Vaginal microbiota alterations in women with recurrent implantation failure and the associated metabolome

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Research

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Abstract

In vitro fertilization-embryo transfer (IVF-ET) is now widely applied in treating infertility. As the number of IVF cycles continues to increase, recurrent implantation failure (RIF) has become a big challenge. The cause of RIF is very complex and remains largely unrevealed, especially for those without any pathological features. It has been proved that vaginal microbiota is associated with many female reproductive diseases, such as pregnancy-related diseases, sexually transmitted diseases, tubal factor infertility, and first trimester miscarriage after in vitro fertilization (IVF) and so on. Hence, vaginal microbiota and its metabolome may also relate to RIF. In this study, we characterized the vaginal microbiota and metabolome of patients with unexplained RIF, while patients who achieved clinical pregnancy in the first IVF cycle were used as controls. Results Based on 16S rDNA sequencing of the vaginal microbiota, the RIF group presented higher microbial α-diversity than the control group (0.80±0.50 vs 0.50±0.39, P-value=0.016) and harbored more non-Lactobacillus microorganisms, including 25 significantly increased genera of both aerobic and anaerobic bacteria. The metabolomic profile showed that the relative abundances of 37 metabolites among 2,507 metabolites were significantly different between the two groups. Among them, 2',3-cyclic UMP and phosphoinositide were the top two metabolites significantly upregulated in the RIF group, while glycerophospholipids and benzopyran were important metabolites that were significantly downregulated. Lysobisphosphatidic acid (LPA) and prostaglandin (PG) metabolized from glycerophospholipids are key factors affecting implantation and decidualization. Benzopyran, as a selective estrogen receptor modulator (SERM), may affect the outcome of pregnancy. All of the metabolome outcomes may result in or from the differential microbiota composition in the RIF patients. Conclusions In conclusion, significant differences were presented in the vaginal microbiota and metabolome between RIF patients and women who became pregnant in the first IVF cycle, which are related to embryo implantation. This study not only deeply investigates the relationship between RIF and the vaginal microbial community and its metabolites but also provides a profound understanding of the pathogenesis of RIF.

Introduction

Infertility is defined as the inability to become pregnant within a couple who have normal intercourse without contraception for one year [1, 2]. The incidence of infertility is 8%-12% in reproductive-aged couples, which has become a definite global public health issue [3]. In vitro fertilization-embryo transfer (IVF-ET) is currently widely used in the treatment of infertility. In 1978, the first IVF baby was born in the UK. Over the intervening years, the success rate has steadily increased, with a cumulative live birth rate of over 52% after 3 cycles of treatment [4]. Nevertheless, IVF treatment failure still occurs frequently. Among all repeated IVF failure types, recurrent implantation failure (RIF) is a special subgroup. RIF refers to the women under the age of 40 years who received at least four good-quality embryos in a minimum of three fresh or frozen cycles that still failed to achieve a clinical pregnancy [5]. Failed IVF usually occurs in elderly patients and those with a low ovarian reserve or low ovarian response for whom there is a need to screen for high-quality embryos by repeated IVF [5]. As the number of IVF cycles continues to increase,

RIF has become a new big challenge, as its occurrence rate among infertility patients has continued to increase in recent years [6]. RIF also carries a heavy financial burden and causes a deep impact on the patient's body and mind.

The investigation and management of RIF usually focuses on the quality of the embryo and endometrial receptivity. However, the importance of maternal systematical diseases, such as thyroid, thrombophilia and immunological disorders, has also been recognized recently [7-9]. Unfortunately, many RIF patients still cannot become pregnant after the overwhelming diagnosis and treatment. Moreover, a recent paper reported that patients who were not of advanced age still have a very low live-birth rate (36.6%), even when euploid blastocyst transfer cycles are selected by comprehensive chromosome screening (CCS) and after confirming the window of implantation (WOI) by endometrial receptive array (ERA) [10]. Even worse, another group of unexplained RIF patients has no pathological features, whose treatment method is more intractable. Hence, the nosogenesis of RIF is very complex and requires a multidimensional explanation.

The human commensal microbiome, referred to as "the other human genome", coexisted and evolved with the human genome to help maintain human health. Microbial dysbiosis and invasion of pathogens can lead to disease and even threaten human life. It has been proven that metabolic syndrome, diabetes [11, 12], obesity [13, 14], alcoholic liver disease [15], cirrhosis [16], coronary heart disease [17] and some mental diseases [18, 19] are related to intestinal microbial metabolism(8-13). With the development of high-throughput sequencing, the microbiome has also been characterized in the female reproductive tract, which was traditionally considered sterile [20]. Ravel et al. divided the vaginal flora into five community state types (CSTs) by 16S rDNA sequencing in 2011, four of which were classified as *Lactobacillus*-dominated (LD) types [21]. *Lactobacillus* is one of the dominant genera in the vaginal microbiota of healthy females, and its key metabolites, lactate acid and hydrogen peroxide, can maintain the anaerobic and acidic vaginal environment and protect it from pathogen infection. A series of pregnancy-related diseases, such as premature rupture of membranes, premature delivery and chorioamnion infection [22-26]; diseases associated with infertility, such as diabetes, obesity, pelvic inflammatory disease [27] and sexually transmitted diseases [28]; and even cervical cancer [29] have been discovered to be associated with vaginal microbial disorders.

A previous systematic review reported that an abnormal vaginal microbiota is associated with tubal factor infertility and early spontaneous abortion in IVF patients. However, that article also pointed out that the quality of evidence was very low and needs further research [30]. In addition, since a microbiota continuum exists along the female reproductive tract and is associated with pregnancy-related diseases, sexually transmitted diseases, infertility-related metabolic diseases, gynecological tumors and so on [28, 31-33], we assume that the microbiota and its metabolites might associate with every step of IVF, including gamete formation, implantation and delivery [34, 35]. Moreover, the correlation between the vaginal microbiome and RIF remains totally unclear. To explore the association between vaginal microbial function and IVF/RIF, metabolomic analysis should be used as an advanced tool. Metabolomics is untargeted identification and quantification of all low molecular weight end-products of

cellular biological processes [36]. The levels of metabolites ultimately reflect the integrated response of a biological system and directly influence the host.

