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# Integrated metagenomic and metabolomic analyses reveal tenacissoside G as a potential non-antimicrobial treatment for bovine endometritis

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## Abstract

**Background** Bovine endometritis is a prevalent uterine disease that directly curtails reproductive performance and indirectly reduces milk production by increasing calving intervals. Postpartum uterine bacterial infection is the primary cause of bovine endometritis, which is typically treated with prostaglandin F2 $\alpha$  and antimicrobials. However, abuse of antimicrobials has led to the emergence of multidrug-resistant bacteria, threatening both human and animal health. To explore alternatives to antimicrobial therapy for bovine endometritis, we integrated uterine metagenomic and metabolomic analyses and identified a novel bioactive metabolite with therapeutic potential. The potential antibacterial and anti-inflammatory effects of this metabolite against bovine endometritis were evaluated by assessing its inhibitory effect on the growth of *F. necrophorum* in vitro, and by quantifying histopathological scores and inflammatory cytokine expression levels in an in vivo mouse model of endometritis, respectively.

**Results** A total of 40 Holstein dairy cows at 21 days to 30 days postpartum were assigned into healthy cows ( $n = 15$ ), subclinical endometritis cows ( $n = 12$ ) and clinical endometritis cows ( $n = 13$ ) according to clinical signs and laboratory tests for bovine endometritis. The uterine fluid was collected aseptically for metagenomics and metabolomics sequencing to identify bacterial species associated with bovine endometritis and metabolites that could potentially be used for treatment of bovine endometritis. A total of 17 bacterial species were significantly associated with bovine endometritis, with *Fusobacterium necrophorum* as the most significantly enriched in cows with clinical endometritis compared to healthy counterparts. In total, 391 metabolites were significantly differentially abundant between healthy and clinical endometritis cows. Among these, a plant-derived compound, tenacissoside G was significantly enriched in healthy cows. Notably, the abundance of *F. necrophorum* was significantly negatively associated with the concentration of tenacissoside G in clinical endometritis cows. Moreover, tenacissoside G significantly inhibited the growth of *F. necrophorum* in vitro and ameliorated inflammation in endometritis caused by *F. necrophorum* in a mice model.

**Conclusion** This study provides new insights into the relationship between uterine microbiome and metabolites in bovine endometritis, potentially leading to novel strategies for treating bovine endometritis. Furthermore,

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tenacissoside G exhibits therapeutic effects against endometritis induced by *F. necrophorum*, and could serve as a potential alternative to antimicrobials for treating endometritis.

**Keywords** Bovine endometritis, Uterine metagenomics, Uterine metabolomics, *Fusobacterium necrophorum*, Tenacissoside G

## Background

Dairy products, rich in high-quality protein, calcium, and vitamins, play a substantial role in satisfying the growing demands for essential nutrients worldwide [1, 2]. Global demand for dairy products is projected to increase substantially in the coming decades, particularly in low- and middle-income countries [3]. However, reproductive failures and diseases in dairy cows contribute significantly to the limited supply of dairy products [4]. Endometritis is a prevalent postpartum uterine disease in dairy herds, leading to markedly reduced reproductive performance, milk production and animal welfare [5–7], as well as elevated treatment costs [8]. Bovine endometritis can be classified into clinical and subclinical endometritis. Clinical endometritis is characterized by purulent or mucopurulent vaginal discharge and endometrial inflammation, typically diagnosed through vaginal examination and cytology. In contrast, subclinical endometritis presents no visible clinical signs but it is identified by endometrial cytology, often marked by an elevated proportion of polymorphonuclear neutrophils (PMNs) [6, 9, 10]. The prevalence of bovine endometritis (including clinical and subclinical endometritis) during the post-partum period ranges from 12% to more than 50%, with substantial regional variation [11–13]. The annual cost of endometritis was estimated between €160 to €420 per case in Europe [14]. These figures underscore the importance of implementing effective control measures for bovine endometritis in the dairy industry.

Uterine bacterial infection is the primary cause of bovine endometritis [15]. Traditional culture-based studies found *Fusobacterium necrophorum* [16] and *Trueperella pyogenes* [17] to be associated with endometritis. More recently, culture independent-based studies have expanded this view, revealing that a broader array of bacterial taxa including *Bacteroides pyogenes*, *Prevotella heparinolytica*, *Helcococcus spp.*, *Peptoniphilus spp.*, and *Porphyromonas levii* are associated with uterine disease in cows [5, 18, 19]. Moreover, emerging research highlights that it is not merely the presence of specific pathogens but rather the disruption of microbial homeostasis within the uterine environment that plays a critical role in the onset and progression of endometritis [20]. A healthy uterine microbiome maintains a balanced microbial community that supports immune tolerance and tissue integrity, thereby preventing opportunistic

infections [21]. In contrast, dysbiosis, characterized by the loss of beneficial microbes, overgrowth of pathogens, and altered metabolic activity, can trigger inflammation, impair uterine recovery postpartum, and contribute to subfertility or infertility [21]. In humans, the composition and stability of the uterine microbiome are closely linked to reproductive success and resistance to diseases such as endometriosis and endometrial cancer [22, 23]. However, pathogens can overcome this protective environment through virulence mechanisms such as biofilm formation, mucin degradation, and antimicrobial resistance, allowing them to persist and disrupt host-microbiota interactions [24]. Although considerable work has investigated the relationship between bovine endometritis and the uterine microbiome, the interactions among endometritis and uterine microbiome as well as uterine metabolome remain poorly understood. A deeper understanding of how microbial homeostasis is maintained or lost following parturition is urgently needed to inform the development of targeted, microbiome-based strategies for early diagnosis, prevention, and treatment. This need is especially pressing in the context of antimicrobial stewardship and the global effort to reduce reliance on antibiotics in livestock production.

The treatment of endometritis in dairy cows worldwide largely relies on antimicrobials and prostaglandin F<sub>2α</sub> [25, 26]. However, current evidence does not support the efficacy of prostaglandin F<sub>2α</sub> in the treatment of bovine endometritis [27]. Although effective, antimicrobials can disrupt microbiome homeostasis and contribute to the emergence of multidrug-resistant bacteria, posing long-term risks to both animal and human health [28]. Therefore, there is an urgent need to develop effective non-antimicrobial and non-hormonal alternatives for the treatment of endometritis. The human gut microbiome can produce a wide range of bioactive small molecule metabolites, including amino acids, oligosaccharides and glycolipids [29], many of which have demonstrated therapeutic potential in diseases such as inflammatory bowel disease [30]. Beyond their metabolic roles, human studies have revealed that the gut and vaginal microbiomes interact bidirectionally with steroids hormones (e.g., estrogens and progestins), forming a regulatory axis that significantly influences women's health across the lifespan [31]. In line with these findings, a recent study identified 49 uterine metabolites whose abundance was

significantly associated with the abundance of pathogenic bacterial species in metritis cows [32]. Notably, 17 of these metabolites were implicated in the overgrowth of opportunistic pathogenic bacteria, such as *Fusobacterium*, *Porphyromonas*, and *Bacteroides*, and were linked to pathological processes including the suppression of biofilm formation by commensal bacteria, immune evasion, dysregulation of host immunity, tissue damage, and inflammation [32]. These findings highlight the potential of specific uterine metabolites as novel therapeutic targets for non-antimicrobial intervention strategies in bovine metritis [32]. However, the potential protective effects of such metabolites against bovine endometritis remain poorly understood and require further investigation.

Tenacissoside G (TG) is a C21 steroidal glycoside extracted from the stem of *Marsdenia tenacissima*, a plant widely used in traditional Chinese medicine for its anti-cancer properties and therapeutic effects in alleviating osteoarthritis [33–35]. TG exerts anti-osteoarthritis effect by suppressing the activation of NF- $\kappa$ B signaling pathway, thereby downregulating the expression of key inflammatory factors in chondrocytes, including inducible nitric oxide synthase (iNOS), matrix metalloproteinase (MMP)–3, MMP-13, interleukin (IL)–6, and tumor necrosis factor (TNF)- $\alpha$  [35]. Additionally, TG enhances the deposition of type II collagen, thereby preserving the integrity of the extracellular matrix and further supporting its therapeutic efficacy in osteoarthritis [35]. In light of its anti-inflammatory properties, TG might be a promising non-antimicrobial candidate for treating bovine endometritis.

In this study, we aimed to (1) identify the bacterial species and metabolites associated with bovine endometritis through integrated analyses of uterine microbiome and metabolome and characterize their potential complex interactions; and (2) evaluate the therapeutic potential of tenacissoside G against endometritis using a mouse model.

## Materials and methods

The clinical examination and sampling procedures, including rectal palpation and uterine irrigation, were executed by trained veterinarians. All experimental protocols in this study were approved by the Animal Ethics Committee of China Agricultural University and authorized by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (AW50214202-2-01; AW50214202-2-02).

### Cow status assessment and sample collection

Total of 40 Holstein dairy cows at 21 to 30 days postpartum from a commercial dairy farm (Shanxi, China) were

enrolled in this study. All cows were housed under identical environmental conditions, received the same diet with ad libitum access to water, and had not received any prior treatment. Parity, calving date, date of disease diagnosis, and treatment details were recorded for all cows. Cows with vaginal lacerations or history of other clinical diseases were excluded from the study prior to sampling.

We used uterine lavage method to collect uterine fluid samples since it is more sensitive compare to cytobrush technique for detecting uterine inflammation and does not adversely affect reproductive performance [36]. Uterine flush samples were collected between days 21 and 30 postpartum. After cleansing the perineal area with 70% ethanol and drying with sterile wipes, a latex embryo flush catheter was inserted into the cervix. A total volume of 200–300 mL of sterile saline was infused into the uterine lumen, gently agitated, and then the fluid was aspirated into a 50 mL syringe [18, 36]. The recovered fluid was aliquoted into three sterile sampling tubes. Two tubes of uterine fluid were stored at  $-80^{\circ}\text{C}$  for metagenomics and metabolomics sequencing, while the third tube was used for cytological examination. In brief, uterine fluid was centrifuged at  $1000\times g$  for 10 min. The resulting pellet was collected, resuspended in sterile saline, and smeared onto a microscope slide. Slides were stained using Diff Quick stain (Solarbio, Beijing, China) for cytological examination. Vaginal discharge was scored according to the criteria described by Sheldon et al. [9]: score 0, clear or translucent vaginal discharge; score 1, vaginal discharge containing flecks of pus; score 2, discharge with  $<50\%$  pus; score 3, vaginal discharge containing  $\geq 50\%$  purulent material, typically white or occasionally sanguineous. Meanwhile, the proportion of PMN in the uterine irrigating fluid cytology smear was determined by calculating the percentage of PMN in 300 cells (PMN and endometrial epithelial cells) with microscopic counting method. Based on vaginal discharge score and PMN proportions, cows were classified into three groups: clinical endometritis (CE), vaginal discharge score 3 and  $\text{PMN} \geq 18\%$ ; subclinical endometritis (SCE), vaginal discharge score 0 and  $\text{PMN} \geq 18\%$ ; and healthy (H), vaginal discharge score 0 and  $\text{PMN} < 18\%$  [6, 9].

Blood samples (10 mL) were aseptically collected from the coccygeal vein of cows using vacutainer tubes without coagulant. These blood samples were kept on ice for 30 min, followed by centrifugation at  $3000\times \text{rpm/min}$  at  $4^{\circ}\text{C}$  for 10 min to isolate the serum. The resulting serum was then aliquoted into tubes and stored for subsequent quantification of inflammatory cytokine levels.

