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Original Research Article

Feeding citrus flavonoid extracts decreases bacterial endotoxin and systemic inflammation and improves immunometabolic status by modulating hindgut microbiome and metabolome in lactating dairy cows

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ABSTRACT

The objectives of this study were to determine the effects of dietary supplementation with citrus flavonoid extracts (CFE) on milk performance, serum biochemistry parameters, fecal volatile fatty acids, fecal microbial community, and fecal metabolites in dairy cows. Eight multiparous lactating Holstein cows were used in a replicated 4 × 4 Latin square design (21-day period). Cows were fed a basal diet without addition (CON) or basal diet with added CFE at 50 (CFE50), 100 (CFE10), and 150 g/d (CFE150). Feeding CFE up to 150 g/d increased milk yield and milk lactose percentage. Supplementary CFE linearly decreased milk somatic cell count. Serum cytokines interleukin-1β (IL-1β), IL-2, IL-6, and tumor necrosis factor-α (TNF-α) concentrations decreased linearly as the levels of CFE increased. Cows in CFE150 had lower serum lipopolysaccharide and lipopolysaccharide binding protein compared with CON. These results indicate feeding CFE decreased systemic inflammation and endotoxin levels in dairy cows. Furthermore, feeding CFE linearly increased the concentrations of total volatile fatty acids, acetate, and butyrate in feces. The relative abundances of beneficial bacteria *Bifidobacterium* spp., *Clostridium coccoides-Eubacterium rectale* group, and *Faecalibacterium prausnitzii* in feces increased linearly with increasing CFE supplementation. The diversity and community structure of fecal microbiota were unaffected by CFE supplementation. However, supplementing CFE reduced the relative abundances of genera *Ruminococcus_torques*_group, *Roseburia*, and *Lachnospira*, but increased genera *Bacteroides* and *Phascolarctobacterium*. Metabolomics analysis showed that supplementary CFE resulted in a significant modification in the fecal metabolites profile. Compared with CON, fecal naringenin, hesperetin, hippuric acid, and sphingosine concentrations were greater in CFE150 cows, while fecal GlcCer(d18:1/20:0), Cer(d18:0/24:0), Cer(d18:0/22:0), sphinganine, and deoxycholic acid concentrations were less in CFE150 cows. Predicted pathway analysis suggested that "sphingolipid metabolism" was significantly enriched. Overall, these results indicate that citrus flavonoids could exert health-promoting effects by modulating hindgut microbiome and metabolism in lactating cows.

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1. Introduction

Substantial genetic and nutritional advancements have contributed to the increased milk production in the dairy industry. A greater milk production is associated with a greater metabolic load, which increases the risk of and susceptibility toward different production diseases (McArt and Neves, 2020). Previous studies have reported that the inflammatory responses resulting from

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bacterial lipopolysaccharide (LPS) induced by high metabolic load markedly disrupted the metabolic homeostasis in high-yield dairy cows (Zebeli and Ametaj, 2009; Zhao et al., 2018). The application of phytochemicals (e.g., flavonoids), as a potential approach for improving animal health and maintaining metabolic homeostasis in livestock farms has become more widespread due to the growing public concern about the side effects of antibiotics (Kuralkar and Kuralkar, 2021). Flavanones (e.g., hesperidin and naringin) and O-polymethoxylated flavones (e.g., tangeretin and nobiletin) are the main components in citrus-derived flavonoids (Li et al., 2021). These citrus-derived flavonoids, as promising phytochemicals, have various biological properties, such as antimicrobial, antioxidant, anti-stress, and anti-inflammatory in ruminants (Alhidary and Abdelrahman, 2016; Lenehan et al., 2017; Sharif et al., 2018; Simitzis et al., 2019).

Ying et al. (2017) reported that adding citrus extracts at 4.5 g/d reduced plasma non-esterified fatty acids and increased plasma insulin in early-lactation cows, indicating that citrus flavonoids have the potential effect of regulating lipid homeostasis in dairy cows. In another study by Santos et al. (2014), the inclusion of 9% to 19% DM of citrus pulp increased the concentrations of total polyphenols, flavonoids, and ferric reducing antioxidant power in the milk of dairy cows. Additionally, the intramammary administration of hesperidin and naringenin was shown to reduce milk somatic cell count (SCC) in dairy cows with mastitis. Collectively, evidence from these studies suggests that citrus flavonoids have potential health benefits for lactating cows.

Citrus flavonoids could interact with gastrointestinal microbiota to influence host metabolic health (Stevens et al., 2019). Analysis of our own unpublished data showed feeding citrus flavonoid extracts (CFE) did not significantly affect rumen fermentation parameters and ruminal microbial structure in lactating dairy cows. In ruminants, it is unknown if citrus flavonoids are absorbed across the rumen epithelium. It was reported that rumen bacteria are known to have the ability of partially deglycosylating naringin and hesperidin (Gladine et al., 2007). We speculated that the hindgut microorganisms may further enhance the bioavailability of citrus flavonoids and promote flavonoid metabolite production. Thus, determining the effects of citrus flavonoids on hindgut microbiota and metabolism is essential for characterizing the role of these flavonoids and their influence on dairy cows' immune status.

We hypothesized that supplementing CFE in the diets of lactating dairy cows might enhance carbohydrate fermentation in the hindgut and improve immune status by altering hindgut microbiota and metabolites. These modifications would have a beneficial influence on the productivity of dairy cows. Accordingly, the objectives were to evaluate the effects of dietary supplementation with CFE on milk production, serum immune indices, fecal volatile fatty acids (VFA), fecal microbiota, and fecal metabolomic profile.

2. Materials and methods

2.1. Animal ethics statement

Procedures for care and handling of animals required for this experiment were approved by the Ethics Committee on Animal Use at Beijing University of Agriculture. This trial was performed according to the Chinese Guidelines for Experimental Protocols of Animal Care and Animal Welfare.

2.2. Citrus flavonoid extract preparation, animals, and treatments

The product CFE was purchased from Shaanxi Xiazhou Biotechnology Co., Ltd. (Xi'an, China). Citrus flavonoids were extracted from the peel powder of *Citrus reticulata* Blanco. In brief, 1 kg of citrus peel powder was extracted twice with 15 L of calcium carbonate solution (0.1%) at 100 °C for 1.5 h. The extraction was then evaporated to dryness using rotary evaporation at 37 °C under reduced pressure. The total flavonoids were enriched by AB-8 macroporous absorption resin columns and eluted with 80% ethanol (2-fold column volume). The eluents were collected and concentrated to dryness for use. The total flavonoid content (56.83%) of CFE was determined using aluminum nitrate spectrophotometry (510 nm) with rutin equivalents (Pitz et al., 2016). The concentrations of major flavonoids, including naringin, hesperidin, neohesperidin, nobiletin, and tangeretin in CFE were analyzed through a HPLC system (1290 Infinity; Agilent Technologies, Inc.) according to the method of Jiang et al. (2019). The chemical composition of CFE is presented in Table S1.

Eight multiparous lactating Chinese Holstein cows (36.1 ± 3.79 kg/d of milk yield [MY], 662 ± 57.1 kg of BW, 160 ± 22.4 day in milk at the start of the experiment) were blocked by parity and MY and randomly allocated to a replicated 4 × 4 Latin square design. Each experimental period lasted 21 d, with 14 d for dietary adaptation (d 1–14) and 7 d for data acquisition and sample collection (d 15–21). Dairy cows in each group were randomly assigned to 4 treatments: the basal diet (CON) and CFE supplementation at 50 g/d (CFE50), 100 g/d (CFE100), and 150 g/d (CFE150).

All cows were kept in a tie stall barn with straw and sawdust bedding equipped with individual feed bins during the entire period and free access to water. Cows were milked 3 times daily at 05:30, 14:00 and 22:00. Milk production was recorded at each milking throughout the trial. Diets were formulated according to NRC (2001). The ingredient and nutritional composition of the basal diet are depicted in Table S2. The fresh TMR was fed twice daily at 07:00 and 15:30. Amounts of feed were adjusted to allow for a minimum of 5% refusals (as-fed basis). During the experiment, the supplied CFE were top-dressed on the first daily meal immediately after total mixed ration (TMR) delivery and mixed with a small amount of the feed. We monitored the animals after feeding to ensure that extracts were completely consumed.

2.3. Feed intake, milk production and composition

Daily feed provision and orts were weighed to calculate dry matter intake (DMI). Weekly samples from all forages and concentrate grains included in the TMR were dried and ground, and a single composite of each ingredient representing the entire study was kept at −20 °C until chemical determination. Samples of TMR and orts were dried in a forced-air oven at 55 °C for 48 h then ground through a 1-mm screen (Wiley mill, Arthur H. Thomas) before analysis. Samples were analyzed for DM (method 935.29; AOAC, 2006), ash (method 942.05; AOAC, 2006), ether extract (method 920.39; AOAC, 2006), crude protein (method 990.03; AOAC, 2006), and starch (method 996.11; AOAC, 2006). Concentrations of neutral detergent fiber and acid detergent fiber were analyzed according to Van Soest et al. (1991), with the use of heat stable α-amylase and sodium sulfite for neutral detergent fiber determination. The NE_L was calculated from tabulated feed values based on NRC (2001).

Milk samples were collected during the last 3 consecutive days (d 19–21) from all 3 milkings and were preserved with 2-bromo-2-nitropropane-1,3-diol. Daily milk samples of each cow were composited in proportion based on MY and transported to Beijing Dairy Cattle Center (Beijing, China). Milk fat, protein, lactose, SCC, and urea nitrogen were analyzed using the infrared spectroscopy (MilkoScan 4000, Foss Electric, Hillerød, Denmark). The 3.5% fat-corrected milk (FCM) was calculated according to Sklan et al.

(1992). Energy-corrected milk (ECM) was calculated based on the equation of Sjaunja et al. (1990).