Here, we characterized the vaginal microbiota and metabolome of patients with unexplained RIF. Additionally, we used individuals who achieved a successful pregnancy in the first IVF cycle as controls to explore the relationship of the microbial community and metabolites with RIF.

Results

The diversity and composition of the vaginal microbiota

A total of 67 samples were analyzed by 16S rDNA sequencing to investigate the vaginal microbiota, including 27 samples from the RIF group and 40 from the control group. The clinical information of the subjects is shown in Table 1a, which indicates that there was no significant difference between the RIF group and the control group (*P*-value > 0.05). In total, 2,824,185 reads were obtained from these 67 samples, and on average 42,152±10,415 reads per sample and 424±7 bp per read were achieved. After clustering, the rarefaction curve of the OTU number was almost a straight horizontal line, which demonstrated that the samples were sequenced with enough depth in this study (Supplementary Figure 1).

A total of 804 OTUs were obtained in the two groups. The RIF group contained 730, and the control group contained 429, among which 355 were shared between the two groups. The number of OTUs in the RIF group was much larger than that in the control group, indicating that the microbial composition of the RIF group was richer. The α-diversity of the microbiota was calculated by the Shannon-Wiener index (0.80±0.50 for the RIF group and 0.50±0.39 for the control group, *P*-value<0.01), which demonstrated that the microbial diversity in the vaginal environment was significantly higher in the RIF patients than in the control individuals (Figure 1).

Taxonomic classification at the phylum level showed similar pattern in the two groups that both dominated by Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and Tenericutes (Mollicutes), while the relative abundances of Firmicutes and Bacteroidetes were significantly different (*P*-value 0.05) (Figure 2a). At the genus level, genera with the top 15 abundances are shown in Figure 2b. Among them, the abundances of *Lactobacillus, Gardnerella*, *Atopobium, Streptococcus* and *Prevotella* were higher than 1% in both the control and RIF groups, while those of *Bifidobacterium*, *Scardovia*, *Mycoplasma*, and *Escherichia* were above 1% only in the RIF group. There were 26 genera that were significantly different between the two groups, of which 5 were aerobes, 12 were anaerobes and 9 were unclassified (Supplementary Table 1). *Lactobacillus*, as a main dominant genus, was also dissected in this study, and 12 species were discovered in the samples. The results showed that *Lactobacillus crispatus* and *Lactobacillus iners* were the most abundant *Lactobacillus* species in the RIF and control groups, respectively. The abundance of *L. iners* was significantly lower in the RIF group than in the control group (*P*-value < 0.05) (Supplementary Table 2).

The distribution of the vaginal microbiota in all the samples

PCA was applied to illustrate the distribution of the microbial community in the samples (Figure 3a). The spots belonging to the RIF group were dispersed, and half of them were scattered in the opposite direction of *Lactobacillus*, which is widely regarded as the probiotic that dominates the healthy female reproductive tract. Additionally, based on LDA, *Lactobacillus* was significantly decreased in the RIF group and contributed mostly to group differentiation (Figure 3b). The dominant genus of both groups was *Lactobacillus*. The relative abundance of *Lactobacillus* was 85.766 ± 28.787% in the control group and significantly decreased (*P*-value=0.013) in the RIF group (61.833 ± 41.849%). Additionally, we defined the subjects for which *Lactobacillus* accounted for greater than 90% relative abundance as *Lactobacillus*-dominated (LD) samples and defined those for which *Lactobacillus* accounted for ≤ 90% as non-*Lactobacillus*-dominated (NLD) samples. The pregnancy rates of LD and NLD individuals were 72.723% and 34.723%, respectively, which presented a significant difference (*P*-value = 0.006).

Comparative metabolomics in the vaginal environment

Twenty-five samples were subjected to metabolic analysis, including 10 RIF group samples and 15 control samples. As well as the sequenced samples, the clinical information of the subjects showed no statistical differences between the two groups (*P*-value>0.05) (Table 1b). Both PCA results of the test and QC samples and correlation analyses of metabolites between the QC samples inferred the stability of the instrument and the good quality of the data (Supplementary Figure 2). In this study, a total of 11,380 peaks of cations were obtained after noise removal; 449 nonredundant metabolites were annotated according to the mass-to-charge ratio (MS1), while 1,541 nonredundant metabolites were detected based on a molecular weight database (MS2). For the anions, 6,658 peaks were obtained, including 146 and 786 nonredundant metabolites according to MS1 and MS2, respectively. After removing the duplicated metabolites, we annotated 573 and 1,934 metabolites based on MS1 and MS2, respectively. As a result, we obtained 2,507 metabolites.

We utilized OPLS-DA to observe metabolites that were differentially abundant between these two groups and classify them into group-related and group-independent metabolites. The results elaborated that the RIF group samples were clustered together and distinct from the grouped control samples (Figure 4a). Of 2,507 annotated metabolites, 37 metabolites were found to have significant differences between the two groups, with variable importance for the projection (VIP) values > 1 and *P*-values < 0.05 (Figure 4b, Supplementary Table 3). To quantify the up/down-regulation of differentially abundant metabolites, we calculated the fold-change in 37 differentially abundant metabolites. The results revealed that 16 metabolites were significantly upregulated in the RIF group, among which 2',3-cyclic UMP and phosphoinositide were the top two metabolites; 21 metabolites were significantly downregulated in the RIF group, and 5 substances were downregulated by 4-fold or more: benzopyran, fatty alcohol, pyrimidine nucleoside, glycerophospholipid and naphthopyran (Figure 4c). The network of the metabolites that significantly correlated with each other was also investigated under the proposing of Spearman's correlation coefficients (R ≥ 0.6 or R ≤ -0.6) and *P*-values (P□0.05).

Correlation between the microbiota and metabolome

In the RIF group, the correlation between the top 17 abundant genera and 37 differentially abundant metabolites was calculated (Figure 5). A significant positive correlation of benzopyran and glycerophospholipids with *Lactobacillus* (*P*-value<0.05, R=0.714) was discovered. Coincidently, the relative abundance of *Lactobacillus* was significantly reduced in the RIF group compared to that in the control group. This result indicated that benzopyran and glycerophospholipids might be functioned metabolites during IVF driven by *Lactobacillus* or related bacteria in the vaginal microbiota. Interestingly, *L. iners* was also positively correlated with benzopyran in the RIF group (R=0.738). For the control group, consistent changes in benzopyrans with the relative abundance of *L. iners* were identified (R=0.527).

