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Integrated microbiome and metabolome analysis reveals altered gut microbial communities and metabolite profiles in dairy cows with subclinical mastitis

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Abstract

Background Dairy cow mastitis is a common and prevalent disease arose by various complicated pathogeny, which poses serious threat to the health of cows, safety of dairy product and economic benefits for pastures. Due to the high stealthiness and long incubation period, subclinical mastitis (SM) of cows causes enormous economic losses. Besides the infection by exogenous pathogenic microorganisms, previous studies demonstrated that gastrointestinal microbial dysbiosis is one of the crucial causes for occurrence and development of mastitis based on the theory of entero-mammary axis. Whereas, limited researches have been conducted on potential pathological metabolic mechanisms underlying the relationship between gut microbiota and SM in cows.

Results The differences in blood parameters, gut microbiome, plasma and fecal metabolome between healthy and SM cows were compared by performing 16 S rDNA sequencing and non-targeted metabolomic analysis in the current study. The content of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and activity of catalase (CAT), total antioxidant capacity(T-AOC) were significantly decreased, while malondialdehyde (MDA) concentration was dramatically increased in serum of SM cows in comparison with healthy cows. The gut of cows with SM harbored more abundant *Cyanobacteria*, *Proteobacteria*, *Succinivibrio* and *Lactobacillus_iners*. Moreover, the abundance of *Paraprevotella*, *Coprococcus*, *Succiniclasticum*, *Desulfovibrio* and *Bifidobacterium_pseudolongum* were observably reduced in the gut of SM cows. Furthermore, higher abundance of pro-inflammatory metabolites were observed in feces (9(S)-HPODE, 25-hydroxycholesterol, dodecanedioic acid, etc.) and plasma (9-hydroxy-10,12-octadecadienoic acid, 13,14-dihydro PGF1α, 5,6-dehydro arachidonic acid, myristic acid, histamine, etc.) of SM cows. The abundance of certain metabolites with anti-inflammatory and antioxidant properties (mandelic acid, gamma-tocotrienol, deoxycholic acid, etc.) were notably decreased in feces or plasma of cows with SM.

Conclusions The intestinal microbial composition and metabolic profiles of healthy and SM cows were significantly distinct, that were characterized by decreased abundance of intestinal symbiotic bacteria, potential probiotics and

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anti-inflammatory, antioxidant compounds, along with increased abundance of potential pro-inflammatory bacteria, lipid metabolites, and the occurrence of oxidative stress in cows suffered from SM. The results of this study further enriched our understanding of the correlations between gut microbiota and metabolic profiles and SM, which provided insight into the formulation of management strategies for SM in cows.

Keywords Dairy cows, Subclinical mastitis, Biochemical indicators, Gut microbial community, Metabolomics

Background

Mastitis of dairy cows is a common disease with high incidence and prevalence in pasture, which leads to the reduction of milk yield and quality, as well as increased culling rate and treatment expenses, causing huge economic losses to the producer worldwide [1]. The mastitis in cows can be categorized into SM and clinical mastitis (CM) according to the presence or absence of clinical symptoms [2]. In practice, the CM is usually well managed in pasture, however, the prevention and treatment of SM is easily to be ignored due to its invisible clinical symptoms and high concealment [3]. Additionally, the SM cows persistently existing in herd have long incubation period and may spread the pathogens to healthy cows, causing periodic mastitis attacks that may eventually escalate into CM [4]. Thus, the incidence of SM and economic losses caused by SM are much higher than that of CM in cows [2]. The occurrence of mastitis is correlated with multiple factors, such as genetic characteristics, environmental hygiene, feeding management, dietary ingredients and improper milking practices, etc [5]. It is generally accepted that the infection by various pathogenic microorganisms derived from environment is the leading cause of mastitis in cows [6]. Whereas, a growing body of recent studies in human, mice and cows have pinpointed that the disturbance of gastrointestinal microbiota is associated with the occurrence of mastitis and may be an important endogenous factor causing mastitis, thus proposing the concept of "gastroenterogenic mastitis" [[7–10].

The multitudinous and diverse microbiota which habitat in the gastrointestinal tract of dairy cows are critical regulators for nutrient digestion, metabolism and immune response of host [11]. It is well-known that gastrointestinal microbiota and their metabolites, including short chain fatty acids(SCFAs), bile acids, products of tryptophan metabolism, could regulate intestinal barrier function by modulating the expression of tight-junction protein and function of immune cell [12–16]. The gut flora disorders can increase the permeability of intestinal mucosa and lead to the disruption of intestinal barrier function, which allows the pathogenic microorganisms and metabolites to migrate to the extraintestinal distal tissues or organs through endogenous pathways including blood and lymphatic circulation, contributing to the inflammatory response in various organs of host, such as pancreas, spleen, liver and mammary gland [8, 17]. Transplanting the fecal microflora from mastitic dairy cows to germ-free mice was proved to induce the up-regulation of expression of endotoxins and inflammatory cytokines in serum, as well as the inflammation of mammary gland, spleen and colon in mice [8]. Moreover, fecal microbiota transplantation from cows with mastitis(M-FMT) to recipient mice promoted the transfer of pathogenic bacteria from gut to mammary gland and destroyed the blood-milk barrier, the butyric acid produced by commensal *Roseburia intestinalis* could restrict the translocation of bacteria to alleviate M-FMT-induced mastitis in mice [18]. Subacute rumen acidosis (SARA), a typical model of rumen microbiota dysbiosis arose by long-term feeding of high-concentrate diet, has been proved to decrease the diversity of ruminal and intestinal flora, and increase the permeability of rumen barrier, which lead to the release of lipopolysaccharide(LPS) into blood, significantly enhancing the concentration of inflammatory factors in circulatory system, thus inducing oxidative stress, apoptosis and inflammation in the mammary gland [19–24]. Hence, the gastrointestinal microbiota and their metabolites can directly or indirectly affect the occurrence and development of mastitis by regulating the intestinal barrier function and immune response of host.

Recently, multiple studies have identified the ruminal or intestinal microbes associated with somatic cell count (SCC), a widely used indicator for the diagnosis of mastitis in dairy cows. The genera *unclassified_f_RF16*, *genera Paenibacillus* and *c_Deltaproteobacteria*, etc., were remarkably enriched in the rumen of dairy cows with high SCC [19–24]. The abundance of microflora and metabolites correlated with inflammation in the rumen of dairy cows with mastitis were markedly changed, *Pseudobutyrivibrio*, *Gastranaerophilale*s and *Moraxella*, etc., with high abundance were characterized in the rumen of clinical mastitic dairy cows along with the elevation of 12-oxo-20-dihy-droxy-leukotriene B4 and 10beta-hydroxy-6beta-isobu-tyrylfuranoeremophilane, *Ruminiclostridium_9* and *Enterorhabdus* were abundant in the rumen of SM cows accompanied by the increase of methenamine and 5-HMF [26]. In addition, the abundance of *Campylobacteraceae*, *Campylobacter*, *unidentified-Christensenellaceae*, *Lactobacillus-reuteri* and *Bacteroides-vulgatus* were prominently enhanced in the feces of dairy cows with CM [27]. Since the composition and function of gastrointestinal microbiota can be

shaped by many factors, such as the host genetic background, physiological condition, growth stage, geographical environment, feeding management, etc [28], distinct gastrointestinal microorganisms associated with dairy cow mastitis has been identified across different studies. Furthermore, the pathological metabolic mechanisms for the relationship between digestive tract microbes, metabolites and mammary health status remain not fully understood. Herein, we obtained feces and plasma samples from healthy and SM cows raised in a farm of Wuhan, and compared the differences in gut microbiome, plasma and fecal metabolome by performing 16 S rDNA sequencing and untargeted metabolomics technology, which enhance the understanding of the relationship between SM and gut microbiota and metabolic profiles, as well as laying the theoretical foundation for formulating the prevention strategies and accurate diagnosis of SM in dairy cows.

Materials and methods

Experimental animals and sample collection

The experimental cows used in this study were selected form the dairy farm owned by Institute of Animal Science and Veterinary Medicine, Wuhan Academy of Agricultural Sciences. The Holstein dairy cows which were fed ad libitum and had free access to water, received the same total mixed ration (TMR) composed of 40% roughage and 60% concentrate twice a day at 5:00 and 17:00. The composition and nutritional levels of TMR are shown in Supplementary Table S1. In this study, Holstein dairy cows with similar parity, lactation days and milk yield were preliminarily selected. Subsequently, the udder health status of cows was comprehensively judged according to milk SCC for nearly one month and clinical symptoms of udder based on the degree of inflammation, which were examined by veterinary surgeon from Wuhan Academy of Agricultural Sciences. Currently, it is generally accepted that 20×10^4 cells/mL is the optimal SCC threshold value to distinguish between healthy and mastitis cows [29–31]. Ultimately, 8 dairy cows with healthy udders (SCC $(9.13\pm2.17)\times10^4$ cells/mL, no clinical symptoms of redness, swelling and fever in the udder, designated as CON group, $n=8$) and 8 dairy cows with subclinical mastitis(SCC $(75.25\pm9.50)\times10^4$ cells/mL, no clinical symptoms of redness, swelling and fever in the udder, designated as SM group, $n=8$) were selected for trial. The flow chart of our experimental design is presented in Fig. 1A.

The feces and blood samples were obtained from each experimental dairy cows on the last day after a month of elevated milk SCC (above a threshold of 20×10^4 cells/mL). Fresh stool samples were collected from rectum of dairy cows through sterile long-arm gloves, which were aliquoted into 2 aseptic 5 mL cryovials and snap-frozen in liquid nitrogen, and then transferred to a -80 ℃ refrigerator for storage until analysis of fecal microbiota and metabolites. Approximately 10 mL of fasting blood samples were collected from the tail vein of dairy cows into vacuum tubes containing coagulant or anticoagulant EDTA, which were kept on ice, and then centrifuged to acquire serum or plasma samples at 7000 r/min for 10 min at 4 ℃. The supernatant were retained and divided into 6 sterile cryovials, which were immediately immersed in liquid nitrogen and then stored at -80 °C until subjected to untargeted metabolomic analysis, and detection of biochemical, antioxidant indices and the concentration of inflammatory cytokines.

Determination of biochemical, antioxidant indices and inflammatory cytokines in serum

The content of total protein (TP), albumin (ALB), globulin (GLB), uric acid (UA), triglycerides (TG), TC, HDL-C and low-density lipoprotein cholesterol (LDLC) in the serum of experimental cows were determined by using a fully automatic biochemical analyzer (TBA120FR, Canon, Japan). T-AOC, the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and CAT, as well as the content of MDA, alkline phosphatase (AKP), non-esterified fatty acid (NEFA) and lactate dehydrogenase (LDH) in the serum were detected with commercially available kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of IgA, IgG, IgM, TNF-α, IL-1β, IL-2, IL-8 and IL-10 were measured by ELISA kit in accordance with the protocols provided by manufacturer (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China).

