

Gut Dysbacteriosis Is Associated with an Imbalanced Cytokines Network in Women with Unexplained Miscarriage

Yongjie Liu

Xinhua hospital affiliated to Shanghai Jiao Tong university school of medicine

Hainan Chen

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Dan Chen

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Liping Feng (✉ liping.feng@duke.edu)

Duke University <https://orcid.org/0000-0002-2936-7397>

Jun Zhang

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Research

Keywords: Gut microbial dysbiosis; Metabolite profiles; Imbalanced cytokines;

Posted Date: May 20th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-20415/v2>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: A dysregulation of cytokine networks has been suggested to be involved in the pathogenesis of unexplained pregnancy loss. Gut microbiota affects host immune response and induces an imbalance in cytokine levels. However, how gut microbial dysbiosis affects the disturbance of cellular immune function in spontaneous abortion (SA) patients remains inconclusive.

Results: Serum proinflammatory cytokine levels were significantly increased in SA patients. Microbial diversity in patients with SA was reduced compared with that in the controls, as were the relative abundances of *Prevotella_1*, *Prevotellaceae_UCG_003*, and *Selenomonas_1* were significantly reduced in the cases. Fecal metabolites profiles were altered in SA patients, and the correlation analyses indicated that some microbe-associated metabolites, which were significantly enriched in bile secretion and histidine metabolism pathways, were positively associated with changes in levels of IL-17A, IL-17F, TNF- α , and IFN- γ in the SA group. Moreover, the microbial-associated metabolites, imidazolepropionic acid and 1, 4-Methylimidazoleacetic acid, are associated with recurrent pregnancy loss.

Conclusions: Our study highlights the network among gut microbiota, fecal metabolites and Th1/Th17-mediated immune response in miscarriage patients and identifies potential novel biomarkers and an intervention target for recurrent miscarriage.

Background

Spontaneous abortion (SA) is the most common complication during pregnancy and affects approximately 15% of clinically recognized pregnancies [1]. After ruling out chromosomal, endocrine, infectious, anatomic, and autoimmune factors, more than 50% of SA cases remain unexplained [2]. In recent years, immune cell dysfunction has been shown to be a risk factor for the pathogenesis of unexplained pregnancy loss [3]. This dysfunction may involve imbalances in cytokines, growth factors and immunosuppressive factors at the maternal-fetal interface [4]. The role of cytokines in pregnancy loss is an emerging research field, which contributes to the understanding of many SA without obvious etiologies.

Among the leukocytes that populate the maternal-fetal interface, CD4⁺ helper T cells (Th cells) can be activated and then differentiate into Th1, Th2 and Th17 cells and produce corresponding types of cytokines. It has been hypothesized that during pregnancy there is a subtle immunological shift to the Th2-type cytokine responses that would suppress the potential harmful effects of the cell-mediated (Th1-type) immune system [5]. Thus, pregnancy has been labeled a 'Th2 phenomenon'. Blois et al. showed that Th1 cytokines led to pregnancy loss in mice [6], and Chaouat et al. demonstrated that this loss can be prevented by Th2 cytokines [7]. Inflammatory processes alter the balance of Th1 and Th2 cytokines and lead to a shift toward Th1 predominance. This abnormal shift of Th1/Th2 cytokines during early pregnancy initiates and intensifies the cascade of proinflammatory cytokine production involved in SA. However, this concept cannot be applied to the whole steps of pregnancy, such as implantation and the

preparation to parturition. In addition to the classical Th1 and Th2 cells, several novel effector T cell subsets have been recently identified, including Th17 cells. Wang et al. found an accumulation of Th17 cells in the peripheral blood of women with unexplained recurrent pregnancy loss (RPL) as compared to normal pregnant women [8]. These Th17 cells, which secrete interleukin-17A (IL-17A), IL-17F, IL-22, and IL-26, are thought to play a role in autoimmune diseases, allograft rejection, and inflammatory immune responses. It is now evident that Th17, Th1 and Th2 immunity, and regulatory T cell (Tregs) mediated immune regulation are essential for successful embryo implantation and establishment of pregnancy [9, 10].

The gut microbiota plays a crucial role in shaping and modulating the immune system and immune responses. Disruption of the commensal microbiota may alter the gut homeostatic balance and lead to gut microbial dysbiosis, which has been increasingly recognized as one of the risk factors in the development of inflammation, as well as autoimmune and immune-mediated diseases [11-13]. Gut microbial dysbiosis is linked to aberrant immune responses, in large part by producing small molecules that are often accompanied by abnormal production of inflammatory cytokines, such as IL-1 β , IL-6, IL-17, IL-23, and interferon- γ (IFN- γ) [14, 15]. The small molecules including their metabolites and components are not only necessary for immune homeostasis, but also influence the susceptibility of the host to many immune-mediated diseases and disorders. Short-chain fatty acids (SCFAs), the bacterially-derived molecules, have been reported to participate in the modulation of cytokines production and Tregs expansion [16, 17]. Moreover, microbial metabolites can penetrate the epithelial barrier, allowing them to enter and accumulate in the host circulatory system where they are sensed by immune cells [18]. In pregnancy, many of the immunological and metabolic changes that occur at the placental interface serve to inhibit rejection of the fetus. Koren et al. have reported that the gut microbial community composition and structure are profoundly altered in the third trimester (T3), and the transfer of T3 microbiota induces inflammation in germ-free recipient mice to a great extent than that in first trimester (T1) microbiota [19]. However, the contribution of gut host-microbial interactions in promoting inflammatory cytokines production and other immune changes at early stages of pregnancy remains to be evaluated. Further, the underlying mechanisms of the correlation between gut microbial dysbiosis and the disturbance of cellular immune function in SA patients remain inconclusive.

Given the effect of gut microbiota modulation on immune responses, we hypothesized that the imbalanced gut microbiota and their metabolites can be linked to the immune dysfunction in pregnancy loss. In the current study, we aimed to examine the network between gut microbial community composition, microbial metabolites, and proinflammatory cytokine responses by performing multi-omics analyses of subjects that had unexplained pregnancy loss versus controls that underwent elected abortion.

Results

Characteristics of study participants

Participants who indicated a reproductive tract infection and recent antibiotic treatment on the questionnaire were removed from the microbiota study. In total, 41 SA patients and 19 controls were included for 16S rRNA gene sequencing. The general characteristics of the study participants, including age, pre-pregnancy body mass index (BMI), education level, smoking status, alcohol-drinking status, reproductive tract infection, antibodies screen and medical history are presented in Table 1. No observable differences in these characteristics were found between the SA and control groups.

Decreased bacterial diversity in fecal microbiota associated with SA patients

Alpha diversity (α -diversity) of the samples, which reflects the local scale of the microbial species, was measured by Chao1 estimators and the Shannon index. Chao 1 is an index of species richness, unrelated to abundance and evenness [20]. The Shannon index is related to not only species richness but also species evenness. Both Chao1 estimators and Shannon index were significantly decreased in the SA group relative to the control group ($p < 0.001$ and $p < 0.01$, respectively), indicating a lower richness and evenness of gut bacteria in SA patients (Fig. 1A). We then analyzed the beta diversity of the two groups. Both the unweighted and weighted Principal coordinate analysis (PCoA) plots revealed that the gut microbiota in subjects with SA clustered significantly compared to that of controls (Fig. 1B and C). These results indicate that the diversity of gut microbiota is significantly lower with different microbiota profile, in SA patients compared with controls.

Alterations in the composition of fecal microbiota associated with SA

The filtered data set contained 60 samples (41 cases and 19 controls). The 16S rRNA gene targeted sequencing yielded between 14982 and 36470 valid tags with average lengths ranging from 423.18 to 433.71 bp. Clustering of these tags produced between 92 and 1083 operational taxonomic units (OTUs)/sample. The identified OTUs belong to 16 phyla, 30 classes, 55 orders, 96 families, and 268 genera. The relative abundance of 1029 OTUs, 7 phyla, 9 classes, 14 orders, 18 families, and 57 genera were significantly changed in the SA group compared to the control group. *Bacteroidetes* was the most predominant phylum, accounting for 53.3% and 51.9% of the OTUs in the SA and control groups, respectively. In addition, *Firmicutes* was enriched in the SA group compared to the control group, whereas *Proteobacteria* was enriched in the control group (Fig. 2A). Given that an upregulated *Firmicutes/Bacteroidetes* ratio has been suggested as an indicator of several pathological conditions [21], the ratio was of 0.65 in controls and 0.80 in cases ($p = 0.039$), indicating a pathological change occurred in SA patients (Fig. 2B).

We further compared the differences in fecal microbiota between the two groups. At the phylum level, *Spirochaetae* ($p < 0.001$), *Fibrobacteres* ($p < 0.001$), and *Tenericutes* ($p < 0.001$) were significantly more abundant in the control group than in the SA group (Fig. 2C). Fifty-seven genera of bacteria changed in abundance in SA patients (Supplementary Table S1). Specifically, the relative abundance of 55 genera of bacteria, including *Prevotella_1*, *Prevotellaceae_UCG_003*, *Roseburia*, and *Selenomonas_1*, were significantly reduced in the SA group, and *Helicobacter* and *Lachnospiraceae_UCG_001* were markedly increased (Fig. 2D). Considering that this discriminant analysis did not distinguish the predominant

taxon, Linear discriminant analysis coupled with effect size measurements (LEfSe) was used to generate a cladogram to identify the specific bacteria associated with SA (Fig. 3A). It was shown that several opportunistic pathogens including *Prevotellaceae_NK3B31_group*, *Bacteroidales_S24_7_group*, and *Eubacterium ruminantium_group* were all significantly overrepresented (all LDA scores (log10) > 3.0) in the feces of SA patients, whereas *Prevotellaceae*, *Prevotella_1*, and *Gammaproteobacteria* were the most abundant microbiota in the control group (LDA scores (log10) > 4.0) (Fig. 3B). These results indicate that the alterations in the composition of fecal microbiota were associated with SA.

