

Endometriosis induces gut microbiota alterations in mice

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STUDY QUESTION: What happens to the gut microbiota during development of murine endometriosis?

SUMMARY ANSWER: Mice with the persistence of endometrial lesions for 42 days develop a distinct composition of gut microbiota.

WHAT IS KNOWN ALREADY: Disorders in the immune system play fundamental roles in changing the intestinal microbiota. No study has used high-throughput DNA sequencing to show how endometriosis changes the gut microbiota, although endometriosis is accompanied by abnormal cytokine expression and immune cell dysfunction.

STUDY DESIGN, SIZE, DURATION: This study includes a prospective and randomized experiment on an animal endometriosis model induced via the intraperitoneal injection of endometrial tissues.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The mice were divided into endometriosis and mock groups and were sacrificed at four different time points for model confirmation and fecal sample collection. To detect gut microbiota, 16S ribosomal-RNA gene sequencing was performed. Alpha diversity was used to analyze the complexity and species diversity of the samples through six indices. Beta diversity analysis was utilized to evaluate the differences in species complexity. Principal coordinate analysis and unweighted pair-group method with arithmetic means clustering were performed to determine the clustering features. The microbial features differentiating the fecal microbiota were characterized by linear discriminant analysis effect size method.

MAIN RESULTS AND THE ROLE OF CHANCE: The endometriosis and mock mice shared similar diversity and richness of gut microbiota. However, different compositions of gut microbiota were detected 42 days after the modeling. Among the discriminative concrete features, the Firmicutes/Bacteroidetes ratio was elevated in mice with endometriosis, indicating that endometriosis may induce dysbiosis. *Bifidobacterium*, which is known as a commonly used probiotic, was also increased in mice with endometriosis.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: More control groups should be further studied to clarify the specificity of the dysbiosis induced by endometriosis. This study was performed only on mice. Thus, additional data acquired from patients with endometriosis are needed in future research. We only detected the changes of gut microbiota at 42 days after the modeling, while the long-term effect of endometriosis on gut microbiota remains poorly understood. Moreover, we only revealed a single effect of endometriosis on gut microbiota.

WIDER IMPLICATIONS OF THE FINDINGS: This study provided the first comprehensive data on the association of endometriosis and gut microbiota from high-throughput sequencing technology. The gut microbiota changed with the development of endometriosis in a murine model. The communication between the host and the gut microbiota is bidirectional, and further studies should be performed to clarify their relationship.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by Grant (81571417) from the National Science Foundation of China and Grant (2015GSF118092) from the Technology Development Plan of Shandong Province. The authors report no conflict of interest.

Key words: gut microbiota / endometriosis / mouse / 16S ribosomal-RNA gene sequencing / dysbiosis

Introduction

Endometriosis is a chronic and estrogen-dependent disease characterized by the presence of endometrial glands and stroma outside the uterus (Bulun, 2009). Endometriosis affects 5–10% of women of reproductive age by causing chronic pelvic pain, dyspareunia and infertility (Giudice, 2010). Given that the pathogenesis and development of endometriosis are poorly understood, specific non-invasive diagnosis markers and therapies for patients with endometriosis still need to be developed (Falcone and Lebovic, 2011; Vercellini et al., 2014). Therefore, the current mechanistic studies on endometriosis are insufficient.

Patients with endometriosis are at high risk of several chronic diseases, such as autoimmune diseases, cancer, asthma/atopic diseases, cardiovascular diseases (Kvaskoff et al., 2015; Mu et al., 2016) and inflammatory bowel diseases (Jess et al., 2012). Endometriosis shares similar characteristics, such as decreased apoptosis, elevated cytokine levels and cell-mediated abnormalities, with several autoimmune diseases (Eisenberg et al., 2012; Capobianco and Rovere-Querini, 2013; Beste et al., 2014). Thus, endometriosis holds a strong relationship with complex immune disorders (Laschke and Menger, 2016).

With the development of high-throughput DNA sequencing technology, many studies have indicated that reciprocal interactions of the immune system and intestinal microbiota play fundamental roles in maintaining immune homeostasis (Caporaso et al., 2011; Maynard et al., 2012; Belkaid and Hand, 2014; Thaïss et al., 2016). Approximately 10^{14} microorganisms (microbiota) reside in humans, and the majority of the microbiota live within the gastrointestinal tract (Arnold et al., 2016). This immune system–microbiota alliance induces protective responses to pathogens and maintains tolerance of innocuous antigens by regulating associated pathways. Thus, aberrations in the communication between the gut microbiota and the innate immune system are associated with complex diseases, including obesity (Shen et al., 2013), hypertension (Yang et al., 2015), atherosclerosis (Wang et al., 2015), liver diseases (Mouzaki et al., 2013), allergies (Vatanen et al., 2016), *Clostridium difficile* infection (CDI), inflammatory bowel diseases (Chu et al., 2016) and graft-versus-host diseases (Mathewson et al., 2016). Furthermore, the reconstitution of gut microbiota by fecal microbiota transplantation (FMT) is highly effective in eradicating CDI (Borody and Khoruts, 2012) and promotes the anticancer immunotherapy of anti-CD152 (Vetizou et al., 2015) and anti-CD274 (Sivan et al., 2015; Zitvogel et al., 2016). Therefore, endometriosis, identified as a chronic inflammatory disease with abnormalities in immune cells and cytokines (May et al., 2010; Beste et al., 2014; Laux-Biehlmann et al., 2015), may be associated with gut microbiota through the immune system.

