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Research article

Zhibai dihuang pill (ZBDH) exhibits therapeutic effects on idiopathic central sexual precocity in rats by modulating the gut microflora

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ABSTRACT

To reveal the role of gut microbiota (GM) in the occurrence and development of idiopathic central precocious puberty (ICPP) using 16S rDNA sequencing and bioinformatics analysis. The Danazol-induced ICPP model was successfully constructed in this study. ZBDH and GnRHa treatments could effectively inhibit ICPP in rats, as manifested by the delayed vaginal opening time, reduced weight, decreased uterine organ coefficient, and decreased uterine wall thickness and corpus luteum number, as well as remarkably reduced serum hormone (LH, FSH, and E2) levels. According to 16S rDNA sequencing analysis results, there was no significant difference in the GM community diversity across different groups; however, the composition of the microbial community and the abundance of the dominant microbial community were dramatically different among groups. ZBDH and GnRHa treatments could effectively reduce the abundance of *Muribaculateae* and *Lactobacillus* and promote *Prevotella* abundance. ZBDH and GnRHa were effective in treating Danazol-induced ICPP model rats. The therapeutic effects of ZBDH and GnRHa could be related to the changes in GM in rats.

1. Introduction

Precocious puberty (PP) is widely recognized as a common endocrine system disease. PP is a clinical developmental disorder defined as the development of secondary sexual characteristics before the age of 8 in girls and the age of 9 in boys [1]. According to the pathogenesis, PP can be divided into 2 distinct categories: central precocious puberty (CPP) and peripheral precocious puberty (PPP). Idiopathic central precocious puberty (ICPP) is prevalent among girls, accounting for 80%–90% of CPP [2]. Currently, gonadotropin-releasing hormone analogs (GnRHa), ketoconazole, and various hormone drugs are commonly used drugs for PP treatment. Despite the improving effects of these drugs on PP, long-term drug administration may lead to adverse reactions and some complications (such as osteoporosis) [3]. Increasing interest in clinical research is being directed toward targeted therapy as a result of technological and medical advancements [4]. Therefore, exploring the factors initiating puberty and investigating the therapeutic targets of PP should be the main priorities in children's sexual development research.

Gut microbiota (GM) is a complex collection of microorganisms in the intestine that activates the intestinal nervous system to affect

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the human endocrine system through microbial metabolites [5]. GM refers to the microbiota in the gastrointestinal tract and is attached to the surface of the host mucosa. Human GM consists of over 1000 microorganism species, of which *Firmicutes*, *Bacteroidetes*, *Microalgae*, *Actinobacteria*, and Proteobacteria are predominant. These microorganisms interact with the human body and regulate the immunological and endocrine systems, as well as some physiological mechanisms (e.g., energy metabolism and nutrition absorption), in order to maintain the dynamic balance and preserve human health [6,7]. Increasing evidence suggests that disruption of GM may contribute to obesity [8]. Obesity is intricately associated with PP [9]; increased fat accumulation can promote PP among children. A study has also highlighted that increased childhood obesity is closely related to advanced puberty, especially among girls [10]. Recently, the potential relationship between GM and PP has been gradually revealed. As has been evidenced, the characteristics of GM are remarkably different between ICPP girls (n = 25) and healthy girls (n = 23) according to the sequencing results [11]. Moreover, it has been demonstrated that a high-fat diet consumed by the mother during lactation can exert a substantial impact on the GM of rats of the next generation and induce early female puberty in young rats; however, microbiota reconstruction can prevent or treat early female puberty [12]. Therefore, the changes in GM may also exhibit effects on ICPP occurrence and development.

Traditional Chinese Medicine (TCM) has exerted therapeutic effects on various endocrine diseases, such as type 2 diabetes [13], polycystic ovary syndrome [14], and hypothyroidism [15]. TCM dictates that the pathogenesis of PP is due to the imbalance of Yin and Yang in the kidney and liver, thereby further inducing hyper-function of ministerial fire [16]. The Zhibai Dihuang pill (ZBDH) is a common TCM formula comprising eight herbal ingredients. It has the effect of nourishing Yin and decreasing internal heat, which inhibits the synthesis and release of GnRH in animal models [17]. Moreover, ZBDH is a recognized TCM for ICPP treatment [18]. However, the extent to which the changes of GM contribute to the mechanism by which ZBDH treats ICPP remains unknown.

In this study, an ICPP rat model was induced, with ZBDH and GnRHa (leuprolide) treatments as the positive controls. 16S rDNA sequencing and bioinformatics analysis were performed to investigate the role of GM in the occurrence and progression of ICPP.

