial ecosystem has

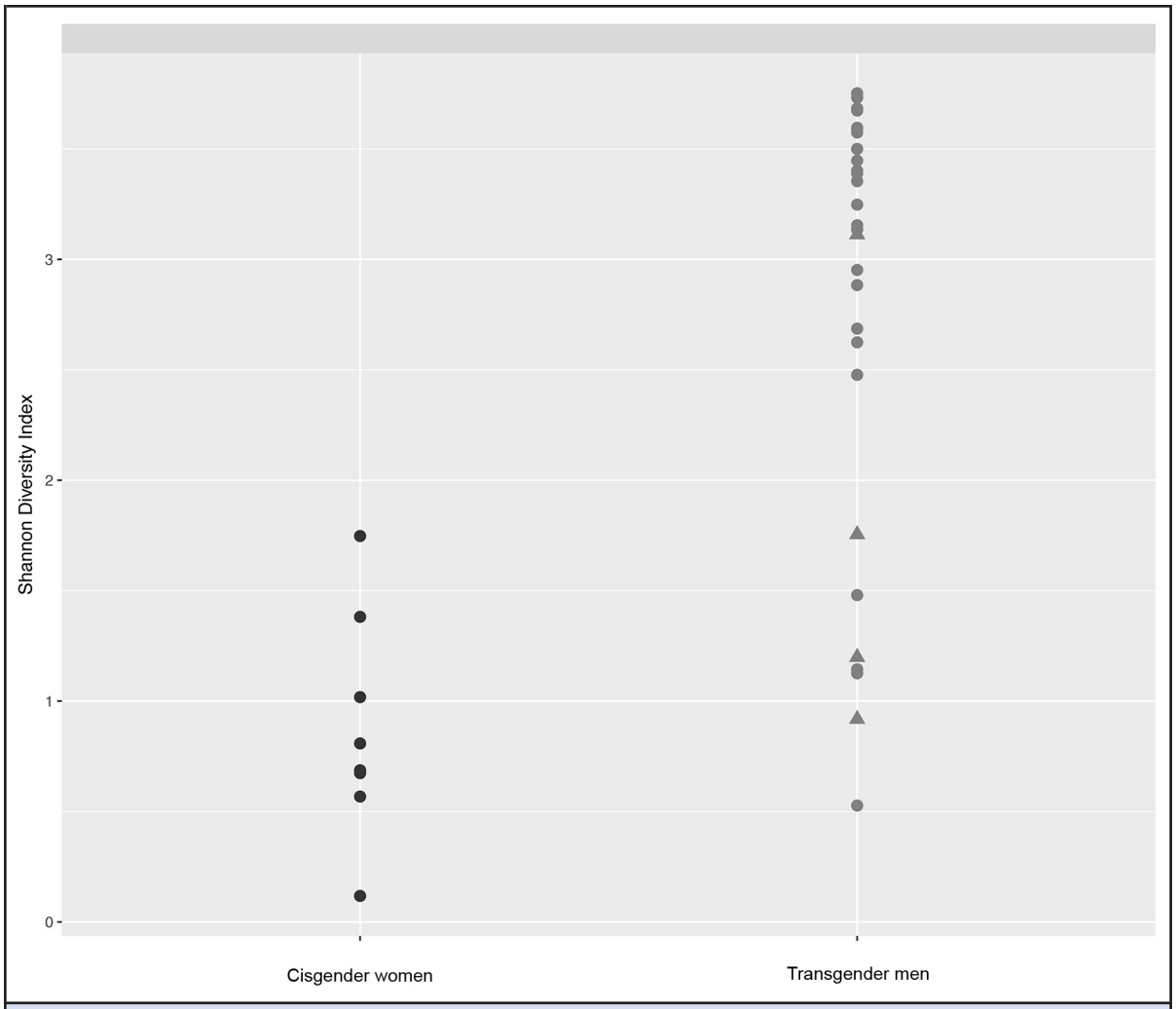

Fig. 4. Interindividual variability measured with Shannon diversity index between cisgender women and transgender men.
Cisgender women are indicated as solid black circles; transgender men who were not administering vaginal estrogen are indicated as solid gray circles, while those who were are indicated as solid gray triangles.

