Effect of the Arabin pessary and natural progesterone on the vaginal microbiome

Antonio G Amorim-Filho (antonio.filho002@hc.fm.usp.br)
Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo
Roberta C R Martins
Instituto de Medicina Tropical, Faculdade de Medicina da Universidade de São Paulo
Lucas A M Franco
Instituto de Medicina Tropical, Faculdade de Medicina da Universidade de São Paulo
Juliana V C Marinelli
Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo
Stela V Peres
Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo
Rossana P V Francisco
Faculdade de Medicina da Universidade de São Paulo
Mário H B Carvalho
Faculdade de Medicina da Universidade de São Paulo

Research Article

Keywords: Obstetric labor, premature. Cervix uteri. Vaginosis, bacterial. Metagenome. Microbiota. Vaginal discharge. Progesterone. Pessaries. High throughput nucleotide sequencing

Posted Date: January 20th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2493078/v1

Abstract

Background

Preterm birth is a leading cause of infant morbidity and mortality. Regardless of its multifactorial nature, it has been demonstrated that vaginal infections, as well instability of the local microbiome, can play a role as risk factors. The aim of the present study was to investigate possible changes in vaginal microbiome composition due to the use of an Arabin pessary or vaginal progesterone tablets in pregnant women as secondary prevention of preterm birth.

Results

We did a prospective analysis of 44 pregnant women at risk of preterm birth for a short cervix (≤ 25 mm) observed on transvaginal ultrasound in the second trimester and randomly assigned to receive an Arabin pessary (PE, n = 22) or vaginal progesterone (PR, n = 22). Vaginal swabs were collected upon diagnosis of short cervix and 4 weeks after treatment initiation to determine the Nugent score and microbiome profiles. The observed microbiomes could be assigned to 3 Community-State Types (CSTs) and most of the samples were characterized by a low-diversity, lactobacilli-dominated microbiota composition that remained stable after the onset of treatment. No treatment-associated change in microbiome alpha diversity was observed in either PE or PR and beta diversity analyses showed no significant dissimilarity between study groups or sampling times. Also, by an analysis of composition (ANCOM) no taxa with differential abundance were demonstrated.

Conclusions

Pessary and progesterone treatment for a short cervix appear to be equivalent regarding stability of the vaginal microbiome and thus patients and practitioners should be reassured about the safety of these methods.

Background

Preterm birth (PTB), defined as that occurring before 37 weeks of gestation, is a major public health concern due to the burden on infant morbidity and mortality. In Brazil its incidence is about 11,5%, held responsible for at least three quarters of infant mortality as well as significant long-lasting sequelae in the survivors [1]. Despite its multifactorial nature, spontaneous PTB (sPTB), *i.e.*, the one occurring without any medical indication, can be associated with bacterial colonization of the amniotic cavity via ascending infection from the vagina and it has been demonstrated that bacterial vaginosis (BV) is a risk factor for sPTB [2]. Another important risk factor is the presence of a short cervix (≤ 25 mm) on transvaginal ultrasound (TVUS) screening in the second trimester of gestation [3]. The use of daily vaginal progesterone tablets (PR) is a well stablished therapy to prevent sPTB in pregnant women with a

short cervix [4] and, more recently, the placement of a flexible silicone ring, the Arabin pessary (PE), around the cervix has been proposed as an alternative and is currently being investigated in clinical trials, with conflicting results [5]. Despite this uncertainty, the use of PE for sPTB prevention is routine in many settings and, although fairly tolerated, is sometimes associated with exacerbated and disturbing vaginal discharge [5, 6]. In addition, the placement of a stitch around the cervix, or cerclage, is reserved for patients at risk for cervical insufficiency [7].

