

ORIGINAL ARTICLE

Hydroxy-selenomethionine supplementation promotes the *in vitro* rumen fermentation of dairy cows by altering the relative abundance of rumen microorganisms

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Abstract

Aims: This study aims to investigate the effect of hydroxy-selenomethionine supplementation on the *in vitro* rumen fermentation characteristics and microorganisms of Holstein cows.

Methods and Results: Five fermentation substrates, including control (without selenium supplementation, CON), sodium selenite supplementation (0.3 mg kg⁻¹ DM, SS03), and hydroxy-selenomethionine supplementation (0.3, 0.6 and 0.9 mg kg⁻¹ DM, SM03, SM06 and SM09, respectively) were incubated with rumen fluid *in vitro*. The results showed that *in vitro* dry matter disappearance and gas production at 48 h was significantly higher in SM06 than SM03, SS03 and CON; propionate and total volatile fatty acid (VFA) production was higher in SM06 than CON. Moreover, higher species richness of rumen fluid was found in SM06 than others. Higher relative abundance of *Prevotella* and *Prevotellaceae-UCG-003* and lower relative abundance of *Ruminococcus-1* were detected in SM06 than CON. Besides, higher relative abundance of *Ruminococcaceae_UCG-005* was found in CON than other treatments.

Conclusions: It is observed that 0.6 mg kg⁻¹ DM hydroxy-selenomethionine supplementation could increase cumulative gas production, propionate, and total VFAs production by altering the relative abundance of *Prevotella*, *Prevotellaceae-UCG-003*, *Ruminococcaceae_UCG-005* and *Ruminococcus-1*, so that it can be used as a rumen fermentation regulator in Holstein cows.

Significance and Impact of the Study: This study provides an optimal addition ratio of hydroxy-selenomethionine on rumen fermentation and bacterial composition via an *in vitro* test.

KEYWORDS

Holstein cow, *in vitro* test, rumen fermentation, rumen microorganism, selenium

INTRODUCTION

Selenium is an indispensable trace element that plays a central role in the metabolism of humans, plants and microorganisms. Inorganic selenium could transform into bioavailable organic forms such as selenomethionine and selenocysteine (Mangiapane et al., 2014). Dietary selenium supplementation improves the antioxidant status of rumen microorganisms and promotes rumen fermentation as well as microbial growth (Mihalikova et al., 2005). The addition of 0.04 (Shi et al., 2011) or 0.07 (Wang et al., 2009) mg kg⁻¹ selenium in the diet can promote the rumen fermentation of dairy cows. Selenium in both inorganic and organic forms is common in dietary supplements used on dairy farms. Inorganic selenium (sodium selenite, SS) is widely used because of its low cost. However, organic selenium has a higher absorption rate (Boldizarova et al., 2005), greater biological activity (Ortman & Pehrson, 1999), higher tissue accumulation rate (Briens et al., 2014) and lower toxicity compared with inorganic selenium.

Hydroxy-selenomethionine (SM) contains the active compound 2-hydroxy-4-methylselenobutanoic acid. In 2013, it was approved for the use as an organic selenium source in some countries (EFSA, 2013), and in July 2021, the US Food and Drug Administration (FDA) issued Document No. 2021-15072, which approved selenomethionine hydroxyl analogues as the source of selenium in the feed of beef cattle and dairy cows (FDA, 2021). Although SM has been shown to improve the relative bioavailability of Se compared with selenium yeast in monogastric animals by several studies (Briens et al., 2013, 2014; Jlali et al., 2013), little research has been conducted regarding the effects of SM on dairy cow production. Wei et al. (2019) found that SM has been shown to promote rumen fermentation and apparent nutrient digestibility compared with SS, suggesting that it has greater apparent absorption. Meanwhile, Sun et al. (2017) demonstrate that SM improves antioxidant status and increases milk and plasma Se concentrations more effectively than SS, indicating it could replace SS as an effective organic Se source for lactating dairy cows. However, whether dietary SM supplementation could regulate rumen microbial composition so as to affect the rumen fermentation of dairy cows remains unclear. Besides, the study indicated that Se-yeast supplementation could mitigate CH₄ emissions from sheep (Pan et al., 2021) and the effect of SM on CH₄ emissions remains unknown. Therefore, this study was performed to analyse the effect of SM on the rumen fermentation, the relative abundance of rumen microorganisms as well as CH₄ emissions of Holstein cows using an *in vitro* gas test.

TABLE 1 Ingredients and nutrient levels of the fermentation substrate

Ingredient (% DM)		Nutrient level (% DM)	
Extruded soybean	0.4	Dry matter	97.8
Steam-flaked corn	5.4	Crude protein	16.7
Spray corn husk	0.2	Ash	8.3
High-fat DDGS	1	Ether extract	4.8
Soybean hull	0.4	Acid detergent fiber	16.3
Xinjiang Whole cottonseed	3.1	Neutral detergent fiber	27.2
MECALIC	0.5		
Fatty powder	0.4		
Beet pellet	0.6		
Water	10.5		
Spanish alfalfa	1.3		
Cane molasses	1		
American alfalfa	2.1		
Oat hay	1.5		
Corn silage	44.8		
Alfalfa silage	4.9		
Concentrate ¹	21.9		
Total	100.0		

¹One kilogram of concentrate contains the following: VA 600000 IU, VD 100000 IU, VE 4000 IU, Fe 3000 mg, Cu 2000 mg, Mn 2500 mg, Zn 8000 mg, Se 60 mg, I 100 mg, and Co 20 mg.

MATERIALS AND METHODS

Materials

SM was obtained from Adisseo Life Science Co. Ltd., and the minimum content of selenium was 2.0%. SS was provided by Beijing Aiweijia Biological Technology Co., Ltd., and the minimum content of selenium was 1.0%. The substrate of fermentation and the diet of the dairy cows that served as rumen fluid donors (Table 1) were provided by Jinyindao Farm of Beijing Capital Agribusiness Group (Beijing, China).

