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Hydrogen cross-feeding among rumen biohydrogenation, propionogenesis and methanogenesis drives the milk fatty acid profile in dairy goats

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Research Article

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Abstract

Rumen microbial biohydrogenation (RBH) is the major factor responsible for the bovine milk rich in saturated fatty acids (FAs). Here, we evaluated the effects of nutritional manipulation of ruminal propionogenesis and methanogenesis, two primary hydrogen sinks, on the RBH and milk FA profiles *in vivo* and *in vitro* using three propionogenesis enhancers (fumarate [FUM], biotin and monensin) and one methanogenesis inhibitor (*N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide [NPD]). The *in vivo* results showed that inclusion of FUM in lactating dairy goat diet could protect dietary unsaturated FAs against RBH with increased proportions of C18:2*n* − 6 (by 33.5%), C18:3*n* − 3 (by 38.1%) and RBH intermediates (e.g. *trans*-10 C18:1 and *trans*-11 C18:1) in rumen contents. Additionally, FUM supplementation increased the milk Δ9 desaturase index (by 15.5%) with higher *cis*-9 monounsaturated FAs in the milk. As a result, FUM increased the proportions of polyunsaturated and monounsaturated FAs in the milk with lower atherogenicity index (by −15.3%) and thrombogenicity index (by −19.5%). Conversely, supplementing NPD increased RBH completeness (by 7.4%) with higher milk atherogenicity index (by 10.5%) and thrombogenicity index (by 8.7%). The adverse effects of NPD on the milk FA profiles can be eliminated when supplemented in combination with FUM. The metagenomic analyses showed that neither FUM nor NPD affect the rumen microbial α- or β-diversity at the strain or gene level. The *in vitro* study showed that the conversion rate of FUM to propionate was increased from 54.7% to 80.6% when FUM supplemented in combination with biotin and monensin, resulting a higher anti-RBH potential. Accordingly, manipulation of ruminal methanogenesis and propionogenesis can redirect hydrogen toward or away from RBH and thereby influence the milk FA profiles. FUM is a promising feed additive in ruminant not only to reduce the methane emissions as previously proved but also to improve the nutritional desirability of the milk FA profiles for human health.

Introduction

Bovine milk and dairy products are important and traditional nutritious foods for humans, especially for infants and elderly people. However, the consumption of milk has raised some health concerns due to its high concentrations of saturated fatty acids (SFAs), which are implicated in cardiovascular diseases, weight gain and obesity (Haug et al. 2007). The high SFA in milk is largely attributed to the rumen microbial biohydrogenation (RBH) of unsaturated FAs (UFAs) (Haug et al. 2007; Yang et al. 2019; Zhao et al. 2022). On the other hand, some RBH intermediates (biohydrogenation intermediates [BIs]), such as *trans*-11 C18:1 and *cis*-9,*trans*-11 C18:2, increase milk conjugated linoleic acids (CLAs), which are considered health-promoting in humans (Zongo et al. 2021).

Some ruminal metabolic processes are interlinked with others due to cross-feeding among microbial species (Lourenço et al. 2010; Mizrahi et al. 2021). Molecular (H_2) and metabolic ([H]) hydrogen are important rumen fermentation intermediates and can be released by microbes when they ferment dietary carbohydrates to acetate and butyrate (Ellis et al. 2008; Janssen 2010; Ungerfeld and Emilio 2020). Methanogenesis and propionogenesis are the main routes for ruminal hydrogen removal (Hristov et al. 2013; Wang et al. 2013). Although RBH only consumes 1% to 2% of the produced hydrogen (Nagaraja et al. 1997), it can convert most dietary UFAs to SFAs (Jenkins et al. 2008; Lourenço et al. 2010). Our previous researches (Li et al. 2021, 2018b, 2018c) have revealed the hydrogen competition between methanogenesis and propionogenesis. For example, supplying fumarate (FUM), an intermediate in the succinate–propionate pathway, could redirect hydrogen away from methanogenesis to propionogenesis

(Li et al. 2021, 2018b). However, little information is available detailing the influences of manipulation of ruminal propionogenesis and methanogenesis on the RBH and then the milk FA profiles.

Here, *in vivo* and *in vitro* experiments were conducted to fill in the above missing information. The objective of the *in vivo* study was to investigate the persistent and combined effects of FUM (a propionogenesis enhancer) and *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD, a methanogenesis inhibitor) (Jin et al. 2017; Li et al. 2021) on RHB and milk FA profiles in lactating dairy goats over a course of 12 wks. A meta-analysis has showed that only an average of 48% of added FUM was converted to propionate in the *in vitro* rumen fermentation, likely due to its high FUM concentration and low converted capacity (Ungerfeld et al. 2007). Therefore, the objective of the *in vitro* study was to investigate the restrictive factors for the conversion rate of FUM to propionate and the anti-RBH potential of FUM.

Materials and methods

All experimental procedures were approved by the Northwest A&F University Animal Care and Use Committee, in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals.

Animals, experimental design and sample collection

The animals, dietary ingredients and chemical composition, feed intake, rumen fermentation and lactation parameters of this study have been described previously (Li et al. 2021). Briefly, 24 primiparous Guanzhong dairy goats were used in a 12-wk randomized complete block design experiment. Goats were blocked based on days in milk, body weight and daily milk production, and within each block were randomly assigned to one of four treatments: control (CON), a basal diet with a forage-to-concentration ratio of 48:52; basal diet supplemented with FUM ($C_4H_4O_4$, Aladdin®, Shanghai, China) at 34 g/d; basal diet supplemented with NPD ($C_8H_9N_3O_4$, J&K Scientific®, Beijing, China) at 0.5 g/d; and the basal diet supplemented with both FUM and NPD. The supply doses of FUM and NPD were determined based on the published data (Jin et al. 2017; Li et al. 2018b).The goats were separately fed and milked twice daily at 07:30 and 17:30 h, and had free access to water.

The feeding experiment lasted 12 wks, and samples were collected at wk 3, 6, 9 and 12. On d 1–4 of each sample collection week, the morning and evening milk of each goat from each day were mixed, and then 45 mL of subsample was collected. On d 5–6 of each sample collection week, blood samples were collected from an external jugular vein into two 10-mL blood tubes before the morning feeding, and ruminal content samples were collected by an oral rumen tube at 6 h after morning feeding. To minimize saliva contamination, approximately 50 mL of ruminal content was discarded before sample collection. The blood sample in the tube was allowed to clot at room temperature for 30 min and centrifuged (3000 ×*g*, 15 min) thereafter to obtain serum. All samples were stored at −80°C before analysis.

