The rodent vaginal microbiome across the estrous cycle and the effect of genital nerve electrical stimulation

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Abstract

Treatment options are limited for the approximately 40% of postmenopausal women worldwide who suffer from female sexual dysfunction (FSD). Neural stimulation has shown potential as a treatment for genital arousal FSD, however the mechanisms for its improvement are unknown. One potential cause of some cases of genital arousal FSD are changes to the composition of the vaginal microbiome, which is associated with vulvovaginal atrophy. The primary hypothesis of this study was that neural stimulation may induce healthy changes in the vaginal microbiome, thereby improving genital arousal FSD symptoms. This study also sought to examine the composition of the rat vaginal microbiome, which is understudied. Nulliparous female rats were used. Treatment animals (n=5) received 30 minutes of cutaneous electrical stimulation targeting the genital branch of the pudendal nerve, and Control animals (n=4) had 30-minute sessions without stimulation. Vaginal lavage samples were taken during a 14-day baseline period including multiple estrous periods and after twice-weekly 30-minute sessions across a six-week trial period. Samples were sequenced at the University of Michigan Host Microbiome Initiative and analyzed for baseline bacterial trends and for changes due to stimulation. We found that the rat vaginal microbiome is dominated by *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla bacteria, which changed in abundance during the estrous cycle and in relationship to each other. While the overall stimulation effects were unclear, some Treatment animals had lower variance in the microbiome diversity for sequential samples than Control animals, suggesting that stimulation may help normalize the vaginal microbiome. Future studies may consider additional physiological parameters, in addition to the microbiome composition, to further examine vaginal health and the effects of stimulation.

Introduction

Female sexual dysfunction (FSD) affects up to 41% of postmenopausal women worldwide (1), and between 40-50% of all women report experiencing at least one symptom of FSD (2). Between 16-25% of women report having difficulty orgasming, and between 8-28% of women report having low genital arousal and poor lubrication (3)(4). FSD is inversely related to quality of life both physically and psychologically (2)(5). Genital arousal FSD has been associated with Vulvovaginal Atrophy (VVA) in some postmenopausal women (6). Symptoms of VVA include vaginal dryness, itching, irritation, and dyspareunia (6). FSD and VVA stemming from postmenopausal hypoestrogenism induces an elevation of the vaginal pH (7). High pH levels can cause changes in the bacterial communities of the vagina such as reductions of *Lactobacillus* species and an increase in the growth of other bacterial species such as group B *Streptococcus*, *Staphylococcus*, coliforms, and diphtheroids (7). This reduction of a dominating species and overall increase in the diversity of the vaginal microbiome is associated with an overall reduction in vaginal health in postmenopausal women (8). Postmenopausal women are 7.8 times more likely to have a more diverse vaginal microbiome than are premenopausal women (9), and this may be due to decreases in their concentrations of ovarian hormones (10). This suggests that hormone changes associated with menopause may have an effect on the vaginal microbiome.

Though studies suggest that female sexual dysfunction is more prevalent than male sexual dysfunction (11), treatment options for FSD and VVA are limited compared to treatment for male sexual dysfunction. Current treatment options for symptoms of VVA include non-hormonal lubrication and estrogen treatments (7), though hormone therapy is not always recommended for long term dysfunction (12). Various pharmaceutical treatments have been evaluated. While Sildenafil has shown improvements in clitoral blood flow and sexual arousal in postmenopausal women (13), its clinical efficacy and high risk of adverse effects such as headaches, flushing, rhinitis, and nausea have led to concern for its true clinical benefit in women (14,15). Flibanserin can improve sexual desire in postmenopausal women (16), however its efficacy in improving the physiological problems associated with FSD have not been demonstrated (17,18).

Recent studies have shown that peripheral nerve stimulation, a third-line treatment for bladder dysfunction, can improve FSD symptoms. The implant of a sacral neuromodulation stimulator, which targets a sacral spinal nerve that contains proximal pudendal nerve fibers, for incontinence and overactive bladder has had positive benefits for FSD (19). Weekly posterior tibial nerve stimulation (PTNS) sessions with a percutaneous needle electrode for similar patients has also shown promise (20,21). Recently we showed that 30-minute weekly sessions of skin-surface PTNS or stimulation on either side of the clitoris to target the genital branch of the pudendal nerve in women without bladder problems could improve the genital arousal aspects of FSD (22). The underlying mechanisms of these improvements are not known. In preclinical studies, we found that increases in rat vaginal blood flow can be driven by 20-30 minutes of direct pudendal and tibial nerve stimulation (23,24). This stimulation effect may lead to an overall improvement in vaginal and genital health, including a modulation of the vaginal microbiome, particularly with repeated sessions.

The primary hypothesis of this study was that genital arousal FSD-treatment relevant peripheral nerve stimulation modulates the vaginal microbiome. Using a non-invasive approach, we sought to bridge a potential relationship between our clinical findings and blood flow changes observed in rodents. Although rats are a standard model for sexual function research (25), it is not clear if they are a proper animal model for the vaginal microbiome of women. Little is known about the general diversity of bacterial species in the rat vaginal microbiome. Though total bacterial abundance has been reported to vary in relation to the rat estrous cycle (26), changes in individual species diversity and abundance in relation to the estrous cycle has yet to be identified. It is important to understand the composition and dynamics of the rat vaginal microbiome for consideration in reproductive, sexual function, and microbiome studies, and for determining its use as a model for humans. Thus in addition to examining the effect of nerve stimulation on the vaginal microbiome, this exploratory study investigated the composition and cycling of the rat

vaginal microbiome. Although stimulation did not result in a clear shift in microbiota versus a control group, some animals have experienced a relative reduction in diversity, suggesting that nerve stimulation may be a treatment option for normalizing the vaginal microbiome. We also observed that the rat vaginal microbiome is dynamic and includes bacteria that fluctuate in abundance across the estrous cycle and in relation to each other. Finally, this study indicates that rats share characteristics with human vaginal microbiota and lays the groundwork for future research.

Materials and methods

Animals and experimental timeline

All procedures were approved by the University of Michigan Animal Care and Use Committee in accordance with the National Institutes of Health's guidelines for the care and use of laboratory animals. Experiments were conducted in 10 nulliparous female Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA, USA) weighing 200 to 250 g. The animals were individually housed in isolated cages to limit cross-contamination. The 10 animals were randomly sorted into Treatment and Control groups. The experimental timeline for each animal was divided into two parts: first a two-week baseline period and then a six-week trial period.

Lavage sampling and estrous characterization

During the intervals described below, standard procedures were followed to obtain vaginal lavage samples (27,28). Careful technique was used to ensure that sampling did not induce pseudopregnancy in animals which is known to have an effect on the estrous cycle (27). Three 100 µl lavage samples were obtained in sequence and combined in a plastic vial. Approximately 50 µl of the total sample was pipetted onto a glass microscope slide and visualized under a standard light microscope with 10x magnification. Characterization of the vaginal smear was used to determination the estrous stage of each sample (28). Estrous cycle determination was performed without reference to the animal treatment group. Proestrus was identified by the presence of clusters of round nucleated epithelial cells with granular appearances. Estrus was identified by the presence of large numbers of cornified or keratinized cells which appear to have jagged edges. Metestrus was identified as a transitional stage by the presence of cornified or rounded epithelial cells as well as leukocytes. Diestrus was identified by the presence of leukocytes as well as small numbers of epithelial cells suggesting the end of diestrus and the beginning of the next cycle. Samples whose estrous determination was clearly identifiable were used for analysis. Samples whose estrous determination was unclear were not used in analysis. The remaining 250 µl of the vaginal lavage sample was transferred into a beaded well plate, which was kept in a -80 °C freezer until sequencing (29).