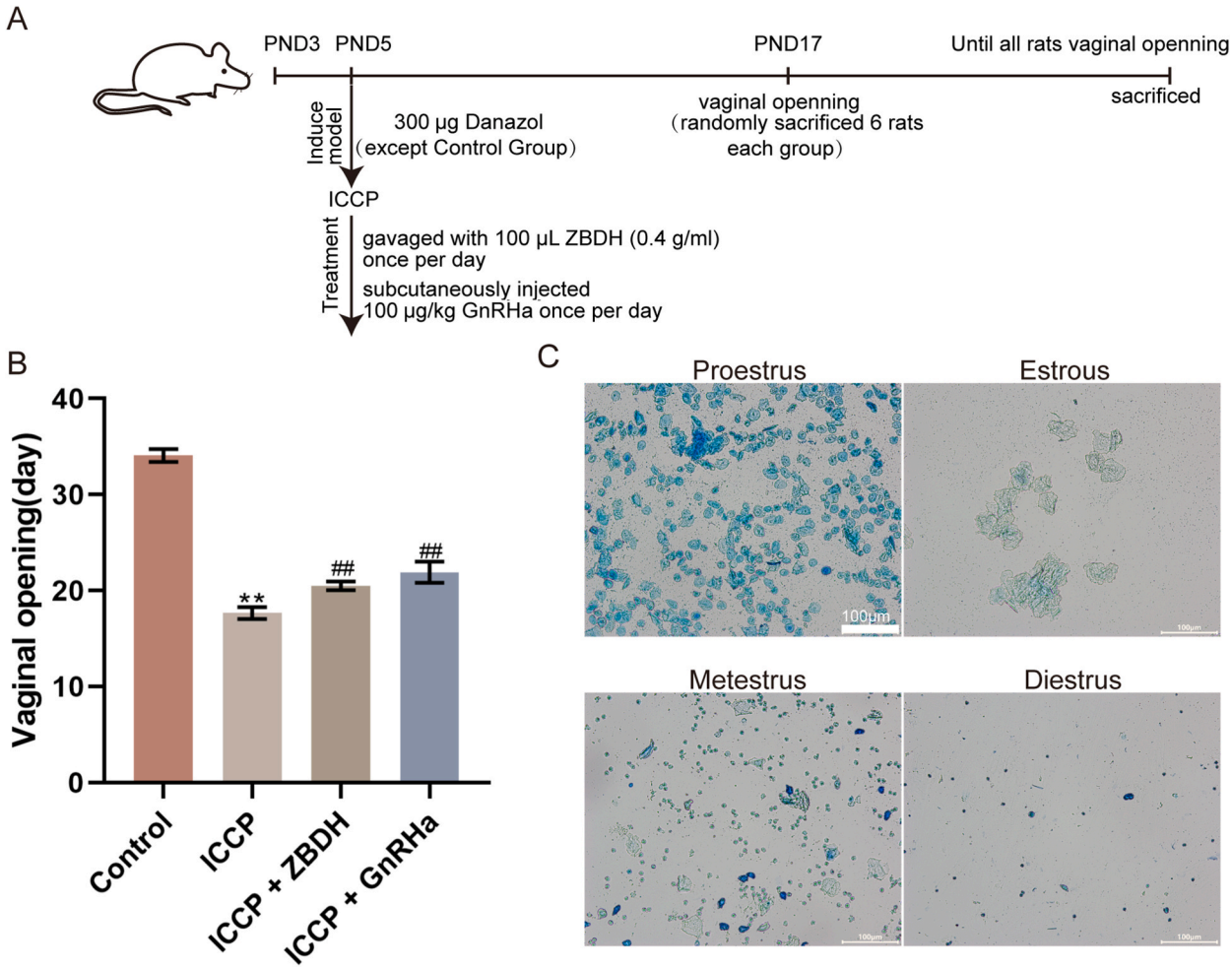

Fig. 1. The Danazol-induced idiopathic central precocious puberty (ICPP) rat model was successfully constructed and ZBDH increased the vaginal opening time. (A) The flow chart of the animal experiment was shown; (B) the vaginal opening time of rats in each group was detected; (C) the normal estrus cycle was displayed. $^{**}p < 0.01$, compared with the control group; $\#\#p < 0.01$, compared with the ICPP group.

2. Materials and methods

2.1. Preparation of ZBDH

ZBDH powers were procured from Jiuzhitang Pharmaceutical Co., Ltd. (Changsha, China). ZBDH is composed of 8 different herbs: Zhi Mu (*Rhizoma Anemarrhenae*), Huang Bai (*Cortex Phellodendri*), Shu Di Huang (*Radix Rehmanniae Preparata*), Shan Zhu Yu (*Fructus Corni*), Shan Yao (*Rhizoma Dioscoreae*), Fu Ling (*Poria*), Mu Dan Pi (*Cx. Moutan*), and Ze Xie (*Rhizoma Alismatis*). The weight ratio of medicinal materials was 1: 1: 4: 2: 2: 1.5: 1.5: 1.5. As stipulated by the manufacturer, 1 g ZBDH contained 2.20 g raw herbs. The qualitative analysis of components of ZBDH was conducted as previously described [19].

2.2. Establishment of ICPP animal model

On the postnatal day (PND) 3, 48 SD rats (supplied by Hunan SJA Laboratory Animal Co., Ltd., Changsha, Hunan, China) were housed in an SPF animal room at a constant temperature (23 ± 2 °C) on a 12-h light:12-h dark cycle, with free access to food and water. After a 2-day period of acclimatization before the experiment, the rats were randomly allocated into 4 groups: control group, ICPP group, ICPP + ZBDH group, and ICPP + GnRHa group (positive control). All rats were weighed on PND5. Rats in the ICPP group were treated with a single subcutaneous injection of 300 μg/25 μL Danazol (MedChemExpress Co., Ltd, Monmouth Junction, NJ, USA). Rats in the control group were injected with 25 μL ethanol/saline as control. In addition to the single subcutaneous injection of 300 μg/25 μL Danazol, rats in the ICPP + ZBDH group were gavaged with 100 μL ZBDH solution (0.4 g/mL) once daily; rats in the ICPP + GnRHa group were subcutaneously injected with 100 μg/kg GnRHa [20] once daily. On PND17, the vaginal opening of rats in the ICPP group was observed, which indicated successful ICPP modeling. The weight of rats in all groups was recorded. All the rats in the ICPP group were sacrificed, and rats in other groups were randomly killed at a ratio of 1:1. When all rat vaginal opening was observed, the estrus time was recorded, followed by the sacrifice of the remaining rats. The schematic of animal treatment is depicted in Fig. 1A.

2.3. Blood and fecal sample collection and organ collection

On PND17, the fecal samples of rats were collected. Rats were euthanized by an intraperitoneal injection of 2% pentobarbital sodium. The blood samples of rats were subsequently collected from the abdominal aorta to measure gonadal hormone levels in serum. The uterus and ovary of rats were weighed to evaluate the organ coefficients (expressed as mg/100 g body weight). The collected organs were then fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining.

2.4. Preparation of vaginal exfoliated cell smear

On PND17, the smears of rat vaginal cells were observed to monitor the normal estrous cycle of rats following the previous methods [21]. The vaginal cells were collected via saline lavage and then stained with 4% methylene blue. Generally, the estrus cycle consists of four phases: proestrus, estrus, metestrus, and diestrus. Proestrus is characterized by a predominance of nucleated epithelial cells; in the estrus stage, there are a great number of cornified squamous epithelial cells; in the metestrus stage, there is a mixture of cornified squamous epithelial and leukocytes; and the diestrus stage is indicated by a predominance of leukocytes.

2.5. H&E staining

The uterus and ovary samples were fixed in 4% paraformaldehyde. After dehydration with gradient ethanol and permeabilization with xylene, the uterus and ovary were embedded in paraffin and sectioned (5 μm). The sections were dried in an oven at 45 °C. Thereafter, the sections were dewaxed using xylene and hydrated through gradient ethanol. Next, the uterus and ovary sections were stained with H&E, subsequently dehydrated with ethanol, and permeated with xylene. The sections were subsequently sealed in neutral resin and observed for uterine wall thickness and follicle growth in the ovary.

