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Original Research Article

Dietary supplementation of *Scutellariae radix* flavonoid extract improves lactation performance in dairy cows by regulating gastrointestinal microbes, antioxidant capacity and immune function

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ABSTRACT

Scutellariae radix flavonoid extract (SFE) has been acknowledged for its antioxidant, anti-inflammatory and antimicrobial properties in enhancing gastrointestinal microbial communities and improving the host's immunity. Nevertheless, the impacts of dietary supplementation with SFE on the gastrointestinal microbes and host metabolism in dairy cows remain uncertain. Therefore, the aim of this study was to assess the effects of dietary supplementation with SFE on the lactation performance, gastrointestinal microbes, and plasma biochemical parameters of dairy cows. Six ruminally and duodenally cannulated multiparous dairy cows were used in a crossover design over 28-d periods that included a 21-d adaptation and a 7-d sample collection period. Cows were fed a basal diet (CON group) or a basal diet supplemented with SFE at 25 g/d (SFE group). SFE supplementation tended to increase milk yield ($P = 0.067$) and milk urea N concentration ($P = 0.079$), and decreased the milk somatic cell counts (SCC, $P = 0.036$). Cows in the SFE group had lower plasma aspartate aminotransferase (AST), malondialdehyde (MDA), tumor necrosis factor (TNF-α), and interleukin-1β concentrations compared with the CON ($P < 0.05$). Meanwhile, SFE supplementation increased butyrate concentration in the rumen ($P = 0.044$). The microbial structure of rumen and duodenum were affected by SFE supplementation ($P = 0.009$ and $P = 0.031$; respectively), resulting in enrichment of *Butyrivibrio* in both parts of the SFE cows ($P = 0.034$ and $P = 0.029$; respectively). However, microbial structure and composition of feces were not affected by SFE supplementation. Overall, our study indicated that dietary supplementation with SFE could enhance lactation performance and milk quality in dairy cows by improving the gastrointestinal inner environment and health status.

1. Introduction

In recent years, significant advancements in genetic, nutrition and farm management have greatly enhanced productivity and fulfilled the demand for dairy products. However, these strategies often necessitate feeding dairy cows high-concentrate diets and maintaining high stocking densities (Abdela, 2016; Zhou et al., 2023). Previous studies have indicated that dairy cows fed high-concentrate diets are constantly exposed to a greater metabolic load, including oxidative stress, which is associated with increased production diseases and culling rate (Ma et al., 2022; Mcart and

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Neves, 2020). Furthermore, it is well recognized that the dairy production chain is a potential transmission route of antibiotic residues, which in turn increases the risk of resistant bacteria in susceptible populations (Jank et al., 2015), and antibiotic-resistant genes and bacteria in manure can promote environmental contamination (Oliver et al., 2020). Given the potential risks associated with antibiotic residues, supplementation with plant extracts, which are rich in bioactive compounds such as polyphenols (Barbato et al., 2019) and flavonoids (Tilahun et al., 2022), may offer a safe and reliable approach to improving the lactation performance and health status of dairy cows (Ciampi et al., 2020; Kolling et al., 2018).

Scutellariae radix is a widely used herb in traditional Chinese medicine, known for its antioxidant, anti-inflammatory, and antimicrobial properties (QY Zhao et al., 2018; Gao et al., 1999). Flavonoids are believed to elicit these effects. Baicalin, baicalein, wogonin, and chrysin are four main flavonoids in *Scutellariae radix* (Li and Chen, 2005). Owing to these beneficial attributes, the application of *Scutellariae radix* has been extended to livestock farming, with numerous studies demonstrating that dietary supplementation with *Scutellari baicalensis* extract can enhance growth performance, nutrient digestibility, and the abundance of beneficial intestinal microbes (Zhang et al., 2022; Zhao et al., 2016). In a recent study, Olagaray et al. (2019) reported that adding *S. baicalensis* extract at a dosage of 60 g/d (containing 33% baicalin) increased milk yield and reduced milk somatic cell counts (SCC). However, most flavonoids exhibit poor aqueous solubility characteristics, extensive metabolism, as well poor systemic absorption, resulting in lower bioavailability and bioactivity (Zhao et al., 2019). In monogastric animals, a series of studies have demonstrated that flavonoids have limited bioavailability after oral administration, with the majority not being absorbed and remain in the colon (Zeng et al., 2020; Song et al., 2020); therefore, it is not clear whether flavonoids are absorbed across the rumen epithelium in ruminants. It was demonstrated that rumen microbes are known to have the capability of partially deglycosylating naringin and hesperidin (Gladine et al., 2007). Hence, a comprehensive determination of the effects of SFE on gastrointestinal (rumen, small intestine, and large intestine) microbes and fermentation profiles can be used to improve lactation performance and the health of dairy cows.

We hypothesized that dietary supplementation with SFE would enhance the fermentation profile and immune status of dairy cows by regulating gastrointestinal microbes, resulting in a positive impact on their lactation performance. Therefore, the current study aimed to evaluate the effects of dietary supplementation with SFE on milk performance, plasma immune indices, gastrointestinal microbes (rumen, duodenum, and feces), and fermentation profiles.

2. Materials and methods

2.1. Animal ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of the China Agricultural University (protocol number: AW42504202-1-2).

2.2. Scutellariae radix flavonoid extract (SFE) preparation, animals, and experimental design

The SFE product was purchased from Shanxi Snout Biotechnology Co., Ltd. (Shanxi, Baoji, China). SFE was extracted from the root of *scutellaria* baicalensis Georgi. In brief, 1 kg *scutellaria* powder was ultrasonically extracted with 8 L ethanol (80%) for 25–40 min. Then, the mixture underwent reflux extraction to obtain the extract, and the extract was concentrated and adjusted to pH 2 with citric acid, filtered and dried (70 °C) to get the crude SFE. The crude SFE was extracted with 8 times distilled water, adjusted to the pH value of 2 with citric acid, and heated at 80 °C for 30 min. Finally, the precipitate was collected by centrifugation ($3500 \times g$ for 10 min at 4 °C) and lyophilized for 24 h to obtain high purity SFE. The total flavonoid content (85.13%) of SFE was determined using the method of Pitz et al. (2016). The concentrations of major flavonoids, including baicalin, chrysin, biochanin A, and apigenin in SFE were determined using a UPLC equipped with a BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters, USA) according to the method of De et al. (2016). The chemical composition of SFE is shown in Table S1.