Literature focusing on the communication between gut microbiota and endometriosis is rare. Initially, altered profiles of intestinal microflora in endometriosis rhesus monkeys were detected by enumerating the isolated and cultured bacteria in differential and selective agars (Bailey and Coe, 2002). However, the indirect and low-throughput detection of *in vitro* cultured gut bacteria cannot represent the original complicated composition of the considerable gut microbiota, as the microbes rarely exist in traditional culture-based approaches. Fortunately, with the emerging development of next-generation high-throughput DNA sequencing technologies, 16S ribosomal-RNA gene sequencing has become the specific standard method for identifying microbiota (Caporaso et al., 2011). Therefore, accurate and comprehensive data on gut microbial composition in endometriosis is in great

demand for achieving an understanding on the intricate relationship between endometriosis and gut microbiota.

To understand what happens to gut microbiota during the development of murine endometriosis, we performed a prospective and randomized study to determine the changes in gut microbiota in a murine endometriosis model by 16S ribosomal-RNA gene sequencing.

Materials and Methods

Animals

All C57BL6 mice were obtained from Beijing HFK Bioscience Company (Beijing, China). All mice were maintained under specific pathogen-free (SPF) conditions with free access to irradiated laboratory food and sterile reverse osmosis water. The cages and bedding were changed weekly to maintain a stable environment. All procedures were approved by the Animal Care and Use Committee of Shandong University (Shandong, China).

Induction and confirmation of endometriosis

The estrous stage was monitored daily by a vaginal smear every morning for 2 weeks, and the mice with normal estrous cycles were used in the following experiments. Endometriosis was established by intraperitoneal injection of endometrial segments as described (Bacci et al., 2009; Long et al., 2016) and used in our previous study (Yuan et al., 2017). Briefly, the mice were initially injected subcutaneously with estradiol benzoate (3 µg/mouse, Aladdin, Shanghai, China). A week later, the treated donor mice were sacrificed, and the uteri were isolated and collected in a Petri dish containing warm 0.01 M phosphate-buffered saline (PBS) (pH 7.2–7.4). The isolated uterine horns were processed identically, including a longitudinal split with a pair of scissors, careful isolation of endometrial tissue and subtle disruption in small, uniform fragments smaller than 1 mm. Thereafter, these fragments were intraperitoneally injected to recipient mice with a 1 ml syringe and a 25 g needle. To eliminate any potential bias, endometrial fragments from every two donor mice were mixed and the mixture was equally injected to four recipient mice. Overall, 15 endometrial segments in 200 µl sterile PBS were injected into each recipient mouse. Finally, four mice each, in the endometriosis or control groups were sacrificed at 7, 14 and 28 days after modeling. The endometriosis group sacrificed at 42 days after modeling kept included mice, whereas the control group included eight mice.

Feces collection

Diet and environmental factors can alter gut microbiome rapidly. Therefore, the SPF facilities, irradiated food and sterile water for mice were controlled strictly in this study. During feces collection, each mouse was kept in one cage. Moreover, high-pressure steam sterilization-treated cages and water, irradiated food, and disposable sterile experimental supplies were used to avoid potential cross contamination. The fecal pellets were collected every hour on Days 7, 14, 28 and 42 before the mice were sacrificed. Finally, 20 fresh fecal pellets from each mouse were collected in a sterile freezing tube and stored on ice temporarily. Thereafter, the samples were all stored at -80°C immediately (Shaw et al., 2016).

Peritoneal macrophage collection, observation and identification

Following fecal sample collection, peritoneal cells were harvested by injecting and shaking 5 ml of ice-bath washing buffer containing Advanced

Roswell Park Memorial Institute 1640 medium (Gibco, USA) and 2% heat-inactivated fetal bovine serum (Gibco, Australia). The collected cell mixture was seeded in a cell-culture plate. To purify the macrophages, the supernatant was discarded at 3 and 6 h twice after seeding. The macrophages were then observed under a microscope. Thereafter, the adherent macrophages were collected by trypsin digesting and scraping for identification.

Flow cytometry (FCM) was utilized to identify peritoneal macrophages. In the present study, F4/80 and CD11b were used as pan-macrophage markers. To avoid adherence of macrophages to the tube wall, macrophages were incubated in 2% paraformaldehyde for 10 min on ice for antibody staining. After centrifugation, fragment crystallizable receptor was blocked by incubation of antimouse CD16/CD32 (93, eBioscience, USA) at a concentration of 1:100 for 10 min on ice. The cells were subsequently incubated with allophycocyanin-conjugated antimouse F4/80 (BM8, eBioscience, USA) at a concentration of 1:25 and phycoerythrin-conjugated antimouse CD11b (M1/70.15, eBioscience, USA) at a concentration of 1:100 for 20 min on ice in the dark. After centrifugation, FCM was performed on Guava easyCyte 6 HT (Merk Millipore, USA), and the results were analyzed by guavaSoft 3.1.1.

Hematoxylin–eosin staining and immunofluorescent staining

The 4 μm -thick sections of tissues were dewaxed and rehydrated in ethanol and water. Then, the slides were stained with hematoxylin (CW BIO, Beijing, China) for 5 min and eosin (CW BIO, Beijing, China) for 2 min.