DNA extraction, 16 S rDNA amplicon sequencing

Total genomic DNA from stool samples were extracted using the MagBeads FastDNA Kit (116570384, MP Biomedicals, USA) following the manufacturer's instructions, and stored at -20 °C prior to further analysis. The quantity and quality of extracted DNA samples were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and detected by agarose gel electrophoresis, respectively.

PCR amplification of the bacterial 16 S rRNA genes V3-V4 region was performed using the forward primer 338 F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of 5× reaction buffer, 5 μL of 5×High GC buffer, 2 μL of dNTP(10mM), 1 μL of each forward and reverse primer (10 μM), 2 μL of DNA template, 8.75 μL of ddH_2O, 0.25 μL of Q5 high-fidelity DNA polymerase. Thermal cycling was comprised of

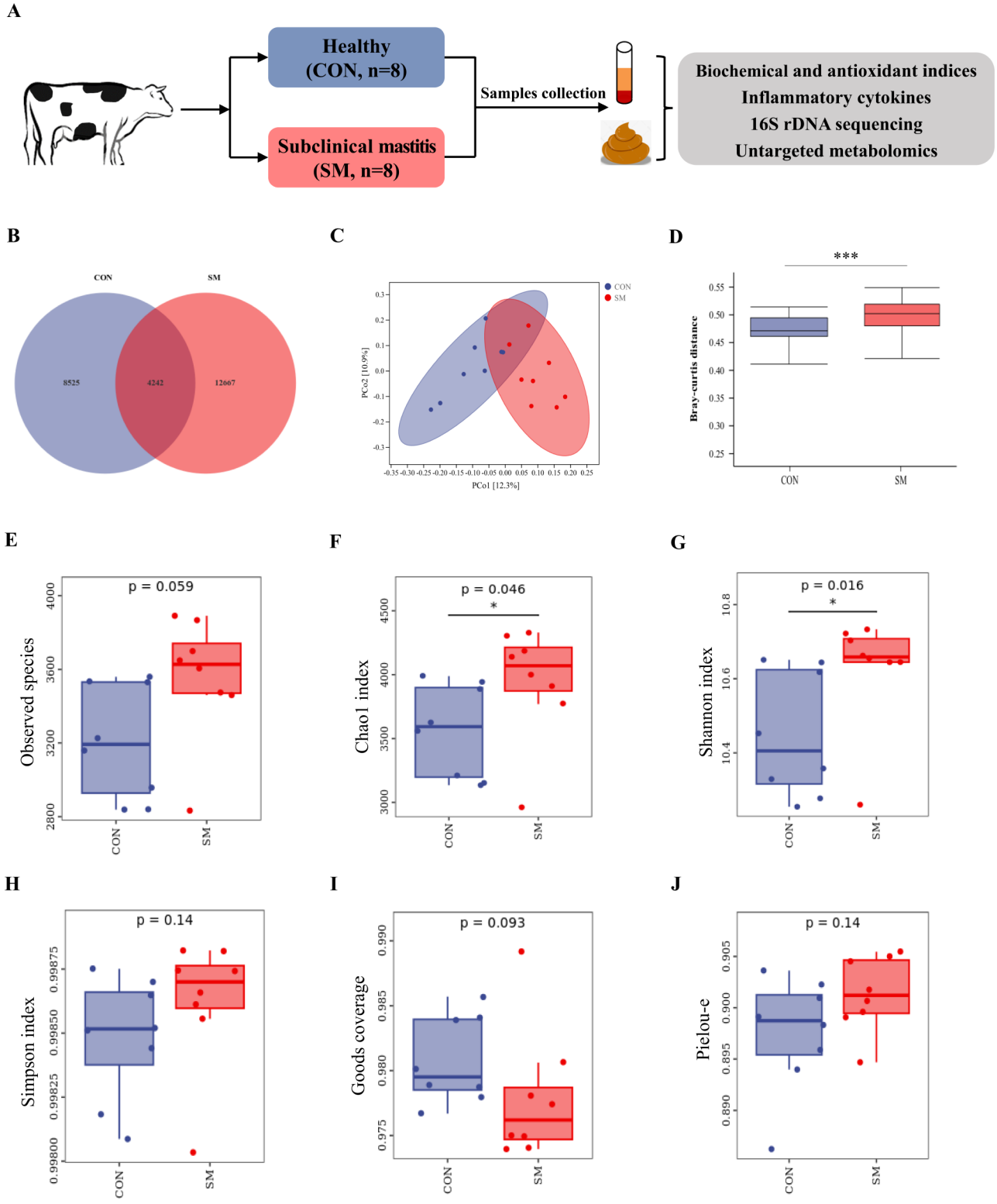

Fig. 1 The gut microbial diversity of healthy and SM cows. (**A**) Experimental design; (**B**) Venn diagram of ASV distribution between groups; (**C**) Beta diversity between groups analyzed by PCoA; (**D**) Bray-curtis distance between groups; (**E-J**) Alpha diversity indexes: observed species, Chao1, Shannon, Simpson, Goods coverage and Pielou-e, respectively. The results are presented as mean ± SEM($n = 8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

initial denaturation at 98 °C for 5 min, followed by 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was carried out using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles).

Processing, bioinformatic analysis of sequencing data

The sequence data were processed and analyzed using QIIME2 (2019.4) with slight modification according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin followed by primers cutting with cutadapt plugin. Sequences were subsequently quality filtered, denoised, merged and chimera removed using the DADA2 plugin. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft and employed to construct a phylogeny with fasttree2. Taxonomy was assigned to ASVs utilizing the classify-sklearn naive Bayes taxonomy classifier within the feature-classifier plugin, referencing the Greengenes 13.8 database.

ASV-level alpha diversity indices, such as Chao1 richness estimator, Observed species, Shannon diversity index, Simpson index, Faith's PD, Pielou's evenness and Good's coverage, were calculated using the diversity plugin with samples rarefied to 32,302 sequences per sample, and displayed as box plots. Beta diversity analysis was conducted to investigate the structural variation of microbial communities across samples using Bray-Curtis metrics and visualized via principal coordinate analysis (PCoA). The significance of difference in microbiota structure among groups was assessed by PERMANOVA (permutational multivariate analysis of variance) using QIIME2. MEGAN and GraPhlAn were engaged to visualize the taxonomic compositions and abundances, respectively. Venn diagram was generated to exhibit the number of shared and unique ASVs among groups by employing R package "VennDiagram", based on the occurrence of ASVs across groups regardless of their relative abundance. Linear discriminant analysis effect size (LEfSe) algorithm was devoted to identify the statistically differential abundant taxa between groups, the value of linear discriminant analysis (LDA) > 2 and $P < 0.05$ were considered significantly different. The prediction of microbial functions was performed by Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) (Gavin M. Douglas, et al., preprint) upon MetaCyc (https://metacyc.org/) databases.

Metabolites extraction, preparation and LC-MS/MS analysis

The plasma samples were thawed at 4 °C, and 100 µL aliquots were mixed with 400 µL of cold extraction solution (methanol: acetonitrile,1:1(v/v)) to remove the protein. The mixed solution was vortexed for 30 s, sonicated for 10 min in water bath at 4 ℃. However, for analysis using frozen stool samples, the thawed feces (50 mg) were mixed with beads and 500 µL of cold extraction solution (methanol: acetonitrile: H_2O, 2:2:1(v/v)) to vortex for 30s. Whereafter, the mixed samples were homogenized (35 Hz,4 min) and sonicated for 5 min in water bath at 4 ℃, and repeated for three times. Both the plasma and feces samples were incubated for 1 h at -40 ℃ to precipitate proteins. Subsequently, the samples were centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was transferred to a fresh glass vial, dried in a vacuum centrifuge and redissolved in 100 µL acetonitrile/water (1:1, v/v) solvent followed by centrifugation at 14,000 g for 15 min at 4 ℃, then the supernatant was injected for LC-MS/MS analysis. To supervise the stability and repeatability of instrument analysis, quality control (QC) samples were prepared by pooling 10 µL of each sample and analyzed together with the other samples. The QC samples were inserted regularly and analyzed in every 5 samples.

Untargeted metabolomic analysis of fecal and plasma samples were performed using an UHPLC (Vanquish UHPLC, Thermo) coupled to a Orbitrap. Chromatography was carried out with an ACQUITY UPLC® HSS T3 (2.1 × 100 mm, 1.8 µm) (Waters, Milford, MA, USA). The column was maintained at 40 ℃. The flow rate and injection volume were set at 0.3 mL/min and 2 µL, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (B2) 0.1% formic acid in acetonitrile (v/v) and (A2) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0 ~ 1 min, 8% B2; 1 ~ 8 min, 8%~98% B2; 8 ~ 10 min, 98% B2; 10 ~ 10.1 min, 98%~8% B2, 10.1 ~ 12 min, 8% B2. For LC-ESI (-)-MS analysis, the analytes were carried out with (B3) acetonitrile and (A3) ammonium formate (5 mM). Separation was conducted under the following gradient: 0 ~ 1 min, 8% B3; 1 ~ 8 min, 8%~98% B3; 8 ~ 10 min, 98% B3; 10 ~ 10.1 min, 98%~8% B3; 10.1 ~ 12 min, 8% B3.

Mass spectrometric detection of metabolites was performed on a Q Exactive HFX Hybrid Quadrupole Orbitrap mass spectrometer equipped with a heated ESI ion source (Thermo Fisher Scientific, USA). Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition methods were used. The parameters were set as follows: sheath gas pressure, 40 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and −2.50 kV for ESI (+) and ESI (-), respectively; capillary temperature, 325 ℃; MS1 range, m/z 100–1000; MS1 resolving power, 70,000 FWHM; number of data dependent scans per cycle, 10; MS/MS resolving power, 17,500

FWHM; normalized collision energy, 30 eV; dynamic exclusion time, automatic.

Untargeted metabolomic data processing and analysis

The raw MS data were converted to MzXML file format using the MSConvert tool in the Proteowizard software package (v3.0.8789) before importing into freely available XCMS software (V3.12.0). For peak picking, the following parameters were used: centWave m/z = 10 ppm, peakwidth = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, minfrac = 0.5 were used. In the extracted ion features, only the variables having more than 50% of the nonzero measurement values in at least one group were kept. The LOESS signal correction method based on QC samples is used to correct data between batches and eliminate instrument batch errors. Substances with RSD > 30% in QC samples are filtered out during data quality control.

