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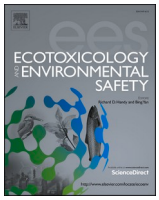

Procyanidin B2 alleviates uterine toxicity induced by cadmium exposure in rats: The effect of oxidative stress, inflammation, and gut microbiota

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ABSTRACT

Environmental exposure to hazardous materials causes enormous socioeconomic problems due to its deleterious impacts on human beings, agriculture and animal husbandry. As an important hazardous material, cadmium can promote uterine oxidative stress and inflammation, leading to reproductive toxicity. Antioxidants have been reported to attenuate the reproductive toxicity associated with cadmium exposure. In this study, we investigated the potential protective effect of procyanidin oligosaccharide B2 (PC-B2) and gut microbiota on uterine toxicity induced by cadmium exposure in rats. The results showed that the expression levels of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were reduced in utero. Proinflammatory cytokines (including tumor necrosis factor-α, interleukin-1β and interleukin-6), the NLRP3 inflammasome, Caspase-1 and pro-IL-1β were all involved in inflammatory-mediated uterine injury. PC-B2 prevented $CdCl_2$-induced oxidative stress and inflammation in uterine tissue by increasing antioxidant enzymes and reducing proinflammatory cytokines. Additionally, PC-B2 significantly reduced cadmium deposition in the uterus, possibly through its significant increase in MT1, MT2, and MT3 mRNA expression. Interestingly, PC-B2 protected the uterus from $CdCl_2$ damage by increasing the abundance of intestinal microbiota, promoting beneficial microbiota, and inhibiting harmful microbiota. This study provides novel mechanistic insights into the toxicity of environmental cadmium exposure and indicates that PC-B2 could be used in the prevention of cadmium exposure-induced uterine toxicity.

1. Introduction

In recent years, urban waste, smelting, mining, metal manufacturing and improper application of fertilizers and pesticides have resulted in continued accumulation of heavy metals in the soil of farmlands and pastures (Ren et al., 2022; Yuan et al., 2021). The contaminated soil not only impede the growth and development of plants and other soil organisms but more importantly, affect the health of humans and/or other animals through the food chain (Peralta-Videa et al., 2009). Among them, cadmium is a ubiquitous heavy metal that is ranked as one of the seventh most toxic industrial carcinogens (Wang and Du, 2013). The average half-life of cadmium in human and other animals is 10–35 years. It is the most common environmental pollution toxin that accumulates in the body, and seriously affects human and animal health (Genchi et al., 2020).

According to World Health Organization (WHO) standards, the allowable concentration of cadmium in drinking water is ~0.05 mg/L (Tchounwou et al., 2012). However, leaching of cadmium from industrial and other sources has bypassed the permissive restrictions in many countries (Hossain et al., 2019; Zhao et al., 2018). Cadmium exposure can lead to a variety of diseases, including renal insufficiency, bone loss and female reproductive disorders (Kumar and Sharma, 2019; Wang et al., 2021). The uterus is considered to be one of the main target organs of cadmium toxicity. Cadmium also indirectly leads to endometrial edema and lipid peroxidation by inducing estrogen disorder (Nasiadek et al., 2018, 2019). However, the effects of cadmium exposure on uterine tissue and the mechanisms of action remain to be investigated.

A growing body of research has focused on finding new ways to

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Table 1
Primer sequences for real-time PCR analysis of cytokines and inflammatory cytokines.

Name	Primer sequences	Product size (bp)
NLRP3	Forward: 5′-GTTCTTCGCTGCTATGTA-3′ Reverse: 5′-AGGTTCTCTCCTGGTTTA-3′	194
IL-1β	Forward: 5′-CCAGGATGAGGACATGAGCA-3′ Reverse: 5′-CGGAGCCTGTAGTGCAGTTG-3′	129
IL-6	Forward: 5′-CCAGGATGAGGACATGAGCA-3′ Reverse: 5′-CGGAGCCTGTAGTGCAGTTG-3′	171
TNF-α	Forward: 5′-GTTGGTCCCCCTTCTC-3′ Reverse: 5′-TCACCCACACCGTCAG-3′	168
Caspase-1	Forward: 5′-TGAAGTTGCTGCTGG-3′ Reverse: 5′-TCTTGTGCTCTGGGC-3′	111
MT1	Forward: 5′-ACTGCCTTCTTGTCGCTTAC-3′ Reverse: 5′-CAGCAGCACTGTTCGTCACTT-3′	256
MT2	Forward: 5′-CCTAGAACTCTACAGCGAT-3′ Reverse: 5′-TTTACACCATTGTGAGGAC-3′	268
MT3	Forward: 5′-GCTTGCCTGGAGGAACTAA-3′ Reverse: 5′-TTGCTGTGCATGGGATTT-3′	346
GAPDH	Forward: 5′-TTCCGTGTTCCTACCCCCA-3′ Reverse: 5′-GCCCAAGATGCCCTTCAGT-3′	129
β-actin	Forward: 5′-TAAAGACCTCTATGCCAACAC-3′ Reverse: 5′-TAAAGCCATGCCAAATGTCTC-3′	350

reduce the effects of cadmium toxicity on organisms, mainly through the use of nutrients to chelate the metal, reduce tissue absorption, and increase body excretion and antioxidant capacity, thereby reducing the likelihood that cadmium induces oxidative stress (Amadi et al., 2019; Flora et al., 2008). Recent results have shown that natural antioxidant molecules from medicinal plants, such as catechol and epigallocatechin gallate, have beneficial effects in mitigating cadmium toxicity (Al Olayan et al., 2020; Chen et al., 2016). Procyanidin oligosaccharide B2 (PC-B2), a natural polyphenolic compound that can be extracted from grape seeds, has attracted great attention due to its antioxidant, anti-inflammation and anticancer properties (Ma et al., 2021; Zhang et al., 2019). Studies have shown that proanthocyanidin extracted from grape seeds can effectively alleviate the toxicity of cadmium chloride to organs, such as renal oxidative damage (Chen et al., 2013). However, the relationship between the natural product-mediated gut microbiota and uterine toxicity induced by environmental cadmium exposure is not clear.

The mammalian gut is a complex environment composed of a rich microbiota (Marchesi et al., 2016). Studies have shown that the gut microbiota is closely related to disease development by affecting host metabolism (Sharon et al., 2014), the immune system (Rooks and Garrett, 2016), the nervous system (Tan et al., 2020), and even carcinogenesis (Yachida et al., 2019) of hosts. The gut microbiota is vulnerable to heavy metals (Duan et al., 2020; Ragan, 1983), and excessive exposure of the gut to the heavy metal cadmium can lead to intestinal dysbiosis (Tinkov et al., 2018). Indeed, there is evidence that changes in gut microbiota are largely associated with uterine inflammation and diseases (Ata et al., 2019; Hu et al., 2021). Therefore, $CdCl_2$ may aggravate its toxic uterine effects by inducing intestinal microbiota disorder, thereby promoting the development of uterine oxidative stress and inflammation and PC-B2 could be a dietary polyphenol supplement playing a physiological regulatory role by affecting the structure of intestinal microbiota (Xiao et al., 2018; Xing et al., 2019). In this study, we used 16S rRNA sequencing and PICRUST2 function prediction analysis to investigate whether PC-B2 alleviates $CdCl_2$-induced uterine toxicity through improving gut microbiota.

We achieved our understanding the protective effect of PC-B2 on uterine toxicity induced by cadmium chloride exposure using rats as an experimental organism. The results indicated that PC-B2 reduced the accumulation of cadmium chloride in rats and alleviated the toxicity induced by cadmium chloride exposure in the rat uterus. Our results provide new insights into the mechanism and alleviation of health problems associated with environmental pollution from hazardous materials.

2. Materials and methods

2.1. Materials

PC-B2 was provided by Cheng du grass source Kang Biotechnology Co., Ltd. Cadmium chloride was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. A myeloperoxidase ELISA kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing Jiancheng Bioengineering Institute). Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) ELISA kits were purchased from Hepeng (Shanghai) Biotechnology Co., Ltd.

