Article

Methane Emissions Regulated by Microbial Community Response to the Addition of Monensin and Fumarate in Different Substrates

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Featured Application: Ruminant livestock contributes significantly to global methane (CH₄) emissions. The CH₄ emissions from ruminants in the Qinghai-Tibet Plateau (QTP) are an important part of China's animal methane emissions. This study evaluated the in vitro effects of CH₄ mitigation additives with different mechanisms and their potential interactions with compounds regularly used in yak feed. Basal substrates (forage and vinasse) and methane mitigation additives (monensin sodium salt (MSS) and disodium fumarate (DF)) have interactions with the in vitro rumen fermentation and microbial composition. The different microbial population was critical to the variations in methane production. Comprehensive analyses of rumen microbiome may help further understand the diet-inhibitor interactions in mitigating methane emissions from ruminants in QTP.

Citation: Xue, D.; Chen, H.; Luo, X. Methane Emissions Regulated by Microbial Community Response to the Addition of Monensin and Fumarate in Different Substrates. *Appl. Sci.* **2021**, *11*, 6282. <https://doi.org/10.3390/app11146282>

Academic Editor: Daniel Cozzolino

Received: 8 May 2021

Accepted: 4 July 2021

Published: 7 July 2021

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Abstract: Ruminants contribute significantly to global methane (CH₄) emissions. This study aimed to evaluate the in vitro effects of monensin sodium salt (MSS) and disodium fumarate (DF) on CH₄ production, rumen fermentation, and microbial community, with different substrates. The addition of MSS and DF, alone and in combination, significantly reduced the concentration and production of CH₄ ($p < 0.05$), and while with vinasse as substrate, the CH₄ production was higher for forage. The highest propionate production and lowest acetate and propionic ratio (A:P) values were all observed in cultures added to the combination of 14 mmol/L DF and 80 mg/kg MSS in both substrates, suggesting that these additives improved the rumen fermentation efficiency. The diversity indexes of prokaryotic microbiota with forage as the substrate were significantly higher than vinasse, and there were different effects on diversity indexes with the addition of MSS and DF depending on the incubated substrate. Supplementation with MSS and DF increased the number of starch degradation and fumarate reducing bacteria, decreased the number of methanogens, but had no significant effect on the number of fibrolytic bacteria. pH, NH₃-N, and rumen volatile fatty acids (VFA) were the main factors influencing prokaryotic community structure. In conclusion, basal substrates (forage and vinasse) and CH₄ mitigation additives (MSS and DF) have interactions on the in vitro rumen fermentation and microbial composition.

Keywords: monensin; disodium fumarate; in vitro; rumen fermentation; microbial community

1. Introduction

Ruminants contribute significantly to global methane (CH₄) emissions. CH₄, a greenhouse gas (GHG), has a major role in climate warming [1]. Agriculture is responsible

for approximately 40–50% of global CH₄ emissions, with the enteric fermentation CH₄ from domestic livestock producing 76–92 Tg/year, accounting for 25% of anthropogenic methane sources [2,3]. CH₄ emissions from domesticated livestock (cattle, sheep, and pigs) in China were 13.21 Tg [4]; however, this data did not include CH₄ emissions from yaks. Yaks, as the main domesticated animals on the Qinghai-Tibet Plateau (QTP) in China, are an important source of income for local herdsman. There are approximately 15 million yak in Chinese territories; this population continues to grow due to the nutritional value of their by-products [5].

Enteric CH₄, which results from the fermentation of feed, is primarily emitted from ruminants by eructation. Methanogens in the rumen mainly use H₂ to reduce CO₂ to CH₄. Studies have shown that H₂ production management is the most important consideration in reducing CH₄ emissions in ruminants [6,7]. There are two main mechanisms to effectively abate CH₄ formation this way [8]: the first is to maintain a low H₂ pressure by inhibiting H₂ liberating reactions. Monensin is an ionophore that can effectively inhibit hydrogen-producing microorganisms (Gram-positive bacteria and protozoa), resulting in more propionate, which means less acetate, formate, butyrate and hydrogen are produced in the rumen [9,10]. Monensin has been shown to decrease the methane emissions of rumen [11,12].

A second option is to promote alternative H₂-using reactions to compete for the hydrogen used to produce methane. This can be achieved by using propionate enhancers, and other electron acceptors, to decrease H₂ availability for CH₄ production. Propionate precursors (i.e., fumarate, malate, or acrylate) consume hydrogen generated in the rumen through the succinate fermentation pathway [13]. Fumarate is one of propionate precursors, and fumarate-reducing bacteria use H₂ to promote the production of rumen propionate [14]. Newbold et al. (2002) noted that fumarate had the most consistent effects, with 50% of added acids being fermented to propionate, resulting in a 14% decrease in methane production [15].