Fecal metabolites profiles were altered in SA patients

The fecal metabolome is a functional readout of the gut microbiome. Fecal metabolic profiling is a novel tool for exploring links between microbiota composition and host phenotypes [22]. Liquid chromatography/mass spectrometry (LC/MS) analysis was used to obtain the fecal metabolic profiles of the 20 subjects. The quality control (QC) samples in the Principle component analysis (PCA) score plot overlapped, which indicates that samples behaved stably for the duration of the run. Using PCA and (orthogonal) partial least-squares-discriminant analysis (OPLS-DA), we found that the SA group was completely separated from the control group (R²Y (cum) = 0.996, Q² (cum) = 0.428), demonstrating that metabolic disturbances exist in these two groups. Significant shifts in the compositions of fecal metabolites were observed in the SA and control groups (Fig. 4A). The permutation test indicated that the analytical platform exhibited excellent stability and repeatability (R² = 0.945, Q² = -0.136), and can be utilized in subsequent metabolomics research (Fig. 4B). In total, 23706 metabolites were detected in these samples. Based on the differential screening strategy, 239 discriminating metabolites were found in the SA group compared with the control group (Fig. 4C and Supplementary Table S2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses indicated that these differentially present metabolites were related to 1) bile secretion (5alpha-androstane-3alpha-ol-17-one sulfate, Deoxycholic acid 3-glucuronide, TXB2, L-Carnitine, and acetylcholine); 2) histidine metabolism (1,4-Methylimidazoleacetic acid, ergothioneine, and imidazolepropionic acid); 3) glycerophospholipid metabolism (acetylcholine, lysoPC(22:1(13Z)), and sn-3-O-(geranylgeranyl)glycerol 1-phosphate); 4) arachidonic acid metabolism pathways (TXB2, 15-Deoxy-d-12,14-PGJ2, and 12(S)-HETE), and 5) steroid hormone biosynthesis (5alpha-androstane-3alpha-ol-17-one sulfate, cortisone, and 7a-Hydroxydehydroepiandrosterone) (Fig. 4D, $p < 0.05$).

Clustering and multivariate analyses reveal distinct metabolites in the SA and control groups

The hierarchical clustering analysis (HCA) of the metabolites that differed between the SA and control groups revealed four large clusters: (i) glycerophospholipids and prenol lipids, which showed higher abundances in the control group than in the SA group, (ii) steroids and steroid derivatives, (iii) amino acids and derivatives, and (iv) alkaloids, drugs and other metabolites, which showed higher abundances in the SA group than in the control group (Fig. 5A). Variable importance in the projection (VIP) values, which were obtained by OPLS-DA analysis, indicate the importance of metabolites for interpreting the differences. The presence of several metabolites, such as hyocholic acid, methyl dihydrophosphate,

cholanoic acid, 3-keto petromyzonol, hydoxycholic acid, oic acid, oxocholanoic acid, THA, isolithocholic acid, and chenodeoxycholic acid sulfate were able to differentiate SA patients from control subjects (Fig. 5B). Specifically, the abundances of hyocholic acid, methyl dihydrophosphate, cholanoic acid, oic acid, oxocholanoic acid, and chenodeoxycholic acid sulfate were significantly higher in SA patients than in controls (Fig. 5C). Taken together, our data clearly demonstrated that SA patients with a unique fecal metabolome, suggesting that there are gut microbiota profiles and metabolites that are associated with SA.

Correlation analysis of fecal microbiota, proinflammatory cytokines, and metabolites

Multiplex analysis revealed markedly increased serum levels of IL-2, IL-17A, IL-17F, tumour necrosis factor- α (TNF- α), and IFN- γ in SA patients as compared to control patients (Fig. 6A). Pearson analysis indicates that the Chao1 index was negatively associated with the changes in IL-17A, and IFN- γ , and the Shannon index was negatively associated with the changes in IL-17A (Fig. 6B). Moreover, the *Bacteroides* abundances were positively associated with the changes in IL-2; the *Helicobacter* abundances were positively associated with the changes in IFN- γ ; the *Prevotella_1* and *Prevotellaceae_UCG_003* abundances were negatively associated with the changes in IL-17A and IFN- γ , and *Selenomonas_1* was negatively associated with the changes in IL-17A, TNF- α , and IFN- γ (Fig. 6C). As shown in the association network (Fig. 6D, and Table 2), the *Bacteroides* abundances were positively associated with the changes in THA, lucidenic acid J, and ergothioneine; both the *Prevotella_1* and *Prevotellaceae_UCG_003* abundances were positively associated with the changes in 7-Hydroxy-3-oxocholanoic acid, and cortisone, and negatively correlated with those of 1,4-Methylimidazoleacetic acid, and imidazolepropionic acid; the *Selenomonas_1* was positively associated with the changes in 7-Hydroxy-3-oxocholanoic acid, 16,16-dimethyl-6-keto Prostaglandin E1, and cortisone, and negatively associated with the changes in 1,4-Methylimidazoleacetic acid, imidazolepropionic acid, adrenic acid, and chenodeoxycholic acid sulfate.

In addition, we identified some microbial-associated metabolites which were significantly enriched in the bile secretion, histidine metabolism, and arachidonic acid metabolism pathways in SA group, suggesting a correlation with an imbalance in gut microbiota or increased serum cytokines. Correlation analysis between these metabolites and cytokines demonstrated that hydoxycholic acid, isolithocholic acid, 7-Hydroxy-3-oxocholanoic acid, TXB2, sn-3-O-(geranylgeranyl) glycerol 1-phosphate, 15-Deoxy-d-12,14-PGJ2, and cortisone, which were decreased in SA patients were negatively associated with the changes in serum levels of IL-17A, IL-17F, TNF- α , and IFN- γ , while the increased fecal chenodeoxycholic acid sulfate, 1,4-Methylimidazoleacetic acid, imidazolepropionic acid, adrenic acid, L-Carnitine, acetylcholine, ergothioneine, and D-Urobilinogen in SA patients were positively associated with the changes in IL-17A, IL-17F, TNF- α , and IFN- γ (Fig. 6E-F, and Table 3). A receiver operating characteristic (ROC) curve analysis indicated that imidazolepropionic acid (area under the curve (AUC), 0.911; Fig. 6G) and 1, 4-Methylimidazoleacetic acid (AUC, 0.930; Fig. 6H) were significantly associated with SA samples.

To investigate whether patients with certain characteristics of gut microbiota and their metabolites are more susceptible to repeated pregnancy loss or infertility, we conducted a follow up survey. Our follow-up

results demonstrated that 17 participants exhibited with a RPL or unsuccessful pregnancy. The ROC analysis also showed higher AUCs for imidazolepropionic acid (0.814; Fig. 6I) and 1, 4-Methylimidazoleacetic acid (0.813; Fig. 6J) for RPL. Thus, these results reveal a link between the distinct metabolites (e.g., imidazolepropionic acid and 1, 4-Methylimidazoleacetic acid) and Th17 immunity in SA patients. Furthermore, our data indicate potential roles of these metabolites as 1) biomarkers to identify women potential risk for recurrent miscarriage and 2) potential targets for prophylactics and intervention.

Discussion

Our study found that the diversity and composition of gut microbiota and metabolites profiles were significantly altered in SA patients. Further analyses revealed that these alterations in gut microbiota were related to the increased Th1- and Th17-related cytokines. The metabolites profiles revealed potential links between gut microbiota and the changes in cytokines and were associated with recurrent miscarriage or delayed pregnancy in the cohort of SA patients. To the best of our knowledge, this is the first study to investigate the association of gut microbiota, fecal metabolites profiles and proinflammatory cytokines in SA patients (Fig. 7).

The Th1 response, especially IL-2, TNF- α , and IFN- γ , is harmful to the survival of the conceptus [23, 24]. A significantly higher level of the Th1 cytokine IFN- γ was present in women with RPL compared with those with normal pregnancies [25]. TNF- α has been reported to inhibit trophoblast invasion, and increased levels of TNF- α have been reported in women with SA [26, 27]. Moreover, Liu et al. found that Th17 cells were significantly increased in RPL women compared with women with normal healthy pregnancies [28]. In mice, abnormal elevation of IL-17 at the maternal-fetal interface led to a miscarriage, while administration of an anti-IL-17 antibody prevented unexplained RPL [29]. The current study also found that serum levels of IL-2, IL-17A, IL-17F, TNF- α , and IFN- γ were markedly increased in SA patients compared to the controls. These results suggest that women with SA have a propensity for proinflammation via Th1- and Th17-mediated immunity.

The gut microbiota of a host is a crucial factor for shaping and modulating the immune responses [30-32]. Gut microbial dysbiosis is a risk factor in the development of inflammation [33, 34]. Reduction of gut microbial diversity has been linked to an increased risk of gastrointestinal diseases and proinflammatory characteristics [35, 36], and low gut bacterial richness is a common hallmark of chronic disease [37]. During pregnancy, the gut microbial within-subject (α) diversity is similar to that of the non-pregnant state at T1, but reduced at T3, after comparing to the 16S rRNA gene sequencing data from the Human Microbiome Project (HMP) [19]. This shift from T1 to T3 includes an increase in the levels of the proinflammatory cytokines IL-2, IL-6, IFN- γ , and TNF- α [19, 38]. Here we report a negative association between systemic Th1- and Th17-associated cytokines and reduced gut microbial diversity during T1, indicating a correlation between gut microbial diversity and the increased proinflammatory cytokines in this cohort of SA patients. One previous study reported that the increased ratio of *Firmicutes* to *Bacteroidetes* is related to chronic inflammation [39]. In our study, we observed both the reduced gut microbiota and the increased ratio of *Firmicutes* to *Bacteroidetes* in the SA group during T1, indicating

that the proinflammatory effects of the microbiome in patients with SA are likely caused by holistic dysbiosis, rather than by a specific pathogen.

