Original Article
The regulation of intestinal flora on host's genes may play an essential role in the development of endometrial hyperplastic processes in yang deficiency individuals

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Abstract: Yang-deficiency constitution (YADC) is linked to a higher vulnerability to various diseases, such as cold coagulation and blood stasis (CCBS) syndrome and infertility. Endometrial hyperplastic processes (EHPs) are a leading cause of infertility in women and are characterized by CCBS. However, it remains unclear whether YADC is related to the development of EHPs. Methods: We recruited 202 EHPs patients including 147 with YADC (YEH group) and 55 with non-YADC (NYEH group). Fecal samples were collected from 8 YEH patients and 3 NYEH patients and analyzed using 16S rRNA V3-V4 sequencing for gut microbiota analysis. We obtained constitution survey data and a differential gut microbiota dataset from the literature for further analysis. Bioinformatics analysis was conducted using gut microbiota-related genes from public databases. Results: YADC was significantly more prevalent in EHPs than non-YADC (P < 0.001), suggesting it as a potential risk factor for EHPs occurrence ($OR_{population survey}$ = 13.471; $OR_{healthy women}$ = 5.173). The YEH group had higher levels of inflammation, estrogen, and tamoxifen-related flora compared to NYEH and healthy YADC groups. There was an interaction between inflammation, estrogen, differential flora, and EHPs-related genes, particularly the TNF gene (related to inflammation) and the EGFR gene (related to estrogen), which may play a crucial role in EHPs development. Conclusion: YEH individuals exhibit significant changes in their gut microbiota compared to NYEH and healthy YADC. The interaction between specific microbiota and host genes is believed to play a critical role in the progression of EHPs.

Keywords: Yang deficiency constitution, endometrial hyperplastic processes, intestinal flora, inflammation, estrogen

Introduction

Endometrial hyperplasia and endometrial polyps both involve endometrial hyperplastic processes (EHPs), which encompass various morphological changes characterized by an increased ratio of endometrial glands to stroma [1]. Around 50 to 80 million people worldwide suffer from infertility, among whom female factors are responsible for around 50% of all infer-

tility cases [2]. Endometrial abnormalities, such as EHPs, are one of the important reasons in females with primary and secondary infertility [3]. Exploring the mechanism of EHPs and early intervention may be of great significance for the treatment of infertility.

In China, the term 'constitution' (i.e., ti-zhi) is widely used in TCM and refers to a framework for understanding physical well-being [4]. The

constitution is evaluated based on factors such as the shape of the human body, physiological functions, psychology, and other characteristics, and includes the balanced constitution and eight types of unbalanced constitutions: Yang Deficiency, Yin Deficiency, Qi Deficiency, Phlegm Dampness, Stagnant Blood, Damp Heat, Stagnant Qi, and Inherited Special Constitutions [5]. Among those unbalanced constitutions, the Yang-deficiency constitution is a common unbalanced constitution that is mainly characterized by cold intolerance, such as a cold body and limbs, resulting from an insufficiency of "Yang-qi" that fails to warm the body [6]. Individuals with such a constitution imply a diminishing energy level in the physiological function of the body, and they may experience symptoms such as chills, loose stools, and a large volume of urine. In traditional Chinese medicine (TCM), cold-evil and Yang deficiency can lead to cold coagulation and blood stasis (CCBS) syndrome, which includes symptoms such as aversion to cold and cold pain, pain alleviated by warmth, cold limbs, blue color, late menstrual period, dysmenorrhea, darkish purple color with clots, a dark purple tongue, white coating, and a deep, slow, and unsmooth pulse [7, 8]. It has been shown to be closely related to gynecological diseases. Diseases associated with EHPs, such as endometrial polyps and abnormal uterine bleeding (AUB), often exhibit symptoms similar to the syndrome of cold coagulation and blood stasis. These symptoms include dysmenorrhea, menstrual blood carrying clots, and abnormal uterine bleeding [9-11]. It is a prevalent unbalanced constitution among the Chinese population, especially among females who experience infertility [12]. It is often associated with an increased susceptibility to certain diseases, such as infertility [13]. Our previous study found that the gut microbiota might play an essential role in maintaining the health of individuals with a Yang-deficiency constitution [14]. This suggests that Yang deficiency may be an important pathological mechanism in EHPs, but its regulatory mechanism is still unclear.

Therefore, based on the clinical epidemiological analysis of whether Yang-deficiency is an important pathogenesis of EHPs, this study aims to investigate the role of the gut microbiota in susceptibility to EHPs in individuals with a Yang-deficiency constitution. After physical dis-

crimination and hysteroscopic surgical examination, the subjects were divided into two groups: Yang deficiency with EHPs (YEH) and non-Yang deficiency with EHPs (NYEH). The fecal samples were collected before surgery, and the composition of the intestinal flora was checked by sequencing the 16S rRNA V3-V4 region. Two constitution survey data and a differential gut microbiota dataset were also obtained from the literature for further analysis. These findings lay the foundation for the treatment of EHPs, while also providing a theoretical basis for preventing people with Yang deficiency from developing EHPs.

Materials and methods

Patients

The study was conducted at the First Affiliated Hospital of Kunming Medical University in Kunming, China, from September 2021 to December 2023, with approval from the local ethics committee (No. KMMU2020MEC028). Patients received both oral and written information about the study's objectives, methods, materials, and their rights and responsibilities, and provided written consent prior to data collection.

The inclusion criteria for this study were as follows: (1) Patients who underwent an ultrasound examination between 3 to 7 days after menstruation, which revealed uterine endometrial thickening. (2) Patients aged 18-70 years who were capable of communication, willing to participate in the study, and able to give informed consent. The exclusion criteria included patients with conditions known to affect the composition of intestinal flora, such as hypertension, cancer, or diarrhea on the day of sampling. Additionally, patients who had taken medications that could potentially impact the composition of intestinal flora, including metformin, liraglutide, antibiotics, or Chinese herbal medicine, within the three months preceding the collection of fecal samples, were also excluded.

The Traditional Chinese Medicine (TCM) constitution of participants was predicted and confirmed by a TCM clinical doctor using the Chinese Traditional Medicine Association standard (ZYYXH/T157-2009) [14]. In the section on identifying the Yang deficiency constitution,

the standard includes 7 questions (Table S1) that we used to develop a questionnaire for patients to answer. We assigned scores to each answer choice in each question, ranging from "No" to "Always" as 1 to 5 points, respectively. The sum of the scores for all 7 questions was used as the initial score, and the transformation score was calculated using the following formula: Conversion score = [(Raw score - Number of items)/(Number of items × 4)] × 100. We calculated the original score and recorded corresponding conversion score. The conversion score is used to determine whether a volunteer belongs to the yang deficiency constitution. For volunteers with a conversion score between 30-39, an experienced traditional Chinese medicine doctor made a further determination based on the characteristics of individuals with yang deficiency constitution (Table S2).

Hysteroscopy examinations were performed according to standard procedures, and patients without endometrial hyperplasia or with other uterine diseases were excluded. The subjects were then divided into two groups based on examination results: Yang deficiency constitution subjects with endometrial hyperplastic processes (YEH) and non-Yang deficiency constitution subjects with endometrial hyperplastic processes (NYEH). We also downloaded a dataset of population constitution survey in Jilin province, China [15], and a dataset of physical constitution assessment on healthy reproductive-aged women in Shanghai, China [4]. These datasets were merged with the data in our study for risk analysis of endometrial hyperplasia in individuals with a predisposition to Yang deficiency constitution. SPSS Statistics is used for chi-square analysis and risk analysis.