2.6. Determination of serum hormone concentration

The blood samples collected from the abdominal aorta were centrifuged (3000 rpm, 4 °C, 5 min). The level of serum hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol (E2)] was detected using enzyme-linked immunosorbent assay (ELISA) kits as directed by the manufacturer (CUSABIO, Wuhan, China and Ruixin Biotech, Quanzhou, China). The standard curve of each hormone was constructed using Excel software. The serum hormone concentration was determined by the linear method.

2.7. 16S rDNA sequencing analysis of microbial community diversity

The microbial genomic DNA was extracted from the fecal samples using the QiAamp PowerFecal Pro DNA kit (Qiagen, Germany) as directed by the manufacturer. The purity of the extracted DNA was determined using a Qubit spectrophotometer (Thermo Fisher, CA, USA). The V3–V4 region of the 16S rDNA gene from the fecal metagenomic DNA was amplified using the universal primers 341F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT). The PCR products were purified using Agencourt AMPure XP beads as per the manufacturer's instructions. The library was constructed using a TruSeq DNA PCR-Free sample preparation kit. The

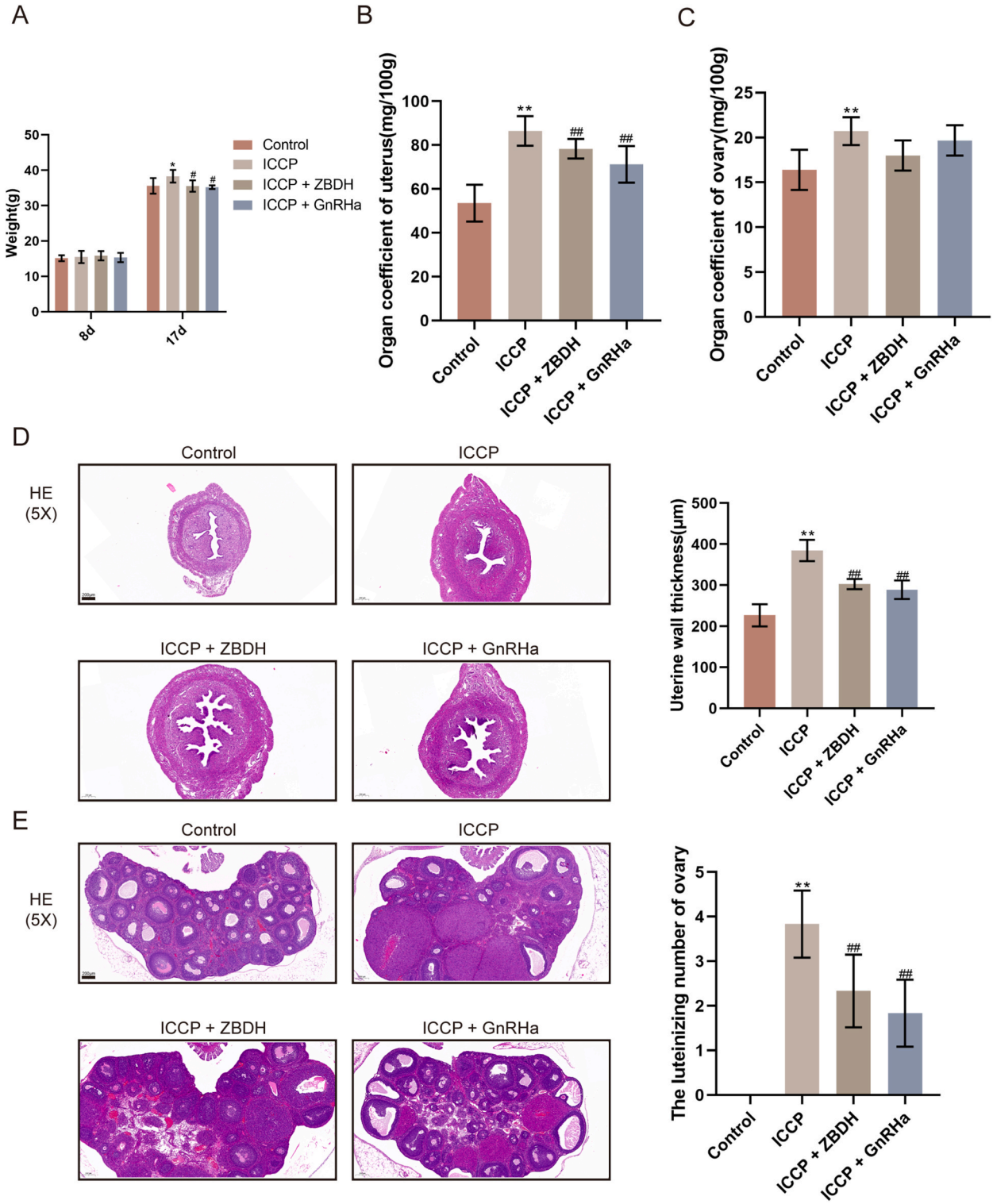

Fig. 2. Phenotypic changes in ICPP rats were detected. (A) Body weight of rats on postnatal day (PND) 5 and PND17 was detected; (B) the organ coefficient of uterine was detected; (C) the organ coefficient of the ovary was detected; hematoxylin and eosin (H&E) staining was used to observe (D) uterine wall thickness and (E) the luteinizing rate of the ovary. $*p < 0.05$, $**p < 0.01$, compared with the control group; $\#p < 0.05$, $\#\#p < 0.01$, compared with the ICPP group.

constructed library was quantified by Qubit and q-PCR. The library was subsequently sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated. The original data were preprocessed and analyzed using QIIME (version 1.9) software. The low-quality reads were filtered using UCHIME. The barcode sequence and primer sequence at both ends of the tags were removed, and the chimeric sequence was removed to yield the clean tags. The sequences were compared, and those having ≥97% identity were clustered into an operational taxonomic unit (OTU) using the UCLUST method in QIIME software (v.1.9). The related statistical diagrams of fecal microbial community structure were plotted, and the dominant microbial community was analyzed.

2.8. Alpha and beta diversity analysis

Alpha diversity reflects the species abundance, diversity, and evenness in a single sample. Chao1, ACE, Shannon, Simpson, and J: Pielou indexes were examined for alpha diversity analysis. Beta diversity refers to the variation of species composition among different communities, reflecting species replacement along an environmental gradient. The beta diversity was analyzed with the analysis of similarity (ANOSIM), principal coordinate analysis (PCA), and non-metric multidimensional scaling (NMDS). The beta diversity analysis was conducted through QIIME2 and R package.