2.2. Establishment of animal model

SD female rats (6–8 weeks old, weighing 180–210 g) were purchased from Chengdu Dossy Experimental Animals Co., Ltd. The rats were placed in a controlled environment (temperature 25 ± 3 °C, humidity 75 ± 5%), given sufficient food and water, and treated with 12 h light and 12 h dark. The disposal of animals during the experiment was approved by the Ethics Committee of Chengdu Normal University (Grant No: CNDU-20210912034R) and met the standards of animal ethics. After seven days of adaptation, the rats were randomly divided to four groups with 10 replicates (i.e. 10 rats in each group). The negative control

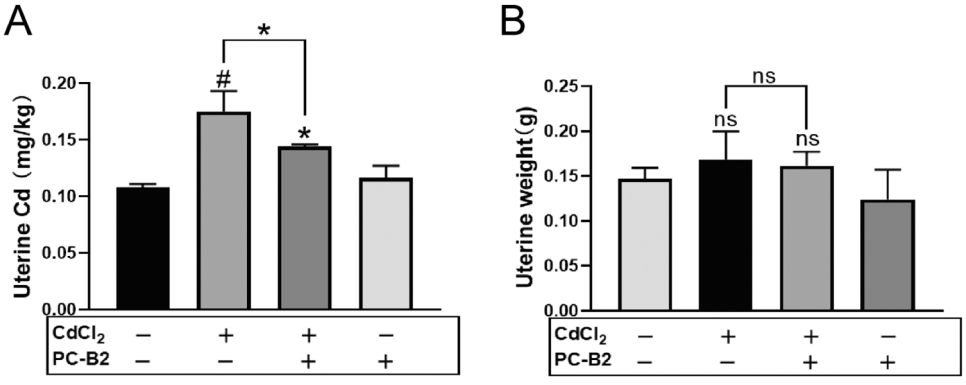

Fig. 1. Changes in uterine cadmium content and uterine weight in SD rats. (A) The content of cadmium in the uterus; (B) Weight of the uterus. Data was presented as mean ± SD (n = 3). In this figure, "* " means significant difference between the two groups ($P < 0.05$), "#" means most significant difference ($P < 0.01$), and while "ns" means no significant difference ($P > 0.05$).

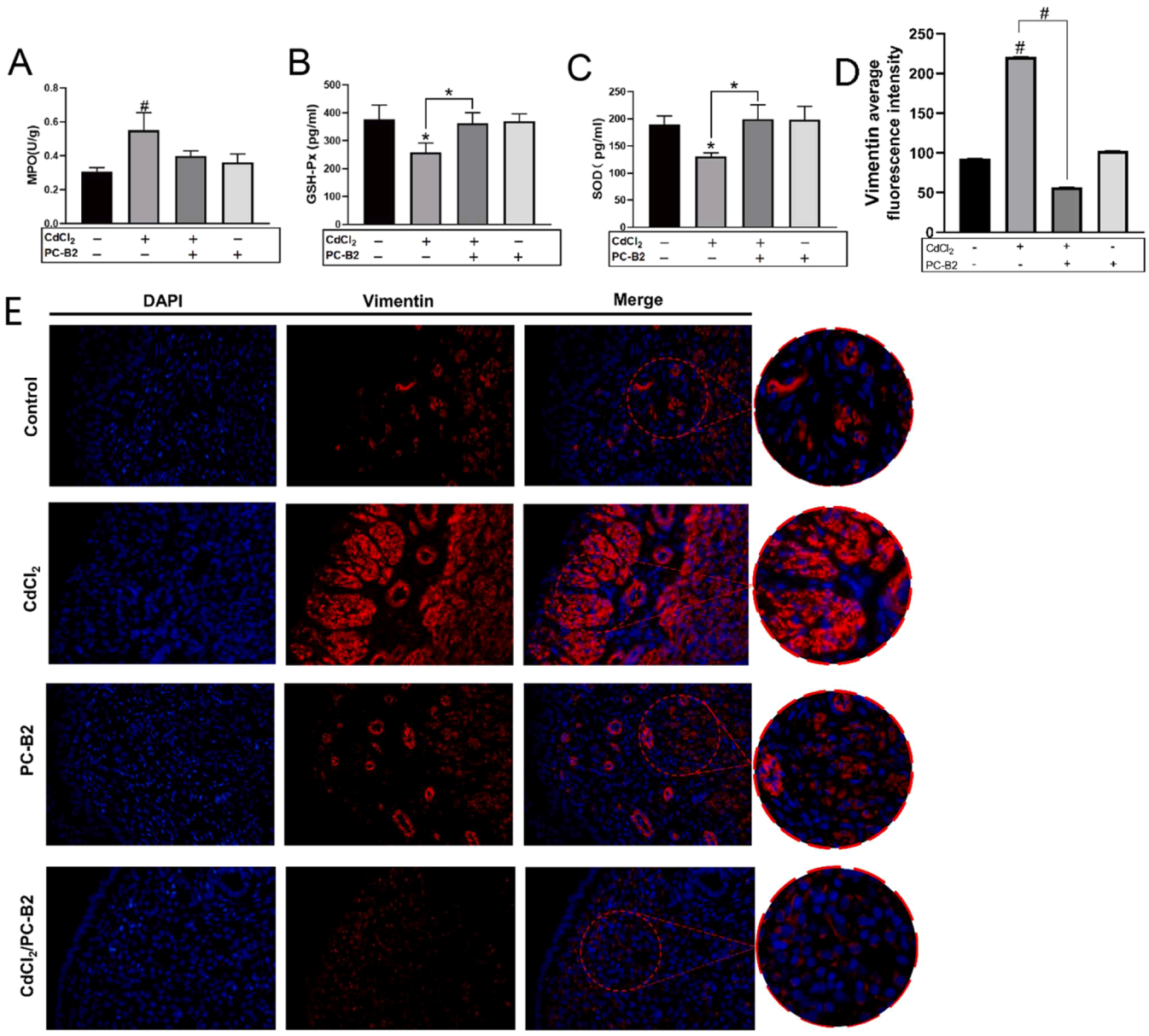

Fig. 2. Expression of antioxidant status in uterine tissue and Immunofluorescence analysis of uterine tissue. (A) MPO activity; (B) The expression levels of GSH-Px; and (C) The expression levels of SOD. Data were presented as mean ± SD (n = 3). (D)The average fluorescence intensity of vimentin, and the data are expressed as the mean ± SD (n = 3). (E)The results of vimentin immunofluorescence. DAPI staining shows the nucleus, and red fluorescence shows vimentin. In this figure, "*" means significant difference between the two groups ($P < 0.05$), while "#" means highly significant difference ($P < 0.01$).

group was given regular gavage with an equal amount of normal saline. The three treatment groups were treated with $CdCl_2$, $CdCl_2$/PC-B2, and PC-B2 respectively. In the $CdCl_2$ group, the chemical (5 mg/kg body weight) was daily fed with diluted food by gastric infusion. In the $CdCl_2$/PC-B2 group, the rats were intragastrically administered each day with $CdCl_2$ (5 mg/kg body weight) firstly and followed by PC-B2 (100 mg/kg body weight) after 12 h (Chen et al., 2013). In the PC-B2 group, the chemical at the rate of 100 mg/kg body weight was also intragastrically administered every day. All chemicals were dissolved in water before being fed to the animals and the experiments were lasted 60 days. Some studies have shown that cadmium began to have effect on hormone circulation of rats (Nasiadek et al., 2018). Considering the long deposition time of cadmium in animals, we used an exposure time of 60 days in our experiments. In the end of the exposure experiment, SD rats were dislocated, and their uteri were harvested, rinsed with ice-cold saline, and stored at − 80 ℃ until further use.