This study was conducted at the Institute of Yuexiu Huishan Dairy Research Farm in Shenyang City, China, from July to September 2023. Six ruminally and duodenally cannulated multiparous Holstein dairy cows (2.5 ± 0.5 parity, 680 ± 36 kg of BW, and 84 ± 7 days in milk) were used in a crossover design with 28-d periods that included a 21-d adaptation and a 7-d sample collection period. Cannulation was performed using a 10-cm internal diameter ruminal cannula (Anscitech Farming Technology Co., Ltd., Wuhan, China) and a gutter-type T cannula placed approximately 10 cm distal to the pylorus (duodenally) (Wang et al., 2023) approximately 40 d postpartum. Cows were randomly assigned to two dietary treatments: basal diet (CON group) or basal diet supplemented with 25 g/d of SFE per cow (SFE group). The dose of SFE used in the current study was determined according to an in vitro test conducted by a team member through a rumen simulation technique. The results showed that 0.1% DM SFE in the TMR had the best effect on rumen fluid incubation performance in dairy cows (unpublished data). Therefore, the dose in the current study was calculated as 0.1% of the average DMI (25 kg/d per cow), resulting in 25 g/d per cow. The SFE product was top-dressed daily and mixed with a small amount of total mixed ration (TMR) during morning feeding according to a previous study (Oh et al., 2015). Each cow was housed separately, and the diets were fed twice daily at 08:00 and 15:00 for ad libitum intake at approximately 110% of actual feed intake. Water was available ad libitum through individual water troughs. All cows were milked at 08:00 and 20:00 daily using a hand-pushed milking machine (Zibo Zhisong Electric Motor Co., China), and milk weights were manually recorded. The experimental diet contained 27.74% starch, 28.59% neutral detergent fiber (NDF), 18.64% acid detergent fiber (ADF), and other nutrients to meet the NRC (2021) nutrient requirements (Table 1).

2.3. Sampling and analysis

During the 7-d sample collection period (d 22-28), both orts and TMR were recorded for the determination of DMI. TMR samples were collected on three consecutive days weekly from Friday to Sunday to get a representative sample of the diet fed to the animals, and orts were obtained daily from each cow at 07:30. TMR and orts samples were dried in a forced-air oven (DGG-9240B; Shanghai-ShenXin Inc., Shanghai, China) at 55 °C for 48 h, then ground through a 1-mm screen before analysis. Standard procedures of the Association of Official Analytical Chemists were used to determine dry matter (method 935.29; AOAC, 2006) and crude ash (method 942.05; AOAC, 2006). The organic matter (OM) content of the feeds was calculated by DM subtracting crude ash content. The crude protein (CP) contents of the TMR and orts were analysed according to the Macro-Kjeldahl procedure (method 990.03; AOAC, 2006). The concentration of ether extract (EE) was analysed according to the method 920.39 of AOAC (2006). Neutral detergent fiber and ADF contents were determined according to the method described by Van Soest et al. (1991), using a heat-stable α-amylase and

Table 1
The ingredients and chemical composition of the experimental diets.

Item	Contents
Ingredients, % of DM	
Corn silage	26.63
Alfalfa hay	12.77
Ground corn grain	27.92
Whole cotton seed	4.42
Molasses skins	0.72
Oyogold	0.84
Soybean meal	13.62
Canola meal	3.63
Glucose	1.88
Calcium fatty acid	2.79
$CaCO_3$	0.65
$NaHCO_3$	0.52
Nacl	0.49
Premix[1]	3.12
Chemical composition, % of DM, unless otherwise stated	
DM, % as fed	48.95
OM	91.12
CP	17.73
NDF	28.59
ADF	18.64
EE	4.67
Starch	27.74
NE_L[2], Mcal/kg of DM	1.69

DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NE_L = Net energy for lactation.
[1] Formulated to provide (per kg of dry matter): 250,000 IU of vitamin A, 50,000 IU of vitamin D_3, 1100 IU of vitamin E, 250 mg of Cu, 500 mg of Mn, 1,000 mg of Zn, 20 mg of Se, 40 mg of I, 24 mg of Co.
[2] Net energy for lactation was estimated using NRC (2001) model.

sodium sulphite. Starch content was determined using a total starch assay kit (Megazyme) based on the method 996.11, AOAC (2006).

