Evaluation of ferric oxide and ferric citrate for their effects on fermentation, production of sulfide and methane, and abundance of select microbial populations using *in vitro* rumen cultures

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Abstract

This study systematically [evaluated](#) the effect of ferric [iron](#) on sulfate reduction to sulfide, feed digestion and fermentation, methane production, and populations of select ruminal microbes using *in vitro* rumen cultures. [Ferric oxide](#) (Fe_2O_3) and ferric citrate ($\text{C}_6\text{H}_5\text{FeO}_7$) at six concentrations ([0, 25, 50, 100, 150, and 200 mg/L as \$\text{Fe}^{3+}\$](#)) were tested. [Ferric iron](#) decreased [production of](#) both H_2S gas [in culture headspace](#) (up to 71.9%) and aqueous sulfide (up to 80.8%), without [adversely affecting](#) other fermentation parameters, with ferric citrate being more effective than ferric oxide. Total archaeal population was increased by ferric citrate, [but methane](#) production was not [affected](#) significantly. The population of sulfate reducing bacteria was affected differently by ferric oxide than [by](#) ferric citrate. The results of this study could guide future *in vivo* studies to develop effective solutions to abate sulfur-associated [polioencephalomalacia](#) in cattle fed [high-sulfur diet such as distiller's dried grains with solubles](#).

Key words: ferric iron, hydrogen sulfide, methane, *Archaea*, sulfate reducing bacteria

Introduction

With the expansion of biofuel industry, output of byproducts such as dried distiller's grain with solubles (DDGS) increased dramatically in the recent years. In 2013, nearly 200 operating plants [produced](#) an estimated [50.5](#) billion [liters](#) of ethanol, which was accompanied with nearly 35 million tons of co-products as DDGS in the United States. The nutritive value of DDGS can be as high as 3 [times](#) of that of corn because of depletion of starch [but enrichment of lipid and protein](#), which makes DDGS a [good](#) protein source for livestock (Janicek et al., 2008). However, the sulfur content of DDGS is much higher than [that of](#) other feedstuffs, ranging from 0.45% to

more than 1.10%, with an average value of 0.84% (Batal and Dale, 2003; [Klopfenstein et al., 2007](#)). Dietary sulfate is biochemically reduced to sulfide by sulfate reducing bacteria (SRB) in the rumen, and sulfide can be protonated to [form](#) H_2S , which is the cause of sulfur-induced polioencephalomalacia (PEM) in cattle (Gould, 1998; Felix et al., 2012). Therefore, cattle ration can only contain a certain proportion of DDGS to avoid the risk of PEM. The excessive sulfate in DDGS comes from sulfuric acid that is used for fermenter pH control and cleaning in the bioethanol production process (Klopfenstein et al., 2007). Also, the increased sulfur concentration of DDGS in ration can depress feed intake, decrease average daily gain (ADG), and have negative health effects (Felix et al., 2012).

It has been reported that [production](#) of H_2S is pH dependent, and low ruminal pH favors formation of H_2S due to increased protonation of sulfide ([Morine et al., 2014](#)). Although the effects of ruminal pH on H_2S production have been studied both *in vitro* and *in vivo*, the results were conflicting ([Morine et al., 2014](#); Wu et al., 2015). Conceptually, incidence of sulfur-induced PEM can be decreased by decreasing sulfide production and by immobilizing sulfide as insoluble sulfide precipitate. Kung et al. (2000) reported that some chemical compounds, such as 9,10-anthraquinone, molybdenum (Mo), copper (Cu), and some antimicrobials, such as chlortetracycline, oxytetracycline, ionophores, decreased H_2S [production](#) in *in vitro* rumen cultures. In other anaerobic environments, such as marine sediments and groundwater, dissimilatory ferric reduction was found to inhibit sulfate reduction to sulfide and methane production (Lovely and Phillips, 1987). This is because ferric iron has a much higher redox potential ($\text{Fe}^{3+}/\text{Fe}^{2+}$: +0.77 V) than sulfate ($\text{SO}_4^{2-}/\text{HS}^{1-}$: -0.22 V) and carbon dioxide (CO_2/CH_4 : -0.24 V), and thus can outcompete the latter two electron acceptors for electrons (primarily [as \[H\]](#) in the rumen). Besides, ferrous iron can combine with sulfide forming insoluble iron sulfide

(FeS), further decreasing H₂S eructation from the rumen. In a recent study, Drewnoski et al. (2014) showed that adding ferric citrate to diet of high sulfate [diet](#) decreased ruminal H₂S concentration [in vitro and](#) in feedlot cattle. No analysis [for rumen microbes or for aqueous sulfide in the in vitro cultures or in the rumen fluid was done in that study](#). Because ferric iron [thermodynamically](#) outcompetes CO₂ for hydrogen, ferric iron can also inhibit methanogenesis. This is demonstrated in a study where iron ochre (hydrated iron oxides, Fe(OH)₃ and FeO(OH)) lowered [methane](#) production by 64% and greater than 90% in constructed wetland in field and in laboratory, respectively (Pangala et al., 2010). Based on the results of the above studies and the thermodynamics of biochemical reduction of ferric iron, we hypothesize that ferric iron can decrease H₂S eructation and CH₄ production and shift the microbial populations involved in sulfidogenesis and methanogenesis. In the present study, the above hypothesis was tested using a series of *in vitro* rumen fermentation cultures and two forms of ferric iron, ferric oxide, and ferric citrate, at varying [ferric iron](#) concentrations. The effects on feed degradation and fermentation characteristics were also evaluated.