Fecal sample collection, processing, RNA sequencing and data processing

A fasting fecal sample was collected between 6:00-9:00 am within 24 hours of a vaginal ultrasound and immediately stored at -80°C. After applying the exclusion criteria, we obtained only 3 stool samples from patients within the NYEH group. Therefore, we selected 8 stool samples from patients within the YEH group, matching them based on age and BMI.

DNA was extracted from the 11 fecal samples using stool collection kits. The V3-V4 region of the 16S rRNA gene was amplified with the Primer set 341 F/806 R to determine the gut bacterial community structure [16]. The amplified products were sequenced using the Illumina MiSeq platform at Novogene Bioinformatics Technology Co., Ltd., Tianjin, China. To obtain clean reads, we used the Cutadapt software (V1.9.1) and UCHIME software to remove barcodes and primers, low-quality reads, and chimera sequences [17-19]. The original sequencing data has been submitted to the NCBI Sequence Read Archive (accession number: PRJNA 1027096). The high-quality reads were analyzed using QIIME (Version 2.15.3) [20]. Similarities among samples between the groups were estimated using principal coordinates analysis plots (PCoA) and displayed using the ggplot2 package in R software (Version 2.15.3). The significant bacterial taxa with differential abundance between groups were identified by the linear discriminate analysis (LDA) effect size (LEfSe) method [21], with a LDA threshold value of 2.0 and MetaStats at 95% confidence interval, respectively. A Venn diagram was generated by VennDiagram package in R. Additionally, we obtained differential flora information between yang deficiency and non-yang deficiency subjects without significant organic lesions from literature [14].

The collection and bioinformatics analysis of EHPs, inflammation, estrogen, and differential flora-related genes

Bioinformatic analysis was conducted to investigate the role of differential flora in endometrial hyperplasia (EHPs). Differentially expressed genes (DEGs) associated with differential flora, estrogen, and treatments that promote or inhibit endometrial growth were downloaded from the Gene Expression Omnibus (GEO) database. The GEO2R tool was used to analyze gene differential expression. $P < 0.05$ and $|logFC| > 0.6$ were considered significant. We took the intersection of differential gene data related to the same differential flora or treatment measures and obtained the union of differentially expressed genes with similar expression trends in two or more datasets. This was considered as the set of differential genes associated with the differential flora or treat-

ment measures. Genes with the same expression trend in the same treatment were combined, and the final logFC value was averaged. Common DEGs with opposite expression trends among the treatment measures to promote and inhibit endometrial growth were then screened.

Additionally, we searched GeneCards (https://www.genecards.org/) for "endometrial hyperplasia processes" and "inflammation" and screened for genes related to EHPs and inflammation with a correlation coefficient score cutoff > 5.0. The identified EHPs-related genes were then compared to genes showing opposite expression trends in growth-promoting and growth-inhibiting measures. Although no available DEGs dataset specifically related to EHPs in GEO, it is known that oral administration of estrogen and tamoxifen promotes endometrial growth [22], while progestin administration or the use of progestin-releasing intrauterine devices inhibits abnormal endometrial growth to some extent [23, 24]. These genes not only exhibit opposite expression trends in measures of proliferation promotion and inhibition but are also associated with EHPs, suggesting their potential importance in endometrial hyperplasia and defining them as EHPs-related genes (EHPs).

After that, protein interaction analysis on differential genes from EHPs, inflammation, and the different flora was performed using String (https://string-db.org/) [25]. We first performed an intersection analysis of the three gene datasets related to EHPs, inflammation, and estrogen. Due to a large number of genes associated with inflammation and estrogen, and the initial analysis showed low interaction values for only these genes, indicating that they are not core genes, we removed them in the final analysis. The remaining intersected genes were combined with the EHPs-related dataset to create a gene list. Since the String database (https://string-db.org/) cannot display genes without corresponding proteins, and the maximum input limit in String is 2000 genes, we first copied the gene list into the "List of Names" box in String, converting gene names to protein names, and removed gene loci that do not have corresponding proteins in String. Then, we performed an interaction analysis by selecting "multiple proteins" and choosing "Homo sapiens" in the "Orga-

nisms" section. The resulting interaction network was exported as a "short tabular text output".

Next, we took the intersection of the differential flora-related gene list with the EHPs, inflammation, and estrogen-related gene datasets once again. Similarly, due to a large number of genes associated with inflammation, estrogen, and the specific microbial species *Lactobacillus rhamnosus*, and the initial analysis showed low interaction values for these genes, indicating that they are not core genes, we removed them during the interaction analysis. Then, we performed an interaction analysis between the differential microbial community-related proteins and the EHPs, inflammation, and estrogen-related proteins.

The interaction results were then visualized using Cytoscape [26]. We created grouping information lists for the protein interactions from the two analysis steps mentioned above. The protein grouping information tables, along with the String interaction results, were imported into Cytoscape to visualize the interaction analysis results.

Results

Epidemiological statistics

In patients with endometrial hyperplastic processes (EHPs), the proportion of a Yang deficiency constitution is higher than those with a non-Yang deficiency constitution. In this study, a total of 537 patients with possible endometrial thickening and endometrial polyps detected by B-ultrasound were included. Patients who had cancer or had taken antibiotics within the past three months, as well as those experiencing diarrhea, were excluded. After further TCM constitution identification and hysteroscopy and histopathological examination, 202 patients were diagnosed with a EHPs (including endometrial polyps, polypoid endometrial hyperplasia, simple hyperplasia, and complex hyperplasia) (**Figure 1**). Among them, 147 patients had a Yang deficiency constitution, accounting for 72.8%, while 55 patients have a non-Yang deficiency constitution, accounting for 27.2% (**Figure 2**). In addition, we downloaded two sets of data on the identification of constitution types in the Chinese population. One set came from a population-based survey on
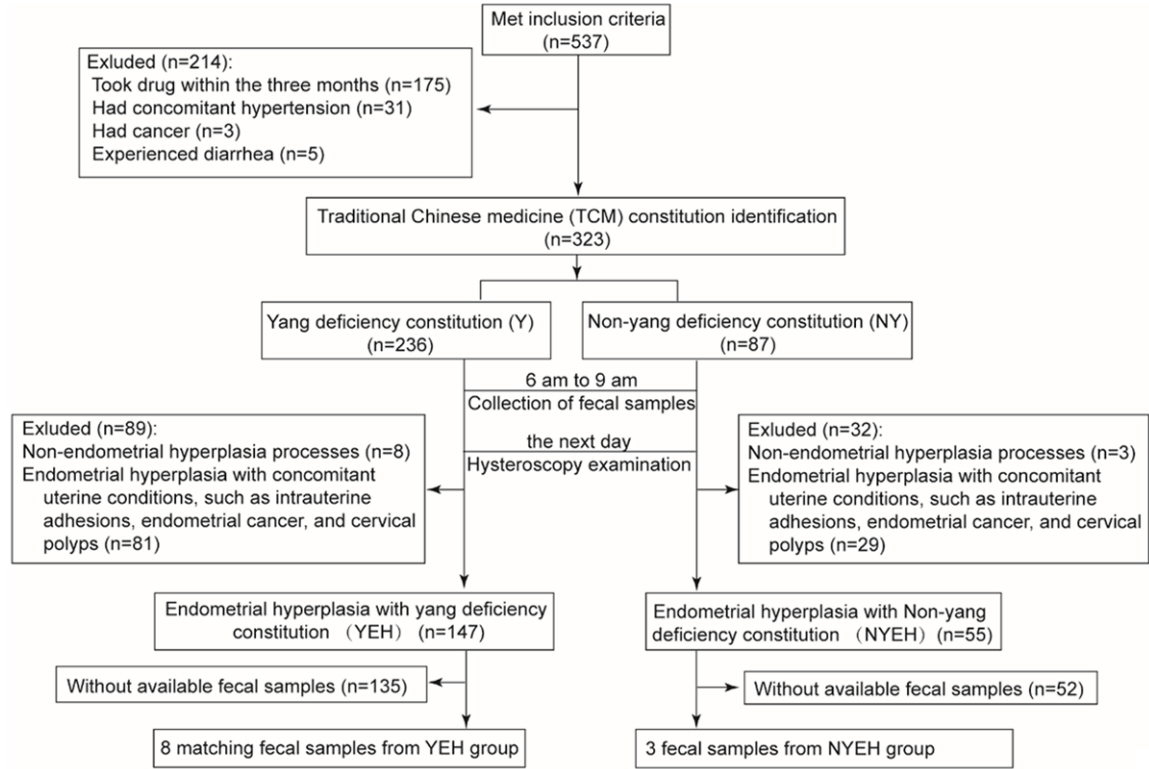
Figure 1. Study design of sample collection. Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; NYEH, non-Yang deficiency subjects with endometrial hyperplasia processes.
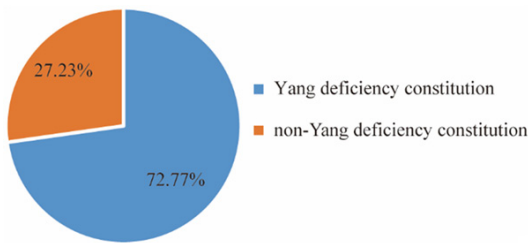