Milk weight was manually recorded during the 7-d sample collection period. Milk samples were collected in a 50 mL centrifuge tube with a preservative (2-bromo-2-nitropropane-1,3-diol) during the last 3 d (d 26-28) of morning and afternoon milking. Milk samples collected twice a day were mixed at a ratio of 6:4 of morning and afternoon (Shan et al., 2018). The pooled samples were quickly sent to the Beijing Dairy Cow Center for determination of milk composition, including fat, protein, and lactose, using a near-infrared reflectance spectroscopy analyser (Seris300 Combi-FOSS; Foss Electric). Energy-corrected milk (ECM) was calculated as follows: ECM = (12.95 × fat yield) + (7.65 × protein yield) + (0.327 × milk yield), and 3.5% fat-corrected milk (FCM) was calculated as follows: 3.5% FCM = (0.4324 × milk yield) + (16.23 × fat yield) according to a previous study (Liu and Vandehaar, 2020). Blood samples were collected from the coccygeal vein 2 h after morning feeding on d 28 of each period. Blood samples (approximately 10 mL) were collected into vacuumed tubes, incubated at room temperature for about 30 min, and centrifuged at 3000 × *g* at 4 °C for 15 min to obtain plasma, which was separated into 2.0-mL tubes, frozen, and stored at −20 °C until further analysis. Plasma concentrations of glucose (GLU), insulin (INS), urea nitrogen (UN), aspartate aminotransferase (AST), and alanine transaminase (ALT) were determined using a GF-D200 automatic biochemical analyser (Jiangsu Zecheng Bioengineering Institute, CLS880, Jiangyin, China), as described by Zhang et al. (2023). Plasma beta-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) following the method described by Sun et al. (2021). Plasma levels of malondialdehyde (MDA), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute), according to a previous study (Zou et al., 2012). The concentrations of tumour necrosis factor-α (TNF-α), amyloid-A (SAA), haptoglobin (HPT), and interleukin-1β (IL-1β) in plasma were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturers' guidelines. Samples of rumen fluid, duodenal digesta, and faecal samples were collected 2 h after morning feeding on d 28. Approximately 50 mL each of rumen fluid and duodenal digesta, and 50 g of faecal sample were collected from each cow, and ruminal pH was immediately determined using a portable pH meter (Testo206, Schwarzwald, Germany). Then, every sample was divided into three plastic containers and frozen immediately in liquid nitrogen. One was used for microbiota analysis and the other was used to determine the volatile fatty acids (VFA). The VFA concentration in rumen fluid and duodenal digesta samples were determined according to the method described by Dai et al. (2023). Briefly, 0.4 mL of 25% ortho-phosphoric acid and 0.3 mL internal standard 4-methylvaleric acid (internal standard) were added into the 2 mL thawed fluid, and the mixture was centrifuged at 15,000 × *g* for 15 min at 4 °C. Subsequently, the supernatant was analysed for VFA composition using a gas chromatograph (Agilent 6890N, Agilent Technology, Inc, Beijing, China) equipped with a DB-FFAP capillary column (30 m × 0.32 mm × 0.5 μm). The conditions were as follows: the temperatures of the injector and detector were maintained at 220 and 250 °C, respectively, with a splitting ratio of 30:1 and high-purity nitrogen flow of 40 mL/min. The concentrations of VFA in the faecal samples were measured according to a previous study by Petri et al. (2019) with minor modifications. Briefly, 2 g of thawed faeces from each sample was mixed with 2 mL of distilled water. Then, 0.6 mL of an internal standard (4-methylvaleric acid; Sigma–Aldrich, St. Louis, MO, USA) and 0.4 mL of 25% phosphoric acid were added. The subsequent procedure was consistent with the rumen fluid determination method. Regarding the rumen fluid samples, the ammonia-nitrogen (NH_3–N) concentration was determined according to a phenol hypochlorite assay as described by Broderick and Kang (1980), and ruminal microbial protein (MCP) concentration was determined based on the method of Makkar et al. (1982).

2.4. DNA extraction, PCR amplification, and high-throughput sequencing

Total genomic DNA from rumen fluid, duodenal digesta, and faecal samples were extracted using an E.Z.N.A Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) (Kong et al., 2022). DNA concentration and purity were determined using a NanoDrop 2000 UV–visible spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and DNA quality was monitored using 1% agarose gel electrophoresis. The V3 and V4 hypervariable regions of the 16S rRNA gene were amplified with a specific primer pair: 338F (5′-ACTCCTACGG-GAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using ABI GeneAmp 9700 PCR thermal cycler (ABI), as per the method described in a previous study (Liu et al., 2023a). The PCR products were visualised in a 2% agarose gel, purified and quantified by AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA) and QuantiFluor-ST (Promega, USA), respectively. Finally, paired-end sequencing libraries were generated using an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) at Paysono Bioinformatics Technology Co., Ltd. (Shanghai, China). All raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) (Accession Number: PRJNA1103999).

2.5. Sequence processing and analysis

Paired-end reads were assigned to the samples based on their unique barcodes and truncated by removing the barcodes and primer sequences. The raw read pairs were overlapped and merged using FLASH (v1.2.11) (Magoc and Salzberg, 2011), then imported to QIIME2 (Version 2022.2) for demultiplexing (Bolyen et al., 2019). Quality control, denoising, removal of chimeric sequences, and generation of amplicon sequencing variants (ASV) were performed using the QIIME2 plugin, DADA2 (Callahan et al., 2016). The SILVA database (version 138) was used for ASV classification labelling analysis, and the microbial species composition at different taxonomic levels (phylum, class, order, family, genus, and species) was obtained. Several alpha diversity indices, including Sobs, ACE, Chao1, Simpson, and Shannon indices, were calculated using QIIME 2. The microbial β-diversity was determined using the distance matrices generated from Bray–Curtis analysis, principal coordinate analysis (PCoA), and ANOSIM analysis. LEfSe was used to identify significantly different taxa between the different treatment groups (Segata et al., 2011).

2.6. Statistical analysis

The analysis of milk performance, fermentation parameters, inflammatory cytokines, and oxidative stress indices data in plasma were conducted with SAS (version 9.4, SAS Institute Inc., USA) using the PROC MIXED statement and the following model:

$$Y_{ijkl} = \mu + T_i + P_j + B_k + C_l(_k) + e_{ijkl,}$$

where Y_{iikl} is the observations for dependent variables, μ is the overall mean, T_i is the fixed effect of treatment (i = CON, SFE), P_j is the fixed effect of the period (j = 1-2), B_k is the fixed effect of square (k = 1-2), $C_l(_k)$ is the random effect of cow nested within the square (l = 1-6), and e_{ijkl} is the residual error. A t-test was used to identify differences in the means of milk performance, fermentation parameters, inflammatory cytokines, and oxidative stress indices data in plasma. Differential microbial composition and α-diversity were compared using a Welch's *t*-test between the two groups, and the *P*-value was corrected by false-discovery rate adjustment. All results were reported as means ± standard error of the mean, and significant differences were declared when $P \leq 0.05$, and $0.05 < P < 0.10$ was defined as a trend of difference.

3. Results

3.1. Feed intake, milk production and composition

As shown in Table 2, SFE supplementation did not influence the DMI of the cows ($P = 0.165$). Similarly, no differences were observed between the CON and SFE groups regarding milk composition parameters, including milk fat, protein, and lactose contents ($P > 0.10$). Milk yield tended to increase ($P = 0.067$) with SFE supplementation compared to the CON group (35.35 vs. 32.84 kg/d). Cows that received SFE tended to have greater milk MUN concentration ($P = 0.079$; 10.93 vs. 9.63 mg/dL). Moreover, milk SCC was significantly lower ($P = 0.026$) in SFE cows than in the CON cows.