2.4. Serum immune parameters

Blood was taken from the tail vessels of cows into vacuum tubes without clot activator (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) after the morning milking and before feeding on d 16 of each period. Serum was separated by centrifugation at 2,000 × *g* for 15 min at room temperature, and then stored at −20 °C until analysis. Concentrations of immunoglobulin A (IgA), IgG, IgM, interleukin-1β (IL-1β), IL-2, IL-4, IL-6, tumor necrosis factor-α (TNF-α), interferon γ (IFN-γ), LPS, LBP, soluble LPS receptor CD14 (sCD14), fibroblast growth factor 21 (FGF21), and adiponectin (ADPN) were analyzed using commercial ELISA kits according to the supplier's instructions (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). All ELISA data were recorded by a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Feces sampling and fecal volatile fatty acids analysis

Fresh fecal samples were taken manually using new gloves for every collection into sterile tubes after the morning milking and before feeding on d 16 of each period. Fecal tubes were immediately flash frozen in liquid nitrogen and stored at −80 °C.

The VFA concentrations in fecal samples were analyzed using the method of Petri et al. (2019) with minor modification. Briefly, 2 g of thawed feces from each sample was mixed with 2 mL of distilled water. Then, 600 μL of the internal standard 4-methylvaleric acid (Sigma−Aldrich, Saint Louis, MO, USA) and 0.4 mL of 25% phosphoric acid were added to the suspension. Those mixtures were centrifuged at 15,000 × *g* at 4 °C for 20 min. Subsequently, the supernatant was analyzed for VFA composition by a gas chromatograph (GC-7890B; Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector and a DB-FFAP capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Wilmington, DE, USA). The temperatures of the injector and detector were 170 and 190 °C, respectively. The oven temperature was increased from 100 to 250 °C.

2.6. Quantitative real-time PCR amplification

Total microbial DNA from each fecal sample was extracted using an E.Z.N.A. Stool DNA Kit (D4015, Omega, Inc., USA) following the manufacturer's protocol. Concentration and integrity of DNA were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.1% agarose gel electrophoresis. The extracted DNA samples were stored at −80 °C until further processing.

The relative abundances of 8 fecal bacteria, including *Bacteroides-Prevotella-Porphyromonas* group, *Bifidobacterium* spp., *Clostridium coccoides-Eubacterium rectale* group, *Clostridium* clusters I and XIVab, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Lactobacillus* spp. were quantified using real-time PCR. Primer sequences of these targeted bacteria are listed in Table S3. The extracted DNA of each sample was standardized to 8 ng/μL for qPCR. The qPCR reaction was performed in triplicate with a total volume of 25 μL. Each reaction system consisted of 12.5 μL of 2 × SYBR Green Master Mix (Life Technologies, Foster City, CA, USA), 1 μL of each primer, 2 μL of extracted DNA samples, and 9.5 μL of DNase/RNase free water. The qPCR reactions were conducted in a Roche LightCycler 96 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). The cycling protocol was 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at the respective annealing temperature, and 15 min at 72 °C for the final extension. The abundance of each target bacteria was expressed as the proportion (%) of the abundance of 16S rRNA genes of each bacterial target against that of the total bacteria using the efficiency-corrected $2^{\Delta\Delta-Ct}$ method (Ramirez-Farias et al., 2009).

2.7. Fecal bacterial 16S rRNA sequencing and bioinformatics analysis

Based on the results of serum immune indices, we selected the samples of CON and CFE150 to determine the effects of CFE on the fecal microbiome and metabolite profiling. Amplification of the hypervariable regions V3 and V4 of bacterial 16S rRNA gene was performed with the 338F (5′-barcode- ACTCCTRCGGGAGGCAG-CAG-3′) and 806R (5′-GGACTACCVGGGTATCTAAT-3′) primer set. The PCR amplification reaction consisted of 1 μL of forward index primer (10 mM), 1 μL of reverse index primer (10 mM), 1 μL of DNA template (10 ng/μL), and 17 μL of Pfx AccuPrime master mix. The amplification started with a denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final elongation at 72 °C for 5 min. The amplicon was performed in triplicate for each sample, and then PCR amplicons were further purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) in accordance with the protocol of the manufacturer. Subsequently, the quality of PCR product was evaluated using the QuantiFluor-ST (Promega, Durham, NC, USA). Paired-end sequencing (2 × 300 bp) was conducted in an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA). The 16S rRNA gene amplicon sequencing data were deposited into the National Center for Biotechnology Information database under accession number PRJNA805345.

The Quantitative Insights into Microbial Ecology version 2 (QIIME 2) pipeline was used to filter and identify the operational taxonomic unit (OTU) of sequenced reads (Caporaso et al., 2010). The sequence with a quality score <20 was removed before downstream analysis. Screening for chimeric sequences was conducted via USEARCH and its database (USEARCH version 8.1; Edgar et al., 2011). Sequence alignment and clustering were performed by PyNAST (Caporaso et al., 2010) and the SILVA database (version 123) of 16S rRNA gene database using the Basic Local Alignment Search Tool. The identity of OTU was determined based on 3% dissimilarity of sequences to the database, and OTU which clustered with more 10 reads were retained. Taxonomy was assigned to each OTU using the RDP classifier (version 2.2) and the GreenGenes database. Abundance estimates were calculated by summing read counts of OTU with identical taxonomic assignments from kingdom to genus taxonomic level. Shannon diversity index, Chao richness estimator, and all other OTU-level alpha diversity indices were computed using MOTHUR (version 1.30.1). Analysis of similarity (ANOSIM) in MOTHUR was performed to determine the difference in bacterial community structure. Beta diversity was calculated using the unweighted and weighted UniFrac distances and visualized using the principal coordinate analysis (PCoA) plot. The functional features of each sample were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (version 2.3.0-b). The final *P*-values were corrected for false discovery rate (FDR) by the Bonferroni method with a significance threshold of $P < 0.05$ adopted.

2.8. Fecal metabolite extraction, LC-MS/MS analysis, and data processing

Fecal metabolites were extracted according to Yu et al. (2017) with modifications. One hundred milligram of fecal sample was dissolved in 1 mL of ice-cold water from a Milli-Q water purification

system (Millipore Corp, Bedford, MA, USA). The mixture was vortexed for 60 s and centrifuged at 13,000 × *g* and 4 °C for 15 min. The supernatant was collected and kept on ice, whereas the remaining fecal pellet was further extracted by adding 1 mL of ice-cold LC-MS grade-methanol. The mixture was vortexed and centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatants were combined and subsequently transferred to a fresh Eppendorf tube with a 0.22-mm filter, and then centrifuged at 15,000 × *g* at 4 °C for 10 min. Finally, fecal filtrate was injected into the UHPLC system (1290; Agilent Technologies, USA) coupled to Triple TOF 5600 (Q-TOF, AB Sciex, USA). The liquid chromatography (LC) separation was performed with a UPLC BEH Amide column (100 mm × 2.1 mm × 1.7 μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.40 mL/min. The elution gradient was 5% B for 0 to 2 min, 5% to 95% B for 2 to 12 min, 95% B for 12 to 15 min, and 95% to 5% B for 15 to 17 min. Mass spectrum was operated in either positive or negative ion mode as described by Wang et al. (2019).

Raw data was extracted and preprocessed using Compound Discoverer software (version 2.0; Thermo Fisher Scientific, Waltham, MA, USA). The metabolites in these harvested data were annotated according to the accurate molecular weight (MW) by searching the exact MW against Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolome Database (HMDB). The principal component analysis (PCA) and supervised orthogonal partial least squares-discriminate analysis (OPLS-DA) were conducted in SIMCA-P software (version 11.0; Umetrics AB, Umeå, Sweden). The differentially expressed metabolites (DEM) were evaluated by the combination of the variable importance in the projection (VIP > 1.5) and the corrected *P*-values (< 0.05) via Student's *t*-test. The value of fold-change (FC) was obtained by comparing the peak intensity of each metabolite between two groups. The online platform, MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to conduct the metabolic pathway analysis (MetPA) based on DEM via the library of *Bos Taurus* (cow) of KEGG (Pang et al., 2021).

2.9. Statistical analyses

The analysis of DMI, MY and composition, serum immune indices, fecal VFA, and 8 fecal bacteria species were conducted with SAS (version 9.4, SAS Institute Inc., USA) using the PROC MIXED statement and the following model:

$$Y_{ijkl} = \mu + S_i + P_j + T_k + C(S)_{l(i)} + e_{ijkl},$$

in which, Y_{ijkl} is the dependent variable; μ is the overall mean; S_i is the fixed effect of square ($i = 1$ to 2); P_j is the fixed effect of period ($j = 1$ to 4); T_k is the fixed effect of treatment ($k = 1$ to 4); $C(S)_{l(i)}$ is the random effect of cow nested within square ($l = 1$ to 4), and e_{ijkl} is residual error. The interactions between period and square, period and treatment, and square and treatment were initially included in the model and removed when $P > 0.20$ (de Souza et al., 2020).

The Kenward-Roger procedure was used to determine the degrees of freedom for the *F* tests. The linear and quadratic effects of treatments were assessed by orthogonal polynomial contrasts. The Tukey–Kramer post hoc test adjusting for multiple comparisons was used to identify the differences among the means. All values presented in the manuscript are means ± standard error of the mean. Effects were assumed to be significant at $P < 0.05$, whereas tendencies toward significance were assumed when $0.05 \leq P < 0.10$. Spearman's rank correlation coefficients between fecal differentially abundant bacteria (DAB) and DEM, DAB and fecal VFA, and DAB and serum immune indices were evaluated in R. These correlations were visualized using the R package 'ggplot'.

3. Results

3.1. Feed intake, milk yield and composition

Feeding CFE up to 150 g/d did not affect DMI of dairy cows (Table S4). Dietary supplementation with CFE significantly affected MY ($P = 0.028$), ECM ($P = 0.040$), lactose percentage ($P < 0.001$), yield ($P < 0.001$), and SCC ($P = 0.003$). Dairy cows with CFE100 or CFE150 had greater MY ($P < 0.05$) compared with CON. The ECM was significantly higher ($P < 0.05$) in the cows supplemented with CFE100 in comparison with CON. The milk lactose yield (Lin $P = 0.028$; Quad $P = 0.027$) and percentage (Lin $P < 0.001$; Quad $P < 0.001$) increased linearly and quadratically with increasing levels of CFE supplementation. The SCC linearly decreased ($P = 0.043$) with increasing amounts of CFE. The milk efficiency (expressed as ECM/DMI) tended to quadratically increase ($P = 0.090$) with increasing CFE. The yields or percentages of milk fat and protein, milk efficiency (expressed as MY/DMI, FCM/DMI), and MUN were similar ($P > 0.10$) among treatments.