In addition to the decreased diversity, we also found that *Prevotella_1*, *Prevotellaceae_UCG_003*, and *Selenomonas_1*, genera known as the dominant bacteria community in the gastroenteric environment of healthy humans, were significantly reduced in SA patients. Studies have reported that the death of *Prevotella* can result in systemic inflammation by inducing increased plasma lipopolysaccharides (LPS) levels [40, 41]. Moreover, a decrease in *Prevotella* and *Prevotellaceae* may cause a degeneration in the abundance of SCFAs, particularly butyrate, which can serve as the energy substrates for epithelial cells of the gut [42]. Thus, a decrease in *Prevotella* and *Prevotellaceae* leads to the reduction of inducible regulatory T cells (iTregs) and activation of proinflammatory cells [43, 44]. The negative correlation between the decreased abundance of *Prevotella_1*, *Prevotellaceae_UCG_003* and serum levels of IL-17A found in our study provide further evidence for this association. Many previous studies have reported on the benefits of *Bifidobacterium* for the reduction of inflammation. Miyauchi et al. found that *Bifidobacterium longum subsp.* alleviated intestinal inflammatory reactions through inhibition of IL-17A production by intestinal epithelial cells [45]. Although no significant decrease of *Bifidobacterium* abundance was found in SA patients in our study, a positive correlation of *Prevotella_1* and *Bifidobacterium* (Supplementary Figure S1) might indicate a regulatory role for *Prevotella_1* on Th17 cytokines. Moreover, we found that both *Prevotella_1* and *Prevotellaceae_UCG_003* correlated highly with enriched imidazolepropionic acid and 1, 4-Methylimidazoleacetic acid, both of which had higher abundance in the SA group. And these two metabolites were positively associated with the level of IL-17A and IL-17F. These data suggested that the gut microbiome has biologically relevant effects on the modulation of Th17 cytokine production in women with early miscarriage.

Modulation of host defense by the microbiota may be exerted mainly through the release of intermediary common mediators (such as metabolites) rather than direct interaction between specific microorganisms and immune cells. An important role for metabolites in microbiota-cytokine interactions is supported by the fact that a large proportion of the metabolites in the blood originate from the gut [46, 47], and our findings of the strong impact of microbial metabolic processes on cytokines production. We observed that several microbiota-associated metabolites that were enriched in the bile secretion pathway, were also significantly altered in the feces of SA patients. It is well-documented that bile acid plays a potent regulation role on intestinal immunity cells [48]. A recently published paper in *Nature* demonstrated that bile acid metabolites can control Th17 and Treg cell differentiation [49]. In the present study, we found negative associations between hyodeoxycholic acid, isolithocholic acid, and TXB2, which were significantly decreased in the SA group, with serum cytokines, IL-17, TNF- α , and IFN- γ , and positive associations for chenodeoxycholic acid sulfate, L-Carnitine, acetylcholine, and cholic acid metabolites. Our results suggest that the bile acid metabolism of the gut microbiota strongly influences Th17-associated cytokine production. Moreover, we found that the fecal concentrations of 1, 4-Methylimidazoleacetic acid, and imidazolepropionic acid that were enriched in histidine metabolism were also significantly increased in the SA group and aligned well with the increased IL-17, TNF- α , and IFN- γ in this group. Microbially produced imidazole propionate, which has been shown to have systemic effects

and is present at higher concentrations in subjects with type 2 diabetes, impairs insulin signaling through mammalian target of rapamycin1 (mTORC1) [50]. A high level of imidazole propionate was reported to be involved in immune activation and low-grade inflammation [51, 52]. Intriguingly, ROC analysis indicated that the two imidazole propionic acids we examined are associated with SA and have a positive predictive value for RPL. Collectively, these results indicate that the gut microbiota has biologically relevant effects on the modulation of Th17 cytokine production through their metabolites in miscarriage. Our results also identified two imidazole propionic acids as not only potential novel biomarkers or risk factors for recurrent miscarriage but targets for prophylactics and intervention.

The strength of this study is that we applied 16S rRNA gene sequencing, metabolomics and host serum cytokines networks, which allowed us to gain more information about host–gut microbiota metabolic interactions in response to the imbalanced cytokines network in unexplained miscarriage. One limitation of this study is that we did not obtain completed questionnaires about the dietary habits of the participants, and therefore could not determine if the diet was a factor in the gut bacterial dysbiosis. In addition, we cannot rule out other etiologies of SA patients such as PCOS and IR, which might be indirectly associated with the proinflammatory cytokines. Another limitation is that we cannot determine the causal relationship between gut microbiota and fecal metabolites and the associated changes in cytokines. Our follow up results support the causal relationship although the sample size is small.

Conclusions

In summary, we demonstrated an association between gut bacterial dysbiosis and a Th1/Th17–mediated proinflammatory state in SA patients with unknown etiology. This study provided insights into the potential roles of gut microbiota in the pathogenesis of some unexplained miscarriages and the potential underlying mechanisms. Our study highlighted the network among gut microbiota, fecal metabolites and immune response in SA patients and identified potential novel biomarkers and an intervention target for recurrent miscarriage. Further study is warrant to determine the casual relationship between gut microbiota and inflammation induced miscarriage and whether the microbiota associated metabolites are the underlying mechanism of the cytokine network imbalance through larger prospective cohort studies and animal studies.

Materials And Methods

Participant enrollment

Eligible cases were women (i) who were less than 35 years of age with a pre-pregnancy BMI of 18.5-23.9 kg/m² (normal reference value standards of BMI for Chinese); (ii) who had no successful pregnancies (their abortions occurred before 20 weeks of gestation); and (iii) whose current male partner had normal semen testing by computer-assisted analysis. Age and gestational week-matched controls were women with normal early pregnancy who elected to have an abortion. All controls had at least one successful pregnancy and no history of SA, preterm labor, or pre-eclampsia. Subjects with a verifiable cause of

miscarriage were excluded. Exclusion criteria were: (i) abnormality of the uterus or cervical incompetence confirmed by hysterosalpingography. (ii) karyotype abnormality in either the participant or the fetus's biological father. (iii) luteal phase defect (diagnosed by basal body temperature in combination with serum progesterone levels < 10 ng/mL), hyperprolactinemia, or hyperandrogenemia. (iv) presence of autoantibodies-like antinuclear antibodies (ANA), anticardiolipin antibodies (ACL), irregular antibody, and extractable nuclear antigens antibodies associated with systemic lupus erythematosus (SLE). Forty-one cases and 19 controls were recruited and samples were collected between September 2017 and October 2018. Follow-up of pregnancy outcomes was conducted for all participants during the end of 2019. A metadata including sample collection and follow up time for each participant was summarized in **Supplementary File 1**. The study protocol was approved by the Ethics Committees of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2017-073). Informed consent was obtained from all participants.

Stool sampling, DNA extraction and 16S rRNA gene sequencing

In total, 41 cases and 19 controls were included in *16S rRNA gene sequencing*. Participants collected stool samples at home using sterilized containers the day before or the day of curettage. Participants were asked to store their stool sample in the refrigerator right after collection and before delivering the samples to the hospital where they were aliquoted and stored at -80°C until DNA extraction. Genomic DNA was extracted from stool samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's instructions. The quality of genome DNA was evaluated with 1% agarose gel. The V3-V4 variable regions of the 16S rRNA gene were amplified using bacterium-specific primers 343F (5'-TACGGRAGGCAGCAG-3') and 798R (5'-AGGGTATCTAATCCT-3') [53]. The PCR products were purified, and the concentrations were adjusted for sequencing on an Illumina MiSeq PE300 (OEbiotech Co., Ltd., Shanghai, China).

Bioinformatic analysis of the fecal microbiota

The raw data obtained from high-throughput sequencing were stored in FASTQ format. Low-quality sequences that had an average quality score below 20 were cut off using Trimmomatic software [54]. After trimming, paired-end reads were assembled with FLASH software [55]. The parameters of assembly were as follows: 10 bp of minimal overlapping, 200 bp of maximum overlapping and a 20% maximum mismatch rate. Further de-noising including the removing of ambiguous base (N), sequences below 200 bp, and chimeras was performed with QIIME software (version 1.8.0) [56]. The obtained clean reads were then subjected to primer-sequence removal and clustering to generate OTUs with a 97% similarity cutoff using Vsearch software [57]. The most abundant one was selected as the representative read of each OTU. All representative reads were annotated and blasted against the Silva database (v. 123) or Greengens (16s rDNA) using RDP classifier (at a confidence threshold of 70%). The α -diversity indices evaluating gut microbial community richness and evenness were performed by mother [58]. PCoA based on the Binary-Jaccard distance was performed to compare the global microbiota composition (β -diversity) between the SA and control groups, and statistically significant differences between the two

groups were calculated by Adonis analyses. LEfSe was applied to identify the bacterial species that differed between samples.

Fecal metabolic profiling and data analysis

Ten SA patients and 10 matched control subjects were randomly chosen for the metabolomics study. Accurately weighed 60 mg fecal sample was transferred to a 1.5-mL Eppendorf tube, and then 20 μ L each of L-2-chlorophenylalanine solution (0.3 mg/mL) and Lyso PC17:0 (0.01 mg/mL) were added as an internal standard. The QC samples (n = 4) were pooled ones in which aliquots of each sample (n = 20) were mixed together. Following ultrasonication and centrifugation, the supernatants from each tube were collected using crystal syringes, filtered through 0.22 μ m microfilters, transferred into a LC/MS glass vial, and analyzed by an ACQUITY UHPLC system (Waters Corporation, Milford, USA) coupled with an AB SCIEX Triple TOF 6600 System (AB SCIEX, Framingham, MA) as described previously [59].

The acquired raw data were analyzed by the Progenesis Q1 software (Waters Corporation, Milford, USA) using the following parameters: precursor tolerance at 5 ppm, fragment tolerance at 10 ppm, and product ion threshold at 5%. Metabolites were identified based on public databases such as the Human Metabolome Database (HMDB), Lipidmaps (v2.3), and METLIN. PCA and OPLS-DA models were carried out to visualize the metabolic alterations among experimental groups. A permutation test (n = 200) was performed to validate the model and avoid over fitting. HCA was applied on Pearson distances using PermutMatrix [60]. Differential metabolites contributing to the separation were identified using VIP value, fold change values and the corresponding *p* values. In general, metabolites with VIP > 1 were considered as relevant for interpreting the discrimination, a fold change value ≥ 1.5 or ≤ 0.667 was the cutoff for up or down-regulation in concentration, and *p* value < 0.05 was believed to be a significant difference.