3.2. Plasma metabolites, liver enzymes, oxidative status, inflammation and acute-phase proteins

Regarding the plasma metabolites, SFE supplementation did not affect the plasma levels of GLU, INS, UN, BHB, or NEFA ($P > 0.10$; Table 3). Compared to the CON group, SFE supplementation significantly increased the plasma concentrations of T-AOC ($P = 0.032$), while decreased the plasma AST ($P = 0.013$) and MDA ($P = 0.013$) levels. Regarding plasma inflammation and acute-phase proteins, SFE supplementation significantly reduced plasma TNF-α ($P = 0.010$) and IL-1β ($P = 0.001$) levels; however, plasma SAA and HPT levels were not affected by SFE supplementation ($P > 0.10$).

Table 2
Effects of dietary with *Scutellariae radix* flavonoid extracts (SFE) supplementation on feed intake, milk production and composition in dairy cows.

Item	Treatment groups[1]		SEM	*P*-value
	CON	SFE		
DMI, kg/d	23.28	24.09	0.289	0.165
Milk yield, kg/d	32.84	35.35	0.687	0.067
ECM[2], kg/d	35.81	39.04	2.333	0.515
3.5% FCM[3], kg/d	34.98	37.85	2.350	0.566
Milk fat, %	3.85	3.92	0.143	0.824
Milk fat, kg/d	1.28	1.39	0.100	0.613
Milk protein, %	3.37	3.52	0.083	0.384
Milk protein, kg/d	1.11	1.24	0.068	0.374
Milk lactose, %	5.07	5.23	0.084	0.363
Milk lactose, kg/d	1.66	1.85	0.096	0.353
MUN, mg/dL	9.63	10.93	0.373	0.079
SCC, $\times 10^3$ cells/mL	159.16[a]	82.83[b]	18.037	0.026

SEM = standard error of the mean; DMI = dry matter intake; ECM = energy corrected milk; FCM = fat corrected milk; MUN = milk urea nitrogen; SCC = somatic cell count.
[a,b] Mean values within a row with different superscripts differed ($P < 0.05$).
[1] CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.
[2] ECM = 12.95 × fat yield + 7.65 × protein yield + 0.327 × milk yield.
[3] 3.5% FCM = 0.4324 × milk yield + 16.23 × fat yield.

Table 3
Effects of dietary with *Scutellariae radix* flavonoid extract (SFE) supplementation on plasma metabolites, liver enzymes, oxidative status, inflammation and acute-phase proteins of dairy cows.

Item	Treatment groups[1]		SEM	*P*-value
	CON	SFE		
Metabolites				
GLU, mmol/L	3.64	3.68	0.104	0.875
INS, mIU/L	15.10	15.61	1.130	0.833
UN, mmol/L	4.13	4.26	0.317	0.852
BHB, mmol/L	0.34	0.38	0.017	0.249
NEFA, μmol/L	38.93	42.57	1.225	0.144
Liver enzymes				
AST, mmol/L	80.75[a]	66.9[b]	3.032	0.013
GGT, mmol/L	13.50	12.91	0.720	0.706
Oxidative status				
GSH-Px activity, U/mL	7.96	8.50	0.324	0.426
T-AOC activity, U/mL	9.09[b]	10.03[a]	0.227	0.032
MDA activity, nmol/mL	1.75[a]	1.52[b]	0.055	0.030
Inflammation and acute-phase proteins				
TNF-α, ng/L	174.46[a]	136.88[b]	8.012	0.010
IL-1β, ng/L	64.28[a]	51.71[b]	2.278	0.001
SAA, μg/L	25.81	25.71	1.244	0.970
HPT, ng/L	42.74	42.29	2.092	0.922

SEM = standard error of the mean; GLU = glucose; INS = insulin; UN = urea nitrogen; BHB = β-hydroxybutyric acid; NEFA = non-esterified fatty acids; AST = aspartate aminotransferase; GG = γ-glutamyl transferase; GSH-Px = glutathione peroxidase; T-AOC = total antioxidant capacity; MDA = malondialdehyde; TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; SAA = amyloid-A; HPT = haptoglobin.
[a,b] Mean values within a row with different superscripts differed ($P < 0.05$).
[1] CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.

3.3. Fermentation profile in the ruminal, duodenal, and fecal contents

Regarding the ruminal fermentation profile, SFE supplementation significantly increased the butyrate concentration ($P = 0.044$; Table 4), whereas the TVFA, acetate, propionate, isobutyrate,

Table 4
Effects of dietary with *Scutellariae radix* flavonoid extract (SFE) supplementation on fermentation profile in the rumen, duodenal, and fecal of dairy cows.

Item	Treatment groups[1]		SEM	*P*-value
	CON	SFE		
Rumen				
pH	6.31	6.14	0.134	0.548
NH_3–N, mg/dL	8.87	9.43	0.491	0.594
MCP, mg/dL	2.33[b]	2.81[a]	0.120	0.037
TVFA, mmol/L	100.41	107.21	7.801	0.687
Acetate, mmol/L	60.02	64.97	3.964	0.570
Propionate, mmol/L	25.52	26.84	2.261	0.789
Isobutyrate, mmol/L	0.60	0.83	0.110	0.342
Butyrate, mmol/L	12.78[b]	18.08[a]	1.360	0.044
Isovalerate, mmol/L	0.48	0.67	0.097	0.323
Valerate, mmol/L	1.01	1.31	0.128	0.267
Duodenal				
pH	4.13	4.02	0.119	0.665
TVFA, mmol/L	4.97	4.75	0.526	0.880
Acetate, mmol/L	3.23	3.19	0.344	0.959
Propionate, mmol/L	1.37	1.10	0.179	0.485
Butyrate, mmol/L	0.36	0.50	0.063	0.290
Fecal				
pH	6.73	6.68	0.096	0.815
TVFA, mmol/L	75.51	84.79	6.12	0.456
Acetate, mmol/L	63.22	72.22	4.89	0.382
Propionate, mmol/L	7.29	7.98	0.93	0.725
Butyrate, mmol/L	4.60	4.59	0.42	0.988

SEM = standard error of the mean; TVFA = total volatile fatty acids; NH_3–N = ammonia-nitrogen; MCP = ruminal microbial protein.
[a,b] Mean values within a row with different superscripts differed ($P < 0.05$).
[1] CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.

valerate, and isovalerate concentrations were not altered by SFE supplementation ($P > 0.10$). Moreover, supplementing SFE did not significantly affect the rumen fluid pH and NH_3–N concentration ($P > 0.10$), while the ruminal MCP concentration was increased ($P = 0.037$) with SFE supplementation. SFE supplementation did not significantly affect the concentrations of TVFA, acetate, propionate, or butyrate in the duodenum or faeces ($P > 0.10$), and the pH was unaffected by SFE addition ($P > 0.10$).