Microbiome studies have previously shown that the vaginal ecosystem of asymptomatic reproductive-age women can be classified into 5 basic Community-State Types (CSTs), 4 of them characterized by a low-diversity, *Lactobacillus*-dominated composition, which differ from each other by the dominant *Lactobacillus* species, *L.crispatus* (CST-I), *L.gasseri* (CST-II), *L.iners* (CST-III) and *L.jenseni* (CST-V), plus one with higher richness, no *Lactobacillus* dominance and presence of BV-associated anaerobes (CST-IV) [8]. Distribution of these CSTs displays considerable variation regarding ethnicity and physiologic conditions [9]. For instance, during uncomplicated pregnancy the vaginal microbiome becomes highly stable, less rich and even more dominated by *Lactobacillus* species [10–13], which are believed to confer protection against infectious microorganisms to the vaginal environment [14]. On the other hand, it has been demonstrated that sPTB is associated with vaginal microbiome dysbiosis and some signatures have been identified in different populations, like loss of stability, increased diversity, and reduced *Lactobacillus* content [15–20].

Our proposal was to investigate if treatment for sPTB in at-risk women could itself drive modifications to the vaginal microbiome, either by local chemical action (in case of PR) or by presence of a foreign body in the vagina (as in PE use), that would further jeopardize these pregnancies. That answer would be valuable helping clinicians choose the appropriate treatment and thus avoid infectious complications. Two previous studies addressed this question. Firstly, Kindinger et al. [17] observed no progesterone-associated modification of vaginal microbiome in an English population of mainly caucasian ancestry. Secondly, Vargas et al. [21], studying a European mixed-ancestry population, demonstrated that patients with a cerclage stitch, which can also be considered a vaginal foreign body, had higher vaginal microbiome diversity as well as reduced *Lactobacillus* content, whereas patients with a PE had no such microbiome modifications.

Methods

Study Design and subjects

The present study was an arm of an ongoing randomized trial comparing PE and PR for sPTB prevention in single pregnancies with a short cervix diagnosed by TVUS between 20–24 wk gestation (NCT02511574, *clinicaltrials.gov*), aiming to investigate if treatment was associated with vaginal microbiome dysbiosis. Subjects with a cervix length ≤ 25 mm and intact membranes at the time of second trimester TVUS cervical screening were enrolled at the prenatal care clinic of Hospital das Clínicas in São Paulo, Brazil. Randomization was performed and patients were allocated to either have an Arabin

pessary (Dr. Arabin GmbH & Co., Germany) placed around cervix (PE group) or receive daily 200 mg of natural PR in form of vaginal tablets (PR group). Of these, 44 patients, 22 in PE and 22 in PR groups were chosen for microbiome determination by 16S rRNA gene amplicon sequencing at two timepoints: before treatment, at the time of randomization (T_0) and after treatment, 4 weeks later (T_1).

In descriptive analysis categorical variables were compared by Chi-square or Fisher's exact tests, and for continuous variables we used Mann-Whitney or Student's t-test. For all analyses a significance level of 95% was adopted. Baseline characteristics of participants, as shown in **Table 1**, were similar for the two study groups.

	PE (n = 22)	PR (n = 22)	p
Demographics			
Mean age + SD (range), yr	30 ± 7 (15–42)	28 ± 6 (17–37)	0.315 [1]
Ethnicity			
White, n	15 (68.2%)	16 (72.7%)	0.741 [2]
Black/Mixed, n	7 (31.8%)	6 (27.3%)	
Mean BMI + SD (range), kg/m2	26.5 ± 4.3 (18.6–36.1)	27.7 ± 5.1 (20.5–37.7)	0.453 [3]
Obstetric history			
Nulliparous, n	13 (59.1%)	8 (36.4%)	0.131 [2]
Miscarriage, n	7 (31.8%)	8 (36.4%)	0.750 [2]
Preterm birth, n	4 (18.2%)	3 (13.6%)	1.000 [4]
Sample collection			
Mean GA at T0 + SD (range), wk	22.3 ± 1.1 (20.6–23.9)	22.9 ± 0.8 (21.6–24.9)	0.107 [3]
Mean cervix length at T_0 + SD (range), mm	16.0 ± 6.0 (5.0–24.0)	17.0 ± 5.0 (7.0–23.0)	0.494 [3]
Nugent score > 3, n	4 (18.2%)	3 (13.6%)	1.000 [4]
Pregnancy outcome			
Mean GA at birth + SD (range), wk	37.4 ± 4.0 (25.4–40.3)	37.4 ± 3.4 (25.9–40.7)	0.677 [3]
Sponteneous preterm birth, n	2 (9.1%)	6 (30.0%)	0.123 [4]

PE: pessary group; PR: progesterone group; BMI: body mass index; GA: gestational age

[1] Student's t-test

[2] Chi-square test

[3] Mann-Whitney test

[4] Fisher's exact test

Table 1 – Baseline characteristics of the study population.