Cytokines quantification by flow cytometry

A bead-based multiplex panel assay (Biolegend, San Diego, CA, USA) was used for simultaneous quantification of 13 human cytokines including IL-2, -4, -5, -6, -9, -10, -13, -17A, -17F, -21, -22, IFN- γ , and TNF- α in the corresponding 41 vs 19 subjects. Briefly, the peripheral blood was collected and centrifuged for 20 min at 1,000 \times g to collect serum. Serum samples were diluted two fold with Assay Buffer before being tested. Next, 25 μ L of mixed beads, detection antibodies, and streptavidin-phycoerythrin were added stepwise to each well. The plate was placed on a plate shaker for shaking at 500 rpm for 30 min at room temperature. After washing the beads three times with 200 μ L of 1 \times Wash Buffer, the beads were resuspended in 150 μ L of 1 \times Wash Buffer, placed on a plate shaker for 1 min and read on a flow cytometer.

Statistical analysis

Data are presented as the mean \pm standard deviation (SD). Significant differences in clinical characteristics were evaluated with Pearson's Chi-square test or Fisher's exact test. A one-way ANOVA or Kruskal-Wallis was used for the significant difference analysis in genus relative abundance. Student's t

test was performed to analyze the differential in metabolites abundance between the SA and control groups. Spearman's rank test or Pearson's correlation test was used to analyze the correlation between changes in genus relative abundance, fecal metabolites, and the levels of serum cytokines. $P < 0.05$ was considered statistically significant.

Availability of data and materials

Sequencing data, metabolites profiles, and metadata for all samples used in this study have been deposited in Figshare (<http://dx.doi.org/10.6084/m9.figshare.12254363>; 12263678; 12263705 respectively). A patient metadata file, which includes the time of sample collection and following up of the last pregnancies, was included in Supplementary file 1. A full record of all statistical analysis and R scripts used in this paper are included in Supplementary file 2.

Abbreviations

SA: Spontaneous abortion; RPL: Recurrent pregnancy loss; Tregs: Regulatory T cell; TNF- α : Tumour necrosis factor- α ; IFN- γ : Interferon- γ ; SCFAs: Short-chain fatty acids; BMI: Body mass index; ROC: Receiver operating characteristic; LPS: Lipopolysaccharides; HMP: Human microbiome project; VIP: Variable importance in projection; HCA: Hierarchical clustering analysis; PCA: Principle component analysis; OPLS-DA: (Orthogonal) partial least-squares-discriminant analysis; LEfSe: Linear discriminant analysis coupled with effect size measurements; SLE: Systemic lupus erythematosus

Declarations

Acknowledgements

We thank the obstetrician in the Department of Obstetrics and Gynecology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine for helpful sampling assistance.

Contributions

LF, YL and JZ conceived and designed the experiments. HC performed sample processing. DC performed the measurement of cytokines levels. YL performed experimental processing of the samples and analyzed the data. YL wrote the manuscript. All authors reviewed the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81903325), and the China Postdoctoral Science Foundation (2018M632138).

Ethics declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committees of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Informed consent or parental permission was obtained for all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hertz-Picciotto I, Samuels S. Incidence of early loss of pregnancy. *N Engl J Med.* 1988; 319:1483-4.
2. D. Ware B, Mark G, Silver RM. Recurrent Miscarriage. *N Engl J Med.* 2010; 363:1740-7.
3. King A. Uterine leukocytes and decidualization. *Hum Reprod Update.* 2000; 6:28-36.
4. Calleja-Agius J, Brincat MP. Recurrent miscarriages: What is the role of cytokines? *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology.* 2009; 24:663-8.
5. Wegmann TG, Hui L, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today.* 1993; 14:353-6.
6. Blois SM, Olmos S, Zenclussen AC, Soto CA, Chuluyan E, Arias K, et al. Therapy with Dendritic Cells (DCs) control fetal abortion rate in the CBA/J×DBA/2J mouse model. *Am J Reprod Immunol.* 2015; 48:149-50.
7. Chaouat G, Meliani A, Assal, Martal J, Raghupathy R, Elliott JF, Elliot J, et al. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *J Immunol.* 1995; 154:4261.
8. Wen-Juan W, Cui-Fang H, Guang-Jie Y, Shi-Hua B, Li-Hua Q, Qi-De L. Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients. *J Reprod Immunol.* 2010; 84:164-70.
9. Jasper, J. M. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Mol Hum Reprod.* 2006; 12:301-8.
10. Pongcharoen S, Somran J, Sritippayawan S, Niumsup P, Chanchan P, Butkhamchot P, et al. Interleukin-17 Expression in the Human Placenta. *Placenta.* 2007; 28:59-63.
11. Kosiewicz MM, Zirnheld AL, Alard P. Gut Microbiota, Immunity, and Disease: A Complex Relationship. *Front Microbiol.* 2011; 2:180.
12. Gevers D, Kugathasan S, Denson L, Vázquez-Baeza Y, VanTreuren W, Ren B, et al. The Treatment-Naive Microbiome in New-Onset Crohn's Disease. *Cell Host Microbe.* 2014; 15:382-92.

13. Paun A, Yau C, Danska JS. Immune recognition and response to the intestinal microbiome in type 1 diabetes. *J Autoimmun.* 2016; 71:10-8.
14. Donia M, Fischbach M. HUMAN MICROBIOTA. Small molecules from the human microbiota. *Science.* 2015; 349:1254766.
15. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. *Cell.* 2016; 167:1125-36 e8.
16. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature.* 2013; 500:232-6.
17. Smith P, Howitt M, Panikov N, Michaud M, Gallini C, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science.* 2013; 341:569-73.
18. Dorrestein PC, Mazmanian SK, Rob K. Finding the missing links among metabolites, microbes, and the host. *Immunity.* 2014; 40:824-32.
19. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Backhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell.* 2012; 150:470-80.
20. Bermon S, Petriz B, Kajénienè A, Prestes J, Castell L, Franco O. The microbiota: an exercise immunology perspective. *Exerc Immunol Rev.* 2015; 21:70-9.
21. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* 2006; 444:1027-131.
22. Zierer J, Jackson MA, Kastenmüller G, Mangino M, Long T, Telenti A, et al. The fecal metabolome as a functional readout of the gut microbiome. *Nat Genet.* 2018; 50:790-5.
23. Hill J, Polgar K, Harlow B, Anderson D. Evidence of embryo- and trophoblast-toxic cellular immune response(s) in women with recurrent spontaneous abortion. *Am J Obstet Gynecol.* 1992; 166:1044-52.
24. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. *Immunol Today.* 1997; 18:478-82.
25. Raghupathy R, Makhseed M, Azizieh F, Hassan N, Al-Azemi M, Al-Shamali E. Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. *Cell Immunol.* 1999; 196:122-30.
26. Otun H, Lash G, Innes B, Bulmer J, Naruse K, Hannon T, et al. Effect of tumour necrosis factor- α in combination with interferon- γ on first trimester extravillous trophoblast invasion. *J Reprod Immunol.* 2011; 88:1-11.
27. Sun C, Zhang Y, Tang C, Wang S, Piao H, Tao Y, et al. Chemokine CCL28 induces apoptosis of decidual stromal cells via binding CCR3/CCR10 in human spontaneous abortion. *Mol Hum Reprod.* 2013; 19:676-86.
28. Liu Y, Wu L, Tong X, Wu L, He G, Zhou G, et al. Study on the relationship between Th17 cells and unexplained recurrent spontaneous abortion. *Am J Reprod Immunol.* 2011; 65:503-11.

29. Wang W, Liu F, Liu X, Hao C, Bao H, Qu Q, et al. Adoptive transfer of pregnancy-induced CD4+CD25+ regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/JxBALB/c mouse model. *Hum Reprod.* 2014; 29:946-52.
30. Stokholm J, Blaser MJ, Thorsen J, Rasmussen MA, Waage J, Vinding RK, et al. Maturation of the gut microbiome and risk of asthma in childhood. *Nat Commun.* 2018; 9:141.
31. Liu R, Hong J, Xu X, Feng Q, Zhang D, Gu Y, et al. Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. *Nat Med.* 2017; 23:859-68.
32. Zhao L, Feng Z, Ding X, Wu G, Zhang C. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. *Science.* 2018; 359:1151-6.
33. Grigg JB, Sonnenberg GF. Host-Microbiota Interactions Shape Local and Systemic Inflammatory Diseases. *J Immunol.* 2017; 198:564-71.
34. M S, SP S, H V, M J, M O, EA F, et al. Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. *Cell.* 2016; 167:1125-36.e8.
35. Ott S, Musfeldt M, Wenderoth D, Hampe J, Brant O, Fölsch U, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut.* 2004; 53:685-93.
36. Claesson M, Jeffery I, Conde S, Power S, O'Connor E, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. *Nature.* 2012; 488:178-84.
37. Pascal V, Pozuelo M, Borrueal N, Casellas F, Campos D, Santiago A, et al. A microbial signature for Crohn's disease. *Gut.* 2017; 66:813-22.
38. Mukhopadhyay I, Hansen R, El-Omar E, Hold G. IBD-what role do Proteobacteria play? *Nat Rev Gastro Hepat.* 2012; 9:219-30.
39. Masumoto S, Terao A, Yamamoto Y, Mukai T, Miura T, Shoji T. Non-absorbable apple procyanidins prevent obesity associated with gut microbial and metabolomic changes. *Sci Rep-Uk.* 2016; 6:31208.
40. Wang S, Huang XF, Zhang P, Wang H, Zhang Q, Yu S, et al. Chronic rhein treatment improves recognition memory in high-fat diet-induced obese male mice. *J Nutr Biochem.* 2016; 36:42-50.
41. Larsen, Madura J. The Immune Response to Prevotella Bacteria in Chronic Inflammatory Disease. *Immunology.* 2017; 151:363-74.
42. Gargari G, Taverniti V, Balzaretto S, Ferrario C, Gardana C, Simonetti P, et al. Consumption of a bifidobacterium bifidum strain for 4 weeks modulates dominant intestinal bacterial taxa and fecal butyrate in healthy adults. *Appl Environ Microbiol.* 2016; 82:5850.
43. Gonçalves P, Araújo J, Di Santo J. A Cross-Talk Between Microbiota-Derived Short-Chain Fatty Acids and the Host Mucosal Immune System Regulates Intestinal Homeostasis and Inflammatory Bowel Disease. *Inflamm Bowel Dis.* 2018; 24:558-72.
44. Nakajima A, Kaga N, Nakanishi Y, Ohno H, Miyamoto J, Kimura I, et al. Maternal High Fiber Diet during Pregnancy and Lactation Influences Regulatory T Cell Differentiation in Offspring in Mice. *J Immunol.* 2017; 199:3516-24.