Fatty acid analysis

The composition of fatty acid (FA) in the milk and rumen content samples was analyzed as described previously (Sun and Gibbs 2012) with some modifications. Briefly, the milk samples of each goat were freeze-dried and mixed for each week, so were the rumen content samples and blood samples. Each mixed sample (500 mg) was directly methylated using 4 mL of 0.5 mol/L NaOH/methanol (15 min at 50°C) and remethylated using 4 mL of 5% HCl/methanol (1 h at 50°C). The FA methyl esters in each sample were extracted with 2 mL of heptane and then analyzed using gas chromatography (GC 7820A; Agilent Technologies, Diegem, Belgium) equipped with a fused silica capillary column (SP-2560, 100 m × 0.25 mm × 0.2 μm; Supelco Inc., Bellefonte, PA, USA) and an iron trap mass detector system (MSD 5977E; Agilent Technologies), with helium as the carrier gas. The split ratios were 20:1, 10:1 and 5:1 for the milk, rumen content and blood samples, respectively. The parameters and conditions of GC-MS were set as described previously (Alves and Bessa 2007; Sun and Gibbs 2012). C19:0 was used as an internal standard. A 37-component FAME mix (Sigma Chemical Co, Saint Louis, USA) and other four FAME mixes (ME 61, ME93, BR2 and BR3, Larodan Fine Chemicals AB, Malmo, Sweden) were used as external standards.

Calculation and statistical analysis

The RBH completeness was calculated as described by Alves et al. (2017), assuming a complete RBH of the C18 FA from the diet:

RBH completeness (%) = $C18{:}0_R/[(cis\text{-}9\ C18{:}1_D - cis\text{-}9\ C18{:}1_R) + (C18{:}2n-6_D - C18{:}2n-6_R) + (C18{:}3n-3_D - C18{:}3n-3_R) + C18{:}0_D] \times 100$

where $C18{:}0_{D/R}$, cis-9 $C18{:}1_D/_R$, $C18{:}2n-6_{D/R}$ and $C18{:}3n-3_{D/R}$ are C18:0, *cis*-9 C18:1, C18:2*n* − 6 and 18:3*n* − 3 in the diet or the rumen as a percentage of total C18 FAs, respectively.

The thrombogenicity index and atherogenicity index of milk FA were calculated as described by Ulbricht and Southgate (1991):

Thrombogenicity index = (C14:0 + C16:0 + C18:0)/[(MUFA + *n* − 6 PUFA)/2 + 3(*n* − 3 PUFA) + (*n* − 3 PUFA/*n* − 6 PUFA)]

Atherogenicity index = [C12:0 + 4 (C14:0) + C16:0]/UFA

where MUFA and PUFA are total monounsaturated and polyunsaturated FAs in the milk, respectively.

The milk Δ9 desaturase index was calculated as *cis*-9 C17:1/(C17:0 + *cis*-9 C17:1), because *cis*-9 C17:1 is assumed to be exclusively synthesized endogenously (Natalello et al. 2019).

All data related to FA were analyzed by a repeated-measures ANOVA, which considers the time-dependent effects of treatments, using the PROC MIXED program in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The statistical model included NPD, FUM, wk and NPD × FUM, NPD × wk, FUM × wk and NPD × FUM × wk interactions as fixed factors, and goat and block as random effects. The sampling week was treated as a repeated measure and the goat as a subject. When there was a treatment × wk interaction, differences among treatments at each sampling week were reanalyzed using the MIXED procedure with NPD, FUM and NPD × FUM interaction as fixed factors, and block as a random effect. When there was an NPD × FUM interaction, Tukey's multiple comparison test was used to assess differences among treatment means.

Metagenomic sequencing and genome catalog constructing

Microbial DNA was extracted from the rumen samples at wk 6 following the protocol of Yu and Morrison (2004), and then sequenced by an Illumina HiSeq X Ten platform with 150-bp paired-end reads (14 G per sample). To obtain a more complete and high-quality metagenome-assembled genome (MAG) catalog,

we also collected 24 published rumen metagenomes (Shen et al. 2020; Shi et al. 2022) from dairy goats in the same farm.

The metagenomic data were individually cleaned, assembled (–use-megahit option), binned (–maxbin2 –concoct –metabat2 options) and bin-refined (-c 50 -x 10 options) using the metaWRAP pipeline (Uritskiy et al. 2018). The metagenomes in each treatment group were co-assembled and co-binned as described above. The acquired MAGs with CheckM (Parks et al. 2015) estimated genome quality score (completeness – 5*contamination + log(N50)) ≥ 50, completeness >50% and contamination <10% were retained. These MAGs were dereplicated twice by <99% average nucleotide identity (ANI) to obtain the strain-level MAG catalog using dRep (Olm et al. 2017) with options –S_algorithm ANImf -sa 0.99 -nc 0.2 -comW 1 -conW 5 -N50W 1.

The MAGs were taxonomically annotated using the GTDB-Tk v2.0.0 (Parks et al. 2018). The relative abundance of MAGs in samples was quantified using Quant_bins module of metawrap (Uritskiy et al. 2018). The relative abundance is expressed as CPM (genome copies per million reads), a similar way like TPM (transcripts per million reads) in RNAseq analysis.

Gene catalog construction and functional annotation

Gene coding sequences of the assembled contigs from megahit were predicted using Annotate_bins module of metawrap (Uritskiy et al. 2018). The predicted genes were clustered using CD-HIT [47] with parameter -n 9 -c 0.95 -G 0 -aS 0.9 to construct the nonredundant gene catalog. Functional annotation (KEGG, GO and COG) of the gene catalog was performed using eggNOG mapper (v2.1.7) with the DIAMOND mapping mode, based on the eggNOG 5.0 orthology data (Huerta-Cepas et al. 2019). The relative abundance of genes in samples was quantified using CoverM (parameter: –min-read-percent-identity 95, –minread-aligned-percent 60, –min-covered-fraction 0.7, -m tpm).

Microbial and gene diversity analysis

Based on the above MAG and gene abundance, the α-diversities of samples were estimated using the abundance-based coverage estimator (community richness) and Shannon index (community diversity) using vegan package in R v.4.1.1. β-Diversity of the MAG and gene abundance were computed and visualized with principal coordinate analysis plots with Bray–Curtis distance (Bray and Curtis 1957) in R. A permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis() function in the vegan package to compare the statistical difference in microbial composition. The differentially abundant MAGs and genes among groups were identified using edgeR (Robinson et al. 2010).

In vitro rumen fermentation

The *in vitro* rumen fermentation procedure was conducted according to the method described by Lin et al. (2013). Seven treatments (5 replicates each) were evaluated: (1) control (CON); (2) 40 mg FUM (FUM); (3) 5 μg biotin (BIO); (4) 50 μg monension (MON); (5) FUM + biotin (FB); (6) FUM + monension (FM); and (7) FUM + biotin + monension (FBM). The doses of treatments were determined based on the previous studies or *in vivo* dose (Chen et al. 2011; Shen and Liu et al. 2017; Ungerfeld et al. 2007). The 0.5 g substrate (including 70% alfalfa hay, 20% corn, 8% soybean meal and 2% sunflower oil), 25 mL mixed rumen fluid from four cannulated dairy goats, 25 mL McDougall's buffer and treatment were incubated in 100-mL serum bottles at 39°C for 24 h. At the end of the incubation, the incubation fluid pH and gas production were immediately measured. The incubation fluids were sampled for the analysis of volatile FAs (VFAs) and FA profiles, and the gas was sampled for the analysis of methane production according to the previously described methods (Li et al. 2018a; Liu et al. 2017; Zheng et al. 2020). The data were analyzed using the MIXED procedure of SAS. The main effects of FUM, biotin, monension and their interactions were investigated.