Sample collection, DNA extraction, library preparation and DNA sequencing

Vaginal specimens were collected at prenatal care visits by speculum examination, with a standard plastic brush and dispersed in 2 ml of sterile 0,9% saline and immediately stored at -80ºC for further analysis. A second sample was collected in appropriate transfer medium for Gram staining and determination of the Nugent score [22]. Bacterial DNA was extracted from thawed samples in a clean, sterile environment using PowerSoil kit (MoBio Laboratories, USA) according to the manufacturer recommendations.

The V4 region of the rRNA 16S gene was amplified by PCR using primers F515 (5'-CACGGTCGKCGGCGCCATT-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3 ') [23]. Sequencing adapters and barcodes were added to the primers (full primer sequences are detailed in **Figure S1**, supplementary material). PCR was carried out with PlatinumR PCR SuperMix High Fidelity kit (ThermoFisher, USA) following the steps: 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 68°C for 1 min. Amplicons were purified and quantitated as previously described [24].

Templates were prepared with Ion Chef System (Thermo Fisher Scientific, MA, USA) and DNA sequencing performed with Ion Personal Genome Machine (Thermo Fisher Scientific, MA, USA) using 318 semi-conductor chips according to the manufacture's recommendations.

Microbiome profiling of vaginal samples

Microbiome analysis was performed with Quantitative Insights into Microbial Ecology (QIIME 2) version 2020.2. Sequences were demultiplexed, denoised and truncated at 240 bp size with a minimum Phred score of 33 to generate amplicon sequence variants (ASVs) using DADA2 algorithm [25]. The average number of ASVs per sample was 232.965 (min 55.588 - max 544.234). ASVs were taxonomically classified in operational taxonomic units (OTUs) to a minimum 97% similarity, and a phylogenetic tree was built, both using Greengenes 13_8 (https://greengenes.secondgenome.com) as reference. Data was then summarized in a feature table containing the relative abundance of OTUs at each taxonomic rank.

Microbial communities were clustered into CSTs by constructing a dendrogram with Microbiome Analyst (https://www.microbiomeanalyst.ca) using complete linkage algorithm and Bray-Curtis dissimilarity as distance metrics (**Figure S2**, supplementary material). Since our sequencing approach did not allow the identification of all *Lactobacillus* species, only three CSTs could be distinguished: CST-I/II/V (non-*L.iners* dominance), CST-IIII (*L.iners* dominance) and CST-IV (no *Lactobacillus* dominance.

Statistical analysis of microbiomes

CST profiles present in each study group were compared between the two sampling timepoints using McNemar-Bowker test. Rarefied samples were analyzed with QIIME2 to determine alpha diversity indices (richness and Shannon index) and Bray-Curtis dissimilarity (beta diversity). Alpha diversity was compared between PE and PR groups at two timepoints (T_0 and T_1) by Kruskal-Wallis test using the R package and the syntax provided by Chen et al. [26]. Comparisons of beta diversity were performed with PERMANOVA analysis and a Principal Coordinates Analysis (PCoA) plot generated with Emperor [27]. All statistical analyses considered a level of significance of 95%.

Differential abundance of taxa was performed by analysis of composition (ANCOM) using Qiime).

Results

As shown in Fig. 1A and as expected in pregnancy, most analyzed samples exhibited low-diversity, *Lactobacillus*-dominated compositions (80 samples). From these, 42 were dominated by *L.iners* (CST-III) and 38 dominated by other *Lactobacillus* species (CST-I/II/V). The remaining 8 samples showed higher diversity, no *Lactobacillus* dominance and higher content of anaerobes, including VB-associated *Gardnerella* (CST-IV), which could be regarded as a state of dysbiosis. The observed CST distribution was in accordance with was previously observed in a larger, similar Brazilian population [28].

An altered Nugent score (> 3) was frequently associated with CST-IV and, although some CST transitions were observed between the two sampling timepoints, two of them towards CST-IV (AF10 and AF71), no significant difference in CST profile was observed in either PE or PR groups (Fig. 1B).