Baseline procedure

During the baseline period, vaginal lavage samples were taken across a 14-day period to record baseline microbiota changes and relative abundances over multiple estrous cycles. An average of 10 samples were taken for each animal during this period. Baseline procedures were the same for Treatment and Control groups. Baseline sampling was performed at the same time of day for each animal to limit the possibility of mischaracterizing the estrous phase during transition periods (30). The animals were anesthetized with 5% Isoflurane in oxygen. Vaginal lavage samples were taken for each animal, at which point Isoflurane was turned off and the animals were allowed to wake up on their own. Samples were characterized under the microscope as described above and transferred to the beaded well plate for storage until sequencing.

Experimental procedures

Trial sessions were performed twice a week for six weeks. Both Treatment and Control animals were anesthetized with intraperitoneal injections of ketamine and xylazine (50 mg/kg, 5 mg/kg respectively) in a cocktail diluted to 20 mg/ml and 2 mg/ml respectively. Ketamine was the preferred anesthetic, as it has been used in prior studies anesthetized rats for evaluating sexual arousal (24,31–33) Respiratory rate and skin temperature were monitored every five minutes for the duration of the procedure to ensure that the animal remained properly sedated. Respiratory rate was monitored by counting chest contractions, and skin temperature was monitored using a laser thermometer (IRT-2, Thermoworks, Salt Lake City, Utah, USA).

Each animal was placed on its back onto a surgical pad. Once visibly sedated, a toe pinch was performed to ensure that the rat had no visible response to pain sensation. A pre-trial vaginal lavage was taken, estrous phase was visualized under the microscope, and the sample was transferred to the beaded well plate. A sterilized bipolar stimulation hook (PNB0.8x2/90, Xi'an Friendship Medical Electronics, Xi'an, Shaanxi, China) was placed with the top hook directly on the clitoris and the bottom hook just below the vaginal opening. The hook was held in place by a micromanipulator (KITE-M3-R, World Precision Instruments, Sarasota, FL, USA). The probe was connected to a stimulus pulse generator (4100, AM Systems, Sequim, WA, USA) which was set to biphasic, current-controlled stimulation.

To set the stimulation amplitude, pudendo-pudendal reflex activation of the external anal sphincter was identified in Treatment animals (34). The pulse generator was set to a pulse width of 1000 µs, a frequency of 1 Hz, and an amplitude of 2.0 mA in order to test for a visible response. The probe position was adjusted until a reflex anal sphincter response was identified in order to ensure that the probe was making proper contact with the genital nerve region. The frequency was then increased to 10 Hz and the amplitude was reduced to 0.1 mA. The amplitude was steadily increased until a muscle response was observed and then the amplitude was lowered just below that threshold. Subsequently, stimulation was applied continuously for 30 minutes. Sterile gauze was used to wipe away any urine that accumulated during the 30-minute session as to not interfere with the electrical conduction. Control animals were positioned just as Treatment animals, with the hook in place, but stimulation was not delivered. Treatment and Control animals were taken off anesthesia using an intramuscular injection of antisedan (5 mg/ml) and placed in individual cages until recovery, at which point they were transferred back to their housing cages.

Data sequencing and microbiome analysis

All sequencing and microbiome analysis were performed at the University of Michigan Host Microbiome Initiative, using standard steps for amplification and sequencing of the microbiome. DNA was isolated in an Eppendorf EpMotion 5075 liquid handling system, using a MagAttract PowerMicrobiome DNA/RNA Kit (27500-4 EP/27500-4 EP-BP, Qiagen, Hilden, Germany). The V4 region of the 16S rRNA gene was amplified for each sample using the Dual indexing sequencing strategy (35). Sequencing was done on the Illumina MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina cat# MS102-2003), according to the manufacturer's instructions with some modifications. Accuprime High Fidelity Taq (Life Technologies cat # 12346094) was used instead of Accuprime Pfx supermix.

PCR was performed using Standard PCR (5 µl of DNA), followed by Touchdown PCR (7 µl of DNA) for samples which did not initially amplify during Standard PCR (36). PCR products were visualized using an E-Gel 96 with SYBR Safe DNA Gel Stain, 2% (G7208-02, Life Technologies. Waltham, Massachusetts, USA). Libraries were normalized using SequalPrep Normalization Plate Kit (A10510-01, Life Technologies, Waltham, Massachusetts, USA) following the manufacturer's protocol for sequential elution. The concentration of the pooled samples was determined using the Kapa Biosystems Library Quantification kit for Illumina platforms (KK4824, Kapa Biosystems. Wilmington, Massachusetts, USA). The sizes of the amplicons in the library were determined using the Agilent Bioanalyzer High Sensitivity DNA analysis kit (5067-4626, Agilent. Santa Clara, California, USA).

The 16S rRNA gene sequence data was processed and analyzed using the software package mothur (v.1.40.2) and the most recent MiSeq SOP (35,37). After sequence processing and alignment to the SILVA reference alignment (release 128) (38), sequences were binned into operational taxonomic units (OTUs) based on 97% sequence similarity using the OptiClust method (Westcott and Schloss, 2017). The Sequence count for each OTU was determined, and Thetayc (Θyc) distances, taking into account relative abundances of both shared and non-shared OTU, were calculated between sample communities (39). Θyc distance is a beta diversity metric, measuring relative abundance and community membership between two sample (40), and was used as a metric of overall sample diversity.

Statistical analysis

Two forms of data were used in statistical analysis: sequence count correlating to relative abundance of each OTU, and Θyc distances. The sequence counts for each OTU had excessive zeros, as is common in microbiome studies (41), which causes most OTU data to not be distributed normally (42), In keeping with this assumption of non-normality, the sequence count data was analyzed using nonparametric statistical tests (43). Samples were identified as either control or treatment, baseline or trial, and by stage of estrous cycle. Community compositions bar plots were made to visualize the relative abundance of the 40 most abundant taxa for each animal over the baseline and trial periods. Principal coordinate analysis (PCoA) (44,45) was used to visualize Θyc distances between samples and to observe potential clustering of baseline samples based on estrous characterization. Analysis of Molecular Variance (AMOVA) (46) of Θyc distances was used to detect significant differences between the diversity of baseline samples based on estrous characterization. Kruskal Wallis analysis was used to detect significant differences in the abundance of each OTU based on estrous characterization. The significance level α was set to 0.05, but Mann Whitney post hoc analyses were run on all samples with a p-value < 0.1 to also evaluate samples that showed a trend toward statistical significance. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to confirm differential abundance of OTU based on estrous stage (47). Correlation analysis was run to determine if the relative abundance of pairs of OTU was significantly correlated over time.

To determine the effects of stimulation on sample diversity, a Mann Whitney analysis was used to detect significant changes in the abundance of each OTU between baseline and trial periods. Kruskal Wallis and Mann Whitney post hoc tests were used to determine if there were changes in OTU abundance based on estrous cycling between baseline and trial periods. Lastly, Θyc distances between consecutive samples were plotted across each animal's experimental timeline to determine the changes in diversity from one sample to the next across time. Mean Θyc distances were calculated for baseline and trial periods and a Trial:Baseline ratio was used to determine mean changes in the diversity between baseline and trial periods for each animal. Chi Squared analysis was used to detect significant variance within Control and Treatment groups. Kruskal Wallis, Mann Whitney, and Spearman tests were run using SPSS (IBM SPSS Statistics for Windows, Version 25.0. IBM Corp, Armonk, NY, USA). Community compositions bar plots, PCoA, and sequence count boxplots were made using R (Version 3.4.4. R Foundation for Statistical Computing, Vienna, Austria). AMOVA and LEfSe analyses were run using Mothur (Version 1.40.2, University of Michigan, Ann Arbor, Michigan, USA). The significance level α for all tests was set at 0.05.