The 4 μm -thick sections of uterine and ectopic regions were dewaxed and rehydrated in ethanol and water. Antigen retrieval was performed in citrate buffer (pH 6.0, 15 min). Then, the sections were washed thrice in PBS. Non-specific binding was blocked with 10% donkey serum (CW BIO, Beijing, China). The sections were incubated for 2 h at 37°C with the primary antibodies, E-cadherin (1:200, R&D Systems, Minneapolis, USA) or vimentin (1:200, EPR3776, Abcam, UK), or IgG control antibodies, washed with PBS thrice, and incubated at room temperature with Dylight 488-conjugated donkey anti-rabbit secondary antibody (1:400, Abcam, UK) or Dylight 594-conjugated donkey anti-goat secondary antibody (1:400, Abcam, UK) for 1 h. After the sections were washed, the cell nuclei were counterstained with fluorescent mounting medium with 4',6-diamidino-2-phenylindole (Abcam, Cambridge, UK).

DNA extraction and sequencing

Total genomic DNA from samples was extracted using CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels (Thermo Fisher Scientific, USA). According to the concentration, DNA was diluted to 1 ng/ μl using sterile water. Genes of the 16S V4 region were amplified using specific 515F-806R primer with barcode (Caporaso *et al.*, 2011). Polymerase chain reaction (PCR) was conducted with Phusion[®] High-Fidelity PCR Master Mix (New England Biolabs). Samples with a bright main strip between 400 and 450 bp after electrophoresis on 2% agarose gel were selected for further experiments. PCR products were mixed in equidensity ratios. Thereafter, the PCR product mixtures were purified with the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq[®] DNA PCR-free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. Library quality was assessed on the Qubit[®] 2.0 Fluorometer (Thermo Fisher Scientific, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Finally, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

Data analysis and presentation

Paired-end reads were assigned to different samples on the basis of their unique barcode and truncated by cutting off the primer sequence and barcode. Paired-end reads were merged using FLASH (V1.2.7) and the splicing sequences were raw tags. To obtain high-quality clean tags, we performed quality filtering of the raw tags under specific filtering conditions in accordance with the QIIME (V1.7.0) quality control process. To detect chimera sequences, the tags were compared with the reference database (Gold database) by UCHIME algorithm, and the chimera sequences were removed. Finally, we obtained the effective tags. Sequence analysis was performed by Uparse software (Uparse v7.0.1001). Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic unit (OTU). The representative sequence for each OTU was screened for further annotation. For each representative sequence, the GreenGene Database was used on the basis of RDP classifier (version 2.2) algorithm to annotate taxonomic information. Multiple sequence alignment was conducted using the MUSCLE software (version 3.8.31) for studying the phylogenetic relationship of different OTUs and the differences in the dominant species in different samples or groups. OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least number of sequences. Subsequent analysis of alpha diversity and beta diversity was performed on the basis of this output-normalized data.

Alpha diversity was applied in analyzing the complexity and diversity of species for samples through six indices, including observed species, Chao 1, Shannon, Simpson, ACE and Good's coverage. The OTUs that reach a 97% nucleotide similarity level were used for alpha diversity. The above indices were calculated with QIIME (version 1.7.0) and displayed with R software (version 2.15.3). Beta diversity analysis was performed using QIIME software (version 1.7.0) to evaluate the differences in species complexity of the samples. Unweighted pair-group method with arithmetic means (UPGMA) clustering was conducted as a type of hierarchical clustering method to interpret the distance matrix using average linkage. UPGMA clustering and beta diversity on both weighted and unweighted unifracs were all conducted using QIIME software (version 1.7.0). Principal coordinate analysis (PCoA) was performed to obtain principal coordinates and visualize complex, multidimensional data. The result of PCoA was achieved by the WGCNA package, stat packages and the ggplot2 package in the R software (version 2.15.3).

Microbial features differentiating the fecal microbiota were characterized using linear discriminant analysis effect size (LEfSe) method for biomarker discovery. This method emphasizes both statistical significance and biological relevance. An effect size threshold of 4 and a significance alpha of 0.05 were used for all the biomarkers discussed in this study.

Results

Similar diversity and richness of gut microbiota in the endometriosis and mock mice

We induced endometriosis models by intraperitoneal injection of endometrial tissues to minimize the disturbance to the delicate peritoneal immune environment and mimic endometriosis formation in humans. The mice were divided into the endometriosis and mock groups. After fecal sample collection and body weight measurement, the mice were sacrificed to evaluate the model formation at four time points (7, 14, 28 and 42 days) (Fig. 1A). The confirmation of the endometriosis model in the experimental groups and elimination of contamination in mock groups were assessed by observing the ectopic endometrial foci (Fig. 1B) and morphological changes of peritoneal