Fresh bovine uterine tissues were collected from healthy, subclinical endometritis, and clinical endometritis cows at slaughterhouse, and fixed in 4%

paraformaldehyde. After fixation, tissues were processed, embedded in paraffin and sectioned at 4  $\mu\text{m}$  thickness. Sections were then dewaxed, hydrated, and stained with haematoxylin and eosin. Histological evaluation was performed using a panoramic digital slide scanner (3DHISTECH, Hungary).

#### Identification of bacterial species in uterine fluid

Aliquots of each uterine fluid sample were streaked onto Columbia Blood Agar Base supplemented with 5% defibrinated sheep blood (Catalog No. BNCC352241, BeNa Culture Collection, Henan, China) and incubated under both aerobic and anaerobic conditions. Aerobic plates were incubated at 37 °C in ambient air supplemented with 5% CO<sub>2</sub> for 12–24 h, while anaerobic plates were incubated at 37 °C in an anaerobic chamber (10 vol % CO<sub>2</sub>, 10 vol % H<sub>2</sub>, 80 vol % N<sub>2</sub>) for 36–72 h. Anaerobic conditions were established by flushing the AnaeroPack jar (Model C-32, Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) with a custom gas mix of 10% CO<sub>2</sub>, 10% H<sub>2</sub>, and 80% N<sub>2</sub> (Hongwei Gas Technology Co., Ltd., Shaanxi, China).

After incubation, colonies with distinct morphologies were sub-cultured to obtain pure isolates. The sub-cultured colonies were subjected to species identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using Autof ms1000 (Autobio, Zhengzhou, China). For each unique colony morphology, a single sub-cultured colony was smeared onto a target plate, overlaid with 1  $\mu\text{L}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) matrix solution, and air-dried, followed by species identification using MALDI-TOF MS according to the manufacturer's instructions. Isolates with identification score  $\geq 9.0$  were considered as successful identifications.

#### Measurement of bovine serum inflammation levels

Serum levels of the inflammatory indicators TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, and myeloperoxidase (MPO) were measured using bovine-specific ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Catalog Nos. TNF- $\alpha$ : H052-1-2, IL-1 $\beta$ : H002-1-2, IL-6: H007-1-2, IL-8: H008-1-2, IL-10: H009-1-2, MPO: H508-1-2). Absorbance was recorded at a wavelength of 450 nm according to the manufacturer's instructions.

#### DNA extraction, metagenome sequencing, and metagenomics data analysis

Bovine uterine fluid samples were lyophilized prior to DNA extraction to improve the DNA extraction efficiency. Genomic DNA was extracted from these lyophilized samples using the Fecal Genome DNA Extraction Kit (AU46111-96, BioTeke, Beijing, China) following the

manufacturer's instructions. Metagenomic libraries were prepared using the TruSeq Nano DNA Library Preparation Kit-Set (#FC-121-4001, Illumina, San Diego, CA, USA). Paired-end sequencing (150 bp) was performed on the Illumina NovaSeq 6000 platform (LC-Bio Technology Co., Ltd., Hangzhou, China). Raw metagenomic reads were processed using KneadData (v0.10.0) for quality control and host contamination removal, as previously described [37, 38]. Adapter sequences were trimmed with Trimmomatic (v0.39) [39], and quality-filtered reads were aligned to the bovine reference genome (bosTau8 3.7, [https://doi.org/10.18129/B9.bioc.BSgen\\_ome.Btaurus.UCSC.bosTau8](https://doi.org/10.18129/B9.bioc.BSgen_ome.Btaurus.UCSC.bosTau8)) using bowtie2 [40] (v 2.5.1) to eliminate the host reads.

#### Taxonomic classification and functional annotation

Taxonomic profiling was performed using MetaPhlAn4 [41], and functional annotation was conducted with HUMAnN3 [42]. Alpha diversity indices, including Simpson, Shannon, richness and gini, and beta diversity (Bray–Curtis distance) were calculated using MetaPhlAn4. The bacterial functional pathways were annotated with HUMAnN3 based on the ChocoPhlAn database. Gene families were regrouped into Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) Orthologies using the humann\_regroup\_table script with the -g uniref90\_ko option. Gene abundance was then normalized to copies per million (CPM). Bacterial species with relative abundance >0.01% and functional features with CPM values >0.01 that presented in >8 samples were retained for downstream analyses.

#### Analysis of uterine fluid metabolome

The LC–MS/MS analysis of uterine fluid was performed using a high-resolution tandem mass spectrometer Q-Exactive (ThermoFisher Scientific, Bremen, Germany) coupled with an UltiMate 3000 UPLC System (ThermoFisher Scientific, Bremen, Germany). Chromatographic separation was carried out on the UPLC system, and data were acquired in both positive and negative ionization modes. Raw mass spectrometry data were pre-processed using XCMS (v 3.9.3, <https://github.com/sneumann/xcms>) [43]. Further data processing, including peak annotation and feature alignment, was conducted using CAMERA (<https://github.com/sneumann/CAMERA>) [44] and metaX toolbox (v 1.4.19, <https://github.com/wenbostar/metaX>) [45] implemented in R (v 4.4.0). Metabolites were identified and annotated by reference to the Human Metabolome Database (HMDB, <http://www.hmdb.ca/>) and KEGG Database (<http://www.genome.jp/kegg/>). The identified metabolites were further validated using an in-house metabolite fragment

spectrum library provided by LC-Bio Technology Co., Ltd (Hangzhou, China).

#### The inhibitory effect of TG against *F. necrophorum*

*Fusobacterium necrophorum* subsp. *necrophorum* (Catalog No. BNCC336998) was obtained from the BeNa Culture Collection ([https://www.bncc.com/pro/p2/8/p\\_336998.html](https://www.bncc.com/pro/p2/8/p_336998.html)), China. The strain was cultured on Columbia Blood Agar Base supplemented with 5% defibrinated sheep blood (Catalog No. BNCC352241, BeNa Culture Collection, Henan, China) under anaerobic conditions (10 vol % CO<sub>2</sub>, 10 vol % H<sub>2</sub>, and 80 vol % N<sub>2</sub>) at 37 °C. Anaerobic conditions were established as described above. After cultivation for 72 h, bacterial colonies were collected and purified. Genomic DNA was extracted using a commercial kit, the TIANamp Bacteria DNA kit (DP302-02, Tiangen, Beijing, China). Details of the whole genome sequencing and analysis of *Fusobacterium necrophorum* subsp. *necrophorum* are provided in Additional file 8.

The minimum inhibitory concentration (MIC) of TG against *F. necrophorum* was determined using a microdilution assay in 96-well microplates, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [46]. Tenacissoside G was initially dissolved in sterile saline containing 40% polyethylene glycol 300 (PEG300) and 5% Tween-80 (Sigma-Aldrich, San Louis, MO, USA) to achieve a stock concentration of 3000 µg/mL. This solution was subsequently diluted in thioglycollate broth to prepare a working concentration range. A two-fold serial dilution was performed to obtain final TG concentrations ranging from 3 to 1500 µg/mL. Each well was inoculated with 100 µL of bacterial suspension containing 1 × 10<sup>6</sup> CFU/mL of *F. necrophorum*. The microplates were incubated at 37 °C for 48 h anaerobically (10 vol % CO<sub>2</sub>, 10 vol % H<sub>2</sub>, and 80 vol % N<sub>2</sub>) in the AnaeroPack jar (Model C-32, Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Anaerobic conditions were achieved as described in the previous section, and the MIC was determined according to the CLSI criteria.

The inhibitory effect of TG on the growth of *F. necrophorum* was evaluated by analyzing growth curve parameters, including maximum growth rate, carrying capacity, and duration of lag phase, across different treatment groups. TG was diluted in fresh thioglycollate broth to prepare working concentrations of 0 (positive control), ½ × MIC, 1 × MIC, 2 × MIC, and 4 × MIC, with each concentration prepared in a total volume of 7 mL. A 140 µL aliquot of *F. necrophorum* culture (OD<sub>600</sub> = 0.5) was inoculated into each tube. Meanwhile, a negative control containing only broth was also included. All tubes were incubated at 37 °C anaerobically (10 vol % CO<sub>2</sub>, 10 vol % H<sub>2</sub>, and 80 vol % N<sub>2</sub>).

Optical density at 600 nm (OD<sub>600</sub>) of *F. necrophorum* culture was measured every 2 h over a 48-h period with three technical replicates for each group. Maximum growth rate and carrying capacity were estimated with GrowthRates package (v 0.8.4, <https://github.com/tpetzoldt/growthrates>), while duration of lag phase was calculated with miLAG package (v 1.0.4, [https://github.com/bognabognabogna/microbial\\_lag\\_calculator](https://github.com/bognabognabogna/microbial_lag_calculator)). Growth curve parameters were statistically compared between the ½ × MIC and the positive control groups using a t-test following the Shapiro–Wilk's test for normality.

#### Murine treatment and sample collection

Six-week-old ICR female mice (Sipeifu Biotechnology, Beijing, China) were housed under specific pathogen-free (SPF) conditions (temperature: 23 ± 1 °C; humidity: 55 ± 5%) on a 12-h/12-h light/dark cycle, with four animals per cage. After one week of acclimatization, mice were randomly assigned into six groups (n = 8 per group), including (1) control group (Con): mice received 100 µL sterile saline injected into both uterine horn using a syringe with a 27-gauge blunt needle at day 0 and from day 2 to day 6; (2) *F. necrophorum* group (Fn): mice were injected with 100 µL *F. necrophorum* suspension (1 × 10<sup>6</sup> CFU/mouse) into uterine at day 0 to induce endometritis, followed by 100 µL sterile saline into each horn of mice from day 2 to day 6; (3) 10 mg/kg TG treated group (10 mg/kg): mice were injected with 100 µL *F. necrophorum* suspension (1 × 10<sup>6</sup> CFU/mouse) into uterine at day 0, followed by intrauterine administration of 100 µL 10 mg/kg TG per horn daily from day 2 to day 6 for endometritis treatment; (4) 20 mg/kg TG treated group (20 mg/kg): same procedure as the 10 mg/kg group, with TG administered at 20 mg/kg; (5) 40 mg/kg TG treated group (40 mg/kg): same procedure as the 10 mg/kg group, with TG administered at 40 mg/kg TG; (6) antimicrobial ceftiofur treatment group (Cef; ceftiofur, a third generation cephalosporin, is an antimicrobial commonly used for bovine endometritis treatment on farms): mice were injected with 100 µL *F. necrophorum* suspension (1 × 10<sup>6</sup> CFU/mouse) into uterine at day 0, followed by 100 µL ceftiofur injection in each horn of the mice daily from day 2 to day 6. All mice were anesthetized with isoflurane prior to each injection and euthanized on day 7. Body weight was measured daily from day 0 to day 7, while the uterus weight was measured at day 7 at euthanasia. The uterus index was calculated as uterus weight (mg) divided by body weight (g) at day 7. Uterine tissues were collected and stored at −80 °C for subsequent quantification of inflammatory cytokines.

### Gross pathology and histological analysis of mice uterine tissues

Fresh uterine tissues were collected from euthanized mice and fixed in 4% paraformaldehyde. After fixation, tissues were processed, embedded in paraffin and sectioned at 4  $\mu\text{m}$  thickness. Sections were then dewaxed, hydrated, and stained with haematoxylin and eosin. Histological evaluation was performed using a panoramic digital slide scanner (3DHISTECH, Hungary).