Results

Six Treatment animals and four Control animals were used in this study. One Treatment animal died after baseline sampling due to complications from anesthesia and its data was only used for baseline analysis. A total of 348 samples, including empty and saline control samples, were submitted to the University of Michigan Host Microbiome Initiative for sequence analysis. DNA was ultimately isolated, amplified, and sequenced in 338 samples. The initial loss of samples may have been due to insufficient bacterial populations present during sampling or a loss of sample after freezing and thawing. Subsampling (45,48) to 1678 sequences per sample resulted in a loss of 32 samples which did not meet the threshold. A

final count of 306 samples were used for analysis. Each of the nine full-study animals provided a mean sample count of 32.8 ± 1.45 samples (range 30-34), excluding the animal used only for baseline analysis, which only provided 10 samples.

A total of 1,591 different OTU were identified in the dataset. Only the first seven OTU had consistently abundant sequence counts and were therefore used in full data analysis. Taxonomic nomenclature for the seven OTU used in the primary analyses is reported in Table 1. The OTU size reports the number of sequences binned into each OTU, a metric of relative abundance. The confidence of the taxonomic ranking for these first seven OTU was 100%. The community composition of each sample, representing the bacterial phyla and genus level rankings present at a relative abundance greater than 1% in the animals in the study are shown in Fig 1. The most abundant phyla across all animals were *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. The relative abundance of these phyla appeared to change over time for each animal during baseline and trial periods, suggesting a dynamic microbiome. Control animals show a relatively consistent and highly diverse composition over time. Treatment animals show greater changes to their individual compositions over time, with varying increases and decreases in diversity.

Table 1. Taxonomic ranking of seven most abundant OTU.

OTU	Size	Kingdom	Phylum	Class	Order	Family	Genus
OTU1	2187427	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Proteus*
OTU2	1982590	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia/Shigella*
OTU3	1492377	*Bacteria*	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
OTU4	785165	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Morganella*
OTU5	366068	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Pasteurellaceae* (unclassified)
OTU6	359287	*Bacteria*	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*
OTU7	82607	*Bacteria*	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*

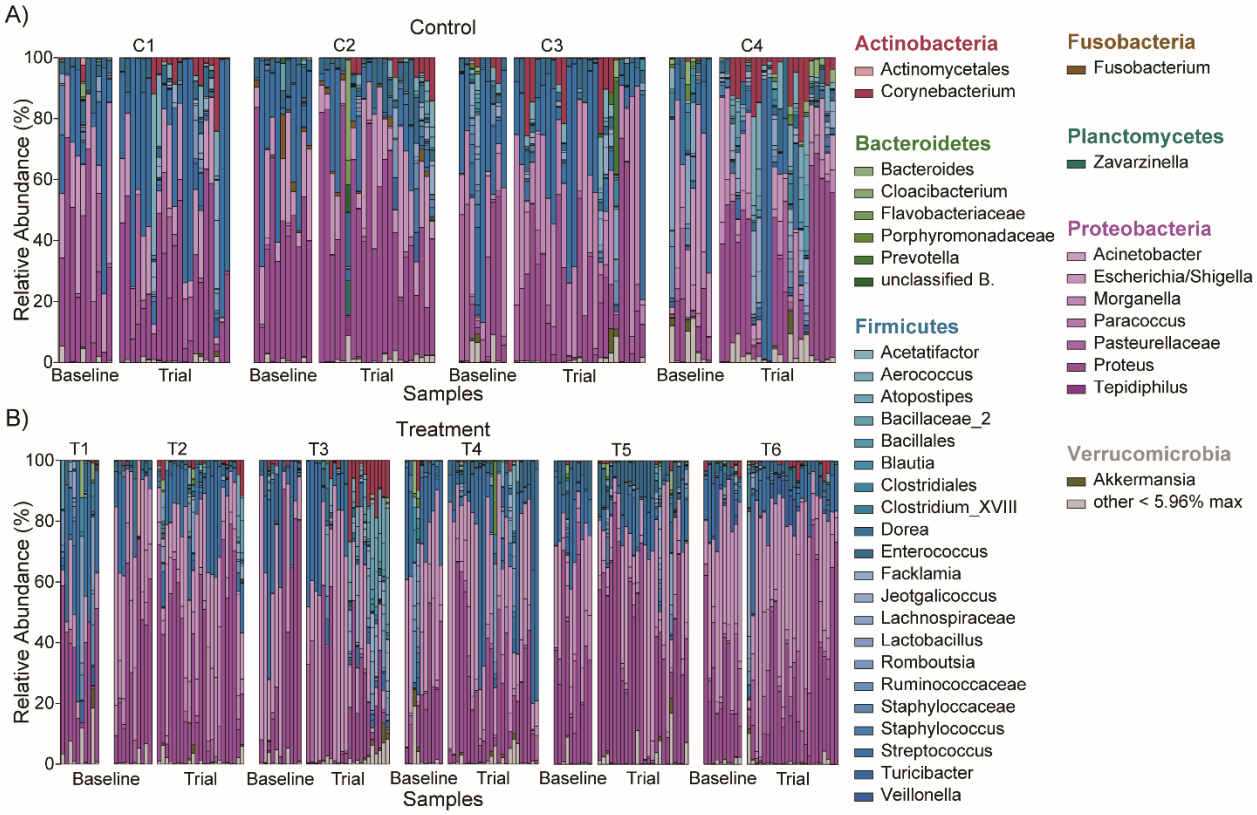

Fig 1. Community composition of Control and Treatment samples over time. Changes in relative abundance of OTU plotted over the baseline and trial periods for A) Control and B) Treatment animals. Colors relate to specific OTU based on genus-level taxonomy rankings.

Non-Stimulation Effects

Principal Coordinate Analysis (PCoA) was used to visualize clustering of samples by estrous stage during the baseline period (Fig 2). Samples were identified by estrous stage characterization and were plotted based on 2 dimensional distances indicating dissimilarities between samples. Dissimilarities were based on the relative abundance of each OTU. The ten OTUs with the lowest axis 1 *p*-values, suggesting the greatest variation between samples, (and a predetermined minimum sequence count per OTU) were used to identify distances between samples. Samples showed a degree of clustering during estrus and diestrus, though no clear sign of clustering was apparent during proestrus and metestrus. Samples in estrus clustered around OTU3, while samples in diestrus clustered around OTU1 and OTU2, suggesting that OTU3 is more abundant on estrus and that OTU1 is more abundant during diestrus. Together, this is evidence of changing OTU abundances based on the estrous cycle.

