Alterations in Vaginal Microbiota among Pregnant Women with COVID-19

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Abstract

Objective: To describe the alterations of the composition of vaginal microbiota in pregnant women with COVID-19. Design: Prospective observational single-centre study Setting: Tertiary referral hospital Participants: Pregnant women with COVID-19 Methods: The vaginal swabs were collected during the active phase of infection and consecutively, within a month after recovering from infection. In three patients, longitudinal samples before, in the course, and after infection were also obtained. The microbiome alterations were examined by 16S rRNA gene sequencing. Main outcome measures: Vaginal microbiota profiles in pregnant women with COVID-19 Results: Nineteen pregnant women with COVID-19 and 28 healthy controls who were matched according to the maternal age and gestational week were recruited. Shannon index and inverse Simpson index for cross-sectional cohort indicate that alpha diversity is significantly higher in women with COVID-19 (P=0.007 and P=0.006, respectively). There was a significantly decrease in Firmicutes (P=0.007) and Lactobacillus (P=0.019) with an increase in Bacteroidetes (P=0.024) in women with COVID-19 when compared to those of healthy controls. The higher amounts of Ureaplasma were found in women with the moderate/severe disease, compared to those of the asymptomatic/mild disease (P=0.001). Lactobacillus gasseri disappeared in women with the moderate/severe disease. Prevotella timonensis was identified only in the COVID-19 group. In longitudinal analysis, Actinobacteria was elevated, Firmicutes and Bacteroides depleted during the active phase. Conclusion: The study revealed that vaginal dysbiosis with a low abundance of Lactobacillus and an increase in Bacteroidetes is associated with COVID-19.

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ABSTRACT

Objective: To describe the alterations of the composition of vaginal microbiota in pregnant women with COVID-19.

Design: Prospective observational single-centre study

Setting: Tertiary referral hospital

Participants: Pregnant women with COVID-19

Methods: The vaginal swabs were collected during the active phase of infection and consecutively, within a month after recovering from infection. In three patients, longitudinal samples before, in the course, and after infection were also obtained. The microbiome alterations were examined by 16S rRNA gene sequencing.

Main outcome measures: Vaginal microbiota profiles in pregnant women with COVID-19

Results: Nineteen pregnant women with COVID-19 and 28 healthy controls who were matched according to the maternal age and gestational week were recruited. Shannon index and inverse Simpson index for cross-sectional cohort indicate that alpha diversity is significantly higher in women with COVID-19 (P=0.007 and P=0.006, respectively). There was a significantly decrease in Firmicutes (P=0.007) and Lactobacillus (P=0.019) with an increase in Bacteroidetes (P=0.024) in women with COVID-19 when compared to those of healthy controls. The higher amounts of Ureaplasma were found in women with the moderate/severe disease, compared to those of the asymptomatic/mild disease (P=0.001). Lactobacillus gasseri disappeared in women with the moderate/severe disease. Prevotella timonensis was identified only in the COVID-19 group. In longitudinal analysis, Actinobacteria was elevated, Firmicutes and Bacteroides depleted during the active phase.

Conclusion: The study revealed that vaginal dysbiosis with a low abundance of Lactobacillus and an increase in Bacteroidetes is associated with COVID-19.

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Keywords: Vaginal microbiome, vaginal microbiota, COVID-19, pregnancy

Introduction

Coronavirus disease 2019 (COVID-19) has emerged worldwide while a subset of people was infected severely, the others recovered from the disease without symptoms. COVID-19 has been shown to have potential adverse effects on pregnancy and neonatal outcomes. Pregnancy itself is a risk factor for the severity of COVID-19 disease, with an increased risk of ICU admission, maternal morbidity, and mortality[1,2]. Furthermore, pregnancy complications such as preeclampsia and preterm birth are more likely to occur in women diagnosed with COVID-19 [3,4].

The predominance of Lactobacillus species (spp.) plays a key role in the inhibition of binding non-domestic and potentially harmful microorganisms to epithelial cells [5–7].*Lactobacillus* maintains the protective low vaginal pH through secreting of lactic acid [8]. Pregnant women with decreased amounts of *Lactobacillus crispatus, Lactobacillus gasseri,* and *Lactobacillus jensenii* in the vaginal microbiota are more likely to deliver preterm [9]. Likewise, the abundance of *Gardnerella vaginalis* increases the risk of preterm birth (PTB) [5,10].