The severity of endometritis was assessed using a previously described uterine scoring method [47, 48]. Briefly, uterine horn dilatation was graded on a scale from 0 to 4: score 0, no visible dilatation; score 1, mild dilatation in a single cross-section; score 2, one to three dilated cross-sections; score 3, more than three dilated cross sections; score 4, confluent and pronounced dilation. Histopathologic scoring of the uterus was based on the extent of endometrial edema, inflammatory cell infiltration, vascular hyperplasia, and tissue damage, following established criteria [49, 50]. Scores ranged from 0 to 4: score 0, no visible histopathologic pathological changes or changes within normal limits; score 1, subtle histopathologic changes that barely exceeded the normal range; score 2, mild but evident histopathologic changes; score 3, clearly pronounced histopathologic alteration; and score 4, severe and extensive histopathologic changes. All scoring was performed in a double-blinded manner by the same experienced evaluator.

### Quantification of murine uterine inflammation levels

The mRNA levels of *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* in murine uterus were quantified by real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA from uterine tissues was extracted with AIPzol total RNA extraction reagent (i-presci, RE205-02, Beijing, China). One microgram of purified RNA was reverse-transcribed into cDNA using ABScript Neo RT Master Mix for qPCR with gDNA Remover (ABclonal Technology, RK20433, Wuhan, China). RT-qPCR was then performed using the 2X Universal SYBR Green Fast qPCR Mix (ABclonal Technology, RK21203, Wuhan, China). Gene transcription levels were analyzed using the  $2^{-\Delta\Delta C_t}$  method [51]. Primer sequences used for RT-qPCR are provided in Table S1.

The expression levels of murine uterine inflammatory indicators including *TNF- $\alpha$* , *IL-1 $\beta$*  and *IL-6* were measured using the ELISA kits (Jianglai Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

### Statistical analysis

Statistical analyses of alpha diversity, cytokine levels and histological scores were conducted with one-way ANOVA and Tukey's HSD test for multiple comparisons

using R (v 4.4.0, <https://www.r-project.org/>) [52]. Differences in microbial community composition (beta diversity) were assessed using PERMANOVA with 999 permutations via the *adonis2* function in the *Vegan* package (v 2.6–4) [53]. Pairwise comparisons were performed using the *pairwise.adonis* function with Benjamini-Hochberg (BH) correction in the *pairwiseAdonis* package (v 0.4.1, <https://github.com/pmartinezarbizu/pairwiseAdonis>). A principal coordinate analysis (PCoA) plot was produced to visualize clustering of samples in the multi-dimensional spaces based on bacterial species abundance. Differential abundance analysis of bacterial species associated with endometritis was performed using *MaAsLin2* (v1.7.3, <https://github.com/biobakery/Maaslin2>) with default settings, applying the Benjamini-Hochberg method for multiple testing correction [54]. KEGG pathway-level functional differences were analyzed using the *LEfSe* (Linear Discriminant Analysis Effect Size) method, which combines non-parametric statistical tests and linear discriminant analysis (LDA). Significance thresholds were set at  $p < 0.05$  for both the Kruskal-Wallis and Wilcoxon tests, and an LDA score  $> 2.0$  was considered indicative of meaningful enrichment. Spearman's rank correlation coefficient ( $\rho$ ) was calculated to evaluate associations between bacterial species abundance, their corresponding function abundance, and concentration of metabolites in uterus using *cor.test* function in *stats* package (v3.6.2), with associations considered significant when  $|\rho| \geq 0.50$  and  $p < 0.05$  (<https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/cor.test>).

Partial least squares discriminant analysis (PLS-DA) was conducted with the *ropls* package (v 1.38.0) [55] in R and the variable importance for projection (VIP) value of each metabolite was calculated using *ropls*. Metabolites were considered significantly differentially abundant if they met all of the following criteria: fold change (FC)  $\geq 1.2$  or  $\text{FC} \leq 1/1.2$ ,  $\text{VIP} > 1$  and  $p < 0.05$  (based on t-test). KEGG pathway enrichment analysis was performed using the *OmicStudio* tools (<https://www.omicsstudio.cn/tool>).

### Results

Based on uterine discharge scores and PMNs in the uterine irrigating fluid cytology smear, 15 healthy cows (H), 12 cows with subclinical endometritis (SCE), and 13 cows with clinical endometritis (CE) were identified.

### Bacterial culture and histology of bovine uterus

A total of 14, 18, and 34 distinct bacterial species were isolated from samples collected from healthy, subclinical, and clinical endometritis cows, respectively. Detailed culture results under aerobic and anaerobic conditions are provided in Table S2. Histology of bovine uterus from

healthy group revealed that the endometrial epithelium exhibited an intact and continuous structure, with no significant inflammatory cell infiltration observed in the stroma. The tissue architecture appeared normal and well-preserved in healthy cows. An increase in inflammatory cell infiltration was observed in the endometrial stroma in SCE cows. The nuclei of these inflammatory cells were densely stained and increased in number, indicating an ongoing inflammatory response in SCE cows. Severe disruption of the endometrial epithelial structure, accompanied by extensive inflammatory cell infiltration were evident in CE cows. Moreover, the epithelial layer appeared damaged or detached in several areas in CE cows (Fig. 1A).

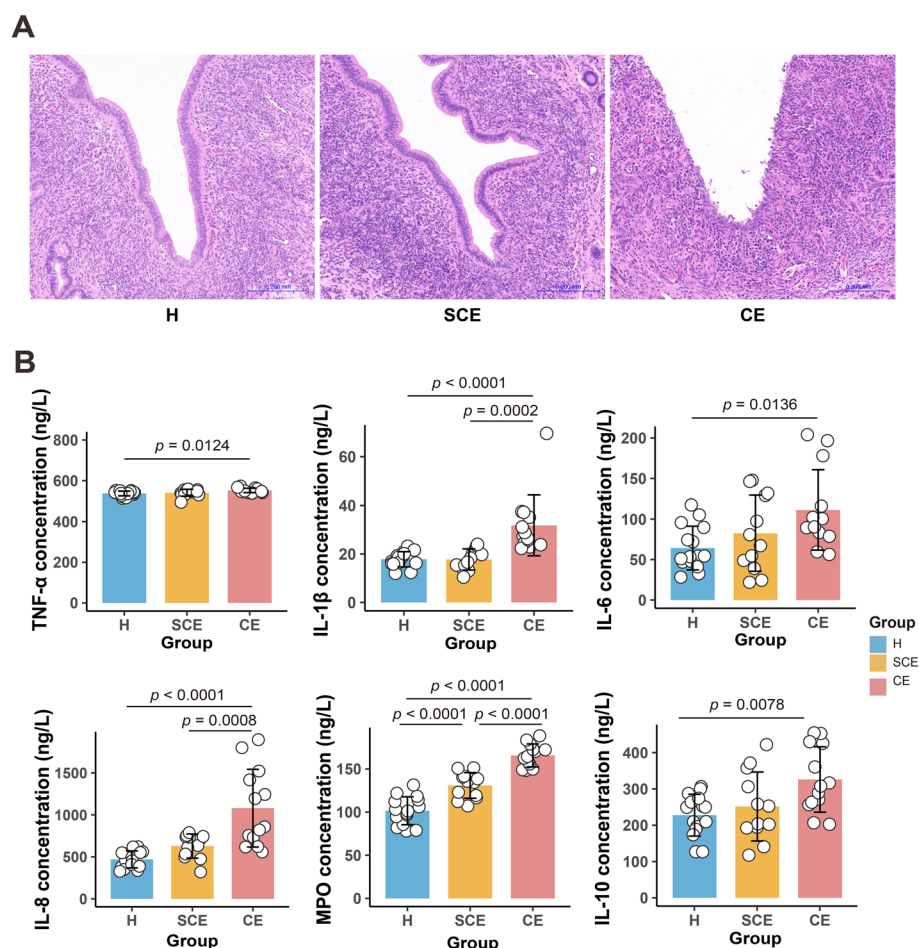
#### Concentration of serum inflammatory mediators increased in endometritis cows

To assess the inflammatory status of dairy cows across groups, serum levels of pro-inflammatory cytokines,

including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, the pro-inflammatory enzyme MPO, and the anti-inflammatory cytokine IL-10 were quantified. The concentrations of TNF- $\alpha$  ( $p=0.0124$ ), IL-1 $\beta$  ( $p<0.0001$ ), IL-6 ( $p=0.0136$ ), IL-8 ( $p<0.0001$ ), MPO ( $p<0.0001$ ) and IL-10 ( $p=0.0078$ ) were significantly higher in CE compared to H group (Fig. 1B, Table S3). Moreover, the concentrations of IL-1 $\beta$  ( $p=0.0002$ ), IL-8 ( $p=0.0008$ ), and MPO ( $p<0.0001$ ) were significantly higher in CE compared with SCE cows (Fig. 1B, Table S3).

#### Compositional profiles of uterine microbials and taxonomic differentiation in three groups

Metagenomic sequencing of the 40 uterine fluid samples generated a total of 4,760,284,400 reads with an average of  $119,007,110 \pm 20,236,517$  (mean  $\pm$  SD) reads per sample (Table S4). After quality trimming and removal of host reads, a total of 163,459,874 clean reads were obtained