been correlated with BV, which has been shown to increase the risk of HIV transmission *(23)*. Limited studies evaluating sexually transmitted infection prevalence in transgender men have suggested they have a higher rate of HIV infection than the general population *(24, 25)*. In BV, the diversity of organisms increases *(26)*, and the onset of pathological symptoms can be caused by a variety of organisms *(27)*. The Shannon diversity index is commonly used to describe the microbial diversity within a specimen *(28–30)*. In this study, the transgender men had a significantly higher Shannon diversity index, which in other studies has been associated with BV. Of note, several of the bacteria found in relative excess in the transgender men have been associated with BV, such as *Atopobium*, *Prevotella*, *Peptostreptococcus*, *Sneathia*, and *Finegoldia* (Fig. 3) *(27)*. *Gardnerella*, a genus commonly associated with BV, was detected in a third of the transgender men (n = 10 of 28) and a fourth of the cisgender women (n = 2 of 8). However, it is noteworthy that the relative abundance of *Gardnerella* in the cisgender women (2.5% and 0.5%) was less than observed in the transgender men, in which 2 of the participants had a relatively high abundance (>40%), 4 had a relatively low abundance (1%–2%), and 4 had a very low relative abundance of <1%. Similarly, *Lactobacillus* species was not observed to be in relative excess in cisgender women relative to transgender men because a subset of the transgender men (n = 12) harbored the bacteria. The relative abundance of *Lactobacillus* differed widely between these men, with 3 of them having >90%, 3

having 15%–25%, 3 having 1%–10%, and 3 having <1%. The only covariate tracking with *Lactobacillus* abundance in the transgender men was the use of an estrogen ring, which 2 of the 3 men with >90% *Lactobacillus* were administering. Incidentally, the participant using a vaginal estrogen tablet did not harbor *Lactobacillus* and was experiencing irritable vaginal symptoms during the encounter for which the swabs were collected. As a group, the transgender men with *Lactobacillus* colonization were significantly more likely to have a lower Shannon diversity index relative to the transgender men with no detectable *Lactobacillus*, and in this respect more closely resembled the vaginal microbiomes of cisgender women.

Although the data presented here allow for new insight into the vaginal microbiome of transgender men, there are important limitations of our study that should be considered. The sample size was relatively small, limiting the exploration of covariates and our power to identify subtle differences between groups. Further, the lack of power in our study increases the likelihood of a type-II statistical error. For example, although the *U*-statistic did not show an association between hormone concentration and vaginal flora, the study was potentially insufficiently powered to conclude that there is a lack of association between these variables. Second, information on probiotic use, race, ethnicity, or body mass index (beyond exclusion of obese individuals) was not available from the study cohort, and the effect of these covariates may also prove significant. Third, the classification approach used in this study would not be expected to identify organisms reported elsewhere as having an important role in the vaginal environment but without representative organisms in NCBI (e.g., BVAB1, BVAB2) *(31)*. Fourth, participants were not recruited specifically on the basis of vaginal health, disease, or sexual preferences. The latter potentially confounded our results because 23 out of 28 of our transgender participants had sexual contact with a vagina, while only 3 of our cisgender controls had similar contact. Further, participants were recruited from a study in which the objective was to establish transgender-specific reference intervals for hematology, chemistry, and special chemistry testing (unpublished observations), and some individuals were consequently excluded from this study for reasons unrelated to sexual health and/or vaginal concerns. Therefore, participants were excluded from this study who may not have been if the sole focus of participant recruitment been on sexual health and/or vaginal concerns. Future work should seek to address these deficiencies and should consider more closely examining the role of vaginal estrogen in promoting *Lactobacillus* colonization, ideally by longitudinally evaluating the vaginal flora of transgender men before and after administration.

In conclusion, the vaginal microbiome of transgender men may differ from that of cisgender women. Inclusion of transgender men in future studies evaluating vaginal flora of healthy people can help elucidate the magnitude and potential clinical implications of these pilot observations.

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