Figure 2. Proportion of Yang-deficiency constitution in patients with endometrial hyperplastic processes.

constitution types in Jilin Province, China [15], which showed that out of 1755 volunteers, 177 had a Yang deficiency constitution. The other set came from a survey on constitution types among 708 healthy women of childbearing age in Shanghai [4], which indicated that out of 724 healthy volunteers, 163 had a Yang deficiency constitution. Combining these two datasets with our study data, the results showed that compared to the general population and healthy women of childbearing age, the proportion of patients with Yang deficiency constitution among EHPs is much higher than

that of non-Yang deficiency constitution ($\chi^2_{general\ population}$ = 515.277, P < 0.001; $\chi^2_{healthy\ women}$ = 173.178, P < 0.001). A yang deficiency constitution may be a risk factor for EHPs ($\chi^2_{Compared\ to\ population\ survey}$ = 515.277, < 0.001; $\chi^2_{Compared\ to\ healthy\ women\ of\ childbearing\ age}$ = 173.178, < 0.001) (**Table 1**).

Subject groups and fecal sample collection

Eight fecal samples were collected from Yang deficiency subjects with endometrial hyperplasia processes (YEH), while three fecal samples were collected from non-Yang deficiency subjects with endometrial hyperplasia processes (NYEH). The age and body mass index (BMI) of the two groups were matched, and their distribution was not significantly different (**Tables 2**, S3).

Fecal sample sequencing quality control

A total of 757,776 reads were generated from the 11 samples using the v3-v4 16S rRNA sequencing method. After filtering and merging, 753,021 high-quality reads were used for con-

Table 1. Analysis of the proportion of Yang deficiency constitution in patients with endometrial hyperplastic processes and the risk of endometrial hyperplastic processes in subjects with Yang deficiency constitution

	EHPs (n=202)	Population physical survey data from Jilin, China (n=1755)	Healthy women of childbearing age from Shanghai, China (n=708)
Yang-deficiency constitution	147	177	163
Non-Yang-deficiency constitution	55	1578	545
χ^2		515.277	173.178
P		< 0.001	< 0.001
OR, 95% CI		13.471, 10.121-17.930	5.173, 3.918-6.830

Abbreviations: EHPs, endometrial hyperplastic processes; OR, odd ratio; 95% CI, 95% confidence interval.

Table 2. Demographic characteristics of participants

Characteristic	YEH (n=8)	NYEH (n=3)	P value
Age (m ± sd)	52.6±14.2	50.3±7.2	0.838
BMI (m ± sd)	25.1±5.2	22.5±2.5	0.54

Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; NYEH, non-Yang deficiency subjects with endometrial hyperplasia processes.

structing OTUs. The rarefaction curves indicated that the sequencing depth was sufficient and the amount of data was reasonable and the species accumulation curve showed that sample size was adequate for capturing species richness (Figure S1A, S1B).

The microbiota complexity of the YEH and NYEH groups

The Venn diagram analysis revealed that there were 701 common OTUs between the two groups (**Figure 3A**). The rank abundance curve indicated similar species richness and evenness of microbial communities (**Figure 3B**). The α-diversity, as measured by observed species, Shannon, Simpson, chao1, ace, and goods coverage indices, was also similar between the two groups (P > 0.05) (Table S4; **Figure 3C**). Furthermore, the β-diversity, based on weighted UniFrac distances, did not show any significant differences between the microbial communities of the YEH and NYEH groups. This was supported by the principal coordinates analysis (PCoA), which did not reveal any distinct separation between the groups (**Figure 3D**).

Significant differential floras between the YEH and NYEH groups

Metastats and linear discriminant analysis effect size (LEfSe) analyses were conducted to identify different bacterial taxa between the two groups. Results showed that 1 phylum, 3 classes, 6 orders, 16 families, 28 genera, and 13 species were enriched in the YEH group, while 1 family, 2 genera, and 1 species were enriched in the NYEH group (log10 LDA score > 2.0 and/or P < 0.05) (Table S5). Among them, the g_*Allobaculum*; g_*Alloprevotella*; g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*; g_*Anaerotruncus*; g_*Bradyrhizobium*; g_Christensenellaceae_R-7_group; g_Defluviitaleaceae_UCG-011; g_*Eggerthella*; g_*Faecalibaculum*; g_Family_XIII_AD3011_group; g_*Frisingicoccus*; g_Lachnospiraceae_UCG-003; g_*Lacticaseibacillus*; g_*Methylobacterium-Methylorubrum*; g_*Mycobacterium*; g_NK4A214_group; g_*Paraprevotella*; g_*Parasutterella*; g_Prevotellaceae_NK3B31_group; g_Prevotellaceae_UCG-001; g_*Pseudoflavonifractor*; g_*Sellimonas*; g_*Sphingobium*; g_*Staphylococcus*; g_UBA1819; g_UCG_005; g_UCG-009; g_*Veillonella*; s_*Alistipes obesi*; s_*Alistipes shahii*; s_bacterium_YE57; s_*Bifidobacterium animalis*; s_*Bradyrhizobium elkanii*; s_Clostridiaceae_bacterium_DJF_VR76; s_*Lactobacillus mucosae*; s_*Lactobacillus rhamnosus*; s_*Megasphaera micronuciformis*; s_*Parabacteroides merdae*; s_*Parabacteroides* sp.; s_*Sutterella wadsworthensis* were *up-regulated in* YEH, while the g_*Parvimonas*; g_*Tyzzerella*; s_*Blautia obeum* were *up-regulated in NYEH*.