Discussion

This study is an attempt to explain the influence of the vaginal microbiota and its metabolites on RIF patients while using the single-procedure successful IVF cases as controls. The vaginal microbiota of the RIF group presented a higher diversity and harbored more potential pathogens than that of the control group. Moreover, the abundance of *Lactobacillus* was also significantly decreased in the RIF group. The metabolite profile delineated that benzopyran and glycerophospholipids were the significantly differentially abundant metabolites between the two groups and may result in or from changes in the microbiota composition.

An increase in vaginal microbial diversity in RIF patients was observed in this study, which is consistent with the study of Richard et al. in 2012. That report was the first study to suggest that the species diversity index of the vaginal microbiota distinguished women who had live births from those who did not [35]. Another study also showed that the clinical pregnancy rate and live-birth rate were significantly reduced (*P*-value = 0.01) in a cohort with a vaginal Shannon-Wiener index higher than 0.93 [37]. To date, few vaginal microbiota studies have investigated in RIF patients, but association studies on an abnormal vaginal microbiota with IVF have been discovered. A review concluded that culture-dependent studies indicated that an abnormal vaginal microbiota was not linked to the outcome of IVF. However, this conclusion was overturned by high-throughput sequencing results, which elaborated that microbiota disorders negatively influenced the IVF outcome [38]. Another review demonstrated that the incidence of bacterial vaginosis (BV) was associated with early abortion of IVF pregnancies and infertility due to tubal factors and was not associated with pregnancy rate and live-birth rate [30]. However, the association between the vaginal microbiota and preterm birth has been well determined in obstetrics. Hyman et al. undertook a cohort study of 88 patients, with 46 patients with a high risk of preterm birth and 42 patients with a low risk of preterm birth, suggesting that vaginal microbiota diversity is significantly increased in preterm women compared with that in women who achieve full-term delivery [26]. Freitas et al. selected 46 preterm birth patients and 170 full-term pregnant women as research subjects and analyzed the vaginal microbiota at 11-16 weeks of gestation. They found that the vaginal bacterial diversity of premature birth patients increased significantly [39].

The abundances of 26 genera detected in the current study were significantly different between the two groups (P<0.05). In the RIF group, all of the genera were significantly increased, especially the aerobic bacteria (8.593% for the RIF group and 2.349% for the control group, *P*-value < 0.05), while only *Lactobacillus* abundance was significantly reduced. Among the 25 increased genera, many anaerobic bacteria, such as *Gardnerella*, *Prevotella* and *Atopobium*, are the main pathogens for BV, while the abundance of many aerobic bacteria, such as *Escherichia*, *Enterococcus*, *Streptococcus* and *Corynebacterium*, are closely related to the risk of aerobic vaginitis (AV) and urinary tract infection (UTI). A study of asymptomatic reproductive tract infections in couples undergoing IVF showed that 29.1% (83/285) of the males and 26.3% (75/285) of the females carried at least one potential pathogen for reproductive tract disease, and more positive cases were found in the IVF pregnancy failure group than in the IVF control group. Additionally, *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus agalactiae*, and *Gardnerella vaginalis* were significantly associated with decreased levels of *Lactobacillus* (P<0.01) [40]. Similarly, the abundance of *Lactobacillus* was negatively related to the abundance of potential BV/AV pathogens in this study, including *Gardnerella*, *Prevotella*, *Atopobium*, and *Streptococcus*, which indicated the protective function of *Lactobacillus*. Our results also indicated that *Lactobacillus* was losing its dominant position in the RIF group which may be associated with the occurrence of repeated pregnancy failures. What's more, the pregnancy rate of NLD subjects was significantly decreased compared with that of LD subjects. Although the relative abundance of *Lactobacillus* was significantly lower in the RIF group than in the control group, the LDA value was still too low to be identified as a biomarker to predict the occurrence of RIF. However, this conclusion may also be manipulated by the research sample size of the study.

Glycerophospholipids and benzopyrans were the key significantly downregulated metabolites in the RIF group discovered in this study. Glycerophospholipids are important structural and regulatory components of biofilms and serve as precursors for many active biomolecules, such as arachidonic acid (AA) and lysobisphosphatidic acid (LPA), which are catalyzed by phospholipase A_2 (PLA_2) [41, 42]. AA then produces prostaglandin (PG) under the action of cyclooxygenase 2 (COX-2). PG and LPA are the terminal products of glycerophospholipids that play key roles in embryo implantation. According to a previous study, LPA plays an essential role in maintaining the normal size and spacing of the embryo, which is positively correlated with embryo implantation in mice [43]. LPA_3, a G protein-coupled receptor that functions in the uterine epithelium, also regulates the activity of COX-2 and the level of PGs, which could directly affect implantation and decidualization [44, 45]. Mice lacking LPA_3 showed defects such as deferred on-time implantation, delayed fetal development, embryo crowding, and sharing of one placenta by several embryos. Additionally, deviation in the PG-producing pathway has a significant impact on the implantation process, resulting in a reduction in the likelihood of achieving pregnancy [46]. Besides, it has been reported that levels of endometrial LPA_3 were reduced in RIF patients [46] and that its activation induces decidualization [45]. More importantly, phospholipase could be stimulated by lipopolysaccharide, which is the key component of the outer membrane of gram-negative bacteria, such as *Lactobacillus*. In addition, glycerophospholipids were positively correlated with the abundance of *Lactobacillus*. Hence, a

balanced vaginal microbiota would result in the normal regulation of glycerophospholipids, which might lead to successful embryo implantation.

Sex hormones are potential metabolites functioning during IVF. In this study, androgens, 5α-androstane and other androgen derivatives were all upregulated in the RIF group, antagonizing estrogen secretion, inhibiting endometrial hyperplasia, depressing ovarian and pituitary function, and in turn causing a negative effect on embryo implantation. Benzopyrans, flavonoids and naphthopyrans, as selective estrogen receptor modulators (SERMs), were all downregulated in the RIF group. Furthermore, benzopyran, as a secondary metabolite in bacteria, could act against fungi and protozoa [47, 48], and its down regulation may be one of the reasons for the propagation of potential pathogens. In our study, benzopyran was positively correlated with *L. iners*, the abundance of which significantly decreased in the RIF group. Hence, the combined function of benzopyran and *L. iners* might contribute to successful embryo implantation.