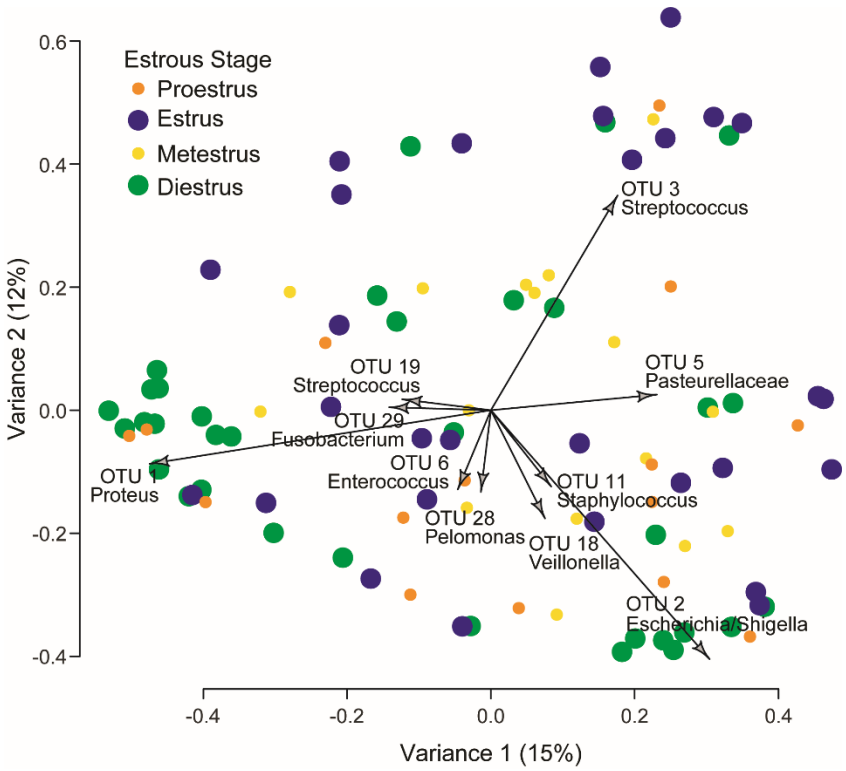

Fig 2. Principal Coordinate Analysis (PCoA) of all animal samples during baseline period. Two-dimensional distances identify dissimilarities between samples based on relative abundances of OTU. Biplot arrows indicate specific OTU which drive samples to different locations on the plot. The first PCoA axis (x-axis) included 15% of all variance. The second PCoA axis (y-axis) included 12% of all variance. Icons for estrus and diestrus are enlarged to help visualize the relative clustering of each.

The visualization of clustering of samples by estrous stage within OTU suggested that the vaginal microbiome changes in diversity based on the estrous phase. Θyc distances for samples from all animals during the baseline period were used to test for differences in the overall diversity of samples based on the estrous phase. AMOVA detected a significant difference in the Θyc distances between samples based on the estrous phase (Fs = 2.57, p = 0.005). Pairwise analysis showed a significant difference between estrus and diestrus samples (Fs = 5.29, p = 0.002) and a near-significant difference between metestrus and diestrus samples (Fs = 2.28, p = 0.059). Other comparisons were not significant (Fs = 1.01–2.09, p = 0.111–0.122).

After determining that the microbiome diversity may change based on the estrous cycle, tests were run to determine if specific OTU had varying abundances based on the estrous stage using both Control and Treatment samples during the baseline, trial, and combined baseline & trial periods. For OTUs that had a significant or near-significant change in abundance across the estrous cycle, Estrous Ratios were calculated between the median sequence count abundances of estrous states that were significantly different according to the Mann Whitney post hoc test. Tables 2-4 give Kruskal-Wallis test results indicating whether each OTU varied based on the estrous stage, separated by animal group and test period, with Estrous Ratios provided for significant post hoc tests. OTU3 was frequently more abundant during estrus, and OTU7 was often more abundant during diestrus(Fig 3). This correlation between OTU abundance and estrous stage was confirmed though Linear Discriminant Analysis Effect Size (LEfSe). LEfSe tests were run using all samples during the baseline period and showed significant differences for only OTU3 and OTU7 across the estrous cycle. OTU3 was significantly more abundant during estrus compared to any of the other three stages (LDA = 5.182, p = 0.0001). OTU7 was significantly more abundant during diestrus compared to any of the other three stages (LDA = 4.543, p = 0.0003).

Table 2. Variation in OTU abundance based on estrous stage for Control animals.

.	Baseline			Trial			Baseline & Trial		
	KW *p*-value	Estrous Ratio	MW *p*-value	KW *p*-value	Estrous Ratio	MW *p*-value	KW *p*-value	Estrous Ratio	MW *p*-value
OTU1	0.683	--	--	0.618	--	--	0.455	--	--
OTU2	0.267	--	--	0.296	--	--	0.754	--	--
OTU3	0.053	E:P 8.521 E:D 3.366	0.046 0.021	0.403	--	--	0.045	E:D 10.730	0.008
OTU4	0.636	--	--	0.934	--	--	0.732	--	--
OTU5	0.171	--	--	0.097	E:D 76.0	0.030	0.137	--	--
OTU6	0.937	--	--	0.844	--	--	0.263	--	--
OTU7	0.060	D:E 1.5:0	0.018	0.032	D:E 44.25	0.021	0.010	D:E 13:0	0.002

KW: Kruskal-Wallis test result. MW: significant Mann Whitney test results for KW *p*-value < 0.10.

Table 3. Variation in OTU abundance based on estrous stage for Treatment animals.

.	Baseline			Trial			Baseline & Trial		
	KW *p*-value	Estrous Ratio	MW *p*-value	KW *p*-value	Estrous Ratio	MW *p*-value	KW *p*-value	Estrous Ratio	MW *p*-value
OTU1	0.424	--	--	0.113	--	--	0.096	D:E 2.450	0.013
OTU2	0.670	--	--	0.023	E:D 4.263	0.007	0.074	E:D 2.448	0.022
OTU3	0.005	E:D 8.663	< 0.001	0.728	--	--	0.003	E:D 5.770	0.001
OTU4	0.730	--	--	0.310	--	--	0.617	--	--
OTU5	0.613	--	--	0.500	--	--	0.556	--	--
OTU6	0.823	--	--	0.074	--	--	0.079	--	--
OTU7	0.001	D:E 6:0	0.014	0.032	P:M 2:0	0.033	0.001	D:E 14:0	0.001

KW: Kruskal-Wallis test result. MW: significant Mann Whitney test results for KW *p*-value < 0.10.

Table 4. Variation in OTU abundance based on estrous stage for Control and Treatment animals combined.

.	Baseline		
	KW *p*-value	Estrous Ratio	MW *p*-value
OTU1	0.198	--	--
OTU2	0.421	--	--
OTU3	< 0.001	E:D 7.386	< 0.001
OTU4	0.651	--	--
OTU5	0.243	--	--
OTU6	0.703	--	--
OTU7	< 0.001	D:P 9:0 D:E 9:0 D:M 9:0	0.025 < 0.001 0.030

KW: Kruskal-Wallis test result. MW: significant Mann Whitney test results for KW *p*-value < 0.10.