45. Miyauchi E, Ogita T, Miyamoto J, Kawamoto S, Morita H, Ohno H, et al. Bifidobacterium longum Alleviates Dextran Sulfate Sodium-Induced Colitis by Suppressing IL-17A Response: Involvement of Intestinal Epithelial Costimulatory Molecules. *Plos One*. 2013; 8.
46. Sridharan G, Choi K, Klemashevich C, Wu C, Prabakaran D, Pan L, et al. Prediction and quantification of bioactive microbiota metabolites in the mouse gut. *Nat Commun*. 2014; 5:5492.
47. Wikoff W, Anfora A, Liu J, Schultz P, Lesley S, Peters E, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *P Natl Acad Sci USA*. 2009; 106:3698-703.
48. Jia W, Xie G, Jia W. Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat Rev Gastro Hepat*. 2018; 15:111-28.
49. Hang S, Paik D, Yao L, Kim E, Jamma T, Lu J, et al. Bile acid metabolites control T17 and T cell differentiation. *Nature*. 2019; 576:143-8.
50. Koh A, Molinaro A, Ståhlman M, Khan M, Schmidt C, Mannerås-Holm L, et al. Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. *Cell*. 2018; 175:947-61.e17.
51. Deiteren A, De Man J, Pelckmans P, De Winter B. Histamine H₂ receptors in the gastrointestinal tract. *Brit J Pharmacol*. 2015; 172:1165-78.
52. Yamamoto M, Pinto-Sanchez M, Bercik P, Britz-McKibbin P. Metabolomics reveals elevated urinary excretion of collagen degradation and epithelial cell turnover products in irritable bowel syndrome patients. *Metabolomics*. 2019; 15:82.
53. Dennis KL, Yunwei W, Blatner NR, Shuya W, Abdulrahman S, Erin T, et al. Adenomatous polyps are driven by microbe-instigated focal inflammation and are controlled by IL-10-producing T cells. *Cancer Res*. 2013; 73:5905-13.
54. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014; 30:2114-20.
55. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. 2011; 27:2957-63.
56. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010; 7:335-6.
57. Torbjørn R, Tomáš F, Ben N, Christopher Q, Frédéric M. VSEARCH: A versatile open source tool for metagenomics. *PeerJ*. 2016; 4.
58. Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microb*. 2009; 75:7537-41.
59. Li H, Cai J, Chen R, Zhao Z, Kan H. Particulate Matter Exposure and Stress Hormone Levels: A Randomized, Double-Blind, Crossover Trial of Air Purification. *Circulation*. 2017; 136:618-27.
60. Caraux G, Pinloche S. PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics*. 2005; 21:1280-1.

Tables

Table 1 Demographic and clinical characteristics of women with spontaneous abortion and the controls

Variables	Spontaneous abortion (n = 41)	Control (n = 19)	<i>P</i> -value
Age (years) ^a	31.3 ± 5.0	32.4 ± 4.7	0.43
Pre-pregnancy BMI (kg/m ²)	21.5 ± 2.7	22.4 ± 4.2	0.39
Education			
Illiterate	0 (0%) ^b	0 (0%)	NE ^c
High school or lower	4 (10%)	4 (21%)	0.27
College	31 (76%)	14 (74%)	0.87
Postgraduate or higher	6 (14%)	1 (5%)	0.54
History of smoking	0 (0%)	0 (0%)	NE
History of drinking	0 (0%)	0 (0%)	NE
Bacterial vaginosis	0 (0%)	0 (0%)	NE
Ureaplasma urealyticum	0 (0%)	0 (0%)	NE
Chlamydia trachomatis	0 (0%)	0 (0%)	NE
Trichomoniasis	0 (0%)	0 (0%)	NE
Colpomycosis	0 (0%)	0 (0%)	NE
Virus			
HIV+	0 (0%)	0 (0%)	NE
HPV+	0 (0%)	0 (0%)	NE
Syphilis	0 (0%)	0 (0%)	NE
Antibody			
Anticardiolipin antibody (IgA, IgM, and IgG)	0 (0%)	0 (0%)	NE
Anti DNA antibody (single and double stranded)	0 (0%)	0 (0%)	NE
Anti ENA (Extractable nuclear antigen, 7 subtypes) antibody	0 (0%)	0 (0%)	NE
Irregular antibody	0 (0%)	0 (0%)	NE
Medical history			
Endometriosis	0 (0%)	0 (0%)	NE
Uterine fibroids	4 (8%)	1 (5%)	0.93
Endometrial polyps	0 (0%)	0 (0%)	NE
Intrauterine adhesion	0 (0%)	0 (0%)	NE
Ovarian cysts	0 (0%)	0 (0%)	NE

Pelvic inflammation	0 (0%)	0 (0%)	NE
DUB (Dysfunctional Uterine Bleeding)	0 (0%)	0 (0%)	NE
Rheumatoid arthritis	0 (0%)	0 (0%)	NE
SLE(Systemic Lupus Erythematosus)	0 (0%)	0 (0%)	NE

a, Data are presented as mean ± SD;

b, Data are presented as n (%);

c. NE, not estimable (due to nullity of category in both groups).

Table 2 Correlation analyses between the genera and abnormal metabolites

	<i>Bacteroides</i>		<i>Prevotella_1</i>		<i>Prevotellaceae_UCG_003</i>		<i>Selenomonas_1</i>	
	r	p	r	p	r	p	r	p
	0.74	< 0.001	-0.21	0.27	-0.27	0.26	-0.24	0.31
acid J	0.78	< 0.001	-0.35	0.13	-0.38	0.10	-0.32	0.17
ine	0.45	0.04	-0.37	0.10	-0.35	0.13	-0.31	0.18
3-oxocholanoic acid	-0.48	0.03	0.61	0.004	0.69	< 0.001	0.66	0.001
	-0.27	0.26	0.46	0.04	0.48	0.03	0.46	0.04
midazoleacetic acid	0.31	0.11	-0.65	0.002	-0.65	0.002	-0.60	0.005
opionic acid	0.34	0.09	-0.68	0.001	-0.67	0.002	-0.66	0.002
ne	0.26	0.26	-0.45	0.004	-0.38	0.09	-0.48	0.03
carboxy-leukotriene B4	0.49	0.03	-0.61	0.004	-0.62	0.003	-0.60	0.006
d	0.50	0.02	-0.43	0.06	-0.48	0.03	-0.49	0.03
thyl-6-keto Prostaglandin	-0.35	0.13	0.54	0.01	0.59	0.007	0.62	0.003
ycholic acid sulfate	0.41	0.08	-0.42	0.06	-0.42	0.06	-0.48	0.03

r, represents the correlation coefficient; p, represents the p values.

Table 3 Correlation analyses between the abnormal metabolites and inflammatory cytokines

Metabolites	Correlation coefficient (r)				
	IL-2	IL-17A	IL-17F	TNF- α	IFN- γ
Hyocholic acid	0.27	0.36	0.29	0.26	0.39
Ursocholic acid	0.29	0.35	0.36	0.28	0.35
Chenodeoxycholic acid sulfate	0.20	0.32	0.45*	0.32	0.35
Hyodeoxycholic acid	-0.09	-0.20	-0.05	-0.13	-0.65**
Isolithocholic acid	-0.09	-0.04	-0.11	-0.23	-0.50*
1,4-Methylimidazoleacetic acid	0.13	0.45*	0.41	0.58**	0.59**
Imidazolepropionic acid	0.15	0.47*	0.47*	0.59**	0.53*
Adrenic acid	0.04	0.43	0.42	0.36	0.38
7-Hydroxy-3-oxocholanoic acid	-0.30	-0.62**	-0.35	-0.44	-0.46*
Lucidenic acid J	0.17	0.23	0.15	0.14	0.44
THA	0.21	0.27	0.17	0.16	0.43
Deoxycholic acid 3-glucuronide	0.20	0.06	0.01	0.20	0.40
L-Carnitine	0.17	0.30	-0.06	0.34	0.54*
Acetylcholine	0.07	0.11	0.07	0.35	0.52*
5 α -androstane-3 α -ol-17-one sulfate	0.18	0.23	0.28	0.10	0.31
TXB2	-0.12	-0.47*	-0.32	-0.49*	-0.40
Ergothioneine	0.11	-0.02	-0.01	0.08	0.50*
LysoPC(22:1(13Z))	0.20	0.15	0.34	0.28	0.34
sn-3-O-(geranylgeranyl) glycerol 1-phosphate	-0.30	-0.54*	-0.33	-0.39	-0.18
15-Deoxy-d-12,14-PGJ2	-0.23	-0.50*	-0.26	-0.55*	-0.52*
12(S)-HETE	-0.17	-0.02	-0.37	-0.33	-0.20
Cortisone	-0.19	-0.26	-0.22	-0.34	-0.54*
D-Urobilinogen	0.12	0.25	0.16	0.31	0.46*
12-Oxo-20-carboxy-leukotriene B4	0.19	0.39	0.39	0.42	0.26
7 α -Hydroxydehydroepiandrosterone	0.16	0.14	0.09	0.26	0.37
16,16-dimethyl-6-keto Prostaglandin E1	-0.28	-0.37	-0.23	-0.42	-0.32

IL, interleukin; TNF- α ,
tumour necrosis factor-
 α ; IFN- γ , interferon- γ .