The mechanism of action of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) on pregnancy varies and remains unknown. The SARS-CoV-2 genome has been identified in the vaginal mucosa of a pregnant woman [11]. Indeed, the role of SARS-CoV-2 infection in vaginal microbiome composition in pregnant women with COVID-19 has not yet been investigated. Therefore, we anticipate that COVID-19 may unfavorably affect the composition of the vaginal microbiota, resulting in adverse pregnancy outcomes. We aimed to describe the alterations of the composition of vaginal microbiota in pregnant women with COVID-19.

Materials and Methods

Study population:

A prospective study was conducted at Koc University Hospital between August 2020 and August 2021. Pregnant women with active or recently infected within one month with SARS-CoV-2 were included in the study. SARS-CoV-2 infection was confirmed by a positive nasopharyngeal polymerase chain reaction (PCR) test. The signs and symptoms of COVID-19 were evaluated in all women who had positive PCR tests for SARS-CoV-2 infection. COVID-19 was classified according to NIH COVID-19 clinical guidelines [12]. Patients with symptoms including fever, myalgia, or gastrointestinal system symptoms were categorized as a mild disease, those who required oxygen (O_2) supplementation as moderate or severe disease (MSD). Recovery was defined as clinical improvement in combination with a negative nasopharyngeal PCR test. Single vaginal swab was collected from each participant at the time of COVID-19 and within one month after recovery from COVID-19. In three patients, longitudinal microbiota analysis was performed with a collection of vaginal swabs before, during active infection, and two months after recovery. The overview of patients was presented in Figure 1.

The healthy controls (HC) were recruited from the prospective study entitled "Vaginal, Placental and Neonatal Buccal Mycobiota, and Microbiome in Preterm Birth". The study was initiated in April 2020 (ClinicalTrials.gov Identifier: NCT04165252). Maternal age and gestational week-matched pregnant women were selected for the healthy controls. Inclusion criteria for the healthy controls are as follows: age older than 18 years with a singleton pregnancy. The exclusion criteria were multiple pregnancies, major fetal structural defects and/or chromosomal abnormalities, stillbirth, having used antibiotics and/or antifungal medication within two weeks at the time of sample collection, the presence of vaginal bleeding at the time of sample collection, and sexual intercourse within 72 hours of sample collection.

Maternal characteristics, medical and obstetrical history were recorded for all participants. Maternal height and weight were measured at the same time with vaginal swabs collection. Gestational age was determined from the last menstrual period and confirmed from the measurement of fetal crown-rump length at the first-trimester scan. The written informed consent was obtained from all participants. Koç University Research Ethics Board approved the study protocol. The study complies with the decleration of Helsinki.

Sample collection, processing, and sequencing

Vaginal samples were collected with REMEL ESwabs. Vaginal swabs were placed into the sterile tube then stored at -80degC until DNA extraction. Frozen vaginal swabs were immersed in sterile PBS then DNA extraction was performed using the Qiagen DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), as described by the manufacturer. DNA concentration was quantified by Qubit (ThermoFisher Scientific, Waltham MA).

Library preparation was performed using QIAseq 16S/ITS Panel Kit (Qiagen, Hilden, Germany) for sequencing the V1–V9 region of the 16S rRNA genebacterial gene. Library quantification was done using QIAseq Library Quant Assay (Qiagen, Hilden, Germany) kit following the manufacturer's instructions with Applied Biosystems QuantStudio 7 Flex Real-Time PCR (Applied Biosystems Inc., Foster City, California).

Sequencing was performed with the Illumina MiSeq platform using the MiSeq v3 Reagent Kit (Illumina, San Diego, CA, United States).

Bioinformatics

FASTQ files were demultiplexed by different regions using the module in the GeneGlobe Data Analysis Center. (https://geneglobe.qiagen.com/tr/analyze). The resulting paired-end FASTQ files were used to profile the microbiota of the samples with DADA2 using default parameters. Sequencing of 16S rRNA gene(V1-V2) regions was performed [13]. Amplicon sequence variants (ASV) were picked out and assigned with taxonomic annotations using the naive Bayesian classifier method embedded in DADA2 [14]. The Silva (v138.1) was used as a reference database (v138.1)[15].

Statistical analysis

Continuous variables were expressed as a median and interquartile range (IQR), whereas categorical variables were expressed as percentages. Mann-Whitney U and Fisher exact tests were applied for comparison of maternal demographic and clinical characteristics between COVID-19 patients with healthy controls. Data were analyzed using IBM SPSS Statistics for Windows, version 26.0 (Armonk, NY: IBM Corp.).