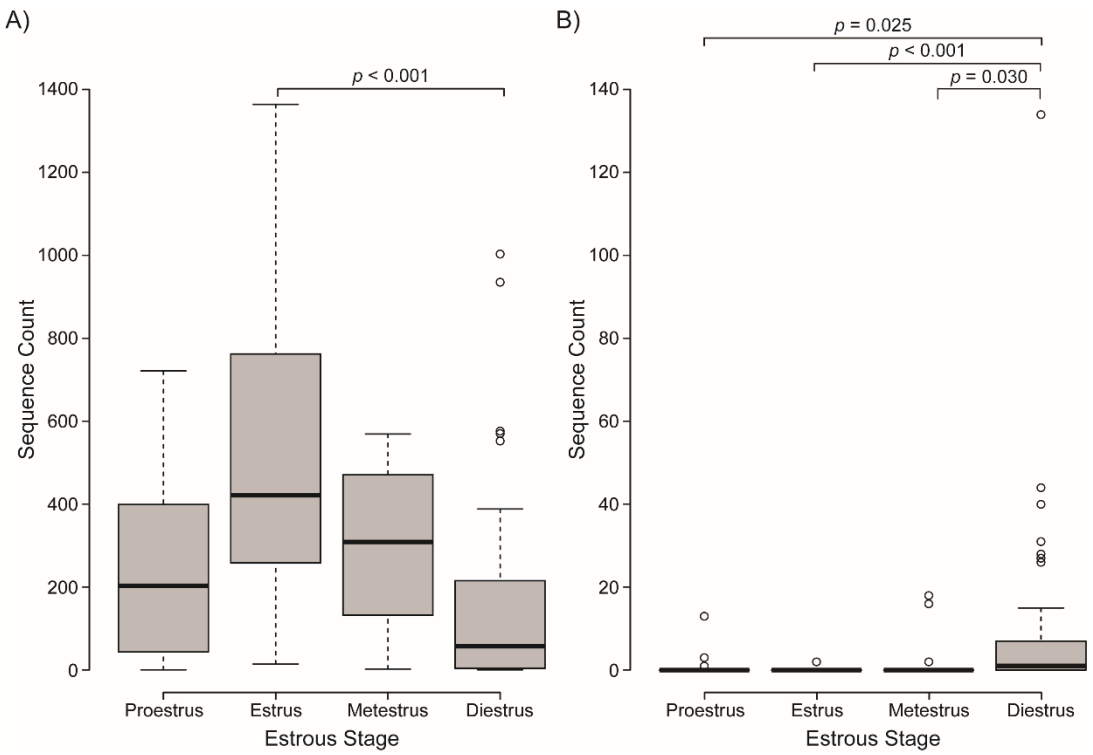

Fig 3. Variations in OTU abundances based on estrous phase characterization. A) OTU3 for Control and Treatment animals during baseline period. B) OTU7 for Control and Treatment animals during baseline period. Similar trends were observed for both OTU3 and OTU7 when separated into Control-only and Treatment-only analyses.

To better understand how the microbiome can change over time, it was important to also understand how OTU abundance changes in relationship to one another. For this, Spearman correlation tests were run for each combination of the first 7 OTUs over time. Correlations were determined for both Control and Treatment groups during the baseline, trial, and combined baseline & trial periods (Table 5). Comparisons were not performed between Control and Treatment animals during the Trial period due to the possible effect of stimulation. Three example OTU-pair correlations showing typical strong and weak relationships are given in Fig 4. All of the correlations found in the Control samples were consistent in their direction with correlations found in the Treatment samples, except for OTU1 paired with OTU2, where stimulation resulted in negatively correlated relationship, whereas tCcontrol animals showed a positively correlated relationship

Table 5. Spearman correlation coefficients (R) for OTU abundance over time between OTU pairs

	Control Animals						Treatment Animals						All Animals	
	Baseline		Trial		Baseline & Trial		Baseline		Trial		Baseline & Trial		Baseline	
OTU set	*p-value*	R	*p-value*	R	*p-value*	R	*p-value*	R	*p-value*	R	*p-value*	R	*p-value*	R
1 & 2	0.622	0.083	< 0.001	0.452	< 0.001	0.308	< 0.001	-0.569	< 0.001	-0.332	< 0.001	-0.394	0.012	-0.265
1 & 3	0.057	-0.312	0.001	-0.352	< 0.001	-0.356	0.004	-0.395	< 0.001	-0.416	< 0.001	-0.400	0.001	-0.352
1 & 4	0.001	-0.516	0.112	-0.169	0.007	-0.239	0.489	0.099	0.029	0.202	0.041	0.158	0.091	-0.180
1 & 5	< 0.001	-0.571	0.005	-0.296	< 0.001	-0.379	0.055	-0.270	< 0.001	-0.35	< 0.001	-0.318	< 0.001	-0.414
1 & 6	0.004	0.461	0.001	0.358	< 0.001	0.378	0.996	-0.001	0.001	0.316	0.004	0.220	0.107	0.172
1 & 7	0.375	0.148	0.301	-0.110	0.961	-0.004	0.805	0.035	0.700	0.036	0.744	0.025	0.579	0.060
2 & 3	0.036	-0.341	0.024	-0.237	0.045	-0.178	0.485	0.100	0.175	0.126	0.068	0.141	0.307	-0.109
2 & 4	0.153	-0.237	< 0.001	-0.523	< 0.001	-0.469	0.545	-0.087	0.279	-0.101	0.224	-0.094	0.932	-0.09
2 & 5	0.264	-0.186	0.023	-0.239	0.031	-0.191	0.261	0.160	0.164	0.129	0.044	0.155	0.649	0.049
2 & 6	0.800	0.043	0.007	0.283	0.009	0.231	0.210	0.179	0.346	0.088	0.137	0.115	0.421	0.086
2 & 7	0.513	-0.109	0.330	-1.104	0.018	-0.209	0.232	-0.170	0.096	-0.155	0.013	-0.192	0.061	-0.199
3 & 4	0.603	0.087	0.790	0.028	0.987	-0.001	0.998	0.000	0.068	0.169	0.163	0.108	0.962	0.005
3 & 5	0.098	0.272	< 0.001	0.465	< 0.001	0.401	0.066	0.259	0.001	0.308	< 0.001	0.306	0.012	0.261
3 & 6	0.102	-0.269	0.033	-0.225	0.012	-0.222	0.725	-0.050	0.431	-0.073	0.436	-0.060	0.421	-0.086
3 & 7	0.049	-0.321	0.005	-0.293	< 0.001	-0.328	0.279	-0.155	0.032	-0.198	0.009	-0.202	0.081	-0.186
4 & 5	0.001	0.518	0.052	0.206	0.001	0.280	0.897	0.019	< 0.001	0.368	0.004	0.219	0.010	0.271
4 & 6	0.373	-0.149	0.354	0.099	0.804	0.022	0.038	0.292	0.009	0.240	< 0.001	0.267	0.458	0.080
4 & 7	0.270	0.184	0.918	0.011	0.218	0.110	0.168	-0.196	0.002	-0.286	0.002	-0.235	0.449	-0.081
5 & 6	0.459	-0.127	0.575	0.060	0.907	0.010	0.091	0.239	0.525	0.059	0.168	0.107	0.458	0.080
5 & 7	0.651	-0.076	0.101	-0.174	0.106	-0.143	0.548	-0.086	0.002	-0.284	0.003	-0.229	0.546	-0.065
6 & 7	0.441	-0.129	0.014	-0.259	0.007	-0.238	0.031	-0.302	< 0.001	-0.376	< 0.001	-0.332	0.115	-0.168