The effects of monensin sodium salt (MSS) or disodium fumarate (DF) on in vitro fermentation and methanogenesis have been comprehensively reported [16–18]. However, few studies have explored the combination effects of these two additives on rumen ecosystem function. MSS and DF have different mechanisms of action for reducing rumen methane emissions; therefore, it was hypothesized that, in combination, rumen methane production would be further decreased, as compared to the use of a single compound.

Here, the effect of supplementation with MSS and DF (alone and in combination) on rumen fermentation, methanogenesis, and the inventory of “microbial types” is reported in vitro. The objective of this work was to study: (a) the effect of MSS and/or DF on rumen fermentation and CH₄ production of two different substrates; (b) the relationships between microbial community, ruminal fermentation parameters and CH₄ production.

2. Materials and Methods

2.1. Sampling

Rumen fluid was sampled from six Maiwa yak (age: 4.00 ± 0.25 years old; BW: 270.17 ± 37.27 kg). The animals were on a 153 day (19 May 2015 to 28 October 2015) short term fattening trial in Songpan County, Sichuan Province, China. The animal experiment was conducted according to the Regulation of the Administration of Laboratory Animals (2017 Revision) promulgated by Decree No. 676 of the State Council. All procedures of animal care and management followed the National Beef Cattle and Yak Industrial Technical System (CARS-37). The animals were fed twice daily at 8:00 am and 6:00 pm at the livestock shed, and their average daily feed intake was recorded. The average daily intake included 13.08 ± 0.67 kg of forage (F), 12.98 ± 0.89 kg of vinasse (V), and 0.65 kg of concentrate supplement (Sichuan Tongda Feed Science Application Research Institute). V was fermented with white distiller’s grains, corn and wheat bran as solid state fermentation substrates, inoculated with *Candida utilis*, *Aspergillus Niger*, *Aspergillus oryzae* and *Bacillus subtilis* for 72 h at 32 °C and with 45% moisture. All animals were dewormed

and immunized in the preliminary feeding period. Sample collection and processing was according to the guidelines of Chengdu Institute of Biology, Chinese Academy of Sciences. The nutritional chemical content of diets and live-weight changes of yaks during the experimental period are shown in Supplementary Materials Table S1. Rumen fluid was collected immediately after slaughtering and filtered through four layers of sterilized gauze into a glass flask and kept under anaerobic conditions by filling it with N₂. The glass flask was preserved at 39 °C and immediately transported to the laboratory.

2.2. In Vitro Rumen Incubation

Collected rumen fluid was diluted (1:2) with artificial saliva that had been flushed with N₂. The protocol to prepare the artificial rumen inoculum was described by Adesogan et al. [19]. The diluted rumen fluid was maintained under anaerobic conditions, and used as the artificial rumen inoculum. F and V were used as the substrates for supplementation of MSS and/or DF. The experimental was designed with a 3 × 3 factorial arrangement of treatments: T represent treatments with different supplementation, VT represents the different treatments with V as substrate, and FT represents the different treatments with F as substrate. MSS was supplemented at 0, 40, 80 mg/kg (dry matter of substrate); DF was added into serum bottles in vitro, and the final treatment concentrations were 0, 7, and 14 mmol/L (Table 1). The concentrations of MSS and DF were slightly adjusted according to previous studies [10,12,14,17]. Incubation substrate (450 mg) was dispensed into a 100 mL serum bottle with an appropriate amount of MSS and/or DF, and then 45 mL artificial rumen inoculum was transferred into each bottle under anaerobic conditions. Inoculated serum bottles (T1–T9; *n* = 4) and non-substrate control (TB; *n* = 3) were sealed with butyl rubber plugs and aluminum caps, and were incubated anaerobically for 24 h at 39 °C. Gas was collected at 2, 4, 6, 9, 12, 20, and 24 h using the water displacement method [20], and 1 mL fermentation gas was simultaneously extracted from the anaerobic serum bottles. The methane content at each time point was measured immediately by gas chromatography. After incubation for 24 h, all samples were cooled on ice to stop the fermentation process, then sampled for DNA extraction, pH, NH₃-N and volatile fatty acids (VFA) measurements.

Table 1. Experiment design in vitro.

Monensin Sodium Salt (mg/kg)	Disodium Fumarate (mmol/L)		
	0	7	14
0	T1	T2	T3
40	T4	T5	T6
80	T7	T8	T9

2.3. Chemical Analyses

Methane was analyzed using gas chromatography (Agilent 7890A, Agilent Co., Santa Clara, CA, USA). Ruminant fluid pH was measured using an acidity meter (Sartorius PB-10, Göttingen, Germany). NH₃-N was detected by a continuous flow analyzer (San++, Skalar, Breda, The Netherlands). VFAs were measured using gas chromatography (Agilent 6850, Agilent Co., Santa Clara, CA, USA) with a polar capillary column (HP-FFAP, 30 m × 0.25 mm × 0.25 µm) and a flame ionization detector (FID) [21]. The nutrient contents of F and V diets were measured as described previously [22].