Results

Effects on FA composition in the rumen contents

Supplementation of the diet with FUM greatly changed the FA compositions of rumen contents (Table 1) such that the proportion of odd carbon and branched-chain FA (OBCFA, by 15.5%, $P = 0.022$), short-chain FA (SCFA, $P = 0.002$), long-chain FA (LCFA, $P = 0.003$) and PUFA (by 32.4%, $P < 0.001$) were increased, while that of MCFA ($P = 0.002$) and SFA (by 3.1%, $P < 0.001$) were decreased. A total of 15 individual FAs were increased by FUM, while only C16:0 (by 4.9%, $P = 0.004$) and C18:0 (by 9.6%, $P = 0.015$), two major RBH end products, were decreased. Compared with CON, FUM increased the proportions of C18:2*n* − 6 (by 33.5%, $P = 0.004$) and C18:3*n* − 3 (by 38.1%, $P < 0.001$), two major RBH substrates. Regarding the proportion of BIs, *trans*-10 C18:1, *trans*-11 C18:1 and *cis*-11 C18:1 were increased ($P < 0.05$) by FUM, and *cis*-11 C18:1 tended to be increased ($P = 0.051$), while *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA and the ratio of *trans*-10 to *trans*-11 were unchanged. RBH completeness was reduced by 12.5% ($P = 0.002$) in the animals receiving FUM (Fig. 1a).

Compared with FUM, NPD had a limited effect on the rumen FA profile. The feeding of NPD increased ($P < 0.05$) the proportions of C6:0, *cis*-9 C17:1, *trans*-11 C20:1 and C18:0, resulting in a lower PUFA (by 10.0%, $P = 0.045$) and a higher SCFA ($P = 0.006$). RBH completeness was increased by 7.4% ($P = 0.036$) in the animals receiving NPD (Fig. 1a).

Interactions ($P < 0.05$) between FUM and NPD were detected with respect to *trans*-11 C20:1, C20:4*n* − 6 and C18:3*n* − 3 (Table 1 and Fig. 1b). A FUM × time interaction ($P = 0.015$) was detected with respect to *trans*-11 C18:1 (Fig. 1c), and the increasing effect of FUM was apparent from wk 6 of the treatment and became stronger over time.

Effects on FA composition in the blood and milk

The responses to FUM for the sum of OBCFA, SCFA, MCFA, LCFA, SFA and PUFA in the blood (Table S1) were consistent with those in the rumen contents. Compared with CON, the proportions of C18:3*n* − 3, C20:0 and C20:4*n* − 6 in blood were increased ($P < 0.05$) by FUM supplementation, while that of C16:0 was decreased ($P = 0.038$). In addition, the proportions of *trans*-11 C18:1 ($P = 0.076$) and C18:2*n* − 6 ($P = 0.051$) tended to be increased by FUM supplementation, while those of C14:0 ($P = 0.098$) and C18:0 ($P = 0.057$) tended to be decreased. Supplementation of diet with NPD

Table 1. Effect of dietary treatments on rumen digesta fatty acid composition of dairy goats

	Treatment					*P*-value					
Fatty acid,g/kg total FA	CON	FUM	NPD	FN	SEM	wk	FUM	NPD	F × N	F × wk	N × wk
C6:0	1.30	1.64	1.56	2.40	0.15	0.013	0.001	0.004	0.118	0.712	0.771
C8:0	0.18	0.23	0.21	0.25	0.02	0.733	0.045	0.240	0.897	0.619	0.443
C10:0	1.51	1.77	2.13	1.45	0.31	0.077	0.501	0.646	0.147	0.355	0.925
C11:0	0.75	1.05	0.98	0.97	0.09	0.008	0.113	0.389	0.094	0.810	0.248
C12:0	14.5	14.0	14.6	14.4	1.07	0.104	0.745	0.848	0.891	0.383	0.040
C13:0	6.87	5.98	6.04	5.70	0.97	0.011	0.534	0.574	0.780	0.335	0.104
C14:0	46.0	42.0	49.1	42.0	3.81	0.007	0.167	0.689	0.687	0.932	0.349
Cis-9 C14:1	0.68	0.74	0.70	0.74	0.05	0.415	0.293	0.866	0.866	0.927	0.897
C15:0	33.7	40.0	35.7	39.8	1.65	<0.001	0.006	0.586	0.518	0.263	0.102
Iso C15:0	18.7	21.9	21.7	22.1	2.61	0.879	0.486	0.546	0.597	0.313	0.612
Cis-10 C15:1	1.62	1.98	1.62	1.77	0.13	0.029	0.071	0.427	0.442	0.304	0.476
C16:0	529	503	509	502	4.82	0.420	0.004	0.055	0.067	0.240	0.850
Cis-9 C16:1	5.03	4.49	4.46	4.28	0.31	0.047	0.266	0.234	0.576	0.159	0.139
C17:0	12.4	13.3	12.6	12.9	0.52	0.001	0.265	0.821	0.507	0.144	0.199
Iso C17:0	4.55	6.46	5.81	6.87	0.68	0.041	0.045	0.239	0.536	0.332	0.382
Cis-10 C17:1	0.60	0.49	0.80	0.68	0.08	0.477	0.163	0.031	0.935	0.286	0.103
C18:0	115	104	126	114	4.06	0.021	0.015	0.020	0.847	0.333	0.681
Trans-9 C18:1	0.94	0.88	0.92	0.91	0.05	<0.001	0.512	0.958	0.717	0.100	0.178
Trans-10 C18:1	4.92	6.54	5.85	6.88	0.54	0.103	0.027	0.259	0.589	0.467	0.169
Trans-11 C18:1	26.0	28.7	25.3	26.6	0.75	0.001	0.015	0.086	0.366	0.015	0.957
Cis-9 C18:1	67.1	58.8	71.2	64.6	5.17	0.122	0.169	0.357	0.877	0.283	0.343
Cis-11 C18:1	8.39	10.3	8.92	10.5	0.83	0.053	0.051	0.650	0.868	0.521	0.022
Cis-9,*trans*-11 CLA	0.51	0.55	0.55	0.65	0.05	0.009	0.190	0.218	0.559	0.696	0.195
Trans-10,*cis*-12 CLA	0.53	0.59	0.54	0.60	0.04	0.519	0.197	0.781	0.940	0.271	0.523
C18:2*n* − 6	36.9	49.2	31.0	48.1	4.38	0.216	0.004	0.442	0.594	0.465	0.473
C18:3*n* − 6	1.60	1.67	1.45	1.80	0.15	0.342	0.179	0.950	0.348	0.687	0.914
C18:3*n* − 3	32.7[b]	45.1[a]	29.4[b]	33.3[b]	1.41	0.002	<0.001	<0.001	0.008	0.545	0.155
C20:0	4.96	5.81	5.40	5.82	0.23	<0.001	0.013	0.336	0.348	0.562	0.564
Trans-11 C20:1	0.70[c]	1.20[a]	0.93[b]	1.01[ab]	0.07	0.518	0.001	0.756	0.010	0.593	0.860
Cis-11 C20:1	0.82	1.02	0.87	1.05	0.10	0.001	0.084	0.693	0.894	0.054	0.375
C20:2*n* − 6	4.99	5.07	6.09	5.28	1.16	<0.001	0.757	0.582	0.709	0.936	0.942
C20:3*n* − 6	0.19	0.13	0.18	0.12	0.08	<0.001	0.448	0.930	0.992	0.722	0.999
C20:4*n* − 6	0.24[b]	0.45[a]	0.34[b]	0.24[b]	0.07	0.417	0.414	0.456	0.045	0.098	0.458
C20:5*n* − 3	3.46	4.29	3.27	4.01	0.24	0.501	0.005	0.338	0.857	0.629	0.701
C21:0	2.47	3.03	2.54	2.73	0.15	0.010	0.020	0.457	0.220	0.719	0.428
C22:0	4.04	4.98	4.61	5.05	0.26	<0.001	0.018	0.240	0.359	0.798	0.302
Trans-13 C22:1	0.44	0.49	0.42	0.46	0.05	0.015	0.460	0.631	0.925	0.616	0.487
Cis-13 C22:1	0.74	0.59	0.64	0.55	0.07	0.097	0.100	0.290	0.670	0.314	0.772
C22:2*n* − 6	1.07	1.28	1.05	1.26	0.15	0.009	0.164	0.888	0.989	0.475	0.807
C22:6*n* − 3	0.19	0.43	0.23	0.28	0.07	0.640	0.063	0.490	0.196	0.419	0.470
C23:0	0.44	0.45	0.49	0.40	0.07	0.085	0.625	0.982	0.470	0.790	0.945