The alpha diversity, beta diversity, and vaginal microbiota composition of the COVID-19 group, and healthy control were compared. Alpha-diversity metrics were calculated as the number of phyla, genus, and species. The vaginal microbiota of asymptomatic or mild, moderate, or severe cases and healthy controls were compared. Beta-diversity was defined by using Bray-Curtis distance. The differences in alpha diversity metrics were performed by Wilcoxon signed-rank test using Python 3.7 (32-bit) and statistical significance was set as P value $<.05$. Statistical data were visualized with GraphPad Prism 8.0.2.

Data availability: All data generated or analyzed during this study are included in this published article.

Results

Study cohort demographics

There were no differences in maternal age, BMI, gestational age at delivery, and birth weight between women with the COVID-19 group with healthy controls. (Table 1). Of 19 women with COVID-19, 13 women had the asymptomatic or mild disease (AMD) (68.4%), and 6 of those had moderate or severe disease (31.6%). The rate of preterm birth (PTB) was 15.3% (n=3) in the COVID-19 group; 2 of 6 women (33.3%) with moderate or severe disease and 1 of 13 women (7.7%) with asymptomatic or mild disease (Table 2).

In asymptomatic or mild disease, 61.5% of those had infection in the second trimester and 38.5% in the third trimester (Table 2). Two women with moderate or severe disease received both antibiotic and antiviral medications. Seven patients received low molecular weight heparin during the active period of COVID-19 disease (Table 2).

The composition of vaginal microbiota composition in the healthy controls and women with COVID-19

Alpha diversity was evaluated by using Shannon and Simpson Index. In the COVID-19 group, the Shannon index was significantly elevated, compared to those of the healthy controls (1.09 vs. 0.75; P=0.007), and the Simpson index was significantly lower in the COVID-19 group than those of healthy controls (0.4 vs. 0.61; P=0.006) (Figure 2A). In pregnant women with asymptomatic/mild and moderate/severe disease, the Shannon index was found to be significantly higher than those of the healthy controls (1.06 and 1.16 vs. 0.75; P<0.05), whereas the Simpson index was significantly decreased, compared to the healthy controls (0.42 and 0.49 respectively, vs. 0.61; P<0.05) (Figure 2A). Beta diversity (Bray-Curtis) results indicated that there were no compositional differences between COVID-19 status and healthy controls (P=0.49) (Figure 2B).

There were no statistically significant differences in the alpha diversity of vaginal microbiota between the second and third trimesters of healthy controls according to Shannon index and Simpson index (0.41 vs. 0.5; P= 0.22 and, 0.8 vs. 0.75; P= 0.08, respectively).

The vaginal microbiota composition and relative abundances of the bacterial phylum, genera and species for two groups were summarized in Figure 3. The three phyla; Firmicutes (86.02%), Actinobacteria (12.12%) and Bacteriodata (Bacteroidetes) (0.54%) accounted for 99.8% of the bacterial species in COVID-19 group. In a comparison of COVID-19 group with healthy controls, Firmicutes significantly decreased (85.9% vs. 96.11, P=0.049) while the amounts of Bacteroidetes significantly increased (0.53% vs. 0.44%; P=0.024) (Figure 3A).

At the genus level, *Lactobacillus sp.* significantly decreased in the COVID-19 group compared to the healthy controls (80.6% vs. 93.98%; P=0.019). Particularly, *Lactobacillus delbrueckii (L. delbrueckii) was not detected* in the COVID-19 group, although its amount was 1.5% in the healthy controls (P=0.046) (Figure 3B).

Lactobacillus iners (L.iners) , *Lactobacillus crispatus (L. crispatus)* , and *Lactobacillus jensenii (L. jensenii)* showed trends towards a decline in COVID-19 group (26.5%, 3.2%, and 7.1%, respectively) when compared to the healthy controls (32.8%, 3.9%, and 7.5%, respectively) but the differences were not statistically significant (P>0.05) (Figure 3C).

In the COVID-19 group, among anaerobe taxa, particularly *Prevotella timonensis* (0.04%) and *Dialister propionicifaciens (0.02%)* were identified but none of these were detected in the healthy controls. In addition to these, the COVID-19-infected group showed a higher abundance of Gardnerella *vaginalis* (6.4% vs. 1.98%; P=0.213). Also, *Ureaplasma*(0.69% vs. 0.04%; P=0.054) was found to be higher in the COVID-19 group compared to the healthy controls.

The variations of vaginal microbiota composition in relation to the severity of COVID-19

Mycoplasma hominis , *Fusobacterium nucleatum* and *Anaerococcus tetradius* were only identified in patients with moderate or severe disease (8%, 0.03%, and 0.003%, respectively) (Figure 4). In a comparison of the patients with asymptomatic or mild and moderate or severe disease, there was no significant difference in abundance of *Prevotella timonensis* (0.05% and 0.02%, p=0.379); however, *Ureaplasma spp.* was significantly higher in the moderate or severe group (2.09%) than those of the asymptomatic or mild (0.04%, p=0.005). In addition, *Gardnerella vaginalis* showed a gradual increase in both two groups (1.99% and 5.11%) compared to the healthy controls (9.33%) (Figure 4).