Experimental design

Rumen fluid was obtained from three healthy Holstein cows (116 ± 20 days in milk) with similar body weight and permanent rumen fistulas. The cows had the same diet and free access to feed and water. There were five treatments with five replicates for each treatment: control (without selenium supplementation, CON), sodium selenite supplementation (0.3 mg kg⁻¹ DM, SS03), hydroxy-selenomethionine supplementation (0.3, 0.6 and 0.9 mg kg⁻¹ DM, SM03, SM06 and SM09). In this regard,

0.3, 0.6 and 0.9 mg kg⁻¹ DM were the final amount of selenium added to the substrate. Fermentation substrate and fluid samples were collected at 3, 6, 12, 24 and 48 h. In detail, the fermentation substrate samples were collected to analyse *in vitro* dry matter disappearance (IVDMD). The fermentation fluid samples were collected to detect the content of ammonia-N (NH₃-N), microbial crude protein (MCP) as well as volatile fatty acid (VFA) and chose some samples to do 16S rRNA gene sequencing to detect microbiological compositions ($n = 2, 3, 4, 3, 3$ in CON, SS03, SM03, SM06 and SM09, respectively). A three-way valve was used to collect the vented gas by connecting it to pre-empted gasbags for the detection of methane production. The studies involving animals were reviewed and approved by China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical (No. AW62701202-1-1).

***In vitro* batch incubation**

An AGRS-III simulated culture device (Beijing, China) was used to conduct *in vitro* anaerobic culture. The rumen fluid collected at 7:00 AM before feeding was filtered to remove feed residue and poured into thermos flasks. Equal amounts of the rumen fluid of three cows were mixed evenly at 39°C, and the air was removed with carbon dioxide. Next, 500 mg of the substrate was added into glass bottles, and buffer solution (50 ml) and rumen fluid (25 ml) were preheated to 39°C and placed into fermentation bottles. Bottles connected to the AGRS-III system were cultured for 48 h at 39°C to record cumulative gas production, and the other bottles were incubated for 3, 6, 12 and 24 h in a thermostatic tank at 39°C (Zheng et al., 2021). To ensure the consistency of rumen fluid, the substrates and buffer were added into the bottle first. Then rumen fluids were added into the bottles in the following order to avoid the experimental error: CON→SS03→SM03→SM06→SM09→CON→SS03→SM03→SM06→SM09....

Analyses

The content of dry matter, crude protein, ash, ether extract, acid detergent fibre and neutral detergent fibre was analysed following the method of AOAC (1999). The content of NH₃-N and MCP was detected through bright blue colourimetry (Verdouw et al., 1978) and Coomassie brilliant blue colourimetry (Makkar et al., 1982) under a wavelength of 595 nm using Microplate Reader (ELx800 light absorption photoenzyme reader, BioTek). The content of VFA was detected through gas chromatography (Erwin et al., 1961) using a gas chromatography (TP-2060,

R.F.TianPu). The detailed operation of NH₃-N, MCP, VFA and pH determination was followed (Zheng et al., 2021). The content of methane was analysed using the gas chromatographic method (Yang et al., 2014).

16S rRNA gene sequencing was performed as the following: After genomic DNA extraction, 1% agarose gel (Thermo Scientific) was used to detect the extracted genomic DNA. The primers (V338F (5'-ACTCCTACGGGAGGCAGCAG-3') and V806R (5'-GGACTACHVGGGTWTCTAAT-3')) targeting variable region V3-V4 were used (Peris et al., 2015). PCR amplification was performed in a MasterCycler Gradient thermocycler (Eppendorf), and the Miseq platform (Allwegene) was used for deep sequencing. The Illumina Analysis Pipeline Version 2.6 was used for image analysis, base calling and error estimation. Raw data were screened by eliminating sequences <230 bp in length, with quality scores <20, with ambiguous bases, or with poor matches between the primer sequence and barcode label. All qualified reads were clustered into operational taxonomic units (OTUs) with a similarity threshold of 97% (Edgar, 2013); sparse curves were generated, and richness and diversity indexes were calculated. Alpha diversity and beta diversity were tested by QIIME (University of California, San Diego, USA).

Calculations

In vitro dry matter disappearance (IVDMD) and gas production were calculated by Equation (1; Zheng et al., 2021) and Equation (2) (Groot et al., 1996); the time corresponding to the maximum rate of substrate degradation (TRmaxS, h), the maximum rate of substrate digestion (RmaxS, h), the time corresponding to RmaxG (TRmaxG, h), and the maximum gas production rate (RmaxG, ml/h) were calculated by Equations (3–6), respectively (Yang et al., 2005).

$$\text{IVDMD} = \text{DM before incubation} - \text{DM after incubation}(\%, \text{ air - dry basis}), \quad (1)$$

$$\text{GP}_t = A / (1 + (C/t)^B), \quad (2)$$

where 'GP_t' is the cumulative gas production (ml g⁻¹ DM) at incubation time 't' (h), 'A' is the asymptotic gas production (ml g⁻¹ DM), 'B' is a sharpness parameter determining the shape of the curve, 'C' is the time (h) at which half of 'A' is reached and 't' is the *in vitro* incubation time.

$$\text{TRmaxS} = C \times (B-1)^{(1/B)}, \quad (3)$$

$$\text{RmaxS} = (B \times \text{TRmaxS}^{(B-1)}) / (C^B + \text{TRmaxS}^B), \quad (4)$$

$$\text{TRmaxG} = C \times ((B-1)/(B+1))^{(1/B)}, \quad (5)$$

$$\text{RmaxG} = (A \times C^B \times B \times \text{TRmaxG}^{-B-1}) / (1 + C^B \times \text{TRmaxG}^{(-B)})^2, \quad (6)$$

Statistical analysis

Data were processed using Excel 2016. The NLIN procedure in SAS 9.2 (SAS Institute Inc.) was used to calculate kinetic parameters in Equations (2–6). The rarefaction curves were based on the OTU clustering results, and Mothur was used for rarefaction analysis. The Shannon–Wiener Curves were calculated using Mothur based on the OTU clustering results, and the Shannon values were calculated using different random samples. Venn, rarefaction curves, and Shannon–Wiener curves were made using R software (version 2.15.3). Significant differences between groups were defined as $p \leq 0.05$ using SAS 9.2 (SAS Institute Inc.). Spearman's rank correlation analysis was used to analyse the correlation between the *in vitro* rumen microbiota and rumen fermentation characteristics of Holstein cows under different sources of selenium.