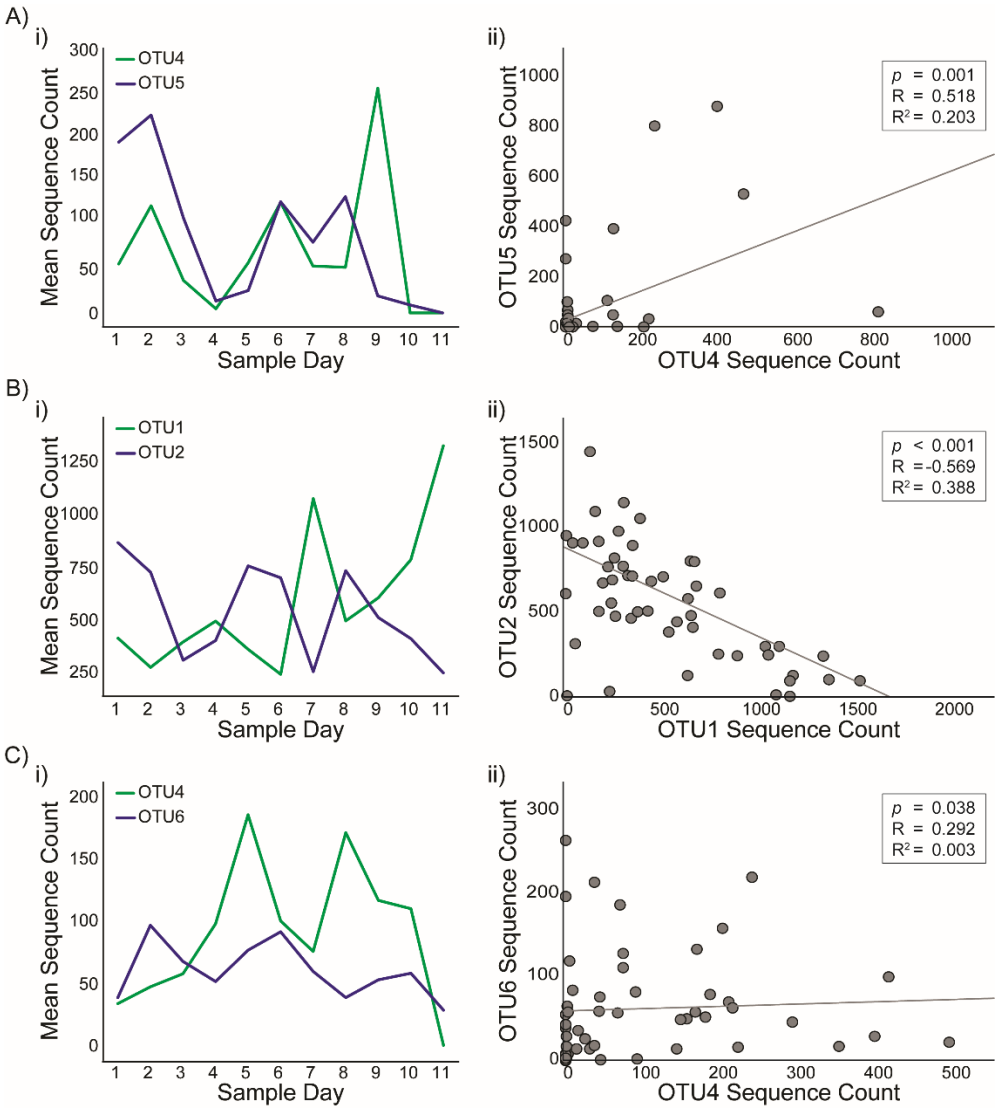

Fig 4. Examples of strong and weak correlations in paired OTU sequence counts during baseline period. A) Strong positive correlation between OTU4 and OTU5 for Control animals i) mean sequence counts and ii) paired scatter plot. B) Strong negative correlation over time between OTU1 and OTU2 in Treatment animals, i) mean sequence counts and ii) paired scatter plot. C) Weak correlation between OTU4 and OTU6 in Treatment animals, i) mean sequence counts and ii) paired scatter plot.

Stimulation effects

To determine the potential effects of stimulation on the microbiome diversity, Control and Treatment samples were tested for significant changes in OTU abundances from baseline to trial periods. Mann Whitney tests were used to determine changes for each animal between periods and between each phase of the estrous cycle for each of the 7 primary OTU. The ratios of the Mann Whitney mean rank values for the baseline period to the mean rank values for the trial period were calculated (B:T Ratio), to determine which experimental segment showed the greatest abundance of each OTU (Tables 6-8). Individual OTU had significant changes in abundance from baseline to trial periods among both Control and Treatment animals and during several different days of the estrous cycle. No consistent trend in overall abundance for any OTU was observed in either Control or Treatment groups. Similarly, both control and treatment groups showed no effect of stimulation on the change in OTU abundance acrouss the estrous cycle (Tables 2 & 3).

Table 6. Change in OTU abundance from baseline to treatment across all samples.

	Control Animals		Treatment Animals	
	B:T Ratio	*p*-value	B:T Ratio	*p*-value
OTU1	0.913	0.491	1.066	0.560
OTU2	1.342	0.027	0.997	0.976
OTU3	1.313	0.041	1.236	0.056
OTU4	0.857	0.227	0.813	0.057
OTU5	1.119	0.396	1.027	0.799
OTU6	1.036	0.791	0.831	0.090
OTU7	1.622	< 0.001	1.187	0.049

Table 7. Change in OTU abundance from baseline to treatment for Control animals across estrous cycle.

	Proestrus		Estrus		Metestrus		Diestrus	
	B:T Ratio	*p*-value	B:T Ratio	*p*-value	B:T Ratio	*p*-value	B:T Ratio	*p*-value
OTU1	1.000	1.000	0.952	0.852	0.758	0.315	0.963	0.912
OTU2	2.100	0.030	1.403	0.152	1.500	0.156	0.850	0.529
OTU3	0.732	0.429	1.350	0.205	1.704	0.043	1.295	0.315
OTU4	0.885	0.792	0.832	0.437	0.919	0.780	0.765	0.315
OTU5	1.848	0.082	0.801	0.347	1.344	0.278	1.132	0.631
OTU6	1.063	0.931	0.994	0.979	0.979	0.968	1.199	0.481
OTU7	0.805	0.537	0.577	0.016	0.488	0.008	0.641	0.089

Table 8. Change in OTU abundance from baseline to treatment for Treatment animals across estrous cycle.

	Proestrus		Estrus		Metestrus		Diestrus	
	B:T Ratio	*p*-value	B:T Ratio	*p*-value	B:T Ratio	*p*-value	B:T Ratio	*p*-value
OTU1	1.375	0.371	1.308	0.241	0.772	0.368	1.068	0.699
OTU2	1.214	0.594	0.703	0.100	0.788	0.407	1.151	0.413
OTU3	1.058	0.859	1.695	0.020	1.123	0.680	0.920	0.624
OTU4	1.318	0.440	0.914	0.698	0.725	0.267	0.650	0.011
OTU5	0.963	0.953	0.854	0.478	1.152	0.581	1.198	0.273
OTU6	0.318	0.440	0.905	0.664	0.756	0.332	0.799	0.187
OTU7	0.517	0.019	0.788	0.280	0.877	0.630	0.895	0.481

To determine if stimulation had longitudinal effects on the diversity of the microbiome over time, Θyc distances were tracked between consecutive samples across the baseline and trial periods for Control and Treatment animals. Each sample was identified by its estrous stage characterization, with samples transitioning between estrous stage identified by an intermediate estrous stage characterization. Individual animal Θyc scatterplots did not show trends in either Control or Treatment groups, based on differences between baseline and trial periods or based on differences due to estrous stage (Fig 5A). Average Θyc distances were determined for each animal's baseline and trial periods. To visualize the sample-to-sample variability differences between baseline and trial periods, the ratio ($R_{T:B}$) of the trial average Θyc distance to the baseline average Θyc distance was then calculated (Fig 5B). The mean $R_{T:B}$ for Control animals (1.264) and Treatment animals (1.308) was not significantly different ($p = 0.730$). All Control animals had a similar increase in mean Θyc distances from baseline to trial periods with a coefficient of variance of 0.91%. Four of the five Treatment animals had increases in mean Θyc distances, though the degree of change was more variable than Control animals, with a coefficient of variance of 29.4%. The variance in

$R_{T:B}$ among Treatment animals was significantly higher than that of Control animals, as confirmed by a chi squared analysis (p = 0.00005).