3.4. Taxonomic configurations of ruminal, duodenal, and faecal bacteria

The effect of SFE supplementation on gastrointestinal microbes in cows was evaluated using 16S rRNA high-throughput sequencing. Regarding ruminal diversity indices, including the Chao1 ($P = 0.427$) and Shannon ($P = 0.264$) indices, no differences were found between the CON and SFE groups (Fig. 1A and B). Furthermore, the beta diversity of rumen bacteria was determined by principal coordinate analysis, explaining 27.0% and 15.5% of the variance, respectively, and a distinct separation was found between the CON and SFE groups (weighted UniFrac, ANOSIM: $P = 0.009$; Fig. 1C), indicating that feeding SFE modified ruminal microbial structure significantly. Bacteroidetes (49.84%), Firmicutes (42.22%), Actinobacteria (1.18%), Proteobacteria (0.53%), and Tenericutes (1.09%) were the dominant bacterial phyla in the two groups (Fig. 1D), and feeding SFE to dairy cows significantly reduced the relative abundances of Tenericutes ($P = 0.005$) and Spirochaetes ($P = 0.041$) in comparison with that of cows fed the CON diet (Table 5). The ruminal microbiome was dominated by *Prevotella* (23.20%), *Ruminococcaceae_Ruminococcus* (8.97%), *Succiniclasticum* (5.22%), *Butyrivibrio9* (2.34%), *YRC229* (1.16%), and *Oscillospira* (0.97%) at the genus level (Fig. 1E). Feeding SFE to dairy cows significantly increased the relative abundance of *Butyrivibrio* ($P = 0.034$) in the SFE group when compared to that in the CON group, and a tendency of lower abundance of CF231 ($P = 0.084$) was found in SFE group when compared to that of the CON group (Table 6). We used LEfSe analysis (LDA $\geq$3, $P < 0.05$) to identify significantly differential bacterial taxa between the CON and SFE groups. Thirteen biomarkers were identified. Fibrobacteria, Fibrobacteres, Fibrobacteraceae, Fibrobacterales, *Bifidobacterium*, *Fibrobacter*, Spirochaetales, Spirochaetaceae, *Treponema*, Spirochaetes, Spirochaetes, and *RFN20* were enriched in the CON group, and Lachnospiraceae was enriched in the SFE group (Fig. 1F).

No differences were found in the alpha diversity indices, including the Chao1($P = 0.348$) and Shannon ($P = 0.752$) indices, between the duodenum in the CON and SFE groups (Fig. S1A, B), whereas PCoA analysis based on weighted UniFrac metrics showed that feeding SFE significantly altered the duodenal microbial structure (weighted UniFrac, ANOSIM: $P = 0.031$; Fig. S1C). As diagrammed in Fig. S1D, Firmicutes (70.85%), Proteobacteria (11.2%), Actinobacteria (9.69%), and Bacteroidetes (6.53%) were the predominant phyla in both the groups, whereas SFE-supplemented cows had a lower relative abundance of Spirochaetes ($P = 0.034$; Table S2) compared to the cows in the CON group. The dominant genera were *Butyrivibrio* (8.85%), *Ruminococcaceae_Ruminococcus* (5.84%), *Prevotella* (4.23%), *Oscillospira* (3.49%), *Sharpea* (3.23%), *Coprococcus* (3.20%), and *Succiniclasticum* (2.75%) in both the groups (Fig. S1E), and the relative abundance of *Butyrivibrio* was higher in the SFE cows than in the CON cows ($P = 0.03$). However, the relative abundances of *Ruminococcaceae_Ruminococcus* ($P = 0.010$), *Coprococcus* ($P = 0.029$), and *Clostridiaceae_Clostridium* ($P = 0.004$) were decreased by SFE supplementation compared to the CON group (Table S3). Furthermore, we used LEfSe analysis to discover and elaborate marker microbes between the CON and SFE groups (LDA $\geq$3.5, $P < 0.05$). Compared to the CON group, the relative abundances of Coriobacteriales, Christensenellaceae, *Pseudobutyrivibrio*, Moryella, and Nocardiopsaceae were higher in the SFE group, whereas those of Ruminococcaceae, Erysipelotrichi, Erysipelotrichaceae, Eiysipelotrichales, Sharpea, Ruminococcus, Coprococcus, Ruminobacter, Lactococcus, Neisseriaceae, Clostridiaceae, Clostridium, Moryella, Pseudobutyrivibrio, and Christensenellaceae were higher in the CON group (Fig. S1F).

A remarkable difference was found in the alpha diversity indices of the faeces, including the Chao1 ($P = 0.019$) and Shannon ($P = 0.008$) indices, which were higher in the CON group than in the SFE group (Fig. S2A, B), whereas we did not observe a distinct separation between CON and SFE, indicating that feeding did not alter the faecal microbial structure (weighted UniFrac, ANOSIM: $P = 0.10$; Fig. S2C). As illustrated in Fig. S2D, Firmicutes (63.09%), Bacteroidetes (29.81%), Actinobacteria (2.85%), Spirochaetes (1.99%), and Proteobacteria (1.03%) were the dominant bacterial phyla in both the groups (Fig. S2D), and no significant differences in the phyla were observed between the CON and SFE groups ($P > 0.05$, Table S4). *Clostridiaceae_Clostridium* (3.24%), *Bifidobacterium* (2.57%), *5-7N15* (2.55%), *Roseburia* (2.45%), and *Treponema* (1.99%) were the dominant genera in both the groups (Fig. S2E), and no significant differences were found between the CON and SFE groups ($P > 0.10$; Table S5). We used LEfSe analysis to identify significantly differential bacterial taxa between the CON and SFE groups (LDA $\geq$3, $P < 0.05$). Erysipelotrichi, Erysipelotrichaceae, Erysipelotrichales, Fusobacteria, Tenericutes, Fusobacteriaceae, Mollicutes, Oscillospira, Phascolarctobacterium, Cetobacterium, Anaeroplasmataceae, and Anaeroplasmatales were enriched in the SFE group (Fig. S2F).