2.2.1. *Tissue sample determination*

An Agilent ICPMS 7700 (U.S.A) instrument was used to analyze cadmium concentrations in blood and uterine tissues according to standard operating procedures of the instrument. MPO activity, GSH-Px and SOD in uterine tissue were determined according to the kit instructions.

2.2.2. *Cell Viability Assay*

IEC-18 cells (YaJi Biological, Shanghai, China) were selected and Cell Counting Kit-8 (CCK-8) (Macklin Biochemical, Shanghai, China) was utilized to assess cell viability. The culture medium for the cells consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), following the manufacturer's instructions. The logarithmic growth stage cell suspension was uniformly inoculated into 96-well plates at a concentration of 5×10^3 cells/mL and cultured at 37 ℃ with 5% CO_2. $CdCl_2$ concentrations (Cao

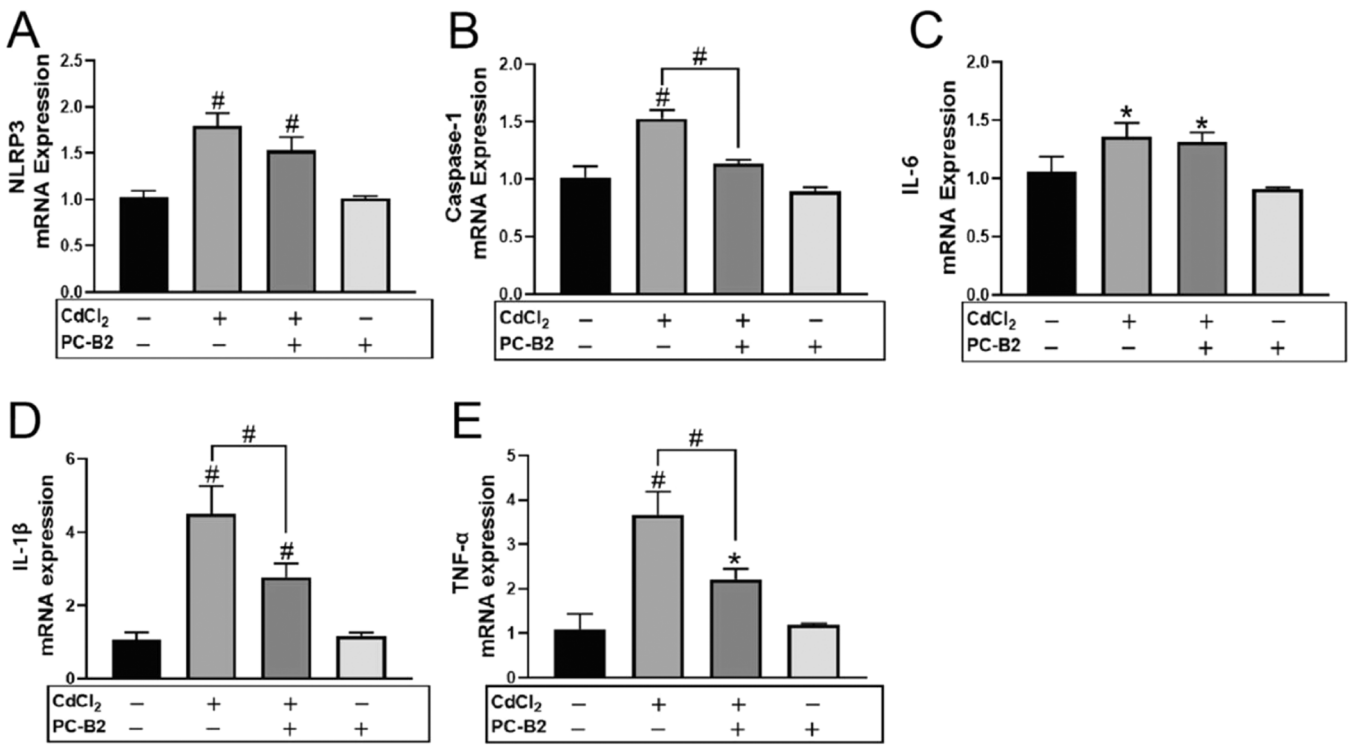

Fig. 3. Expression of inflammatory cytokines by qRT—PCR analysis. (A) - (E) is the relative expression levels of NLRP3, caspase-1, IL-6, IL-1β and TNF-α, respectively. Data were presented as mean ± SD (n = 3). In this figure, "*" means significant difference between the two groups ($P < 0.05$), while "#" means highly significant difference ($P < 0.01$).

et al., 2021) were set to 5, 10, 20, 40, and 80 μM while PC-B2 concentrations (Zheng et al., 2022) were set to 25, 50, 100, 200, and 400 μg/mL. After 24 h of incubation, CCK-8 was added. Following exposure to cadmium at a concentration of 20 μM for 24 h, PC-B2 with varying gradients was added and incubated for an additional 24 h before the addition of CCK-8 solution to each well. The cells were incubated for 1–4 h, and the OD values were measured at 450 nm using an enzyme-labeled instrument. The protective effect of PC-B2 on cadmium-damaged IEC-18 cells was determined by calculating their relative cell viability.

2.2.3. qRT—PCR and Western blot analysis

Total RNA from uterine tissue and IEC-18 cells was extracted with TRIzol reagent according to the manufacturer's instructions and reverse transcribed into cDNA using a Transcriptor cDNA Synth kit (Roche). Real-time PCR was performed with 1 μL cDNA template using specific primers (Table 1) designed with the reference Primer Premier 5.0, and GAPDH and β-actin was used as an internal control. The amplification conditions were as follows: 95 °C incubations for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s. The relative expression level of the target gene was determined by the 2-ΔΔCt method (Hu et al., 2021).

Total protein was extracted from each uterus and IEC-18 cells and quantified using a dioctinic acid assay (Thermo Fisher Scientific, USA). The target proteins cytokines and inflammatory cytokines separated from the total protein (25 μg) by SDS—PAGE were transferred to a PVDF membrane (Millipore Corporation, USA). The membrane was blocked with 5% skim milk (W/V) at room temperature for 90 min and then incubated with primary antibody at 4 °C overnight. After washing with 1% Tween 20 in TBS, the membrane was incubated with the secondary antibody at room temperature for 50 min, and then the target protein was visualized using the LiCorOdyssey system (Li-Cor Biosciences, USA).

2.2.4. Flow cytometry

We employed flow cytometry to quantify apoptosis. The uterine tissue was first dissected and then subjected to enzymatic digestion with 0.35% collagenase and 0.025% DNase I at 37 ℃ for one hour, followed by incubation in pancreatic enzyme at the same temperature for another hour to obtain a single cell suspension(Kassis et al., 1984). The resulting cell suspension was filtered through a 200-mesh cell filter, followed by centrifugation at 4 ℃ for 5 min at 300–400g and subsequent removal of the supernatant. After that, an appropriate amount of PBS was added to wash the cells, which were then centrifuged at room temperature for another 5 min at 300–400g before discarding the PBS. This washing process was repeated once more before loading the cells into a sample tube. The uterine cell apoptosis was assessed using flow cytometry. For each sample, one blank tube and two single-stained tubes were prepared, and a small number of cells were transferred from the sample tubes to the corresponding tubes. After washing with precooled PBS, the cells were resuspended in 500 μL of precooled 1 × Binding Buffer working solution. Subsequently, Annexin V-FITC (5 μL) and PI staining solution (10 μL) were added to each sample tube as well as each single stained tube. The samples were gently vortexed and incubated for 5 min at room temperature in the absence of light. Apoptosis rate of cells was analyzed using a CytoFLEX flow cytometer (Beckman Coulter, China).

2.2.5. Immunofluorescence

Vimentin expression in rat endometrial epithelium was analyzed using a 1:100 dilution of Vimentin polyclonal antibody (product: PA5–116538) according to the manufacturer's instructions. Blue: DAPI was used for nuclear staining and visualization of fluorescence using an EVOS fluorescence microscope imaging system. (Invitrogen, AMF4300, USA).