RESULTS

In vitro rumen degradability and kinetic parameters

IVDMD and cumulative gas production at 48 h (GP_{48}) was higher in SM06 than in CON, SS03 and SM03 ($p < 0.01$, $p = 0.04$), and increased linearly with the SM dose ($p = 0.02$, 0.03). Besides, after cultivation for 6 h, IVDMD was significantly higher in SM03 than in SS03 ($p = 0.04$) whilst no significant differences between each treatment and the control were observed when cultured for 3, 12, and 24 h ($p = 0.08$, 0.26 and 0.10). Other gas production kinetic parameters were not affected by treatments ($p > 0.05$; Table 2).

In vitro fermentation characteristics

The *in vitro* fermentation characteristics $\text{NH}_3\text{-N}$, MCP, pH, VFA pattern and methane are shown in Table 3. Higher $\text{NH}_3\text{-N}$ concentrate was observed in SM06 and SS03 compared with CON at 3 h ($p = 0.04$), and no significant differences were observed under *in vitro* culture for 6, 12, 24 and 48 h ($p = 0.84$, 0.98 , 0.70 and 0.45 , respectively). MCP concentrate was higher in all treatments compared with CON at 3 h ($p < 0.01$), and it increased quadratically and cubically with the SM dose ($p < 0.01$). MCP concentrate was higher in SM03 than in SM09 ($p = 0.04$) at 6 h, MCP

concentrate was lower in SM03 than in other treatments ($p = 0.01$) at 12 h, and MCP concentrate was significantly higher at 24 h in SM06 than in SM03 and SM09 ($p = 0.02$). However, under *in vitro* culture for 48 h, the MCP concentrate was higher in CON than in SM03, SM06 and SM09 ($p = 0.02$). Propionate concentrate was significantly higher in SM06 compared with CON, and it increased linearly with the SM dose ($p = 0.02$). Total VFA concentrate was significantly higher in all treatments compared with CON ($p = 0.04$), and it increased linearly and quadratically with the SM dose ($p < 0.01$). Furthermore, no significant differences were observed in pH, acetate concentrate, butyrate concentrate, acetate/propionate and CH_4 production ($p = 0.86$, 0.29 , 0.64 , 0.93 , and 0.23 , respectively).

Rumen microbiome

There were 1,881 operational taxonomic units (OTUs) obtained from CON, SS03, SM03, SM06, and SM09, 70 of which were common OTUs among the five treatments (Figure 1). Rarefaction curves revealed that the sequencing depth of rumen fluid samples of dairy cows enough in each treatment (Figure 2a). The Shannon–Wiener curve also indicated that the sequencing data were sufficient for reflecting most of the microbial information in the samples (Figure 2b). This was also different from the Chao1, Shannon and Observed_species diversity index, all of which indicated that species richness was significantly higher in SM06 compared with the other treatments (Figure 3a–c, $p < 0.05$).

The relative abundances of the four most common taxa in different treatments at the phylum level are shown in Figure 4. *Firmicutes* and *Bacteroidetes* were the most abundant, followed by *Proteobacteria* and *Verrucomicrobia*. The relative abundance of *Firmicutes* was higher in CON (52%) than in other treatments (SS03, SM03, SM06, SM09 = 48%, 48%, 50%, 48%, respectively). The relative abundance of *Bacteroidetes* was higher in SM06 (35%) than in CON (31%) and significantly higher than SM03 (33%, $p = 0.01$), and its relative abundance was also higher in SS03 (34%), SM03 (33%) and SM09 (33%) than in CON (31%). The relative abundance of *Proteobacteria*, *Verrucomicrobia*, and other species did not significantly differ among treatments ($p > 0.05$).

The relative abundances of the four most common taxa in different treatments at the genus level are shown in Figure 5. Significant differences among treatments were observed for *Prevotella_1*, *Ruminococcus_1*, *Ruminococcaceae_UCG-005* and *Prevotellaceae_UCG-003*. The relative abundance of *Prevotella_1* was higher in SM06 (8.20%) than in CON (5.48) and significantly higher than SM09 (6.90%, $p = 0.02$); its relative abundance was also higher in SM03 (7.92) than in CON (5.48%). The relative abundance of *Ruminococcus_1* and

TABLE 2 *In vitro* rumen degradability and kinetic parameters of Holstein cows under different sources of selenium¹