The identification of metabolites was performed by comparing accurate m/z value (< 10 ppm) and MS/MS spectra with public databases including HMDB, MassBank, LipidMaps, mzCloud, KEGG, as well as an in-house database established with available authentic standards. Quantified data were output into excel format and subjected to univariate statistical analysis using R package. Dimension reduction analyses, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were conducted on the sample datasets using R package "Ropls", which generated score plots to visually depict the differences in metabolite compositions among various samples. The variable importance in projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. The metabolites with VIP > 1, *P*-value < 0.05 and fold change (FC) > 1.2 or < 0.83 were designated as statistically significantly upregulated or downregulated differential metabolites, respectively. Cluster analysis of the abundance values of differential metabolite was conducted by employing the Pheatmap package (v1.0.12) within R to plot a heatmap, which effectively illustrates the abundance profiles of metabolites across different samples. Functional analysis of the differential metabolites was primarily conducted through KEGG enrichment analysis using the clusterProfiler package (v 4.6.0), which was aimed at obtaining the significant enriched metabolic pathways, thus providing insights into the biological processes influenced by the differential metabolites The advanced analysis of differential metabolites was undertaken through machine learning techniques provided by the mlr3verse package (v0.2.7), which facilitated feature selection and model interpretation. Receiver operating characteristic (ROC) curves were constructed using the pROC package (v1.18.2) to assess the predictive power of the identified differential metabolites, ultimately yielding crucial information about key metabolites within the differential set that could serve as biomarkers or indicators of specific biological states. The Spearman correlation analysis between differential metabolites in feces and plasma and fecal microflora were performed by applying the correlation function in R package, and represented by a heatmap.

Statistic analysis

The sample size of this study was verified to be sufficient by employing SPSS27.0 software. A post hoc power calculation indicated that the sample size in this study achieved a power of 1.0 at 0.05 α level for a 2- sided test. The experimental data are presented as mean ± standard error of the mean (SEM). The statistical analyses were carried out by IBM SPSS26.0 software (IBM, Chicago, USA). The comparison of serum biochemical and antioxidant indices, inflammatory cytokines concentration and alpha diversity index between two groups were performed using unpaired two-tailed t test (Student's t-test). The relative abundance of bacterial taxa between two groups were analyzed by Mann-Whitney U test. Results with $P < 0.05$ were regarded as significantly different, $0.05 < P < 0.10$ were interpreted as a tendency.

Results

The serum biochemical, antioxidant indices, and inflammatory cytokines concentrations in healthy and SM cows

The content of TC and HDLC in the serum of SM cows were significantly lower than those in CON group ($P < 0.05$), the UA ($P = 0.063$) and LDLC ($P = 0.055$) concentration tended to decrease in SM group. Whereas, there were no significant differences in serum levels of TP, ALB, GLB, AKP, TG, NEFA and LDH between CON and SM groups ($P > 0.05$) (Table 1). T-AOC ($P < 0.05$) and CAT ($P < 0.01$) activities were remarkably reduced, while the concentration of MDA ($P < 0.05$) was notably increased in the serum of SM cows compared with CON group. No significant differences of SOD and GSH-PX activities were observed among CON and SM groups ($P > 0.05$) (Table 2). In addition, the serum IgA, IgG, IgM, TNF-α, IL-1β, IL-2, IL-8 and IL-10 concentration showed no significant differences between CON and SM groups ($P > 0.05$) (Table 3).

The gut microbial diversity and composition of healthy and SM co

A total of 1,550,335 raw reads were acquired from 16 stool samples through 16 S rDNA sequencing, which were then low-quality filtered, denoised, merged and chimera removed to yield 809,370 high-quality sequences.

Table 1 Serum biochemical indices of healthy and SM cows

Items	Group		*P*-Value
	CON	SM	
TP (g/L)	71.28 ± 1.87	73.40 ± 2.58	0.516
ALB (g/L)	34.58 ± 0.63	35.55 ± 0.75	0.336
GLB (g/L)	36.70 ± 1.60	37.85 ± 2.57	0.71
AKP (U/L)	517.45 ± 33.21	563.58 ± 81.24	0.607
UA (μmol/L)	64.00 ± 3.41	54.53 ± 3.23	0.063
TG (mmol/L)	0.16 ± 0.016	0.15 ± 0.020	0.775
TC (mmol/L)	4.80 ± 0.42[a]	3.51 ± 0.30[b]	0.025
HDLC (mmol/L)	2.58 ± 0.10[a]	1.99 ± 0.21[b]	0.023
LDLC (mmol/L)	1.43 ± 0.19	0.95 ± 0.13	0.055
NEFA (mmol/L)	0.10 ± 0.025	0.11 ± 0.033	0.97
LDH (U/L)	1580.40 ± 63.20	1418.36 ± 101.14	0.196

[a, b] Values within a row with different letters differed significantly ($P < 0.05$). Data are presented as mean ± SEM. Abbreviations: TP, total protein; ALB, albumin; GLB, globulin; AKP, alkline phosphatase; UA, uric acid; TG, triglycerides; TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; NEFA, non-esterified fatty acid; LDH, lactate dehydrogenase

Table 2 Antioxidant capacity in the serum of healthy and SM cows

Items	Group		*P*-Value
	CON	SM	
T-AOC (mM)	0.82 ± 0.042[a]	0.63 ± 0.042[b]	0.011
SOD (U/mL)	14.56 ± 0.67	12.92 ± 0.81	0.143
GSH-PX (U/mL)	215.88 ± 26.15	205.29 ± 42.14	0.834
CAT (U/mL)	3.73 ± 0.47[a]	1.84 ± 0.32[b]	0.005
MDA (nmol/mL)	2.05 ± 0.30[a]	3.32 ± 0.37[b]	0.023

[a, b] Values within a row with different letters differed significantly ($P < 0.05$). Data are presented as mean ± SEM. Abbreviations: T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde

Table 3 Inflammatory cytokines concentration in the serum of healthy and SM cows

Items	Group		*P*-Value
	CON	SM	
IgA (g/L)	295.56 ± 13.49	323.46 ± 25.09	0.344
IgG (g/L)	643.22 ± 21.98	712.64 ± 70.58	0.364
IgM (g/L)	154.54 ± 6.55	198.15 ± 24.24	0.104
TNF-α (ng/L)	262.39 ± 19.67	286.71 ± 19.17	0.391
IL-1β (ng/L)	557.02 ± 31.66	619.03 ± 52.73	0.33
IL-2 (ng/L)	351.65 ± 19.79	372.68 ± 27.55	0.545
IL-8 (ng/L)	182.89 ± 7.51	178.74 ± 10.71	0.756
IL-10 (ng/L)	114.95 ± 4.41	119.43 ± 5.93	0.554

Data are presented as mean ± SEM. Abbreviations: TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; IL-2, interleukin 2; IL-8, interleukin 8; IL-10, interleukin 10

Whereafter, 26,634 ASVs were obtained from each sample after normalization based on a 97% sequence similarity threshold. The rarefaction curves of observed species tended to flatten gradually as the depth of sequencing increased, which indicated that the sequencing in this study have covered and reflected the vast majority of microbial species in samples, increasing the number of sequencing furtherly could detect no more microorganisms (Supplementary Fig. S1). Namely, the sequencing datasets were credible, that could be applied for succedent analyses. The result of Venn diagram showed that the total intestinal ASVs of cows in SM group was higher than that of CON group, the overlapped ASVs between two groups were 4242 (Fig. 1B). PcoA of beta diversity based on Bray-Curtis distance presented an obvious separation of fecal microbial communities between CON and SM groups, indicating that the significant difference existed in gut microbial composition profiles of healthy and SM cows (Fig. 1C-D). Alpha diversity analysis, a metric used to estimate the richness and evenness of species within samples, demonstrated that the chao and shannon index of intestinal microorganisms of SM cows were significantly higher than those in CON group ($P < 0.05$). There were no significant differences for observed species, pielou-e, goods_coverage and simpson indexes between CON and SM groups ($P > 0.05$) (Fig. 1E-J). The microbial composition and structure analysis was performed to compare the relative abundance of fecal microflora at different taxonomic levels. 26,634 identified ASVs were clustered and assigned to 22 phyla and 279 genera of bacteria (Supplementary Tables S2 and S3). At phyla level, the dominant bacteria in the feces of cows in CON and SM groups were *Firmicutes*(59.42 ± 0.86%), *Bacteroidetes*(36.35 ± 0.96%) and *Tenericutes*(1.40 ± 0.07%), the sum of their abundance accounted for 97.17% of the overall abundance (Supplementary Table S2; Supplementary Fig. S2A). At genus level, the bacteria with relative abundance > 1% were deemed to be predominant, which occupied more than 87% of the total abundance. The *unidentified_Ruminococcaceae* (31.45 ± 0.68), *unidentified_Bacteroidales* (15.68 ± 0.46), *unclassified_Clostridiales* (4.23 ± 0.14), *unidentified_Rikenellaceae* (3.54 ± 0.16), *unidentified_RF16* (3.30 ± 0.21), *unidentified_Lachnospiraceae* (2.24 ± 0.12), *Oscillospira* (2.09 ± 0.070), *f__Clostridiaceae__g__Clostridium* (1.92 ± 0.12), *Ruminococcus* (1.88 ± 0.078) and *Paludibacter* (1.12 ± 0.045) were the most abundant genus in the feces of cows in CON and SM groups(Supplementary Table S3; Supplementary Fig. S2B).

The differential fecal microbiota between healthy and SM cows

LEfSe analysis and wilcoxon rank-sum test were further devoted to identify the representative differential microbes between CON and SM groups. The cladogram and score plot of LEfSe revealed that the bacteria of phyla *Cyanobacteria* ($P < 0.05$) and *Proteobacteria* ($P < 0.05$) were significantly enriched in the gut of cows in SM group (Fig. 2A-B). At genus level, *Coprococcus* ($P < 0.05$), *Succiniclasticum* ($P < 0.05$), *Desulfovibrio* ($P < 0.05$) and *Paraprevotella* ($P < 0.01$) were remarkably more abundant