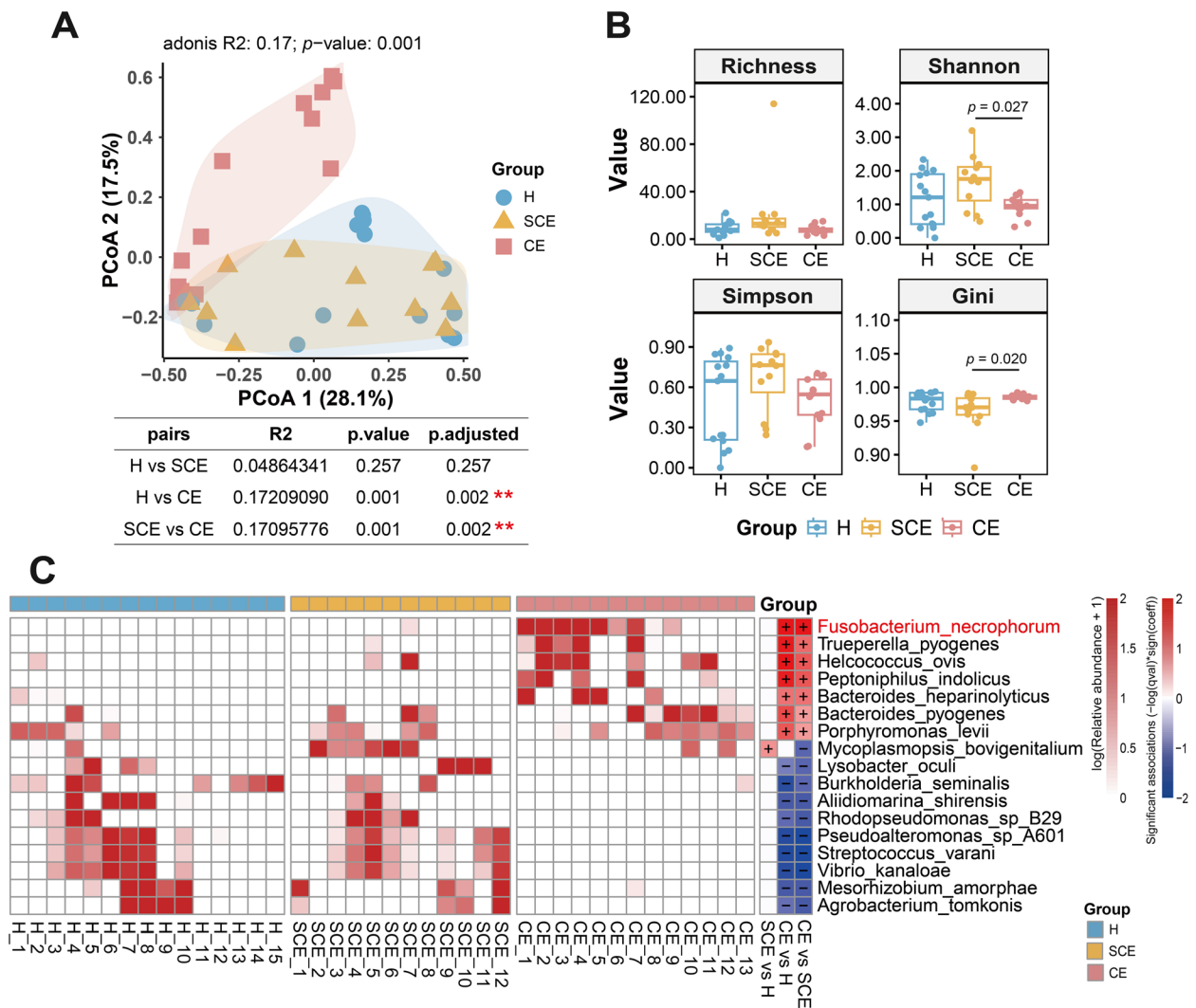


**Fig. 1** Histological analysis of bovine uterine tissue and concentrations of inflammatory mediators in bovine serum. **A** Histological section of bovine uterine tissue stained with hematoxylin and eosin (H&E). **B** Serum concentrations of inflammatory mediators in different groups of cows. H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows

with an average of  $4,086,497 \pm 7,860,113$  (mean  $\pm$  SD) reads per sample (Table S4).

Microbial domain profiling of the uterine metagenome revealed that 39 samples consisted entirely of bacterial sequences, while one sample from the SCE group contained 99.76% bacteria, 0.045% archaea, and 0.19% eukaryotes (Table S5). In total, six significantly differential bacterial phyla were detected in the three groups of bovine uterine microbiome, including *Fusobacteri*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Tenericutes* and *Proteobacteria* (Table S6). Among these, the predominant bacterial genera were *Fusobacterium*, *Bacteroides*, and *Helcococcus* in CE group (Table S6). At the species level,

the five most abundant bacterial species were *Porphyromonas levii*, *E. necrophorum*, *Mycoplasma bovis*, *Bacteroides pyogenes*, and *Burkholderia seminalis* (Table S6). Principal coordinate analysis based on Bray–Curtis distance demonstrated a significant shift in bacterial species composition in CE cows compared to both H and SCE cows ( $p=0.002$  for both comparisons; Fig. 2A). Notably, Shannon and Gini diversity indices were significantly different between the SCE and CE groups (Fig. 2B). In total, 17 bacterial species were found to be significantly associated with endometritis. Among them, 16 bacterial species were significantly differentially abundant between H and CE cows, and 17 bacterial species



**Fig. 2** Alternations of uterine microbiota at species level in cows with endometritis. **A** Bacterial abundance at species level visualized using principal coordinate analysis (PCoA) and Bray–Curtis distance matrix. The calculation of significance ( $p < 0.05$ ) by using PERMANOVA and the pairwise test. **B** Alpha diversity indexes in three groups. **C** The bacterial species significantly different in SCE and CE compared with H using MaAsLin 2 with linear mixed effects model on log transformed relative abundance of bacterial species. H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows. \*\* represents  $p_{\text{adjusted}} < 0.01$

significant different in relative abundance between SCE and CE groups, only one bacterial species *Mycoplasma bovis* was significantly higher in SCE compared with H group (Fig. 2C, Table S6). Several species were significantly abundant in the CE group compared to both H and SCE, *F. necrophorum*, *T. pyogenes*, *Helcococcus ovis*, *Peptoniphilus indolicus*, *Bacteroides heparinolyticus*, *Bacteroides pyogenes*, and *Porphyromonas levii*. These findings suggest that *F. necrophorum*, *T. pyogenes*, and *Bacteroides pyogenes*, may be potential pathogenic bacteria that induce clinical endometritis, while *Helcococcus ovis*, *Bacteroides heparinolyticus* and *Peptoniphilus indolicus* might be novel pathogens for bovine endometritis. Conversely, the relative abundance of several species including *Lysobacter oculi*, *Burkholderia seminalis*, *Aliidiomarina shirensis*, *Rhodopseudomonas* sp B29, *Pseudoalteromonas* sp A601, *Streptococcus varani*, *Vibrio kanaloae*, *Mesorhizobium amorphae*, and *Agrobacterium tomkonis* were significantly decreased in CE compared to H and SCE groups (Fig. 2C, Table S6).

#### Functional profiling of the uterine microbiome

To investigate functional differences in the uterine microbiome, metagenomic sequencing reads were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. At KEGG level 1, the categories “Environmental Information Processing” and “Cellular Processes” were significantly decreased in CE compared to the H and SCE groups (LDA > 2,  $p < 0.05$ , Fig. S1A, Table S7). KEGG level 2 analysis identified 12 pathways that were significantly different between H and CE and 12 KEGG pathways significantly different between SCE and CE (Fig. S1B, Table S7). However, there was no significantly differential KEGG pathway between the H and SCE groups (Fig. S1B, Table S7). The major KEGG level 3 pathways were classified as “Carbohydrate metabolism”, “Glycan biosynthesis and metabolism” and “Amino acid metabolism”, respectively (Fig. 3A, Table S7). In the “Glycan biosynthesis and metabolism” category, “Lipopolysaccharide biosynthesis”, “Other glycan degradation”, “Peptidoglycan biosynthesis”, and “Various types of N-glycan biosynthesis” were significantly upregulated in CE compared H cows. Moreover, multiple carbohydrate metabolism pathways, such as “Starch and sucrose metabolism”, “Pentose phosphate pathway”, “Glycolysis/Gluconeogenesis”, “Galactose metabolism”, and “Fructose and mannose metabolism”, all categorized under “Carbohydrate metabolism”, were also significantly enriched in CE cows compared to H and SCE cows. Alterations were further observed in pathways related to “Amino acid metabolism”, “Lipid metabolism” and “Energy metabolism” with several level 3 pathways significantly dysregulated in the CE group (LDA > 2,  $p < 0.05$ , Fig. 3A, Table S7).

#### Correlation between abundance of uterine bacterial species and uterine microbial functional pathways

Spearman's rank correlation coefficient was calculated to examine the correlation between the abundance of differential bacterial species and their associated functional pathways. A total of 75 significant correlations were identified, comprising 61 positive correlations and 14 negative correlations. The abundance of *F. necrophorum*, which was predominant in the CE group, was significantly negatively associated with “Bacterial motility proteins” ( $\rho = 0.55$ ,  $p < 0.01$ ). In contrast, it was positively correlated with both “Fructose and mannose metabolism” ( $\rho = 0.60$ ,  $p < 0.01$ ), “Pentose phosphate pathway” ( $\rho = 0.60$ ,  $p < 0.01$ ) and “Galactose metabolism” ( $\rho = 0.64$ ,  $p < 0.01$ ), which are classified to carbohydrate metabolism. (Fig. 3B, Table S8).

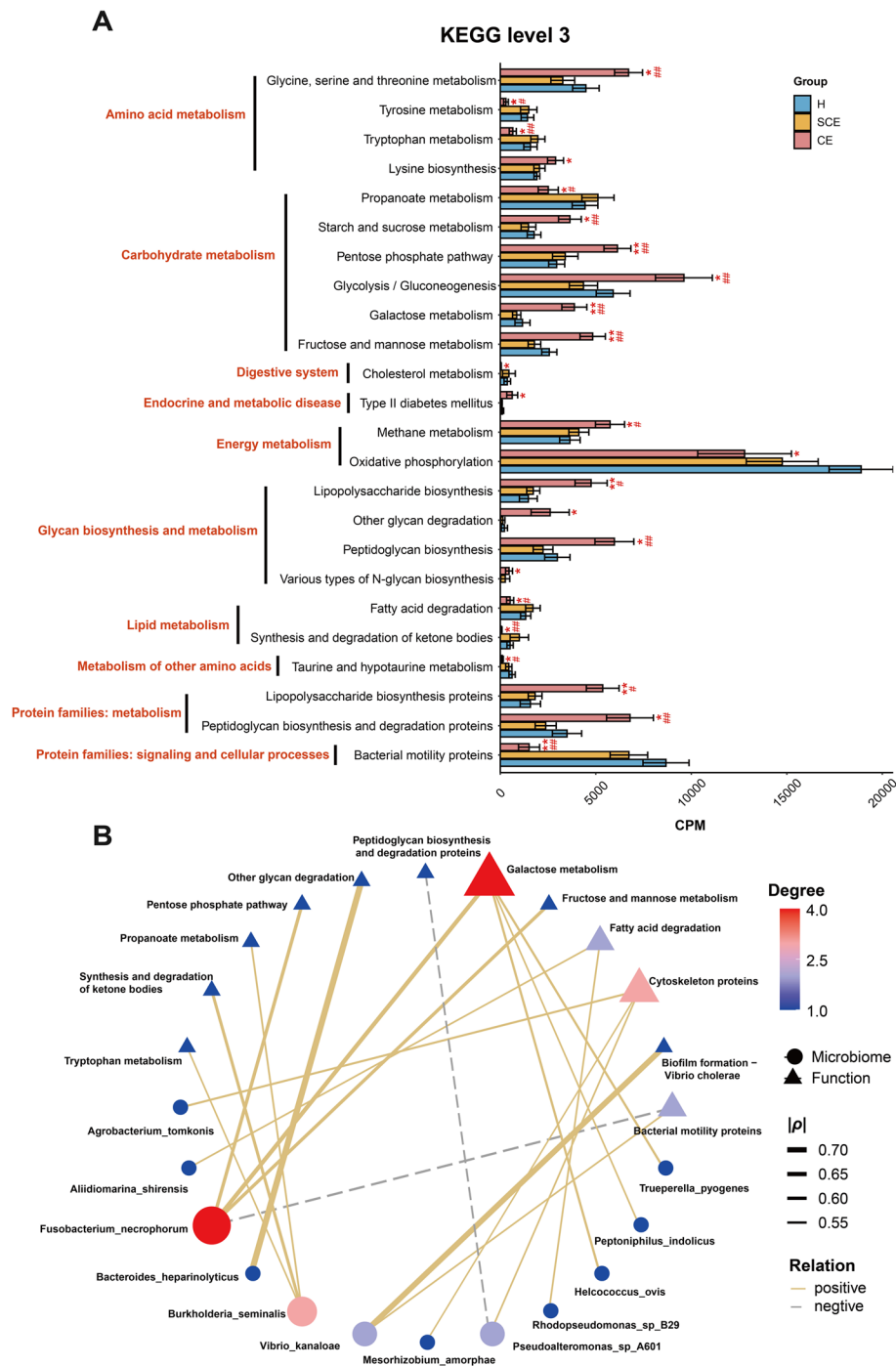
#### Uterine metabolomics profiling differed among healthy and endometritis cows

Uterine metabolite profiles across the three groups were visualized using PLS-DA (Fig. 4A). A total of 391 differential uterine metabolites were identified between the H and CE groups (VIP > 1,  $p < 0.05$ , Table S9), with 363 metabolites enriched in CE and 28 metabolites enriched in H (Fig. 4B, Table S9). Most major metabolites enriched in CE were classified as glycerophospholipids, fatty acyls, steroids and steroid derivatives, and carboxylic acids and derivatives (Table S9). In contrast, the major up-regulated metabolites in H group included tenacissoside G, 5a,6a-Epoxy-7E-megastigmene-3b,9e-diol 9-glucoside, 3-indoxylsulfate potassium salt, harmol glucuronide, and semilicoisoflavone B (Fig. 4C, Table S9). The PLS-DA model also identified 91 uterine metabolites that significantly differentiated H from SCE cows (VIP > 1,  $p < 0.05$ , Table S10), with 85 metabolites enriched in SCE and only 6 enriched in H (Fig. S2A, Table S10). For SCE versus CE, 315 differential metabolites were identified, including 300 upregulated and 15 downregulated in CE cows (Fig. S2B, Table S11). The most significantly altered metabolites in each comparison are presented in Fig. S2 C-D.