2.9. Statistical analysis

All the experimental data were expressed as mean ± standard deviation (SD). The *t*-test and one-way analysis of variance (ANOVA) was performed using GraphPad Prism 8.0 software. Tukey's post-hoc test was used for multiple comparisons. A *p*-value of less than 0.05 indicates a statistically significant difference.

3. Results

3.1. ZBDH increased the vaginal opening time of the danazol-induced ICPP rat model

The rats were injected with Danazol on PND5 to induce the ICPP rat model of ICPP. The ICPP rats in the positive control group were further treated with ZBDH and GnRHa, respectively (Fig. 1A). As shown by the results, the vaginal opening of Danazol-induced ICPP rats occurred on the PND17, which was significantly earlier than that of normal control rats; however, ZBDH and GnRHa treatments could effectively delay the vaginal opening time of ICPP rats ($p < 0.05$) (Fig. 1B). The above results showed that the Danazol-induced ICPP rat model was successfully constructed, and ZBDH and GnRHa could effectively delay the vaginal opening time of ICPP rats. The normal estrus cycle is shown in Fig. 1C.

3.2. ZBDH and GnRHa could alleviate the phenotypic changes in ICPP rats

The phenotypic changes in ICPP rats were then further observed. Firstly, the weight growth [from the modeling time (PND5) to the estrus phase (PND17)] of rats in each group was observed. As indicated by the results, rats in the ICPP group exhibited remarkably increased weight growth relative to rats in the control group; ZBDH and GnRHa treatment could notably reduce the weight growth of ICPP rats ($p < 0.05$) (Fig. 2A). The organ coefficient of the uterus and ovary was subsequently detected. It was found that ICPP rats had dramatically increased organ coefficient of the uterus and ovary compared with control rats ($p < 0.01$); both ZBDH and GnRHa treatments markedly reduced the uterus organ coefficient of ICPP rats ($p < 0.01$), which, however, showed no significant effects on the organ coefficient of the ovary (Fig. 2B/C). Subsequently, H&E staining was performed to observe the uterine wall thickness and the

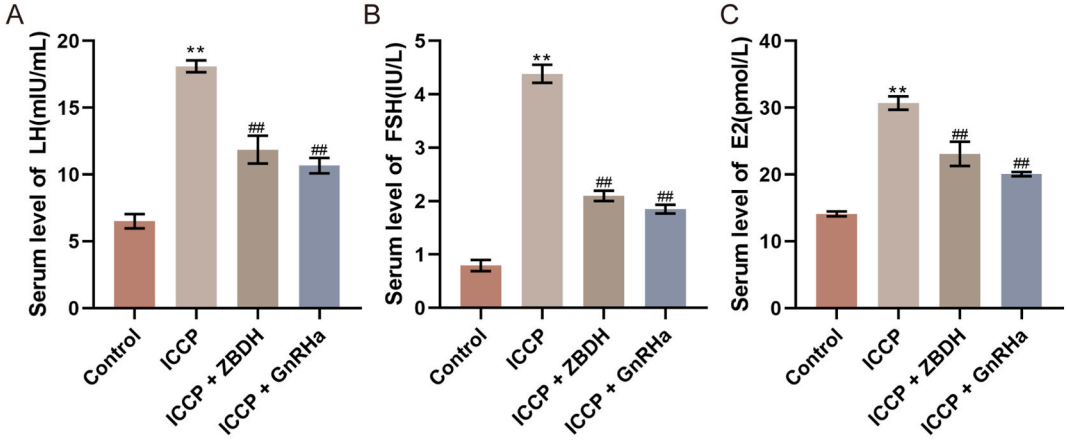

Fig. 3. Changes in serum hormones in ICPP rats were detected. The serum level of (A) luteinizing hormone (LH), (B) follicle-stimulating hormone (FSH), and (C) estradiol (E2) was detected. $^{**}p < 0.01$, compared with the control group; $\#\#p < 0.01$, compared with the ICPP group.

luteinizing rate of the ovary. According to the staining results, normal control rats showed clear histological layer and normal cell morphology, and there were no obvious pathological changes; however, ICPP rats had noticeably increased uterine wall thickness; ZBDH and GnRHa treatments effectively decreased uterine wall thickness of ICPP rats (all $p < 0.01$) (Fig. 2D). Moreover, compared