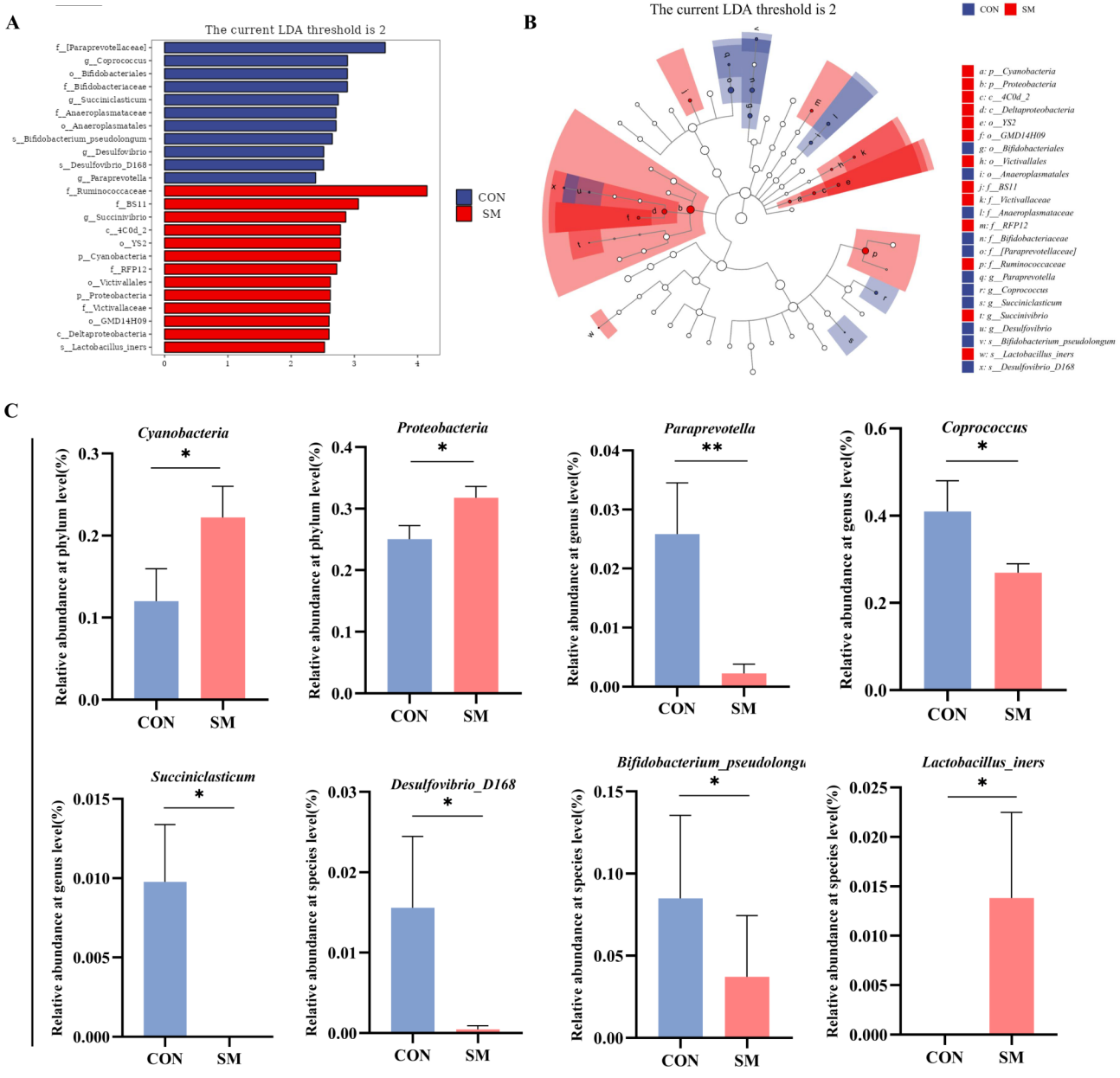

Fig. 2 Analysis of fecal differential microbes between healthy and SM cows. (**A**) The linear discriminant analysis (LDA) score histogram of differential intestinal microbes between groups; (**B**) The cladogram of linear discriminant analysis effect size (LEfSe) of differential intestinal microbes between groups; (**C**) Differential intestinal bacteria between groups at phylum, genus, species level. Data are presented as mean ± SEM($n = 8$). *$P < 0.05$, **$P < 0.01$

in the feces samples of CON group. Whereas, the gut of cows in SM group harbored more *Succinivibrio* ($P < 0.05$) in comparison with CON group (Fig. 2A-B). Moreover, the relative abundance of *Desulfovibrio_D168* ($P < 0.05$) and *Bifidobacterium_pseudolongum* ($P < 0.05$) in SM group were significantly lower than that of CON group, while *Lactobacillus_iners* ($P < 0.05$) showed higher relative abundance in the gut of cows in SM group compared with CON group (Fig. 2C).

Difference of gut microbial function between healthy and SM cows

The predicted function of gut microbiota in healthy and SM cows were inferred by PICRUSt2, which indicated that the microflora related to fatty acid and lipid biosynthesis was significantly enhanced in SM group ($P < 0.0001$), the capacity of secondary metabolite degradation was dramatically declined in SM group ($P < 0.001$). Additionally, the gut of cows in CON group tended to enrich more abundant microbes correlated with nucleic acid processing ($P = 0.065$) and

alcohol degradation($P = 0.097$). Nevertheless, the relative abundance of microflora associated with pyrimidine deoxyribonucleotides de novo biosynthesis ($P = 0.088$), superpathway of L-aspartate and L-asparagine biosynthesis($P = 0.089$) tended to be predominant in the gut of cows in SM group (Fig. 3).

Fecal metabolites profiling in healthy and SM cows

Non-targeted metabolomics technique using ultra performance liquid chromatography coupled to high-resolution mass spectrometry was employed to investigate the fecal metabolomic characteristics of healthy and SM cows. In the current study, a total of 623 metabolites including 503 at positive and 120 at negative ion mode, were identified in the feces samples of dairy cows in CON and SM groups. Subsequently, the identified metabolites were statistically analyzed at subclass level, which was displayed as a donut chart (Supplementary Fiure S3). The amino acids, peptides, and analogues (9.0%), fatty acids and conjugates (6.1%), carbohydrates and carbohydrate conjugates (3.7%), eicosanoids (3.0%), purines and purine derivatives (1.9%), benzoic acids and derivatives (1.9%), lineolic acids and derivatives (1.6%), triterpenoids (1.4%), bile acids, alcohols and derivatives (1.3%), hydroxycinnamic acids and derivatives (1.0%), etc. constituted the major components of metabolites in feces samples.

The PLS-DA analysis, based on the abundance of identified metabolites in each sample, suggested that the contribution of PC1 and PC2 to the variation were 20.5% and 14.2%, respectively, under the positive ion mode. In the negative ion mode, PC1 and PC2 accounted for 18.7% and 12.2% of the variation, respectively (Fig. 4A-B). In addition, the PLS-DA score plots showed that the samples from CON and SM groups can be effectively distinguished under both positive and negative ion modes, indicating that the fecal metabolite profiles differentiated between CON and SM groups (Fig. 4A-B). In total, 57 significant differential metabolites comprising 27 upregulated and 30 downregulated metabolites were screened in the feces samples of CON and SM groups, that were visualized by constructing a volcano plot to exhibit the distribution and fold changes of differential metabolites (Fig. 4C). The detailed information of all identified significantly differential metabolites in feces are listed in Supplementary Table S4. A heatmap depicting the fecal differential metabolites between CON and SM groups is shown in Fig. 4D, which revealed that there were obvious partitions in the composition of differential metabolites between the two groups. Thereafter, we conducted KEGG pathway enrichment analysis of the differential metabolites. The fecal differential metabolites identified between two groups were mainly enriched in the pathways, such as linoleic acid metabolism, steroid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, mTOR and PPAR signaling pathway, arachidonic acid metabolism and amino acid metabolism (Fig. 4E).

Identification and characterization of fecal signature differential metabolites between healthy and SM cows

In order to screen for the metabolites with significant representative characteristics, the machine learning analysis was performed on the fecal differential metabolites between CON and SM groups. The results showed that the abundance of glycylleucine, L-3-cyanoalanine, 3-methyl-L-tyrosine, 2-(Methylamino)benzoic acid, N(6)-methyllysine, and Gamma-tocotrienol etc. in the feces of cows in CON group were markedly higher than those in SM group. Nevertheless, the abundance of 3-epiecdysone, 25-hydroxycholesterol, 9(S)-HPODE, 2-amino-2-deoxyisochorismate, etc. were observably increased in SM group compared with CON group (Fig. 5A). Additionally, the feature importance of these differential metabolites in random forest model and its contribution for classification were relatively high, that might be signature differential metabolites in the feces of cows in CON and SM groups (Fig. 5A). ROC analysis, an established statistical approach for assessing the predictive power and diagnostic value, also identified several differential metabolites exhibiting potential as biomarkers of SM in dairy cows. The area under curve (AUC)

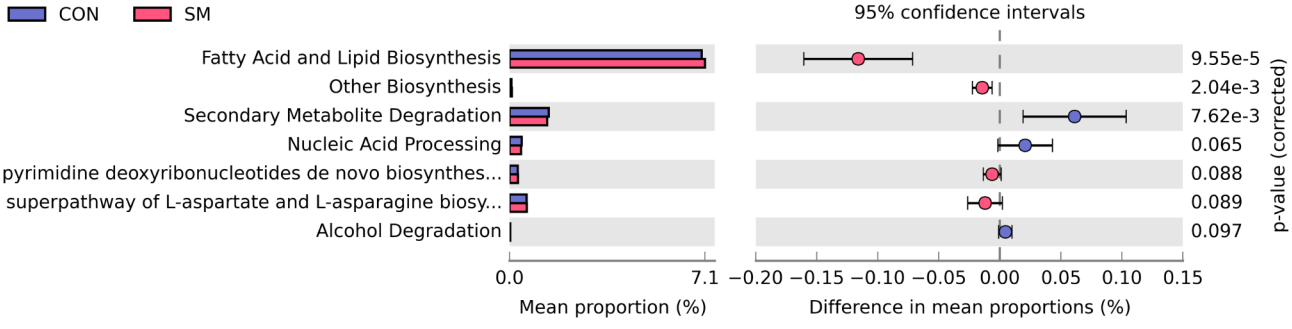

Fig. 3 Analysis on the difference of gut microbial function between healthy and SM cows. The abundance proportion of different functional pathways of gut microbiota with a corrected *P*-value < 0.1 are presented. The central plot denotes the difference in proportion of functional abundance within a 95% confidence interval