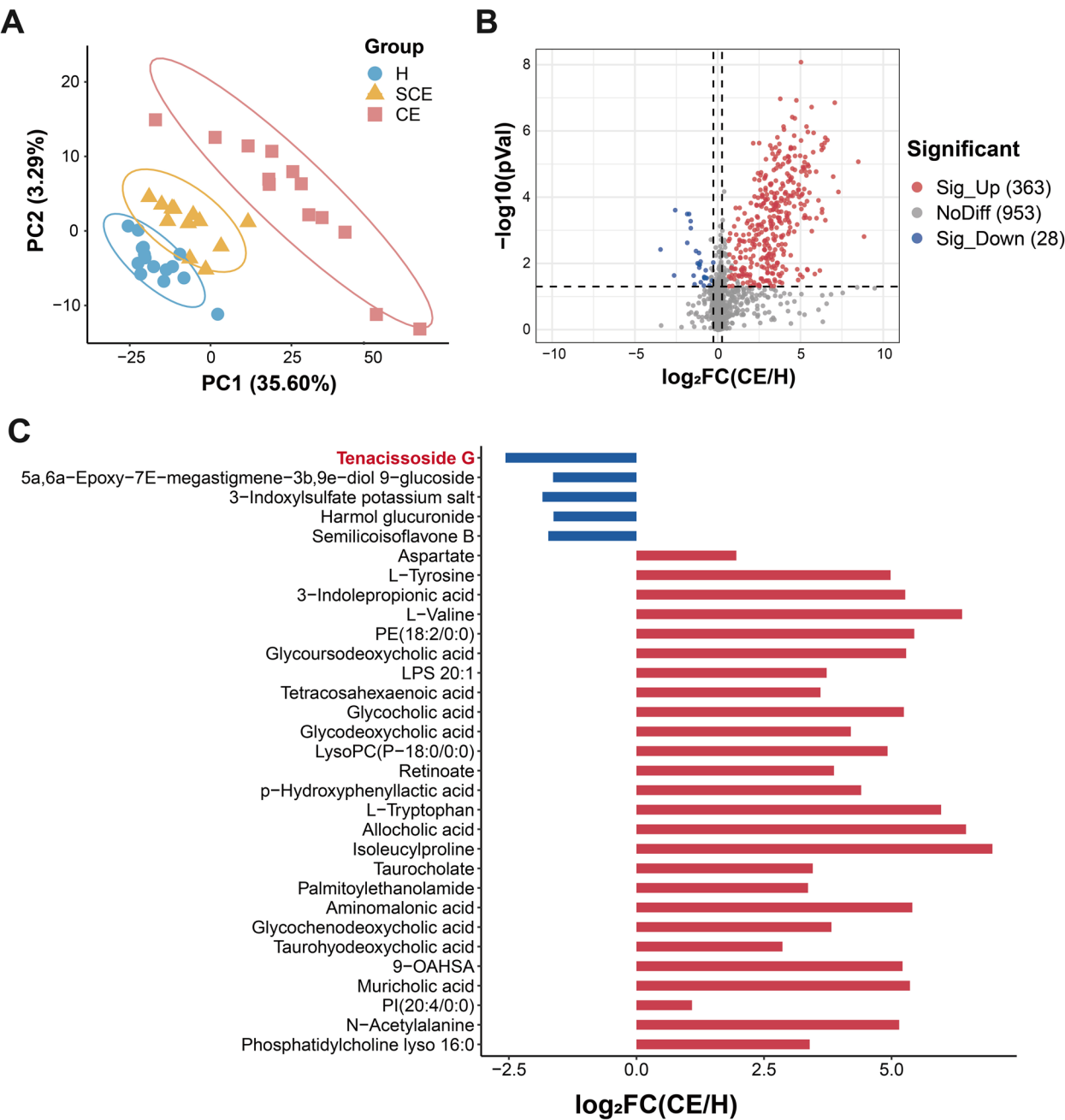
A total of 236 differential metabolites were shared between the CE vs. H and CE vs. SCE comparisons (Fig. S3A). Analysis of metabolite distribution patterns revealed a distinct separation between the CE and H groups, with the SCE group displaying intermediate levels, in agreement with the clustering pattern identified in the PLS-DA analysis (Fig. 4A, Fig. S3B).

#### Function of uterine metabolites differed between healthy and endometritis cows

To assess global functional changes in uterine metabolites, KEGG enrichment analysis was performed on the total set of detected metabolites. The “Metabolism” category showed the highest overall enrichment,



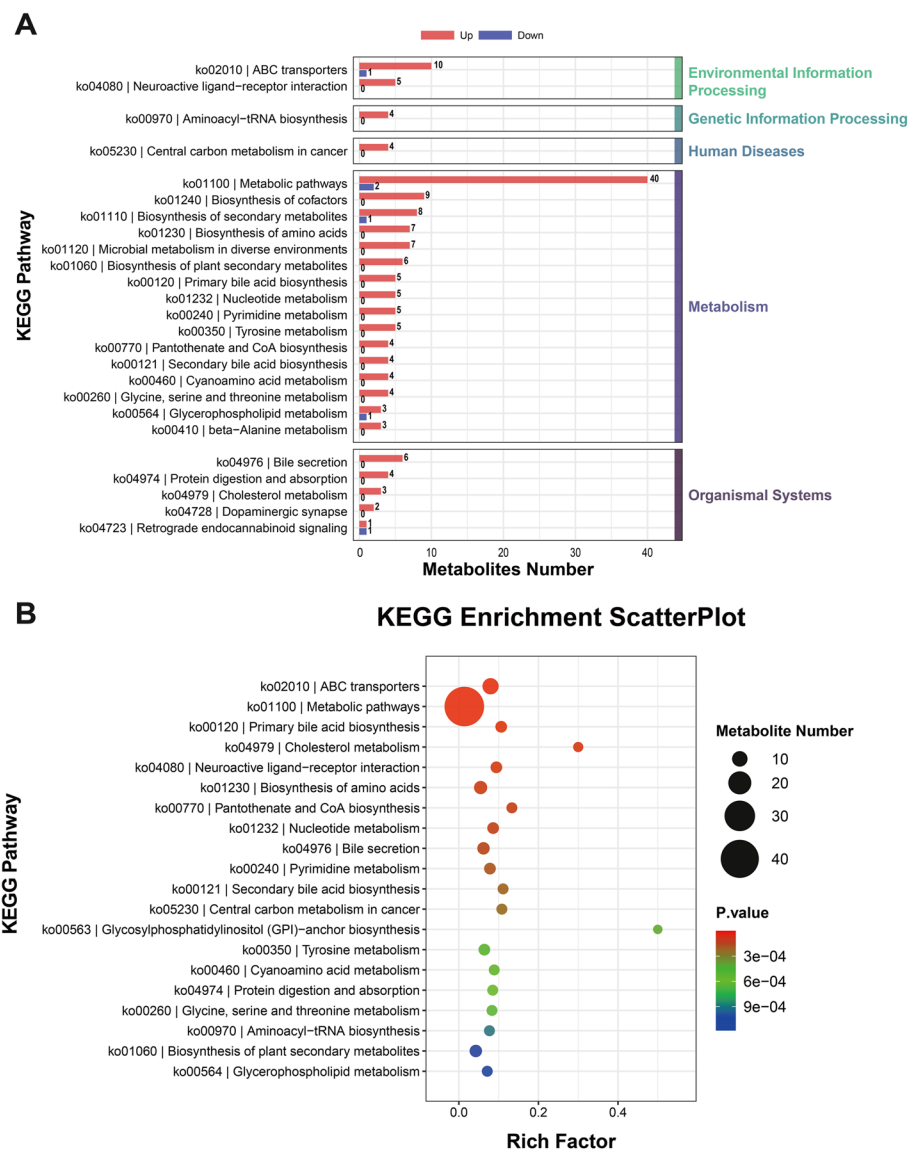
**Fig. 3** Microbial functions identified in metagenomes and the relationships with microbiota. **A** Major differential KEGG level 3 pathways were compared through linear discriminant analysis (LDA) effect size (LEfSe) with LDA > 2 and  $p$ -value < 0.05. **B** Correlations network between the significantly changed microbes and functions (Spearman's correlation, Spearman's  $|\rho| \geq 0.50$  and  $p < 0.05$ ). H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows. \* Represents LDA score > 2 and  $p$ -value < 0.05 compared with H, \*\* represent LDA > 2 and  $p$ -value < 0.01 compared with H. # Represents LDA score > 2 and  $p$ -value < 0.05 compared with SCE, ## represent LDA > 2 and  $p$ -value < 0.01 compared with SCE



**Fig. 4** Uterine differential abundant metabolites in three groups. **A** Partial least squares discriminant analysis (PLS-DA) of metabolites. **B** Volcano plot of uterine differential abundant metabolites between H and CE ( $FC \geq 1.2$  or  $FC \leq 1/1.2$ ,  $VIP > 1$ ,  $p < 0.05$ ). **C** Log<sub>2</sub>-fold change of major differential metabolites of cows in H and CE. FC: fold change; H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows

primarily involving pathways such as “Metabolic pathways” (with the largest number of enriched metabolites), “Biosynthesis of cofactors”, “Biosynthesis of secondary metabolites” and “Biosynthesis of amino acids”. (Fig. 5A, Fig. 5B). Compared to healthy cows, the CE group showed significantly higher enrichment

in “Glycosylphosphatidylinositol (GPI)-anchor biosynthesis”, “Cholesterol metabolism”, “Pantothenate and CoA biosynthesis”, “Primary bile acid biosynthesis”, and “Secondary bile acid biosynthesis” (Fig. 5B, Table S12). Similarly, “Glycosylphosphatidylinositol (GPI)-anchor biosynthesis”, “Cholesterol metabolism”, and “Secondary bile acid biosynthesis” were elevated in SCE relative



**Fig. 5** Analysis of uterine metabolites function. **A** The number of up- and down-regulated metabolites of differential KEGG pathways in CE compared with H. **B** Enrichment analysis of KEGG pathways in CE compared with H

to healthy cows, and further increased in the CE group compared to the SCE group (Table S13, Table S14).