As for community alpha diversity, either comparing the two sampling timepoints inside PE and PR groups or each study group (Fig. 2A and **B**) at T_0 and T_1 (Fig. 2C and **D**), no significant difference was observed, indicating composition stability during the observation period.

Similarly, no significant dissimilarity was observed between sampling timepoints by PERMANOVA in either PE (Fig. 3A) or PR (Fig. 3B) groups.

Finally, we performed an ANCOM analysis to further explore possible differences in taxa abundance between study groups and sampling timepoints, including the dominant feature *Lactobacillus*. Thus, as demonstrated for community diversity, no taxa with differential abundance between T_0 and T_1 was found in PE (Fig. 4A) and PR (Fig. 4B).

Discussion

Much effort has been undertaken lately to identify sPTB signatures in the vaginal microbiome and current evidence supports the idea of an association with vaginal ecosystem instability. The characteristic low diversity, Lactobacillus dominance hampers differential analysis. Our demonstration that vaginal microbiome of women at high risk for sPTB remains stable after pessary placement or progesterone treatment is in accordance with the results of the two previous studies addressing the issue [17, 21]. An

important limitation of our study is the relatively small number of cases enrolled (n = 44), but still comparable to the studies of Kindinger et al. [17] (n = 25, PR group) and Vargas et al. [21] (n = 26, PE group). The small number of cases also makes difficult to draw conclusions about the association between vaginal dysbiosis and sPTB in our study population. Also, we chose to directly compare PE and PR, whereas the others included a control, no intervention group (cervix > 25 mm). It could be argued that microbial composition associated with a short cervix already represents a state of dysbiosis and thus rising sPTB risk even before treatment initiation. Nevertheless, in the study of Witkin et al. [28], which analyzed a larger Brazilian population (n = 340), mostly (90%) presenting a normal cervix, the CST distribution was similar to what we observed. Furthermore, the authors found CST-III to be a risk factor for developing a short cervix, probably due to biochemical modifications related to *L.iners* lactic acid secretion. A similar *L.iners* effect was described by Kindinger et al. [17] rising the possibility that CST-III represents an intermediate state of dysbiosis. Hence, the high prevalence of CST-III in our high-risk population certainly warrants further investigation.

We believe that our results add significant information to the current knowledge on the impact of the vaginal microbiome in patients at risk of sPTB and will help clinical management of these patients.

Conclusions

Vaginal progesterone tablets and Arabin pessaries are widely prescribed for sPTB prevention in at-risk women for a short cervix identified in the second trimester. Since vaginal infection, such as BV, or even subtle alterations in vaginal microbiota, as loss of stability, increased diversity and decreased content of *Lactobacillus* have been assigned as risk factors for sPTB [29], we sought to investigate if those treatments could themselves be implicated in local dysbiosis, and thus posing extra threats to pregnancy. We demonstrated that treatment is not associated with significant changes on the vaginal microbiome, so patients and clinicians should be reassured about safety regarding infectious complications. As far as we know the present study is one of a few to address the impact of PE and PR on the vaginal ecosystem, the first to analyze it in a Brazilian population and we believe that knowledge on that issue will certainly improve care to patients at risk for sPTB.

List Of Abreviations

BV: bacterial vaginiosis

CST: Community-state type

PE: pessary

PR: progesterone

PTB: preterm birth

sPTB: spontaneous preterm birth

TVUS: transvaginal ultrasound

Declarations

Ethics approval and consent

The present study was approved by the “Ethics Committee for Analysis of Research Projects (CAPPesq)” from Hospital das Clínicas, University of São Paulo (https://www.hc.fm.usp.br/hc/cappesq/cappesq) under the number CAAE 20611813.0.0000.0068.

All research subjects signed the institutional consent for participation and data publication. All signed forms are kept at our department for further reference.

Availability of data

All datasets generated or analyzed during the current study are publicly available in https://drive.google.com/drive/folders/12uEFU2X0AuTpqToRhMOuoMJnSOYYTvpp?usp=share_link.

Competing interests

The authors declare no financial or non-financial conflicts of interest.

Funding

This research was funded with internal department resources, no specific grant was involved.