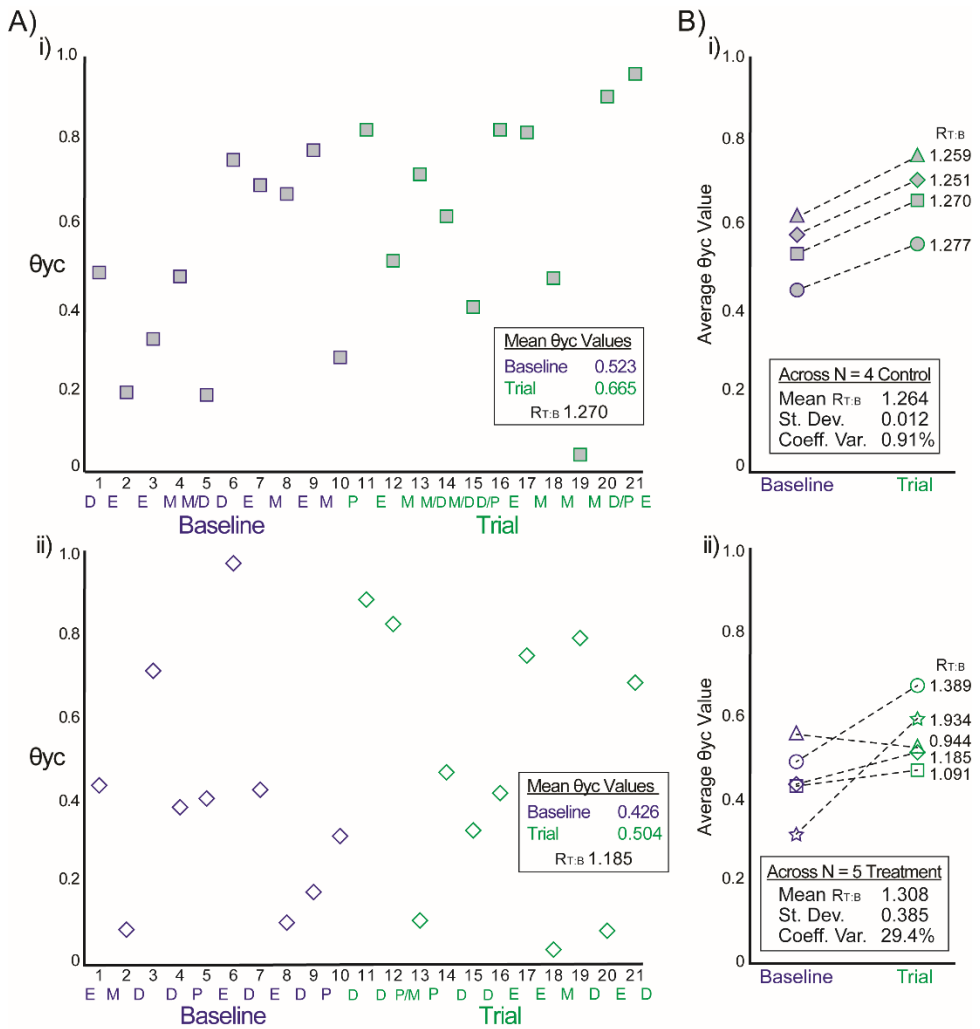

Figure 5. Θyc distances between consecutive samples for Control and Treatment groups. A) Example scatterplots of Θyc distances for consecutive samples for i) Control 1and ii) Treatment 2 animals. Baseline period in blue; Trial period in green. Estrous period (P/E/M/D for states) indicated at bottom of each plot. B) Mean Θyc distances during baseline and trial periods for i) Control and ii) Treatment animals. Icons in (A) correspond to same animals in (B).

Discussion

To our knowledge, this is the first study to explore the complexity of the changing vaginal microbiome diversity in rats across the estrous cycle. Previous studies have made broad claims about the overall abundance of bacteria during the estrous cycle (49), but this study is the first to identify changes in specific OTU abundances and identify correlations between OTU across time. Our results suggest a dynamic vaginal microbiome (Table 1, Fig 1) that shifts based on both changes in specific OTU abundances by estrous stage (Tables 2-4, Fig 2-3) and in correlations among the OTU (Table 5, Fig 4). Additionally, this is the first study to our knowledge that examines the effect of neural stimulation on the microbiome of any organ in any species. Specifically, this study evaluated the effect of a specific form of neural stimulation on the rat vaginal microbiome. Though the effect of stimulation was ambiguous (Tables 6-8), analysis of

Θyc changes over time showed differences in variance among Treatment animals when compared to Control animals (Fig 5), suggesting a potential effect of stimulation on vaginal microbiome diversity.

Several statistical tests confirmed that specific OTU abundance levels varied based on the stage of the estrous cycle. Initial community compositions showed that OTU abundance can change across relatively short time periods (Fig 1). PCoA clustering suggested that the changing of OTU abundance may be related to the estrous cycle (Fig 2). Certain OTU were more abundant during specific estrous stages compared to other stages. Though the total variance reported by PCoA was low (27%), current literature suggests that, even when yielding a low percent variation, clustering can yield biological insight and patterns can be revealed (45). This was confirmed by AMOVA analysis of Θyc which showed that the Θyc distances, which correlate to the overall diversity among samples, were significantly different between estrus and diestrus. Clearly, estrous cycling in the rat vagina has an effect on the overall diversity of the microbiome.

Further analysis was done to identify which specific OTU showed significant differences in abundance based on the estrous cycling. Kruskal Wallis and post hoc Mann Whitney tests showed that, across multiple time periods, OTU3, OTU5, and OTU7 were significantly different between estrus and diestrus. OTU3 and OTU5 were most abundant during estrus and OTU7 was most abundant during diestrus (Fig 3). This was confirmed, though not for OTU5, using LEfSe analysis showing that OTU 3 was significantly more abundant during estrus than any other stage and OTU7 was significantly more abundant during diestrus than any other stage. Together, this suggests that the reason for the overall difference in bacterial abundance between estrous stages (26) is not a global increasing or decreasing of all bacterial abundances but is due to several specific OTU adjusting in relative abundance based on the estrous cycle.

Changes in the vaginal microbiome due to estrous cycling are thought to be correlated to several factors such as pH, hormones, presence of mucin, and other biological conditions (26,50). During estrus, mucus secretion from glands located in the cervix of the uterus increase the mucus in the environment (51,52). It is theorized that the microbiome flourishes during this time because the mucus acts as a medium for bacterial growth (26). Estrus would appear to be the optimal estrous stage for bacterial growth, suggesting that there is a global increase in bacterial composition during estrus. This assumption, however, does not take into account the dynamics of each OTU. Our study suggests that while some species favor estrus, other favor diestrus. Several OTU, for example, showed predominance during diestrus compared to estrus. OTU1 showed clear clustering in PCoA during diestrus(Fig 2), and OTU7 was significantly more abundant during diestrus compared to estrus across multiple forms of analysis (Fig 3, Tables 2-4). This would suggest that several OTU, including OTU1 the most abundant OTU found in our study, tend to favor diestrus. It was also interesting to observe that the vast majority of differences in OTU abundance occurred between estrus and diestrus, suggesting that proestrus and metestrus act as transitions between points of significant bacterial growth or decay. While the data do not show that all of the OTU were significantly different in abundance across the estrous cycle, it is theorized that most of the OTU are changing slightly from estrous stage to stage and certain OTU change more dramatically than others. It is not understood why specific OTU showed significant differential abundances depending on the estrous state and other OTU did not. Further studies are necessary to understand this phenomenon.

There were many significant correlations between OTU (Fig 4, Table 5), suggesting that OTU can be related to each other. It was interesting to note that positive and negative correlations were found between both OTU of similar and different taxonomic ranking. For example, OTU1, OTU2, and OTU4 were all in the *Enterobacteriaceae* family. In Control animals during the trial period, a significant positive correlation was found between OTU1 and OTU2 but a significant negative correlation was found between OTU2 and OTU4. Inversely, in Control animals during the trial period, a significant positive correlation was found between OTU3 (*Firmicutes*) and OTU5 (*Proteobacteria*) and a significant negative correlation was found between OTU1 (*Proteobacteria*) and OTU3. No clear trend was identified suggesting that more similar OTU or more different OTU have any predetermination for positive or negative correlations (Table 5). Further analysis is necessary to understand the relationship between OTU species and competition for resources within the vaginal microbiome of rats.