The asterisk represents the statistical significance between metabolites and cytokines. * $p < 0.05$, ** $p < 0.01$.

Supplementary Files

Supplementary Figure S1 Correlation analyses in bacterial genera.

Supplementary File 1 Metadata associated with all samples used in this study.

Supplementary File 2 Full account of statistical analysis performed in R.

Figures

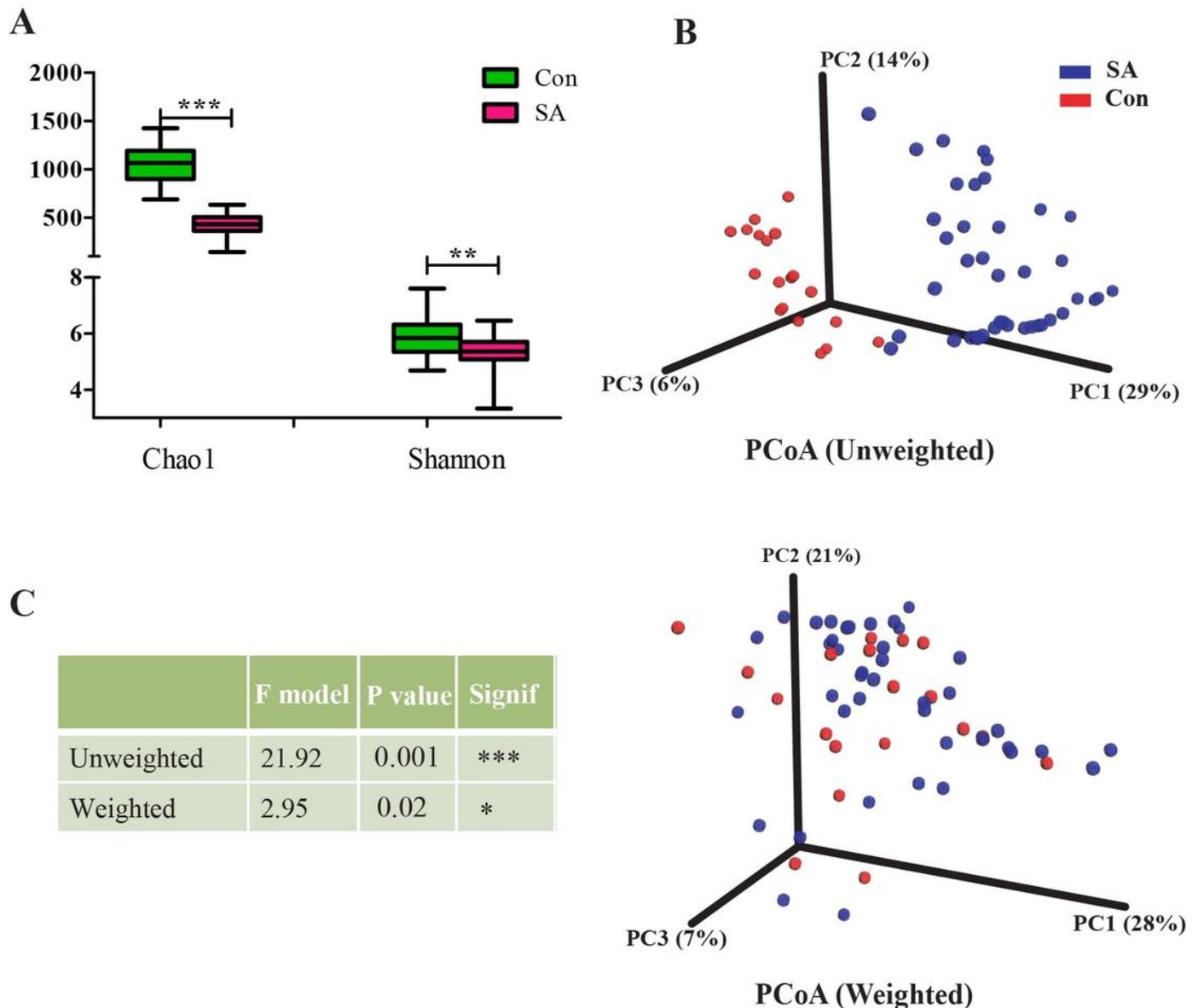


Figure 1

Gut microbiota diversity analyses. (A) Species diversity differences between the SA and control groups were estimated by the Chao1, and Shannon indices. $**p < 0.01$, $***p < 0.001$. (B) PCoA plot base of the relative abundance of OTUs showing bacterial structural clustering. (i) Unweighted UniFrac PCoA plots; (ii) Weighted UniFrac PCoA plots. SA group (blue dots), Con group (red dots), where dots represent individual samples. (C) Adonis analysis of statistical summary of different groups. F Model, represents F-test Value; $*p < 0.05$, $***p < 0.001$.

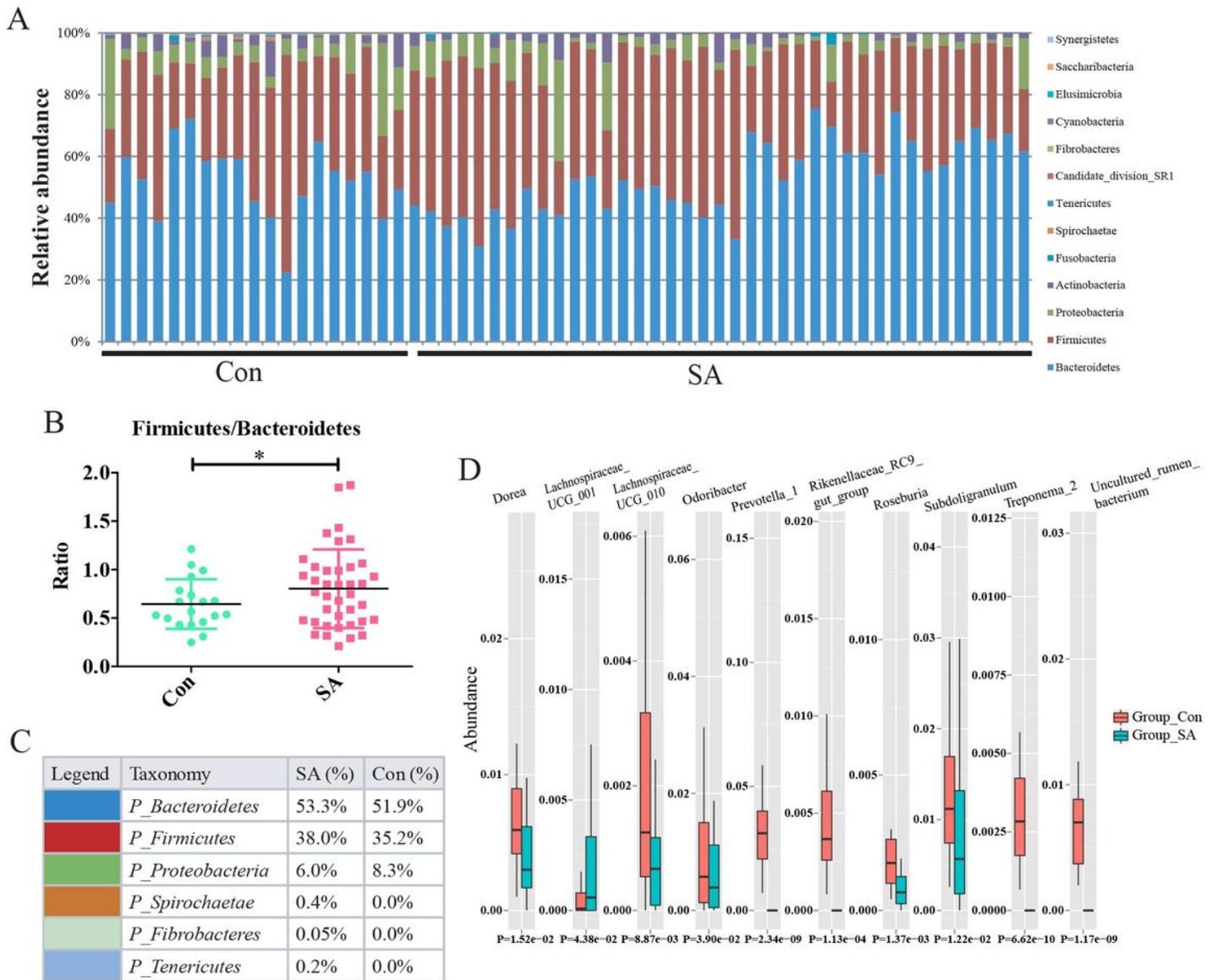


Figure 2

Gut microbial dysbiosis in SA patients. (A) Relative abundance of the main bacterial phylum in each group. (B) The ratio of Firmicutes/Bacteroidetes in each group. * $p < 0.05$. (C) Component proportion of bacterial phylum in each group. $n = 41$ for the SA group and $n = 19$ for the control group. (D) The top 10 significantly different genera in the relative abundances between the control and SA groups; Kruskal–Wallis, all $p < 0.05$).