2.4. Microbial Analyses

2.4.1. 16S rRNA Sequencing

Total DNA was extracted from 1 mL fermented rumen fluid by the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Universal primers 515F/909R were used for amplification of the V4–V5 hypervariable region of 16S rRNA genes. The methods and procedures of MiSeq sequencing and data processing were the same as those described by

Xue et al. [23]. The sequencing data were deposited in the NCBI Sequence Read Archive, accession number SRP104034.

2.4.2. Real-Time PCR Analysis

Eight primer pairs were used to detect populations of selected species in each rumen sample (Table S2). Real-time PCR assays were performed on Chromo 4 (Bio-Rad Laboratories Inc., Hercules, CA, USA) with fluorescence detection by SYBR green dye (Tiangen, Beijing, China). The amplification reactions were performed in a total volume of 20 μ L, containing 1 μ L dilutions (1:10) of template DNA, 10 μ L 2 $\times$ SYBR Green SuperReal Premix, 0.6 μ L of each primer (10 μ M) and 7.8 μ L sterilized distilled water. Amplification consisted of one cycle of 95 $^{\circ}$ C (15 min), 40 cycles of denaturation at 95 $^{\circ}$ C (15 s), and annealing/extension at 60 $^{\circ}$ C (1 min). Fluorescence detection was performed after each extension step. For melting curve detection, the temperature was increased by 1 $^{\circ}$ C every 30 s from 60 to 95 $^{\circ}$ C. The standard curves were generated using 10-fold dilution series of the linearized plasmid DNA from clones identified as bacteria, methanogenic archaea, ciliate protozoa, anaerobic fungi, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Ruminococcus flavefaciens* and *Ruminococcus albus*. All data were presented as the average copy number of the gene targeted per milliliter of rumen fluid and analyzed using CFX Manager software version 3.1 (Bio-Rad).

2.5. Data Analysis

The overall structure of rumen microbiota was assessed by principal coordinate analysis (PCoA). Statistical significance among groups was evaluated by PerMANOVA analysis using “adonis” in the R vegan package [24]. Mantel test and the BioEnv procedure were used to evaluate the relationships between environmental variables and prokaryotic communities, and then selected the environmental variables with the highest Spearman correlation were selected for the variance partitioning analysis (VPA) by “varpart” in the R vegan package. Redundancy analysis (RDA) was performed in Canoco 5, and the significance was tested using the ANOVA procedure in the R vegan package ($p < 0.05$).

3. Results

3.1. Effect of MSS and DF on In Vitro Rumen Fermentation and Methanogenesis

The effects of MSS and DF supplementation on in vitro rumen fermentation and methanogenesis are shown in Figure 1 and Table 2. The addition of MSS alone (40 mg/kg) or DF (7 and 14 mmol/L) significantly increased 24 h total gas production ($p < 0.05$), whereas the addition of only MSS (80 mg/kg) and the co-addition of MSS and DF caused a linear reduction ($p < 0.05$). The addition of MSS and DF, alone and in combination, was found to significantly decrease methane concentration and production ($p < 0.05$). When V was the substrate, methane production was higher than for F; however, the addition of 7 mmol/L DF and 80 mg/kg MSS with V as the substrate showed low methane production compared to control cultures ($p < 0.01$), so did cultures with F as the substrate (80 mg/kg MSS) ($p < 0.05$). Compared to the control group, MSS and DF supplementation alone or in combination significantly increased propionate production and decreased acetate:propionate (A:P) ($p < 0.05$). The highest propionate production and lowest A:P values were all observed in cultures supplemented with the combination of 14 mmol/L DF and 80 mg/kg MSS, in both substrates.

3.2. Effect of MSS and DF Supplementation on Prokaryotic Community Diversity and Structure

Methane production in different groups was used to analyze the prokaryotic microbial communities under the supplementation of MSS and DF, alone (14 mmol/L DF or 80 mg/kg MSS) and in combination (7 mmol/L DF and 80 mg/kg MSS), in both substrates. In total, 182,276 high-quality reads of 16S rRNA gene in 28 samples were obtained by MiSeq sequencing; a standard sequence number of 6238 was used to calculate the prokaryotic community characteristics.

The alpha diversity of prokaryotic microbiota from different groups was calculated (Table S3). The Chao1 index, observed OTUs and Shannon diversity of prokaryotic microbiota with F as the substrate were significantly higher than V ($p < 0.05$). MSS and DF supplementation alone or in combination significantly increased the Shannon diversity and Chao1 index compared to control cultures with F as the substrate ($p < 0.05$), whereas these values decreased with V ($p < 0.05$). The Chao1 index, observed OTUs and Shannon diversity showed significant positive associations with pH, $\text{NH}_3\text{-N}$, isobutyrate and iso-valerate, whereas they had negative associations with butyrate (Table S4). Differences in the prokaryotic community structure under different substrates were evident ($R^2 = 0.612$, $p < 0.001$) based on the weighted UniFrac distance metrics (Figure 2). The first two axes explained 74.61% of variations (PCo1-axis 64.84% and PCo2-axis 9.77%). Supplementation with MSS and DF had a significant effect on the prokaryotic community structure with V ($R^2 = 0.615$, $p < 0.001$) or F ($R^2 = 0.472$, $p < 0.001$) as substrate.