3.2. Serum immune parameters

Serum IgG ($P = 0.037$), IL-1β ($P = 0.029$), IL-2 ($P = 0.034$), IL-6 ($P = 0.011$), and LPS ($P = 0.034$) concentrations were significantly affected by supplementary CFE (Table 1).

Dietary supplementation with CFE quadratically affected ($P = 0.024$) serum IgG concentration, and the greatest value of IgG was found at CFE50. Adding CFE tended to affect ($P = 0.082$) IgA concentration, whereas the dosage effect of CFE was not observed. Increasing CFE inclusion linearly reduced the concentrations of IL-1β ($P = 0.020$), IL-2 ($P = 0.018$), IL-6 ($P = 0.019$), and TNF-α ($P = 0.047$) in serum. Serum LPS was lower ($P < 0.05$) in the cows with CFE50 or CFE150 compared with CON. Serum LBP displayed a quadratic effect ($P = 0.017$), with the lowest value at CFE150. Serum ADPN increased linearly ($P = 0.043$) with increasing CFE levels. Serum concentrations of IgM, IL-4, IFN-γ, sCD14, and FGF21 were unaffected ($P > 0.10$) by CFE addition.

3.3. Fecal volatile fatty acids

Feeding CFE to dairy cows significantly affected fecal butyrate concentration ($P = 0.010$), and tended to change the concentrations of total volatile fatty acids (TVFA; $P = 0.058$) and acetate ($P = 0.062$; Table 2). The production of propionate, isobutyrate, valerate, and isovalerate were not different ($P > 0.10$) across treatments. The concentrations of TVFA ($P = 0.014$), acetate ($P = 0.014$), and butyrate ($P = 0.002$) in feces increased linearly with increasing CFE addition. Additionally, the proportion of butyrate linearly increased ($P = 0.030$) with increasing supplementation of CFE.

3.4. Fecal microbial flora

Dietary supplementation with CFE significantly influenced the relative abundances of *C. coccoides-E. rectale* group ($P < 0.001$) and *F. prausnitzii* ($P = 0.003$) in feces (Table 3). No differences ($P > 0.10$) were noted in the relative abundances of *Bacteroides-Prevotella-Porphyromonas* group, *Clostridium* cluster I, and *Lactobacillus* spp. among treatments. The relative abundances of *Bifidobacterium* spp. ($P = 0.048$), *Clostridium* cluster XIVab ($P = 0.041$), and *F. prausnitzii* ($P = 0.002$) linearly increased with increasing CEF addition amounts; but *Clostridium* cluster XIVab linearly decreased ($P = 0.041$) as CFE increased. Feeding CFE quadratically affected ($P = 0.002$) *C. coccoides-E. rectale* group, and the greatest value was observed at CFE50. In addition, supplementary CFE tended to linearly reduce ($P = 0.063$) the relative abundance of *E. coli*.

Table 1
Effects of feeding citrus flavonoid extracts (CFE) on serum immune indices in dairy cows.

Item	CFE supplementation, g/d				SEM	*P*-value[1]		
	0	50	100	150		T	L	Q
IgA, μg/mL	180.72	189.57	183.43	194.01	3.747	0.082	0.107	0.851
IgG, μg/mL	1,736.88^{b}	1,869.24^{a}	1,771.05^{ab}	1,699.46^{b}	39.997	0.037	0.281	0.024
IgM, μg/mL	109.33	111.02	114.03	109.08	3.135	0.653	0.887	0.347
IL-1β, ng/L	55.39^{a}	55.11^{ab}	51.71^{bc}	51.24^{c}	1.165	0.029	0.020	0.944
IL-2, ng/L	345.33^{a}	332.50^{ab}	319.46^{b}	320.00^{b}	6.758	0.034	0.018	0.407
IL-4, ng/L	83.40	84.68	85.45	83.20	2.814	0.916	0.986	0.502
IL-6, ng/L	19.58^{a}	17.71^{b}	18.42^{ab}	17.84^{b}	0.417	0.011	0.019	0.124
TNF-α, ng/L	256.86	245.32	240.53	238.53	5.404	0.106	0.047	0.464
IFN-γ, ng/L	941.63	886.11	911.94	900.00	20.676	0.263	0.324	0.332
LPS, ng/L	711.05^{a}	658.72^{b}	681.60^{ab}	672.0^{b}	12.632	0.034	0.103	0.098
LBP, ng/mL	14.39	15.35	14.90	13.97	0.406	0.074	0.320	0.017
sCD14, μg/L	44.52	43.46	43.32	42.08	0.950	0.305	0.221	0.206
FGF21, ng/L	924.09	900.86	895.74	864.82	26.192	0.403	0.103	0.876
ADPN, μg/L	168.43	174.89	177.95	177.38	3.038	0.109	0.043	0.276

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; IL = interleukin; TNF-α = tumor necrosis factor-α; IFN-γ = interferon-γ; LPS = lipopolysaccharide; LBP = lipopolysaccharide binding protein; sCD14 = soluble lipopolysaccharide receptor CD14; FGF21 = fibroblast growth factor 21; ADPN = adiponectin.
[a,b] Means within a row with different superscripts differ ($P < 0.05$).
[1] T = treatment; L = linear; Q = quadratic.

Table 2
Effects of feeding citrus flavonoid extracts (CFE) on fecal volatile fatty acids in dairy cows.

Item	CFE supplementation, g/d				SEM	*P*-value[1]		
	0	50	100	150		T	L	Q
TVFA, μmol/g	39.72	40.04	44.93	46.03	1.964	0.058	0.014	0.849
Acetate, μmol/g	28.46	28.72	32.17	32.86	1.416	0.062	0.014	0.884
Propionate, μmol/g	5.76	5.74	6.36	6.49	0.340	0.276	0.131	0.848
Butyrate, μmol/g	3.63^{c}	3.68^{bc}	4.48^{ab}	4.64^{a}	0.275	0.010	0.002	0.824
Isobutyrate, μmol/g	0.84	0.94	0.89	0.94	0.056	0.514	0.440	0.665
Valerate, μmol/g	0.62	0.62	0.66	0.65	0.044	0.857	0.605	0.996
Isovalerate, μmol/g	0.41	0.35	0.37	0.46	0.052	0.439	0.553	0.169
Percentage of TVFA, %								
Acetate	71.73	71.88	71.59	71.40	0.641	0.956	0.660	0.790
Propionate	14.45	14.24	14.15	14.01	0.372	0.863	0.509	0.946
Butyrate	9.15	9.09	9.94	10.13	0.392	0.124	0.030	0.746
Isobutyrate	2.11	2.36	2.01	2.05	0.122	0.137	0.322	0.381
Valerate	1.54	1.55	1.47	1.42	0.105	0.781	0.434	0.842
Isovalerate	1.01	0.87	0.83	0.99	0.111	0.569	0.819	0.179

TVFA = total volatile fatty acids.
[a-c] Means within a row with different superscripts differ ($P < 0.05$).
[1] T = treatment; L = linear; Q = quadratic.

3.5. Diversity and divergence of fecal bacterial community

The amplicon sequencing of 16 fecal samples generated a total of 663,411 high-quality reads, an average of 41,463 sequences per sample, and the average sequencing read length was 412 bp. High-quality reads were clustered into 1,247 microbial OTU, among which 1,116 OTU were found in all groups and accounted for 89.5% of the total OTU, indicating the presence of an extensive common microbiome (Fig. S1A). The rarefaction curves indicated the sequencing depth was sufficient to describe the microbial composition of each treatment (Fig. S1B). No difference was found in the α-diversity indices, including ACE, Chao, Shannon, and Simpson among treatments (Fig. S2). The PCoA analysis based on unweighted and weighted UniFrac metrics showed that

Table 3
Effects of feeding citrus flavonoid extracts (CFE) on the relative abundances (% of total bacteria) of several specific fecal microbiota in dairy cows.

Item	CFE supplementation, g/d				SEM	*P*-value[1]		
	0	50	100	150		T	L	Q
Bacteroides-Prevotella-Porphyromonas group	17.92	15.02	15.20	16.73	1.169	0.261	0.535	0.077
Bifidobacterium spp., $\times 10^{-2}$	14.31	21.83	19.80	22.41	2.430	0.062	0.048	0.331
Clostridium coccoides-Eubacterium rectale group	3.85^{c}	8.84^{a}	6.62^{b}	7.10^{ab}	0.636	<0.001	0.015	0.002
Clostridium cluster I, $\times 10^{-2}$	16.53	11.96	16.45	17.11	1.647	0.131	0.569	0.299
Clostridium cluster XIVab	4.45	4.09	3.39	2.97	0.492	0.154	0.041	0.967
Escherichia coli, $\times 10^{-2}$	2.91	2.80	1.99	2.06	0.361	0.169	0.063	0.815
Faecalibacterium prausnitzii	1.03^{b}	1.74^{a}	1.72^{a}	1.81^{a}	0.216	0.003	0.002	0.400
Lactobacillus spp., $\times 10^{-2}$	0.49	0.42	0.51	0.40	0.077	0.663	0.664	0.846

[a-c] Means within a row with different superscripts differ ($P < 0.05$).
[1] T = treatment; L = linear; Q = quadratic.

feeding CFE did not modify ($P > 0.05$) fecal microbial structure (Fig. 1).

Phylogenetic analysis of the sequences of fecal bacteria identified 15 phyla. Firmicutes and Bacteroidota were the most abundant phyla, which accounted for an average of 93.4% of the community (Fig. S3A). The relative abundance of phylum Bacteroidota was greater ($P = 0.024$) in the feces of cows with CFE150 compared with that of CON (Fig. 2A).