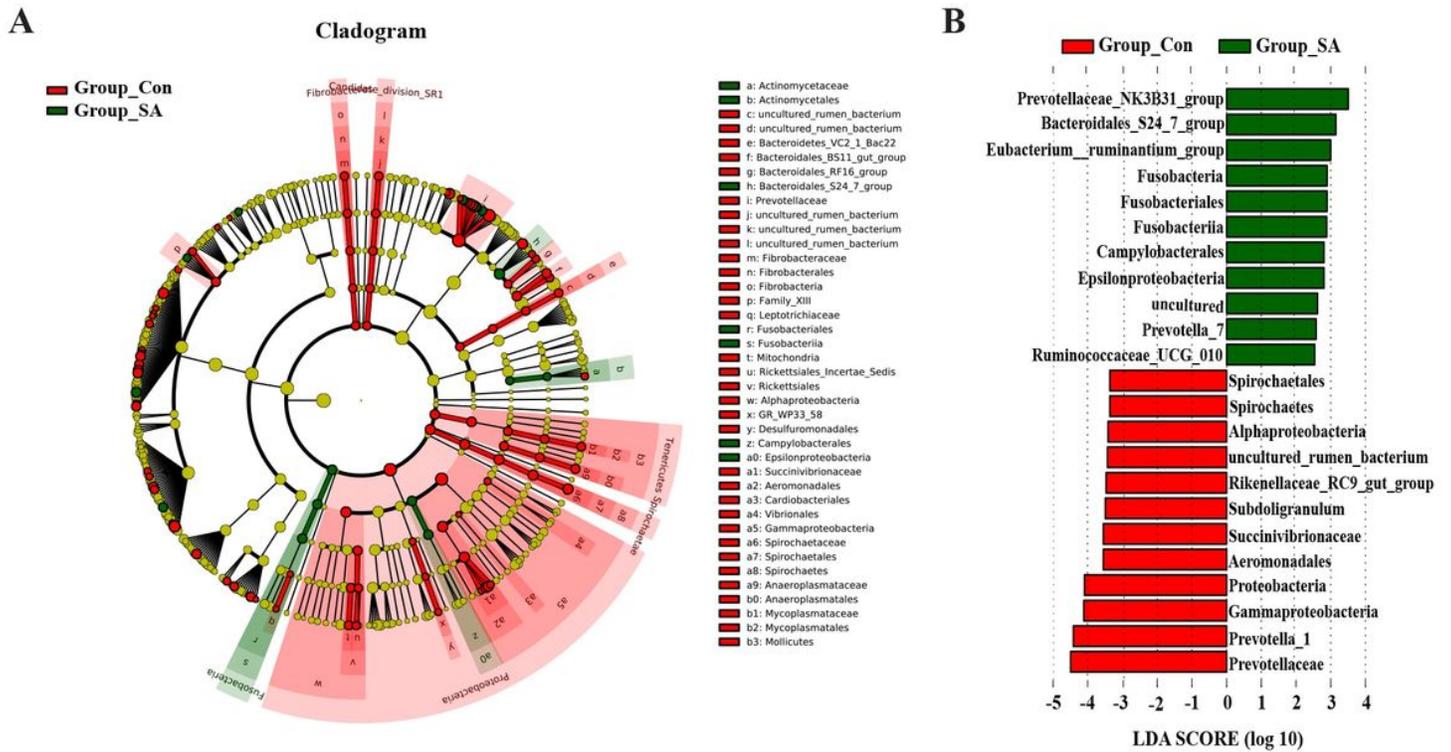


Figure 3

Compositions of different species in the control and SA groups by LEfSe analyses. (A) Taxonomic representation of statistically and biologically consistent differences between the control and SA groups. Significant differences are represented with different colors (red and green represent core microbes in the control and SA groups, respectively; yellow represents microbes shared between the control and SA groups). (B) Histogram of LDA scores for differentially abundant genera between the control and SA groups.

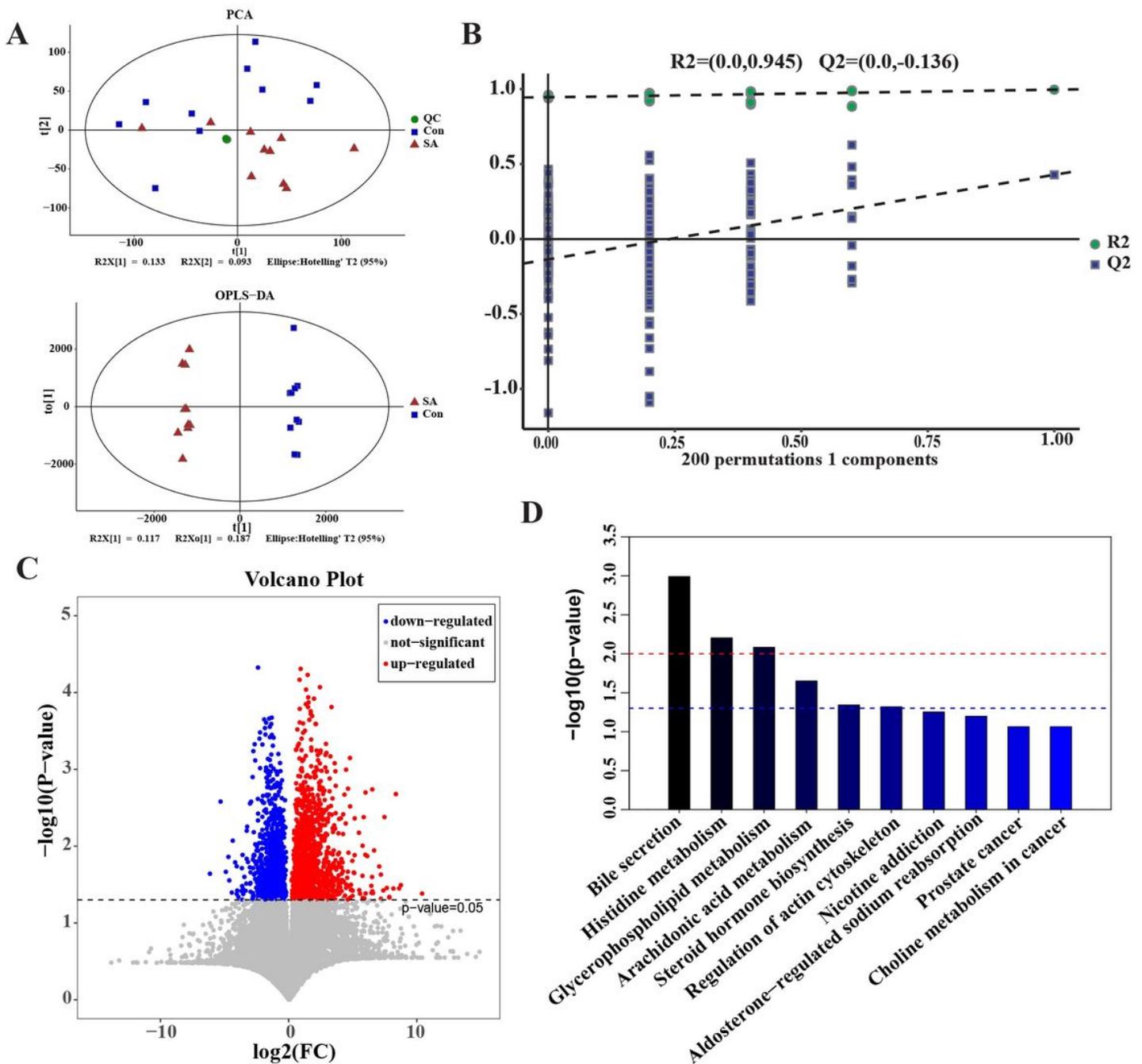


Figure 4

Fecal metabolomics for quantification of metabolites in the SA and control groups. (A) PCA and OPLS-DA score plots for differentiating the metabolites in each group. (B) The corresponding permutation test (200 times) for the OPLS-DA model. (C) Volcano plot showing the differentially accumulated metabolites in the SA and control groups. (D) Enriched KEGG pathways in the SA group compared with the control group.

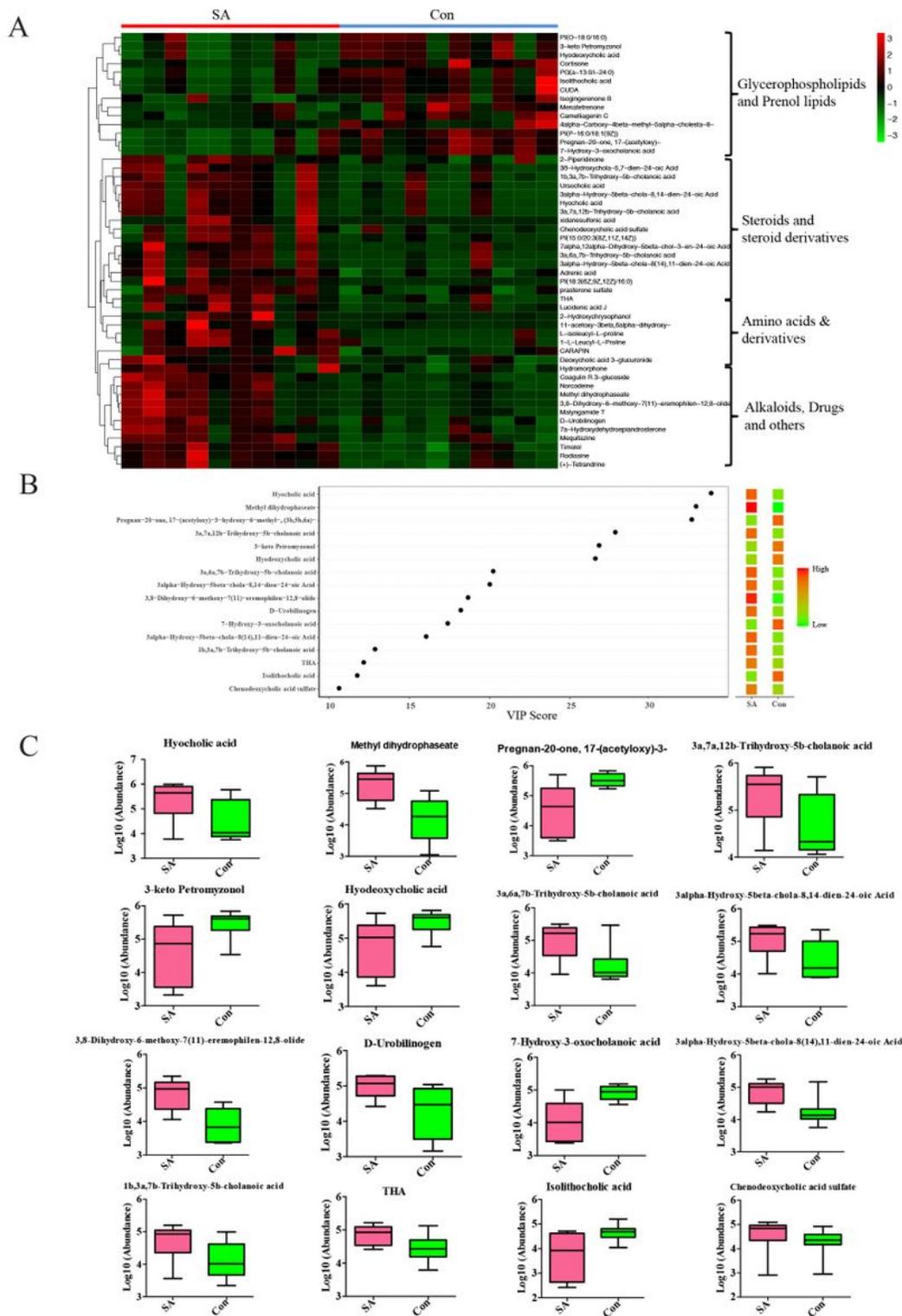


Figure 5

Distinct metabolites, as identified by clustering and multivariate analyses, between the SA and control groups. (A) Hierarchical clustering analysis (HCA) for the SA and control group metabolites based on their z-normalized abundances. (B) The discriminatory metabolites of top 16 VIP scores which obtained from the OPLS-DA models. (C) Abundance comparisons of metabolites between the SA and control groups.