Figure 1. Effect of monensin sodium salt (MSS) and/or disodium fumarate (DF) supplementation on in vitro methanogenesis under different substrates (forage or vinasse). Inoculated bottles with a no-supplementation control (T1) and non-substrate control bottles (TB).

3.3. Effect of MSS and DF Supplementation on the Composition of Rumen Prokaryotic Community

The dominant phyla across all samples were Bacteroidetes (average relative abundance 34.14–58.96%), Firmicutes (6.81–26.89%), Tenericutes (4.46–40.75%), Euryarchaeota (2.22–10.12%) and Spirochaetes (1.30–11.43%) (Figure 3). Different substrates resulted in variations in the overall prokaryotic community composition. The relative abundances of Euryarchaeota and Tenericutes were significantly influenced by substrate ($p < 0.05$). The relative abundance of Euryarchaeota with F as substrate increased significantly by 2.91% compared to V, but Tenericutes was reduced by 28.93% ($p < 0.05$). Supplementation with MSS and DF, alone and in combination, caused a substantial increase in the relative abundance of Bacteroidetes, Fusobacteria, and Proteobacteria, and decrease in the relative abundance of Euryarchaeota and Firmicutes.

Table 2. Effect of monensin and fumarate supplementation on the in vitro rumen fermentation under different substrates.

Parameters	Substrates	T1	T2	T3	T4	T5	T6	T7	T8	T9
pH value	Forage	7.35 ± 0.04 ab	7.28 ± 0.08 ab	7.4 ± 0.06 a	7.22 ± 0.24 ab	7.41 ± 0.04 a	7.39 ± 0.08 ab	7.33 ± 0.12 ab	7.25 ± 0.09 b	7.35 ± 0.05 ab
	Vinasse	7.21 ± 0.00 bc	7.32 ± 0.01 a	7.27 ± 0.04 ab	7.17 ± 0.08 c	7.24 ± 0.04 bc	7.22 ± 0.06 bc	7.16 ± 0.03 d	7.25 ± 0.03 bc	7.23 ± 0.1 bc
NH ₃ -N (mg/dL)	Forage	31.95 ± 3.07 a	28.93 ± 2.31 a	29.31 ± 1.99 a	34.83 ± 6.78 a	29 ± 0.63 a	28.74 ± 0.96 a	26.29 ± 3.54 a	36.07 ± 3.18 a	31.16 ± 3.56 a
	Vinasse	22.61 ± 0.06 a	17.95 ± 0.86 c	20.49 ± 0.38 b	23.05 ± 1.13 ab	21.72 ± 1.2 ab	23.77 ± 3.02 ab	24.93 ± 1.00 a	23.41 ± 0.45 a	23.31 ± 1.32 ab
Acetate (mmol/L)	Forage	36.75 ± 0.2 a	38.61 ± 4.67 a	41.22 ± 5.1 a	44.31 ± 10.94 a	34.54 ± 1.2 a	36.23 ± 3.14 a	30.37 ± 1.6 a	37.74 ± 4.63 a	38.55 ± 1.21 a
	Vinasse	33.27 ± 0.83 abc	34.67 ± 2.54 ab	36.11 ± 0.85 a	33.66 ± 2.27 abc	37.03 ± 2.07 a	32.42 ± 3.51 abc	31.35 ± 2.54 bc	26.66 ± 2.47 D	30.91 ± 2.39 c
Propionate (mmol/L)	Forage	8.32 ± 0.33 b	15.37 ± 0.89 a	17.91 ± 2.43 a	12.11 ± 3.71 ab	13.76 ± 1.33 ab	17.91 ± 0.77 a	8.45 ± 0.49 b	14.87 ± 2.25 ab	19.82 ± 2.22 a
	Vinasse	6.32 ± 0.03 b	15.76 ± 2.86 ab	14.91 ± 6.18 ab	9.58 ± 1.3 b	15.69 ± 0.27 a	16.7 ± 2.65 ab	10.67 ± 0.86 b	13.11 ± 2.42 ab	18.03 ± 0.98 a
Isobutyrate (mmol/L)	Forage	0.77 ± 0.04 a	0.71 ± 0.16 a	0.75 ± 0.09 a	0.99 ± 0.28 a	0.7 ± 0.02 a	0.71 ± 0.09 a	0.73 ± 0.05 a	0.87 ± 0.11 a	0.82 ± 0.06 a
	Vinasse	0.64 ± 0.02 ab	0.60 ± 0.03 ab	0.63 ± 0.05 a	0.65 ± 0.02 a	0.64 ± 0.02 a	0.56 ± 0.06 b	0.62 ± 0.05 ab	0.56 ± 0.03 c	0.59 ± 0.03 ab
Butyrate (mmol/L)	Forage	6.73 ± 0.28 ab	5.65 ± 1.46 c	5.97 ± 0.83 c	9.79 ± 3.34 a	6.25 ± 0.21 b	6.16 ± 0.92 b	7.27 ± 0.56 ab	8.34 ± 1.24 a	7.53 ± 0.61 a
	Vinasse	11.23 ± 0.08 a	8.11 ± 0.06 d	9.39 ± 1.24 bc	10.31 ± 0.29 ab	9.58 ± 0.52 bc	7.93 ± 0.65 d	9.38 ± 0.67 bc	8.03 ± 0.8 d	8.8 ± 0.91 cd
Isovalerate (mmol/L)	Forage	1.42 ± 0.06 c	1.54 ± 0.17 b	1.61 ± 0.14 b	2.05 ± 0.75 ab	1.79 ± 0.24 ab	1.7 ± 0.21 ab	1.63 ± 0.13 ab	2.31 ± 0.24 a	2.23 ± 0.39 ab
	Vinasse	1.14 ± 0.06 c	1.38 ± 0.12 ab	1.42 ± 0.09 a	1.15 ± 0.06 c	1.25 ± 0.11 bc	1.11 ± 0.17 c	1.1 ± 0.09 c	1.05 ± 0.06 d	1.2 ± 0.12 c
Valerate (mmol/L)	Forage	1.25 ± 0.03 ab	1.13 ± 0.27 b	1.19 ± 0.15 ab	1.65 ± 0.52 a	1.14 ± 0.05 ab	1.13 ± 0.16 b	1.06 ± 0.11 c	1.28 ± 0.16 ab	1.24 ± 0.1 ab
	Vinasse	1.08 ± 0.03 ab	1.04 ± 0.08 b	1.05 ± 0.02 b	1.24 ± 0.13 a	1.27 ± 0.07 a	1.01 ± 0.13 b	1.21 ± 0.09 a	0.99 ± 0.09 b	1.14 ± 0.18 ab
Acetate:propionate	Forage	4.42 ± 0.2 a	2.52 ± 0.32 ab	2.31 ± 0.03 ab	3.69 ± 0.23 a	2.52 ± 0.18 ab	2.02 ± 0.09 ab	3.59 ± 0.1 a	2.55 ± 0.23 ab	1.97 ± 0.27 b
	Vinasse	5.27 ± 0.11 a	2.23 ± 0.25 ab	3.01 ± 1.93 ab	3.53 ± 0.23 ab	2.36 ± 0.12 ab	1.95 ± 0.14 b	2.94 ± 0.04 ab	2.06 ± 0.17 b	1.72 ± 0.06 b