Item ³	Treatment ²					SEM	p value ⁴			
	CON	SS0.3	SM0.3	SM0.6	SM0.9		Dose ⁵			
							Treatment	Linear	Quadratic	Cubic
IVDMD (%)										
3 h	57.89	58.61	55.20	57.22	59.21	0.47	0.08	0.82	0.17	0.17
6 h	58.70 ^{ab}	57.09 ^b	63.30 ^a	58.06 ^{ab}	57.70 ^{ab}	0.81	0.04	0.94	0.12	0.14
12 h	63.57	65.59	65.40	64.85	65.37	0.49	0.26	0.27	0.52	0.52
24 h	75.59	78.24	77.85	78.30	77.84	0.34	0.10	0.07	0.15	0.15
48 h	87.02 ^c	87.89 ^c	88.06 ^{bc}	89.98 ^a	88.32 ^{ab}	0.38	0.04	0.03	0.33	0.34
GP ₄₈ (ml g ⁻¹ DM)	64.73 ^c	64.63 ^c	71.66 ^{bc}	86.04 ^a	81.81 ^{ab}	9.81	<0.01	0.02	0.08	0.08
Kinetic parameters										
A (ml g ⁻¹ DM)	72.29	71.37	78.65	90.01	90.37	8.79	0.13	0.06	0.18	0.25
B	1.16	1.47	1.35	1.39	1.37	0.18	0.67	0.14	0.34	0.34
C (h)	7.62	7.39	8.48	8.15	7.50	1.07	0.91	0.72	0.11	0.11
TRmaxG (h)	0.89	2.01	2.01	2.15	1.92	0.80	0.62	0.17	0.36	0.36
RmaxG (ml h ⁻¹)	7.45	6.52	5.92	7.61	7.35	1.27	0.66	0.98	0.61	0.64
TRmaxS (h)	1.69	3.71	3.76	4.00	3.60	1.46	0.61	0.17	0.35	0.35
RmaxS (h ⁻¹)	0.11	0.12	0.09	0.10	0.10	0.03	0.84	0.25	0.21	0.21

¹Means in the same row with different superscripts differ significantly ($p \leq 0.05$); SEM: standard error among five treatments ($n = 5$).

²CON, SS03, SM03, SM06 and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6 and 0.9 mg kg⁻¹ DM, respectively); SS: sodium selenite, SM: hydroxy-selenomethionine.

³IVDMD: *in vitro* dry matter degradability (%); GP₄₈: cumulative gas production (ml g⁻¹ DM) at 48 h; A: asymptotic gas production (ml g⁻¹ DM); B: a sharpness parameter determining the shape of the curve; C: the time (h) at which half of A is reached, and t is the *in vitro* incubation time; TRmaxS: the time at which the maximum rate of substrate degradation is reached (h); RmaxS: the maximum rate of substrate digestion (h⁻¹); TRmaxG: the time at which RmaxG is reached (h); and RmaxG: the maximum gas production rate (ml h⁻¹).

⁴Linear, Quadratic, and Cubic: linear quadratic and cubic effect of dose.

⁵Dose: CON, SM03, SM06, SM09.

Ruminococcaceae_UCG-005 was higher in CON (1.61% and 1.07) than in the other treatments (SS03, SM03, SM06, SM09 = 1.26%, 1.17%, 1.12%, 1.34% and = 0.73%, 0.74%, 0.67%, 0.70%). The relative abundance of *Prevotellaceae_UCG-003* was higher in SM06 (1.14%) than in CON (0.69%) and significantly higher than SM09 (0.83%, $p = 0.01$); its relative abundance was also higher in SS03 (0.97%) and SM03 (1.01%) than in CON (0.69%).

Using Spearman correlation analysis to explore the relationship between the *in vitro* rumen microbiota and rumen fermentation characteristics of Holstein cows under different sources of selenium (Figure 6). The relative abundance of *Ruminococcaceae_UCG-005* was negatively related with propionate, total VFA, and IVDMD (correlation index = -0.9, $p = 0.04$). The relative abundance of *Ruminococcaceae_UCG-005* was positively related with MCP-48h (correlation index = 0.9, $p = 0.04$). The relative abundance of *Ruminococcus_1* was negatively related with NH₃-N-3h (correlation index = -0.9,

$p = 0.04$). Moreover, NH₃-N-3h was positively related with the relative abundance of *Prevotella_1* and *Prevotellaceae_UCG-003* (correlation index = 0.9, $p = 0.04$).

DISCUSSION

Soluble carbohydrates produce gas during the *in vitro* fermentation process. In this study, gas production was significantly higher in SM06 than in CON, SS03 and SM03 under *in vitro* culture for 48 h. This may stem from the addition of SM in the fermentation substrate, which improves the antioxidant status of rumen microorganisms, thereby promoting microbial growth as well as rumen fermentation (Hidiroglou et al., 1968; Mihalikova et al., 2005), including the fermentation of soluble carbohydrates. 16S rRNA gene sequencing revealed higher microbial diversity in SM06 than in other treatments; higher relative abundance of *Prevotella* in SM06 than in CON, SS03 and SM03;

TABLE 3 Rumens fermentation characteristics of Holstein cows under different sources of selenium¹

Item ³	Treatment ²						p value ⁴			
	CON	SS0.3	SM0.3	SM0.6	SM0.9	SEM	Treatment	Dose ⁵		
								Linear	Quadratic	Cubic
NH ₃ -N (mg dl ⁻¹)										
3 h	3.74 ^b	4.62 ^a	4.33 ^{ab}	4.73 ^a	4.19 ^{ab}	0.11	0.04	0.08	0.13	0.12
6 h	5.82	5.41	5.56	5.31	5.12	0.16	0.84	0.26	0.50	0.50
12 h	6.92	6.67	6.77	6.78	6.95	0.14	0.98	0.93	0.92	0.92
24 h	16.11	15.44	15.74	15.74	15.61	0.16	0.70	0.25	0.52	0.52
48 h	18.98	20.32	20.00	19.46	19.74	0.22	0.45	0.19	0.31	0.31
MCP (mg dl ⁻¹)										
3 h	4.62 ^b	5.88 ^a	5.87 ^a	5.41 ^a	5.48 ^a	0.10	<0.01	0.02	<0.01	<0.01
6 h	6.25 ^{ab}	6.38 ^{ab}	6.77 ^a	6.29 ^{ab}	5.52 ^b	0.12	0.04	0.32	<0.01	<0.01
12 h	5.61 ^a	5.60 ^a	4.75 ^b	5.95 ^a	5.45 ^a	0.10	0.01	0.92	0.26	0.33
24 h	6.18 ^{ab}	6.22 ^{ab}	5.73 ^b	6.50 ^a	5.67 ^b	0.08	0.02	0.38	0.66	0.61
48 h	7.41 ^a	6.57 ^{ab}	6.36 ^b	5.75 ^b	6.30 ^b	0.13	0.02	<0.01	<0.01	<0.01
Final pH										
	6.99	7.02	6.70	7.05	7.02	0.01	0.86	0.46	0.76	0.76
VFA (mmol L ⁻¹)										
Acetate	37.36	38.50	38.80	38.92	38.70	0.21	0.29	0.07	0.09	0.17
Propionate	14.82 ^b	15.60 ^{ab}	15.66 ^{ab}	15.98 ^a	15.82 ^a	0.12	0.02	0.02	0.07	0.07
Butyrate	6.21	6.36	6.39	6.43	6.54	0.05	0.64	0.13	0.32	0.32
Total VFA	58.39 ^b	60.31 ^a	60.85 ^a	61.33 ^a	61.02 ^a	0.63	0.04	<0.01	0.02	0.05
Acetate/ Propionate	2.52	2.47	2.48	2.44	2.45	0.01	0.93	0.46	0.64	0.65
Methane (ml)										
	7.29	8.20	7.73	6.86	7.06	0.20	0.23	0.70	0.58	0.60