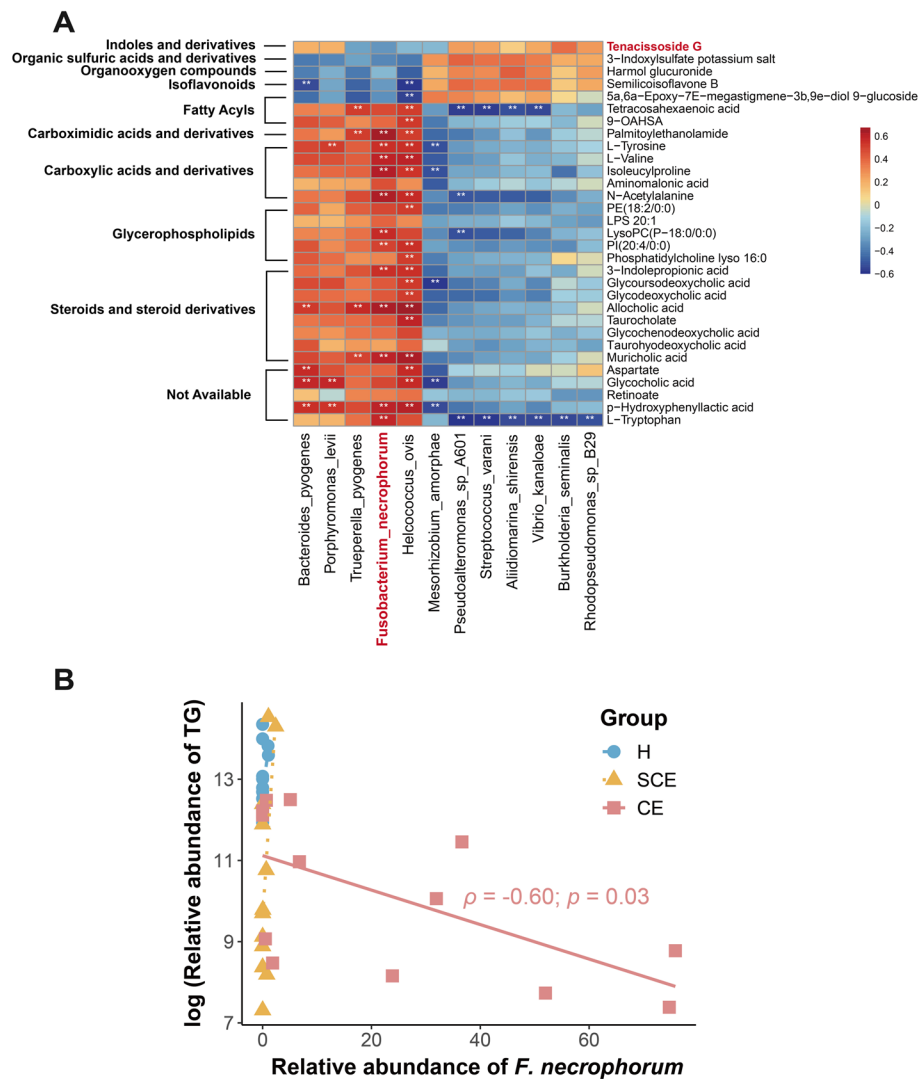
#### Correlation between the abundance of uterine microbial species and uterine metabolites

Subsequently, we explored the associations between the microbial abundance and the corresponding metabolite profiles in the same uterine samples. *F. necrophorum* abundance was positively correlated with several metabolites, such as palmitoylethanolamide ( $\rho=0.69$ ,  $p<0.01$ ), isoleucylproline ( $\rho=0.63$ ,  $p<0.01$ ), N-Acetylalanine ( $\rho=0.64$ ,  $p<0.01$ ), L-Tryptophan ( $\rho=0.57$ ,  $p<0.01$ ), allocholic acid ( $\rho=0.66$ ,  $p<0.01$ ), muricholic

acid ( $\rho=0.60$ ,  $p<0.01$ ), and p-Hydroxyphenyllactic acid ( $\rho=0.60$ ,  $p<0.01$ ) (Fig. 6A, Table S15). Notably, a significant negative association between the abundance of *F. necrophorum* and TG abundance ( $\rho=-0.60$ ,  $p=0.03$ ) was identified only in the CE group (Fig. 6B, Table S16), while no relationship was found in the H or SCE groups.

#### TG inhibited the growth of *F. necrophorum* in vitro and ameliorated endometritis in mice induced by *F. necrophorum* in vivo

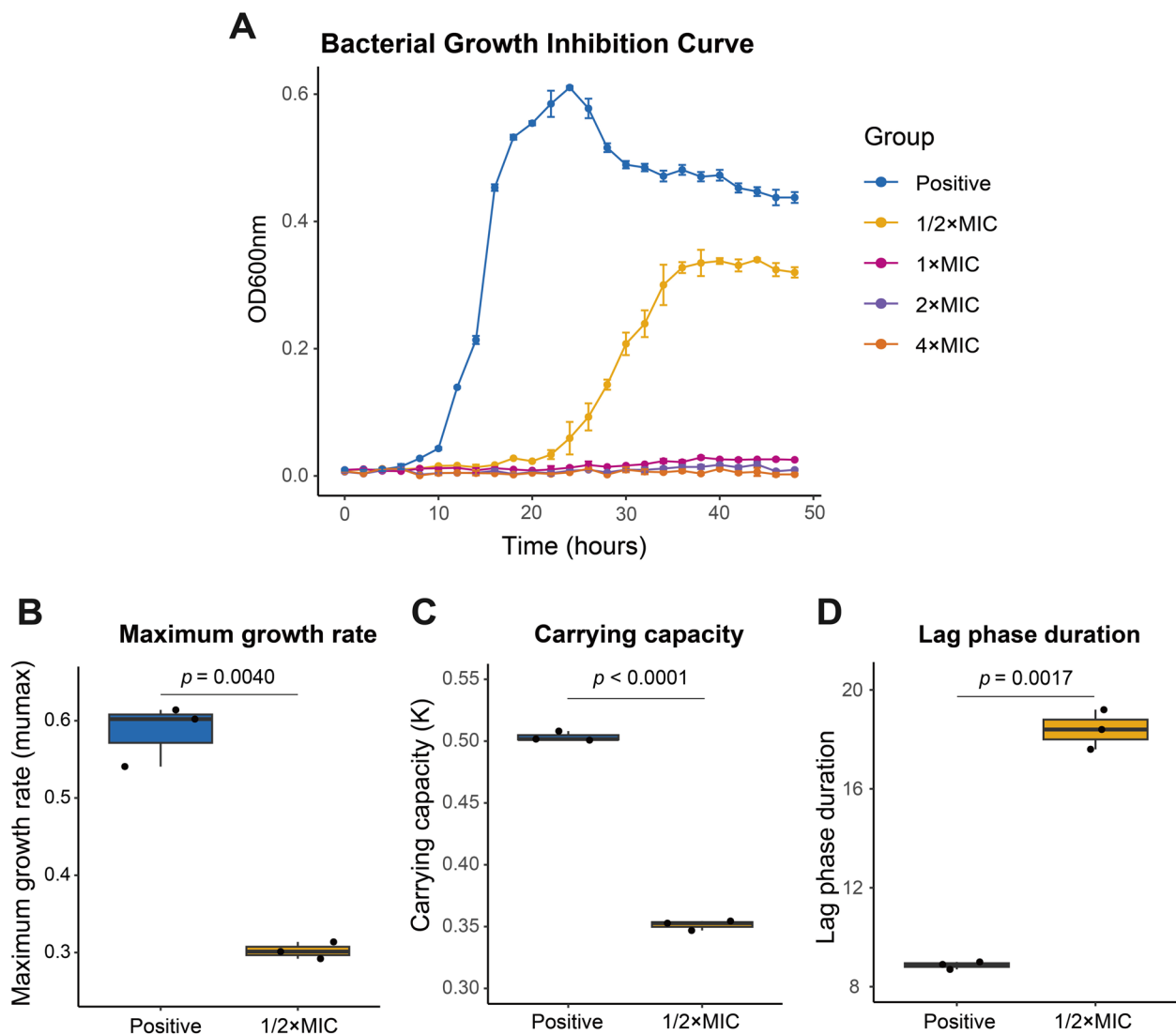
To evaluate the anti-bacterial effect of TG against *F. necrophorum*, we determined the MIC and growth parameters of *F. necrophorum* under different concentrations



**Fig. 6** The relationships between uterine differential abundant metabolites and bacterial species. **A** Correlation matrix between abundance of bacterial species and metabolites that were significantly influenced in CE (Spearman's correlation, \* means Spearman's  $|\rho| \geq 0.50$  and  $p < 0.05$ , \*\* means Spearman's  $|\rho| \geq 0.50$  and  $p < 0.01$ ). **B** Scatter plot of the abundance of *Fusobacterium necrophorum* and abundance of Tenacissoside G (TG). H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows

of TG. The MIC value of TG against *F. necrophorum* was 187.5  $\mu\text{g/mL}$  (Table S17). TG significantly inhibited the growth of *F. necrophorum* (Fig. 7A), specifically, the  $\frac{1}{2} \times \text{MIC}$  group exhibited a significantly lower maximum growth rate and carrying capacity, along with a significantly prolonged duration of lag phase compared to the positive control group (Fig. 7B-D, Fig. S4, Table S18). To further validate the potential anti-bacterial effect of TG in vivo, a randomized clinical trial was conducted in mice treated with varying doses of TG or ceftiofur for five days, starting 48 h post-infection with *F. necrophorum*. The ratio of uterus/body weight was significantly elevated in the Fn group compared to the Con group ( $p < 0.0001$ ).

This ratio was significantly reduced following treatment with TG at 10 mg/kg ( $p = 0.0003$ ), 20 mg/kg ( $p < 0.0001$ ), 40 mg/kg ( $p < 0.0001$ ), and with ceftiofur ( $p < 0.0001$ ) (Fig. 8A, Table S19). No significant differences in overall body weight were observed among the treatment groups (TG and ceftiofur) ( $p > 0.05$ , Fig. 8B, Table S19). Histological assessment revealed moderate to severe uterine edema in the Fn group, with significantly increased uterine scores in the Fn and 10 mg/kg TG groups relative to the control (both  $p < 0.001$ ). In contrast, uterine scores were significantly reduced in the 20 mg/kg, 40 mg/kg TG, and ceftiofur groups compared to the Fn group ( $p < 0.001$  for all) (Fig. 8C, Table S20). Severe endometrial damage,