Lowercase $p < 0.05$ and uppercase $p < 0.01$.

Figure 2. The principal coordinate analysis of prokaryotic communities of 14 mmol/L disodium fumarate (DF) groups (FT3 and VT3), 80 mg/kg monensin sodium salt (MSS) groups (FT7 and VT7), 7 mmol/L DF and 80 mg/kg MSS groups (FT8 and VT8), and control groups (FT1 and VT1).

Figure 3. The relative abundance of prokaryotic phyla in 14 mmol/L disodium fumarate (DF) groups (FT3 and VT3), 80 mg/kg monensin sodium salt (MSS) groups (FT7 and VT7), 7 mmol/L DF and 80 mg/kg MSS groups (FT8 and VT8), and control groups (FT1 and VT1).

At the genus level, the seven most abundant genera (average relative abundance >1%) included *Prevotella* (26.34%), *Anaeroplasma* (20.37%), *Methanobrevibacter* (4.31%), *Treponema* (3.30%), *Butyrivibrio* (1.41%) and *Succiniclasicum* (1.39%) (Table S5). *Methanobrevibacter* was the dominant methanogen genus across all samples, which mainly use H_2/CO_2 for growth and methanogenesis. The addition of MSS and DF, alone and in combination, increased the relative abundance of *Prevotella*, *Fibrobacter*, *Selenomonas*, *Succiniclasicum* and *Succinivibrio*, but decreased the relative abundance of *Methanobrevibacter*.

3.4. Relationships between Rumen Fermentation Parameters and Prokaryotic Communities

An RDA ordination plot was created to explore the relationships between prokaryotic community structure and ruminal fermentation parameters. The results showed that 51.22% and 12.52% of the variation in prokaryotic community could be explained by axis 1 and axis 2 (Figure 4); there was a significant correlation between the community structure of

prokaryotes and rumen fermentation parameters. The Mantel test further showed that there was a significant correlation between rumen fermentation parameters (pH, NH₃-N, acetate, isobutyric, butyrate, and isovaleric) and prokaryotic community structure (Table S6).

Figure 4. Redundancy analysis (RDA) of ruminal fermentation parameters and prokaryotic community compositions at the phylum level. Arrows indicate the direction and magnitude of parameters associated with prokaryotic community structures. A:P, acetate and propionic ratio; TVFAs, total volatile fatty acids.