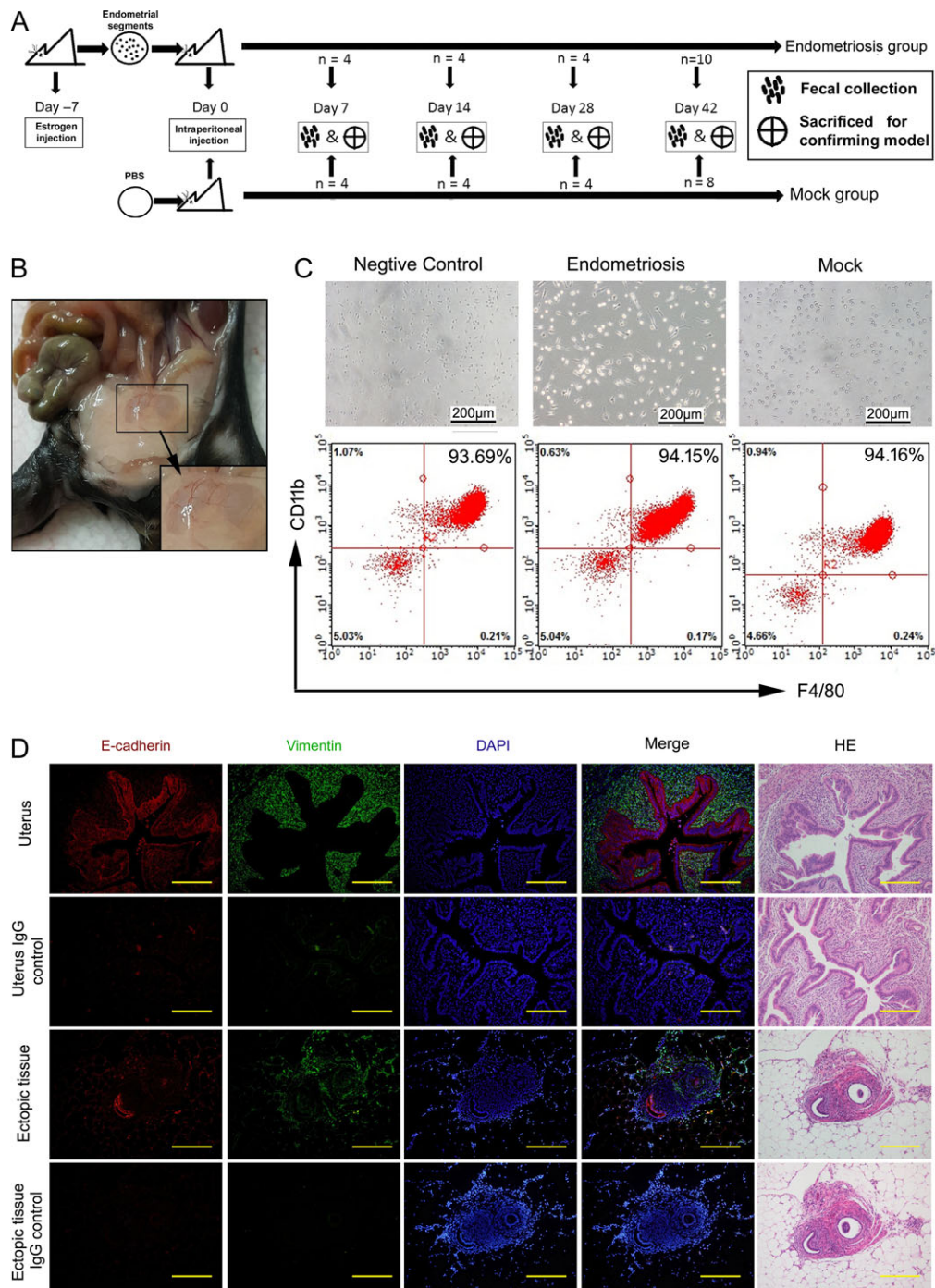


Figure 1 (A) Flow diagram of this study. (B) Typical ectopic endometrial foci could be found in peritoneal cavity of endometriosis mice at 42 days after modeling. (C) Compared with those in the negative control and mock groups, the morphology of peritoneal macrophages in endometriosis mice was abnormal as observed under a light microscope. Peritoneal macrophages were identified by flow cytometry in mice with endometriosis. (D) HE staining and immunofluorescent staining of E-cadherin (red) and vimentin (green) in eutopic and ectopic endometrial tissues (bar = 200 µm).

macrophages (sensitive immune cells corresponding to peritoneal stimulation) (Fig. 1C). Peritoneal macrophages expressing F4/80 and CD11b were identified by FCM detection (Fig. 1C). Adherent and classical ectopic endometrial foci were found in the fatty tissues around the bladder in all endometriosis mice sacrificed at 14, 28 and 42 days after modeling. Most of the injected endometrial segments

remained isolated in the peritoneal cavity 7 days after modeling. Abnormal peritoneal macrophages could be detected in all endometriosis mice. Meanwhile, all peritoneal macrophages from mock groups maintained normal morphology which was similar to macrophages isolated from untreated mouse. Both hematoxylin–eosin (HE) staining and immunofluorescent staining of E-cadherin and vimentin were

performed to identify the existence of ectopic endometrial–glandular epithelial cells and stromal cells (Fig. 1D).

No difference in body weight was observed in the two groups sacrificed at the same time points (Fig. 2A). No significant structural

changes and inflammatory cell infiltration was observed during HE staining of the colon (Fig. 2B). On the basis of the result of data analysis, no significant differences were observed using Shannon (Fig. 2C) and Simpson (Fig. 2D) analyses, indicating that the alpha diversity of