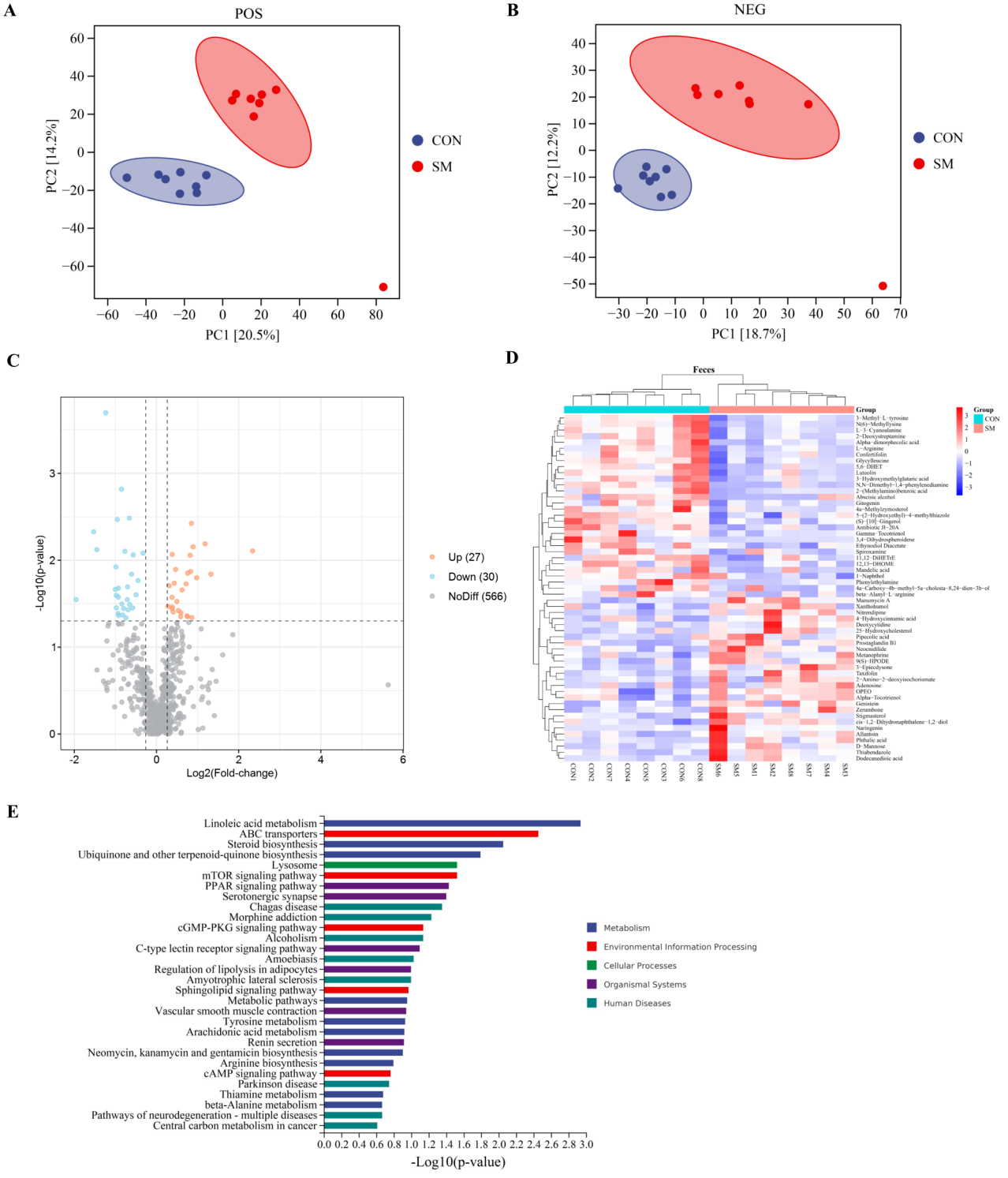

Fig. 4 Analysis of fecal differential metabolites between healthy and SM cows. (**A**) PLS-DA score plot of metabolic profiling of fecal samples under positive ion mode; (**B**) PLS-DA score plot of metabolic profiling of fecal samples under negative ion mode; (**C**) Volcano plot of fecal differential metabolites between healthy and SM cows; (**D**) Clustering heatmap of fecal differential metabolites between healthy and SM cows; (**E**) KEGG enrichment analysis of fecal differential metabolites between healthy and SM cows

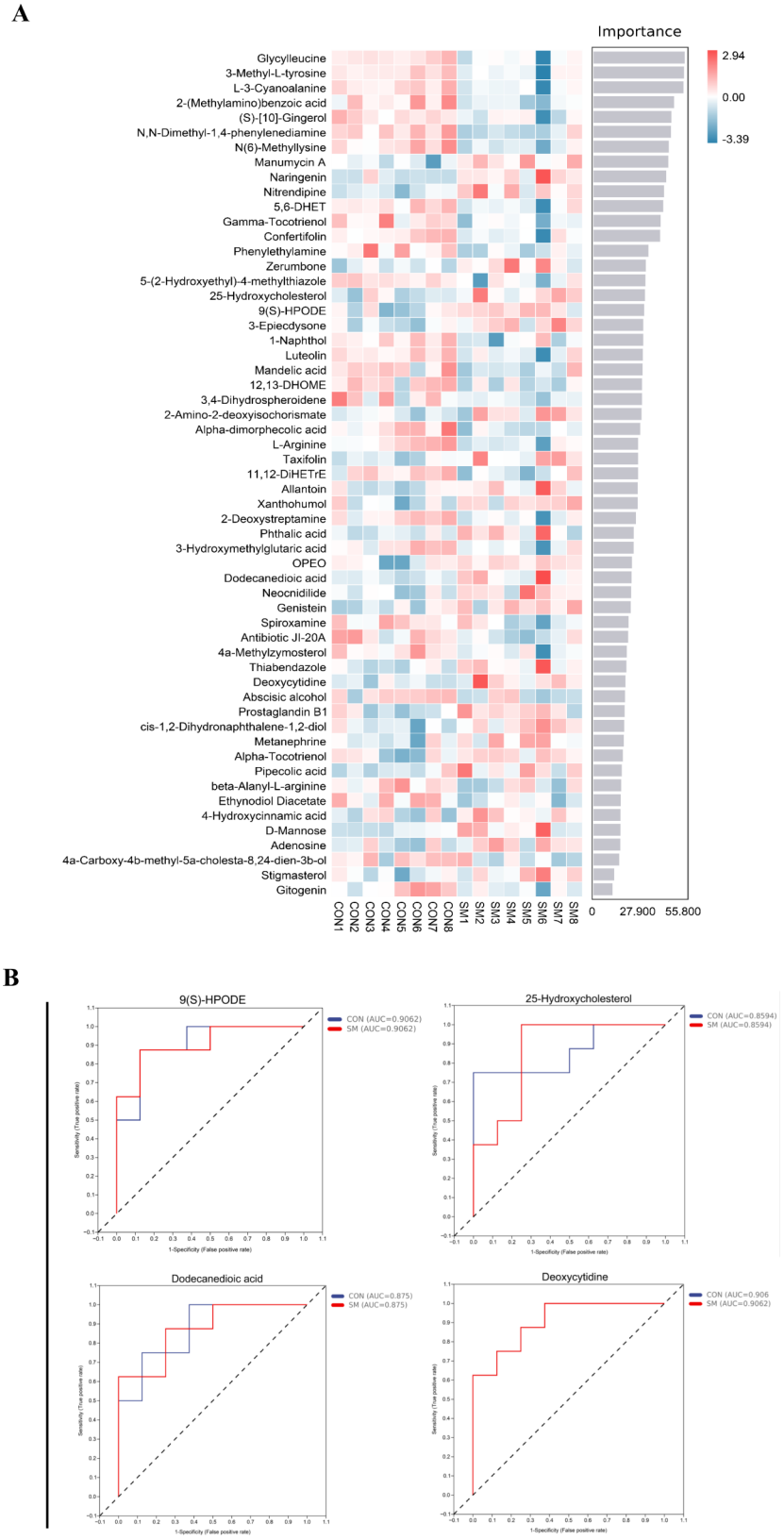

Fig. 5 Identification and characterization of fecal marker differential metabolites for the prediction of SM. (**A**) Machine-learning analysis of fecal differential metabolites between healthy and SM cows; (**B**) ROC curves of fecal differential metabolites between healthy and SM cows

of 9(S)-HPODE, 25-hydroxycholesterol, deoxycytidine, dodecanedioic acid and are all greater than 0.85, which suggested that these 4 fecal differential metabolites may be valuable indicators for SM in dairy cows (Fig. 5B).

Plasma metabolites profiling in healthy and SM cows

The metabolic profile in blood can accurately and quickly reflect the physiological and pathological state of the body, and the changes in blood metabolites composition are closely correlated with the overall health status of body. Therefore, the characteristics of plasma metabolomics of healthy and SM cows were also profiled in the current study. 6753 metabolites containing 3103 at positive and 3650 at negative ion mode, were identified in the plasma samples of cows in CON and SM groups through mapping with the public and a self-established database. At subclass level, these metabolites can be classified in to several major categories, such as amino acids, peptides, and analogues (4.78%), fatty acids and conjugates (2.47%), carbohydrates and carbohydrate conjugates (1.36%), sesquiterpenoids (0.77%), benzoic acids and derivatives (0.58%), bile acids, alcohols and derivatives (0.52%), glycerophosphocholines(0.46%), eicosanoids(0.41%), lineolic acids and derivatives(0.41%), purines and purine derivatives(0.31%) (Supplementary Fiure S4).

The score plots of PLS-DA analysis showed that the symbols representing plasma samples were completely separated under both positive and negative mode, suggesting significant differences in the composition of plasma metabolites between CON and SM groups (Fig. 6A-B). A total of 905 significantly differential metabolites were identified between the two groups, among which 457 metabolites were upregulated and 448 metabolites were downregulated (Fig. 6C). Moreover, the volcano plot illustrating differential metabolites between groups demonstrated that the vast majority of differential metabolites had relatively concentrated FC, and some of significantly upregulated or downregulated metabolites presented a larger range of FC (Fig. 6C). The details of all significantly differential metabolites identified in plasma are displayed in Supplementary Table S5. A heatmap plotted by hierarchical clustering analysis revealed changes in the abundance of plasma differential metabolites between two groups (Fig. 6D). KEGG enrichment analysis indicated that the plasma differential metabolites were mainly involved in protein digestion and absorption, taurine and hypotaurine metabolism, amino acids metabolism, pyrimidine metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism, etc. (Fig. 6E).

Identification and characterization of plasma signature differential metabolites between healthy and SM cows

We conducted machine learning analysis on the top100 differential metabolites in plasma to further distinguish the signature differential metabolites between healthy and SM cows. The results showed that the abundance of monolinolein, N-cyclopropyl-3-(4-fluorophenoxy) propane-1-sulfonamide, diethylthiophosphate, L-cysteine, 9-hydroxy-10,12-octadecadienoic acid in the plasma of cows in SM groups were significantly higher than those in CON group(Fig. 7A). While, the abundance of 2,5-dihydro-2,4,5-trimethyloxazole, 2-(2-amino-4-ethyl-6-oxopyrimidin-1-yl)-N-butylacetamide, 12,13-EODE, Glu Thr Phe Arg, Ala Ile Gln Arg Thr etc. were prominently elevated in the plasma of cows in CON group compared with SM group (Fig. 7A). All these differential metabolites with relatively high feature importance scores in random forest model could potentially serve as signature differential metabolites in the plasma of cows in CON and SM groups (Fig. 7A). Furthermore, we estimated the signature and predictive power of differential metabolites by applying ROC analysis, among which the AUC of hippuric acid, monolinolein, 9-hydroxy-10,12-octadecadienoic acid, myristic acid, 12-hydroxydodecanoic acid, 5,6-dihydroxy PGF1α, 5,6-dehydro arachidonic acid and stearoyl serotonin were all above 0.85, indicating their potential as promising biomarkers for SM in dairy cows (Fig. 7B).