2.3. 16 S sequencing and microbiota analysis

The cDNA was extracted using a kit, and the library was double-

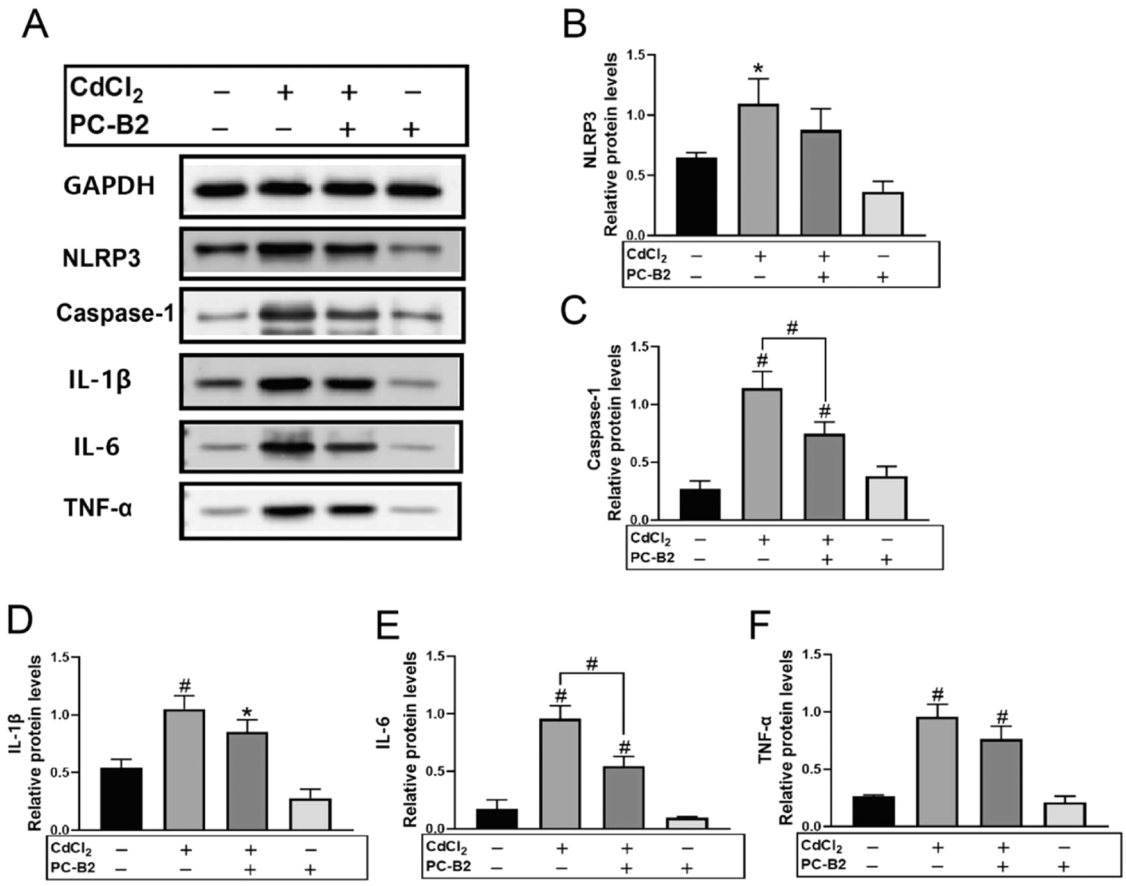

Fig. 4. Expression of inflammatory cytokines by Western blot analysis. (A) The protein expression of NLRP3, caspase-1, IL-1β, IL-6 and TNF-α was detected by immunoblotting, the experiment was repeated for at least 3 times with each group; and (B) - (F) was the expression of NLRP3, caspase-1, IL-1β, IL-6 and TNF-α protein, respectively. Data were presented as mean ± SD (n = 3). In this figure, "*" means significant difference between the two groups ($P < 0.05$), while "#" means highly significant difference ($P < 0.01$).

terminally sequenced based on the Illumina NovaSeq sequencing platform. Off-machine data were imported into QIIME2 for denoising and quality control. After annotation, α and β diversity were calculated using the R program package. Shannon and Simpson indices were used to measure α diversity, and ANOVA was used to detect differences. β diversity was analyzed by NMDS, and permutation multivariate analysis of variance (PERMANOVA) was used to test community differences. In addition, Picrust2 was used to predict flora function.

2.4. Statistical analysis

Numerical results were expressed as the mean ± standard deviation and analyzed by using SPSS statistical software (version 23.0, IBM). One-way analysis of variance (ANOVA) was used for statistical analysis, and multiple comparisons were used to analyze the differences between the means of normally distributed data. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. $CdCl_2$ deposition in the uterus and changes in uterine wet weight in SD rats

Excessive deposition of cadmium in the uterus can result in uterine lesions. Therefore, we compared changes in cadmium concentration among the four groups and found that $CdCl_2$ significantly increased cadmium concentration in the uterus while PC-B2 application significantly decreased it (Fig. 1A). Meanwhile, we observed that the group exposed to cadmium did not exhibit a significant increase in uterine weight (Fig. 1B).

3.2. Uterine toxicity analysis of SD rats induced by $CdCl_2$ exposure

3.2.1. MPO activity, GSH-Px and SOD expression were quantified using ELISA and Immunofluorescence assays

To test the hypothesis that cadmium exposure causes uterine damage by destroying the oxidative stress level in SD rats and PC-B2 has a protective effect, uterine tissue samples of the SD rats were collected to detect the expression of MPO, GSH-Px and SOD by ELISA. As shown in Fig. 2A–C, $CdCl_2$ treatment significantly increased the MPO concentration in the uterine tissue, while $CdCl_2$/PC-B2 treatment decreased the MPO concentration (Fig. 2A). The ELISA results showed that compared with the control group, the expression levels of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in the $CdCl_2$ group were significantly decreased, while their expression levels in the $CdCl_2$/PC-B2 group were significantly increased (Fig. 3B and C). Meanwhile, we conducted an analysis and evaluation of the extent of uterine tissue damage based on the immunofluorescence results. As shown in Fig. 2E, $CdCl_2$ significantly increased the vimentin content in the uterus, while PC-B2 application resulted in no significant difference compared to the control group (Fig. 2D). These findings suggest that $CdCl_2$ can induce toxic damage to rat uterus by disrupting oxidative stress levels, whereas PC-B2 can alleviate such damage.