Author’s contributions

Study design: AGAF, MHBC, JVCM, RPVF

Patient enrollment and sampling: AGAF, MHBC, JVCM

DNA sequencing: AGAF, LAMF, RCRM

Statistical analysis: RCRM, SVP

Paper writing/review: AGAF, MHBC

Acknowledgements

The authors wish to acknowledge Drs. Esther C Sabino, Mara Hoshida and Priscila G Amaral for their invaluable contribution to the present work.

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Figures

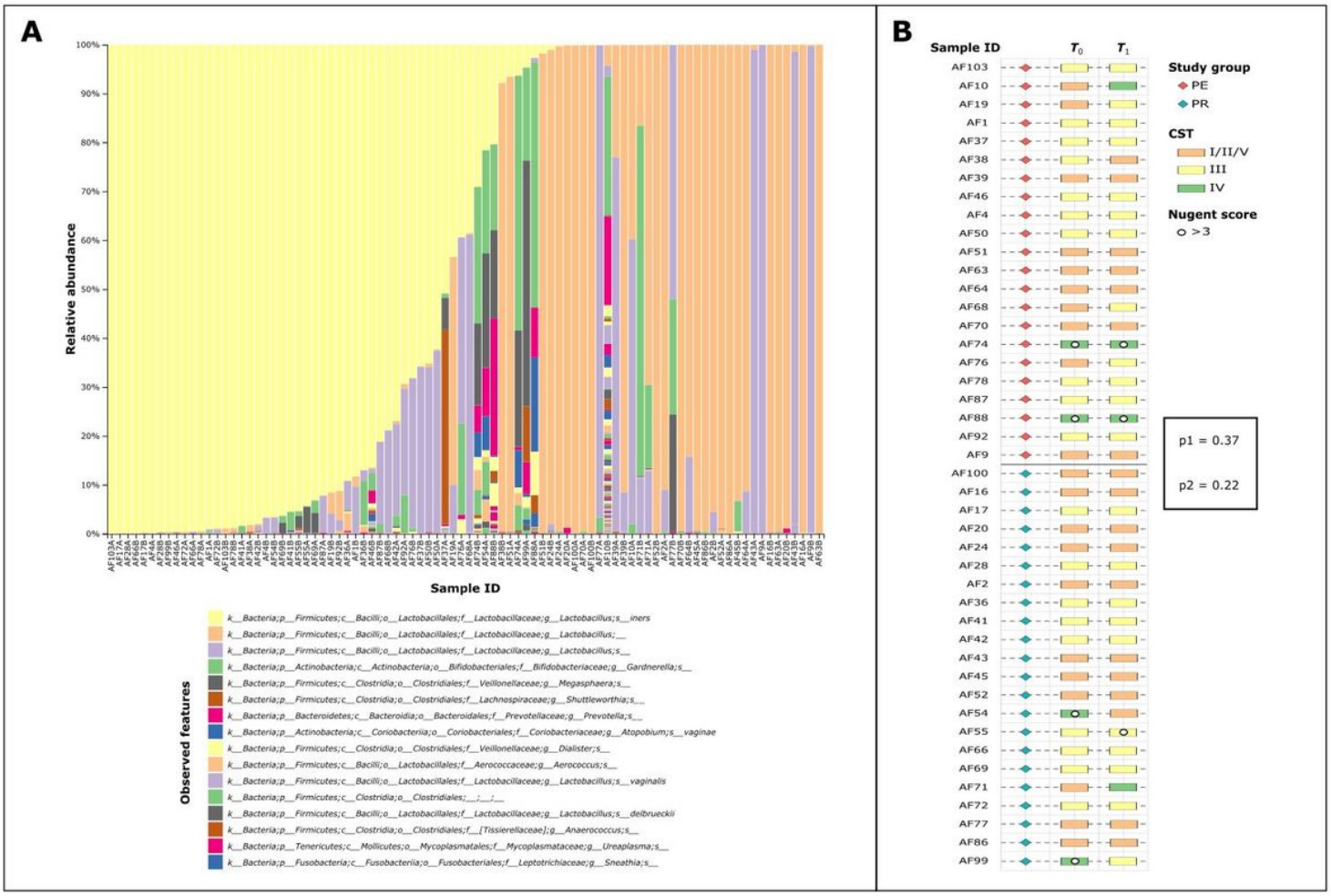

Figure 1

Composition and stability of vaginal microbiome. **A** taxa-barplot of features identified. Bottom: legend for the most abundant features. Samples were ordinated according to *L.iners* abundance. The letters A and B after each sample ID