The correlation analysis of differential intestinal microbes with metabolites in feces and plasma

To further explore the relationships between gut microbiome, fecal or plasma metabolome and SM in dairy cows, Spearman's correlation analysis and significance test were performed. The heatmap of correlations between fecal differential metabolites and differential microorganisms at several taxonomic levels in feces of dairy cows in CON and SM groups was shown in Fig. 8A. As for the intestinal predominant bacteria in dairy cows of SM group, *Cyanobacteria* and *Succinivibrio* were negatively correlated with amino acid metabolites and analogues (L-arginine, glycylleucine, L-3-cyanoalanine, 3-methyl-L-tyrosine). *Proteobacteria* and *Succinivibrio* were positively correlated with 3-epiecdysone, alpha-tocotrienol, dodecanedioic acid, 2-amino-2-deoxyisochorismate and adenosine, but negatively correlated with glycylleucine. With regards to the microorganisms significantly enriched in the intestinal tract of dairy cows in CON group, *Paraprevotella* and *Coprococcus* were negatively associated with manumycin A and cis-1,2-dihydronaphthalene-1,2-diol, and positively associated with fatty acid metabolites (11,12-DiHETrE, 5,6-DHET, 12,13-DHOME), amino acid metabolites and analogues (glycylleucine, 3-methyl-L-tyrosine). Furthermore, *Succiniclasticum* and *Desulfovibrio* were negatively associated with Manumycin A, fatty acid metabolites (9(S)-HPODE, dodecanedioic acid), while positively associated with amino acid metabolites and analogues

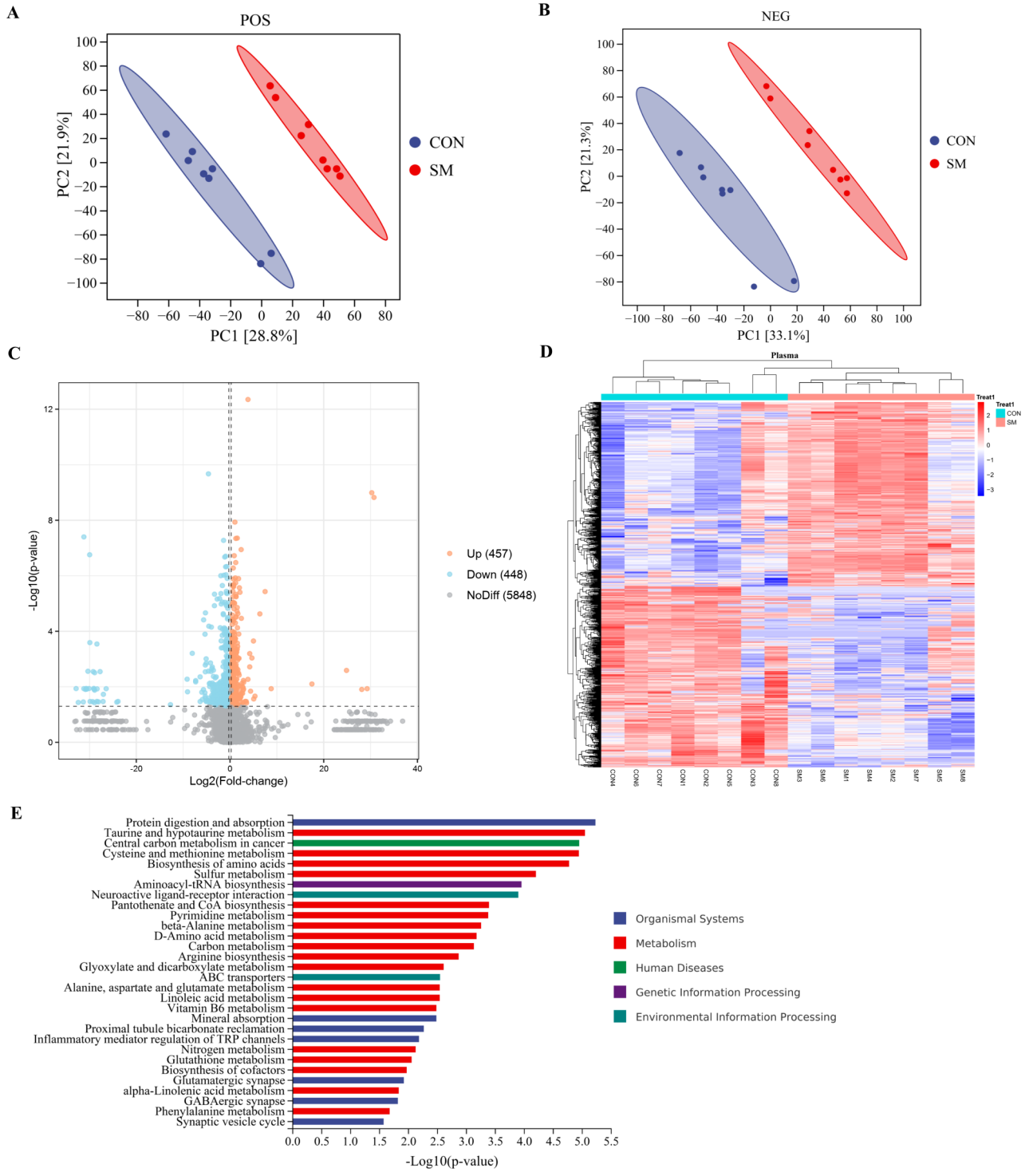

Fig. 6 Analysis of plasma differential metabolites between healthy and SM cows. (**A**) PLS-DA analysis of metabolites in each sample under positive ion mode; (**B**) PLS-DA analysis of metabolites in each sample under negative ion mode; (**C**) Volcano plot of plasma differential metabolites between healthy and SM cows; (**D**) Clustering heatmap of plasma differential metabolites between healthy and SM cows; (**E**) KEGG enrichment analysis of plasma differential metabolites between healthy and SM cows

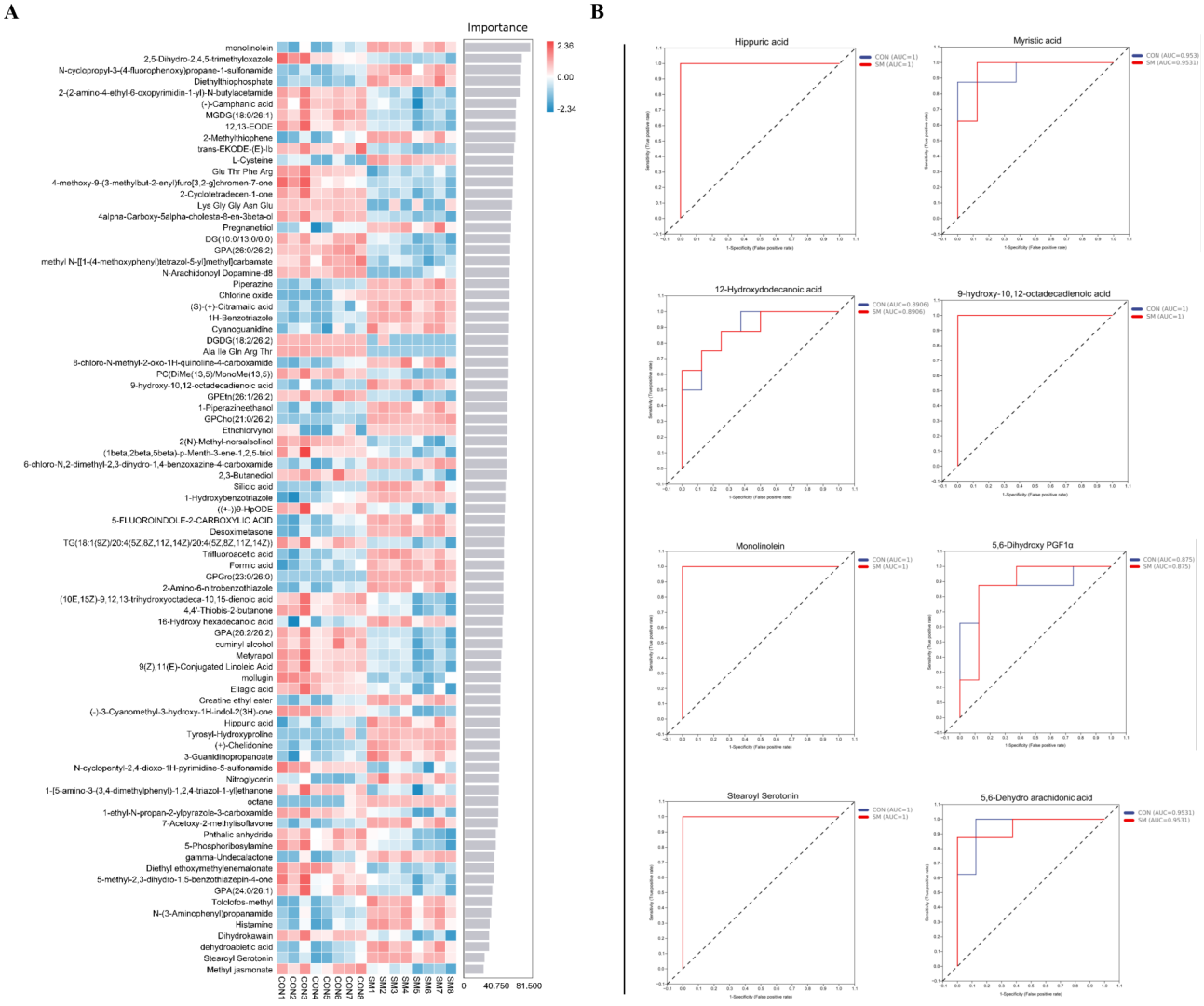

Fig. 7 Identification and characterization of plasma marker differential metabolites for the prediction of SM. (**A**) Machine-learning analysis of plasma differential metabolites (TOP100) between healthy and SM cows; (**B**) ROC curves of plasma differential metabolites between healthy and SM cows

(L-arginine, glycylleucine, 3-methyl-L-tyrosine). *Bifidobacterium_pseudolongum* showed negative correlations with manumycin A and deoxycytidine in feces (Fig. 8A).