3.2.2. qRT—PCR and Western blot analysis

To further validate the extent of uterine damage caused by exposure

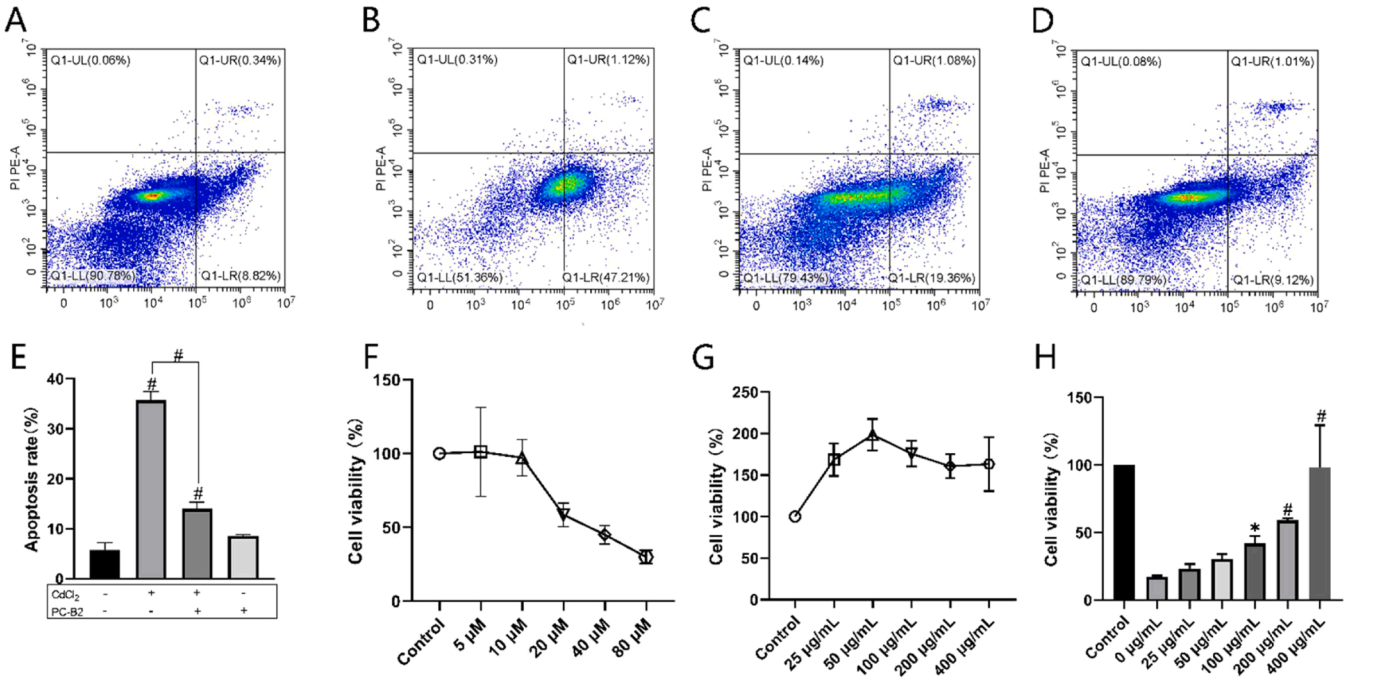

Fig. 5. An experimental study on the survival and apoptosis of IEC-18 cells was conducted. (A) - (D) was the result of flow cytometry for the Control group, the $CdCl_2$ group, the $CdCl_2$/PC-B2 group, the PC-B2 group, respectively; and (E) The apoptosis rate analyzed by flow cytometry. The data were expressed as the mean ± SD (n = 3). Effects on the viability of IEC-18 cells were assessed, with (F) $CdCl_2$ (n = 5) and (G)PC-B2 (n = 5) tested separately. Additionally, the impact of varying concentrations of PC-B2 on cell viability was evaluated in conjunction with 20 μM $CdCl_2$ exposure (H). In this figure, "*" means significant difference between the two groups ($P < 0.05$), while "#" means highly significant difference ($P < 0.01$).

to $CdCl_2$ and assess the protective effect of PC-B2, we conducted an analysis of gene expression and protein levels associated with the inflammatory response. The mRNA expression levels of NLRP3, caspase-1, IL-1β, IL-6 and TNF-α were significantly upregulated in the $CdCl_2$-treated group. However, the expression levels of caspase-1, IL-6 and TNF-α in the $CdCl_2$/PC-B2 co-treatment group were significantly reduced compared to those in the $CdCl_2$-treated group (Fig. 3A–E). The protein expression levels of NLRP3, caspase-1, IL-1β, IL-6 and TNF-α were significantly increased in the $CdCl_2$ group. However, compared to the $CdCl_2$ group, the expression levels of caspase-1 and IL-6 were significantly decreased in the $CdCl_2$/PC-B2 group (Fig. 4A–F). Therefore, our findings suggest that $CdCl_2$ not only disrupts uterine oxidation levels but also induces inflammation in the uterus. Additionally, PC-B2 inflammatory factor exhibits a certain inhibitory effect on alleviating $CdCl_2$ toxicity in the uterus.

3.3. Effect of PC-B2 on uterine epithelial and IEC-18 cells induced by $CdCl_2$ exposure in SD rats

In addition to the inflammatory response, cadmium exposure in the uterus can also cause a large amount of apoptosis of uterine cells and further aggravate uterine diseases. Therefore, we examined the apoptosis of four groups of uterine epithelial cells. The effects of $CdCl_2$ exposure and PC-B2 on IEC-18 cells were detected (Fig. 5A-D). The results showed that the total apoptosis rate was 9.16% in the control group (Fig. 5A), 48.33% in the $CdCl_2$ group (Fig. 5B), 20.44% in the $CdCl_2$/PC-B2 group (Fig. 5C), and 10.13% in the PC-B2 group (Fig. 5D). Compared with the control group, the $CdCl_2$ group significantly increased apoptosis rate in uterine epithelial cells. The $CdCl_2$/PC-B2 group significantly reduced apoptosis rate compared with the $CdCl_2$ group. There was no difference between the control group and the PC-B2 group in the apoptosis rate (Fig. 5E). Further examination of the effect of $CdCl_2$ exposure on cell viability showed that the cell viability began to decrease at the concentration of 20 μM $CdCl_2$ (Fig. 5F). In the determination of the concentration of PC-B2 on cell viability, we found that 50 μg/mL had the best effect on cell viability (Fig. 5G). We exposed IEC-18 cells to a concentration of 20 μM $CdCl_2$ for 24 h, and then added PC-B2 with different concentration gradients for cell viability test. It was found that PC-B2 tended to reduce the decline in IEC-18 cell viability caused by $CdCl_2$ exposure in a dose-dependent manner (Fig. 5H).

3.4. Metallothionein gene (Mt) expression

We further determined the mRNA expression levels of MT1, MT2, and MT3 proteins in uterine tissue and IEC-18 cells. The results demonstrated that PC-B2 significantly upregulated the mRNA expressions of all three MT proteins in IEC-18 cells (Fig. 6A–C). In contrast, $CdCl_2$ exposure only induced a non-significant increase in the mRNA expressions of MT1 and MT2 proteins (Fig. 6A and B), while significantly increasing that of MT3 protein (Fig. 6C). Moreover, all three types of MT proteins were markedly elevated in uterine tissue. There was no statistically significant difference observed between the PC-B2 group and the Control group (Fig. 6D–F). The PC-B2 treatment resulted in a significant enrichment of three metallothionein (MT) proteins, with MT3 protein showing particularly high levels of enrichment (Fig. 6F).

3.5. Effect of PC-B2 on intestinal microbiota structure in SD rats exposed to $CdCl_2$

After 16S rRNA sequencing, an operational taxonomic unit (OTU) was generated by denoising the DADA2 channel of QIIME2. In each treatment group, *Firmicutes*, *Bacteroidota* and *Verrucomicrobiota* were dominant at the phylum level. Others, including *Desulfobacterota* and *Actinobacteriota*, were detected at lower levels (Fig. 7A). However, the major bacterial species in the microbiota did not differ significantly among the treatment groups (Fig. 7B). We further investigated the