¹Means in the same row with different superscripts differ significantly ($p \leq 0.05$); SEM: standard error among five treatments ($n = 5$).

²CON, SS03, SM03, SM06, and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6, and 0.9 mg kg⁻¹ DM); SS: sodium selenite, SM: hydroxy-selenomethionine.

³NH₃-N: ammonia N; MCP: microbial proteins; Final pH: pH at 48 h; VFA: volatile fatty acids, is calculated by the sum of acetate, propionate and butyrate.

⁴Linear, Quadratic, and Cubic: linear quadratic and cubic effect of dose.

⁵Dose: CON, SM03, SM06, SM09.

and higher relative abundance of *Prevotellaceae-UCG-003* in SM06 than in CON. Previous studies have shown that members of the phylum *Bacteroides* are mainly responsible for protein hydrolysis and carbohydrate degradation, whilst members of the phylum *Firmicutes* play an important role in energy utilization. (Chen et al., 2015; Wu et al., 2011). *Prevotella_1* belongs to *Bacteroides* and it closely related to the decomposition of starch. However, the interaction analysis between the relative abundance of *Prevotella_1* showed no relation between them, so that it could be further verified in subsequent studies. *Ruminococcaceae_UCG-005* belongs to *Firmicutes* and the interaction analysis between the relative abundance of *Ruminococcaceae_UCG-005* and IVDMD at 48 h showed that they have significant negative relation. Therefore, the addition of selenium may influence the relative abundance of *Prevotella_1* and *Ruminococcaceae_UCG-005*,

thereby increasing cumulative gas production in the fermentation system after *in vitro* incubation for 48 h.

The concentration of microbial protein not only reflects the ability of microorganisms to utilize NH₃-N but also indirectly reflects the relative abundance of microbes in the fermentation system. In this study, MCP content was significantly higher in SM06 than in SM03 and SM09 under *in vitro* culture for 24 h. 16S rRNA gene sequencing indicated that microbial diversity was significantly higher in SM06 than in the other treatments, which is consistent with the results of previous studies indicating that selenium supplementation may increase the relative abundance and activity of rumen microbes (Faixová et al., 2007; Mihalikova et al., 2014; Wang et al., 2007). However, the MCP content was significantly lower in each SM treatment than in CON following *in vitro* culture for 48 h. This may stem from the increase in the relative abundance and activity of rumen

FIGURE 1 Microbial OTU Venn diagram of the rumen microbiome under different sources of selenium. CON, SS03, SM03, SM06 and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6 and 0.9 mg kg⁻¹ DM, respectively). SS, sodium selenite; SM, hydroxy-selenomethionine

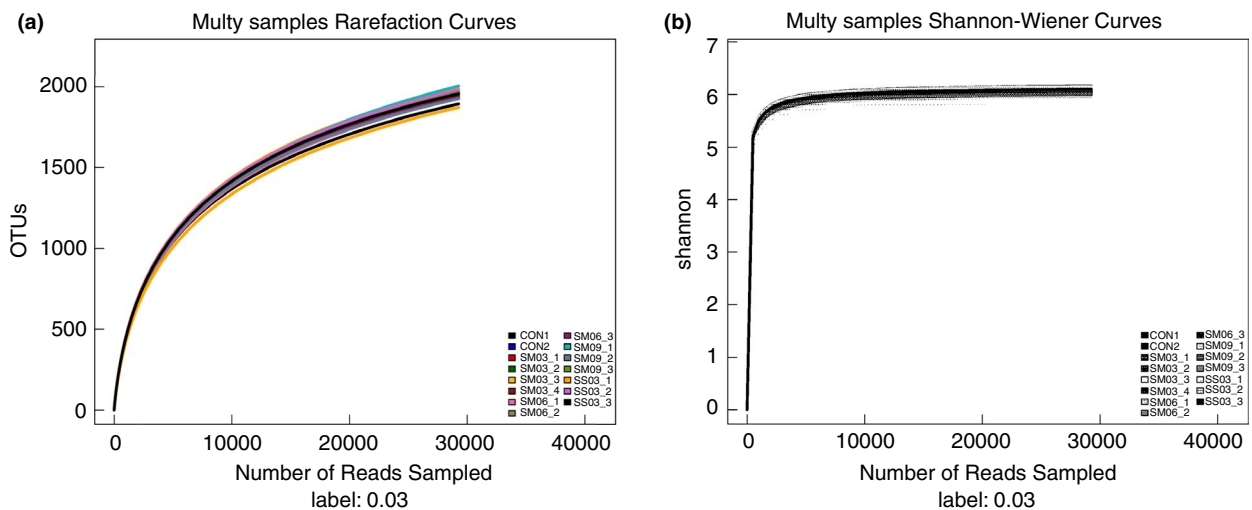
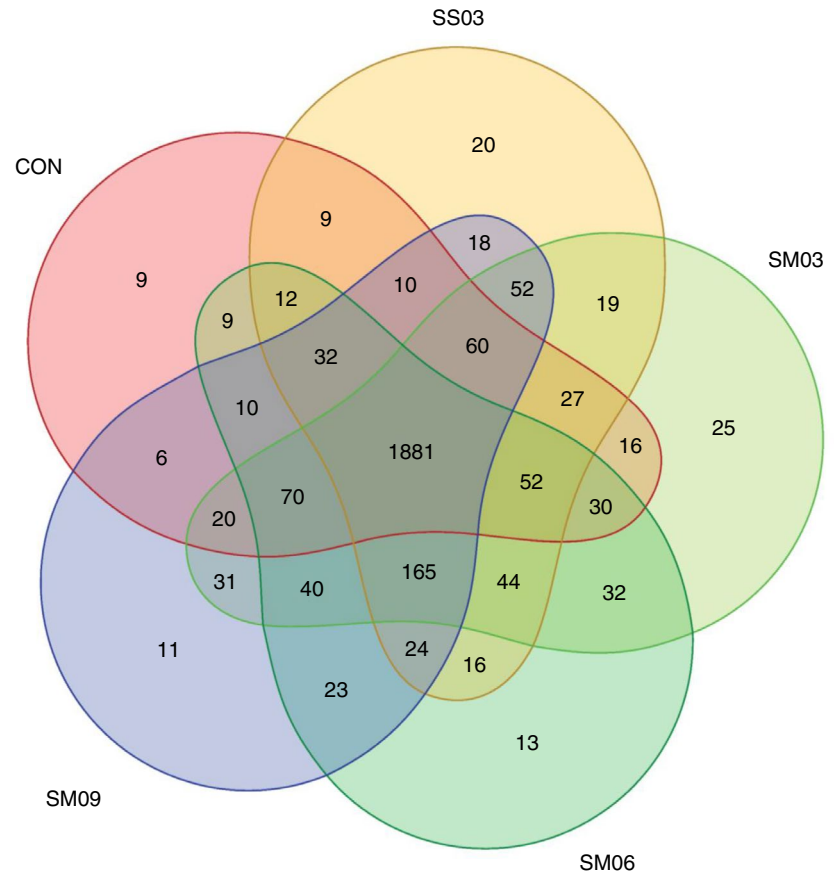