Comparison of gut microbiota differences between Yang deficiency individuals with EHPs and Yang deficiency individuals with no apparent organic lesions

A Venn diagram was used to compare different bacterial taxa between Yang deficiency individuals with EHPs and with no apparent organic lesions from our previous study [14] to identify
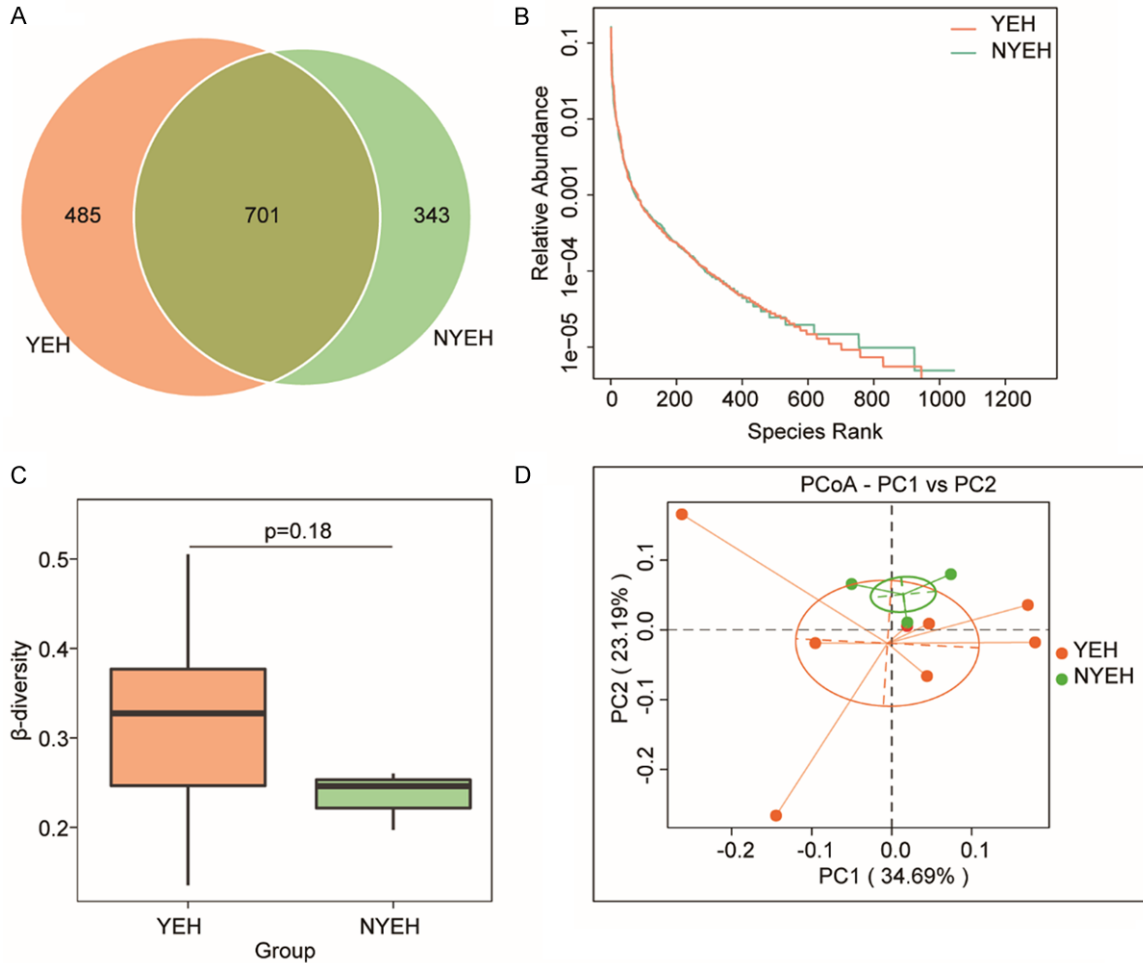
Figure 3. The gut microbiota profile comparison between the YEH and NYEH groups. A. The common OTUs between the two groups were analyzed using Venn diagrams. B. The species richness and evenness of microbial communities between the two groups were demonstrated by the rank abundance curve. C. The β-diversity between the two groups was analyzed based on weighted UniFrac distances. D. Principal coordinate analysis (PCoA) was conducted using weighted UniFrac distances to compare the gut microbiota profiles between the two groups. Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; NYEH, non-Yang deficiency subjects with endometrial hyperplasia processes; OTUs, Operational Taxonomic Units.

important flora associated with morbidity (**Figure 4**). The c_Thermoleophilia and o_Solirubrobacterales were upregulated in both groups, while the *Akkermansia muciniphila* species was upregulated in Yang deficiency individuals with EHPs but downregulated in those without apparent organic lesions (**Table 3**).

Bioinformatics analysis of inflammation-related genes, estrogen-related genes, and EHPs-related genes

From the GEO database, we collected multiple datasets on differentially expressed genes related to endometrial growth. These datasets include tamoxifen, estrogen, and estradiol, which promote endometrial growth, and the levonorgestrel-releasing intrauterine system, levonorgestrel, etonogestrel, medroxyprogesterone acetate, depot medroxyprogesterone acetate, and progesterone, which inhibit endometrial growth to some extent. Additionally, we collected datasets on differential flora (Table S6).

We identified a total of 1700 common DEGs associated with endometrial growth inhibition and 2678 common DEGs associated with endometrial growth promotion (Tables S7, S8).
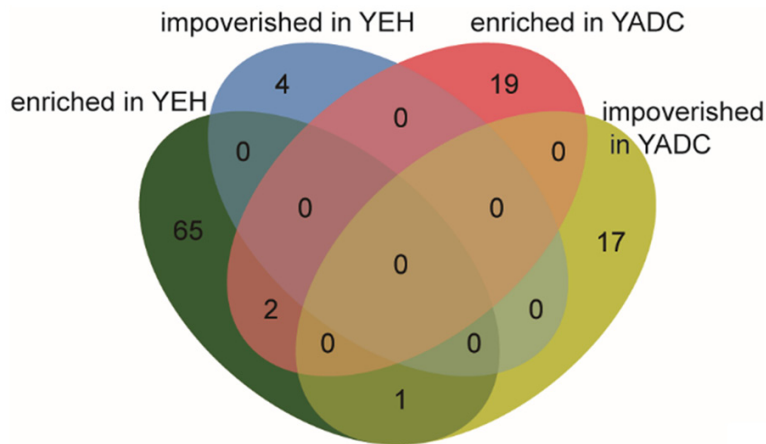
Figure 4. The gut microbiota profiles were compared between YEH and YADC subjects. Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; YADC, the Yang deficiency subjects with no significant organic lesions were observed.

Furthermore, we identified 12 common DEGs for *A. muciniphila*, 11 common DEGs for *B. animalis*, 1730 common DEGs for *L. rhamnosus*, and 23 common DEGs for *P. merdae* (Tables S9, S10, S11, S12). Additionally, we identified 241 DEGs associated with estrogen (Table S13). Among these DEGs, 249 showed opposite expression trends between promoting and inhibiting endometrial growth (Table S14). We also identified 1970 genes related to endometrial hyperplasia processes (EHPs) and 592 inflammation-related genes from GeneCards (Tables S15, S16).

Furthermore, we found that 30 EHPs-related DEGs exhibited opposite expression trends between promoting and inhibiting endometrial growth (Table S17). The inflammation-related genes and estrogen-related DEGs have 12 and 2 common genes with EHPs-related genes, respectively (**Figure 5A**). Similarly, the *L. rhamnosus*-related DEGs and *B. animalis*-related DEGs have 8 and 1 common gene with EHPs-related genes, respectively (**Figure 5B**). Compared to estrogen, EHPs may be more associated with inflammation, while compared to *B. animalis* and other flora, EHPs may be more associated with *L. rhamnosus.*

There is a close interaction between inflammation, estrogen, and EHP-related DEGs, as shown in **Figure 5C**. Among these interactions, *TNF* and *EGFR* appear to be the core genes in EHPs due to their high interaction values.

There is also a close interaction between different flora-related DEGs and EHP-related DEGs, as depicted in **Figure 5D**. This suggests that changes in the microbiota may be involved in endometrial hyperplastic processes. Specifically, *L. rhamnosus* directly regulates the core genes TNF, while *B. animalis* and *A. muciniphila* indirectly regulate these core genes. It is worth noting that the regulation of TNF expression by *L. rhamnosus* is consistent with the regulation of TNF expression in inhibiting endometrial growth. This suggests that *L. rhamnosus* may have a beneficial effect on endometrial hyperplastic processes and could be a potential research direction for the prevention and treatment of such conditions.