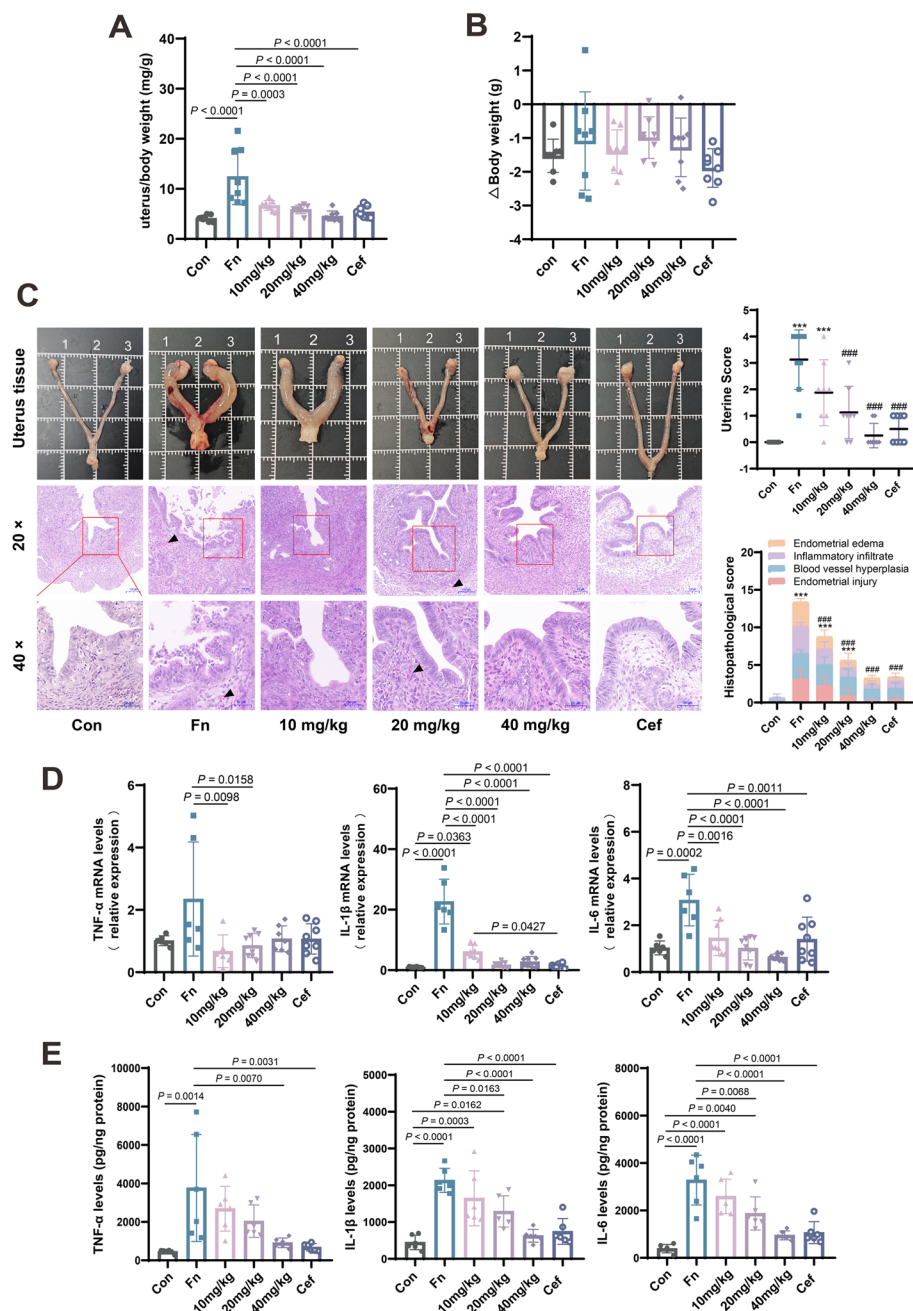
**Fig. 7** Effects of TG on the growth of *F. necrophorum* in vitro. **A** The growth curve of *F. necrophorum* at different TG concentrations. **B** The maximum growth rate of *F. necrophorum* for positive and 1/2 × MIC groups. **C** The carrying capacity of *F. necrophorum* for positive and 1/2 × MIC groups. **D** The duration of lag phase of *F. necrophorum* for positive and 1/2 × MIC groups. Positive: positive control group without antimicrobials or TG; 1/2 × MIC: 1/2 × MIC TG treated group; 1 × MIC: group treated with MIC concentration of TG; 2 × MIC: 2 × MIC TG treated group; 4 × MIC: 4 × MIC TG treated group

characterized by epithelial detachment and fragmentation, mucosal thickening, glandular hyperplasia, and inflammatory infiltration, was observed in the Fn group, while these pathological changes were significantly alleviated in TG- and ceftiofur-treated groups (Fig. 8C). Additionally, TG and ceftiofur treatments significantly reduced *F. necrophorum*-induced inflammation, as evidenced by decreased expression of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 at both transcript and protein levels (Fig. 8D, E, Table S21). Together, these results demonstrate that TG inhibits *F. necrophorum* growth in vitro and in vivo. TG treatment alleviated *F.*

*necrophorum*-induced endometritis in mice in a dose-dependent manner, with 20 mg/kg and 40 mg/kg showing the most pronounced effects. Importantly, there was no significant difference in tissue-protective effects between TG and ceftiofur, suggesting that TG may serve as a promising non-antimicrobial alternative for treating endometritis.

## Discussion

The role of the uterine microbiome in bovine reproductive health has been previously explored, particularly in relation to uterine metabolites and their role in



**Fig. 8** Effects of TG on *F. necrophorum* induced endometritis in mice. **A** Alteration of uterus index = uterus/body weight (mg/g). **B** Changes in body weight of mice during the experiment. **C** Gross pathology and histological analysis of uterine tissues. **D** The mRNA expressions of inflammatory factors of the uterus in mice. **E** The concentrations of inflammatory factors of the uterus in mice. The red box highlights the uterine endometrial epithelial layer, while the black arrow points to the blood vessels. Con: control group; Fn: *F. necrophorum* group; 10 mg/kg: 10 mg/kg TG treatment group; 20 mg/kg: 20 mg/kg TG treatment group; 40 mg/kg: 40 mg/kg TG treatment group; Cef: antimicrobial ceftiofur treatment group. \*\*\* represents  $p$ -value  $< 0.001$  compared with control; ### represents  $p$ -value  $< 0.001$  compared with Fn

disease progression [32]. Although numerous studies have characterized the uterine microbiome and differential metabolites in dairy cows [5, 18, 56], few have integrated metagenomic and metabolomic data to investigate

the functional interactions between the uterine microbiome and bovine endometritis. In this study, we employed metagenomic sequencing analysis to characterize the taxonomic and functional landscape of the uterine

microbiome in cows with endometritis. In parallel, untargeted metabolomic profiling was conducted to identify metabolites with potential therapeutic effect. Furthermore, an in vivo mouse model was used to validate the anti-endometritis effects of TG against *F. necrophorum*-induced uterine inflammation.

Our study identified *F. necrophorum*, *T. pyogenes*, *Bacteroides pyogenes*, and *Porphyromonas levii* to be associated with endometritis, which was consistent with previous reports [5, 20]. Experimental intrauterine infusion with *F. necrophorum*, *E. coli*, and *T. pyogenes* (each at  $1.0 \times 10^6$  CFU) has been shown to induce metritis in dairy cows [57]. Among these pathogens, *F. necrophorum* emerged as a predominant species in clinical endometritis in our study and was also shown to induce endometritis in a murine model, highlighting its pathogenic potential. In addition, *Helcococcus ovis*, *Bacteroides heparinolyticus*, and *Peptoniphilus indolicus* were identified as candidate novel pathogens for endometritis. Notably, *H. ovis* and *B. heparinolyticus* have also been implicated in bovine metritis in recent studies [20, 58, 59], suggesting their emerging role in uterine infections.

Integrating microbial composition with functional analysis is essential for uncovering mechanistic links between the microbiome and host disease processes [60]. In our study, functional profiling revealed increased glycan biosynthesis and metabolism of uterine microbiome in the CE cows, indicating enhanced metabolic activity of uterine pathogens. This was supported by the enrichment of related pathways such as peptidoglycan biosynthesis, and lipopolysaccharide biosynthesis and associated proteins of uterine microbiome in CE cows compared to healthy controls. The increased abundance of glycan biosynthesis and metabolism pathways in CE cows is consistent with the known biology of *F. necrophorum*, whose surface is enriched with glycan-binding proteins that facilitate host tissue attachment and evasion of host immune defenses [61]. Furthermore, *F. necrophorum* is capable of metabolizing amino acids into indole derivatives, which act as bacterial signaling molecules that regulate motility, sporulation, virulence, and biofilm formation, thereby suppressing commensal bacterial populations [32]. Functional annotation using HUMAnN3 revealed increased lysine biosynthesis and glycine, serine, and threonine metabolism, both classified under amino acid metabolism in CE cows (Fig. 3A, Table S7). These findings were in line with our whole-genome sequencing of *F. necrophorum* (Fig. S5C, Table S22) and supported by a previous study [61]. Additionally, carbohydrate metabolism pathways such as starch and sucrose metabolism, glycolysis/gluconeogenesis, galactose metabolism, and fructose and mannose metabolism were also enriched and validated by *F. necrophorum* genome content, in

agreement with an earlier study [61]. Three significant correlations were identified between the abundance of *F. necrophorum* and functional pathways. Notably, a negative correlation with bacterial motility proteins was observed, aligning with the fact that *F. necrophorum* is a non-motile anaerobe [61]. Interestingly, *Fusobacterium nucleatum* which shares ecological niches with other *Fusobacterium* species, produces adhesins that mediate galactose-sensitive binding to mammalian cells, highlighting the importance of glycan interactions in 4.1 *Fusobacterium* spp. pathogenesis [61].

Metabolomic analysis provides valuable insights into disease mechanisms by identifying key metabolites derived from both host and pathogen sources [62]. In our study, uterine metabolite profiling revealed a large number of differentially abundant metabolites in the CE cows, suggesting elevated metabolic activity within the uterine environment. These uterine metabolites might serve as potential biomarkers of endometritis. Similarly, the differentially enriched KEGG pathways observed in the CE group aligned with the overall distribution trends of both metabolites and microbial functions, indicating a strong microbiome-metabolome interaction. Among the metabolite classes, glycerophospholipids, critical components of cell membranes, were prominently enriched and are known to be closely linked to inflammation [63]. In particular, lysophosphatidylcholines (lysoPCs) interact with G-protein-coupled receptors to regulate immune cell activation and contribute to inflammatory responses [56]. As reliable indicators of inflammation, elevated lysoPC levels are associated with membrane damage and disruptions in phospholipid metabolism [64]. In this study, four lysoPC, including LysoPC (P-18:0/0:0), LysoPC(18:1(11Z)/0:0), LysoPC(22:5(4Z,7Z,10Z,13Z,16Z)/0:0), and LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0), were significantly upregulated in the CE group, indicating their potential role in modulating glycerophospholipid metabolism and inflammatory progression. Additionally, we observed an increased abundance of indole derivatives in the CE group. Indole compounds, previously shown to be elevated in cows with metritis, are known to influence bacterial signaling, immune evasion, and host inflammation, suggesting a potential contribution to the sustained inflammatory status of the uterus [32].

Tenacissoside G, a C21 steroidal glycoside derived from *Marsdenia tenacissima*, possesses both anti-cancer and anti-inflammatory properties [34, 35]. Its higher abundance in healthy cows suggests a potential protective role against uterine inflammation in the postpartum period. Further mechanistic studies are warranted to elucidate how TG contributes to uterine immune homeostasis and whether it could serve as a novel therapeutic agent.