FIGURE 2 Diversity index of the rumen microbiome under different sources of selenium. (a) Rarefaction results based on OTUs (3% divergence) for each sample. (b) Shannon–Wiener curve (3% divergence). CON, SS03, SM03, SM06, and SM09 (addition of SS at a ratio of 0 or 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6 and 0.9 mg kg⁻¹ DM, respectively). SS, sodium selenite; SM, hydroxy-selenomethionine

microbes, which accelerates the rumen consumption of NH₃-N, leads to energy and nitrogen imbalances in the fermentation system, and inhibits MCP synthesis. In addition, rumen microorganisms, especially cellulolytic bacteria that synthesize protein using NH₃-N, use carbohydrate

fermentation to provide energy and carbon (Reynolds and Kristensen, 2008). *Rumencoccus* is composed of two powerful fibre-degrading bacteria that can decompose fibre into hemicellulase and cellulose through rumen fermentation, and cellulose can be further degraded into VFAs

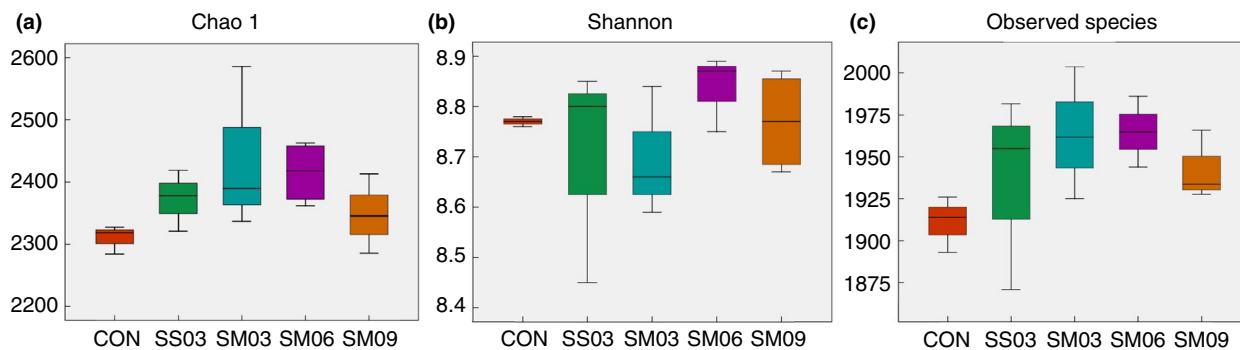


FIGURE 3 Diversity estimates based on the rumen bacterial 16S rRNA gene under different sources of selenium. (a) Chao 1; (b) Shannon; and (c) Observed_species. CON, SS03, SM03, SM06 and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6 and 0.9 mg kg⁻¹ DM, respectively). SS, sodium selenite; SM, hydroxy-selenomethionine