The top 25 bacterial genera of the fecal microbiome among treatments are shown in Fig. S3B, among which 23 genera (with relative abundance > 1%) were considered abundant core genera. Genus UCG-005 (average 15.07%) was the most abundant taxa from the phylum Firmicutes, followed by *unclassified_f__Lachnospiraceae* (average 4.18%), and *Christensenellaceae_R-7_*group (average 3.63%); whereas *Rikenellaceae_RC9_gut_*group (average 18.14%) was the most abundant genus from the phylum Bacteroidota, followed by *Prevotellaceae_UCG-003* (average 4.53%), *norank_f__Muribaculaceae* (average 4.19%), and *Bacteroides* (average 3.69%). Feeding CFE at 150 g/d to dairy cows increased the relative abundances of genera *Bacteroides* ($P = 0.014$), *Phascolarctobacterium* ($P = 0.031$), *norank_f__norank_o__Bacteroidales* ($P = 0.034$), *dgA-11_gut_*group ($P = 0.040$), *norank_f__Paludibacteraceae* ($P = 0.016$), *Anaerovorax* ($P = 0.046$), and *Hydrogenoanaerobacterium* ($P = 0.047$) in feces in comparison with CON (Fig. 2B). However, the relative abundances of fecal bacterial genera of *Ruminococcus_torques_*group ($P = 0.024$), *Roseburia* ($P = 0.027$), *Lachnospira* ($P = 0.026$), *norank_f__norank_o__Coriobacteriales* ($P = 0.004$), and *norank_f__Prevotellaceae* ($P = 0.032$) were lower in cows receiving the CFE150 compared with CON.

Linear discriminant analysis (LDA) with effect size (LEfSe) was used to identify significantly differential bacterial taxa between CON and CFE150. The LDA histogram (Fig. S4) manifested that there were 21 biomarkers with LDA scores > 3, including 11 biomarkers in the CON group and 10 biomarkers in CFE150, respectively.

Several correlations between the concentrations of fecal VFA and serum immune indices and the abundances of DAB are shown in Fig. 3. The concentrations of fecal TVFA and acetate were both positively correlated with *Bacteroides* and *Ruminococcus_torques_*group. The concentration of fecal butyrate was positively correlated with *Bifidobacterium* spp., *C. coccoides-E. rectale* group, and *F. prausnitzii*, and negatively correlated with *Clostridium* cluster XIVab. Serum IL-1β was negatively correlated with *Bifidobacterium* spp. Serum IL-6 was negatively correlated with *Bifidobacterium* spp. and *C. coccoides-E. rectale* group, and positively correlated with *Clostridium* cluster XIVab. Serum TNF-α was positively correlated with *Clostridium* cluster XIVab. Serum ADPN was positively correlated with *Bifidobacterium* spp.

The predicted "metabolism" functions at KEGG level 2 of the fecal bacterial microbiota are shown in Fig. S5A. Compared with CON, the relative abundances of 'glycan biosynthesis and metabolism and 'energy metabolism' were greater in CFE150. However, the relative abundance of 'lipid metabolism' was less in CFE150 compared with CON. At KEGG level 3, the categories of 'metabolism' were further predicted. Compared with CON, the functions involved with 'lipoic acid metabolism', 'one carbon pool by folate', 'ubiquinone and other terpenoid-quinone biosynthesis', 'citrate cycle', 'glycosaminoglycan degradation', 'histidine metabolism', 'phenylalanine metabolism', 'phosphonate and phosphinate metabolism', 'glyoxylate and dicarboxylate metabolism', 'other glycan degradation', and 'oxidative phosphorylation' were more prevalent in CFE150. Compared with CON, 'lysine biosynthesis', 'pentose phosphate pathway', 'sphingolipid metabolism', 'lipopolysaccharide biosynthesis', 'starch and sucrose metabolism', 'limonene and pinene degradation', 'secondary bile acid biosynthesis', and 'glycerolipid metabolism' were less prevalent in CFE150.

3.6. Fecal metabolites

A total of 685 valid peaks were detected in the fecal samples, and 548 compounds were obtained against KEGG and HMDB. According to the unsupervised PCA plot, no noticeable separation was found between CON and CFE150 (Fig. 4A and B). The OPLS-DA results in Fig. 4D and E showed distinct separations for fecal metabolites between the CON and CFE150. In each plot of the OPLS-DA, all samples were within the 95% Hotelling's T^2 ellipse. The parameters of the permutation test showed a satisfactory fitness and prediction effectiveness ($R^2 > 0.80$) of the model, which can be used to identify DEM.

As shown in Table 4, 44 DEM between CON and CFE150 were identified ($P < 0.05$; fold change [FC] > 1.20 or < 0.83, and VIP > 1.5). Among these metabolites, 18 were classified as lipids and lipid-like molecules, 8 as organoheterocyclic compounds, and 5 as benzenoids; others were classified as organic acids and derivatives, phenylpropanoids and polyketides, organic nitrogen compounds, nucleosides, organic oxygen compounds, benzenoids, nucleosides, nucleotides, and analogues, benzoic acids and derivatives, and carboxylic acids and derivatives. Compared with CON, dietary addition with CFE at 150 g/d increased ($P < 0.05$) the fecal

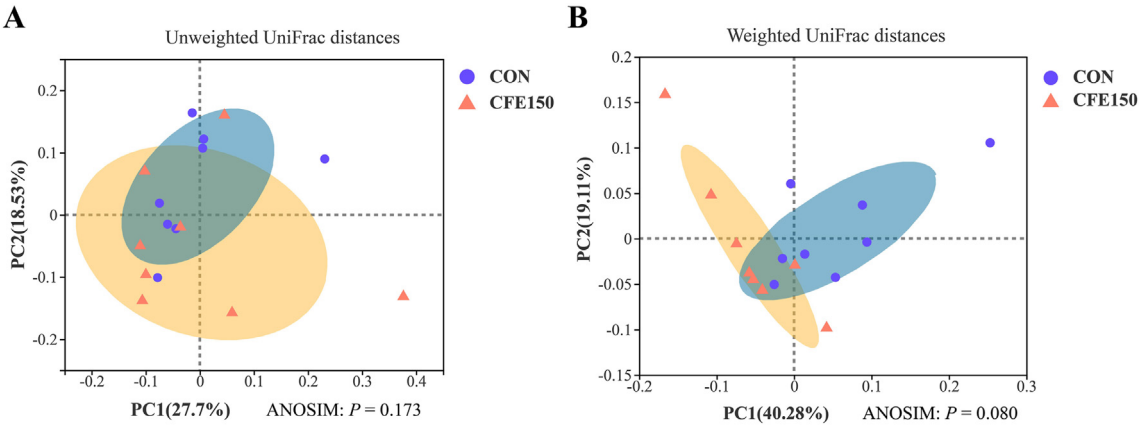

Fig. 1. Similarity of the fecal bacterial structure between dairy cows fed citrus flavonoid extracts at 0 (CON) and 150 g/d (CFE150). (A) PCoA plot based on unweighted UniFrac. (B) PCoA plot based on weighted UniFrac distance. The effect of CFE supplementation on the clustering pattern of fecal microbiota was evaluated via ANOSIM. Significance was declared with $P < 0.05$.

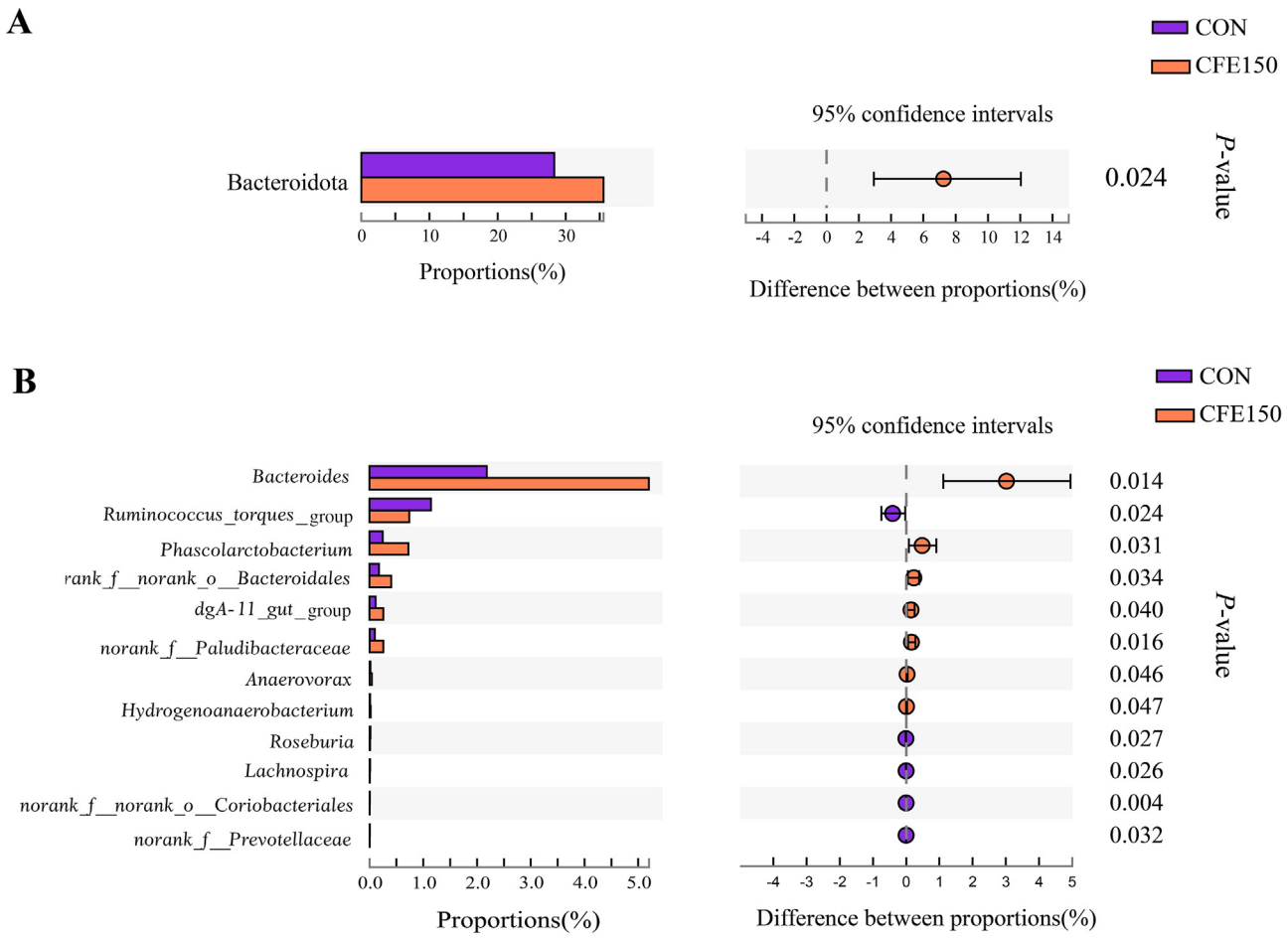

Fig. 2. The fecal bacteria taxa that had significant differences between dairy cows fed citrus flavonoid extracts at 0 (CON) and 150 g/d (CFE150). (A) Differential taxa at phylum level. (B) Differential taxa at genus level. Positive differences denote greater relative abundances of fecal bacteria at the phylum level and at the genus level in CFE150, while negative differences denote fewer relative abundances in CFE150.