The VPA showed that 78.3% of the variation could be explained by three major variables: pH, NH₃-N, and VFA could independently explain 0.2, 1.6, and 22% of the total variation. In addition, there were significant interactions among three main variables, such as the interaction of VFAs with pH (19%), VFAs with NH₃-N (33.1%), and three major variables (2.4%), were observed (Figure S1). Spearman's correlation analysis found CH₄ production were positively correlated with acetate ($p < 0.01$) and TVFAs content ($p < 0.05$) (Table S7). In conclusion, the pH, NH₃-N and VFAs were the main factors that influenced prokaryotic community structure.

3.5. Effect of MSS and DF Supplementation on the Abundance of Selected Microbes

MSS and DF addition, alone or in combination, significantly increased the population density of *F. succinogenes* and *S. ruminantium* compared to control cultures with different substrates ($p < 0.05$) but decreased the population density of methanogenic archaea ($p < 0.05$) (Table 3). The lowest density of methanogens was found in cultures with 14 mmol/L added DF in F as the substrate, but when V was the substrate, the lowest density of methanogens was found in cultures added with the combination of 7 mmol/L DF and 80 mg/kg MSS. The population density of total bacteria, methanogenic archaea, ciliate protozoa and *S. ruminantium* with F as the substrate were lower than V, but the number of fibrolytic bacteria (e.g., *R. flavefaciens*, *F. succinogenes* and *R. albus*) with F as the substrate were higher than V, demonstrating that F can provide more material for fibrolytic bacteria growth.

Table 3. Effect of monensin and fumarate supplementation on selected microbial populations under different substrates.

Target Species	Forage				Vinasse			
	T1	T3	T7	T8	T1	T3	T7	T8
Total bacteria ($\times 10^7$ copies/mL)	3.51 $\pm$ 1.07 abc	2.54 $\pm$ 1.23 c	2.83 $\pm$ 1.42 c	3.34 $\pm$ 2.12 bc	9.53 $\pm$ 3.22 ab	10.34 $\pm$ 7.47 a	9.97 $\pm$ 3.01 a	5.43 $\pm$ 1.93 abc
Methanogenic archaea ($\times 10^5$ copies/mL)	2.2 $\pm$ 0.16 bCd	0.93 $\pm$ 0.42 e	1.28 $\pm$ 0.63 d	1.76 $\pm$ 0.99 Cd	4.34 $\pm$ 0.44 a	2.95 $\pm$ 1.74 abc	3.75 $\pm$ 0.63 ab	2.63 $\pm$ 0.65 bCd
Ciliate protozoa ($\times 10^2$ copies/mL)	6.46 $\pm$ 0.22 ab	2.62 $\pm$ 1.73 b	6.36 $\pm$ 4.3 ab	10.24 $\pm$ 6.21 a	7.04 $\pm$ 0.71 ab	9.06 $\pm$ 6.45 ab	9.9 $\pm$ 2.97 a	11.25 $\pm$ 5.79 a
Anaerobic fungi (copies/mL)	7.8 $\pm$ 0.48 a	7.24 $\pm$ 0.91 a	12.23 $\pm$ 5.47 a	8.05 $\pm$ 1.42 a	10.68 $\pm$ 1.47 a	6.65 $\pm$ 2.99 a	6.47 $\pm$ 2.08 a	11.91 $\pm$ 6.39 a
<i>Fibrobacter succinogenes</i> ($\times 10^4$ copies/mL)	13.89 $\pm$ 6.19 ab	16.08 $\pm$ 6.27 a	17.76 $\pm$ 8.31 a	33.65 $\pm$ 21.54 a	0.37 $\pm$ 0.09 D	1.9 $\pm$ 1.48 c	2.91 $\pm$ 1.03 bc	1.21 $\pm$ 0.54 c
<i>Selenomonas ruminantium</i> ($\times 10^5$ copies/mL)	2.15 $\pm$ 0.05 C	2.43 $\pm$ 0.25 C	2.75 $\pm$ 0.73 d	3.55 $\pm$ 1.8 C	7.28 $\pm$ 1.73 BC	15 $\pm$ 8.83 B	98.94 $\pm$ 44.96 a	47.74 $\pm$ 11 aB
<i>Ruminococcus flavefaciens</i> ($\times 10^2$ copies/mL)	5.74 $\pm$ 0.14 a	2.08 $\pm$ 0.47 b	2.34 $\pm$ 1.43 b	1.85 $\pm$ 0.85 b	1.82 $\pm$ 0.11 b	2.84 $\pm$ 1.19 ab	2.87 $\pm$ 0.93 ab	2.09 $\pm$ 0.47 b
<i>Ruminococcus albus</i> ($\times 10^2$ copies/mL)	4.63 $\pm$ 0.56 a	2.42 $\pm$ 0.24 b	1.46 $\pm$ 0.28 C	1.52 $\pm$ 0.26 C	0.76 $\pm$ 0.02 d	1.46 $\pm$ 0.08 C	0.34 $\pm$ 0.16 e	0.50 $\pm$ 0.20 d

Lowercase $p < 0.05$ and uppercase $p < 0.01$.