(*Continued*)

Table 1. (*Continued.*)

	Treatment					*P*-value					
Fatty acid,g/kg total FA	CON	FUM	NPD	FN	SEM	wk	FUM	NPD	F × N	F × wk	N × wk
C24:0	3.77	4.62	4.26	4.61	0.23	<0.001	0.018	0.305	0.281	0.739	0.243
Cis-15 C24:1	0.38	0.50	0.41	0.48	0.04	<0.001	0.052	0.954	0.602	0.579	0.300
Cis-C18:1 BI	75.5	69.1	80.1	75.1	4.90	0.033	0.262	0.297	0.892	0.406	0.525
Trans-C18:1 BI	31.8	36.1	32.1	34.4	0.80	0.004	0.001	0.367	0.241	0.094	0.180
C18:2 BI	1.04	1.14	1.09	1.25	0.09	0.068	0.162	0.380	0.754	0.426	0.281
t10/t11 ratio	0.20	0.23	0.24	0.27	0.02	0.035	0.171	0.152	0.835	0.242	0.273
OBCFA	82.0	94.7	88.3	93.9	3.58	0.083	0.022	0.457	0.343	0.313	0.765
SCFA	1.48	1.87	1.77	2.65	0.17	0.016	0.002	0.006	0.162	0.752	0.794
MCFA	658	637	646	636	4.28	0.040	0.002	0.146	0.233	0.289	0.400
LCFA	341	361	352	362	4.31	0.030	0.003	0.184	0.217	0.297	0.395
SFA	799	774	803	784	4.49	0.001	<0.001	0.172	0.521	0.914	0.573
MUFA	118	117	123	121	5.15	0.005	0.695	0.424	0.930	0.450	0.517
PUFA	82.3	109	74.1	95.6	4.88	0.098	<0.001	0.045	0.617	0.449	0.584

Notes: [a–c]Means with different superscripts within a row differ significantly ($P < 0.05$).
Means by treatment were the pooled data ($n = 6$) of 3, 6, 9 and 12 wk.
CON, control; FUM, fumarate; NPD, *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide; FN, FUM + NPD; SEM, standard error of means; CLA, conjugated linoleic acid; BI, biohydrogenation intermediates; OBCFA, odd carbon and branched-chain fatty acid; SCFA, short-chain fatty acid (C6:0–C8:0); MCFA, medium-chain fatty acid (C10–C16); LCFA, long-chain fatty acid (C17:0–C24); SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

reduced the sum of PUFA (by 32.2%, $P = 0.006$), accompanied with decreased *trans*-11 C18:1, C18:3*n* − 3 and *cis*-11 C20:1.

Inclusion of FUM also greatly changed the FA profile in the milk (Table 2), with the proportion of OBCFA (by 11.9%, $P = 0.001$), PUFA (by 24.1%, $P = 0.004$) and MUFA (by 10.3%, $P = 0.003$) being increased, while that of SFA (by 5.0%, $P = 0.001$) being decreased. A total of 11 individual FAs were changed in response to FUM, and among them only the proportions of C14:0 ($P = 0.042$) and C18:0 ($P = 0.007$) were decreased, leading to a decreased SFA. Increases in C11:0, C13:0 and C15:0 proportions after FUM addition contributed to increased OBCFA. Compared with CON, FUM increased ($P < 0.05$) the proportion of C18:2*n* − 6, C18:3*n* − 3, *trans*-11 C18:1 and *cis*-9 C18:1, and tended ($P = 0.065$) to increase that of *cis*-9,*trans*-11 CLA. The ratio of milk t10 to t11 in the goats receiving FUM was lower ($P = 0.012$) than that in the control. In addition to *cis*-9 C18:1 and *cis*-9,*trans*-11 CLA, other *cis*-9 FAs (i.e., *cis*-9 C14:1 [$P < 0.001$], *cis*-9 C16:1 [$P = 0.099$] and *cis*-9 C17:1 [$P = 0.002$]) were or tended to be increased by FUM, leading to an increased Δ9 desaturase index (by 15.5%, $P = 0.008$) (Fig. 2a). Due to these shifts, FUM supplementation decreased ($P < 0.01$) the milk atherogenicity index by 15.3% and the thrombogenicity index by 19.5% (Fig. 2).

Supplementation with NPD reduced the proportion of MUFA (by 4.9%, $P = 0.003$), PUFA (by 8.8%, $P < 0.001$) and LCFA ($P = 0.031$) in milk while increasing that of SFA ($P < 0.001$) and MCFA ($P = 0.043$) (Table 2). The proportion of C14:0 ($P = 0.031$) was increased, while that of *trans*-10 C18:1, *cis*-9 C18:1, *cis*-9,*trans*-11 CLA and C20:4*n* − 6 was decreased ($P < 0.05$) by NPD. The NPD supplementation increased the milk atherogenicity index (by 10.5%, $P = 0.001$) and thrombogenicity index (by 8.7%, $P < 0.001$) and tended to decrease the Δ9 desaturase index ($P = 0.075$).