concentrations of retinyl beta-glucuronide, 3,7,8,15-scirpenetetrol, pulegone, goshonoside F1, cinncassiol C3, 3-hydroxy-6,8-dimethoxy-7(11)-eremophilen-12,8-olide, taurocholic acid, tafluprost free acid, trigoforin, naringenin, hesperetin, hippuric acid, mandelic acid, homovanillin, pyrogallic acid, glucuronide, penmacric acid, taurine, β-lapachone, riboflavin, guanosine, cytidylic acid, and sphingosine. Compared with CON, dietary addition with CFE at 150 g/d reduced ($P < 0.05$) the fecal concentrations of 3-hydroxy-10′-apo-b,y-carotenal, cerebroside B, GlcCer(d18:1/20:0), diosbulbin H, DG(22:2(13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0), digalactosylceramide, Cer(d18:0/24:0), Cer(d18:0/22:0), ansamitocin P3, deoxycholic acid, methylimidazoleacetic acid, 7-hydroxymethotrexate, uric acid, hypoxanthine, xanthine, 2,8-dihydroxyadenine, ganosporelactone B, N-ribosylhistidine, homogentisic acid, cysteinylglycine, and sphinganine.

Fig. 5 shows the metabolic pathways enriched with 44 DEM that were identified between CON and CFE150. Table S5 depicts the *P*-values and the impact values of pathway analysis. The pathways of 'sphingolipid metabolism' ($P < 0.05$; pathway impact = 0.28), 'taurine and hypotaurine metabolism' ($P < 0.05$; pathway impact = 0.43), and 'purine metabolism' ($P < 0.05$; pathway impact = 0.07) were significantly enriched. Other metabolic pathways ($P > 0.05$) could be neglected.

The Spearman correlation matrix between DAB and DEM is presented in Fig. 6. A portion of up-regulated DEM were positively correlated with *Bifidobacterium* spp., *C. coccoides-E. rectale* group, *F. prausnitzii*, *Bacteroides*, and *Ruminococcus_torques*_group. A portion of down-regulated DEM were positively correlated with *Phascolarctobacterium*, and negatively correlated with *C. coccoides-E. rectale* group and *Bacteroides*.

4. Discussion

Citrus extract has emerged as a promising phytogenic feed additive for improving performance and health in ruminants (Balcells et al., 2012; Paniagua et al., 2021). Naringin, hesperidin, neohesperidin, and nobiletin are usually predominant active flavonoids present in citrus fruits or by-products (Li et al., 2021). In the present study, naringin and hesperidin were the most abundant flavonoids in CFE. Generally, naringin and hesperidin require hydrolysis to their active aglycone forms naringenin and hesperetin to exert various health-promoting activities (Altunayar-Unsalan et al., 2022).

Based on the serum untargeted metabolomic profiling, compared to CON, dairy cows in CFE150 had greater contents of naringenin, hesperetin, tangeretin-4′-glucuronide, and 3′-demethyl-nobiletin in serum (data not shown). These results suggested that more active compounds derived from citrus flavonoids metabolism were available for dairy cows in CFE150.

Although DMI did not differ among treatments, supplementing CFE up to 150 g/d increased MY and milk lactose of dairy cows in comparison with CON. Contrary to what was observed in our

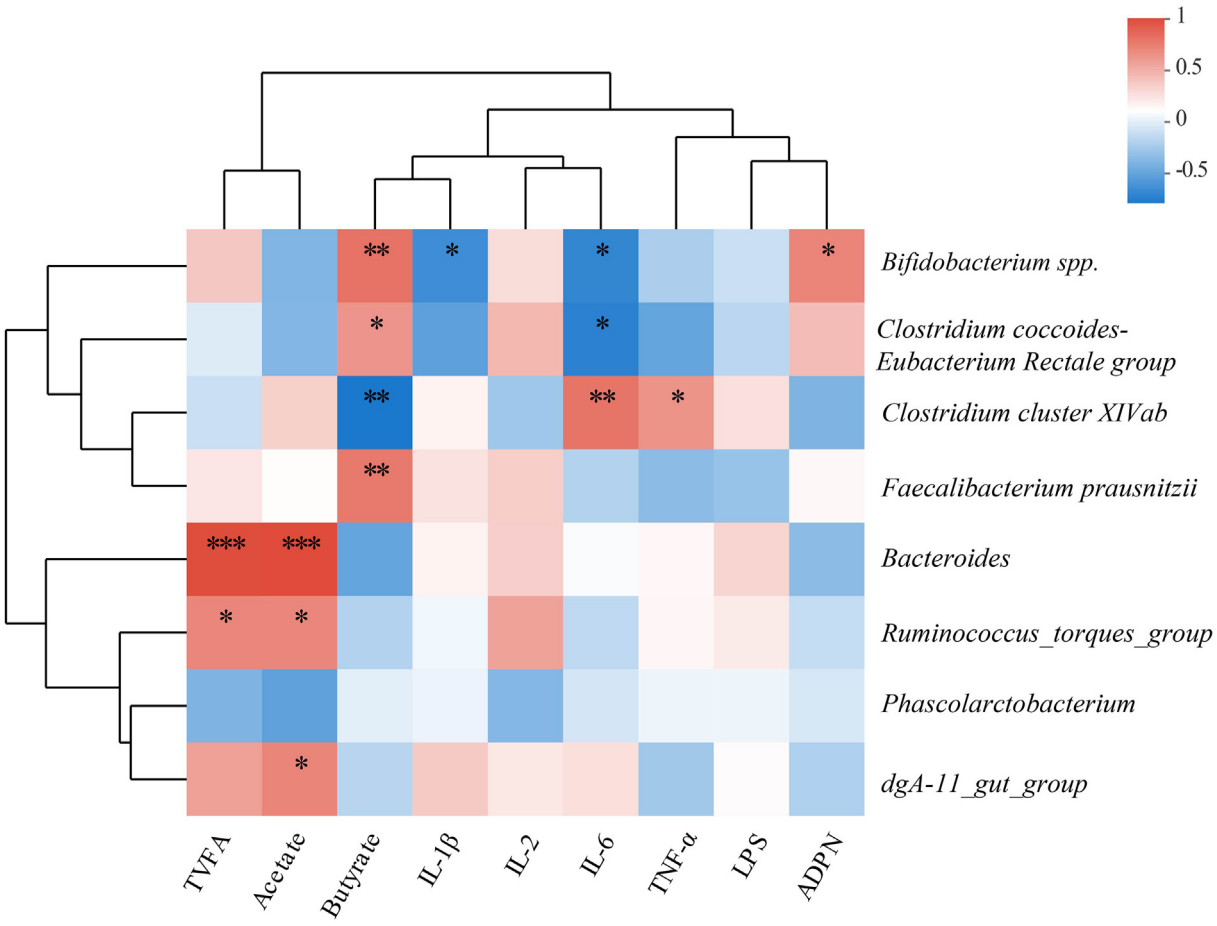

Fig. 3. The Spearman correlation matrix between fecal differentially abundant bacteria and fecal volatile fatty acids and serum indices. Asterisks denote significant difference between fed the control diet (CON) and citrus flavonoid extracts at 150 g/d (CFE150) (*0.01 < $P \leq 0.05$; **0.001< $P \leq 0.01$; ***$P \leq 0.001$). TVFA = total volatile fatty acids; LPS = lipopolysaccharide; ADPN = adiponectin.

experiment, Ying et al. (2017), in the assessment of feed intake or milk performance by citrus extract supplementation, did not observe any effects on DMI, MY or milk composition. The supplementation level of citrus extract (4 g/d) in Ying et al. (2017) was far lower than that of our study; therefore, differences in supplementary amount might account for this disparity in milk performance. In the present study, the reduced milk SCC accompanied by the increased milk yield may be due to the anti-inflammatory effect of flavonoid compounds. Similarly, several studies indicated reduced milk SCC of dairy cows received diets supplemented with flavonoid-rich extracts from bamboo leaf (Zhan et al., 2021), baicalin (Burmanczuk et al., 2021), or quercetin (Burmanczuk et al., 2018) compared with a control. Furthermore, a newly published research study found that the administration of hesperidin and naringenin via the intramammary route reduced the milk SCC of mastitis-affected cows (Burmanczuk et al., 2022). These results suggest that citrus flavonoids have the potential to improve mammary health status of dairy cows.