Discussion

The nature of the disease of endometrial hyperplastic processes (EHPs) is that the blood which is deposited and blocked results in abdominal mass gradually [27]. Yang deficiency constitution can lead to symptoms such as Qi stagnation and blood stasis, suggesting a possible association between Yang deficiency constitution and the onset of EHPs. Our epidemiological statistics show that compared to general population constitution survey data and data on healthy couples of reproductive age, the proportion of Yang deficiency constitution is significantly higher in EHPs patients. Yang deficiency constitution poses a higher risk for developing EHPs compared to non-Yang deficiency constitution.

In the past few years, there has been a growing focus on the potential influence of the gut microbiota on human health and well-being [28]. In this study, we analyzed the gut microbiota composition of the YEH and NYEH groups and found that both groups have similar levels of microbiota complexity. This similarity may be attributed to the presence of similar diseases, despite belonging to different constitutions.

Table 3. The differentially expressed flora among groups

Different bacterial taxa	The trend of bacterial community changes
p_unidentified_Bacteria; c_Alphaproteobacteria; c_Clostridia; o_Burkholderiales; o_Christensenellales; o_Micrococcales; o_Peptococcales; o_Rhizobiales; f_[Eubacterium]_coprostanoligenes_group; f_67-14; f_Beijerinckiaceae; f_Christensenellaceae; f_Defluviitaleaceae; f_Erysipelotrichaceae; f_Microbacteriaceae; f_Muribaculaceae; f_Mycobacteriaceae; f_Nocardioidaceae; f_Oscillospiraceae; f_Peptococcaceae; f_Rhizobiaceae; f_Sphingomonadaceae; f_Sutterellaceae; f_UCG-010; f_Xanthobacteraceae; g_*Allobaculum*; g_*Alloprevotella*; g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*; g_*Anaerotruncus*; g_*Bradyrhizobium*; g_Christensenellaceae_R-7_group; g_Defluviitaleaceae_UCG-011; g_*Eggerthella*; g_*Faecalibaculum*; g_Family_XIII_AD3011_group; g_*Frisingicoccus*; g_Lachnospiraceae_UCG-003; g_*Lacticaseibacillus*; g_*Methylobacterium-Methylorubrum*; g_*Mycobacterium*; g_NK4A214_group; g_*Paraprevotella*; g_*Parasutterella*; g_Prevotellaceae_NK3B31_group; g_Prevotellaceae_UCG-001; g_*Pseudoflavonifractor*; g_*Sellimonas*; g_*Sphingobium*; g_*Staphylococcus*; g_UBA1819; g_UCG_005; g_UCG-009; g_*Veillonella*; s_*Alistipes obesi*; s_*Alistipes shahii*; s_bacterium_YE57; s_*Bifidobacterium animalis*; s_*Bradyrhizobium elkanii*; s_Clostridiaceae_bacterium_DJF_VR76; s_*Lactobacillus mucosae*; s_*Lactobacillus rhamnosus*; s_*Megasphaera micronuciformis*; s_*Parabacteroides merdae*; s_*Parabacteroides* sp.; s_*Sutterella wadsworthensis*	Elevated in YEH (65)
f_Family_XI; g_*Parvimonas*; g_*Tyzzerella*; s_*Blautia obeum*	Reduced in YEH- (7)
c_Thermoleophilia; o_Solirubrobacterales	Elevated in YEH and YADC (2)
s_*Akkermansia muciniphil*a	Elevated in YEH while reduced in YADC (1)

Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; YADC, the Yang deficiency subjects with no significant organic lesions were observed; p, phylum; c, class; o, order; f, family; g, genus; s, species.
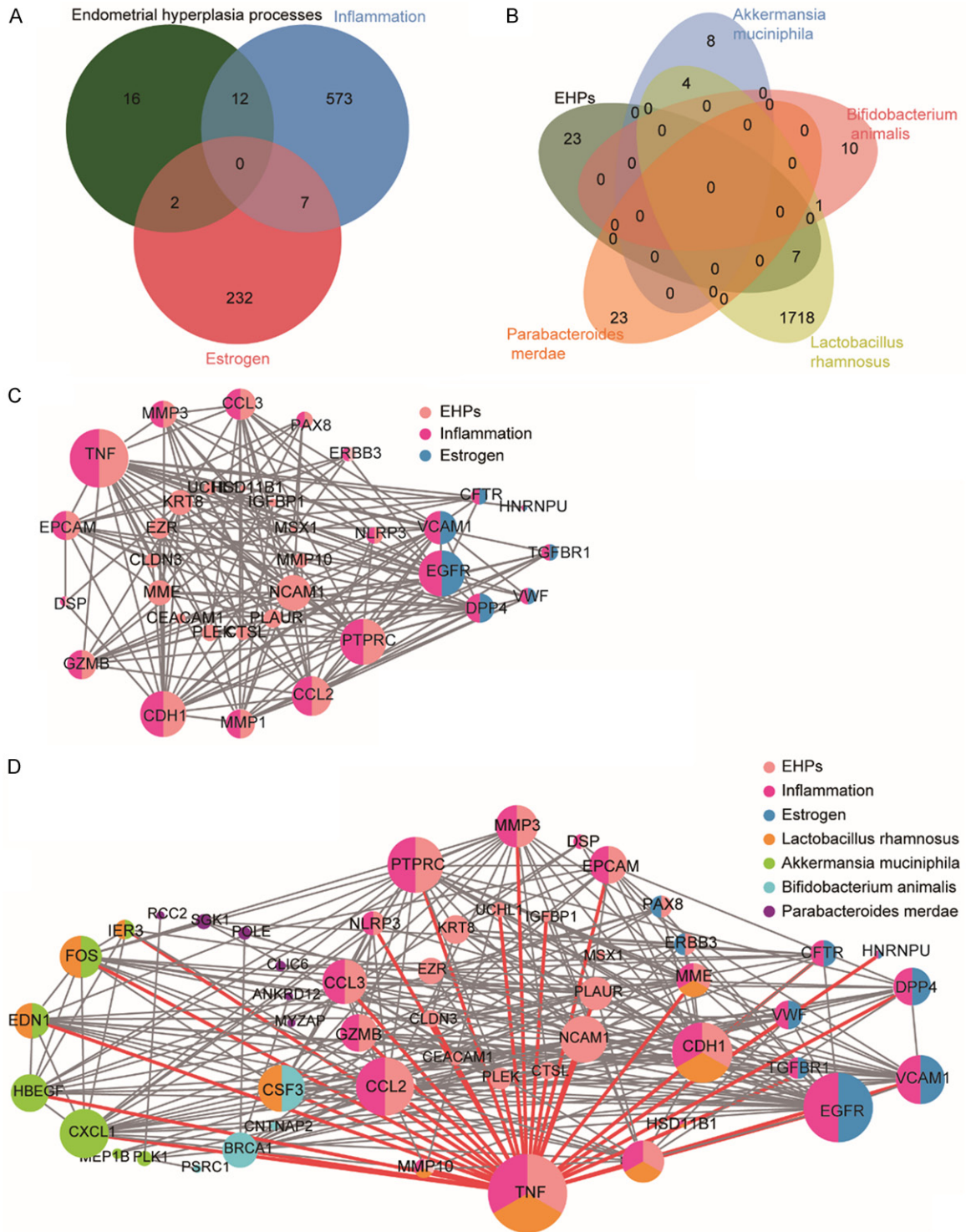
Figure 5. Bioinformatic analysis of EHPs, inflammation, estrogen, and differential flora-related genes. A. A Venn diagram was used to analyze the common genes among estrogen-related DEGs, inflammation-related genes, and endometrial hyperplasia process-related DEGs. B. A Venn diagram was used to analyze the common genes among endometrial hyperplasia process-related genes, *Akkermansia muciniphila*-related DEGs, *Lactobacillus rhamnosus*-related DEGs, *Bifidobacterium animalis*-related DEGs, and *Parabacteroides merdaes*-related DEGs. C. Interaction analysis between inflammation-related genes, estrogen-related genes, and EHP-related genes. D. Interaction analysis between inflammation-related genes, estrogen-related genes, EHP-related genes, and differentially abundant flora-related genes. Abbreviations: DEGs, differentially expressed genes; EHPs, endometrial hyperplasia process-related DEGs; Inflammation, inflammation-related genes; Estrogen, estrogen-related DEGs.