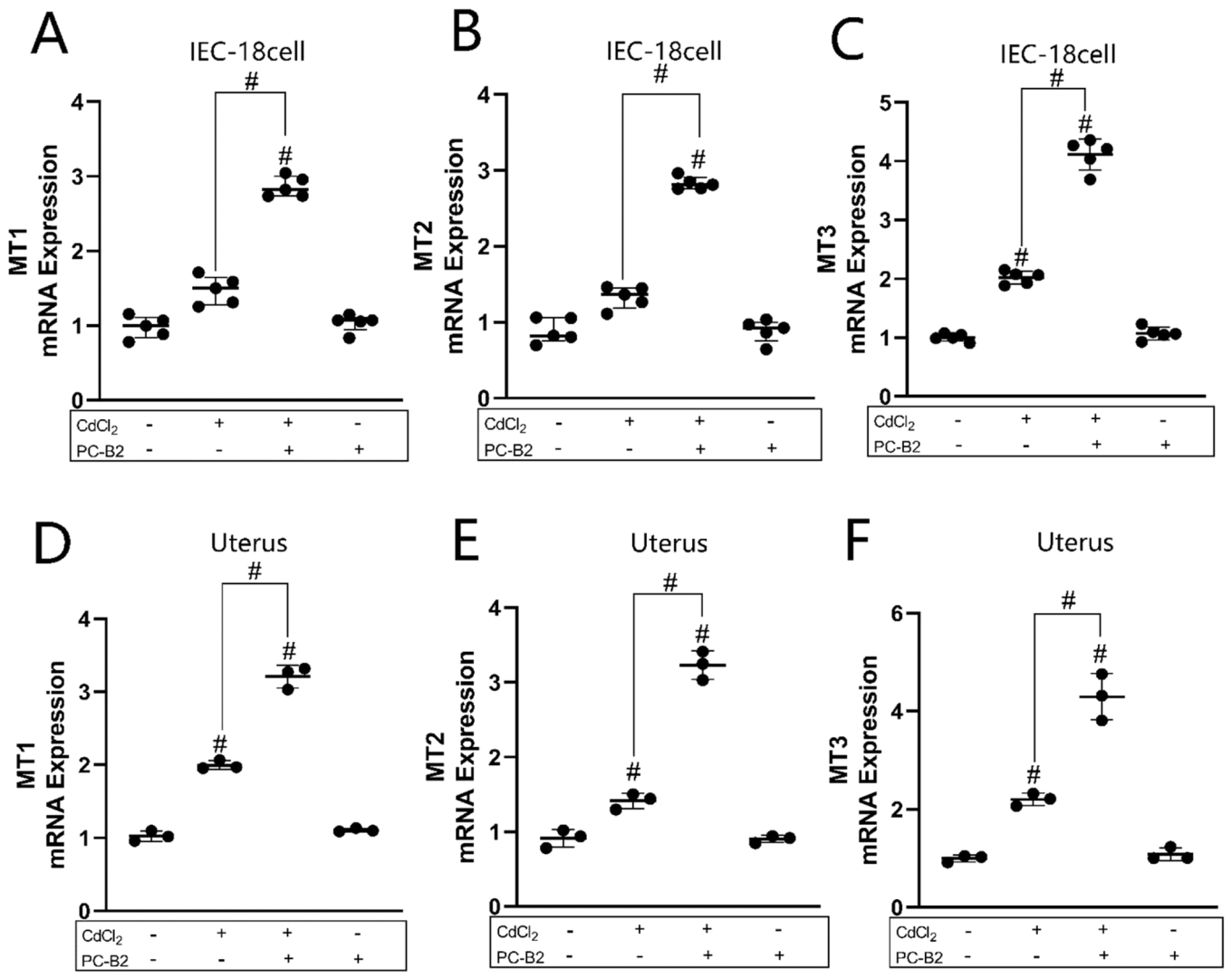

Fig. 6. MT protein expression. (A-C) The mRNA expression levels of MT1, MT2, and MT3 proteins were analyzed in IEC-18 cells, the data were expressed as the mean ± SD (n = 5). (D-F) The mRNA expression levels of MT1, MT2, and MT3 proteins in uterine tissue were analyzed, the data were expressed as the mean ± SD (n = 3). In these figures, "*" means significant difference between the two groups ($P < 0.05$), while "#" means highly significant difference ($P < 0.01$).

differences in microbiota composition among the treatments by analyzing their α and β diversity. Non-metric multidimensional scaling (NMDS) showed that the $CdCl_2$/PC-B2 group had obvious changes compared with the other three groups (Fig. 7C). Shannon value and Simpson value indicated the diversity of gut microbiota , the findings indicated that PC-B2 had a modest impact on the diversity of gut microbiota, albeit not statistically significant. (Fig. 7D and E).

At the genus level, linear discriminant and effect size (LEfSe) analysis showed that the *Lgnatzschineria* was dominant in the control group compared with the $CdCl_2$ group (Fig. 8A). Compared with the PC-B2 group, the main bacteria flora in the control group were *Alistipes, Clostridiales VadinBB60 group* and *Coxiella* (Fig. 8B). Compared with the $CdCl_2$ group, PC-B2 treatment made *Gordonibacter* and *Bacteroidota pectinophilus* become the dominant communities (Fig. 8C). Finally, we conducted a comprehensive comparison and found that the signature species of the PC-B2 group was the beneficial bacterium *Muribaculum* (Fig. 8D), we therefore suggest that biotransformation of gut microbes increases the efficacy of PC-B2.

Changes in galactose metabolism, TCS, bacterial chemotaxis and quorum sensing pathways were observed in the $CdCl_2$/PC-B2 group by PICRUST2 function prediction analysis. The PC-B2 group had changes in RNA degradation, glyoxylate and dicarboxylate metabolism and bacterial secretion system pathways (Fig. 8E). Therefore, we suggest that the alleviation of cadmium toxicity in utero by PC-B2 is correlated with the changes in gut metabolites.

Finally, we analyzed the relationship between the major species components of the gut microbiota and the expression levels of proteins associated with the uterine inflammatory response. The results showed that the expression levels of proteins associated with the uterine inflammatory response were positively correlated. In addition, the expression of NLRP3 and IL-1β was positively correlated with Bacteroides but not with other bacterial groups (Fig. 8F).

4. Discussion

Cadmium released by human excrement, agrochemicals and sewage pollution in farmlands may be absorbed by plants and aquatic organisms (e.g. shellfish) and enter humans and animals through food chains (Amachree et al., 2013). Its accumulation in humans and animals is a particular threat to the uterus, causing tissue damage and oxidative stress (Nasiadek et al., 2014; Peereboom-Stegeman and Jongstra-Spaapen, 1979). The accumulation of reactive oxygen species (ROS) induced by cadmium exposure can cause a series of nonspecific damages, such as DNA strand breaks, lipid peroxidation and protein

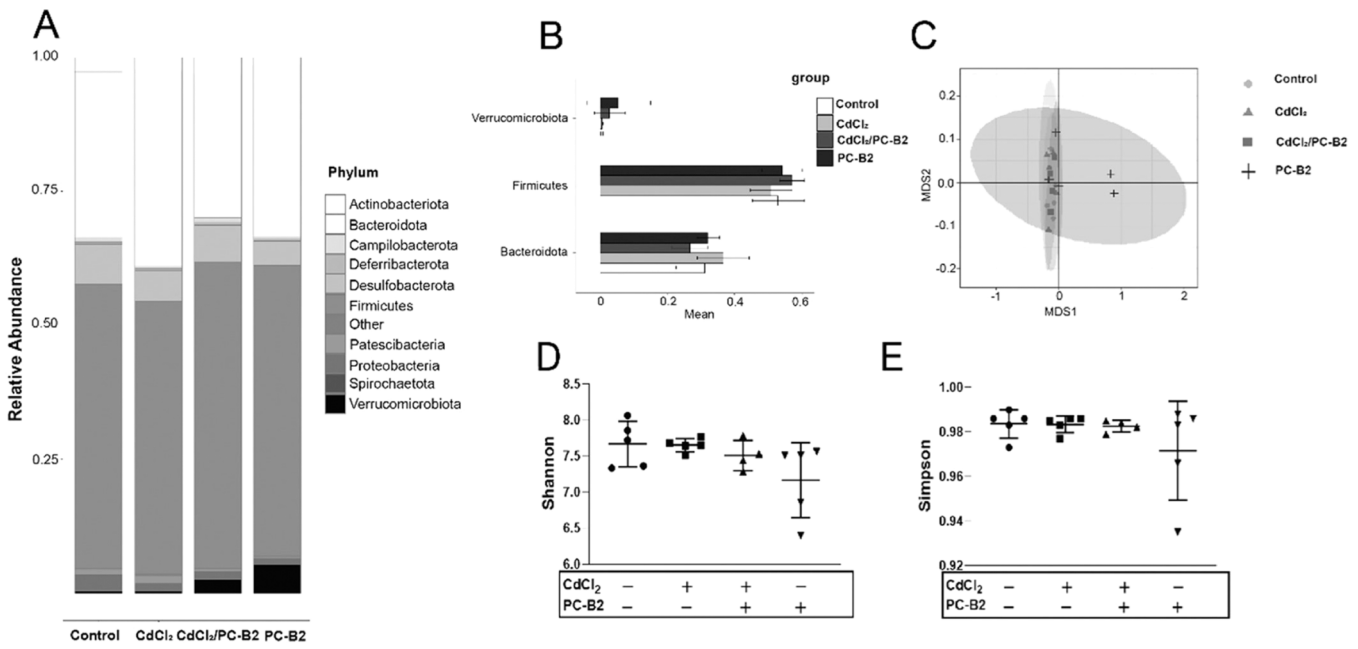

Fig. 7. Species composition in the gut microbiota and differences in intestinal microbiota among treatments. (A) Gut microbiota at the phylum level; (B) the relative abundance comparison of three major taxa; (C) the β diversity of the microbiota estimated by NMDS (Stress = 0.09460455), which was not significant by the PERMANOVA test (P = 0.09004, R^2 = 0.8865); (D) Shannon indices of the gut microbiota; and (E) Simpson indices of the gut microbiota.