Conclusion

In this study, the vaginal microbiota and metabolome of patients with unexplained RIF (RIF group) and women who became pregnant in the first IVF cycle (control group) were investigated. In general, significant differences were discovered in the vaginal microbiota and metabolome between the two groups, which are related to embryo implantation. The RIF group presented lower abundance of Lactobacillus and higher abundance of other aerobic or anaerobic microorganisms than the control group and showed a significantly higher α-diversity. The metabolome profile indicated that LPA and PG metabolized from glycerophospholipids, a metabolite significantly downregulated in the RIF group, are key factors affecting implantation and decidualization, while benzopyran, another metabolite significantly downregulated in the RIF group that is a SERM and pathogen inhibitor, may contribute to the outcome of pregnancy.

Methods

Criteria for recruitment of the research subjects and ethical approval

Patients who underwent frozen embryo transfer (FET) in the Department of Reproductive Medicine, Peking University Shenzhen Hospital from May 2018 to December 2018 were selected and divided into the unexplained RIF group (RIF group) and successful pregnancy in the first IVF cycle group (control group). All the women were less than 40 years of age, and their functional ovarian reserve was assessed with the levels of FSH < 12 mIU/ml and AMH > 1.1 ng/ml. The inclusion criteria for the RIF group were patients who 1) received at least four good-quality embryos in a minimum of three fresh or frozen cycles but had still not become pregnant and 2) were not pregnant in this FET cycle. The patients who 1) had never been conducted a fresh or frozen cycle before or 2) were pregnant in the first FET cycle were counted in the control group.

Patients with simple male factor infertility, ovulation and menstrual disorders, genital tract organic lesions and systemic diseases were excluded. All patients were confirmed to have a normal uterine cavity by hysteroscopy and a normal chromosome karyotype by chromosome G-banding and karyotype analysis. The patients enrolled were all examined for cervical mucus within 3 months before surgery to exclude *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *Neisseria gonorrhoeae* infection and for vaginal secretions to exclude bacterial vaginosis, vulvovaginal candidiasis, trichomonas vaginitis and other vaginal inflammation symptoms, such as vaginal itching and abnormal discharge. All of the above possible infections were reviewed 7 days before surgery. The recruited patients also did not receive any antibiotics (oral or topical), vaginal douching or sexual behavior within 2 weeks before sample collection.

Sample processing

Vaginal specimens were collected on the day of embryo transfer before the operation. A sterile swab with a separated package was immersed into the vagina and maneuvered in a circle in the upper third of the vagina. Care was taken to avoid possible contamination. Three parallel samples were taken from each patient, immediately placed in an ice box and then transferred to a -80 °C freezer within 20 min for subsequent genital flora testing. After the samples were batched, they were sent to Shenzhen We-Health Gene Company for follow-up sequencing and metabolic analysis.

Total DNA extraction and 16S rRNA sequencing

DNA extraction was conducted with a DNeasy PowerSoil kit (Qiagen, Hilden, Germany), followed by DNA purification with VAHTS DNA Clean Beads (Vazyme, Nanjing, China) according to the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the universal primers: 338F□5'-ACTCCTACGGGAGGCAGCAG-3'□ and 806R □5'-GGACTACHVGGGTWTCTAAT -3'□ using a TransStart® FastPfu DNA Polymerase Kit on an ABI GeneAmp® 9700 PCR instrument. The PCR system was 50 μl in total, which contained 4 μl of 5×FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of forward primer, 0.8 μl of reverse primer, 0.4 μl of FastPfu Polymerase, 0.2 μl of BSA, 10 ng of template DNA and ddH_2O to make up the total volume. PCR was performed using the following conditions: 3 min of denaturation at 95 °C; 29 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, and elongation at 72 °C for 45 s; and a final extension at 72 °C for 10 min. The 16S rDNA amplicons were purified and used for MiSeq sequencing library construction following the manufacturer's instructions for the MiSeq Reagent Kit v2.

Microbiota analysis

Clean paired-end sequences were filtered by eliminating low-quality base pairs, contaminated reads, N-containing reads and low-complexity sequences. The operational taxonomic units (OTUs) were clustered under the 97% cutoff and annotated by the Ribosomal Database Project after merging reads via overlaps. The α-diversity inferred by the Shannon-Wiener index was calculated by the R package Vegan, while principal component analysis (PCA) was performed by the ade4 package. Linear discriminant analysis (LDA) was performed using Galaxy software.

Metabolome analysis

Metabolites were extracted from vaginal swabs with 1,000 μL of isolation liquid (methanol:acetonitrile:water = 2:2:1). Then, 10 μL of isolations from all samples were mixed as quality control (QC) samples for stability evaluation during the experiment [49, 50]. Using liquid chromatography (LC) and mass spectrometry (MS, Q-Exactive Orbitrap, Thermo-Fisher Scientific, USA), the signals of metabolites in all samples were examined. Using ProteoWizard (v3.0.9134) and the XCMS package in R (v3.2), the MS raw data were converted to a data matrix that contained the retention time (RT), mass-to-charge ratio (m/z) value, and peak intensity. According to the peak intensity of metabolites, their variable importance in the projection (VIP) was calculated by orthogonal projections to latent structures - discriminant analysis (OPLS-DA). Student's t-test was applied to detect differentially enriched metabolites between the control and RIF groups (VIP>1, *P*-value<0.05). Correlations among the metabolites were evaluated by Spearman correlation analysis, and the network was visualized by Cytoscape software (v3.4.0). In addition, Spearman correlation analysis was also applied to investigate the relationship between the vaginal microbiota and metabolites.