Recent studies suggested that gut microbiome dysbiosis can exacerbate the severity of endometritis induced by *Staphylococcus aureus* in mice, accompanied by a reduction in gut-derived short-chain fatty acids (SCFA) [65]. Conversely, intraperitoneal administration of sodium acetate, propionate, and butyrate led to increased SCFA levels in both blood and uterine tissue [65]. Additionally, anti-inflammatory effects have been reported following both oral [66] and intrauterine [67] administration of *Clostridium butyricum*, likely mediated by its production and systemic distribution of butyrate. These findings suggested a potential regulatory role of microbial metabolites, particularly SCFAs like butyrate, in modulating uterine inflammation, and implied that metabolites originating from the gastrointestinal tract or other systemic sources may influence uterine immune responses. In our study, steroids and steroid derivatives were significantly enriched in the CE cows, with several mapping to inflammation- and metabolism-related KEGG pathways, including secondary bile acid biosynthesis, pyrimidine metabolism, cholesterol metabolism, and bile secretion. While the role of these pathways in endometritis remains poorly understood, bile acids are known to be major microbiota-derived metabolites that play key roles in shaping microbial community structure, abundance, and metabolic activity [68]. Dysregulated bile acid transformation by gut bacteria is a known contributor to various metabolic, infectious, and inflammatory diseases through disruption of bile acid receptor signaling [68]. In accordance with this, our results showed significant dysregulation of bile acid metabolism in cows with endometritis, including enrichment of both primary (ko00120) and secondary (ko00121) bile acid biosynthesis pathways (Fig. 5A-B, Table S12), suggesting that altered bile acid metabolism may contribute to the development of uterine inflammation. Specifically, insights from human studies support a link between cholesterol metabolism and uterine diseases. In human endometriosis, cholesterol is both locally synthesized in endometrial stromal cells and accumulates at higher levels in lesions compared to normal endometrium, suggesting disrupted lipid metabolism and local cholesterol homeostasis [69]. In our study, the cholesterol metabolism pathway (ko04979) was significantly downregulated in the CE group, based on metagenomic functional analysis (Fig. 5A-B, Table S12). Although the mechanistic relationship between cholesterol metabolism and endometritis remains unclear, these findings point to cholesterol and bile acid metabolism as potential regulatory axes in uterine inflammation.

Collectively, our findings suggest that systemic microbial metabolites, particularly bile acids and cholesterol-related compounds, may play a dual role in both reflecting and modulating the pathophysiology of bovine

endometritis, highlighting their potential as novel therapeutic targets worthy of further investigation.

By integrating uterine metabolomic and microbiome data, we observed that the abundance of several bacterial species, particularly *H. ovis*, was positively correlated with a majority of differentially enriched metabolites (Fig. 6A). Additionally, the abundance of *F. necrophorum* and *B. pyogenes* exhibited notable correlation with the abundance of metabolites, with 12 and 5 significant correlations, respectively. Notably, correlation analysis across the three groups revealed a significant negative correlation between *F. necrophorum* abundance and TG levels in CE cows, whereas no such significant correlation was observed in the H or SCE groups. These findings suggest that TG may play a protective role in bovine clinical endometritis associated with *F. necrophorum*.

Our results, including the MIC determination of TG against *F. necrophorum* in vitro, histopathological assessments, and both transcriptional and protein-level analyses of inflammatory markers, demonstrate that TG possesses anti-*F. necrophorum* activity and effectively mitigates endometritis in a murine model. Together, these findings highlight TG as a promising non-antimicrobial alternative for the treatment of bovine endometritis.

## Conclusion

Bovine endometritis is a multifactorial inflammatory disease influenced not only by pathogenic bacterial infections, such as *Fusobacterium necrophorum*, but also by alterations in the uterine metabolic pathways. Our findings suggest that metabolic dysregulation may contribute to disease development; however, whether these metabolic changes are causative or a consequence of endometritis remains to be determined. Notably, we identified tenacissoside G, a plant-derived bioactive compound, as significantly enriched in healthy cows and negatively correlated with *F. necrophorum* abundance in the CE group. This association was further supported by in vivo validation in a murine model of *F. necrophorum*-induced endometritis, where TG demonstrated protective effects, underscoring the potential of TG as an alternative to antimicrobials. Future studies are warranted to elucidate the mechanisms underlying the protective effects of TG against bovine endometritis.

## Abbreviations

|               |                                             |
|---------------|---------------------------------------------|
| TG            | Tenacissoside G                             |
| iNOS          | Inducible nitric oxide synthase             |
| MMP           | Matrix metalloproteinase                    |
| IL-6          | Interleukin-6                               |
| TNF- $\alpha$ | Tumor necrosis factor- $\alpha$             |
| PMN           | Proportion of polymorphonuclear neutrophils |
| MPO           | Myeloperoxidase                             |
| KEGG          | Kyoto Encyclopedia of Genes and Genomes     |
| CPM           | Copies per million                          |

|          |                                                  |
|----------|--------------------------------------------------|
| HMDB     | Human Metabolome Database                        |
| MIC      | Minimum inhibitory concentration                 |
| CLSI     | Clinical and Laboratory Standards Institute      |
| PEG300   | Polyethylene glycol 300                          |
| SPF      | Specific pathogen-free                           |
| Con      | Control group                                    |
| Fn       | <i>F. necrophorum</i> Group                      |
| 10 mg/kg | 10 mg/kg TG treated group                        |
| 20 mg/kg | 20 mg/kg TG treated group                        |
| 40 mg/kg | 40 mg/kg TG treated group                        |
| Cef      | Antimicrobial ceftiofur treatment group          |
| RT-qPCR  | Real-time quantitative polymerase chain reaction |
| PCoA     | Principal coordinate analysis                    |
| LDA      | Linear discriminant analysis                     |
| PLS-DA   | Partial least squares discriminant analysis      |
| VIP      | Variable importance for projection               |
| FC       | Fold change                                      |
| H        | Healthy cows                                     |
| SCE      | Subclinical endometritis                         |
| CE       | Clinical endometritis                            |

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40168-025-02264-x>.

Additional file 1: Table S1. Primers used in this study. Table S2. Bacterial species identified by MALDI-TOF MS from aerobic and anaerobic cultures of bovine uterine fluid samples. Table S3. Concentrations of inflammatory factors in three groups. Table S4. Summary statistics of metagenomics sequence data generated from uterine fluid samples. Table S5. The relative abundance of each domain in uterine fluid samples. Table S6. The relative abundances of the uterine fluid bacterial phyla, genera and species in three groups. Table S7. The abundance of enriched KEGG pathways in uterine microbiome. Table S8. Correlation between abundance of bacterial species and their functions in uterus. Table S9. Significantly differential uterine metabolites between H and CE cows. Table S10. Significantly differential uterine metabolites between H and SCE cows. Table S11. Significantly differential uterine fluid metabolites between SCE and CE cows. Table S12. Significantly differential metabolites KEGG pathways between H and CE cows. Table S13. Significantly differential metabolites KEGG pathways between H and SCE cows. Table S14. Significantly differential metabolites KEGG pathways between SCE and CE cows. Table S15. Correlation between abundance of bacterial species and metabolites in uterus. Table S16. Correlation between *Fusobacterium necrophorum* in metagenomics and tenacisosside G in metabolomics within each of the three groups of cows. Table S17. Minimum inhibitory concentration (MIC) of tenacisosside G against *Fusobacterium necrophorum* *in vitro*. Table S18. Effects of tenacisosside G on the growth of *F. necrophorum* *in vitro* with three replicates. Table S19. Body weight and uterus weight in mice. Table S20. Uterine score and uterine histopathologic score in mice. Table S21. Concentrations of inflammatory factors in mice in each group. Table S22. *Fusobacterium necrophorum* subsp. *necrophorum* KEGG pathway mapping.

Additional file 2: Fig. S1. Uterine microbial functions identified in metagenomes. A KEGG level 1 pathways that were compared through linear discriminant analysis (LDA) effect size (LefSe) with LDA > 2 and *p*-value < 0.05. B Major significantly different KEGG level 2 pathways in three groups. H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows. \* Represents LDA score > 2 and *p*-value < 0.05 compared with H, \*\* represent LDA > 2 and *p*-value < 0.01 compared with H. # Represents LDA score > 2 and *p*-value < 0.05 compared with SCE, ## represent LDA > 2 and *p*-value < 0.01 compared with SCE.

Additional file 3: Fig. S2. Significantly different uterus metabolites of SCE vs H and CE vs SCE. A Volcano plot of uterine differential metabolites between H and SCE (FC ≥ 1.2 or FC ≤ 1/1.2, VIP > 1, *p* < 0.05). B Volcano plot of uterine differential metabolites between SCE and CE (FC ≥ 1.2 or FC ≤ 1/1.2, VIP > 1, *p* < 0.05). C Fold changes of major differential metabolites of cows in H and SCE. D Fold changes of major differential

metabolites of cows in SCE and CE. FC: fold change; H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows.

Additional file 4: Fig. S3. The number and the relative abundance of differential metabolites between groups. A The Venn diagram of the number of significantly different uterine metabolites. B Heatmap of major metabolites in three groups, the values were calculated through Z score transformation normalization. H: healthy cows; SCE: subclinical endometritis cows; CE: clinical endometritis cows.

Additional file 5: Additional file 5: Fig. S4 The growth curve of *F. necrophorum* in different concentrations of TG with three technical replicates. Positive: positive control group without antimicrobials; 1/2 MIC: 1/2 MIC TG treated group; 1 MIC: group treated with 1 MIC TG; 2 MIC: 2 MIC TG treated group; 4 MIC: 4 MIC TG treated group.

Additional file 6: Fig. S5. Genomic annotation of *Fusobacterium necrophorum* subsp. *necrophorum* whole genome. A The number of genes of *Fusobacterium necrophorum* subsp. *necrophorum* in KEGG pathways. Number of genes of *Fusobacterium necrophorum* subsp. *necrophorum* enriched in B carbohydrate metabolism and C amino acid metabolism.

Additional file 7: Fig. S6. Effects of *Fusobacterium necrophorum* on endometritis in mice at different time points. A The appearance of uterus tissue influenced by *Fusobacterium necrophorum* at different time points. B The inflammatory factors levels of uterus tissue detected by ELISA. Con: control; 24h: treated with 10<sup>6</sup> CFU *Fusobacterium necrophorum* for 24h; 36h: treated with 10<sup>6</sup> CFU *Fusobacterium necrophorum* for 36h; 48h: treated with 10<sup>6</sup> CFU *Fusobacterium necrophorum* for 48h. \*\* represents *p*-value < 0.01 compared with control; \*\*\* represents *p*-value < 0.001 compared with control.

Additional file 8: Supplementary materials and results. *F. necrophorum* culture *in vitro* and functional analysis as well as the effects of *F. necrophorum* on endometritis in mice.

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## Authors' contributions

CX designed the study. QC collected the samples and wrote the manuscript. ZD and ML performed bioinformatics and statistical analysis. QC, SZ and YH conducted animal experiment. CX, ZD, BA and AP revised the manuscript. All authors reviewed the manuscript.

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## Data availability

All data required to evaluate the conclusions of the paper are available in the paper and/or the Supplementary Materials. The metagenomics data were deposited into the NCBI Sequence Read Archive (SRA), the submission number is SUB14901938, the bioproject accession number is PRJNA1192973 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1192973>). The metabolomics data that support the findings of this study have been deposited in the China National GeneBank DataBase (CNCBdb) with accession number CNP0006847 (<https://db.cngb.org/search/project/CNP0006847/>), and the metabolism ID is METM0000235. The submission number of whole genome sequencing of *Fusobacterium necrophorum* subsp. *necrophorum* is SUB14921965, the bioproject accession number is PRJNA1196535 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1196535>).

## Declarations

### Ethics approval and consent to participate

All experimental protocols in this study were approved by the Animal Ethics Committee of China Agricultural University and authorized by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (AW50214202-2-01; AW50214202-2-02).

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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