refer to *T0* (before treatment) and *T1*, (after treatment), respectively. **B** Diagram representing community-state types (CSTs) at *T0* and *T1*, as well as samples with altered Nugent scores. Box: p values from McNemar-Bowker test comparing *T0* vs *T1*, p1: pessary (PE), p2: progesterone (PR).

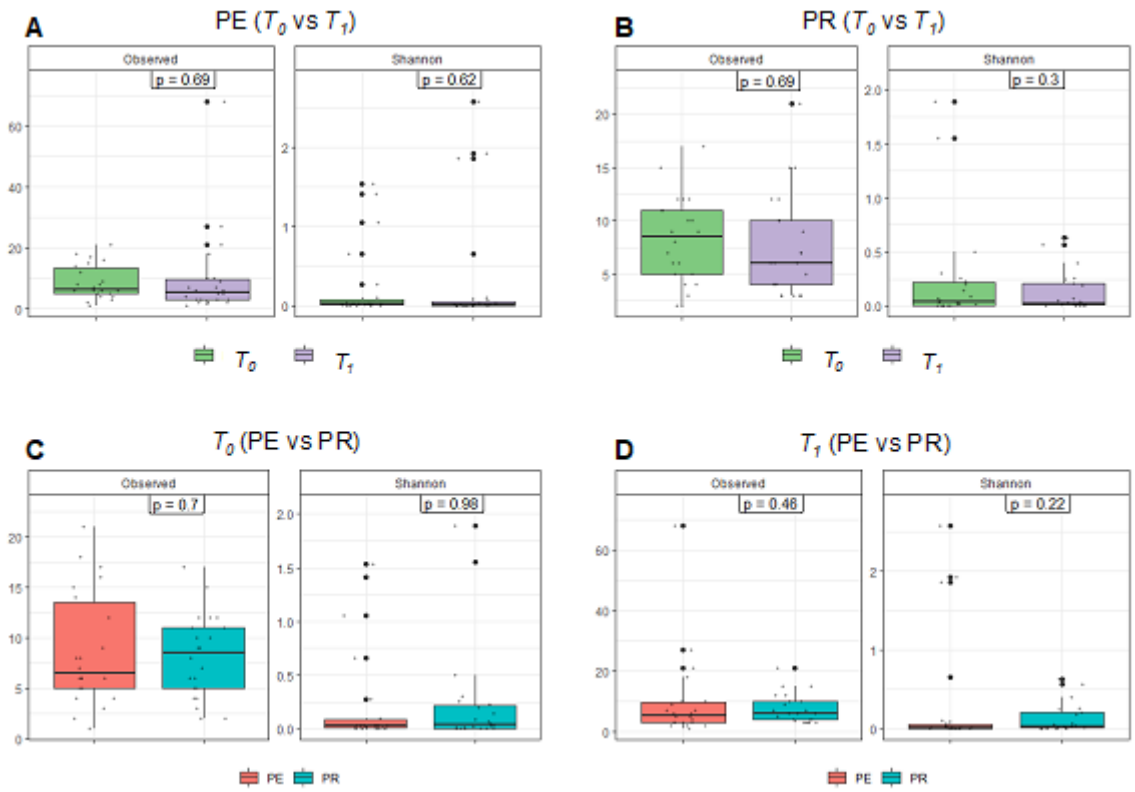

Figure 2

Vaginal microbiome alpha diversity at genus level. Comparison of alpha diversity between T_0 and T_1 in PE (**A**) and PR (**B**). Comparison between PE and PR at T_0. (**C**) and T_1 (**D**). Boxes: p values from Kruskal-Wallis test. Observed: richness. Shannon: Shannon diversity index. PE: pessary group. PR: progesterone group. T_0: before treatment. T_1: after treatment.