Effects on rumen microbial flora structure and function

In this study, we assembled and binned 1.1 Tb rumen metagenomes from dairy goats, generating 1,776 strain-level (<99% ANI) and 1,187 species-level (<95% ANI) MAGs . Of the 1,776 MAGs, 347 were estimated to be near-complete (>90% completeness and <5% contamination), 432 to be high-quality (>80% completeness and >60 quality score), 996 to be moderate-quality (>50% completeness and >50 quality score) (Table S2). A total of 7 MAGs were assigned to 2 phyla of archaea (*Methanobacteriota, Thermoplasmatota*), and the left MAGs were assigned to 19 phyla of bacteria with two-thirds belonging to *Bacteroidetes* (733) and *Firmicutes_A* (527). Additionally, the constructed gene catalog included 598,234 nonredundant genes. Based on the MAG or gene catalog, neither FUM nor NPD affected the microbial α- or β-diversity (Fig. 3).

In vitro rumen fermentation

Both tannin and monensin supplementation had no effect on the total VFA or acetate concentrations, monensin tended to increase the propionate concentration ($P = 0.060$) and reduce the methane production ($P = 0.086$). The addition of FUM increased ($P < 0.05$) the gas production, total VFA, acetate and propionate concentrations, and reduced ($P < 0.05$) the methane production and the ratio of acetate to propionate. However, the actual conversion rate of FUM to propionate was only 54.7%, and it was increased when FUM supplemented in combination with biotin (60.8%) or monensin (77%) alone, and was the highest (80.6%) when in combination with both biotin and monensin (Table 3). Both FUM and monensin reduced ($P < 0.05$) the proportion of SFA, while increasing that of MUFA and PUFA ($P < 0.05$), without changing the t10/t11 ratio (Tables 3 and S3). The proportion of ruminal MUFA and PUFA were highest and the methane production was lowest

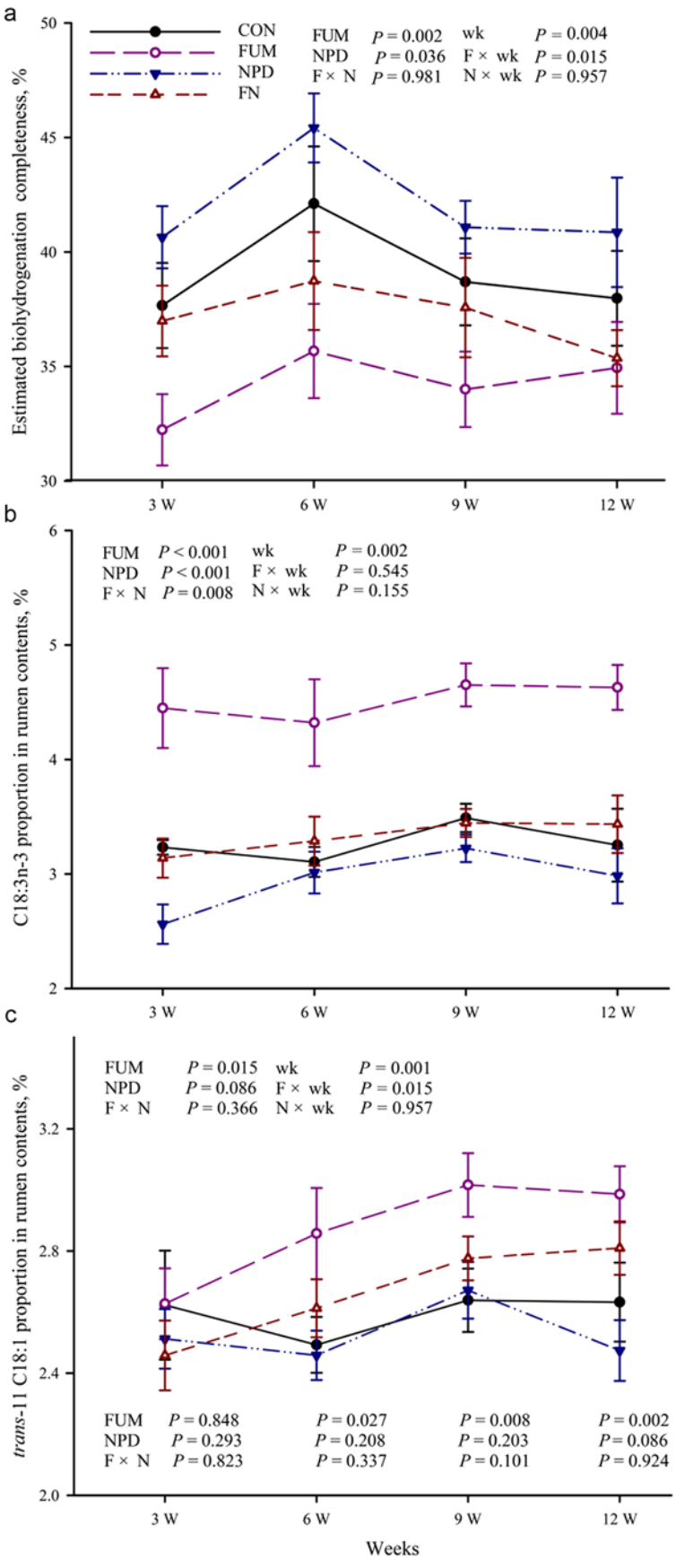

Figure 1. Temporal effects of fumarate (FUM), *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD), and their combination on biohydrogenation completeness (a), proportion of C18:3*n* − 3 (b), and *trans*-11 C18:3*n* − 3 (c) in the rumen. The biohydrogenation completeness (%) was estimated according to the changes of C18 FA contents in diet and rumen digesta (Alves et al. 2017). Note: C, control; FN, FUM + NPD. The *P*-values of ANOVA of the repeated-measures are shown above the curves, while the *P*-values of two-way ANOVA for each week are shown below the curves.

Table 2. Effect of dietary treatments on the milk fatty acid composition of dairy goats