4. Discussion

4.1. Methane Emissions Regulated by Microbial Community under Different Substrates

Methane production is affected by the substrate, microbial communities, rumen pH, etc. [25]. Different substrates induce different conditions during rumen fermentation. Giraldo et al. [26] found the production of methane was affected by substrates with different forage:concentrate ratios, as 0.3:0.7 (LF) was higher than forage 0.7:0.3 (HF). Serment et al. [27] found that high substrate concentrations produced more CO₂ and CH₄ than low concentrations. Martinez et al. [28] reported that in vitro methane production was increased by decreasing the forage:concentrate (F:C) ratio. This study identified that substrate (F vs. V) had the most significant impact on methane production. Compared to F substrate, V had lower NDF and ADF, and higher CP and EE (Table S1), and methane production with V as the substrate was higher than with F. This may be due to the higher fermentation rates of high-concentrate substrates than those of high-forage ones.

Enteric methane is formed during the microbial degradation of food in the rumen, and any variation in its production level relies on changes in the microbial ecosystem [29]. The diversity indexes of prokaryotic microbiota, with F as the substrate were significantly higher than V, and the microbial community structure (e.g., Euryarchaeota and Tenericutes) varied under the two substrates. Hook et al. [30] revealed that feeding ruminants a high-concentrate diet changed the diversity and community structure of methanogens but did not affect the abundance of rumen methanogens. Other studies also found that the type of diet can significantly affect the abundance and community of methanogens [31,32], while Patra and Yu (2015) showed that substrate can cause changes in rumen microbial community in vitro fermentation systems, using the denaturing gradient gel electrophoresis (DGGE) profiles of bacterial and archaeal communities [33]. The structure and/or activity of the microbial community determines the methane production and, partially, the utilization of H₂ [34]. Manatbay et al. [35] reported that the variable forage:concentrate ratios changed the abundances of cellulolytic bacteria, total fungi and counts of protozoa in the in vitro experiment, however did not change the abundance of methanogens and total bacteria. Consistent with the results of this study, the number of *R. flavefaciens*, *F. succinogenes* and *R. albus* were higher with F as substrate than V, but total bacteria, methanogenic archaea, ciliate protozoa and *S. ruminantium* were lower with F as substrate than with V. F can provide more material for fibrolytic bacteria growth. Therefore, the variation of rumen methane production may be due to the differences of rumen microbiome caused by different substrates.

4.2. Effect of Methane Mitigation Additives on In Vitro Methane Production, Rumen Fermentation, and Microbes

Supplementation with MSS and DF, alone and in combination, enhanced propionate production compared to the control and thus decreased methane production and A:P (Table 2). For all substrates, the mean CH₄ production decreased by 6.8 and 14.0% for concentrations of 7 and 14 mmol/L DF, respectively, while MSS (40 and 80 mg/kg) reduced the methane production by 15.2 and 46.3%, respectively. The effects of MSS and DF on methane production were not consistent. Asanuma et al. [36] reported that supplementation with fumarate to cultures that were fermenting hay powder and concentrate, significantly decreased methane production and increased propionate production. Some studies have also found that the addition of fumarate can decrease methane production during rumen fermentation [37,38]. Lopez et al. [39] reported that fumarate can also be decomposed into acetate stoichiometrically. The addition of fumarate decreased the ratio of acetate:propionate, but increased propionate and acetate production. The acetate production in this study also increased with the addition of DF, but most fumarate in this study could have been converted to propionate. Russell and Houlihan [40] found that monensin can also convert acetate to propionate. Wolin [41] demonstrated a significant correlation between methane production and A:P. Odongo et al. [12] revealed significant declines, of

7%, in CH₄ emissions from lactating dairy cows fed 60:40 forage-to-concentrate total mixed ratio (TMR) supplemented with monensin.

The addition of MSS and DF also has a significant impact on the rumen microbial community. The addition of MSS and DF decreased the relative abundance of Euryarchaeota and Firmicutes but increased the relative abundance of Bacteroidetes. These were consistent with Abrar et al. [10], who found that monensin inhibits Gram-positive bacteria and protozoa, while reducing the relative abundance of methanogens. Zhou et al. [42] found that the number of methanogens significantly decreased in the rumen fluid samples of sheep fed 20 g/kg DF. *Methanobrevibacter* was the dominant methanogen genus in this study. Janssen and Kirs [43] showed that the number of *Methanobrevibacter* accounts for nearly two thirds of rumen archaea. Consistent with our previous research result, H₂/CO₂ is the main pathway of methanogenesis [23].