4. Discussion

Flavonoid-rich extract has emerged as a promising phytogenic feed additive for improving the performance and health of

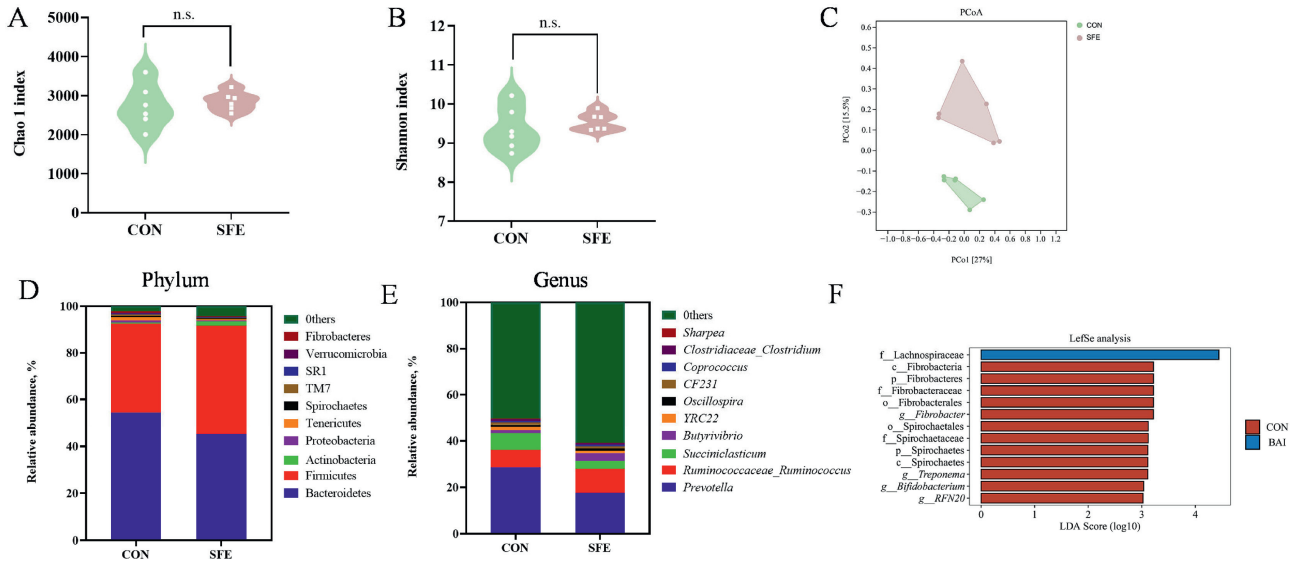

Fig. 1. Effects of dietary with *Scutellariae radix* flavonoid extract (SFE) on diversity and composition of the rumen bacterial community of dairy cows. (A) Chao1 index. (B) Shannon index. (C) Beta diversity based on the principal coordinate analysis (PCoA) using weighted UniFrac distance. (D) Relative abundance of bacteria community at the phylum level. (E) Relative abundance of bacteria community at the genus level. (F) Linear discriminant analysis effect size approach identifying biomarker genera between two groups. CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.

Table 5
Effects of dietary with *Scutellariae radix* flavonoid extract (SFE) supplementation on bacterial abundance in rumen of dairy cows (phylum level, %).

Item	Treatment groups[1]		SEM	*P*-value
	CON	SFE		
Bacteroidetes	54.46	45.22	3.185	0.158
Firmicutes	37.98	46.46	3.286	0.211
Actinobacteria	0.66	1.70	0.350	0.116
Proteobacteria	0.67	0.39	0.152	0.399
Tenericutes	1.54[a]	0.64[b]	0.281	0.005
Spirochaetes	0.59[a]	0.36[b]	0.059	0.041
TM7	0.55	0.20	0.113	0.131
SR1	0.43	0.27	0.118	0.536
Verrucomicrobia	0.41	0.24	0.084	0.355
Fibrobacteres	0.47	0.15	0.096	0.104

SEM = standard error of the mean.
[a,b] Mean values within a row with different superscripts differed ($P < 0.05$).
[1] CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.

Table 6
Effects of dietary with *Scutellariae radix* flavonoid extract (SFE) supplementation on bacterial abundance in rumen of dairy cows (genus level, %).

Item	Treatment groups[1]		SEM	*P*-value
	CON	SFE		
Prevotella	28.68	17.71	3.697	0.150
Ruminococcaceae_Ruminococcus	7.61	10.34	2.121	0.555
Succiniclasticum	7.11	3.32	1.505	0.223
Butyrivibrio	1.21[b]	3.46[a]	0.554	0.034
YRC22	1.35	0.97	0.190	0.346
Oscillospira	0.93	1.01	0.252	0.888
CF231	1.12	0.71	0.142	0.084
Coprococcus	0.68	0.85	0.141	0.588
Clostridiaceae_Clostridium	0.65	0.73	0.061	0.491
Sharpea	0.31	0.08	0.108	0.322

SEM = standard error of the mean.
[a,b] Mean values within a row with different superscripts differed ($P < 0.05$).
[1] CON = control diet with no SFE supplementation; SFE = control diet with 25 g/d SFE supplementation for per cow.