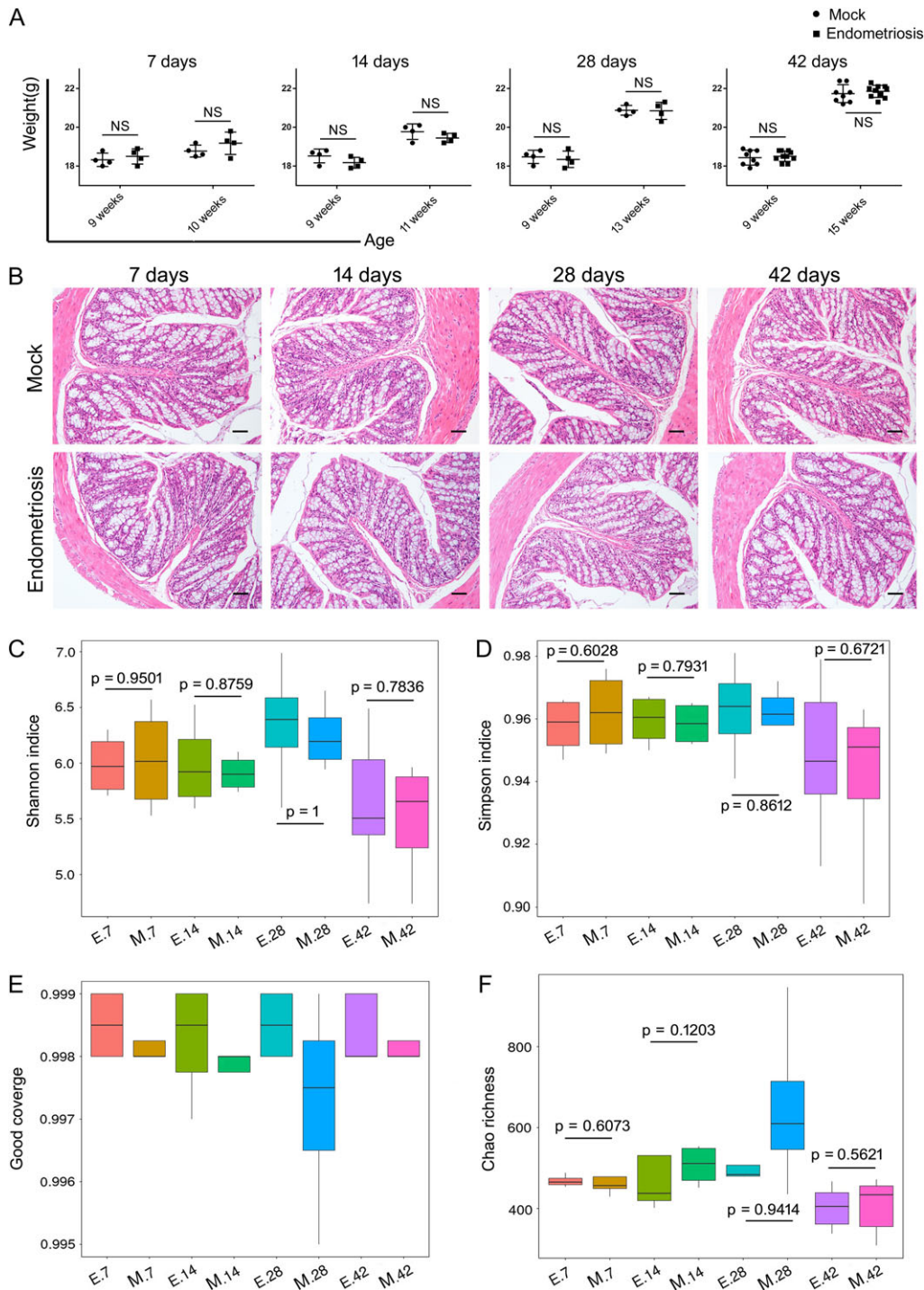


Figure 2 (A) Body weight changes of the mice. (B) HE staining of the colon, bar = 50 μm. (C) Shannon and (D) Simpson indices were used to estimate the diversity of fecal microbiota. (E) Good's coverage index demonstrated that most of the gut bacterial taxa were identified. (F) Chao index was used to estimate the microbial richness. The results were compared using Wilcoxon test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (endometriosis group, E; mock group, M).

gut microbiota was similar in the endometriosis and mock mice. The >99.0% Good's coverage index demonstrated that most of the gut microbial taxa were identified. These data represented the majority of microbial sequences of the fecal samples in the study (Fig. 2E). Chao analysis was used to detect the differences in microbial richness of the two groups at different time points, and no significant change was observed (Fig. 2F).

Endometriosis mice developed a distinct composition of gut microbiota at 42 days after modeling

Beta diversity analysis was performed to detect the differences of microbial composition among all groups (Fig. 3A and B). The endometriosis group sacrificed at 42 days after modeling was different from the mock group and showed an obvious higher beta diversity index than the other groups. To confirm this finding, we conducted UPGMA clustering of groups on weighted unifracs at the phylum level. Consistent with the beta diversity analysis, the endometriosis group at 42 days

was separated clearly. Furthermore, both UPGMA clustering of all groups on the weighted unifracs at the phylum level (Fig. 3C) and the result of PCoA analysis (Fig. 3D) indicated that the mice with endometriosis at 42 days was distinct from others. The analyses mentioned above demonstrated that the microbial composition of endometriosis changed with the persistent existence of ectopic endometrial tissues in the peritoneal cavity.

Dysbiosis of gut microbiota was detected in the endometriosis mice

To identify the distinct gut microbiota associated with endometriosis, we compared the composition of fecal microbiota between the endometriosis and mock groups at 42 days after injection using LEfse method. The cladogram that represented the composition of the fecal microbiota and the predominant microbiota in the two groups at 42 days is shown in Fig. 4A. Six discriminative features are shown in the LEfse analysis (Fig. 4B). Firmicutes were enriched in the endometriosis group, whereas Bacteroidetes were enriched in the mock group. The compositional changes of gut microbiota at different taxon levels