Biomarkers of inflammation status can be used to provide direct information about homeostasis in dairy cows (Shangraw and McFadden, 2022). In our study, increased IgG concentration in serum was observed after CFE supplementation with 50 g/d, indicating the immune response of dairy cows was enhanced by CFE. An endotoxin-associated immune response can be activated by LPS binding to specific cell surface receptors. Lipopolysaccharide interacts with toll-like receptor 4 to stimulate the release of proinflammatory factors such as IL-1β, IL-2, and IL-6 (Sordillo and Mavangira, 2014). In our experiment, dietary supplementation with CFE reduced serum concentrations of LPS, LBP, IL-1β, IL-2, IL-6, and TNF-α, indicating citrus flavonoids could decrease the levels of endotoxin and systemic inflammation in dairy cows. In the study of Paniagua et al. (2019), CFE supplementation down-regulated the gene expression related to inflammation, such as *IL-25*, *TLR4*, and β-defensin1 in the rumen epithelium of Holstein bulls, supporting the modulatory effects of CFE on immune action in ruminants. Additionally, CFE addition linearly increased serum concentration of ADPN. Adiponectin, an adipokine secreted by adipocytes, plays an important role in energy homeostasis regulation, lipid and glucose metabolism, and insulin sensitivity (Li et al., 2019). Thus, these results indicated that CFE modulated lipid metabolism in cows, suggesting the potential effects of reducing liver lipid accumulation during periods of negative energy balance, and the regulatory mechanisms at the molecular level warrants further research.

We speculated that the gastrointestinal microbiota play crucial roles in the metabolism of citrus flavonoids and immune regulation in dairy cows. Our results showed that supplementing CFE tended to linearly increase microbial crude protein and propionate concentrations, but TVFA and ruminal microbial structure and composition were not significantly altered by CFE (data not shown). However, few studies have uncovered the effects of citrus flavonoid intake on hindgut fermentation, microbiome and metabolites in dairy cows. Volatile fatty acids are the end products derived from

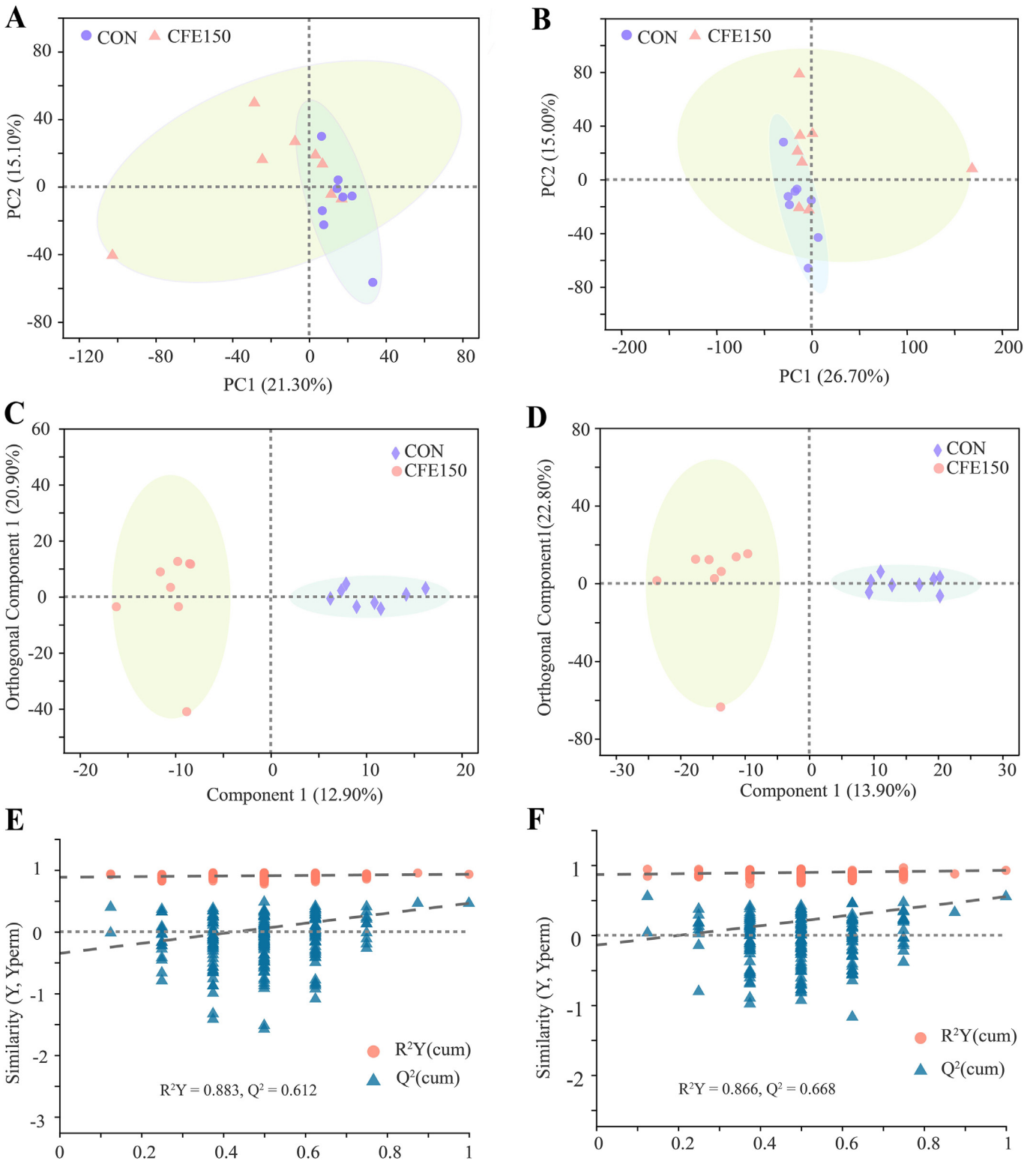

Fig. 4. Analysis of the fecal microbiota in dairy cows fed citrus flavonoid extracts at 0 (CON) and 150 g/d (CFE150). The PCA (A) and OPLS-DA (C), and OPLS-DA permutation test (E) in the positive model, and plot of PCA (B) and OPLS-DA (D) and OPLS-DA permutation test (F) in the negative model, for the fecal metabolites. R^2Y, goodness-of-fit parameter; Q^2, predictive ability parameter.

Table 4
Identification of significant differentially expressed metabolites in feces of dairy cows by comparison of control diet (CON) and citrus flavonoid extracts (CFE150) with variable importance in the projection (VIP) > 1.5 and $P < 0.05$.

Chemical taxonomy		Compounds	VIP[1]	log2FC[2]
Super class	Sub class			
Increased in CFE150				
Lipids and lipid-like molecules	Terpene glycosides	Retinyl beta-glucuronide	3.41	0.69
Lipids and lipid-like molecules	Sesquiterpenoids	3,7,8,15-Scirpenetetrol	4.91	0.65
Lipids and lipid-like molecules	Monoterpenoids	Pulegone	3.50	0.46
Lipids and lipid-like molecules	Terpene glycosides	Goshonoside F1	3.28	0.44
Lipids and lipid-like molecules	Sesquiterpenoids	Cinncassiol C3	3.20	0.37
Lipids and lipid-like molecules	Terpene lactones	3-Hydroxy-6,8-dimethoxy-7(11)-eremophilen-12,8-olide	2.95	0.33
Lipids and lipid-like molecules	Bile acids, alcohols and derivatives	Taurocholic acid	1.85	0.17
Lipids and lipid-like molecules	Eicosanoids	Tafluprost free acid	1.77	0.14
Phenylpropanoids and polyketides	Not available	Trigoforin	5.78	1.90
Phenylpropanoids and polyketides	Flavans	Naringenin	6.14	1.45
Phenylpropanoids and polyketides	O-methylated flavonoids	Hesperetin	4.90	0.75
Benzenoids	Benzoic acids and derivatives	Hippuric acid	6.04	1.39
Benzenoids	Not Available	Mandelic acid	4.33	0.64
Benzenoids	Methoxyphenols	Homovanillin	3.50	0.44
Benzenoids	Phenols and derivatives	Pyrogallic acid	2.40	0.26
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Glucuronide	4.17	0.59
Organic acids and derivatives	Amino acids, peptides, and analogues	Penmacric acid	2.68	0.30
Organic acids and derivatives	Organosulfonic acids and derivatives	Taurine	2.52	0.21
Organoheterocyclic compounds	Naphthopyranones	β-Lapachone	2.08	0.18
Organoheterocyclic compounds	Alloxazines and isoalloxazines	Riboflavin	1.75	0.18
Nucleosides, nucleotides, and analogues	Not Available	Guanosine	2.10	0.15
Nucleosides, nucleotides, and analogues	Pyrimidine ribonucleotides	Cytidylic acid	1.87	0.15
Organic nitrogen compounds	Amines	Sphingosine	1.60	0.28
Decreased in CFE150				
Lipids and lipid-like molecules	Sesterterpenoids	3-Hydroxy-10′-apo-b,y-carotenal	1.81	−0.38
Lipids and lipid-like molecules	Glycosphingolipids	Cerebroside B	1.69	−0.79
Lipids and lipid-like molecules	Glycosphingolipids	GlcCer(d18:1/20:0)	2.17	−0.56
Lipids and lipid-like molecules	Terpene lactones	Diosbulbin H	1.54	−0.29
Lipids and lipid-like molecules	Diradylglycerols	DG(22:2(13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	1.58	−0.40
Lipids and lipid-like molecules	Ceramides	Digalactosylceramide	1.98	−0.53
Lipids and lipid-like molecules	Ceramides	Cer(d18:0/24:0)	1.71	−0.84
Lipids and lipid-like molecules	Ceramides	Cer(d18:0/22:0)	2.04	−0.48
Lipids and lipid-like molecules	Prenol lipids	Ansamitocin P3	2.02	−0.57
Lipids and lipid-like molecules	Bile acids, alcohols and derivatives	Deoxycholic acid	2.22	−0.45
Organoheterocyclic compounds	Imidazoles	Methylimidazoleacetic acid	1.82	−0.87
Organoheterocyclic compounds	Pterins and derivatives	7-Hydroxymethotrexate	1.64	−0.39
Organoheterocyclic compounds	Purines and purine derivatives	Uric acid	1.85	−0.70
Organoheterocyclic compounds	Purines and purine derivatives	Hypoxanthine	1.96	−0.61
Organoheterocyclic compounds	Purines and purine derivatives	Xanthine	1.75	−0.41
Organoheterocyclic compounds	Purines and purine derivatives	2,8-Dihydroxyadenine	1.80	−0.33
Benzoic acids and derivatives	Steroid lactones	Ganosporelactone B	1.62	−0.58
Carboxylic acids and derivatives	Amino acids, peptides, and analogues	N-Ribosylhistidine	1.73	−0.42
Benzenoids	Phenylacetic acids	Homogentisic acid	2.20	−0.88
Organic acids and derivatives	Amino acids, peptides, and analogue	Cysteinylglycine	2.09	−0.47
Organic nitrogen compounds	Amines	Sphinganine	2.52	−0.66

[1] VIP = variable importance in the projection.
[2] log2FC = $\log_2$(fold change).

microbial fermentation of dietary carbohydrates in the gastrointestinal tract, and they are associated with conferring a beneficial influence on the host metabolic homeostasis (Gibson and Roberfroid, 1995). Butyrate, as one of the primary energy sources for the gastrointestinal tract, is expected to inhibit systemic inflammation in the gastrointestinal tract (Hamer et al., 2008). Thus, increased TVFA and butyrate production in the current experiment was beneficial for maintaining energy homeostasis and controlling systemic inflammation.