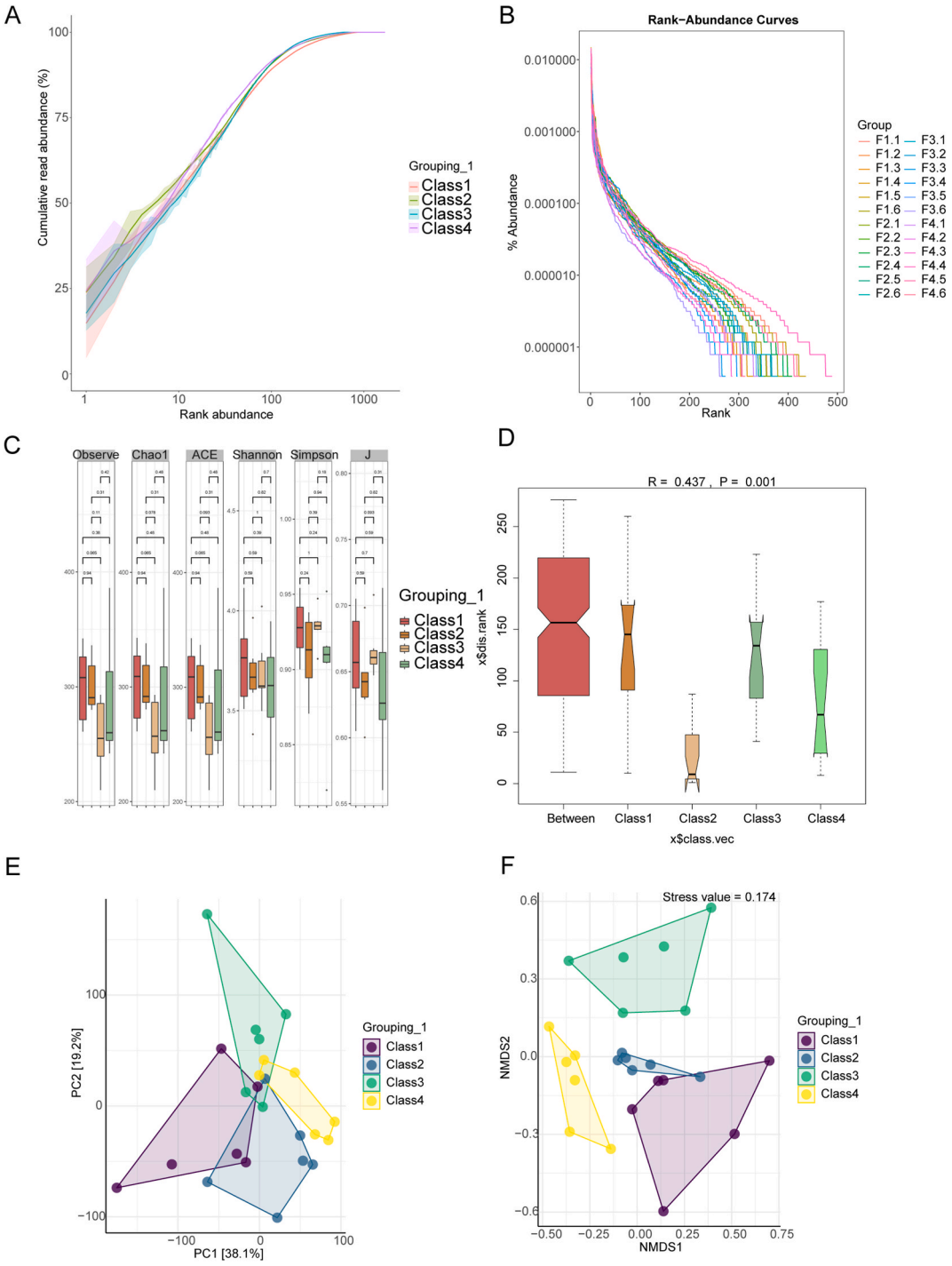

Fig. 4. The overall changes in gut microbiota (GM) in rats were observed. Class 1: Control group; Class 2: ICCP group; Class 3: ICCP + ZBDH group; Class 4: ICCP + GnRHa group; (A) The dilution curve was used to analyze the abundance of species in different samples; (B) the Rank-Abundance curve was used to analyze the sample diversity; (C) alpha diversity analysis was performed; beta diversity analysis, including (D) analysis of similarity (ANOSIM), (E) principal coordinate analysis (PCA), and (F) non-metric multidimensional scaling (NMDS) were performed to analyze the differences of microbial community among groups.

with a small number of primary or secondary follicles in control rats, a great number of follicles and corpus luteum were observed in ICPP rats ($p < 0.01$); there were many follicles, but a little amount of corpus luteum after ZBDH and GnRHa treatments (Fig. 2E). The above results indicated that ZBDH and GnRHa could alleviate the phenotypic changes in Danazol-induced ICPP rats.

3.3. ZBDH and GnRHa could inhibit the increase of serum hormone levels in ICPP rats

Furthermore, the changes in hormones in rat serum were analyzed using ELISA. According to the results, compared with the control rats, ICPP rats showed remarkably elevated serum hormone (LH, FSH, and E2) levels, which was consistent with the symptoms of PP; ZBDH and GnRHa treatments notably reduced the serum LH, FSH, and E2 levels in ICPP rats (all $p < 0.01$) (Fig. 3A–C). The above results revealed that ZBDH and GnRHa could inhibit the increase of serum hormones in Danazol-induced ICPP rats.

3.4. GM community was significantly different across different groups

Firstly, the rarefaction curve (also known as the richness curve) was used to reflect the sequencing depth and coverage of species richness of the test samples. According to the results, the curves of samples in the four groups tended to be flat (Fig. 4A), which indicated that the sequencing depth of this experiment had basically covered all species in the samples. Next, the Rank-Abundance curve was used to analyze the sample diversity. The analysis results revealed that the species composition in each group was rich and uniform (Fig. 4B), which could be used for subsequent analyses. Furthermore, the results of alpha diversity analysis are shown in Fig. 4C. Observe, and Chao1 were used to estimate the OTU number in the sample; the ACE index was used to estimate the OTU number in the community; Shannon and Simpson were used to estimate community diversity; J index was used to measure the closeness of different species in the sample. The results showed that there was no significant difference in the diversity of GM in groups. Beta diversity analysis was performed to investigate the difference in the microbial community among groups (Fig. 4D–F), including ANOSIM analysis (Fig. 4D), PCA analysis (Fig. 4E), and NMDS analysis (Fig. 4F). As shown by results, there were significant differences in GM community across different groups.