ruminants (Alhidary and Abdelrahman, 2016; Yu et al., 2023). However, few studies have investigated the effects of dietary SFE supplementation on lactation performance, gastrointestinal microbes, antioxidant capacity, and immune function in ruminants. DMI was not significantly affected by SFE supplementation, indicating that SFE did not affect palatability. This is in accordance with the findings of Aguiar et al. (2014), who found that cows fed diets supplemented with flavonoid-rich extracts of propolis at rates of 3.81, 3.27, or 1.93 mg/kg of ingested dry matter (DM) did not differ among treatments (Aguiar et al., 2014). The tendency for higher milk yield in SFE-fed cows was similar to that reported by Yu et al. (2023), who found that citrus extract supplementation positively affected milk yield in cows. Similar research reported that adding flavonoid extract to dairy cow diets had a positive effect on milk fat (Totakul et al., 2022) or lactose (Olagaray et al., 2019), which was contrary to what was observed in our experiment, therefore, the lack of consistent results could be attributed to the differences in the types of flavonoid extracts, dietary nutrient levels (energy, CP, etc.), and lactation stage between studies. Increased somatic cells in milk reflect poor breast health and milk quality, which are serious issues in modern dairy cow farming that result in considerable financial losses to the dairy husbandry. Nearly 70% of the subclinical breast infections are associated with a temporary or permanent reduction in milk production, mainly due to inflammatory damage to the mammary tissue (Eckersall et al., 2006). In the present study, a reduced milk SCC was observed after each cow received 25 g/d SFE supplementation, which may, in part, be attributed to SFE improving the antioxidant capacity of dairy cows. This is in accordance with similar studies that reported a positive effect on milk SCC in cows that received diets supplemented with flavonoid-rich extracts, such as bamboo leaves (Zhan et al., 2021), citrus (Yu et al., 2023), and quercetin (Burmańczuk et al., 2018). Moreover, a cellular research study reported that baicalin protected cow mammary epithelial cells from lipopolysaccharide (LPS) induced inflammatory injury and apoptosis by attenuating inflammation response (Yang et al., 2016). The milk MUN concentration is generally used to evaluate the balance between carbohydrates and nitrogen sources in the rumen environment (Aguilar et al., 2012);

SFE supplementation tended to increase milk MUN concentrations in our study. Increased concentrations of MUN could be attributed to the excessive amount of NH_3 being absorbed from the rumen and converted to urea into the blood system which easily passes through to the milk (Broderick and Clayton, 1997). The greater ruminal NH_3–N concentration in the SFE group observed in this study could confirm this, although it was not statistically significant. In contrast, the results of the present study differ from those of Dschaak et al. (2011), who observed a negative effect of condensed tannin extract supplementation on milk MUN. The lack of consistent results can be attributed to the differences in influencing the DMI. A higher DMI was observed in the SFE cows than in the CON cows, which did not reach statistical significance in the present study. In contrast, Dschaak et al. (2011) observed a negative effect on DMI with a decrease in intakes of 1.75 kg/d, when lactating dairy cows were fed diets supplemented with condensed tannin extract. These results demonstrate the potential benefits of SFE supplementation on milk production in dairy cows.

No effect of SFE supplementation on plasma concentrations of GLU, INS, UN, BHBA, or NEFA was observed. Similar to our study, some previous studies have reported no difference in the plasma concentrations of energy and protein in ruminants receiving diets supplemented with flavonoid-rich extracts from propolis (Varela et al., 2023), quercetin (Gruse et al., 2016), or cabbage (Gao et al., 2022) compared to those fed the CON diets. The liver is a critical organ in the metabolism and adaptation of dairy cows, as well as for coordinating the metabolism of the stomach, intestines, adipose tissue, and mammary glands (Drackley, 1999). AST and gamma-glutamyl transferase (GGT) are important aminotransferases in animals and are considered valuable indicators of hepatic injury. In the present study, the plasma AST concentration in SFE cows decreased compared with those in the CON cows, which is consistent with the result that SFE supplementation had a positive effect on protecting liver injury in chicks (Xu et al., 2021). Thus, the results of our study indicated that SFE supplementation improves liver metabolism in cows. The dynamic balance of free radicals in an organism is primarily achieved by antioxidant enzymes that maintain the normal function and metabolism of the animal body (Khan et al., 2020). In fact, as a comprehensive index for evaluating the antioxidant system, plasma T-AOC reflects the cumulative effect of antioxidants in animals, with a higher concentration indicating greater antioxidant capacity (Kampa et al., 2002). In contrast, plasma MDA is a lipid peroxidation product, and a lower MDA concentration reveals that the organism has low oxidative stress (Zhao et al., 2014). Previous studies confirmed that flavonoid-rich extracts improve the antioxidant capacity of animals. For example, Liu et al. (2023b) observed that supplementation with moringa leaf flavonoids in the diet of dairy cows resulted in an enhanced antioxidant capacity and increased plasma SOD and T-AOC levels paralleled with reduced MDA levels. In addition, a previous study on pre-weaning calves fed mulberry leaf flavonoids showed a positive effect on oxidative stress and increased GSH-PX and SOD levels (Wang et al., 2018). In the present study, increased T-AOC paralleled with reduced MDA concentration in plasma was observed after SFE supplementation, indicating that the antioxidative capacity of dairy cows was enhanced by SFE supplementation, which is in line with the above results, potentially due to flavonoids acting as reducing agents and hydrogen donors to neutralise oxygen radicals and remove hydrogen peroxide and superoxide ions (Kahkonen et al., 1999). TNF-α and IL-1β are mainly proinflammatory cytokines and are released during inflammatory processes (Gabay and Kushner, 1999). Indeed, the increase in pro-inflammatory cytokines is a sign of oxidative stress (Dinda et al., 2017) with alterations of mitochondrial functionality and an increase in free radical production (Musco et al., 2020). Notably, a previous study has reported that supplementing citrus flavonoid extract (CFE) can effectively reduce the serum concentrations of inflammatory cytokines (IL-6, TNF-α) in high-grain fed dairy cows, suggesting that CFE could reduce the inflammatory response. Moreover, a recent study evaluated the effect of CFE on systemic inflammation and showed that these extracts had a positive effect on reducing serum concentrations of LPS proinflammatory cytokines (TNF-α and interleukin-6), acute phase proteins (LPS-binding and HPT protein) in dairy cows (Zhao et al., 2023a,b). In the current study, dietary supplementation with SFE reduced plasma TNF-α and IL-1β concentrations, indicating that SFE supplementation could enhance immune function in dairy cows.