In the longitudinal vaginal microbiota analysis of the three pregnant women, the relative abundance of Actinobacteria increased from 10.8% to 59.2% during active infection and returned to 10.8% in the post-COVID-19 phase. There is a declining trend in the abundance of Bacteroidata (Bacteroidetes) (from 65.4% to 17.9%) and Firmicutes (from 59.2% to 28.4%) during the phase of active COVID-19 with no regrowth in the post-COVID-19 phase (17.9% and 10.8%, respectively) (Figure 5).

Discussion

Main Findings

The findings of our study indicated that the composition of vaginal microbiota was unfavorably affected by COVID-19 disease and there was a prominent dysbiosis during active COVID-19 infection. Intrauterine infection is a well-established reason for preterm birth. The most common and genuine pathway is that microorganisms can access the amniotic cavity by ascending from the vagina and cervix, resulting in the development of intrauterine infection and subsequent inflammatory response in fetoplacental tissues that eventuates preterm birth[16,17]. Since evidence on the relation of dysbiosis in vaginal microbiota and preterm birth is accumulating, we can speculate that one of the mechanisms for the explanation of increased rate of COVID-19-associated PTB may be vaginal dysbiosis[5,18,19].

Vaginal dysbiosis is defined as an increase of alpha diversity in vaginal microbiome communities [20]. The study found that the Shannon index was remarkably high (1.16) in the COVID-19 group compared to the healthy controls (0.75). Recent studies have revealed that vaginal dysbiosis has a negative impact on vaginal protective mechanisms via increasing local pro-inflammatory effectors[21,22].

We identified diminished *Lactobacillus* communities in women with COVID-19 disease, particularly more profound in those with moderate or severe disease (77%) when compared to the healthy controls (93%; P=0.04). *Lactobacillus delbrueckii* (P=0.046) significantly decreased among women with COVID-19. Within-subgroup analysis,*Lactobacillus gasseri (L. gasseri)* disappeared in patients with moderate or severe disease. It is well-established that pregnant women with low amounts of *L. crispatus, L. gasseri, or L. jensenii* in their vaginal microbiota are more likely to deliver before term[9,23]. In a case-control study, the abundance of*L. gasseri* was found to be associated with decreased risk of early spontaneous preterm birth [24].

In the longitudinal study with three patients, we didn't perform analysis at the species level because of the small sample size. There was approximately a 40% decline in the relative abundance of Firmicutes and Bacteroides during the active COVID-19 period, which was sustained after recovery. In addition, the abundance of Actinobacteria was the highest in the active disease stage compared to the pre and post COVID-19 periods. Ceccarani et al. revealed that the vaginal flora of healthy women was constituted of mainly Firmicutes and Bacteroidetes, albeit with a low abundance of Actinobacteria [25]. In view of our findings, it would be considered that SARS-CoV-2 infection negatively affects the vaginal compositions of pregnant women.

Strengths and Limitations

The most important strength of the study is the first trial, which elucidated the relation of SARS-CoV-2 infection with the composition of vaginal flora. The vaginal samples were comprehensively collected from the healthy controls and women with COVID-19 disease. The samples from vaginal flora were analyzed exclusively by the three doctors in the group. Furthermore, we had longitudinal samples from three pregnant women, which provided an opportunity for comparison of vaginal composition of pregnant women before and after the disease. Further, detailed clinical data were collected from each participant and reported to consider for an evaluation of each subject.

There are several limitations. Our sample size was small to detect the statistical significance between women with severe COVID-19 and women with asymptomatic or mild disease, albeit with some significant differences. Secondly, a potential confounding factor that may differ among the groups was the use of antibiotics in the severe cases during the active stage at the time of sample collection.