S. ruminantium, *Veillonella parvula*, *F. succinogenes*, and *Wolinella succinogenes* are thought to be the major fumarate reducing bacteria in the rumen [36], while *R. albus* and *R. flavefaciens* are the main cellulolytic bacterial species [44]. Fibrolytic bacteria are the main hydrogen production bacteria in rumen fermentation, while fumarate reducing bacteria are the main hydrogen consuming bacteria. Fumarate can stimulate the competition between fumaric acid reducing bacteria and methanogens for hydrogen or formate, and thus reduce the methane production of rumen. The addition of MSS and DF, alone and in combination, increased the number of starch degrading bacteria (*Prevotella* and *Selenomonas*) and fumarate reducing bacteria (*Fibrobacter* and *Selenomonas*), decreased the number of methanogens, while no significant effect was observed on fibrolytic bacteria (*R. albus* and *R. flavefaciens*). Yang et al. [17] reported that the addition of DF to high-forage diets increased the number of *S. ruminantium* and fumarate-reducing bacteria, but the number of fungi, protozoa, and fibrolytic bacteria were not affected. Asanuma et al. [36] reported the addition of 30 mM fumarate increased the growth of *F. succinogenes*, *S. ruminantium*, *S. lactilytica*, *V. parvula* and *W. succinogenes* in vitro cultures. Giraldo et al. [45] reported no significant impact on the abundance of cellulolytic bacteria by supplementing with fumarate in Rusitec fermenters. Castromontoya et al. [46] found monensin decreased the effectiveness of H₂ to CH₄ formation, mainly by enhancing the amylolytic activity of inhibiting cellulolytic activity.

4.3. Interactions between Substrates and Methane Mitigation Additives

Different substrates may produce different conditions in the rumen, which will further influence how methane inhibitors affect methanogenesis and rumen microbes. The addition of DF was more effective in lowering CH₄ production with V as the substrate, while supplementation with MSS was more effective with F as the substrate. The highest reduction of methane production was 57%, with 80 mg/kg MSS (T7) with F as the substrate, while the addition of 7 mmol/L DF and 80 mg/kg MSS (T8) with V as the substrate reduced methane production by 49%. Zhang et al. [47] showed that total gas production and CH₄ were affected by the interactions between the rare earth element lanthanum and feed mixtures with different levels of NDF. Castromontoya et al. found that “in vitro” methane and VFA production were affected by interactions between feed additives and substrates, but the different microbial populations might be the key to these interaction effects [46]. In this study, the diversity indexes of prokaryotic microbiota with F as the substrate was significantly higher than V, but had no effect on the diversity indexes with the addition of MSS and DF under different incubated substrates. Additives (DF and MSS) have different mechanisms to alleviate enteric CH₄. The effectiveness of these additives is closely related to rumen microbiota. Therefore, a comprehensive analysis of the rumen microbiome can promote the understanding of the interaction between diet and inhibitors in mitigating methane emissions from ruminants in QTP.

5. Conclusions

This study aimed to evaluate the in vitro effects of CH₄ mitigation additives with different working mechanisms, and their possible interaction with feed compounds regularly used in yak feed. This study found that the substrate (F vs. V) has the greatest effect on methane production, and the effects of MSS and DF were not consistent. This research provides evidence that basal substrates (F and V) and methane mitigation additives (MSS and DF) have interactions on the in vitro rumen fermentation and the microbial composition. Differences in the microbial population were the key to the variations in methane production. A comprehensive analysis of rumen microbiome may help further understand the diet-inhibitor interactions in mitigating methane emissions from ruminants in QTP.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11146282/s1>, Figure S1: The relative contributions of ruminal fermentation parameters quantified by variance partitioning analysis (VPA), Table S1: Nutritional chemical contents of diets and live-weight changes of yak during the experimental period, Table S2: Primers used for the quantification of rumen microbes by real-time PCR assay, Table S3: Prokaryotic alpha diversity indices at 97% sequence similarity of 16S rRNA gene sequence (per 6238 sequences), Table S4: Spearman correlation coefficients between alpha diversity indices of prokaryotic microbiota and fermentation parameters, Table S5: Relative abundance of dominant prokaryotic groups at the genus levels (values are means $\pm$ standard error), Table S6: Spearman's correlation of fermentation parameters with prokaryotic community structure as determined by the partial Mantel test ^a, Table S7: Spearman's correlation coefficients between environmental variables.

Author Contributions: D.X.: Conceptualization; software; formal analysis; methodology; validation; writing—original draft preparation; writing—review and editing; H.C.: conceptualization; funding acquisition; writing—review and editing; supervision; X.L.: data curation; formal analysis; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by Key R&D Program of Sichuan Province (2020YFS0024), the National Natural Science Foundation of China (42001093) and the Chinese Academy of Sciences Light of West China Program (2018XBZG_XBQNXZ_B_008).

Institutional Review Board Statement: The present study did not involve species that are endangered or protected in China. All procedures of animal care and management followed the National Beef Cattle and Yak Industrial Technical System (CARS-37).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors give special thanks to Wan Xiong for her editing and valuable comments on the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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