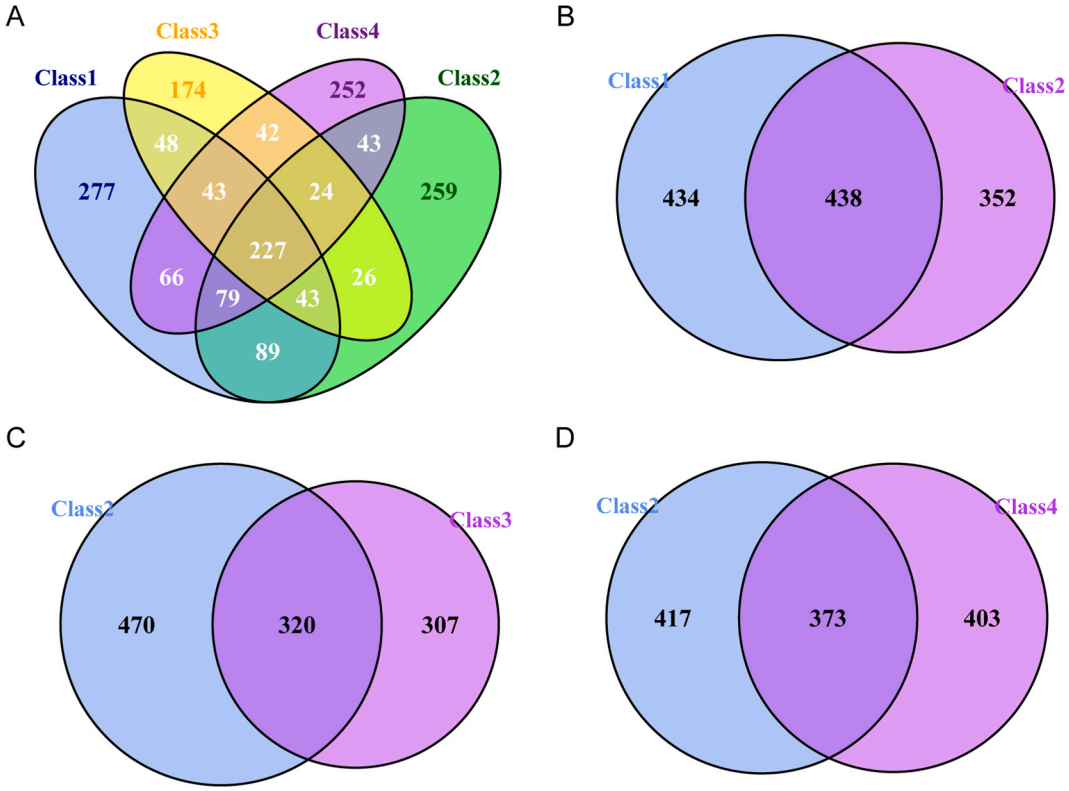

Fig. 5. The intersected operational taxonomic units (OTUs) across different groups were detected. (A) Venn diagram of data in four groups was shown; (B) Venn diagram of data in the control group and ICPP; group; (C) Venn diagram of data in the ICPP group and ICPP + Zhibai Dihuang Wan (ZBDH) group; (D) Venn diagram of data in the ICPP group and the ICPP + GnRHa group. Class 1: Control group; Class 2: ICCP group; Class 3: ICCP + ZBDH group; Class 4: ICCP + GnRHa group;

3.5. ZBDH and GnRHa treatments could affect the species of GM community in rats

The above findings proved that there were differences in the GM community among groups. Subsequently, a Venn diagram was plotted to display the OTU intersection of rats in different groups. It was observed that there were 227 intersected OTUs among the four groups (Fig. 5A). Furthermore, the intersected OTUs between the ICPP group and the other 3 groups were investigated. According to the results, there were 438, 320, and 373 intersected OTUs between the ICPP group and the control group, the ICPP + ZBDH group, and ICPP + GnRHa group, respectively (Fig. 5B–D). These findings demonstrated that Danazol-induced ICPP, as well as ZBDH and GnRHa treatments, could affect the GM community species in rats.

3.6. ZBDH and GnRHa treatments could affect dominant GM in ICPP rats

Next, the microbiotas with high relative abundance in the GM community in each group were displayed using a heat map, histogram, and box plot (Fig. 6A–C). The dominant GM in the four groups was compared. It was found that *Lactobacillus*, *Munbaculaceae*, *Dubosiella*, *Alloprevotella*, *Prevotellaceae_Ga6A1_group*, *Bifidobacterium*, *Romboutsia*, [*Eubacterium*]*_coprostanoligenes_group*, *Prevotella*, and *Prevotellaceae_NK3B31_group* were mainly dominant microbiota in samples in the four groups. Next, the changes in dominant GM

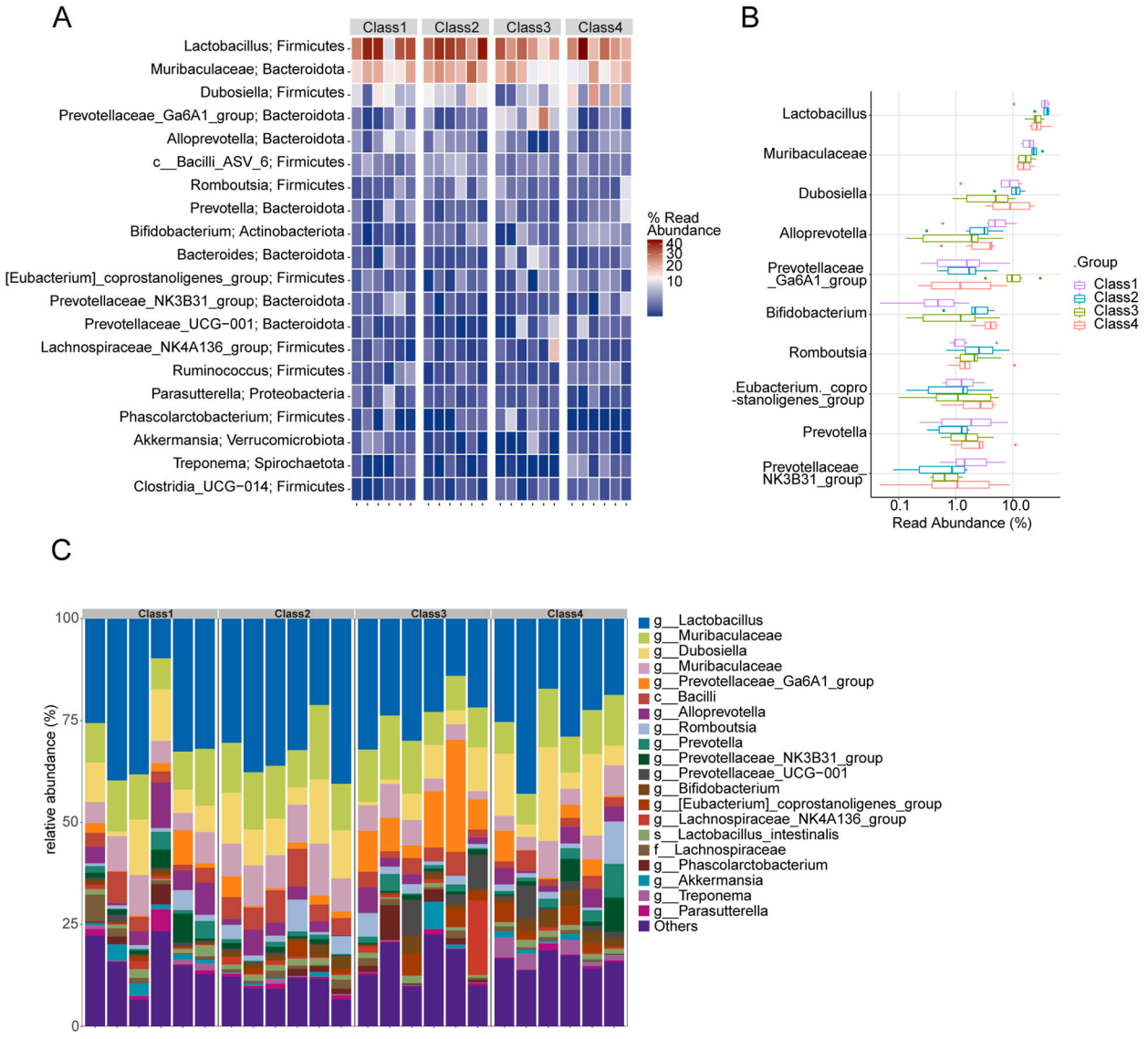

Fig. 6. The dominant GM was significantly different among different groups. (A) A heat map of GM was shown; (B) the histogram of the relative abundance of GM was shown; (C) the box plot of the relative abundance of GM was shown. Class 1: Control group; Class 2: ICCP group; Class 3: ICCP + ZBDH group; Class 4: ICCP + GnRHa group;