List Of Abbreviations

IVF-ET: in vitro fertilization-embryo transfer; RIF: recurrent implantation failure; LPA: Lysobisphosphatidic acid; PG: prostaglandin; SERM: selective estrogen receptor modulator; CCS: comprehensive chromosome screening; WOI: window of implantation; ERA: endometrial receptive array; CSTs: community state types; LD: *Lactobacillus*-dominated; NLD: non-*Lactobacillus*-dominated; FET: frozen embryo transfer; PCR: polymerase chain reaction; OTUs: operational taxonomic units; PCA: principal component analysis; LDA: linear discriminant analysis; QC: quality control; LC: liquid chromatography; MS: mass spectrometry; RT: retention time; m/z: mass-to-charge ratio; VIP: variable importance in the projection; OPLS-DA: orthogonal projections to latent structures - discriminant analysis; BV: bacterial vaginosis; AV: aerobic vaginitis; UTI: urinary tract infection; AA: arachidonic acid; LPA: lysobisphosphatidic acid; PLA_2: phospholipase A_2; COX-2: cyclooxygenase 2.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Peking University Shenzhen Hospital at May 10, 2018. The study is sponsored by the Peking University Shenzhen Hospital [2018]No.017 and was conducted as a single-center study without any investigational product. All the enrolled subjects provided written consent and gave permission to access medical records to obtain their related clinal information and vaginal specimens.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author (Shangrong Fan, fanshangrong@163.com) on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

This paper has 6 authors in total. SF is the correspondence author. MF and XZ are the co-first -authors who contribute equally to the study. SF and MF contributed to the conception and design of the study. MF and WQ contributed to the achieving of ethical approval. MF, WQ and YL conducted the collection of samples. MF, SF and XZ analyzed the data. XZ and MF drafted and SF revised the manuscript. All authors read and approved the final manuscript.

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Table

Table 1a. Clinical characteristics of the participants in the two groups whose samples were submitted for 16S rDNA sequencing of the vaginal microbiota.

Clinical characteristics	RIF group（n=27）	Control group（n=40）	*P* value
age（years）	33.4±3.7	32.0±4.0	0.213
BMI（kg/m^2）	20.9±3.4	22.3±7.6	0.102
AMH（ng/ml）	3.0±1.9	4.4±3.8	0.101
Duration of infertility (years)	4.5±3.0	3.7±2. 7	0.068
Endometrial thickness (mm)	11.5±1.8	12.1±2.2	0.162
Number of oocytes	12.7±5.9	12.2±5.5	0.734
Number of embryos	9.7±5.0	9.7±4.4	0.847
Number of good embryos	2.9±2.1	2.8±2.5	0.568
Good quality embryo rate (%)	48.6±24.2	35.7±31.6	0.045
No. of embryos transferred	1. 9±0.4	2.19±0.4	0.044
No. of qualified embryos transferred	1.3±0.5	1.39±0.9	0.576

Table 1b. Clinical characteristics of the participants in the two groups that were analyzed by metabolic analysis.

Clinical characteristics	RIF group（n=10）	Control group（n=40）	*P* value
Age（years）	33.7±3.8	31.8±3.8	0.189
BMI（kg/m^2）	21.6±3.5	20.9±4.2	0.977
AMH（ng/ml）	3.8±2.3	5.8±4.7	0.338
Duration of infertility (years)	5.3±3.4	3.9±1.4	0.480
Endometrial thickness (mm)	10.2±3.6	11.2±2.1	0.382
Number of oocytes	13.5±7.2	13.3±6.7	0.955
Number of embryos	10.5±7.2	10.5±5.5	0.737
Number of good embryos	2.2±2.9	2.6±2.3	0.498
Good quality embryo rate (%)	42.0±60.5	30.6±28.8	0.933
No. of embryos transferred	2.0±0.5	2.1±0.5	0.658
No. of good embryos transferred	1.3±0.5	1.3±0.9	0.628

Supplementary File Legends

Supplementary Figure 1. Rarefaction curve of the sequenced samples.

Supplementary Figure 2. Quality control results of the metabolomics analysis, including the PCA results of test and QC samples (a) and correlation analysis of metabolites between the QC samples (b).

Supplementary Table 1. Genera in the vaginal microbiota that were significantly differentially abundant between the RIF and control groups.

Supplementary Table 2. Relative abundance of *Lactobacillus* species discovered in the samples that were significantly different between the RIF and control groups.

Supplementary Table 3. Quantities of 37 metabolites that were found to have significant differences between the two groups via the determination of variable importance for the projection (VIP) values and *P*-values.