Our study demonstrates that the rat vaginal microbiome has a dynamic microbiome which can change based on the estrous cycle. This is somewhat unique compared to other animals. Baboons, pigs, and

mice have all been shown to have no difference in vaginal microbiome diversity based on ovulatory cycling (36,53,54). Mice have also been shown to have no shifting of intestinal microbiome diversity due to hormone shifting during estrus (55). Dogs, however, have been shown to have a changing vaginal microbiome based on the estrous cycle, including increased diversity among OTU during estrus (56). Humans do not appear to have changes in vaginal microbiome diversity based on menstrual cycling (57), though small fluctuations have been reported (58). Interestingly, humans do have significant differences in microbiome diversity throughout the three trimesters of pregnancy and when comparing their pre and postpartum microbiome (59,60).

The bacterial composition of the rat microbiome is similar to that of the mouse (49), dog (56), and pig (53), which are dominated by *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Fig 1, Table 1). We observed that the rat vaginal microbiome is relatively different from the baboon vaginal microbiome, which is composed primarily of *Bacteroidetes*, *Fusobacteria*, and *Firmicutes* (54). When compared to the human vaginal microbiome, however, the rat microbiome had some similarities. The human vaginal microbiome is heavily predominated by *Lactobacillus* making it a unique microbiome (61–63), dissimilar to all other animal models tested thus far (64). The human microbiome, however, has high abundances in *Corynebacterium*, *Escherichia*/*Shigella*, *Streptococcus*, and *Staphylococcus* species, as well as several others (65). This study in rats showed a relative predominance of *Proteus*, a species that is found somewhat commonly in the human vaginal microbiome (66). Our study did not show a high predominance of *Lactobacillus*, though it (OTU15 and OTU39) and all of the previously listed common human vaginal bacterial species were found (Fig 1, Table 1). *Lactobacillus* is not uncommonly found in rat microbiome studies (66), though it is not as highly abundant as in human studies. With further research, the rat may be considered a useful model for the human vaginal microbiome.

While our study did not clearly indicate that stimulation of the genital branch of the pudendal nerve drives changes in the vaginal microbiome of rats, there was some evidence suggesting that stimulation may have an effect on microbiome diversity. Tests run using OTU abundance data showed significant changes in abundance from baseline to trial in both Control and Treatment animals (Tables 6-8). This suggests that OTU abundance changes independent of stimulation and therefore stimulation could not be concluded as having a significant effect on the abundance of OTU across time. This may be because OTU abundance by nature is a somewhat variable metric (43). Θyc distances were used to identify more broad changes in overall diversity from baseline to trial periods. Again, both Control and Treatment groups showed similar changes in Θyc distances from baseline to trial periods. The mean value of change in Θyc distances from baseline to trial period did not differ significantly when comparing Control and Treatment groups (Fig 5). What was noteworthy, however, was that the coefficient of variance for Treatment animals was 32.3 times larger than for the Control animals, representing a significant difference between the groups. Additionally, community compositions showed that Treatment groups had more variable changes in the relative abundances of OTU over time compared to Control groups (Fig 1). This suggests that stimulation may have affected the overall diversity of OTU, though without a clear trend.

Interestingly, while all of the Control animals and 4 of the 5 Treatment animals had an increase in the mean Θyc during the trial period, one Treatment animal had a decrease and the increase in two rats was at a lower rate than Control animals (Fig 5B). In humans, a less diverse vaginal microbiome is considered more stable and healthier (67). The human microbiome is said to be healthy when predominated by one or a few bacterial species, such as *Lactobacillus* (68). In a similar vein, studies have shown that women with bladder issues such as urge urinary incontinence have more diverse bladder microbiomes than women without incontinence (69,70). Additionally, studies have shown that women with imbalances in *Lactobacillus* in their vaginal microbiome can have changes in hormones associated with cervicovaginal inflammation, which leads to changes in their vaginal immunity and HIV susceptibility (10,57,71). It is possible that the handling and stress of the study contributed to an increase in the microbiome diversity for Control animals, and that the three Treatment rats with lower rate of increase or a decrease were affected by stimulation. Partial population responses to neuromodulation are often reported in the literature (72) and so a larger study size or use of an animal model of VVA may be useful to examine these relationships further. If genital nerve stimulation can modulate vaginal microbiome diversity, per our original hypothesis,

it offers hope as a treatment for VVA and suggests underlying mechanisms for its potential use in treating genital arousal aspects of female sexual dysfunction.

A possible limitation of this study was the use of excessive zeros in the data analysis. Excessive zeros in microbiome studies are common and can potentially skew data (41,73). Zeros come in multiple forms (74). Outlier zeros are due to extraneous conditions, structural zeros are due to the nature of different experimental groups, and sampling zeros are any other zero that may be due to low sampling depth. Each type of zero can be addressed differently by removing them, using pseudo counts, and other algorithms to minimize data skew (75). This study used nonparametric statistical tests to control for non-normal distributions but did not transform or remove any of the zeros in the data set. Further studies in this field may attempt to perform analysis of microbiome data while controlling for all three types of zeros to determine if this affects the results.

While frozen storage of samples does not have an effect on microbiome diversity (76,77), thawing and refreezing can have a significant effect (78). Therefore, it was important to prevent this occurrence during the experimental periods prior to sequencing. While appropriate steps were taken, it is possible that some samples may have experienced temperature changes which may have affected their bacterial composition. A maternal effect can create changes in the microbiome due to parenting techniques, which can last over several generations (45). Though changes in individual animal microbiome were not specifically controlled for maternal effect in this study, we used young rats that had not spent time with their mothers post-weaning. Therefore, while multi-generation effects may have had a role, a first-generation maternal effect was not a limitation in this study.

This study used transcutaneous electrical stimulation, which only involves skin-contact stimulation. Prior to each stimulation interval we verified that nerve recruitment was occurring, and this skin-surface approach did not require the stress of surgery for an implant. However, our prior rat studies that observed increases in vaginal blood flow used cuff electrodes implanted directly on the pudendal (23) and tibial nerves (24). Furthermore, while some nerve stimulation studies for bladder function have used skin-surface stimulation, most have shown greater efficacy using percutaneous or implanted stimulation approaches (79). Thus our reduced-contact stimulation technique may have limited the efficacy of the nerve recruitment and contributed to the relatively inconclusive stimulation results in Treatment animals. Additionally, our recent clinical study using skin-surface stimulation had greater improvements in sexual function for tibial nerve stimulation than genital nerve stimulation, in a small sample size (22), suggesting that alternate nerve targets may have stronger effects. Future studies with direct neural stimulation comparing genital or pudendal nerve stimulation to other nerve targets may yield more conclusive results.

Conclusions

This study demonstrates that the rat vaginal microbiome can change based on the estrous cycle. The microbiome is dynamic, changes in diversity over time, and is composed of OTU which fluctuate both in synchrony to and in opposition to one another. These findings support continued research into the rat vaginal microbiome and its relationship to the vaginal microbiome of other species including humans. While the rat vaginal microbiome was not completely similar to the human vaginal microbiome, many of the same major bacteria types were present, suggesting that rats may be a relevant animal model for humans in this area. Neural stimulation was not found to have a clear effect on the microbiome, though stimulation may have modulated the microbiome diversity in a subset of animals. This study suggests the potential for further research into the use of neural stimulation to drive changes in the vaginal microbiome and to increase vaginal function and health. Future studies may also examine additional physiological parameters in addition to the microbiome, such as vaginal pH and blood flow as well as hormones and other systemic factors, to obtain a more comprehensive perspective on their interrelationships.

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