in the four groups were analyzed. As indicated by the results (Fig. 7 and Table 1), compared with the ICPP group, the control group showed a notably reduced abundance of *Romboutsia*, *Bifidobacterium*, *Muribaculaceae*, and *Lactobacillus*, and increased abundance of *Prevotellaceae_NK3B31_group*, *Prevotella*, and *Alloprevotella*. The ICPP + ZBDH group exhibited a remarkably decreased abundance of *Bifidobacterium*, *Dubosiella*, *Muribaculaceae*, and *Lactobacillus*, and an increased abundance of *Prevotella*, [*Eubacterium*]*_coprostanoligenes_*group, and *Prevotellaceae_Ga6A1_*group relative to the ICPP group. Compared with those in the ICPP group, the abundance of *Muribaculaceae* and *Lactobacillus* was dramatically reduced, and the abundance of *Prevotellaceae_NK3B31_group*, *Prevotella*, [*Eubacterium*]*_coprostanoligenes_group*, and *Bifidobacterium* was increased in the ICPP + GnRHa group. Collectively, Danazol-induced ICPP downregulated the abundance of *Prevotella* and increased the abundance of *Bifidobacterium*, *Muribaculaceae,* and *lactobacillus*, which could be significantly reversed by ZBDH or GnRHa.

4. Discussion

It has been shown that GM plays a crucial role in human health and disease [22]. Disease development is closely connected to the microecological imbalance of GM; therefore, managing the microbiology of GM could be beneficial in the treatment of numerous disorders. For example, a previous study has highlighted that remodeling the GM can treat type 2 diabetes mellitus and its complications [23]. The regulation of GM can ameliorate gut dysbiosis, cognitive decline, and depressive behaviors [24]. Chlorogenic acid-induced changes in GM can inhibit metabolic endotoxemia in HFD-fed mice [25]. The relationship between GM and PP has been gradually revealed [11,26]. Nevertheless, little is known about the mechanism of GM in the initiation and development of ICPP. In this study, rats were induced for ICPP modeling and treated with ZBDH or GnRHa; the mechanism of GM in ICPP occurrence and development was explored using 16S rDNA sequencing and bioinformatics.

Previous studies have demonstrated that ZBDH and GnRHa could be used for ICPP clinical treatment [18,27]. In this study, the Danazol-induced ICPP rat model was first established to verify the therapeutic effects of ZBDH and GnRHa in the animal model. It was observed that Danazol induction advanced the vaginal opening time of rats, increased rat body weight, uterine organ coefficient, and ovary organ coefficient, and elevated the levels of serum hormones (LH, FSH, and E2). Moreover, consistent findings have also been previously reported [21]. ZBDH and GnRHa treatments could effectively improve Danazol-induced symptoms and the related physiological indicators, as manifested by the delayed vaginal opening time, decreased body weight and uterine organ coefficient, and reduced serum hormone LH, FSH, and E2 levels. These improvements have also been demonstrated in a previous study [21]. These

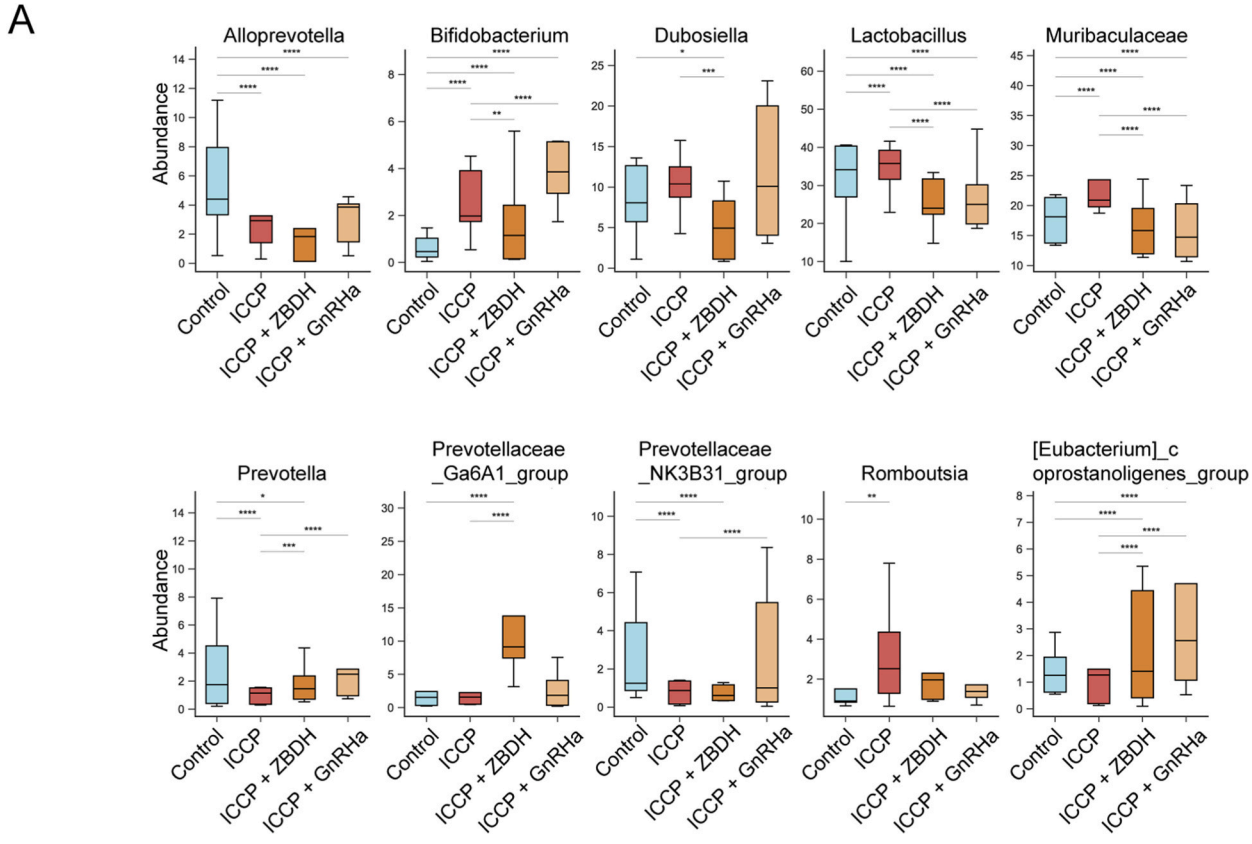

Fig. 7. The abundance of GM in different groups. The changes in dominant GM in the four groups were analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Table 1
Analysis of GM abundance of Control, ICPP, ICPP + ZBDH, ICPP + GnRHa.