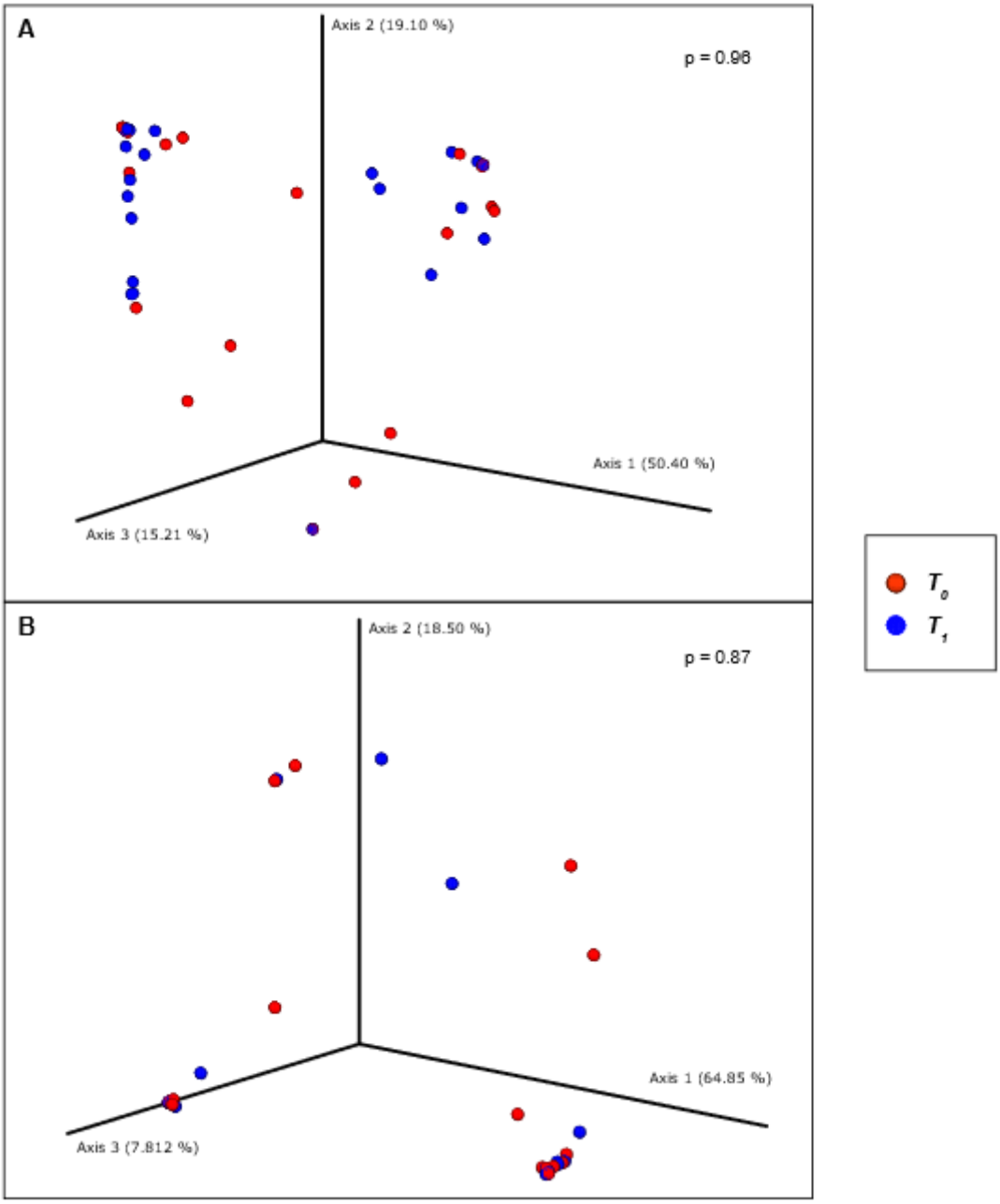

Figure 3

PCoA plot of vaginal microbiome T_0 vs T_1 Bray-Curtis dissimilarity at genus level. **A** pessary group (PE). **B** progesterone group (PR). Top right: p values by PERMANOVA test. T_0: before treatment. T_1: after treatment.