It is widely believed that decreasing hindgut pH with greater VFA concentrations could suppress the growth of pathogenic bacteria, consequently promoting the growth of beneficial microbiota (Brownawell et al., 2012). In this study, feeding CFE to dairy cows increased the proportion of *Bifidobacterium* spp. and *Faecalibaterium prausnitzii*, which are regarded as beneficial bacteria (Unno et al., 2015). Similar responses to increasing the proportions of *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* were also observed when CFE was offered to rats (Unno et al., 2015) or humans (Lima et al., 2019). *Bifidobacterium* spp. are acetate producers (Mandalari et al., 2010); thus, an increase in fecal acetate was expected with a linear increase in the relative abundance of *Bifidobacterium* spp. induced by CFE supplementation. The *Bifidobacterium* spp. was negatively correlated with serum IL-1β and IL-6, indicating CFE may serve to modify the growth of beneficial bacteria via increasing VFA production to improve the inflammatory status of dairy cows. In addition, it has been documented that *Bifidobacterium* spp. could hydrolyze certain rutinose-conjugate flavonoids (e.g., hesperidin), thus releasing their aglycone form

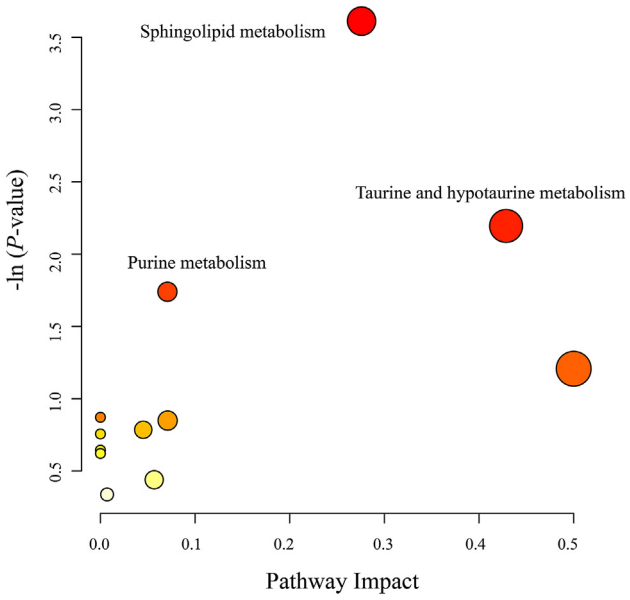

Fig. 5. The pathway analysis of fecal differentially expressed metabolites based on the comparison of dairy cows fed citrus flavonoid extracts at 0 (CON) versus 150 g/d (CFE150). The x axis denotes the impact values in of pathways the topology analysis, and larger bubbles indicate higher impact values. The y axis denotes the *P*-value (-ln*P*) of the metabolic pathway in the enrichment analysis, and darker bubbles indicate higher levels of pathway enrichment.

(Amaretti et al., 2015). Due to this, the increase in the abundance of fecal *Bifidobacterium* spp. may have potentially enhanced the bioavailability of citrus flavonoid compounds in the current study.

Faecalibacterium prausnitzii and *C. coccoides-E. rectale* group have been recognized as producers of butyrate, which provides energy for the intestinal epithelium (Barroso et al., 2014; Jaimes et al., 2019). The increase in fecal butyrate may be ascribed to the increased growth of *F. prausnitzii* and *C. coccoides-E. rectale* group. This is further supported by the positive correlation between fecal butyrate and *F. prausnitzii* or *C. coccoides-E. rectale* group. In the present study, the tendency of linear decrease in the relative abundance of harmful bacterium *E. coli* was due to the inhibition effect of CFE. The effect on *E. coli* was consistent with the decrease in serum content of LPS, as *E. coli* was the main LPS producer (Somerville et al., 1996).

In the present study, more comprehensive information regarding fecal bacteria community were investigated by 16S rRNA sequencing. The Alpha or Beta diversity was not affected by CFE supplementation, indicating that the hindgut microbial system is predominated by a core community whose structure remains stable regardless of CFE dosage. The relative abundances of phylum Bacteroidota and genus *Bacteroides* were greater in CFE150 compared with CON. Phylum Bacteroidetes includes genera known to ferment carbohydrates for the production of VFA, mainly acetate and propionate (Boger et al., 2019). *Bacteroides* was one of the most frequently reported and modified bacterial genus when animals received dietary flavonoid or polyphenol treatment according to a systemic review (Moorthy et al., 2021). Many *Bacteroides* strains are regarded as next-generation probiotics (Dahiya et al., 2019), such as *Bacteroides uniformis*, *Bacteroides acidifaciens*, and *Bacteroides dorei*. In the present study, it can therefore be inferred that the increase in *Bacteroides* proportion was related to the health benefits of CFE.

It was reported that the bacteria belonging to genus *Roseburia*, as butyrate producers, play an important role in controlling intestinal inflammation (Karlsson et al., 2013). However, cows fed CFE150 exhibited a lower abundance of genus *Roseburia* in feces compared with CON. This finding is inconsistent with the promoting influence of CFE on butyrate that we noted, and the reasons for this result are unclear. *Ruminococcus_torques*_group, as the dominant flora, could resolve intestinal mucin damage to the mucosal barrier (Yang et al., 2021). The decrease of *Ruminococcus_torques*_group in CFE150 indicated the pathogenicity factors of pathogens were weakened by CFE. Unlike *Bacteroides*, the bacteria of *Phascolarctobacterium* barely ferment carbohydrates, but use succinate as a substrate to produce propionate (Belda et al., 2021), thus, coexistence with succinic acid-producing bacteria such as *Bacteroides* is essential for the genus *Phascolarctobacterium*. Therefore, the increased fecal *Phascolarctobacterium* abundance of cows fed CFE150 might have resulted from the greater proportion of *Bacteroides*. The metabolic functions of fecal microbiota were predicted using the KEGG database. Compared with CON, some key metabolic pathways, such as 'glycan biosynthesis and metabolism', 'energy metabolism', and 'lipid metabolism' were altered in CFE150, which indicated that CFE could improve the metabolic pattern of hindgut microbiota, thus exerting a health-promoting effect in dairy cows.

Metabolomic data showed that some metabolites belonging to the superclass of benzenoids were increased in CFE150. Naringenin can be catabolized into phenolic products such as 3-(4′-hydroxyphenyl) propionic acid (HPPA), 4′-hydroxybenzoic acid, and hippuric acid by gut bacteria (Mu et al., 2020). Therefore, the increased hippuric acid in CFE150 indicated the interaction between CFE metabolism and gut microbiota may be important to increase citrus flavonoid bioavailability in dairy cows. Meanwhile, we observed that CFE could regulate bile acid metabolism by up-regulating the level of taurocholic acid and down-regulating deoxycholic acid. Cholic acid is converted to deoxycholic acid by gut bacteria, and deoxycholic acid has a high toxicity (Rodríguez-Morató et al., 2018). Therefore, the decreased deoxycholic acid suggested a protective effect of CFE on gastrointestinal health by modulating the levels of secondary bile acids.

In the present study, the pathway of 'sphingolipid metabolism' was significantly enriched based on the DEM between CON vs. CFE150. Sphingolipids, as structural membrane constituents and essential eukaryotic signaling molecules, play an important role in modulating immunity and inflammation status (Brown et al., 2019). On the one hand, inflammatory modulators (LPS or TNF-α) can stimulate de novo ceramide synthesis and contribute to the conversion of sphingomyelin back into ceramides via the various sphingomyelinases (Chaurasia and Summers, 2021). Therefore, in the present study, the decreased ceramide levels in CFE150 could be attributed to the reduction of serum LPS and TNF-α. On the other hand, the decrease in GlcCer(d18:1/20:0), Cer(d18:0/24:0), and Cer(d18:0/22:0) observed in CFE150 can be related to the increase in serum ADPN. It has been demonstrated that ADPN, via ADPN receptors, exerts or enhances ceramidase activity, converting deacylated ceramide to sphingosine and sphingosine-1-phosphate (Vasiliauskaite-Brooks et al., 2017). In this study, sphingosine was increased by CFE150, suggesting that ceramidase activity may be upregulated to promote ceramide degradation. The suppression of ceramide production can also reduce endoplasmic reticulum stress, consequently resulting in inhibition of inflammation (Chaurasia and Summers, 2021). Recently, Brown et al. (2019) found that the lack of *Bacteroides*-derived sphingolipids contributed to intestinal