Species	vs ICPP		
	Control	ICPP + ZBDH	ICPP + GnRHa
*Prevotellaceae_NK3B31*_group	Up*	Down	Up*
Prevotella	Up*	Up*	Up*
*[Eubacterium]_coprostanoligenes*_group	Down	Up*	Up*
Romboutsia	Down*	Down	Down
Bifidobacterium	Down*	Down*	Up*
*Prevotellaceae_Ga6A1*_group	Down	Up*	Up
Alloprevotella	Up*	Down	Up
Dubosiella	Down	Down*	Down
Muribaculaceae	Down*	Down*	Down*
Lactobacillus	Down*	Down*	Down*

*$P < 0.05$.

above results revealed that the Danazol-induced ICPP rat model was successfully established, and ZBDH and GnRHa exerted therapeutic effects on ICPP.

After determining the successful construction of the ICPP rat model and the therapeutic effects of ZBDH and GnRHa on ICPP, 16S rDNA sequencing analysis was performed to investigate the diversity of GM. The results showed that there was no difference in GM diversity among groups; however, there were differences in GM community across different groups. Furthermore, it was observed that the dominant GM of ICPP rats was changed, and the dominant GM of ICPP rats was also affected after ZBDH and GnRHa treatments. These results suggested that ZBDH and GnRHa exerted therapeutic effects on ICPP, which may be mediated by regulating the dominant GM in rats. Noticeably, the results in this study showed that *Bifidobacterium* abundance was increased in the ICPP rats. Consistently, multiple studies have substantiated this result. For example, it has been pointed out that *Bifidobacterium* is increased in girls with CPP [28]. *Bifidobacterium* is positively related to GnRH, which can promote puberty [29]. Additionally, the results in this study also proved that *Bifidobacterium* abundance decreased after ZBDH treatment but increased after GnRHa treatment. This finding indicated that ZBDH may play a role in ICPP treatment by inhibiting *Bifidobacterium*; intestinal microorganisms that GnRHa acted on may be different from those ZBDH acted on. Moreover, it was found that ICPP rats showed upregulated *Muribagulaceae* and *Lactobacillus* abundance and downregulated *Prevotella* abundance; ZBDH and GnRHa treatment could effectively inhibit the abundance of *Muribagulaceae* and *Lactobacillus* and increase the abundance of *Prevotella*. Hence, *Muribaculateae*, *Lactobacillus*, and *Prevotella* served as potential marker microbiota in ICPP, and intervention with them may exert potential therapeutic effects on ICPP. Nevertheless, experimental verification is required for further verification.

In addition to the changes in gut microflora, previous findings suggest that ZBDH and GnRHa exert multifaceted pharmacological effects in treating ICPP. ZBDH is a traditional compound TCM that nourishes Yin and reduces internal heat and is used to treat Yin-deficiency-heat syndrome [30], which prepared from *Rhizoma anemarrhenae*, *Cortex phellodendri*, *Radix rehmanniae preparata*, *Rhizoma dioscoreae*, *Fructus corni*, *Cortex moutan*, *Rhizoma alismatis* and *Poria* [31]. ZBDH, recognized for its role in hormonal regulation, particularly in gynecological health [32] and precocious puberty [17,33], includes components such as Cornus officinalis and Dioscorea opposita, which impact hormonal pathways [34,35]. These components potentially regulate the hypothalamic-pituitary-gonadal axis. Moreover, Kim et al. revealed that *Anemarrhenae rhizome* and *Phellodendri cortex* treatment delayed vaginal opening in rats with precocious puberty by inhibiting the activation of the hypothalamic-pituitary-gonadal axis [36]. Similarly, GnRHa's primary action involves modulating gonadotropin release, thereby regulating LH and FSH secretion, crucial in puberty development [37–39]. The therapeutic efficacy observed in this study likely results from a combination of gut microflora modulation and direct influence on hormonal regulation and the central nervous system.

However, while this study demonstrates the effects of ZBDH and GnRHa on gut microflora in ICPP, it acknowledges certain limitations. The alterations in gut microbiota observed do not definitively establish the complete mechanism of action of these treatments. Gut microflora modulation represents only part of the multifactorial processes involved in ICPP. Further research is warranted to unravel deeper mechanisms beyond the gut-brain axis. Additionally, although there have been clinical observations of ZBDH combined with leuprolide acetate in the treatment of central precocious puberty in children [40], in order to better explore the correlation between gut microbiota changes and ICPP treatment outcomes in humans, more extensive clinical studies with larger sample sizes are essential.

In conclusion, this study demonstrated the therapeutic effects of ZBDH and GnRHa on Danazol-induced ICPP rats. The intestinal ecological disorder was observed in ICPP rats, and the change of *Bifidobacterium* abundance may be tightly implicated in the pathogenesis and treatment of ICPP. This study indicated that intervention with the abundance of *Muribaculaceae*, *Lactobacillus*, and *Prevotella* may have potential therapeutic effects on ICPP.

Ethics statement

This study was approved by the Animal Ethics Committee of Hunan Provincial People's Hospital [Approval number: Ke 2023 (02)].

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Availability of data and materials

The data that support the findings of this study are available are available within the article.

CRediT authorship contribution statement

Canhong Yi: Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Hui Zou:** Writing – original draft, Funding acquisition, Conceptualization. **Xiaojuan Lin:** Data curation. **Shanshan Liu:** Methodology, Investigation. **Juan Wang:** Visualization, Software. **Yuquan Tian:** Resources, Formal analysis. **Xujing Deng:** Methodology, Data curation. **Jianhong Luo:** Validation, Resources. **Chan Li:** Supervision, Software. **Yin Long:** Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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