Interpretations

Disruption of balance of vaginal microbiota causes invades of several facultative or strict anaerobes, including *Gardneralla vaginalis, Mycoplasma hominis, Prevotella spp., Fusobacterium spp* ., Ureoplasma spp., and *Porphyromonas spp.* and replacement of*Lactobacilli* [26–28]. Aligning with the aforementioned results, we identified a significantly higher abundance of Bacteroidetes in COVID-19 group. In particular, *Prevotella timonensis was only identified* in women with COVID-19 (0.04%). Likewise, *Dialister propionicifaciens* appeared in the COVID-19 group (0.02%). The SARS-CoV-2 infection triggers the production of prostaglandins and pro-inflammatory mediators to confer ischemia resulting in widespread tissues [29,30]. Notably, we determined an increase of anaerobic species such as *Gardnerella vaginalis* , *Anaerococcus tetradius* , *Fusobacterium nucleatum* , *Prevotella timonensis* abundance in women with severe disease. Based on these results, we postulated that ischemia in genito-urinary compartments could be a predisposing factor for overgrowth of anaerobes in vaginal microbiota.

Pregnant women are more susceptible to SARS-CoV-2 infection due to physiological, mechanical, and immunological changes during pregnancy[2,31]. Data supported that pregnancy is a risk factor for severe disease related to COVID-19 [4,32]. Recently, in a large population-based cohort study, fetal death and preterm birth occurred more frequently in women with SARS-CoV-2 infection than non-infected women (adjusted odds ratio (aOR), 2.21; 95% confidence interval (CI), 1.58 – 3.11; P< 0.001 and OR, 2.17; 95% CI, 1.96 – 2.4; P<0.001, respectively) [1]. In the study, the prevalence of preterm birth (15.3%) was high, especially in the severe COVID-19 (2 out of 3 preterm birth), as compared to the prevalence reported before COVID-19 (9.6%) [33]. Even with the small sample size of our study, the rate of preterm birth increased in women with severe COVID-19 disease than those with asymptomatic or mild disease, aligning with a recent meta-

analysis[34]. The abundance of *Ureaplasma* and*Mycoplasma* species increased the risk of preterm delivery through chorioamnionitis, salpingitis, bacterial vaginosis, and postpartum endometritis [20,35]. We found that *Mycoplasma hominis* (8% vs. 0%; P=0.01) and *Ureaplasma* spp. (2.09% vs. 0.04%, p=0.001) showed significantly higher abundance in moderate or severe cases compared to those of the healthy controls. Of note, the abundance of *Ureaplasma spp.* was significantly higher in women with moderate or severe disease than those of asymptomatic or mild disease (p=0.005). We claim in light of these findings and previous evidence from microbiota studies that the subsequent preterm birth in women with severe disease could be a consequence of impaired vaginal composition.

Conclusions

COVID-19 disease in pregnant women causes dysbiosis in vaginal microbiota with a significant reduction in the abundance of Lactobacillus combined with an increase of Bacteroidetes, especially in*Prevotella timonensis* . We detected that the severity of the disease was associated with increased *Ureaplasma spp.* Based on these findings, we suggest that COVID-19 promotes an unfavorable vaginal microenvironment, which may provide insight into the risk of adverse pregnancy outcomes such as preterm birth. These results raise clinically relevant questions regarding the use of microbiome associated biomarkers as a risk assessment tool for preterm birth in pregnant women during COVID-19. The implication of findings would postulate possible targeted therapy, comprising modification of the vaginal microbiota composition in pregnant women with COVID-19.

Disclosure of interests: The authors declare that they have no competing interests.

Contribution to authorship: F.C., E.C., G.O. and C.V. designed and conducted the research. F.C., M. K., O.D., E.C., O.E., G.O., C.V., A.G. and O.K. interpreted the results and wrote the manuscript. S.G.C and E.C. collected the samples and clinical outcomes. G.O. and C.V. processed the samples, prepared the DNA for sequencing, and conducted the experiments. E.P., G.O., C.V., E.C. analyzed the data and prepared the figures and tables. E.P. processed the bioinformatic analysis. A.G. and O.K. supervised the bioinformatics analysis.

Details of ethics approval: The ethical approval was obtained for the healthy controls and pregnant women with COVID-19 from Koc University Research Ethics Board (No:2019.093IRB2.030 and No:2020.138.IRB1.028). The written consent forms were obtained from all participants.

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Reference:

1. Gurol-Urganci I, Jardine JE, Carroll F, et al. Maternal and perinatal outcomes of pregnant women with SARS-CoV-2 infection at the time of birth in England: national cohort study. *Am J Obstet Gynecol* . Published online 2021. doi:10.1016/j.ajog.2021.05.016

2. Di Toro F, Gjoka M, Di Lorenzo G, et al. Impact of COVID-19 on maternal and neonatal outcomes: a systematic review and meta-analysis. *Clin Microbiol Infect* . 2021;27(1). doi:10.1016/j.cmi.2020.10.007