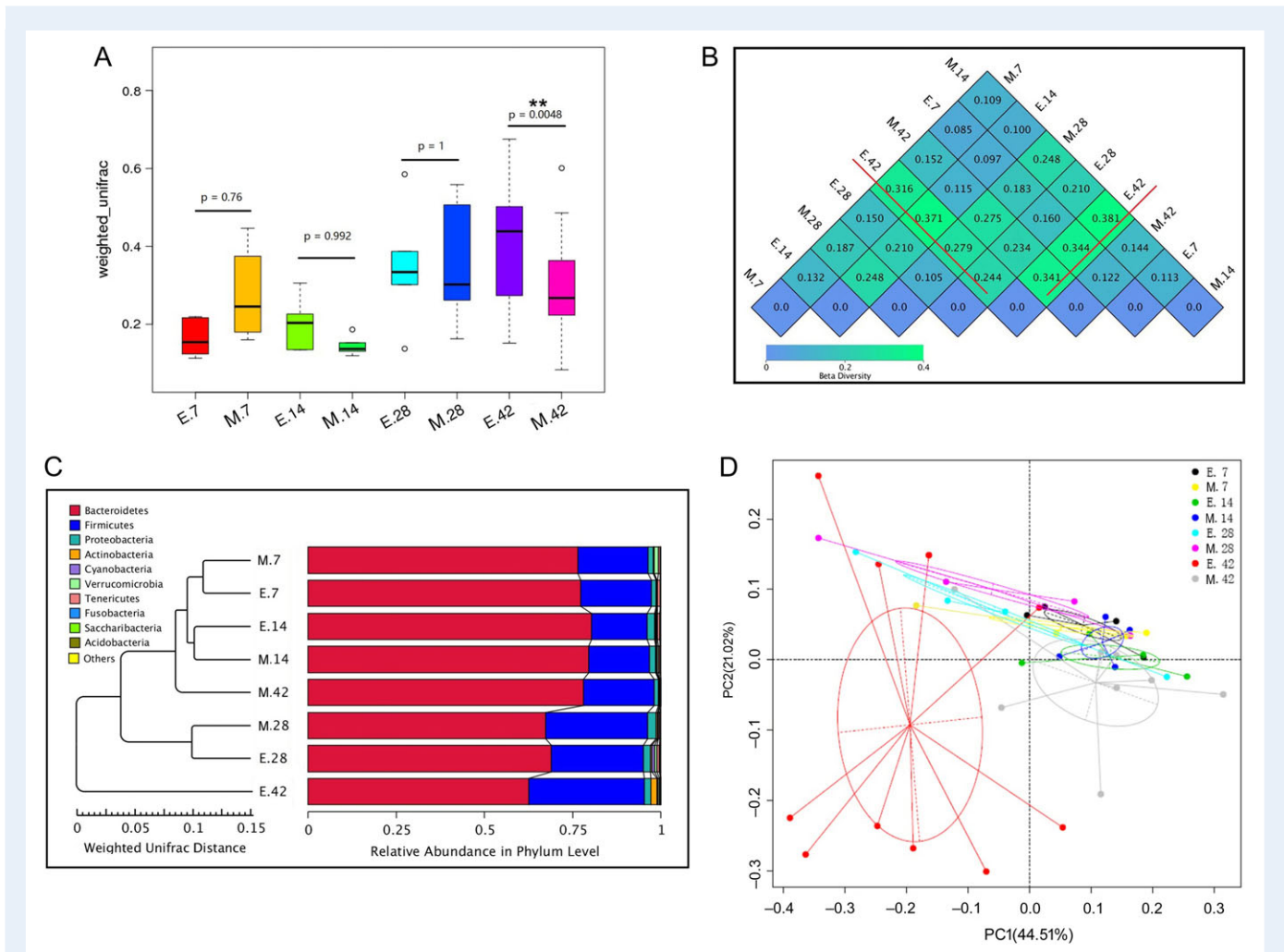


Figure 3 (A and B) Beta diversity indices, (C) UPGMA clustering of groups on weighted unifracs of groups and (D) PCoA analysis all indicated that the mice with endometriosis at 42 days after modeling developed a significantly different gut microbiota compared with the other groups. The results were compared by Wilcoxon test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (endometriosis group, E; mock group, M).