However, there were more upregulated microbial communities in the YEH group. Among them, *Pseudoflavonifractor*, *Paraprevotella*, Prevotellaceae_UCG-00131, *Veillonella* and S*ellimonas* were associated with depression [29-33], while *Parasutterella* was linked to psychological stress [34]. Additionally, *Anaerotruncus* was linked to endometriosis, and the species *Parabacteroides merdae* was associated with chronic fatigue syndrome [35, 36]. The species *Alistipes obesi* and *Alistipes shahii*, both belonging to the *Alistipes* genus, were associated with chronic fatigue syndrome [37]. It is worth noting that all of these diseases exhibit inflammatory features [38-41]. Furthermore, opportunistic pathogens such as *Ruminococcaceae_NK4A214_group*, Christensenellaceae_R-7_group, *Anaerotruncus*, *Mycobacterium* and the species *Megasphaera micronuciformis* were positively correlated with pro-inflammatory cytokines and inflammation [42-46].

The presence of the genera *Methylobacterium-Methylorubrum*, *Staphylococcus Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Veillonella*, was found to be related to abnormal proliferation-related diseases [46-48]. The abundance of the genera *Parasutterella*, Prevotellaceae_UCG-001, and the species *Bifidobacterium animalis*, was positively correlated with estradiol and tamoxifen treatment [49-51]. The upregulation of these flora related to inflammation and estrogen levels, both of which may play essential roles in the occurrence of endometrial hyperplasia and polyps [52, 53]. Therefore, inflammation-related and abnormal proliferation-related gut flora were elevated in the YEH group, which may be involved in the occurrence and development of EHPs.

Nonetheless, the genera Defluviitaleaceae_UCG-011 and species *Allobaculum* sp., *Lactobacillus mucosae*, *Lactobacillus rhamnosus*, and *Akkermansia muciniphila*, which have been reported to have anti-inflammatory functions [54-58], were also found to be elevated in the YEH group. However, due to their relatively small proportion, their beneficial effects may not be evident in the overall outcome of the study.

The Thermoleophilia and Solirubrobacterales are common upregulated flora in Yang deficiency with EHPs and Yang deficiency with no apparent organic lesions. The Thermoleophilia was the predominant class in the nasal microbiota of amyotrophic lateral sclerosis patients [59], which is accompanied by both neuroinflammation, systemic inflammation, and dysregulated peripheral immunity [60, 61]. Additionally, the presence of Thermoleophilia is positively associated with proteinuria [62], which promotes interstitial inflammation [63]. The flora belonging to the Solirubrobacterales order found so far consists of soil microorganisms that are related to the degradation of harmful compounds, such as 3,5-dichloroaniline [64] and polycyclic aromatic hydrocarbons [65, 66]. This suggests that there may be substances harmful to the health of individuals with a Yang deficiency constitution. On the other hand, *A. muciniphila* is downregulated in Yang deficiency with no apparent organic lesions, but upregulated in the Yang deficiency with EHPs. The *A. muciniphila* is known for its anti-inflammatory and immunoregulatory effects, with potential therapeutic benefits for various diseases [67, 68]. However, it is downregulated in individuals with Yang deficiency without organic lesions, potentially hindering its beneficial effects. These changes in intestinal flora and their functions contribute to the susceptibility of individuals with Yang deficiency constitution to diseases. Despite the upregulation of *A. muciniphila* in Yang deficiency subjects with EHPs, the presence of other more pro-inflammatory bacteria may neutralize its anti-inflammatory effect.

Bioinformatic analysis revealed that EHPs share common genes with inflammation and estrogen, and these genes exhibit close interactions, indicating the association of EHPs with both inflammation and estrogen. However, compared to estrogen-related genes, EHPs-related genes show a higher number of shared genes with inflammation-related genes, suggesting a stronger correlation between EHPs and inflammation. This is consistent with the finding of a higher abundance of upregulated inflammation-related microbial species in the gut microbiota. Among the differentially abundant microbial species, EHPs show a greater number of genes associated with *L. rhamnosus*, indicating a closer relationship between EHPs and *L. rhamnosus* compared to other differentially abundant species. Within these

genes, TNF is associated with EHPs, inflammation, and the gut microbiota *L. rhamnosus*, while EGFR is associated with inflammation and estrogen. *TNF* (tumor necrosis factor) is a critical cytokine involved in systemic inflammatory reactions, stimulating the production of other inflammatory cytokines and chemokines, and inducing necrosis and apoptosis in tumor cells [69]. *EGFR* (epidermal growth factor receptor) is a member of the ERBB family of tyrosine kinase receptors and plays a crucial role in regulating cell proliferation, differentiation, division, survival, and cancer development [70]. Therefore, these core genes may play important roles in the development of EHPs.

Conclusion

Yang deficiency constitution may be a risk factor for the occurrence and development of EHPs. The inflammation and estrogen-related intestinal flora of EHPs patients with Yang deficiency were significantly higher than those without Yang deficiency constitution and no obvious organic lesions. These bacteria regulate host genes, such as inflammation-related TNF and estrogen-related EGFR, and thus may play a role in the development of EHPs. Unfortunately, there is a lack of reports of differentially expressed genes associated with harmful flora, which limits the exploration of the molecular mechanisms by which these microbial communities contribute to disease development. Surprisingly, the regulatory trend of TNF gene expression in *L. rhamnosus* is similar to that of inhibiting endometrial proliferation, suggesting that *L. rhamnosus* may be a beneficial microflora for the prevention and treatment of EHPs, but the beneficial effect may not be significant due to the presence of more pro-inflammatory bacteria in patients. However, it also provides a research direction for the treatment of EHPs.

Our study had a small sample size and an uneven distribution between groups, which may impact the accuracy of the conclusions. However, we conducted bioinformatic analysis of differential genes related to intestinal flora from a large sample to improve the reliability of the results. Furthermore, the merging of the proportion data on Yang deficiency constitution from the literature with the data from this study for epidemiological statistical analysis could not match basic characteristics such as age and BMI index, which may have had a certain impact on the formation of conclusions. Future studies should consider larger sample sizes and animal models to further validate these findings and explore their underlying mechanisms.

Disclosure of conflict of interest

None.

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Proposed cause of endometrial hyperplastic processes in Yang deficiency subjects

Table S1. Questionnaire and criteria for identifying yang deficiency constitution

Can you please answer the question based on your experiences and feelings over the past year?	No (absolutely not)	Very few (a little bit)	Sometimes (some)	Frequently (quite)	Always (very)
(1) Do you feel cold in your hands and feet?	1	2	3	4	5
(2) Do you feel cold in the epigastric, back, or lumbar region?	1	2	3	3	3
(3) Do you feel more sensitive to cold and wear more clothes than others?	1	2	3	3	3
(4) Do you have a lower tolerance for cold than most people (such as in cold winter, air conditioning, or fans in summer)?	1	2	3	3	3
(5) Are you more prone to catching a cold than others?	1	2	3	3	3
(6) Do you feel uncomfortable or afraid of consuming cold food or drinks?	1	2	3	3	3
(7) Do you experience diarrhea or stomach discomfort after getting cold or consuming cold food or drinks?	1	2	3	3	3

Note: 1. Original score = Sum of individual item scores. 2. Conversion score = [(Raw score - Number of items)/(Number of items * 4)] × 100. 3. If the conversion score is greater than or equal to 40, it is determined to be a yang deficiency constitution. 4. If the conversion score is between 30-39 points, it may indicate a yang deficiency constitution. 5. If the conversion score is less than 30 points, it is considered a non-yang deficiency constitution.