	Treatment					*P*-value					
Fatty acid,g/kg total FA	CON	FUM	NPD	FN	SEM	wk	FUM	NPD	F × N	F × wk	N × wk
C6:0	12.6	12.8	13	12.7	0.46	<0.001	0.963	0.714	0.620	0.462	0.523
C8:0	18.8	19.2	19.4	18.6	0.87	<0.001	0.819	0.994	0.557	0.284	0.895
C10:0	81.9	77.2	78.1	82.3	3.06	<0.001	0.933	0.833	0.171	0.343	0.853
C11:0	0.75	1.03	0.84	1.07	0.11	<0.001	0.033	0.589	0.819	0.248	0.006
C12:0	51.6	53.3	52.4	51.4	3.02	<0.001	0.920	0.865	0.656	0.924	0.031
C13:0	0.71	0.90	0.73	0.92	0.05	<0.001	0.003	0.751	0.996	0.185	0.022
C14:0	119	109	126	119	3.65	<0.001	0.042	0.031	0.666	0.968	0.614
Cis-9 C14:1	6.99[b]	11.0[a]	6.99[b]	9.03[ab]	0.11	<0.001	<0.001	<0.001	<0.001	0.048	0.051
C15:0	7.57	9.11	7.93	9.25	0.24	<0.001	<0.001	0.326	0.665	0.589	0.529
Iso C15:0	0.46	0.51	0.46	0.52	0.04	0.228	0.244	0.818	0.953	0.680	0.327
C16:0	317	314	331	333	10.6	<0.001	0.975	0.151	0.797	0.808	0.106
Cis-9 C16:1	7.53	8.65	6.67	7.44	0.54	0.001	0.099	0.076	0.761	0.480	0.962
C17:0	7.38	7.27	7.59	7.59	0.25	<0.001	0.785	0.336	0.841	0.912	0.537
Iso C17:0	0.91	0.92	0.90	0.87	0.04	0.001	0.759	0.520	0.642	0.487	0.314
Cis-10 C17:1	1.96	2.32	1.91	2.14	0.08	<0.001	0.002	0.177	0.452	0.155	0.739
C18:0	86.8	65.1	84.4	71.3	0.56	<0.001	0.007	0.734	0.453	0.724	0.702
Trans-10 C18:1	9.12	9.77	8.95	8.96	0.22	0.011	0.151	0.039	0.165	0.980	0.835
Trans-11 C18:1	31.7[b]	35.8[a]	31.1[b]	31.5[b]	0.72	0.006	0.008	0.004	0.023	0.986	0.662
Cis-9 C18:1	190	205	178	188	4.19	0.002	0.009	0.004	0.459	0.896	0.487
Cis-11 C18:1	3.67	3.94	3.62	3.66	0.08	0.006	0.071	0.071	0.183	0.907	0.589
Cis-9,*trans*-11 CLA	5.64	6.64	5.25	5.51	0.32	<0.001	0.065	0.029	0.253	0.939	0.683
Trans-10,*cis*-12 CLA	0.86	0.81	0.72	0.80	0.05	<0.001	0.830	0.203	0.235	0.592	0.134
C18:2*n* − 6	29.3[b]	35.2[a]	26.7[b]	26.6[b]	1.40	<0.001	0.054	0.001	0.049	0.272	0.406
C18:3*n* − 3	3.61[b]	6.11[a]	3.32[b]	3.42[b]	0.20	<0.001	<0.001	<0.001	<0.001	0.022	0.099
C20:0	1.57	1.49	1.53	1.45	0.08	<0.001	0.375	0.608	0.985	0.112	0.312
Cis-11 C20:1	0.34	0.33	0.31	0.33	0.01	0.021	0.598	0.322	0.121	0.439	0.311
C20:4*n* − 6	1.51	1.98	1.25	1.49	0.11	<0.001	0.005	0.003	0.303	0.897	0.787
C21:0	0.40	0.41	0.39	0.39	0.02	<0.001	0.874	0.216	0.662	0.057	0.703
C22:0	0.31	0.29	0.27	0.24	0.03	<0.001	0.327	0.096	0.786	0.225	0.510
t10/t11 ratio	0.29	0.27	0.29	0.28	0.003	0.731	0.012	0.082	0.108	0.732	0.577
OBCFA	20.2	22.6	20.8	22.8	0.51	<0.001	0.001	0.438	0.744	0.321	0.273
SCFA	31.4	31.9	32.3	31.4	1.30	<0.001	0.866	0.901	0.570	0.354	0.840
MCFA	594	585	611	614	10.5	<0.001	0.814	0.043	0.568	0.903	0.513
LCFA	375	384	357	354	10.0	<0.001	0.752	0.031	0.568	0.890	0.568
SFA	707	672	723	710	5.92	<0.001	0.001	<0.001	0.087	0.982	0.454
MUFA	243	268	231	243	5.26	0.003	0.003	0.003	0.236	0.921	0.527
PUFA	41.0	50.9	37.4	37.9	1.54	<0.001	0.004	<0.001	0.009	0.185	0.214

Notes: [a,b]Means with different superscripts within a row differ significantly ($P < 0.05$).
Means by treatment were the pooled data ($n = 6$) of 3, 6, 9 and 12 wk.
CON, control; FUM, fumarate; NPD, *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide; FN, FUM + NPD; SEM, standard error of means; CLA, conjugated linoleic acid; OBCFA, odd carbon and branched-chain fatty acid; SCFA, short-chain fatty acid (C6:0–C8:0); MCFA, medium-chain fatty acid (C10–C16); LCFA, long-chain fatty acid (C17:0–C24); SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