function loss, leading to cell dysfunction, cell death and even tissue damage. However, SOD, CAT and GPx are components of the first line of defense against oxidative stress (Paithankar et al., 2021). Our findings indicate that exposure to cadmium results in a decrease in the levels of GSH-PX and SOD, while simultaneously increasing MPO levels. However, there is a general positive correlation between MPO levels and the severity of disease in the body, indicating that cadmium may impair uterine function by depleting components that provide protection against oxidative stress. Following the application of PC-B2, an increase in SOD and GSH-Px levels was observed, indicating that PC-B2 may effectively prevent $CdCl_2$-induced uterine oxidative stress by inhibiting the depletion of SOD and GSH-Px. Moreover, antioxidants have been shown to alleviate $CdCl_2$-induced oxidative stress by reducing the levels of SOD and GSH-Px (Ren et al., 2021; Wang et al., 2019). SOD and GSH-Px are important factors in the nuclear factor NF-E2-related factor 2 (Nrf2) pathway (He et al., 2020). However, Nrf2 has paradoxical effects on the regulation of NLRP3 inflammasome activity (Ahmed et al., 2017). Nevertheless, previous studies utilizing both natural and synthetic compounds have also demonstrated the inhibitory effect of Nrf2 on the activation of NLRP3 inflammasome. For example, mangiferin has been shown to upregulate Nrf2 and HO-1 expression in a dose-dependent manner. Furthermore, the activation of NLRP3, ASC, caspase-1, IL-1β and TNF-α in the liver induced by LPS/D-GalN was effectively suppressed (Pan et al., 2016). Therefore, the role of Nrf2 in inflammation-related diseases remains unclear. It is significant to elucidate the mechanism by which it activates inflammasome functions in order to gain a more comprehensive understanding of this process. The presence of oxidative stress often coincides with manifestations of inflammation (Ren et al., 2021), such as liver and kidney injury, as well as neuroinflammation (Cai et al., 2021; Li et al., 2021; Reuter et al., 2010). Studies have demonstrated that $CdCl_2$ induces activation of the NLRP3 inflammasome via NF-κB signaling pathway (Reuter et al., 2010), which may be attributed to the initiation of immune response triggered by damage-associated molecular patterns (DAMPs). DAMPs bind to pattern recognition receptors (such as Toll-like receptors) and promote the release of inflammatory cytokines through the activation of NF-κB/p65. It induces inflammatory reactions, including IL-1β, IL-6 and TNF-α (Liu et al., 2019). DAMPs also activate the nucleotide binding oligomerization domain (NOD) receptor family (NLRs), which leads to NLRP3 inflammasome and caspase-1 precursor activation (Turner, 2016). Activated caspase-1 cleaves the progenitor cytokines pro-IL-1β and pro-IL-18, triggering pyroptosis and promoting the maturation and secretion of IL-1β and IL-18, ultimately activating multiple inflammatory pathways (Garcia-Martinez et al., 2015). The production and release of proinflammatory cytokines and the formation of the NLRP3 inflammasome are key processes of uterine toxicity caused by cadmium exposure (Di Nicuolo et al., 2021). $CdCl_2$ exposure increased the expression of TNF-α, IL-1β and IL-6 and promoted the activation of NLRP3 in utero. PC-B2 intervention significantly downregulated the expression of NLRP3, suggesting that PC-B2 may block the activation of the NLRP3 inflammasome and related signaling pathways. In addition, the release of IL-1β was inhibited, suggesting that the harmful effects of $CdCl_2$ exposure can be alleviated.

Moreover, the apoptosis of uterine epithelial cells may be attributed to exposure to $CdCl_2$ due to a positive correlation between the apoptosis index of uterine epithelial cells and duration of $CdCl_2$ exposure (Rani et al., 2014). Natural plant extracts have been demonstrated to possess inhibitory effects on uterine morphological damage induced by cadmium exposure (Sapmaz-Metin et al., 2017). We observed that PC-B2 inhibited the apoptosis of uterine epithelial cells induced by exposure to $CdCl_2$. The apoptotic rate of uterine epithelial cells significantly increased to 48.33% upon treatment with $CdCl_2$, while intervention with PC-B2 effectively reduced it to 20.44%. Moreover, this intervention significantly decreased cadmium deposition in uterine tissue and improved the survival rate of IEC-18 cells exposed to $CdCl_2$. Previous research has demonstrated that dietary intake of licorice can enhance the metabolism of cadmium in the liver and kidneys, leading to its rapid elimination from other tissues and organs (Zheng et al., 2022). Therefore, further investigation is needed to determine whether PC-B2 can exert a similar effect.

Metallothionein (MT) is widely believed to play a crucial role in mitigating the toxic effects of cadmium by reducing its deposition in tissues through binding cadmium ions (Genchi et al., 2020). Our findings indicate a significant upregulation of MT1, MT2, and MT3 protein mRNA expression levels in utero following exposure to cadmium. Moreover, a significant upregulation of MT3 protein mRNA expression