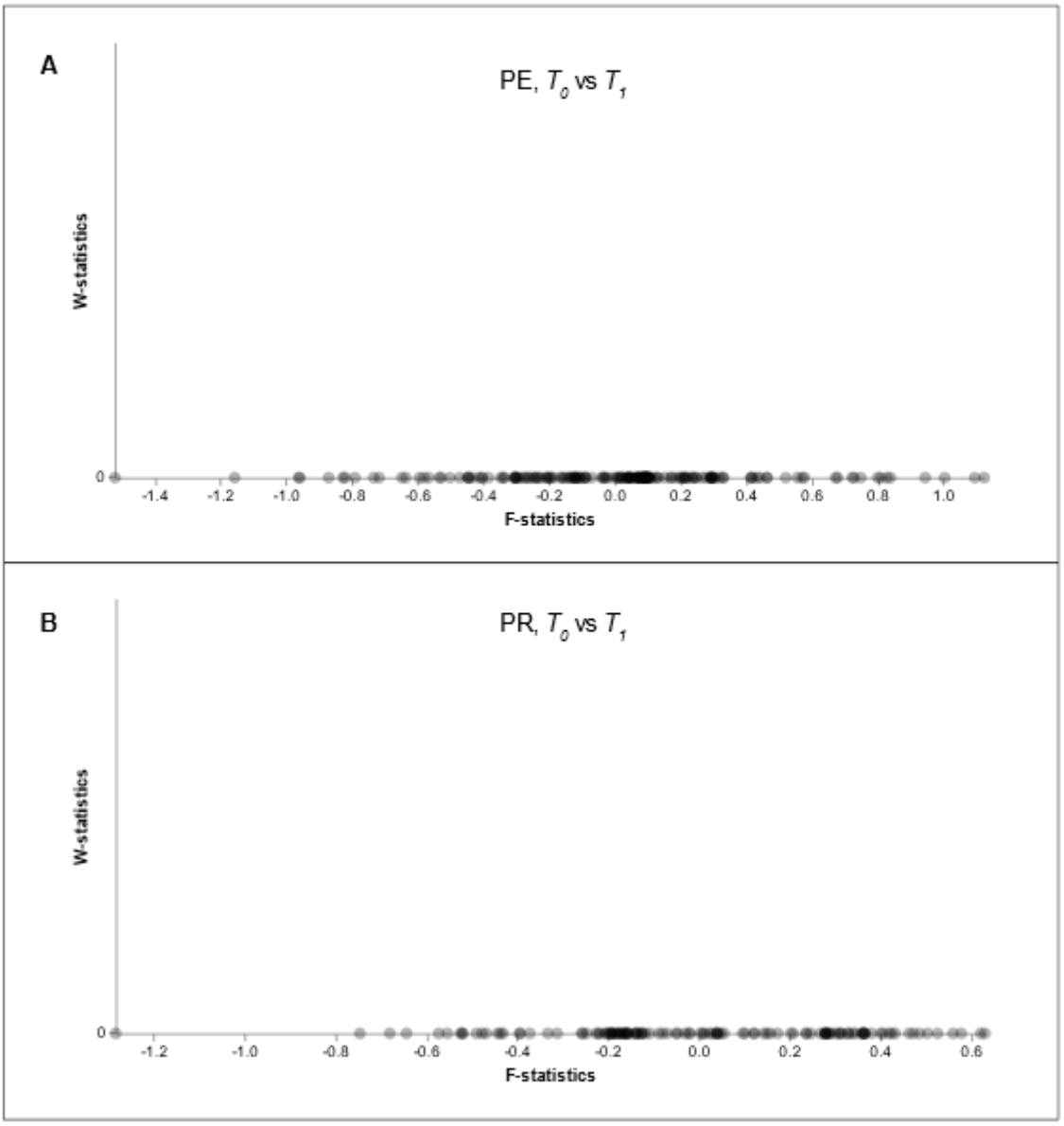

Figure 4

Volcano plot of vaginal microbiome ANCOM. F-statistics are represented on the x-axis and W-statistics on the y-axis. **A** Comparison between T_0: and T_1 in PE group. **B** Comparison between T_0: and T_1 in PR group. T_0: before treatment. T_1: after treatment.

Supplementary Files

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