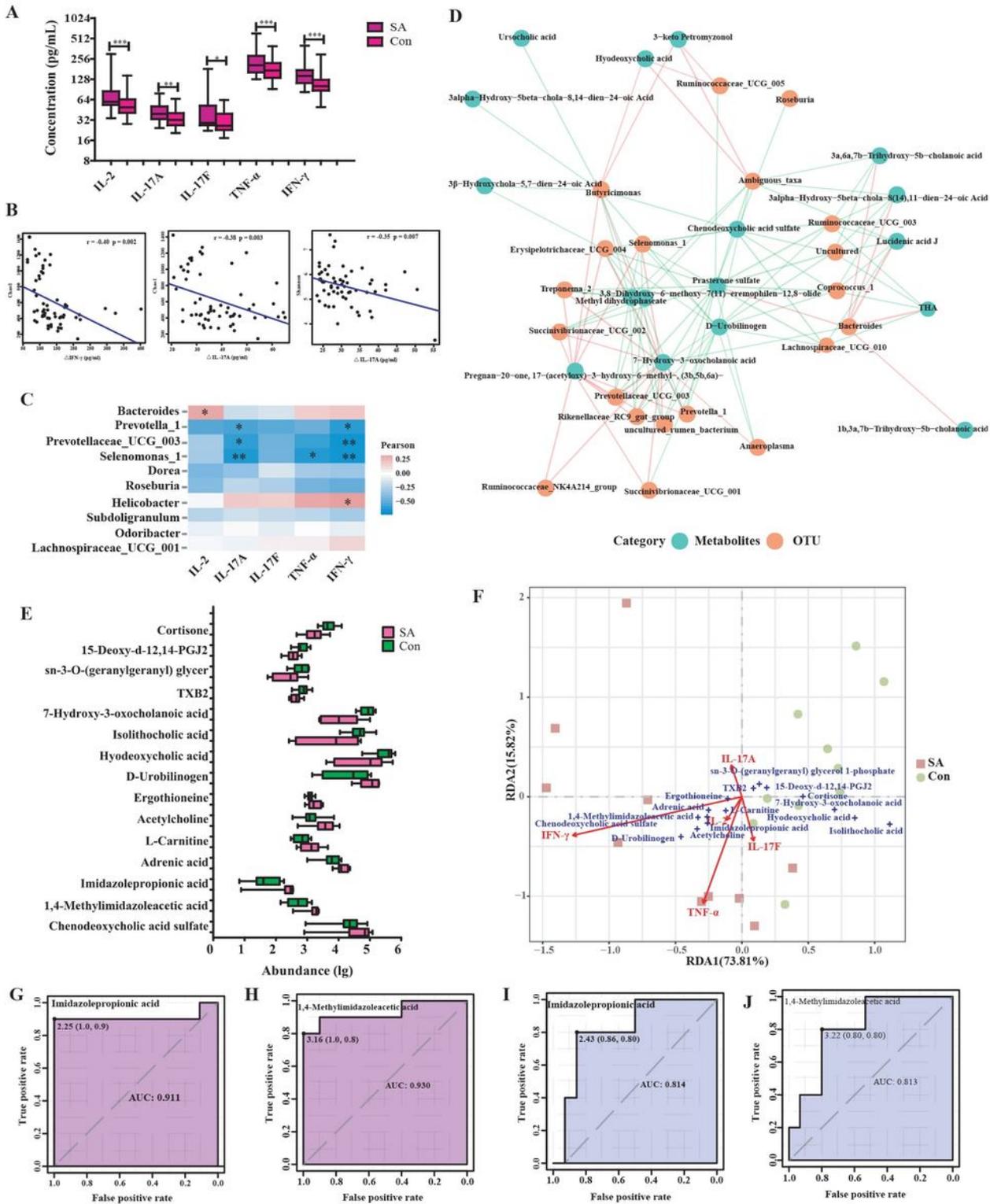


Figure 6

Correlation analyses of fecal microbiota and metabolites and the inflammatory cytokine profiles. (A) The inflammatory cytokine profiles in SA patients (N = 41) and controls (N = 19). *p < 0.05, **p < 0.01, ***p < 0.001. (B) Correlation between Chao1 index, Shannon index and proinflammatory cytokines using Pearson's linear correlation model. (C) Heatmap analysis of the correlation between the top 10 abnormal genera and proinflammatory cytokines. Orange represents positive correlations and blue negative

correlations. * $p < 0.05$, ** $p < 0.01$. (D) Network analysis of the correlation between abnormal genera and discriminatory metabolites. Red line represents positive correlations and green negative correlations. (E) Abundance profiles analysis of the microbe-associated metabolites. (F) RDA analysis of the correlation between inflammatory cytokines and discriminatory metabolites. (G-J) ROC analyses for metabolite abundance showing the high AUCs for imidazolepropionic acid and 1, 4-Methylimidazoleacetic acid in SA patients and their RPL.

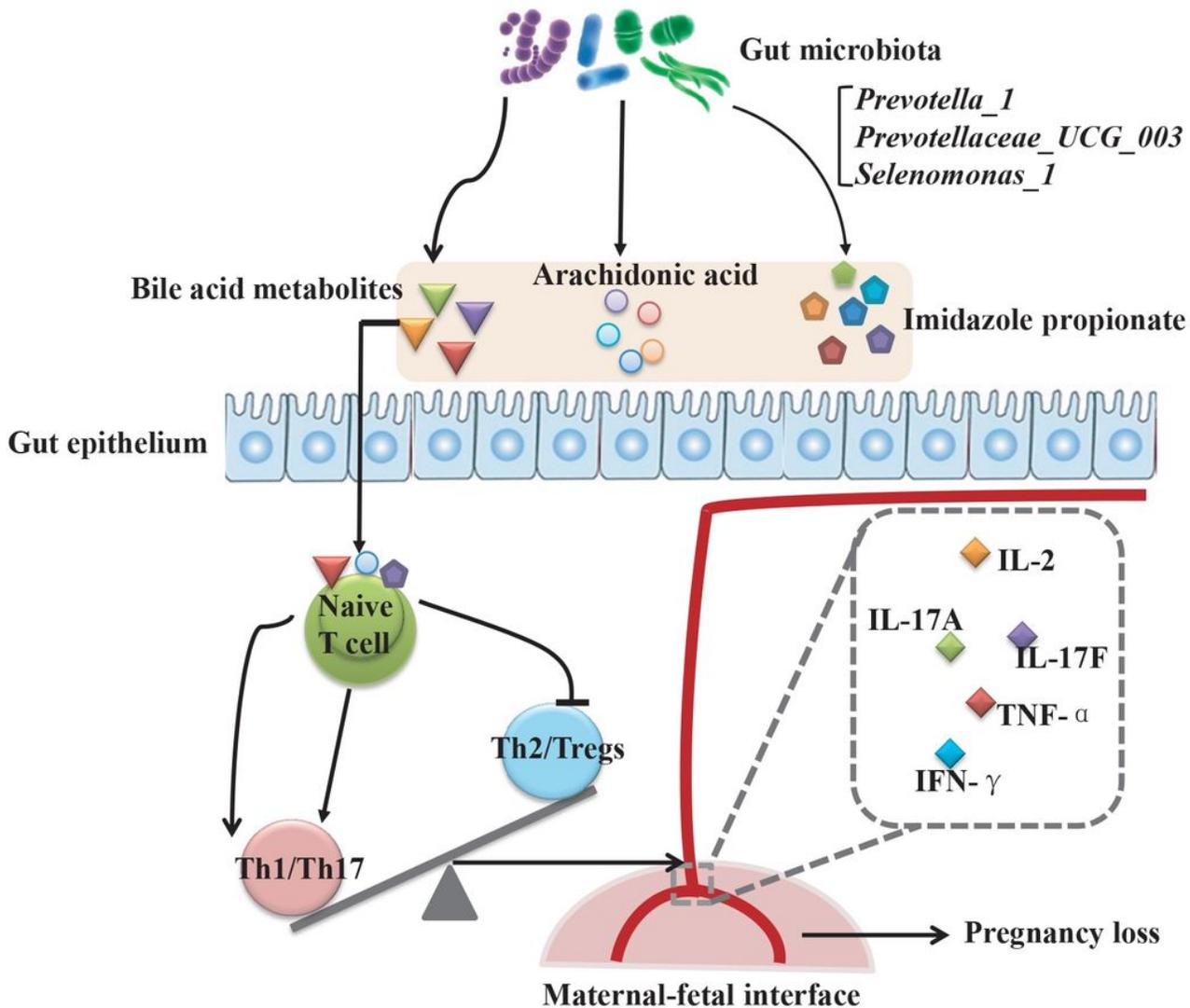


Figure 7

Proposed pathway in gut microbiome mediated pregnancy loss.

Supplementary Files

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

- [SupplementaryTableS1.docx](#)
- [Supplementaryfile2.docx](#)

- [SupplementaryFigureS1.pdf](#)
- [Supplementaryfile1.xlsx](#)
- [SupplementaryTableS2.docx](#)