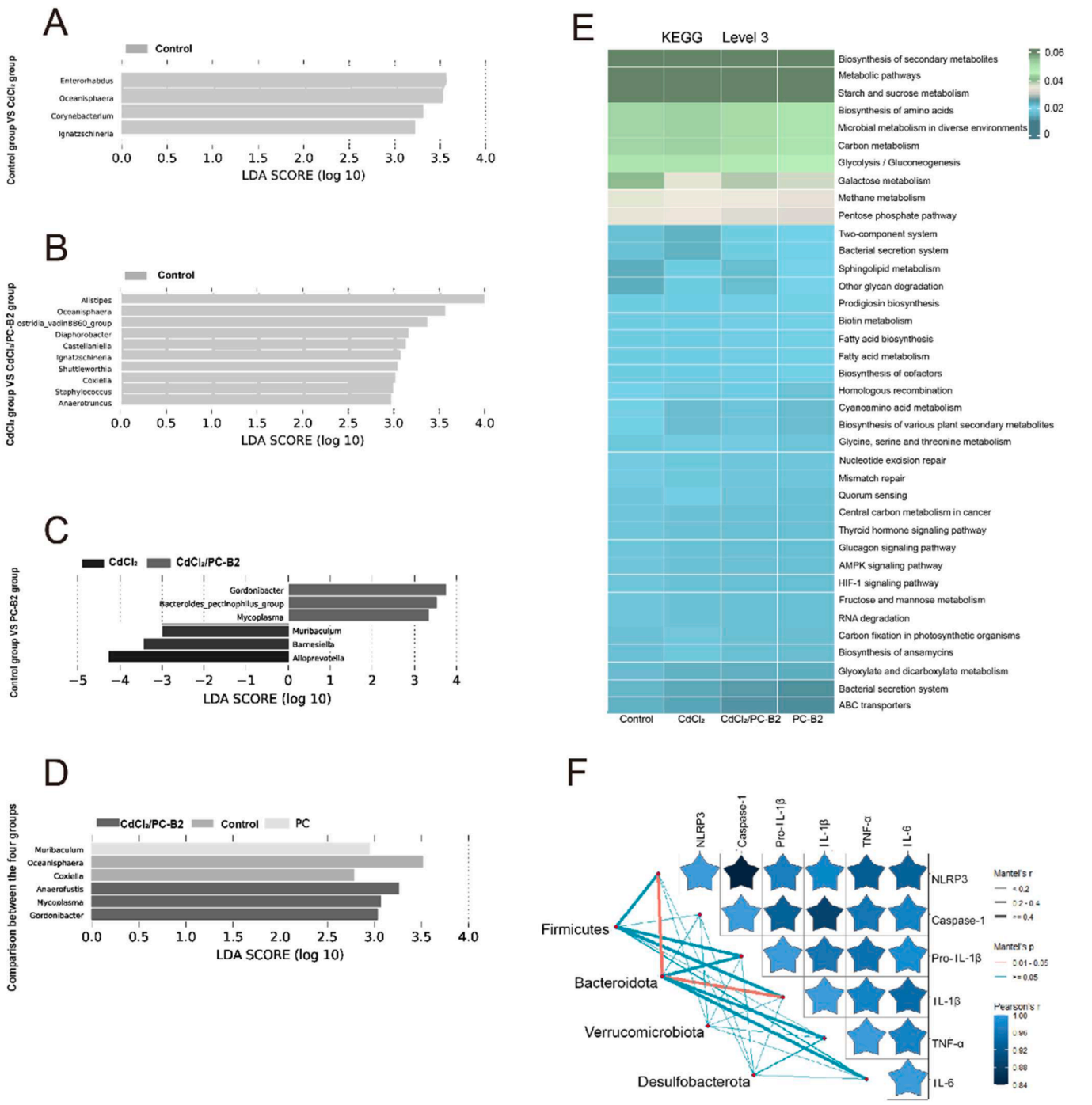

Fig. 8. Linear discriminant analysis at the genus level. (A) Control group and $CdCl_2$ group; (B) Control group and PC-B2 group; (C) $CdCl_2$ group and $CdCl_2$/PC-B2; (D) Control group and all treatment groups; (E) Pathway heatmap of the top 20 functional pathways in the gut microbiota; and (F) Relationship between proteins of the uterine inflammatory response and intestinal microbiota composition by Mantel analysis.

was observed in IEC-18 cells, consistent with previous findings (Nakamura et al., 2012), indicating that enhancing MT protein expression may be a key strategy for mitigating cadmium toxicity (Chai et al., 2020). Notably, exposure to cadmium can significantly induce the synthesis of MT1 and MT2 proteins. Although expression levels are elevated under cadmium exposure, it appears that MT3 proteins may not possess the ability to mitigate cadmium toxicity in utero (Nakamura et al., 2012). The application of PC-B2 significantly increased the mRNA expressions of MT1, MT2, and MT3 proteins in both uterus and IEC-18 cells. This suggests that PC-B2 may induce the expression of MT protein and reduce cadmium deposition in the uterus. Previous studies have also shown that licorice can stimulate the production of MT1 protein, which is closely associated with intestinal microbial metabolism (Akao, 2000; Kiso et al., 1984). As a polyphenolic flavonoid, PC-B2 shares similar structural composition and biological activities (such as antioxidant properties) with dietary licorice. Therefore, it is hypothesized that PC-B2 may reduce cadmium deposition in the uterus and improve intestinal microbiota function. In future studies, we will further investigate its metabolic pathways.

The gut microbiome is considered to be one of the key factors regulating host health (Fan and Pedersen, 2021). Previous studies have shown that the composition and quantity of microbial species is strongly

associated with female reproductive disorders of human females, such as endometriosis (Tsai et al., 2019). However, there is a paucity of research on the impact of intestinal flora on uterine inflammatory injury and its underlying mechanism. Our findings suggest that although cadmium alone do not significantly alter gut flora richness, it have a significant effect on both gut flora composition and uterine injury, which is consistent with previous studies (Zheng et al., 2022). β-diversity is frequently utilized to assess compositional similarity among distinct microbiotas. Therefore, we conducted further analysis on the changes in β-diversity among the four groups and observed that the microbiota in the control group exhibited a tighter clustering with the $CdCl_2$/PC-B2 treatment group as compared to the $CdCl_2$ group. This implies that $CdCl_2$ may disrupt the intestinal microbiota, while PC-B2 intervention can restore it to its original state. This is consistent with previous research indicating that cadmium exposure alters the gut microbiome structure (Tinkov et al., 2018).

Interestingly, when we examined the species composition of the gut microbiota across treatments, we found that $CdCl_2$ exposure reduced the abundance of *Lgnatzschineria* and other beneficial bacteria, while $CdCl_2$/PC-B2 treatment increased the abundance of the beneficial bacteria *Bacteroidota pectinophilus* and the *Gordonibacte* community. *Gordonibacter* is a group of bacteria that contribute to the production of urolitin from dietary polyphenols, which exerts anti-inflammatory activity in human and animals (Quaranta et al., 2019). Dietary polyphenols stimulate the growth of beneficial bacteria and inhibit pathogenic bacteria by regulating intestinal ecological imbalance (García-Villalba et al., 2020). Recent studies have shown that chemicals contained in grapes (e.g., terpenoids, carotenoids, and proanthocyanidins) can ameliorate intestinal dysbiosis and prevent gut-related inflammatory diseases (Molinari et al., 2022). PC-B2 reduced the abundance of the harmful bacteria *Alistipes, Clostridiales Vadinbb60 Group* and *Coxiella*. Among them, *Alistipes* reduces bacteria that produce butyric acid, which has anti-inflammatory effects (Santa et al., 2019). *Coxiella* is a pathogenic bacterium that can manifest as acute diseases such as pneumonia and hepatitis or chronic diseases such as endocarditis in humans (Parker et al., 2020). Our results showed that PC-B2 improved the dysregulation of intestinal flora caused by $CdCl_2$ exposure and increased the abundance of the *Bacteroidota pectinophilus group* and *Gordonibacter*. These dominant bacteria could play anti-inflammatory and antioxidant roles, which may contribute to the improvement of uterine inflammatory damage and oxidative stress. Therefore, we further analyzed the relationship between the main composition of the intestinal microbiota and the expression level of inflammatory response-related proteins in the rat uterus. The results showed that Bacteroides was positively associated with the expression of NLRP3 and IL-1β. PC-B2 alleviated $CdCl_2$-induced uterine injury in mice by reducing Bacteroides abundance. We also performed a PICRUST2 functional prediction analysis. These results suggest that PC-B2 may affect metabolites by modulating some biochemical pathways in the gut microbiota, thereby attenuating $CdCl_2$ toxicity.

In conclusion, our results indicate that PC-B2 can effectively reduce the uterine toxicity caused by $CdCl_2$ in female rats and enhance the antioxidant and anti-inflammatory abilities under $CdCl_2$ stress, possibly through upregulating the expression of MT protein. Moreover, our findings suggest a crucial role of PC-B2 in mitigating $CdCl_2$-induced uterine toxicity through the modulation of gut microbiota composition. This study provides a basis for further investigations into the reproductive toxicity mechanism of cadmium and the protective effect of gut microbiota against cadmium toxicity.

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CRediT authorship contribution statement

Binhong Hu: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Songqing Liu:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing. **Yuanyue Luo:** Data curation, Formal analysis, Methodology, Supervision, Writing – original draft. **Jingyu Pu:** Data curation, Methodology, Supervision, Writing – original draft. **Xin Deng:** Formalanalysis, Methodology, Supervision. **Wenjing Zhou:** Formal analysis, Methodology, Validation. **Yuqing Dong:** Methodology, Software, Writing – review & editing. **Yichuan Ma:** Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Gang Wang:** Investigation. **Fan Yang:** Investigation, Validation. **Tianhui Zhu:** Conceptualization, Data curation, Project administration, Resources, Writing - review & editing. **Jiasui Zhan:** Conceptualization, Supervision, Software, Visualization, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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