Table S2. Diagnostic standards for the Yang deficiency constitution

	Yang deficiency
Main characteristics	Cold intolerance
	Cold hands, feet, stomach, and waist
Secondary characteristics	Calm and introverted personality
	Feeling cold easily, low energy, slow and deep pulse.
	Prefer hot food and drinks
	Susceptible to cold
	Watery stool
	Fat
	Whitish skin
	Nocturia
	Tender and pale tongue

Proposed cause of endometrial hyperplastic processes in Yang deficiency subjects

Table S3. Characteristics of participants in this study (N=8)

Sample ID	Age	Constitution type	Pathological examination results	Group	Height (m)	Weight (kg)	BMI (kg/m^2)
YEH1	59	Yang deficiency consitution	Endometrial polyps, complex hyperplasia with atypia	YEH	1.58	50	20.03
YEH2	35	Yang deficiency consitution	Endometrial polyps	YEH	1.63	48	18.07
YEH3	46	Yang deficiency consitution	Endometrial hyperplasia with endometrial polyps	YEH	1.58	61	24.44
YEH4	44	Yang deficiency consitution	Endometrial polyps	YEH	1.50	49	21.78
YEH5	72	Yang deficiency consitution	Endometrial hyperplasia	YEH	1.48	67	30.59
YEH6	74	Yang deficiency consitution	Endometrial complex hyperplasia with focal atypical hyperplasia	YEH	1.50	75	33.33
YEH7	45	Yang deficiency consitution	Endometrial simple hyperplasia with polypoid growth	YEH	1.55	67	27.89
YEH8	46	Yang deficiency consitution	Endometrial hyperplasia with focal atypical glandular hyperplasia	YEH	1.56	60	24.65
NYEH1	54	non-Yang deficiency consitution	Endometrial polyps	NYEH	1.50	48	21.33
NYEH2	42	non-Yang deficiency consitution	Endometrial simple hyperplasia with endometrial polyps	NYEH	1.55	50	20.81
NYEH3	55	non-Yang deficiency consitution	Endometrial hyperplasia	NYEH	1.55	61	25.39

Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; NYEH, non-Yang deficiency subjects with endometrial hyperplasia processes.

Table S4. The α-diversity was assessed using observed species, Shannon, Simpson, Chao1, ACE, and goods coverage indices to compare the diversity between the two groups

	Observed_species	Shannon	Simpson	Chao1	Ace	Goods_coverage	PD_whole_tree
YEH (m±s)	465.6±125.1	4.8±0.7	0.89±0.05	492.6±134.4	499.6±135.2	0.999+0.0003	46.3±16.0
NYEH (m±s)	558.0±32.1	5.2±0.1	0.92±0.003	594.7±34.4	602.0±38.4	0.999±0.0002	51.3±16.6
p value	0.22	0.22	0.22	0.22	0.22	0.18	0.84

Abbreviations: YEH, the Yang deficiency subjects with endometrial hyperplasia processes; NYEH, non-Yang deficiency subjects with endometrial hyperplasia processes; m, Mean value; s, standard deviation.

Proposed cause of endometrial hyperplastic processes in Yang deficiency subjects

Table S5. Significantly different bacterial taxa identified by Metastats and LefSe analysis at phylum, class, order, family, genus, and species levels between YEH and NYEH

Taxa	level	Significant level	Identify method	Enrichment
f__Family_XI	family	#	LEfSe	0
g__Parvimonas	genus	#	LEfSe	0
g__Tyzzerella	genus	#	LEfSe	0
s__Blautia_obeum	species	*#	Metastats/LEfSe	0
p__unidentified_Bacteria	plylum	*	Metastats	1
c__Alphaproteobacteria	calss	*	Metastats	1
c__Clostridia	calss	*	Metastats	1
c__Thermoleophilia	calss	*	Metastats	1
o__Burkholderiales	order	*	Metastats	1
o__Christensenellales	order	**#	Metastats	1
o__Micrococcales	order	*	Metastats	1
o__Peptococcales	order	*	Metastats	1
o__Rhizobiales	order	*	Metastats	1
o__Solirubrobacterales	order	*	Metastats	1
f__[Eubacterium]_coprostanoligenes_group	family	**#	Metastats/LEfSe	1
f__67-14	family	*	Metastats	1
f__Beijerinckiaceae	family	*	Metastats	1
f__Christensenellaceae	family	**#	Metastats/LEfSe	1
f__Defluviitaleaceae	family	**#	Metastats/LEfSe	1
f__Erysipelotrichaceae	family	*#	Metastats/LEfSe	1
f__Microbacteriaceae	family	*	Metastats	1
f__Muribaculaceae	family	**	Metastats	1
f__Mycobacteriaceae	family	*	Metastats	1
f__Nocardioidaceae	family	*	Metastats	1
f__Oscillospiraceae	family	*#	Metastats/LEfSe	1
f__Peptococcaceae	family	*	Metastats	1
f__Rhizobiaceae	family	*	Metastats	1
f__Sphingomonadaceae	family	*	Metastats	1
f__Sutterellaceae	family	*	Metastats	1
f__UCG-010	family	*	Metastats	1
f__Xanthobacteraceae	family	*	Metastats	1
g__Allobaculum	genus	*	Metastats	1
g__Alloprevotella	genus	*	Metastats	1
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	genus	*	Metastats	1
g__Anaerotruncus	genus	*	Metastats	1
g__Bradyrhizobium	genus	*	Metastats	1
g__Christensenellaceae_R-7_group	genus	**#	Metastats	1
g__Defluviitaleaceae_UCG-011	genus	**#	Metastats	1
g__Eggerthella	genus	*	Metastats	1
g__Faecalibaculum	genus	*	Metastats	1
g__Family_XIII_AD3011_group	genus	*#	Metastats/LEfSe	1
g__Frisingicoccus	genus	**#	Metastats/LEfSe	1
g__Lachnospiraceae_UCG-003	genus	*	Metastats	1
g__Lacticaseibacillus	genus	*	Metastats	1
g__Methylobacterium-Methylorubrum	genus	*	Metastats	1

g__Mycobacterium	genus	*	Metastats	1
g__NK4A214_group	genus	*	Metastats	1
g__Paraprevotella	genus	**#	Metastats/LEfSe	1
g__Parasutterella	genus	*	Metastats	1
g__Prevotellaceae_NK3B31_group	genus	**	Metastats	1
g__Prevotellaceae_UCG-001	genus	*	Metastats	1
g__Pseudoflavonifractor	genus	*	Metastats	1
g__Sellimonas	genus	*	Metastats	1
g__Sphingobium	genus	*	Metastats	1
g__Staphylococcus	genus	*	Metastats	1
g__UBA1819	genus	*	Metastats	1
g__UCG_005	genus	#	LEfSe	1
g__UCG-009	genus	*	Metastats	1
g__Veillonella	genus	*	Metastats	1
s__Akkermansia_muciniphila	species	*	Metastats	1
s__Alistipes_obesi	species	*	Metastats	1
s__Alistipes_shahii	species	*	Metastats	1
s__bacterium_YE57	species	*	Metastats	1
s__Bifidobacterium_animalis	species	*#	Metastats/LEfSe	1
s__Bradyrhizobium_elkanii	species	*	Metastats	1
s__Clostridiaceae_bacterium_DJF_VR76	species	**	Metastats	1
s__Lactobacillus_mucosae	species	**	Metastats	1
s__Lactobacillus_rhamnosus	species	*	Metastats	1
s__Megasphaera_micronuciformis	species	*	Metastats	1
s__Parabacteroides_merdae	species	*#	Metastats/LEfSe	1
s__Parabacteroides_sp	species	*#	Metastats/LEfSe	1
s__Sutterella_wadsworthensis	species	**	Metastats	1