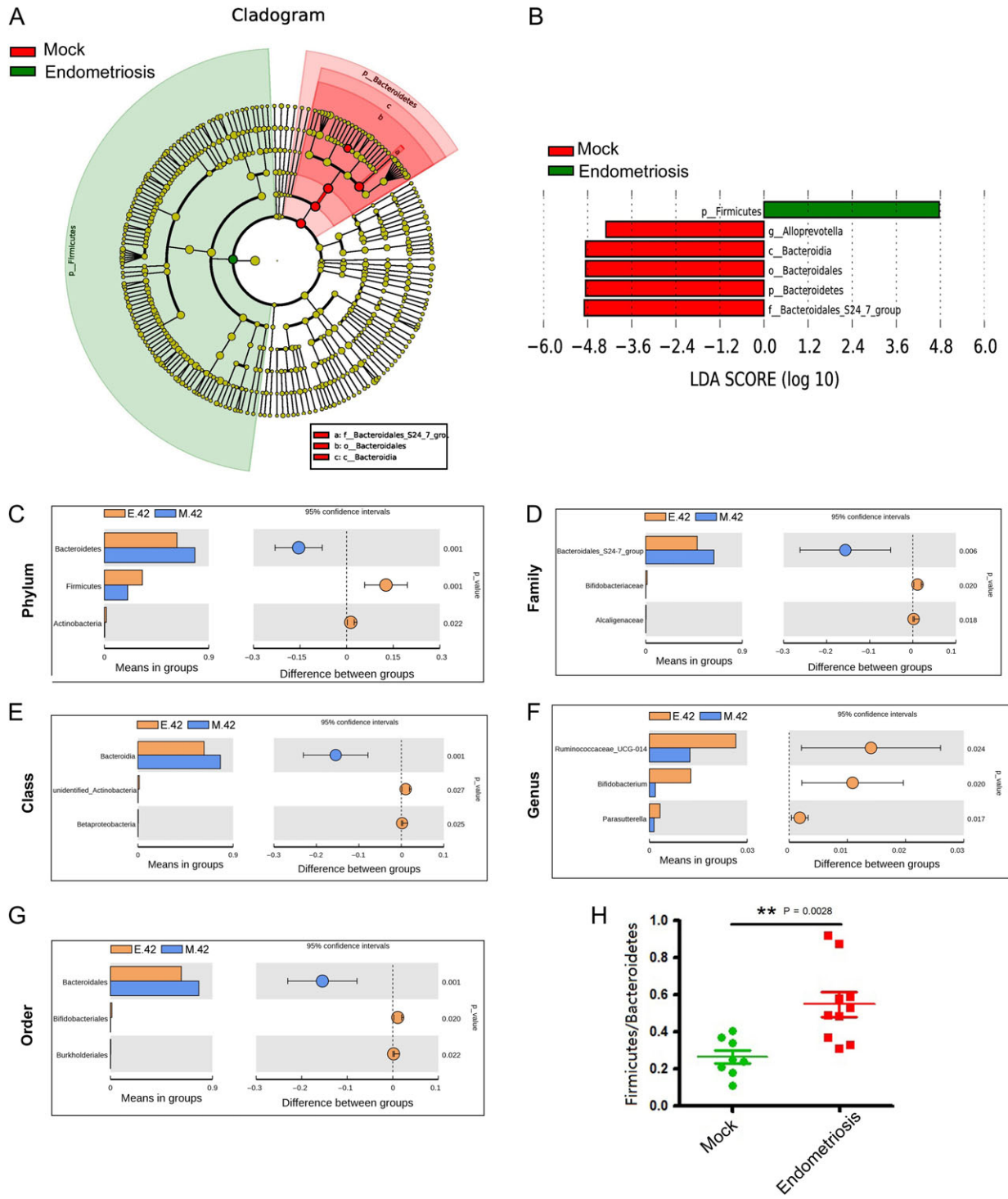


Figure 4 Discriminative features between endometriosis and control mice at 42 days after modeling. **(A)** Cladogram using results of the linear discriminant analysis (LDA) model on the bacterial hierarchy and **(B)** LDA coupled with effect size measurements identified the most differentially abundant taxa between the two groups. Comparison of relative abundance at the bacteria **(C)** phylum, **(D)** family, **(E)** class, **(F)** genus and **(G)** order levels between the two groups. **(H)** Mice with endometriosis showed a higher Firmicutes/Bacteroidetes ratio ($P = 0.0028$). The results were compared by Student's t -test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (endometriosis group, E; mock group, M).

between the two groups were calculated using T -test. At the phylum level, Firmicutes and Actinobacteria were abundant in the endometriosis group, whereas Bacteroidetes were prevalent in the mock group (Fig. 4C). At the class level, the endometriosis mice maintained an abundance of unidentified Actinobacteria and Betaproteobacteria. However, mock mice involved more Bacteroidia members (Fig. 4E)

than endometriosis mice. At the order level, Bifidobacteriales and Burkholderiales were of higher abundance in the endometriosis group than the other member orders, whereas Bacteroidales predominated in the mock group (Fig. 4G). At the family level, the endometriosis mice maintained high numbers of Bifidobacteriaceae and Alcaligenaceae, and the mock mice involved more Bacteroidales members (Fig. 4D). At the genus level, three genera (*Ruminococcaceae*-UGG-014, *Bifidobacterium* and *Parasutterella*) were abundant in the endometriosis mice (Fig. 4F). The Firmicutes/Bacteroidetes ratio, widely considered as a feature of dysbiosis, was compared between the endometriosis and mock groups at 42 days by *T*-test. The endometriosis mice were nearly twofold higher in the ratio than were the mock mice (Fig. 4H).

Discussion

To our knowledge, this study provided the first comprehensive data that reveal the association of endometriosis and gut microbiota, through high-throughput DNA sequencing technology. We did not detect significant changes in microbial richness and diversity in the endometriosis and mock groups. However, at 42 days after the modeling, the endometriosis mice developed fairly different microbial compositions. These results provide the initial evidence for research on the relationship between gut microbiota and endometriosis.

The microbiota, ~10 times more numerous than host cells, has been proven to play a fundamental role in the induction, training, and function of the host immune system (Erny et al., 2015; Lynch and Pedersen, 2016; Thaiss et al., 2016). In return, to maintain the symbiotic relationship of the highly diverse and evolving microbiota with the host, the immune system evolves. The interactions between the immune system and resident microbiota are important and complicated. The overall phylum-level composition of the human gut microbiome is similar to that of the mouse gut microbiome. More than 70% of gut microbiota is composed of Firmicutes, Bacteroidetes and Proteobacteria. Furthermore, the mouse gut microbiome is functionally similar to its human counterpart, with 95.2% of its Kyoto Encyclopedia of Genes and Genomes orthologous groups in common (Xiao et al., 2015). Endometriosis is accompanied by abnormal expression of cytokines and dysfunction of immune cells in ectopic lesions, peritoneal cavity and peripheral blood, which could interact with gut microbial alteration (May et al., 2010; Capobianco and Rovere-Querini, 2013; Borrelli et al., 2014). In this study, several changes to the gut microbiota in different taxon levels were discovered in mice with endometriosis. Among the altered microbiota, Firmicutes/Bacteroidetes ratio and *Bifidobacterium* have been widely studied in other diseases. The ratio of Firmicutes/Bacteroidetes is an important indicator in evaluating the microbial composition. According to previous studies, the Firmicutes/Bacteroidetes ratio is tightly associated with obesity (Cox et al., 2015), hypertension (Yang et al., 2015) and irritable bowel function (Rajilic-Stojanovic et al., 2011). The elevated Firmicutes/Bacteroidetes ratio in mice with endometriosis indicates that endometriosis induced the dysbiosis. The elevated level of *Bifidobacterium* (belonging to the phylum of Acitonobacteria) contributed to the increased level of Acitonobacteria in mice with endometriosis. *Bifidobacterium* is a commonly used probiotic playing a significant role in strengthening of the intestinal barrier, modulation of the immune response and pathogen antagonism either by the production