The correlation heatmap of fecal differential microbes and plasma differential metabolites, milk SCC of dairy cows in CON and SM groups was displayed in Fig. 8B. *Cyanobacteria* and *Lactobacillus_iners* in feces were positively correlated with plasma differential metabolites from linoleic acid metabolism pathway, including 9-hydroxy-10,12-octadecadienoic acid, monolinolein and alpha-linolenic acid, and arachidonic acid metabolism pathway, involving 13,14-dihydro PGF1α, 5,6-dehydro arachidonic acid, as well as metabolites related to fatty acid metabolism, such as myristic acid, 12-hydroxydodecanoic acid, 12-hydroxyoctadecanoic acid, dodecanoic acid. *Proteobacteria* was positively correlated with hippuric acid, myristic acid, taurine, alpha-linolenic acid, 12-hydroxyheptadecanoic acid, 5,6-dehydro arachidonic acid in plasma. *Succinivibrio* was positively associated with plasma amino acid metabolites or analogues (tyrosyl-hydroxyproline, L-methionine), taurine and SCC of milk. *Succiniclasticum* and *Desulfovibrio*, which were dominant in the intestinal tract of dairy cows in CON group, showed negative correlations with plasma linoleic acid metabolites (9-hydroxy-10,12-octadecadienoic acid, alpha-linolenic acid), histamine, 2-hydroxymyristic acid, 12-hydroxyheptadecanoic acid, stearoyl serotonin, taurine and SCC. *Paraprevotella* was positively associated with plasma fatty acid metabolites (9,10,13-TriHOME, myristoleic acid) and oligopeptides (Ile Glu Val His Gly, His Glu Tyr Lys), while negatively associated with histamine, hippuric acid, tyrosyl-hydroxyproline, monolinolein and SCC. Moreover, *Bifidobacterium_pseudolongum* was positively associated

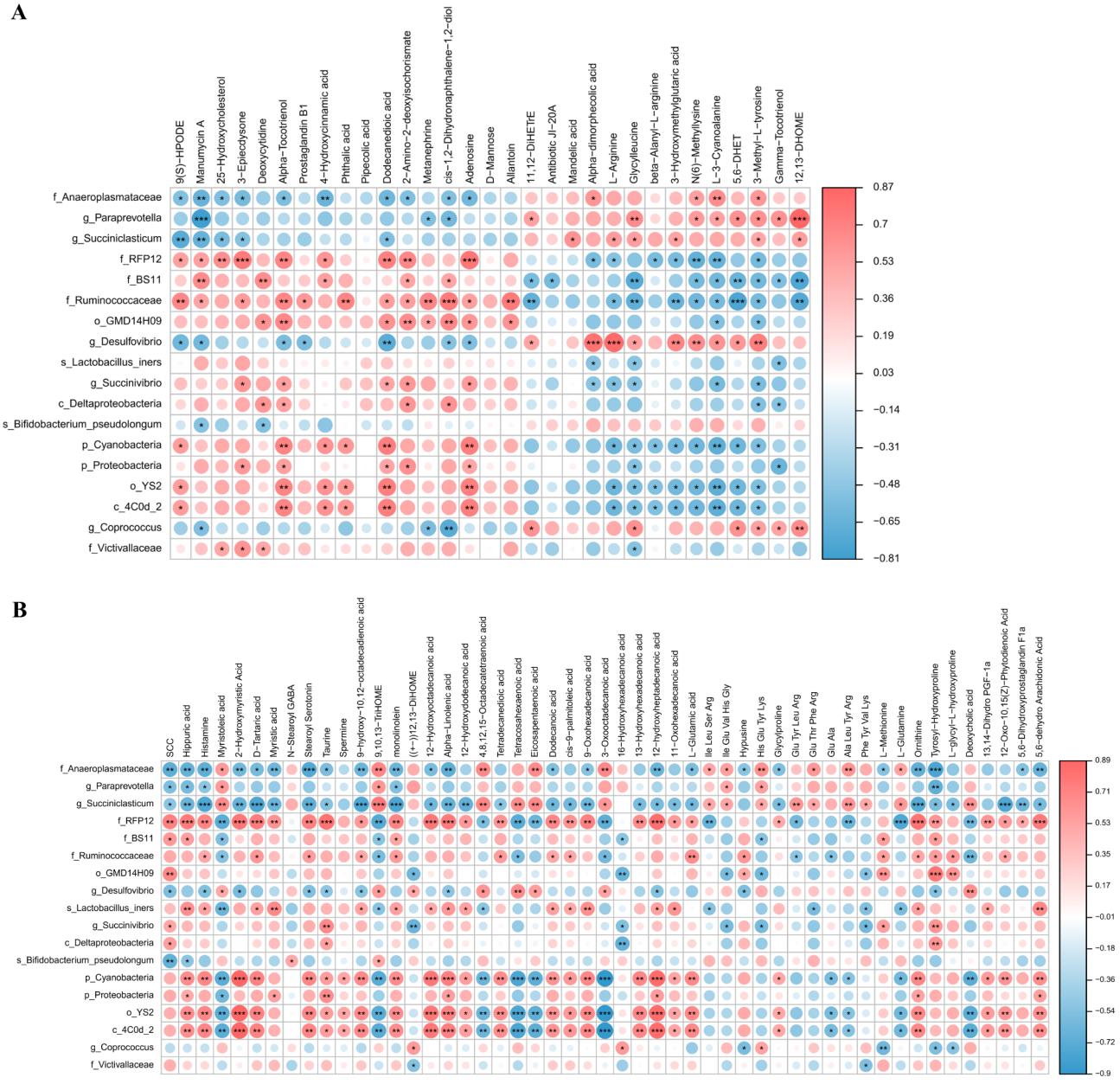

Fig. 8 Spearman correlation analysis between (**A**) gut differential microbes and fecal differential metabolites, (**B**) gut differential microbes, milk SCC and plasma differential metabolites. The legend shows the correlation coefficients values. The red color denotes positive correlation, while blue color represents negative correlation. The intensity of color indicates the strength of correlation. Dot size represents the corresponding P-value, a larger dot denotes a smaller P value. The significance was displayed as "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$

with 9,10,13-TriHOME, N-stearoyl GABA, whereas negatively correlated with hippuric acid and SCC (Fig. 8B). Interestingly, L-3-cyanoalanine, 12,13-DHOME and dodecanedioic acid were overlapped differential metabolites between feces and plasma (Supplementary Fig. S5). Specifically, L-3-cyanoalanine and dodecanedioic acid showed opposite expression patterns in feces and plasma of SM cows, while, the abundance of 12,13-DHOME in both feces and plasma presented the same regulatory trend.

Discussion

In the current study, feces and plasma samples from healthy and SM cows were collected to analyze the differences in gut microbiome and metabolism, thereby exploring the relationship between intestinal microorganisms and their metabolites and udder health status, as well as dissecting the potential pathological metabolic mechanisms of SM.

Firstly, we compared the differences in blood parameters between healthy and SM cows. The results indicated

that the content of TC and HDL-C, the activity of CAT and T-AOC in the serum of SM cows were dramatically lower than those of healthy cows, while, the content of MDA in SM cows was significantly increased, which were consistent with the previous findings from Turk et al. [31] and Sadek et al. [32]. All these results provided evidences for the existence of oxidative damage and redox imbalance in SM cows.

The gastrointestinal microbiota are essential regulators implicated in nutrient digestion, energy metabolism and immune response of host, which have been confirmed to be associated with multiple infectious and metabolic diseases [11]. In the present study, we compared the difference of fecal microbial composition and function between healthy and SM cows. PCoA analysis based on Bray-Curtis distance indicated significant differences in gut microbial composition and structure between the two groups. The alpha diversity analysis revealed that the chao1 index and shannon index of intestinal microorganisms of SM group were significantly higher than those in CON group, indicating the higher diversity and richness of microflora in the gut of SM cows. The investigation on rumen microbial composition in cows with different SCC demonstrated that the shannon index in cows with high SCC was remarkably higher than cows with low SCC [25]. The chao1, sob and ace indices of ruminal microbiota were found to be prominently elevated in mastitic cows compared with healthy cows [33]. The results of the above two studies are similar to our findings.

The taxonomic profiling of intestinal microbiota at phylum level showed that *Firmicutes*, *Bacteroidetes* and *Tenericutes* were the most predominant bacterial phyla in the gut of cows in CON and SM groups, which are in congruency with the results from Hu and Jiang et al. [20, 27]. However, the relative abundance of these three bacterial phyla presented no significant difference between the two groups, which suggested that the microbes playing an essential role in the gut of cows remained relatively stable at phylum level. LEfSe analysis showed that *Cyanobacteria*, *Proteobacteria*, *Succinivibrio* and *Lactobacillus_iners* were significantly enriched in the gut of SM cows, whereas, *Paraprevotella*, *Coprococcus*, *Succiniclasticum*, *Desulfovibrio* and *Bifidobacterium_pseudolongum* were remarkably enhanced in the gut of cows in CON group. These fecal differential microbes might be the marker microorganisms distinguishing SM cows from healthy cows. *Proteobacteria* and *Succinivibrio* with high abundance have also been identified in the gut of cows with CM induced by SARA [20]. Among the 16 differential microbes identified by Zhao et al., which were significantly enriched in the intestines of mastitic cows, 8 belong to *Proteobacteria*, including *Succinivibrionacea* [18]. *Succinivibrio* has been confirmed to be one of the core bacterial taxa in the rumen of dairy cows and negatively correlated with milk yield [34, 35]. *Cyanobacteria* was known as a photosynthetic gram-negative bacteria that were presumed to originate from freshwater or terrestrial environment, has also been proved to be significantly enriched in the rumen of cows with CM [26, 36]. Nevertheless, the investigation on the regulatory role of *Cyanobacteria* as intestinal microbe in host metabolism has been limited, and its correlation with mastitis remains unclear. *Lactobacillus_iners*, an obligate L-lactate-producing bacterium, was discovered to have a markedly higher relative abundance in the gut of mice with diabetic nephropathy [37]. Additionally, it was associated with bacterial vaginitis in females and considered to be an opportunistic pathogen [38, 39]. In this study, the relative abundance of *Paraprevotella*, *Coprococcus*, *Succiniclasticum*, *Desulfovibrio* and *Bifidobacterium_pseudolongum* in the gut of SM cows were prominently reduced. Another previous research has shown that the abundance of *Desulfovibrio* and *Bifidobacterium_pseudolongum* were markedly decreased in the rumen of cows with CM, besides, the administration of *Bifidobacterium_pseudolongum* could restore the weight loss of germ-free mice caused by FMT from mastitic cows, and significantly reduce their mortality [40]. *Paraprevotella* is an intestinal symbiotic bacterium isolated from feces of healthy human dornors recently, which could recruit trypsin to the bacterial surface to protect sIgA from degradation by trypsin, playing a crucial role in anti-infection and intestinal immunity of host [41]. *Succiniclasticum*, a core bacterial community in the rumen of ruminants, which is specialized in fermenting and converting succinate into propionate, as well as enhancing the bioavailability of butyrate and correlating positively with feed efficiency in host [42–44]. *Coprococcus*, being the primary butyrate producing bacteria, which could not only enhance the intestinal barrier function by upregulating the expression of claudin-1 and ZO-1 [45], but also increase the secretion of IL-18 by activating GRP109A in intestinal epithelia cells through its metabolite butyrate, thus inhibiting the occurrence of inflammation [46]. Furthermore, the report from Zhao et al. also proved that *Coprococcus* was notably enriched in the gut of healthy cows [18], which agree with our results. Recently, an investigation on the intestinal microbial characteristics in 45 cohorts involving 2518 patients with inflammatory bowel disease (IBD), indicated that the abundance of *Paraprevotella*, *Coprococcus* and *Desulfovibrio* were memorably decreased in the gut of patients with Crohn's disease or ulcerative colitis [47]. Correlation analysis showed that the genus *Paraprevotella*, *Succiniclasticum* and *Desulfovibrio* were significantly negatively associated with SCC, *Succinivibrio* showed signally positive correlation with SCC. Moreover, species *Bifidobacterium_pseudolongum* was notably negatively associated with SCC,

that was in accordance with the results from Hsieh [40]. Collectively, the reduction in the abundance of these symbiotic bacteria or potential probiotics accompanied by the enrichment of potential pathogenic bacteria in the gut of cows might contribute to the development of SM.