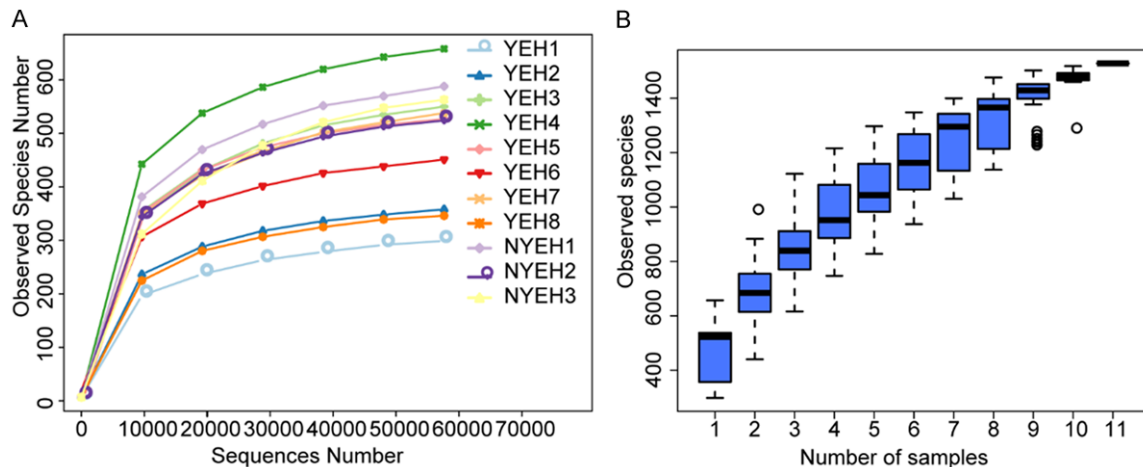Note: *p < 0.05; **p < 0.01; # LDA Score (log 10) > 2.0. 1 = YEH; 0 = NYEH.

Figure S1. Curve charts for samples in this study. A. Rarefaction curves based on the observed species. B. Species accumulation curves. The blue-shaded areas represent confidence intervals of OTUs number, which was determined. Each curve represents one sample.

Table S6. The differentially expressed gene datasets used for bioinformatics analysis in this study

Group	Datasets
Methods to promote endometrial growth	Tamoxifen: GSE106892, GSE3013, GSE14518
	Estrogen: GSE3013, GSE14518, GSE12446 (1)
	Tamoxifen+ estrogen: GSE14518
Methods to inhibit endometrial growth	Levonorgestrel intrauterine syste (LNG-IUS): GSE60129 (1), GSE137765 (1)
	Levonorgestrel-containing oral contraceptive: GSE137765 (2)
	Medorxyprogesterone acetate (MPA): GSE55691 (1), GSE60129 (2)
	Etonogestrol (ETO): GSE55691 (2)
	Progesterone (P): GSE55691 (3)
Akkermansia muciniphila	GSE211506 (1), GSE211506 (2), GSE126730 (1), GSE126730 (2), GSE59644
Bifidobacterium animalis	GSE201075, GSE21930, GSE63531
Lactobacillus rhamnosus	GSE84949 (1), GSE84949 (2), GSE84949 (3), GSE72804 (1), GSE72804 (2), GSE20940 (1), GSE20940 (2), GSE20940 (3), GSE20940 (4), GSE62311(1), GSE62311 (2)
Parabacteroides merdae	GSE88919 (1), GSE88919 (2)

Table S9. Differentially expressed genes related to *Akkermansia muciniphila*

Gene.symbol	logFC
MEP1B	-0.74056821
OTOP2	-0.67806023
EDN1	-0.65471107
PLK1	-6.47E-01
CXCL1	0.60534824
SLC25A25	0.6406248
PLCXD1	0.66350816
PRSS22	0.71493494
YOD1	0.85224912
IER3	0.867574117
FOS	1.13568465
HBEGF	1.45741329

Table S10. Differentially expressed genes related to *Bifidobacterium animalis*

Gene symbol	logFC
CSF3	-2.393778084
TMEM182	-1.777885161
TRIM9	-1.174318055
BAG2	-0.733530429
CNTNAP2	-0.63903156
BRCA1	-0.613215879
PSRC1	-0.605212837
SNORD58B	0.607724955
SNORA61	0.707320904
TMEM144	0.973622086
C8B	2.935571989

Table S12. Differentially expressed genes related to *Parabacteroides merdae*

Gene symbol	logFC
IGH-VJ558	-2.180005858
NLRP9B	-1.076688025
SGK1	-0.918463525
EID3	-0.91317661
PLA2G4C	-0.878965435
ANKRD12	-0.81461154
ZFP991	-0.78085141
ZFP950	-0.766964095
A830080D01RIK	-0.727377545
TAF1D	-0.704520615
MYO9A	-0.658174342
TRDN	-0.646220495
MYZAP	-0.640502115
ADAM4	-0.629779795
DCUN1D5	-0.614755815
PAPPA2	0.648158673
ACPP	0.651200155
GM20831	0.729725196
SSTY1	0.729725196
POLE	0.73599332
RCC2	0.766438735
FRAT1	0.76811958
CLIC6	0.83968738

Proposed cause of endometrial hyperplastic processes in Yang deficiency subjects

Table S17. Differentially expressed genes which might related to endometrial hyperplasia processes

Gene symbol	logFC$_{inhibit}$	logFC$_{promote}$	Relevance score
SLC18A2	-1.942266798	1.304551796	5.285880089
CEACAM1	-1.195350508	1.809247991	5.291052818
MSX1	-6.55E-01	0.993553258	5.339707851
MMP10	1.92456962	-7.83E-01	5.666735172
HTRA1	6.94E-01	-0.83967565	5.672434807
SAMHD1	7.11E-01	-1.06E+00	6.028414726
PLEK	1.578124657	-0.93637934	6.181598186
NCAM1	8.92E-01	-1.339062756	6.327258587
GZMB	1.35379665	-1.498839795	6.482638359
CCL3	1.303188028	-2.294725016	7.032598019
DSP	-9.78E-01	2.304930223	7.064298153
UCHL1	1.08E+00	-0.93101259	7.163775444
NLRP3	8.16E-01	-0.98064053	7.588256359
PLAUR	1.33E+00	-1.690667165	7.789633751
CLDN3	-7.16E-01	1.356962455	8.173981667
KRT8	-0.972470667	1.599805982	8.41655159
PTPRC	1.588444263	-0.788663168	9.130378723
HSD11B1	1.77950448	-0.949267133	11.32518101
CTSL	1.567822663	-0.69553638	11.59943962
EPCAM	-9.48E-01	1.290798653	12.09244442
PAX8	-8.89E-01	6.67E-01	12.15378761
EZR	-6.57E-01	0.96741593	12.93273354
ERBB3	-8.36E-01	1.142784635	14.97532368
MMP3	1.22E+00	-1.634073905	16.92087936
MME	-1.1950781	2.118469214	17.84244537
CCL2	2.490122893	-1.21192065	18.72039795
IGFBP1	2.939555337	-1.49429595	18.73225975
MMP1	1.224754025	-1.882387757	19.2563839
TNF	7.04E-01	-1.282422743	26.31483841
CDH1	-1.02E+00	3.731074497	53.55101013