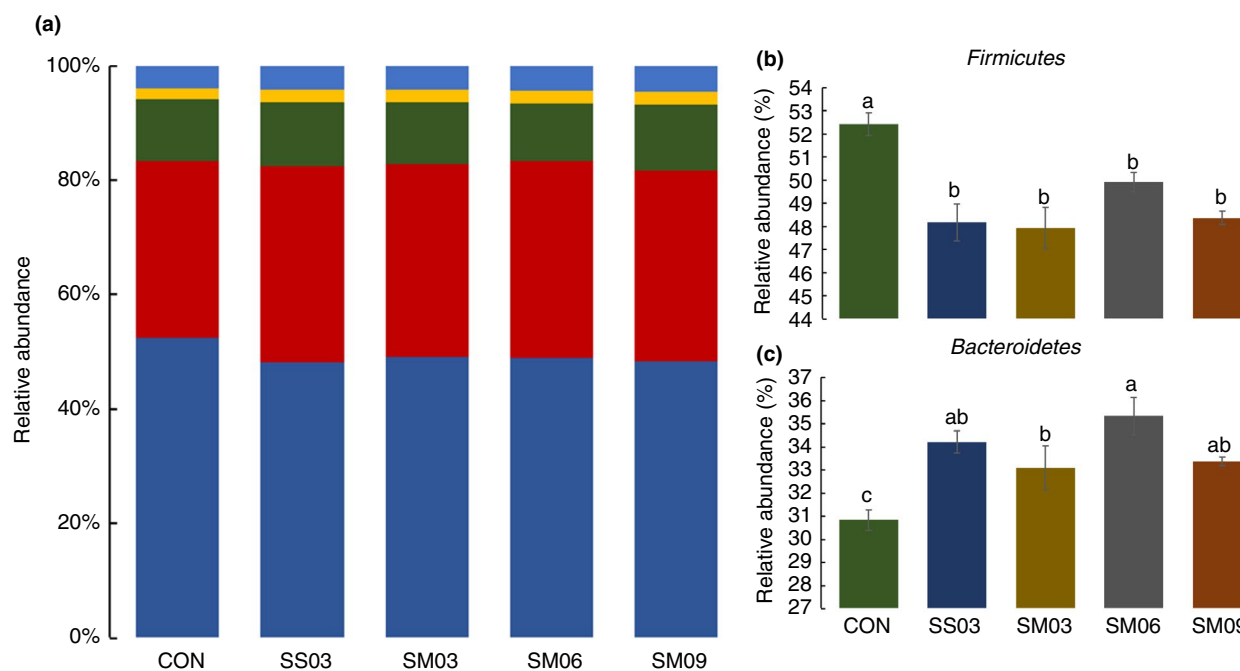


FIGURE 4 The relative abundance of microbial species under different sources of selenium. (a) Histogram of the relative abundances of species of the rumen microbiome in all treatments at the phylum level (abundances <0.1 were grouped into 'Other'). (b) The relative abundance of *Firmicutes* in all treatments. (c) *Bacteroidetes* relative abundance in all treatments. CON, SS03, SM03, SM06, and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6, and 0.9 mg kg⁻¹ DM, respectively). SS, sodium selenite; SM, hydroxy-selenomethionine

(Jami & Mizrahi, 2012). In this study, although the relative abundance of *Ruminococcaceae_UCG-005* was higher in CON than in the other treatments, this result needs to be further verified by *in vivo* test of dairy cows because sample size to do 16S rRNA gene sequencing in CON was small. Moreover, the interaction analysis between the relative abundance of *Ruminococcaceae_UCG-005* and MCP content at 48 h showed that they have a significantly positive relation, which explains the higher MCP content in CON compared with the SM treatments.

Volatile fatty acids are a crucial energy source for Holstein cows. Their composition, production, and ratio in rumen fluid are vital indicators of rumen fermentation function. Rumen acetate and butyrate are materials for milk fat synthesis (Mansson, 2008), and 66.7% of the glucose used to produce milk lactose is derived from propionate gluconeogenesis (Reynolds, 2006). Energy efficiency increases as the yield of propionate increases and the yield of acetate and butyrate decreases (Knapp et al., 2014). In this study, the production of propionate was higher in SM06 and SM09

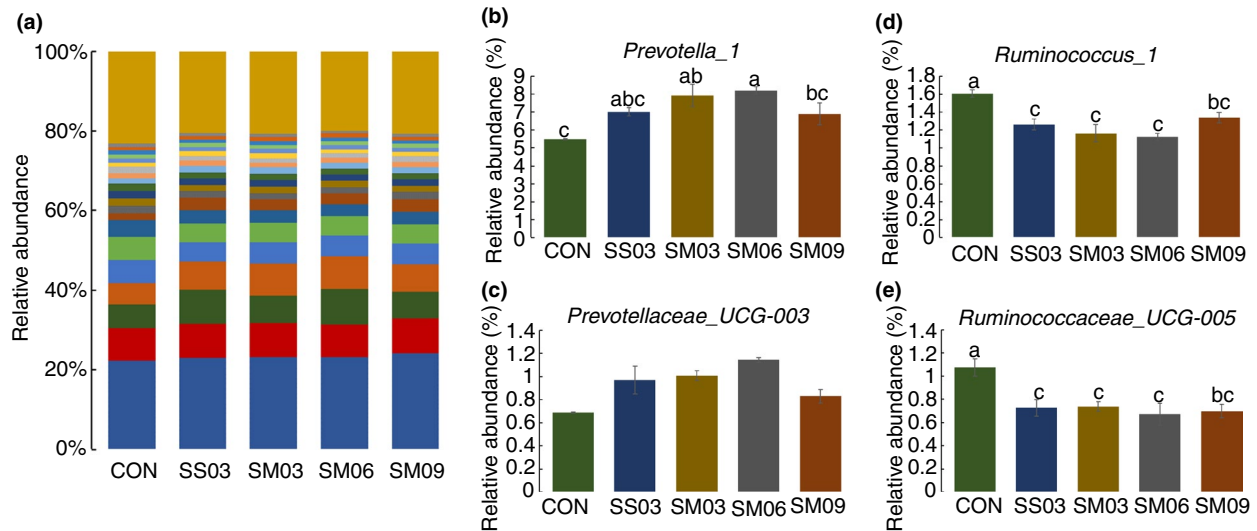


FIGURE 5 The relative abundance of microbial species under different sources of selenium. (a) Histogram of the relative abundances of species in the rumen microbiome in all treatments at the genus level (abundances <0.1 were grouped into ‘Other’). (b) *Prevotella_1* relative abundance in all treatments. (c) *Ruminococcus_1* relative abundance in all treatments. (d) *Prevotellaceae_UCG-003* relative abundance in all treatments. (e) *Ruminococcaceae_UCG-005* relative abundance in all treatments. CON, SS03, SM03, SM06 and SM09 (addition of SS at a ratio of 0 and 0.3 mg kg⁻¹ DM; addition of SM at a ratio of 0.3, 0.6 and 0.9 mg kg⁻¹ DM, respectively). SS, sodium selenite; SM, hydroxy-selenomethionine