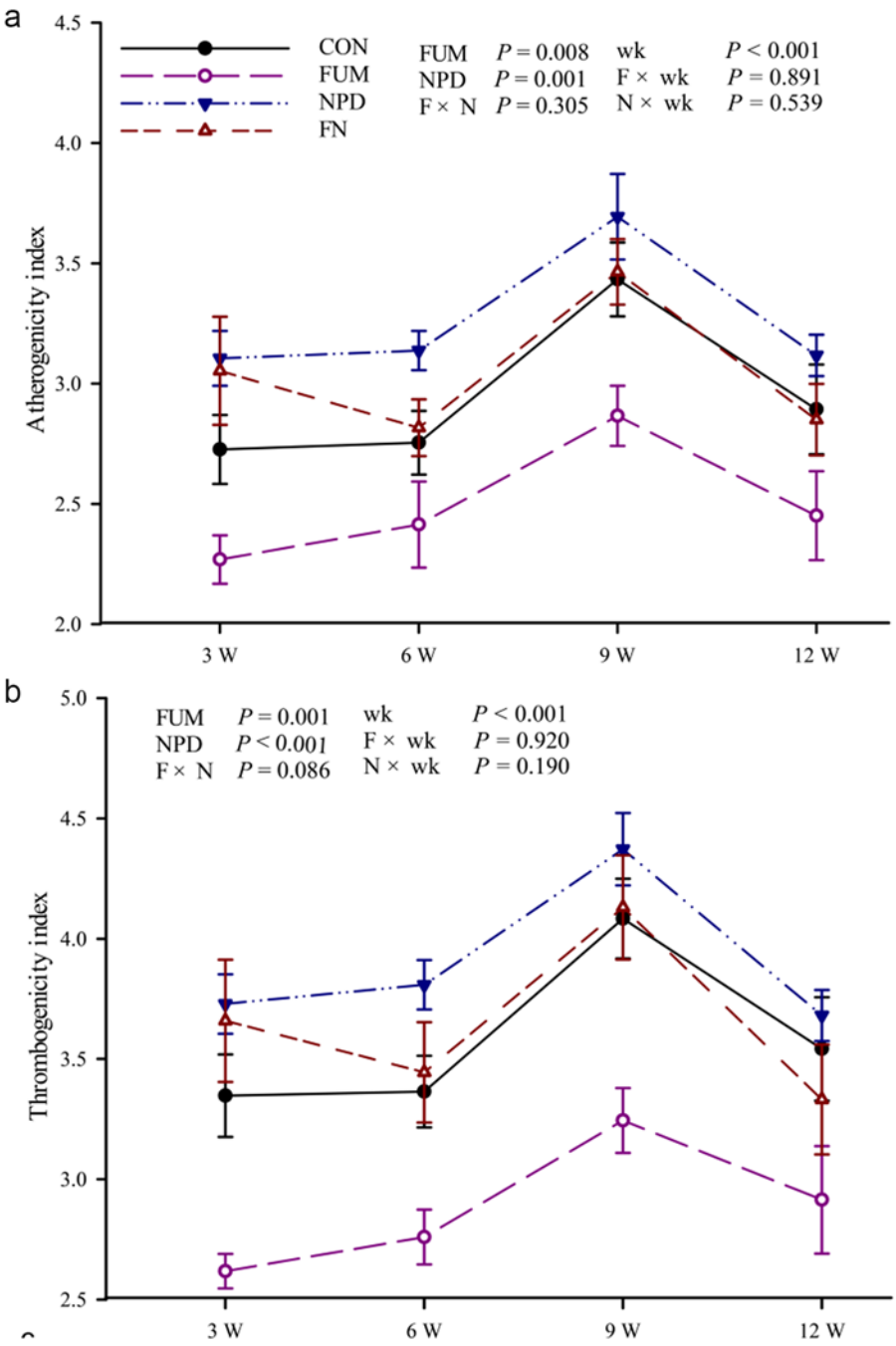

Figure 2. Temporal effects of fumarate (FUM), *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD), and their combination on the atherogenecity index (a), thrombogenecity index (b), and desaturase index (c) in milk. C, control; FN, FUM + NPD. The *P*-value of the repeated-measures ANOVA is presented at the top.

when FUM supplemented in combination with both biotin and monensin.

Discussion

Interdependence among RBH, propionogenesis and methanogenesis

Rumen microbial acetate and butyrate fermentation releases [H], which can be converted to H_2 by hydrogenase (Hegarty 1999) for intercell hydrogen transfer. Methanogenesis, propionogenesis and RBH are primary sinks for ruminal hydrogen. In this study, we proved that manipulation of ruminal methanogenesis and propionogenesis by FUM and NPD could redirect hydrogen toward or away from RBH in dairy goats. The metagenomic analyses showed neither FUM nor NPD affect rumen microbial flora structure and function, suggesting that the FUM and NPD induced hydrogen redirection were likely influenced at the metabolic level rather than at microbial level. Propionogenesis and RBH can both utilize the intracellular [H] transfer and intercellular H_2, while methanogens can only use interspecies-transferred H_2 (Wang et al. 2013). Therefore, RBH is more metabolically interdependent with propionogenesis in hydrogen competition than with methanogenesis, although methanogenesis is the largest sink for ruminal hydrogen. This explains the greater response of rumen FA profiles to FUM than to NPD. Consistently, Yang et al. (2019) observed

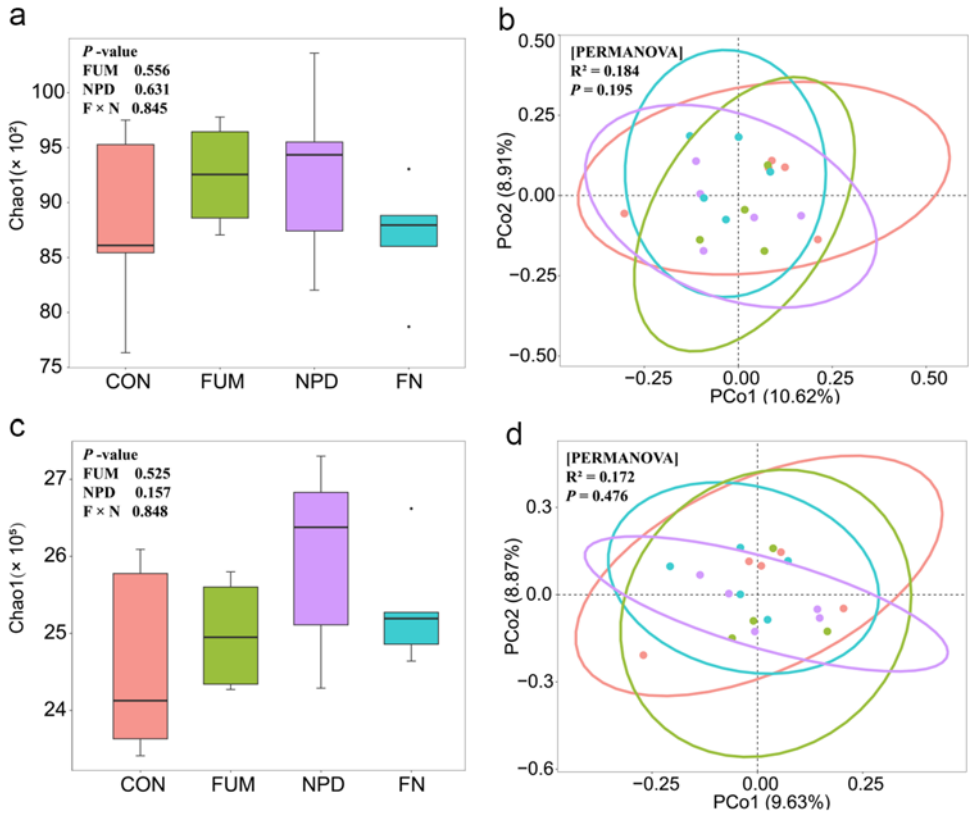

Figure 3. Effects of fumarate (FUM) and *N*-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD), and their combination on the rumen microbial α- or β-diversity at the strain (a, b) or gene level (c, d).

Table 3. Effect of dietary treatments on the *in vitro* methane production, fatty acid composition and fermentation parameters

	Treatment								*P*-value					
Item	CON	FUM	BIO	MEN	FB	FM	FBM	SEM	FUM	BIO	MEN	FB	FM	FBM
pH	6.77	6.65	6.70	6.74	6.73	6.70	6.65	0.051	0.166	0.423	0.491	0.173	0.714	0.101
CH_4 production, mL	11.24	10.55	11.17	10.72	10.66	10.30	10.35	0.387	0.025	0.946	0.086	0.742	0.591	0.902
Gas production, mL	70.40^{b}	78.20^{a}	75.40^{ab}	72.80^{ab}	78.00^{a}	77.00^{a}	77.20^{a}	2.604	0.001	0.067	0.686	0.041	0.156	0.883
Total VFA, mM	75.86	79.70	73.67	75.45	81.22	85.47	85.53	3.123	<0.001	0.784	0.138	0.425	0.150	0.734
Acetate, mM	49.22	49.14	47.92	48.49	49.73	53.07	52.07	1.701	0.014	0.489	0.137	0.589	0.065	0.497
Propionate, mM	14.74	18.56	14.22	15.00	19.02	20.16	20.41	0.860	<0.001	0.971	0.060	0.398	0.235	0.854
Butyrate, mM	8.48	8.52	8.28	8.35	8.85	8.54	9.03	0.577	0.242	0.644	0.999	0.371	0.735	0.841
A: P	3.34	2.65	3.37	3.24	2.62	2.65	2.55	0.090	<0.001	0.608	0.187	0.337	0.470	0.557
Fatty acid proportion, g/kg total FA														
t10/t11 ratio	0.16	0.19	0.19	0.18	0.16	0.16	0.18	0.026	0.121	0.335	0.133	0.218	0.245	0.173
SFA	670.0	620.0	682.1	606.3	622.2	594.5	578.2	21.81	<0.001	0.915	<0.001	0.414	0.221	0.492
MUFA	150.8^{b}	168.6^{b}	150.5^{b}	175.8^{a}	173.5^{a}	179.7^{a}	181.7^{a}	10.08	<0.001	0.518	<0.001	0.539	0.015	0.686
PUFA	179.2	211.4	167.4	217.8	204.3	225.8	240.0	17.23	0.013	0.775	0.007	0.488	0.544	0.408