of antimicrobial compounds or through competition for mucosal binding sites (Veiga et al., 2010). Furthermore, compared with non-*Bifidobacterium*-treated mice, *Bifidobacterium* treatment of mice significantly improves tumor control by modulating the activation of dendritic cells in the steady state, which in turn supports improved effector function of tumor-specific CD8+ T cells (Sivan et al., 2015). However, the particular role of *Bifidobacterium* during endometriosis may be clarified by further FMT experiments.

Gut microbiota could be inherited and influenced by environmental factors (David et al., 2014; Goodrich Julia et al., 2014; Becattini et al., 2016; Gomez de Agüero et al., 2016; Pendse and Hooper, 2016). Abnormal microbial communities disturb immune homeostasis via their metabolites and components (Rooks and Garrett, 2016). A maternal high-fat diet induces changes in the gut microbiome of offspring and leads to behavioral alterations that can be restored by selective reintroduction of a commensal bacterial strain (Buffington et al., 2016). Moreover, in another study, host microbiota vitally altered microglia maturation and function, leading to impaired innate immune responses (Erny et al., 2015). Thus, conversely, an altered gut microbiota induced by endometriosis may be considered an important factor that could influence endometriosis by both genetic and environmental pathways.

Peritoneal macrophages may be a potential important regulators in the communication between endometriosis and gut microbiota. With the existence of peritoneal-distributed endometrial tissues, the monocyte-derived small peritoneal macrophages and resident large peritoneal macrophages are activated. Then, the peritoneal immune environment is remodeled by affecting CD4+ cell differentiation via secreted interleukins (ILs). The proportion of Th17 cells was elevated in the peritoneal cavity in the murine endometriosis model. Moreover, IL-17 concentrations were significantly higher in the peritoneal fluid of patients with minimal-to-mild endometriosis compared with those with moderate-to-severe endometriosis and those without the disease (Zhang et al., 2005). Th17 cells stimulate the production of antimicrobial proteins by intestinal epithelial cells. Th17 cells also form tight junctions between these cells by the secretion of signature cytokines, IL-17A, IL-17F and IL-22. These also mediate immunoglobulin-A transportation and granulocyte recruitment (Honda and Littman, 2016). Conversely, peritoneal macrophages may be influenced by disturbed inflammatory disease in the gut (Thevaranjan et al., 2017). The enhanced gut permeability and leakage of bacterial products from the gut results in dysregulated macrophage function in the peritoneal cavity. This condition may promote the survival of endometrial segments. However, further distinct studies are needed to clearly understand the particular pathways of communication between endometriosis and gut microbiota.

Several limitations and unanswered questions in this study include the following points. (i) Limited by the set of control groups, the specificity of gut microbiota induced by endometriosis was not demonstrated. Moreover, age is an important factor, which changes gut microbiota (Magri and Cerutti, 2016; Yassour et al., 2016). In this study, to avoid the potential disturbance caused by repeated fecal collection and the model quality, we simply divided the mouse cohorts into several observation groups. This factor may decrease the significance of the study (Kim et al., 2017). The changes associated with age were also not discussed. (ii) Although mouse and human share similar gut microbiota composition and function, this study cannot completely

represent the complicated reactions between the human body and gut microbiota. In this study, the mice developed ectopic endometrial tissues only in the fatty tissues around the bladder, but patients with endometriosis still maintain ectopic foci in other organs. Moreover, the persistence of ectopic endometriosis foci stays longer in women than the endometriosis model in this study. Thus studies in women with appropriate inclusion/exclusion criteria should be designated to determine this complicated relationship (Becattini *et al.*, 2016). (iii) We only demonstrated a single effect of endometriosis on gut microbiota. In fact, the association of endometriosis and gut microbiota is bidirectional (Maynard *et al.*, 2012). Thus, FMT experiments from endometriosis mice to germ-free mouse should be performed to illustrate the influence of disturbed gut microbiota on the initiation and development of endometriosis. (iv) Emerging technologies, such as high-throughput culturing, engineered organoids derived from human stem cells, and microfluidic assays, should be utilized to improve the efficiency and quality of microbiome research in endometriosis (Arnold *et al.*, 2016; Browne *et al.*, 2016; Marx, 2016; Scholz *et al.*, 2016).

In summary, we have shown that endometriosis induced changes in gut microbial composition in mice. This finding offers a novel pathway in studying the mechanisms involved in the development of endometriosis.

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Authors' roles

G.W. conceived and designed the study. M.Y. analyzed and interpreted data and drafted the article. M.Y., D.L., Z.Z., H.S. and M.A. performed the experiments. All authors participated in the writing and revision of the article.

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Conflict of interest

The authors report no conflict of interest.

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