3. Flannery DD, Gouma S, Dhudasia MB, et al. Assessment of Maternal and Neonatal Cord Blood SARS-CoV-2 Antibodies and Placental Transfer Ratios. *JAMA Pediatr* . Published online 2021. doi:10.1001/jamapediatrics.2021.0038

4. Villar J, Ariff S, Gunier RB, et al. Maternal and Neonatal Morbidity and Mortality Among Pregnant Women With and Without COVID-19 Infection. *JAMA Pediatr* . Published online April 22, 2021. doi:10.1001/jamapediatrics.2021.1050

5. Fettweis JM, Serrano MG, Brooks JP, et al. The vaginal microbiome and preterm birth. *Nat Med* . 2019;25(6). doi:10.1038/s41591-019-0450-2

6. Di Paola M, Sani C, Clemente AM, et al. Characterization of cervico-vaginal microbiota in women developing persistent high-risk Human Papillomavirus infection. *Sci Rep* . 2017;7(1). doi:10.1038/s41598-017-09842-6

7. Witkin SS. Vaginal microbiome studies in pregnancy must also analyse host factors. *BJOG An Int J Obstet Gynaecol* . 2019;126(3). doi:10.1111/1471-0528.15300

8. Witkin SS, Linhares IM. Why do lactobacilli dominate the human vaginal microbiota? *BJOG An Int J Obstet Gynaecol* . 2017;124(4). doi:10.1111/1471-0528.14390

9. Payne MS, Newnham JP, Doherty DA, et al. A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study). *Am J Obstet Gynecol* . 2021;224(2). doi:10.1016/j.ajog.2020.08.034

10. Donders GG, Van Calsteren K, Bellen G, et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG An Int J Obstet Gynaecol* . 2009;116(10). doi:10.1111/j.1471-0528.2009.02237.x

11. Kirtsman M, Diambomba Y, Poutanen SM, et al. Probable congenital sars-cov-2 infection in a neonate born to a woman with active sars-cov-2 infection. *CMAJ* . 2020;192(24). doi:10.1503/cmaj.200821

12. NIH. COVID-19 Treatment Guidelines Panel. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines. National Institutes of Health.*Nih* . 2020;2019.

13. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data.*Nat Methods* . 2016;13(7). doi:10.1038/nmeth.3869

14. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.*Appl Environ Microbiol* . 2007;73(16). doi:10.1128/AEM.00062-07

15. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools.*Nucleic Acids Res* . 2013;41(D1). doi:10.1093/nar/gks1219

16. Gomez-Lopez N, Romero R, Xu Y, et al. Fetal T Cell Activation in the Amniotic Cavity during Preterm Labor: A Potential Mechanism for a Subset of Idiopathic Preterm Birth. *J Immunol* . 2019;203(7). doi:10.4049/jimmunol.1900621

17. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* . 2008;371(9606). doi:10.1016/S0140-6736(08)60074-4

18. McMillan A, Rulisa S, Sumarah M, et al. A multi-platform metabolomics approach identifies highly specific biomarkers of bacterial diversity in the vagina of pregnant and non-pregnant women. *Sci Rep* . 2015;5. doi:10.1038/srep14174

19. Shetty PK, Menon AG, Rai R. Prevelance, risk factors and adverse perinatal outcomes of bacterial vaginosis in pregnancy. *Int J Reprod Contraception, Obstet Gynecol* . 2021;10(9). doi:10.18203/2320-1770.ijrcog20213460

20. DiGiulio DB, Callahan BJ, McMurdie PJ, et al. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci U S A* . 2015;112(35). doi:10.1073/pnas.1502875112

21. Swidsinski A, Verstraelen H, Loening-Baucke V, Swidsinski S, Mendling W, Halwani Z. Presence of a Polymicrobial Endometrial Biofilm in Patients with Bacterial Vaginosis. *PLoS One* . 2013;8(1). doi:10.1371/journal.pone.0053997

22. Campisciano G, Zanotta N, Licastro D, De Seta F, Comar M. In vivo microbiome and associated immune markers: New insights into the pathogenesis of vaginal dysbiosis. *Sci Rep* . 2018;8(1). doi:10.1038/s41598-018-20649-x

23. Jayaprakash TP, Wagner EC, Van Schalkwyk J, et al. High diversity and variability in the vaginal microbiome in women following Preterm Premature Rupture of Membranes (PPROM): A prospective cohort study.*PLoS One* . 2016;11(11). doi:10.1371/journal.pone.0166794

24. Tabatabaei N, Eren AM, Barreiro LB, et al. Vaginal microbiome in early pregnancy and subsequent risk of spontaneous preterm birth: a case–control study. *BJOG An Int J Obstet Gynaecol* . 2019;126(3). doi:10.1111/1471-0528.15299