VFA act as the major carbon source for ruminal microbes and provide 60%–80% digestible energy for ruminants (Galyean, 2014). Furthermore, concentration and composition are important indicators of rumen fermentation status and microbial composition (Bergman, 1990). Previous studies have demonstrated that dietary supplementation with flavonoid-rich extracts affects the concentration or composition of rumen VFA (Ma et al., 2017; Yu et al., 2023). We observed that supplementation with SFE increased ruminal butyrate concentration, which might be attributed to the greater abundance of *Butyrivibrio*, which has been recognised as a producer of butyrate (Wang et al., 2021). Butyrate, a major energy source in the gastrointestinal tract, is important in regulating the gut immune function and inflammatory reactions (Hamer et al., 2008). Hence, the reduced milk SCC and greater immune function with SFE supplementation were likely the result of the higher ruminal butyrate content. Moreover, the results showed that SFE supplementation increased ruminal MCP concentration. The addition of SFE provided an energy substrate which may have reduced the amount of AA used for the energy supply and thus increased MCP synthesis in the rumen. To the best of our knowledge, no previous study has investigated the effects of dietary flavonoid extract supplementation on the duodenal fermentation profiles of dairy cows. We did not observe differences in the duodenal fermentation profiles among treatments, which may be because flavonoids are degraded by rumen microorganisms and act as carbon sources for ruminants (Gessner et al., 2017). Recently, a study reported that feeding CFE to dairy cows significantly increased faecal butyrate concentration (Zhao et al., 2023a,b), which is not consistent with our results, as we observed no difference in the faecal fermentation profile. Hence, further studies are needed to explore the issues associated with the ratio of ruminal degradation of SFE.

Previous studies have demonstrated the antimicrobial activities of flavonoids against some gram-negative and gram-positive bacteria, as well as protozoa (Dong et al., 2021; Karoline Ferreira Leite et al., 2023). In the present study, the β-diversity analysis of the rumen microbes revealed that the SFE supplementation significantly altered ruminal microbial structure. However, there were no significant differences in the alpha-diversity indices between the CON and SFE groups, consistent with that found in a previous study (Zhao et al., 2023a,b). In the present study, cows fed SFE had reduced relative abundances of Tenericutes and Spirochaetes in the rumen. Previous studies have shown that many *Treponema* spp. are linked to ulcerative mammary dermatitis and bovine digital dermatitis in cattle (Sadet et al., 2007) and contagious ovine digital dermatitis in sheep (Evans et al., 2012), indicating that it has a deleterious impact on host health. Similar responses in reducing the abundance of Spirochaetes were also observed when flavonoids were offered to pigs (T Zhao et al., 2018) or dairy cows (Zhan et al., 2017). It should be noted that various species of Spirochaetes such as *Spirochaetes syphilis* and *Spirochaetes lymeiosis* can induce severe infections in animals (Bonhomme and Werts, 2022). This suggests that SFE could limit the growth of harmful bacteria in the rumen,

consequently exhibiting a health-promoting effect on dairy cows, which was also evidenced by the decline in milk SCC. To the best of our knowledge, few studies have explored the effects of SFE supplementation on small intestinal (duodenal) microbes in dairy cows. In the current study, duodenal samples were used to reflect small intestinal conditions to a certain extent. SFE supplementation altered the microbial structure in the duodenum, with an increase in the relative abundance of *Butyrivibrio* and a reduction in the relative abundances of *Ruminococcus*, *Coprococcus*, and *Clostridium*. *Butyrivibrio* can degrade hemicellulose and pectin to produce butyrate (Palevich et al., 2019), which suppresses the growth of pathogenic bacteria (Brownawell et al., 2012). Wallis et al. (2020) revealed that Ruminococcaceae family microbiota are mainly involved in nitrogen and amino acid metabolism. Therefore, changes in the abundance of these microbiota may affect the microbial amino acid metabolism and may be associated with the host immune system. *Coprococcus* belongs to the phylum Trichoderma, which actively ferments carbohydrates and is an important producer of butyric acid, which is closely related to the gastrointestinal health (Wang et al., 2022). Huang et al. (2022) showed that adding soybean isoflavones could increase the abundance of *Coprococcus* and reduce the body weight gain rate, which is inconsistent with our results. *Clostridium*, belonging to the phylum Firmicutes, prevents inflammation associated with the production of SCFAs, particularly butyrate, through the breakdown of dietary fibre (Guo et al., 2020). Nevertheless, the negative impact of these two beneficial genera remains unclear and requires further investigation. Although some studies have confirmed that dietary supplementation with flavonoids alters the structure and composition of the hindgut microbiota in dairy cows (Zhao et al., 2023a,b), no significant effects were observed when cows were fed SFE in the current study. Consequently, we speculated that the different results on the hindgut microbiota might be attributed to differences in the types of flavonoids, nutrient levels (energy, CP, etc.), and milk production between the studies.

5. Conclusions

The present study provided new insights into the effects of SFE, such as improved milk yield and reduced milk SCC, increased ruminal butyrate concentration, and reduced plasma AST, MDA, TNF-α and IL-1β concentration in dairy cows. Dietary supplementation with SFE altered the structure and composition of the rumen and duodenum by increasing the relative abundance of *Butyrivibrio*, and decreasing the relative abundance of Ruminococcus, Coprococcus, and Clostridium in the duodenum. Thus, SFE could be considered as a potential natural supplement for improving the lactation performance and health status of dairy cows.

CRediT authorship contribution statement

Dongwen Dai: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Chunxiao Dong:** Methodology, Software, Writing – review & editing. **Fanlin Kong:** Methodology, Resources. **Shuo Wang:** Data curation, Formal analysis, Methodology. **Shuxiang Wang:** Formal analysis, Investigation, Methodology. **Wei Wang:** Formal analysis, Funding acquisition, Methodology, Visualization. **Shengli Li:** Conceptualization, Funding acquisition, Investigation, Validation, Writing – original draft, Writing – review & editing.

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 A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.11.019.

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