Figures

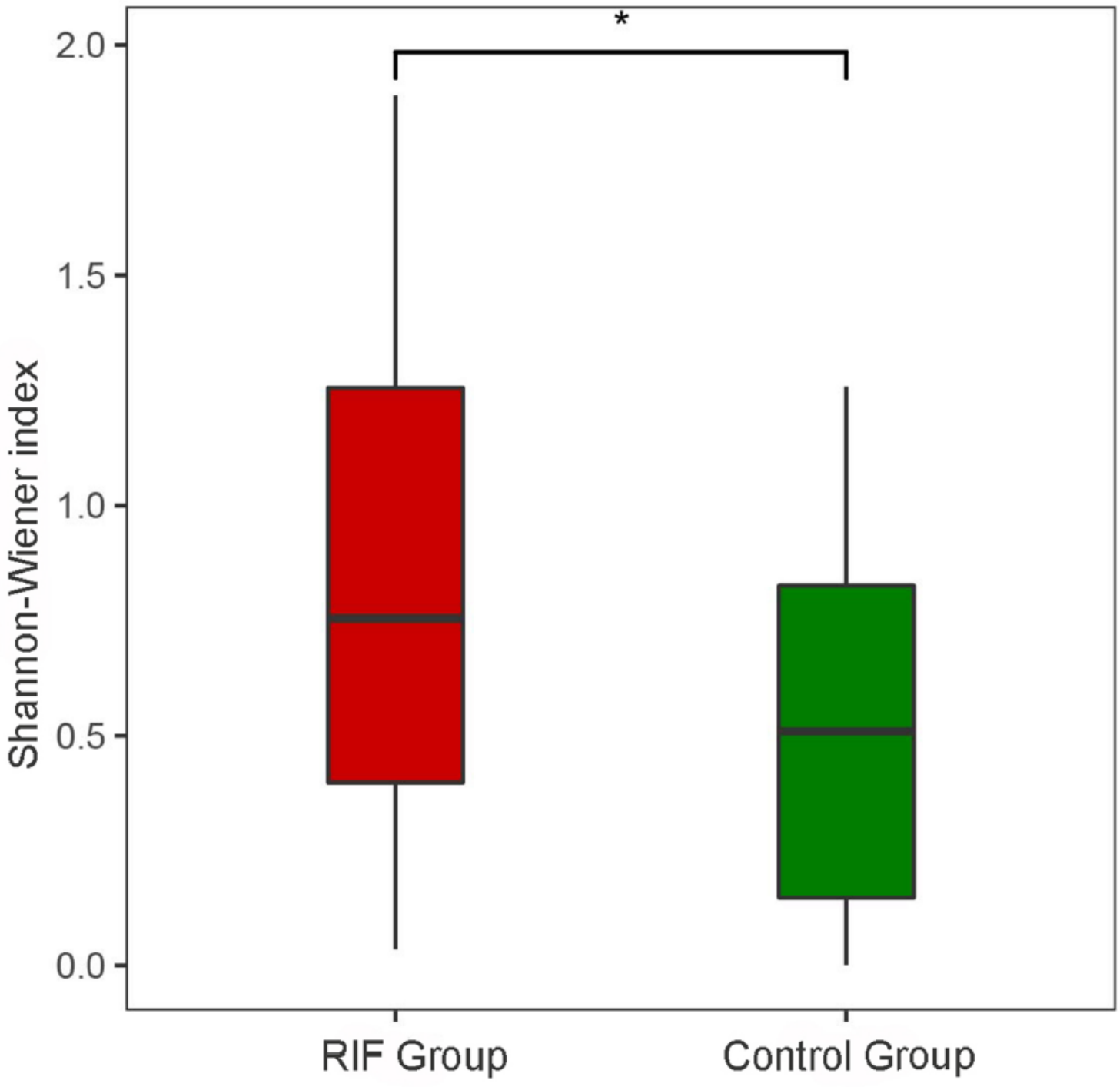

Figure 1

The α-diversity of the vaginal microbiota in the two groups was calculated and is shown by the Shannon-Wiener index.

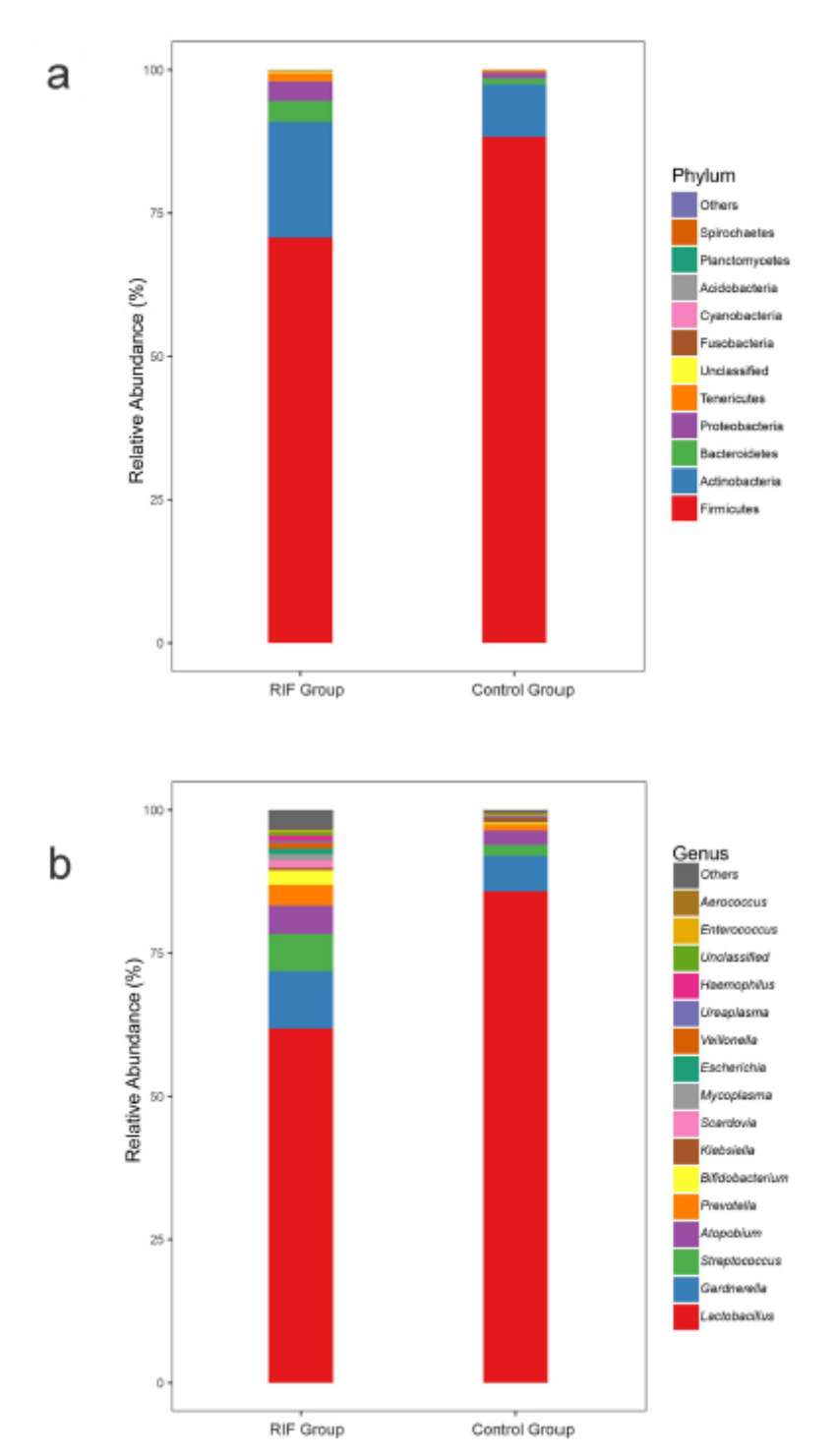

Figure 2

Taxonomic classification of the vaginal microbiota at the phylum level (a) and genus level (b) from the RIF and control groups.