25. Ceccarani C, Foschi C, Parolin C, et al. Diversity of vaginal microbiome and metabolome during genital infections. *Sci Rep* . 2019;9(1). doi:10.1038/s41598-019-50410-x

26. Saraf VS, Sheikh SA, Ahmad A, Gillevet PM, Bokhari H, Javed S. Vaginal microbiome: normalcy vs dysbiosis. *Arch Microbiol* . 2021;203(7). doi:10.1007/s00203-021-02414-3

27. Doyle RM, Harris K, Kamiza S, et al. Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. *PLoS One* . 2017;12(7). doi:10.1371/journal.pone.0180167

28. Hyman RW, Fukushima M, Jiang H, et al. Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* . 2014;21(1). doi:10.1177/1933719113488838

29. Liu Y, Zhang C, Huang F, et al. Elevated plasma levels of selective cytokines in COVID-19 patients reflect viral load and lung injury.*Natl Sci Rev* . 2020;7(6). doi:10.1093/nsr/nwaa037

30. Li G, Fan Y, Lai Y, et al. Coronavirus infections and immune responses. *J Med Virol* . 2020;92(4). doi:10.1002/jmv.25685

31. Liu H, Wang LL, Zhao SJ, Kwak-Kim J, Mor G, Liao AH. Why are pregnant women susceptible to COVID-19? An immunological viewpoint.*J Reprod Immunol* . 2020;139. doi:10.1016/j.jri.2020.103122

32. Oakes MC, Kernberg AS, Carter EB, et al. Pregnancy as a risk factor for severe coronavirus disease 2019 using standardized clinical criteria. *Am J Obstet Gynecol MFM* . 2021;3(3). doi:10.1016/j.ajogmf.2021.100319

33. Hamilton BE, Martin JA, Osterman MJKS. Births: Preliminary Data for 2015 National Vital Statistics Reports. *Natl Vital Stat Reports* . 2015;65(3).

34. Wei SQ, Bilodeau-Bertrand M, Liu S, Auger N. The impact of COVID-19 on pregnancy outcomes: a systematic review and meta-analysis. *Can Med Assoc J* . 2021;193(16). doi:10.1503/cmaj.202604

35. Foxman B, Wen A, Srinivasan U, et al. Mycoplasma, bacterial vaginosis-associated bacteria BVAB3, race, and risk of preterm birth in a high-risk cohort. *Am J Obstet Gynecol* . 2014;210(3). doi:10.1016/j.ajog.2013.10.003

Figure legends

Figure 1. An overview of study design

The cohort includes pregnant women with COVID-19 (n=19) and the healthy controls (n=28), recruited from Clinical Trials. NCT04165252 study. Longitudinal study cases were shown in purple. The three preterm birth cases were shown in black circles.

Figure 2. The comparison of alpha diversity of the vaginal microbiota between healthy controls and pregnant women with COVID-19

TC and HC refer to total COVID-19 patients (n=19) and healthy controls (n=28), respectively. Pregnant women with COVID-19 were divided into two sub-groups according to severity of disease as Asymptomatic/Mild (AMC) (n=13) and Moderate/Severe (MSC) (n=6). Mann Whitney U rank test is performed for comparison of alpha diversity metrics. Data are presented as mean The P value less than 0.05 is accepted

as significant (* P<0.01, ** P<0.05). **B.** Beta diversity was analyzed with Bray-Curtis distances (P=0.49). Blue color refers to the healthy controls and pregnant women with COVID-19 were presented in red colour.

Figure 3. The vaginal microbiome composition of healthy controls and women with COVID-19

Healthy control (n=28) and women with COVID-19 (n=19); A. The most abundant bacteria at the phylum level are represented for two groups. B.The most abundant bacteria at the genus level are represented for two groups. C. The relative abundance of different bacteria species in two groups. Only phyla and genera present at relative abundances >0.01% are reported. Remaining taxa are grouped as "Others".

Figure 4. The comparison of abundance of vaginal microbiota species among Healthy Controls, Asymptomatic/Mild and Moderate/Severe cases

* refers to statistical significance P<0.05; ** represents statistical significance P<0.01; P1 refers the comparison of bacteria species between the healthy controls with women with asymptomatic/mild disease; P2 refers the comparison of bacteria species between the healthy controls with women with moderate/severe disease. P3 refers to the comparison of bacteria species between women with asymptomatic/mild disease with those of moderate/severe disease.