The fecal and blood metabolome are important tools for exploring the linkage between gut microbiome and host health, which were considered to possess important application value in the early prediction of diseases and research on pathological mechanisms [48]. The fecal differential metabolites between healthy and SM cows are mainly enriched in lipid metabolism-related pathways, such as linoleic acid metabolism and steroid synthesis, which mutually corroborates with the results from PICRUSt in predicting the significant enhancement of lipid metabolism ability of gut microbiota in SM cows. The relative abundance of fecal differential metabolites related with lipid metabolism in SM cows were dramatically increased, including 9(S)-HPODE, 25-hydroxycholesterol, dodecanedioic acid and prostaglandin B1. The lipid peroxidation process of linoleic acid is correlated with oxidative stress and many inflammatory diseases, such as atherosclerosis and rheumatoid arthritis [49]. 9(S)-HPODE, one of the lipid peroxidation products of linoleic acid, is rapidly reduced into stable 9-HODE in the body [50]. The content of 9-HODE was memorably elevated in tissues of cows with mastitis caused by intramammary *Streptococcus uberis* infection [51]. The correlation analysis showed that fecal 9(S)-HPODE was positively associated with *Cyanobacteria*, whereas, negatively correlated with *Succiniclasticum* and *Desulfovibrio*. 25-hydroxycholesterol is an oxidation product of cholesterol mediated by 25-hydroxylase, and its secretion is closely related to inflammation and immune status of tissues and organs [52]. 25-hydroxycholesterol secreted by macrophages in response to toll-like receptor activation has been certified to modulate the production of IgA [53]. The secretion of IL-6 and IL-8 can be upregulated by 25-hydroxycholesterol via enhancing NF-κB signaling, thus amplifying the inflammatory response [54, 55, 56]. Dodecanedioic acid, a dicarboxylic acid involved in a metabolic pathway intermediate between lipids and carbohydrates, can be used as an alternate fuel substrate and promptly oxidized to produce succinic acid for gluconeogenesis in type 2 diabetes [57, 58]. Nevertheless, few studies have been reported on the role of dodecanedioic acid in inflammatory diseases. Prostaglandin B1, a derivative of arachidonic acid, is a metabolite of prostaglandin E1. Prostaglandins (PGs) not only mediate the acute inflammatory response, but also implicate in a variety of chronic inflammatory diseases through synergistically interacting with cytokines to activate NF-κB and induce the expression of inflammation-related genes [59]. The fecal *Desulfovibrio* was negatively associated with Prostaglandin B1. Mandelic acid is an alpha hydroxy-carboxylic acid with antioxidant and anti-inflammatory activity that reduces the production of pro-inflammatory mediators and oxygen free radicals [60]. The markedly reduced abundance of mandelic acid in SM cows was positively associated with *Succiniclasticum*. Gamma-tocotrienol has been proved to not only inhibit the activation of MAPK, NF-κB signaling pathway and polarization of M1 macrophage, reduce systemic inflammatory response to improve insulin resistance induced by high fat diet [61], but also ameliorate inflammation in liver via suppressing endoplasmic reticulum stress and the expression of IL-1β [62]. The observably decreased abundance of gamma-tocotrienol in SM cows was negatively associated with *Proteobacteria* and *Lactobacillus_iners*, while positively correlated with *Paraprevotella* and *Coprococcus*.

With regards to the plasma metabolome in the current study, the relative abundance of linolenic acid metabolism related compounds, such as 9-hydroxy-10,12-octadecadienoic acid, monolinolein and alpha-linolenic acid, were significantly enhanced in SM cows. The increased abundance of 9-hydroxy-10,12-octadecadienoic acid(9-HODE) in plasma echoed with the elevated abundance of 9(S)-HPODE in feces. Alpha-linolenic acid, a precursor for the synthesis of DHA and EPA, has been certified to restrain the activation of NF-κB signaling, and reduce the expression of pro-inflammatory cytokines and oxidative stress to alleviate inflammation [63, 64]. The increased abundance of plasma alpha-linolenic acid in SM cows might be related to the defense against inflammation. The relative abundance of arachidonic acid metabolites related with inflammation in the plasma of SM cows were memorably increased, including 13,14-dihydro PGF1α, 12-oxo-10,15(Z)-phytodienoic acid, 5,6-dihydroxy PGF1α and 5,6-dehydro arachidonic acid. Among them, both of 13, 14-dihydro PGF1α and 5,6-dehydro arachidonic acid were positively correlated with *Cyanobacteria* and *Lactobacillus_iners*. Histamine plays a decisive role in immune response and neurotransmission, moreover, the histamine-secreting bacteria are notably enriched in the gut of patients with IBD [65]. The significantly increased abundance of histamine in the plasma of SM cows were positively associated with *Cyanobacteria* and *Lactobacillus_iners*. Myristic acid, a medium-chain saturated fatty acid signally increased in the plasma of SM cows in this study, has been demonstrated to regulate the synthesis of TG in MAC-T cells through the ubiquitination pathway [66], as well as exacerbate the inflammation of adipose tissue induced by high fat diet in mice [67]. Therefore, we presume that the enhancement of myristic acid in plasma of cows fed a high-concentrate diet during lactation chronically might be disadvantageous for the maintenance of udder health. Hippuric acid derived from the metabolism of benzoic acid by gut microbes, is

significantly reduced in the blood and urine of patients with IBD [68, 69]. The colitis of mice induced by DSS is relieved by hippuric acid via increasing the abundance of beneficial gut bacteria and alleviating intestinal barrier damage [70]. The significant increased plasma hippuric acid might be related to the anti-inflammatory self-regulation of the body. Bile acids has been verified to play a fundamental role in the modulation of intestinal microflora, carbohydrate and lipid metabolism, as well as host innate immunity [71]. Deoxycholic acid, one of the most common secondary bile acids produced by intestinal microbes via the metabolism of cholic acid, ameliorate acute and chronic colitis in mice through activating G-protein coupled bile acid receptor 1 (GPBAR1), and downregulating the secretion of inflammatory cytokines and chemokines [72]. In the present study, the relative abundance of deoxycholic acid in plasma of SM cows was prominently descend, that might be unfavourable for the body's early defense against inflammation. Summarily, the enhancement in abundance of pro-inflammatory lipids implicated in linoleic acid, arachidonic acid and cholesterol metabolism, along with the reduction in the abundance of substances with anti-inflammatory and antioxidant activities in the gut and blood of dairy cows might be correlated with the occurrence of SM.

Conclusions

The content of TC, HDL-C and antioxidant capacity in serum of SM cows were significantly reduced compared with healthy cows. Composition of fecal microbiota and metabolic characteristics in feces and plasma differentiated between healthy and SM cows. The lipid metabolism capacity of intestinal microbial community in SM cows was prominently heightened. The decreased abundance of multiple intestinal symbiotic bacteria or potential probiotics (*Paraprevotella*, *Coprococcus*, *Succiniclasticum*, *Desulfovibrio* and *Bifidobacterium_pseudolongum*), and increased abundance of potential pro-inflammatory microbes (*Succinivibrio*, *Lactobacillus_iners*), coupled with the elevation of pro-inflammatory metabolites in feces (9(S)-HPODE, 25-hydroxycholesterol, dodecanedioic acid) and plasma (9-hydroxy-10,12-octadecadienoic acid, 13,14-dihydro PGF1α, 5,6-dehydro arachidonic acid, myristic acid, histamine), as well as reduction in the anti-inflammatory and antioxidative compounds (mandelic acid, gamma-tocotrienol, deoxycholic acid), might be detrimental to the maintenance of udder health and probably related with the development of SM in cows. Nevertheless, the precise regulatory mechanisms by which theses microorganisms and metabolites regulate SM in dairy cows remain largely indistinct, that need to be further clarified in the future research. Our study enhances the understanding of the relationship between gut microbiota, metabolic profiles and SM in dairy cows.

Abbreviations

SM	Subclinical mastitis
TC	Total cholesterol
HDL-C	High-density lipoprotein cholesterol
CAT	Catalase
T-AOC	Total antioxidant capacity
MDA	Malondialdehyde
CM	Clinical mastitis
SCFAs	Short chain fatty acids
M-FMT	Fecal microbiota transplantation from cows with mastitis
SARA	Subacute rumen acidosis
LPS	Lipopolysaccharide
SCC	Somatic cell count
TMR	Total mixed ration
TP	Total protein
ALB	Albumin
GLB	Globulin
UA	Uric acid
TG	Triglycerides
LDLC	Low-density lipoprotein cholesterol
SOD	Superoxide dismutase
GSH-PX	Glutathione peroxidase
AKP	Alkline phosphatase
NEFA	Non-esterified fatty acid
LDH	Lactate dehydrogenase
ASVs	Amplicon sequence variants
LEfSe	Linear discriminant analysis effect size
LDA	Linear discriminant analysis
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
OPLS-DA	Orthogonal partial least squares discriminant analysis
VIP	Variable importance in projection
FC	Fold change
ROC	Receiver operating characteristic
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
AUC	Area under curve
DSS	Dextran sulfate sodium
IBD	Inflammatory bowel disease
GPBAR1	G-protein coupled bile acid receptor 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03810-1.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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Author contributions

Jie Yu, Chenhui Liu, Dingfa Wang and Pingmin Wan performed the animal experiments and analyzed the samples. Jie Yu, Lei Cheng, Xianghua Yan designed this study, analyzed the data, wrote and revised the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Data availability
The sequencing data generated in this study were deposited in the NCBI SRA database (accession number PRJNA1144514).

Declarations

Ethics approval and consent to participate
Studies on the experimental animals were performed with the informed consent of the herd owner. All animal procedures were performed according to Hubei Provincial Regulation on Administration of Laboratory Animals (1 October 2005). The animal study protocol was approved by the Ethics Committee of Institute of Animal Science and Veterinary Medicine, Wuhan Academy of Agricultural Sciences (No.202303007; date of approval: 7 March 2023).

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

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