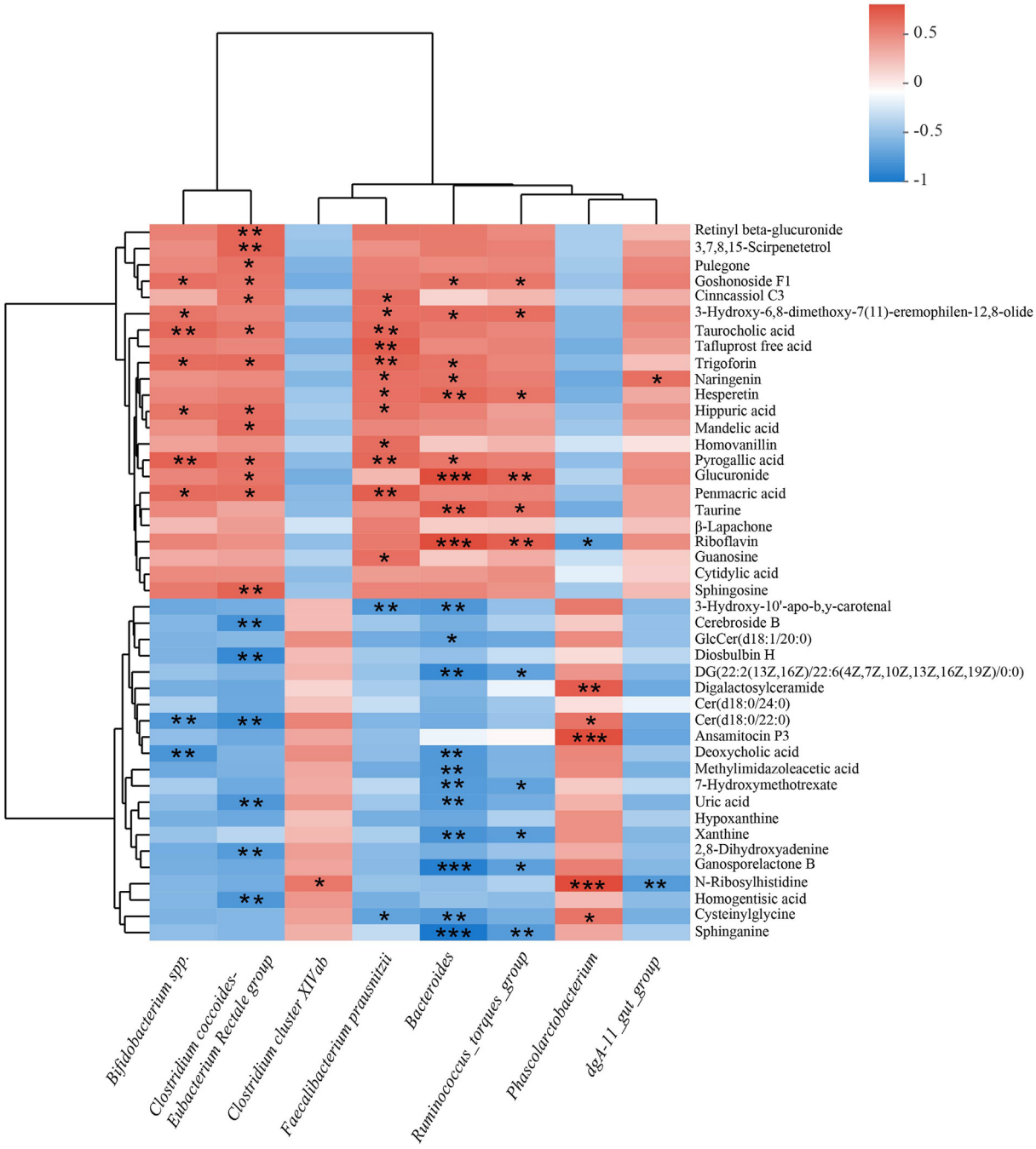

Fig. 6. The Spearman correlation matrix between fecal differentially abundant bacteria and fecal differentially expressed metabolites. Asterisks denote significant difference between dairy cows fed the control diet (CON) and citrus flavonoid extracts at 150 g/d (CFE150), *$0.01 < P \leq 0.05$; **$0.001 < P \leq 0.01$; ***$P \leq 0.001$.

inflammation and increased host ceramide levels in mice and demonstrated that sphingolipid produced by *Bacteroides* species could maintain symbiosis with the host. Although the *Bacteroides*-derived sphingolipids were difficult to quantify in the present study, we speculated that the increase in *Bacteroides* abundance could promote sphingolipid biosynthesis and decrease host ceramides to improve intestinal homeostasis.

Taken together, supplementing CFE in the diet could be a promising approach for decreasing endotoxin and systemic inflammation in lactating dairy cows. We speculated that the significant change in hindgut microbiota and metabolites may be potentially associated with the improvement in immunometabolic status (Fig. 7). Based on current observations, the improvement in milk performance and mammary gland health may also be explained by the decrease in proinflammatory factors as the amount of CFE supplementation increased. Determining the interaction of citrus flavonoids and hindgut microbiota may need more studies concerning the metabolic fate of citrus flavonoids in ruminants. Also, further studies via targeted metabolomics or lipidomics are required to provide accurate quantification of some of the metabolites (e.g., sphingolipid metabolism) whose functions are currently not well elucidated in dairy cows.

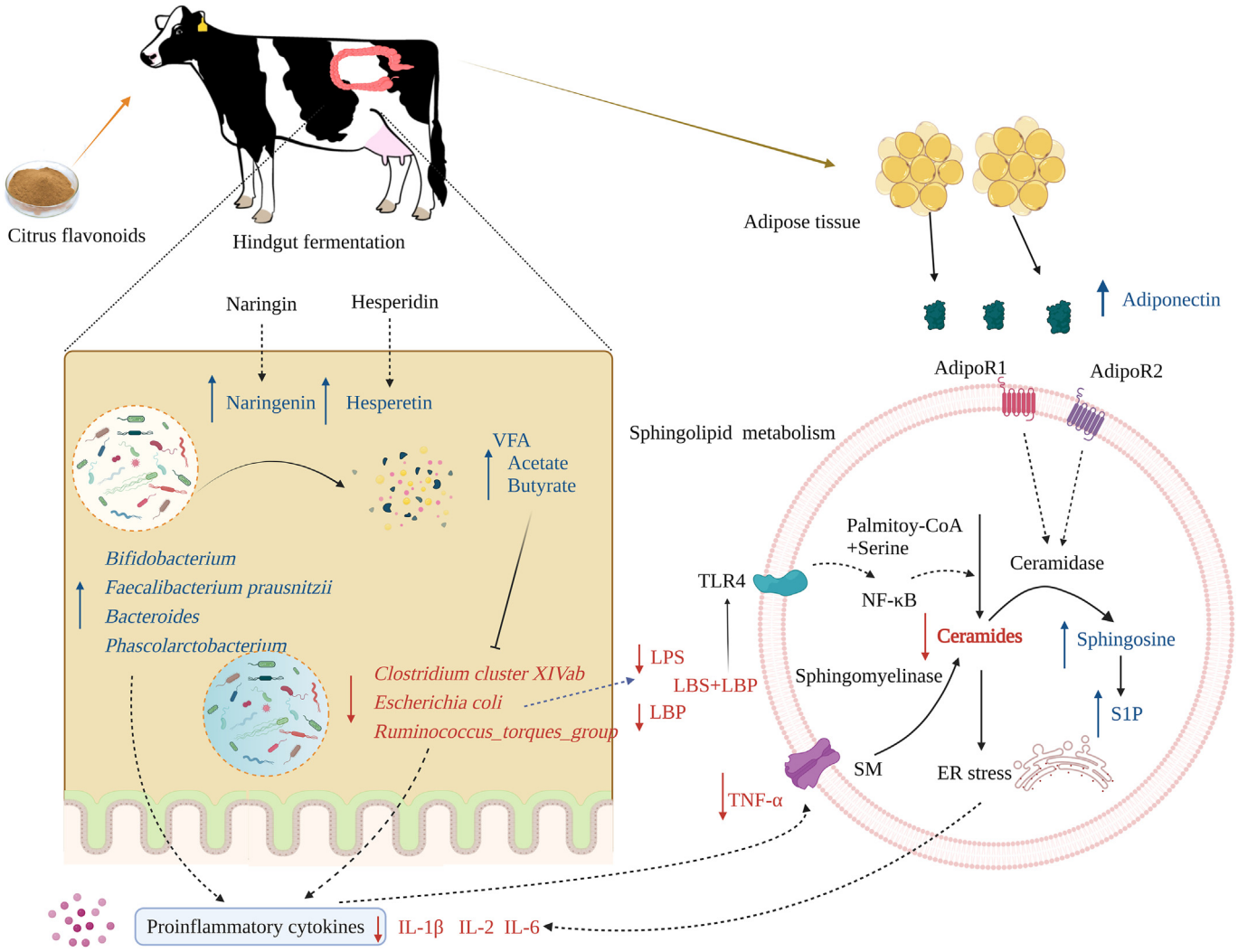

Fig. 7. A working mechanism to illustrate the hindgut bacteria and metabolites that might be associated with the improvement of immunometabolic status in lactating dairy cows. AdipoR = adiponectin receptor; ER = endoplasmic reticulum; IL = interleukin; LBP = lipopolysaccharide binding protein; LPS = lipopolysaccharide; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor α; SM = sphingomyelin; S1P = sphingosine 1-phosphate; VFA = volatile fatty acids.

5. Conclusion

In this study, we revealed that CFE has a clear beneficial effect on immune status and milk performance of mid-lactation dairy cows. Supplementary CFE improved immunometabolic status of dairy cows by regulating serum IgG, IL-1β, IL-2, IL-6, TNF-α, and LPS levels. The anti-inflammatory effects of CFE were associated with promotion of hindgut fermentation and the increase in probiotics *Bacteroides*, *Phascolarctobacterium*, *Bifidobacterium* spp., and *F. prausnitzii*, and the decrease in *Clostridium* cluster XIVab, *E. coli*, and *Ruminococcus_torques*_group. Sphingolipid metabolism and secondary bile acid production in the hindgut were also modulated by CFE supplementation, contributing to the protective effects of CFE on lipid and intestinal homeostasis. Further understanding of the regulatory mechanisms involved in the metabolic health effects of CFE as well as their metabolites in the gastrointestinal tract of dairy cows is crucial for future application of CFE as feed additives in the dairy industry.

Author contributions

Yuchao Zhao and **Shiqiang Yu**: Methodology, Investigation, Writing—Original Draft; **Liuxue Li**, **Huiying Zhao**, and **Yuqin Li**: Investigation, Data curation; **Linshu jiang**: Conceptualization, Methodology, Resources, Funding acquisition, Writing – Review & Editing; **Ming Liu**: Writing – Review & Editing. All authors read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.03.007.

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