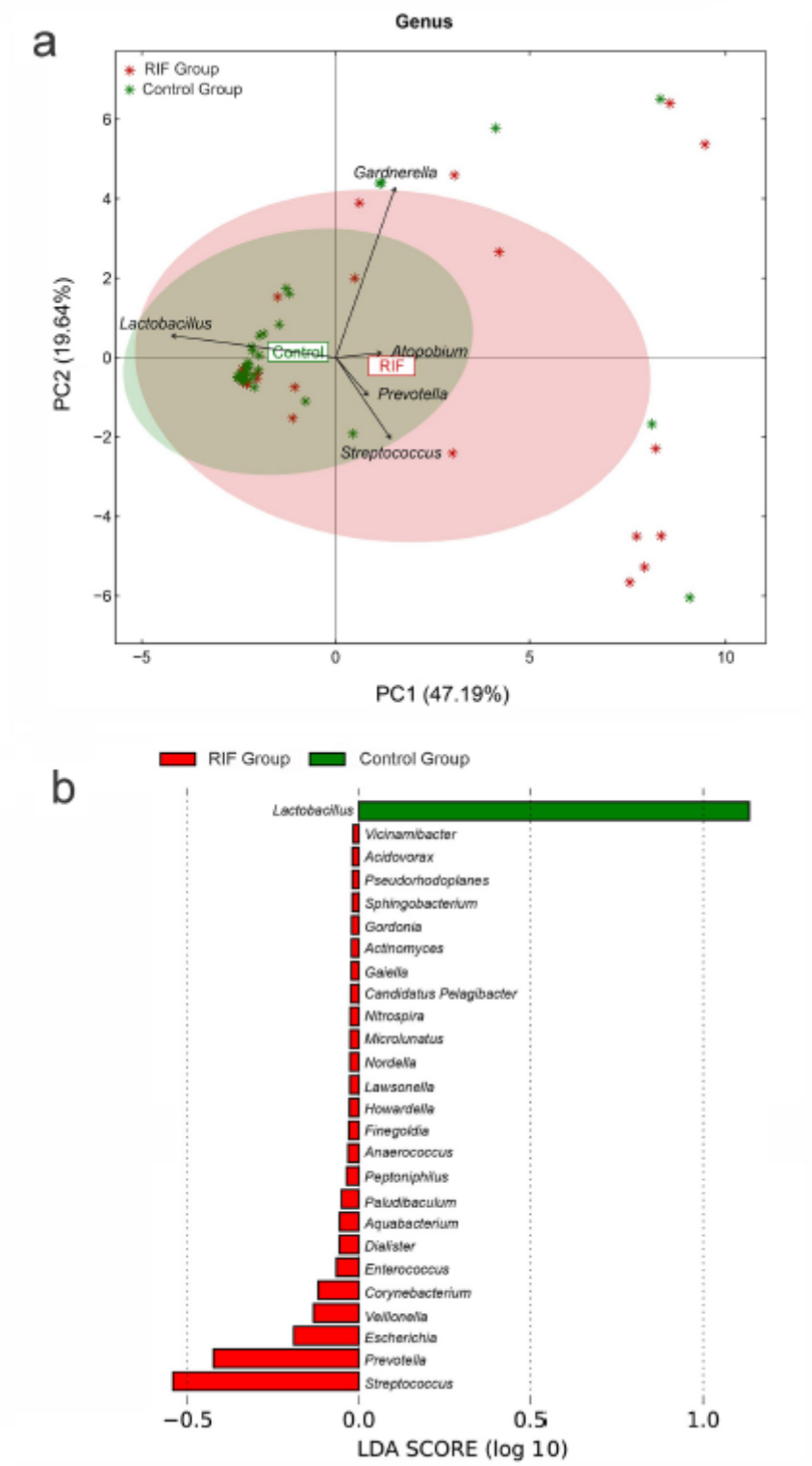

Figure 3

(a) Principal Component Analysis was applied to demonstrate the distribution of the vaginal microbial community in the samples. The red and green stars represent the samples from the RIF and control groups, respectively. The arrows indicate the different genera, and their contributions to the explanation of the sample difference are shown by the arrow length. The angle between the arrows represents the positive correlation (< 90°) or negative correlation (> 90°) among the genera. (b) Linear discriminant

analysis of the differentially abundant genera, which indicated their contribution to group differentiation. The green bar indicates that the genus (Lactobacillus) was more abundant in the control group, while red bars indicate that those genera were more abundant in the RIF group.