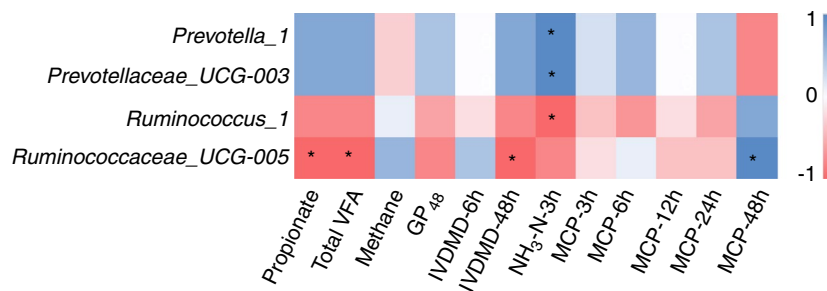


FIGURE 6 Heatmap of the Spearman rank correlations between the *in vitro* rumen microbiota and rumen fermentation characteristics of Holstein cows under different sources of selenium. *Means $p < 0.05$. IVDMD-6h, IVDMD-48h, *in vitro* dry matter degradability (%) at *in vitro* culture for 6 h and 48 h; GP₄₈, cumulative gas production (ml g⁻¹ DM) at 48 h; NH₃-N-3h, ammonia N content at *in vitro* culture for 3 h; MCP-3h, MCP-6h, MCP-12h and MCP-24h, MCP-48h, microbial proteins content at *in vitro* culture for 3 h, 6 h, 12 h, 24 h and 48 h respectively. VFA, volatile fatty acids

than in CON, and total VFA production was significantly higher in each treatment than in CON. This indicates that supplementation of SM in the fermentation substrate increases the production of propionate. In addition, acetate/propionate decreased as more SM was added, indicating that SM supplementation changes the mode of rumen fermentation of dairy cows from acetate to propionate. The decrease in acetate and propionate and increase in total VFA production stemmed from the increase in propionate production; these findings are consistent with those of Shi

et al. (2011), showing that the supplementation of 3 g kg⁻¹ DM of SM in the diet of sheep increased the concentration of total VFAs in the rumen. Wang et al. (2009) also showed that selenium yeast supplementation in the diet of dairy cows increased the total VFA concentration in the rumen.

The significant increase in propionate production in SM06 in this study may be related to the significant increase in the relative abundance of *Prevotella_1* and *Prevotellaceae-UCG-003*. *Prevotella* is a dominant bacteria that is thought to be responsible for starch fermentation to

produce propionate in the rumen. The positive response of the *Prevotella* flora should be due to the antioxidant function of dietary selenium. Studies have shown that rumen microorganisms form protein and cell wall components in the form of selenomethionine by adding selenium (Hidiroglou et al., 1968), and Se could protect cell membrane against oxidative damage by scavenging free radicals (Surai, 2006). Subsequent studies should examine Se content and glutathione peroxidase activity of ruminal bacteria of dairy cows. Previous studies showed that the presence of mixed protozoa (*Entodinium caudatum*, *Isotricha intestinalis*, *Metadinium medium* and *Eudiplodinium maggii*) altered the volatile fatty acids balance with more acetate and butyrate produced at the expense of propionate (Ranilla et al., 2007). Moreover, Cobanova et al. (2017) reported that the abundance of protozoa increased significantly after adding 0.4 mg kg⁻¹ DM selenium to the diet of sheep. Mihalikova et al. (2005) indicated that the addition of 0.3 mg Se kg⁻¹ DM has a positive effect on lamb rumen protozoa growth. Thus, the numerical increase in the production of acetate and butyrate after selenium adding, and the significant increase of propionate in SM 06 treatment in this study may be related to the relative abundance of different kinds of protozoa, so that subsequent studies should examine the effect of SM addition on the abundance and activity of rumen protozoa in the rumen of dairy cows. Significant increases in propionate production in SM06 may also explain the decrease in total methane production in the fermentation system, as propionate production consumes the main substrate for methane synthesis in the rumen: hydrogen (Moss et al., 2000). *In vivo* dairy cow feeding tests are needed to examine the effect of dietary SM on methane production, as such tests could provide new insights into how rumen fermentation could be altered to reduce the methane emissions associated with dairy farming.

Selenium is a key part of glutathione peroxidase and the cellular antioxidant system (NRC, 2001; Surai, 2006). Rumen microbes can absorb selenomethionine from their own cell walls or dietary protein (Hidiroglou et al., 1968), which alleviates oxidative damage by eliminating free radicals (Cobanova et al., 2017; Mihalikova et al., 2005; Surai, 2006). In this study, species richness was significantly higher in SM06 than in the other treatments, which may be related to the antioxidant function of selenium in the rumen fermentation process. The addition of 0.4 mg kg⁻¹ selenium in the diet of sheep has been shown to increase the selenium concentration in the rumen mucosa and microbial GSH-Px activity (Cobanova et al., 2017). Similarly, Hidiroglou et al. (1968) found that adding selenium to sheep feed low in selenium increased the species richness of rumen microorganisms. Thus, the increase in the species richness of microorganisms in SM06 in this study may be related to the addition of SM, which improved the antioxidant status of

rumen microorganisms and thus promoted their growth. This study is also consistent with the findings of Mihalikova et al. (2005) showing that the species richness of rumen microorganisms and protozoa increased after adding selenium to the diet of sheep; the same result was also obtained in dairy calves (Zhang et al., 2020). Subsequent tests are needed to further explore the effect of SM supplementation in the diet of dairy cows on the activity and antioxidant status of rumen microbial GSH-Px.

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CONFLICTS OF INTEREST

No conflict of interest declared.

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