Notes: ^{a,b}Means ($n = 5$) with different superscripts within a row differ significantly ($P < 0.05$).
CON, control; FUM, fumarate; BIO, biotin; MON, monensin; FB, FUM + BIO; FM, FUM + MON; FBM, FUM + BIO + MON; SEM, standard error of means; A:P, the ratio of acetate to propionate; VFA, volatile fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

that methanogenesis and RBH were more independent than previously suggested (Lourenço et al. 2010). Our results suggested that the H_2 sinks and the [H] sinks in the rumen should be treated differently and separately when investigating the interdependence among RBH, VFA profiles and methanogenesis or other hydrogen-utilizing processes.

Although both methanogenesis and propionogenesis could compete hydrogen with RBH, methanogenesis represents a loss

of dietary energy and a cause of greenhouse effect (Hristov et al. 2013) while propionogenesis represents an energy-rendering pathway due to incorporating hydrogen energy and the main precursor of gluconeogenesis in ruminants (Millen et al. 2016). Therefore, it is unwise to reduce RBH by enhancing methanogenesis, while is wise by enhancing propionogenesis.

FUM – a promising RBH inhibitor and milk UFA improver

Because of RBH, the outflow and transfer of dietary UFA from the rumen to milk are limited (Chilliard et al. 2007). For a long time, researchers have been exploring strategies to improve the content and composition of UFA in milk, such as feeding a high fresh grass diet or supplementing vegetable oils or oilseeds (Alves et al. 2017; Bainbridge et al. 2017; Hassan et al. 2020). Our results suggest that supplementation with the propionate enhancer FUM protects dietary UFAs (i.e., C18:2*n* − 6 and C18:3*n* − 3) against RBH by reducing its first step and last step, which results in the accumulation of C18:2*n* − 6, C18:3*n* − 3 and *trans* C18:1 BI. The FUM-increased milk PUFA was partly attributed to the increased supply of PUFA from the gut. In addition, increased milk *cis*-9 FA (i.e., *cis*-9 C14:1, *cis*-9 C16:1, *cis*-9 C17:1 and *cis*-9 C18:1) and Δ-9 desaturase index suggest that higher activity of Δ9 desaturase enzyme occurred in the FUM-fed animals. The biosynthesis of Δ9 UFA involves a multi-enzyme system that includes cytochrome b5 reductase, cytochrome b5 and desaturase, which are all located on the mitochondrial membrane. Cytochrome b5 reductase is well-established as using NADH from tricarboxylic acid cycle as an electron donor to catalyze the reduction of cytochrome b5, which then transfers electrons to activate desaturase (Zhang et al. 2016). A recent study showed that FUM supplementation could enhance the tricarboxylic acid cycle in dairy goats (Dong et al. 2024), which likely explain the enhanced activity of desaturases by FUM. Taken together, the reduced RBH and enhanced activity of Δ9 desaturase by FUM modulated the milk FA composition toward higher PUFA and MUFA proportions with lower thrombogenicity and atherogenicity indexes, which are used to predict the risk of ischemic heart disease in humans (Ulbricht and Southgate 1991). Moreover, these beneficial shifts of the milk FA profiles in response to FUM were persistent throughout the 12-wk of experiment, making FUM a reliable milk UFA improver.

Inclusion of NPD enhanced RBH, resulting in lower milk PUFA and MUFA proportions and higher thrombogenicity and atherogenicity indexes. These changes might be attributed to redirection of the hydrogen spared from methanogenesis by inhibitor was partly diverted to RBH. Consistently, inhibiting methanogenesis with 3-nitrooxypropanol also increased the proportion of SFA in the milk fat of dairy cows (Melgar et al. 2021). Therefore, the adverse effects of methanogenesis inhibitor on milk FA profile need to be taken seriously when the inhibitors were fed to dairy animals in the farm. But the adverse effects of NPD in altering the milk FA profile can be eliminated when supplemented in combination with FUM, suggesting that the hydrogen spared from the inhibited methanogenesis by NPD was more likely used for propionate synthesis rather than for RBH.

Enhancing the recovery of FUM as propionate could improve its anti-RBH and anti-methanogenesis potential *in vitro*

Inconsistent with previous *in vivo* studies (Li et al. 2021), the average recovery of FUM as propionate has been showed only half of added FUM *in vitro* (Ungerfeld et al. 2007) with lower-than-expected anti-methanogenesis potential, which is in accordance with our observations. Previous explanation for the low conversion was that high FUM concentration and short incubation times may exceed the rate of FUM utilization *in vitro*, and inferred that was limited by FUM-reducer, biotin or vitamin B_{12} availability (Ungerfeld et al. 2007). This explanation and inference were proved in this study that monensin and biotin supplementation improved the conversion of added FUM to propionate. As a result, the anti-RBH potential and anti-methanogenesis potential of FUM were improved when supplemented in combination with both biotin and monensin, which further confirmed the hydrogen cross-feeding among RBH, propionogenesis and methanogenesis.

FUM increases the microbial FAs

The increased OBCFA in the milk and the rumen of FUM-fed goats indicate that FUM increased the outflow of bacteria because OBCFA are predominantly of bacterial origin and generally absent from feeds (Or-Rashid et al. 2007). The increased OBCFA are likely associated with FUM-enhanced propionogenesis (Li et al. 2021), as odd-chain FA are formed through elongation of propionate or valerate by rumen microbes (Kaneda 1991; Or-Rashid et al. 2007). As rumen microbes contain a lower UFA content, the increased OBCFA by FUM would provide structural lipids with optimal fluidity for cell membranes because of their lower melting point than the corresponding straight-chain SFA (Buccioni et al. 2012; Or-Rashid et al. 2007).

Conclusion

Manipulation of ruminal methanogenesis and propionogenesis can redirect hydrogen toward or away from RBH and thereby influence the milk FA profiles. The responses of rumen and milk FA profiles to FUM being greater than those to NPD, because RBH is more metabolically interdependent with propionogenesis, due to linking by intracellular [H], than with methanogenesis. Enhancing the recovery of FUM as propionate by monensin and biotin could improve its anti-RBH potential *in vitro*. The inclusion of FUM in the dairy goat diet can be a promising strategy to persistently reduce RBH and enhance the activity of Δ9 desaturase, both of which work together to improve the content of milk UFA and reduce milk thrombogenicity index and atherogenicity index, a more nutritionally desirable milk FA profiles.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2024.22.

Data availability statement. The raw sequencing data are available in the China National GeneBank DataBase (CNGBdb, https://db.cngb.org/cnsa/) under the accession number CNP0005751.

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Conflicts of interests. The authors declare that they have no conflicts of interests.

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