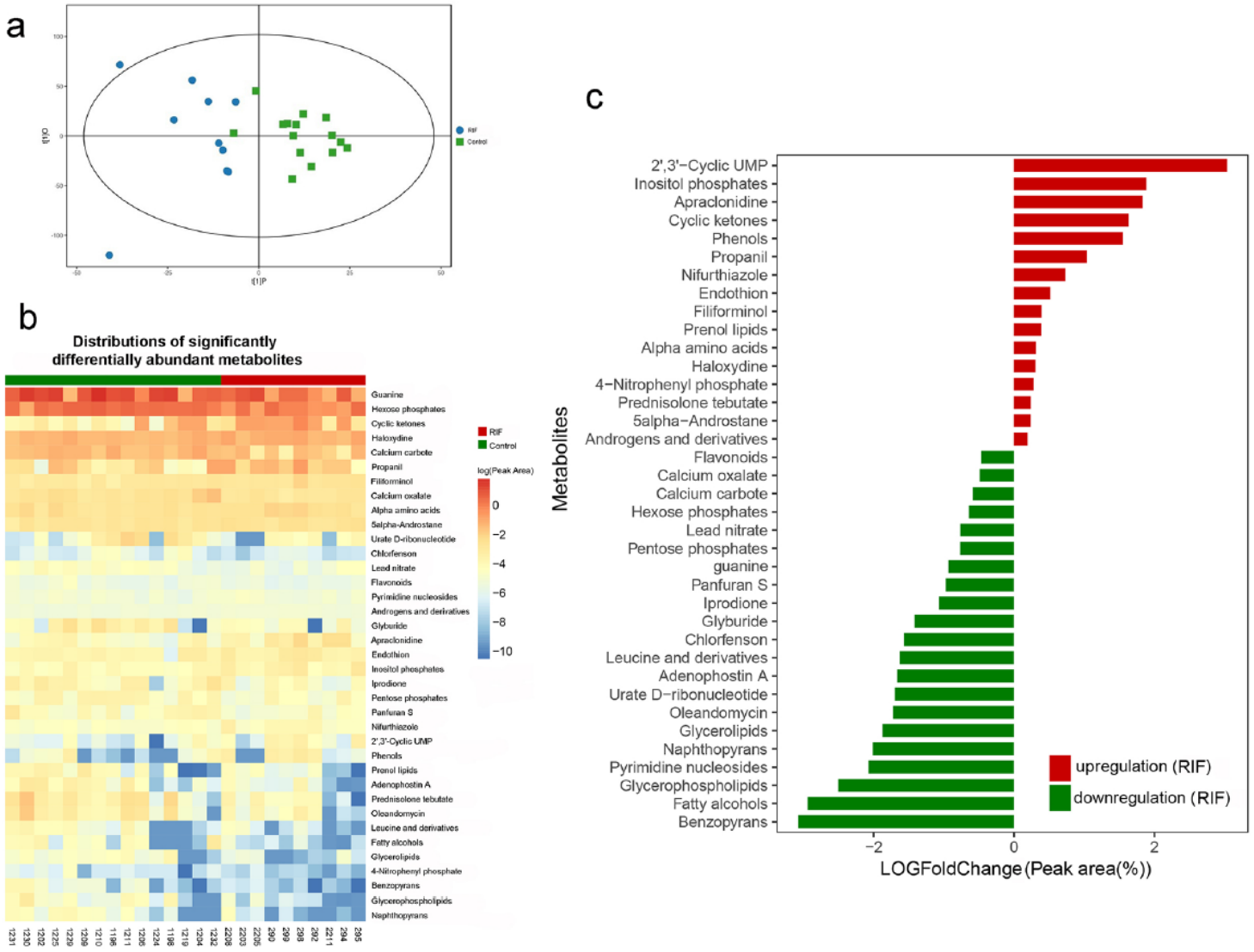

Figure 4

(a) Result of Orthogonal Projections to Latent Structures - Discriminant Analysis of the samples. The red and green stars represent the samples from the RIF and control groups, respectively. The X axis shows the predicted principal component score, which indicates the intergroup difference. The Y axis represents the orthogonal principal component score, which indicates the intragroup difference. (b) Heatmap of the differentially abundant metabolites. (c) Quantitative fold-change in the differentially abundant metabolites. Red bars represent the upregulated metabolites in the RIF group, while green bars represent downregulated metabolites in the RIF group.

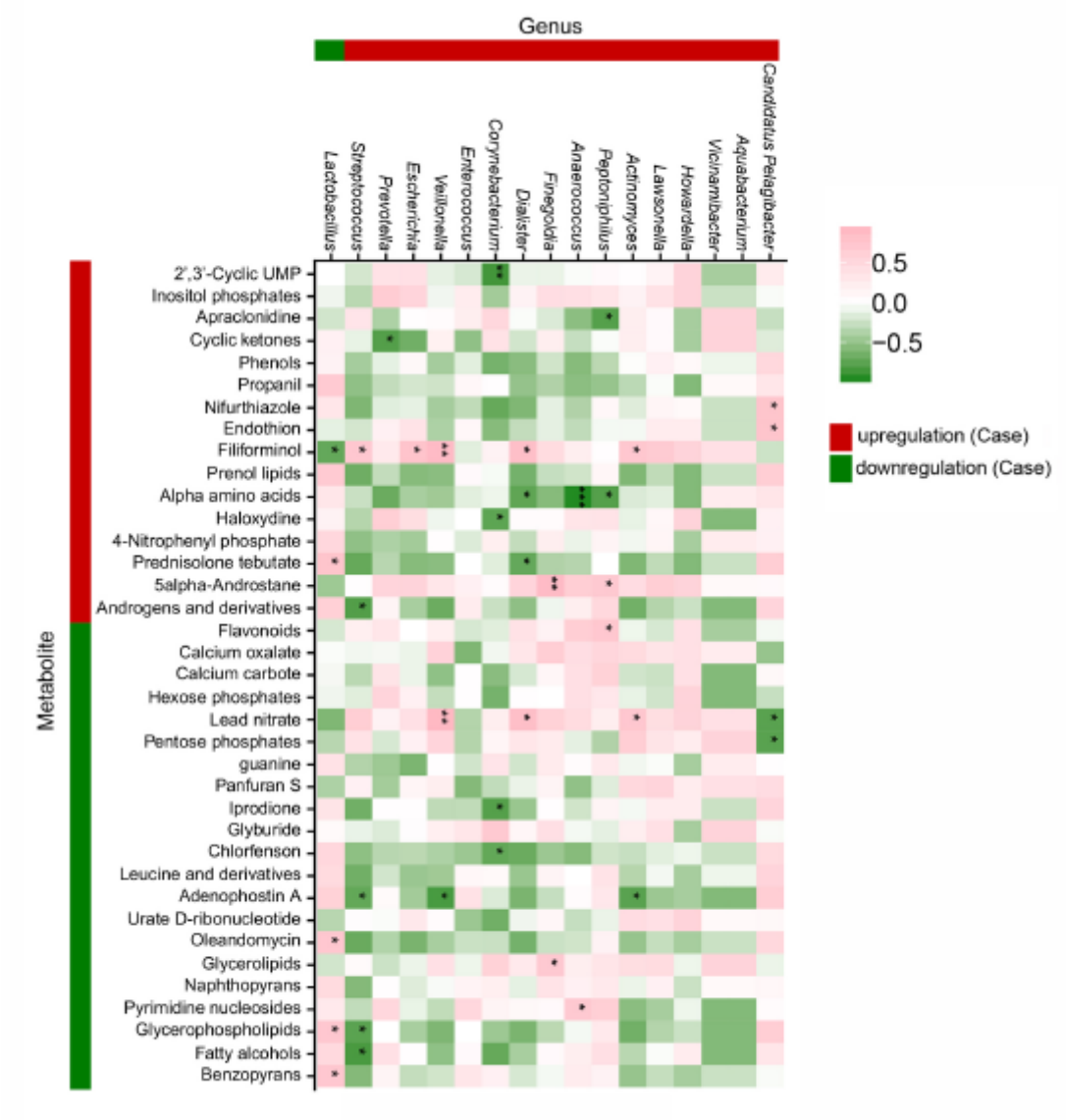

Figure 5

Correlation analysis between the differentially abundant metabolites and differential genera in RIF group. Red color on the bar outside the heatmap represent the metabolite/genus was up-regulated in the RIF group, while green strand for those were down-regulated.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pdf
- SupplementaryFigure2.pdf
- SupplementaryTable2.docx
- SupplementaryTable3.docx
- SupplementaryTable1.docx