Figure 5. The longitudinal analysis of vaginal microbiota

The abundance rate of Phyla in vaginal microbiota before COVID-19, during acute phase and after recovery in three patients (asymptomatic/mild [n=2] and moderate/severe [n=1])

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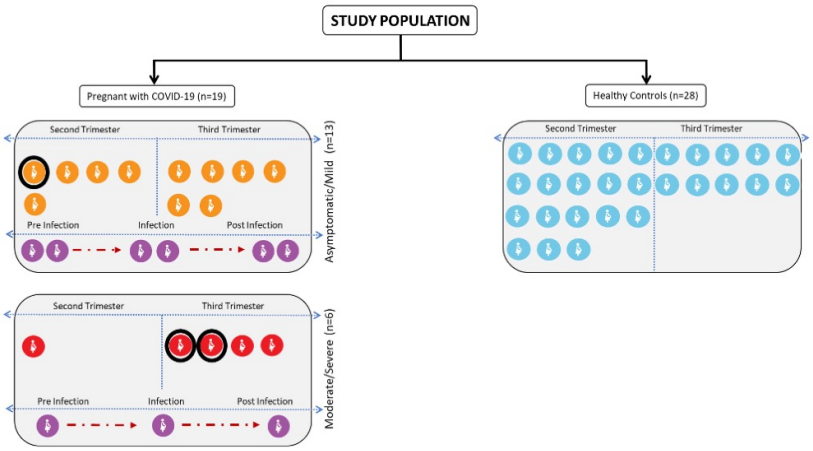

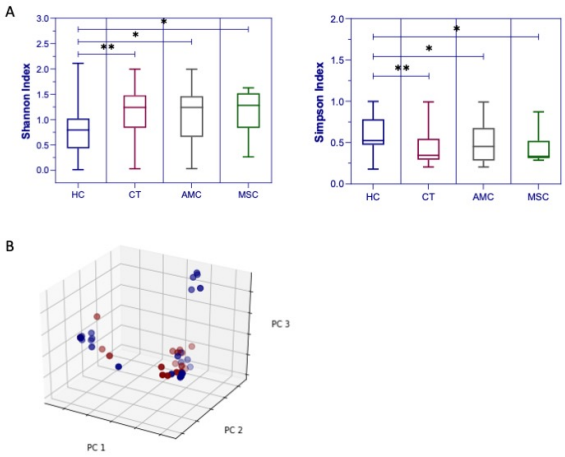

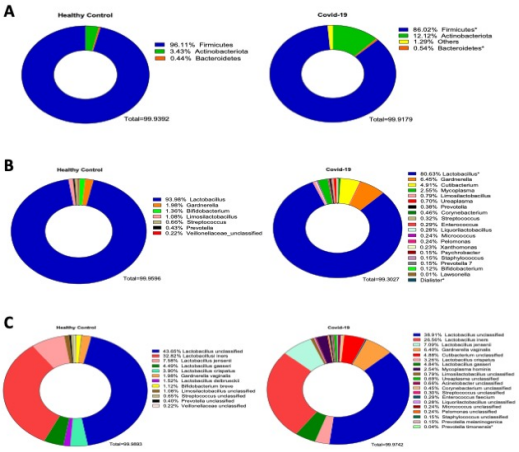

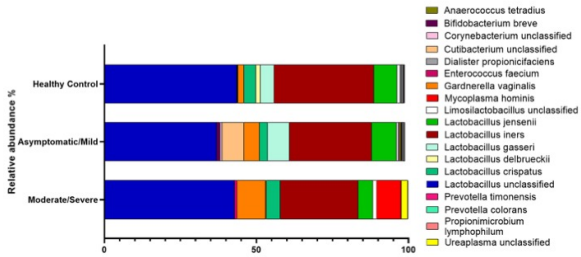

Bacteria Species	Healthy Control (n = 28)	Asymptomatic/Mild Cases (n=13)	Moderate/Severe Cases (n = 6)	P1	P2	P3
Lactobacillus gasseri	4.4709	7.1172	NA	0.381	NA	NA
Ureaplasma unclassified	0.0473	0.0455	2.0946	0.451	0.001**	0.005*
Prevotella colorans	0.0184	0.0039	NA	0.034*	NA	NA
Prevotella timonensis	NA	0.0501	0.0248	NA	NA	0.379
Propionimicrobium lymphophilum	NA	0.0022	NA	NA	NA	NA
Dialister propionicifaciens	NA	0.0314	NA	NA	NA	NA
Anaerococcus tetradius	NA	NA	0.0038	NA	NA	NA
Fusobacterium nucleatum	NA	NA	0.0344	NA	NA	NA
Mycoplasma hominis	NA	NA	8.0019	NA	NA	NA