Materials and methods

Experimental procedures

Two ferric iron types (ferric oxides and ferric citrate) each at six [concentrations](#) were [evaluated](#) in a 2 × 6 factorial design in this *in vitro* study. The ferric iron content of ferric oxide (Fe₂O₃; Sigma-Aldrich) and ferric citrate (C₆H₅FeO₇·H₂O; Sigma-Aldrich) was calculated, 70% and 19.9%, respectively, from their molecular formula so that the same ferric iron concentrations were used for the two types of ferric compounds. The six ferric iron ([Fe³⁺](#)) concentrations were 0, 25, 50, 100, 150, and 200 mg/L *in vitro* culture. The inoculum was prepared essentially as

described previously (Wu et al., 2015). Briefly, fresh rumen fluid was collected at approximately 3 h after the morning feeding from 2 ruminally cannulated lactating Jersey cows that were fed a total mixed ration (TMR) (Table 1) twice a day at 0500 and 1700 h. Rumen content was collected from each cow and squeezed through four layers of cheesecloth. About 250 mL of rumen fluid from each cow was combined into one 500 mL bottle, leaving no headspace in the sample bottle. The rumen fluid inoculum was brought to the laboratory within 10 min (about 2.5 km away) and placed into an anaerobic chamber containing N₂ (95%), H₂ (3%), and CO₂ (2%). During sample collection, the animals were handled following the protocols approved by The Ohio State University Animal Care and Use Committee.

The *in vitro* fermentation was carried out in 120-mL serum bottles in triplicate for each treatment. Alfalfa hay and concentrate in 50:50 ratio was used as feed substrate, with the concentrate containing DDGS (60.0%, on dry matter basis), ground corn (16.28%), soybean meal (11.24%), wheat middlings (3.68%), mineral and vitamin mix (6.01%), and Aminoplus® (2.79%). Total sulfur content of this substrate was 0.21% (DM basis). The artificial saliva was prepared anaerobically as described by Menke (1979) except the reducing solution was not added. Inside of the anaerobic chamber, 30 mL of the anaerobic artificial saliva and 10 mL of the fresh rumen fluid inoculums were dispensed to each of the serum bottles containing 0.4 g of the substrate, which contained the amount of ferric compound required to achieve the working ferric iron concentration. After they were sealed with butyl rubbers plus crimped aluminum seals, the serum bottles were incubated at 39°C for 48 h in a water bath with occasional manual shaking (Menke et al., 1979). A relatively longer incubation time, 48 h rather than 24 h, was used to allow the SRB and ferric-reducing bacteria present in the inoculum, which was collected from

donor cows fed a diet containing low proportion of DGGS and typical iron content and thus likely had low abundance of SRB and ferric-reducing bacteria, to grow in the *in vitro* cultures.

Fermentation characteristics analysis

After 48 h incubation, gas pressure in each bottle was measured using a Traceable® manometer (Fisher Scientific, Pittsburgh, PA, USA) to determine total gas production. Then, 25 mL of headspace gas was withdrawn from each culture bottle to analyze H₂S gas concentration using one Sendidyne® precision gas detector tube, which has a detection range of 0.05 – 1.2% H₂S (Sensidyne, St. Petersburg, FL, USA). Finally, 4 mL of headspace gas was collected from each of the *in vitro* culture bottles into one culture tube completely filled with distilled water by displacement for methane analysis. From each culture bottle, 1.5 mL culture was collected and centrifuged at 16,000 *xg* (5 min, 4°C), and the pellet and the supernatant were immediately preserved at -20°C for DNA extraction and VFA analysis, respectively. The VFA concentration in the culture and the methane concentration in the headspace were analyzed using gas chromatography (HP 5890 series, Agilent Technologies) (Machmuller et al., 2003). The amount of methane and H₂S produced in each culture bottle was calculated from the total gas volume in the headspace and the corresponding concentration of methane and H₂S, respectively.

The pH values and the concentration of aqueous sulfide of the *in vitro* cultures were determined using an Accumet® dual channel pH/ion meter (Fisher Scientific, Suwanee, GA, USA), which is equipped with a silver/sulfide combination electrode. The amount of aqueous sulfide present in each culture bottle was calculated from its total culture volume and the corresponding concentration of aqueous sulfide. The remaining culture of each culture bottle was filtered through a filter bags (Ankon Technology) to determine the disappearance of both dry

matter (DM) and neutral detergent fiber (NDF) of the added substrate. The apparent DM disappearance of the feed substrate was calculated after drying the original feed substrate and the residues in the filter bags at 105°C for 18 h (AOAC, 1990). The NDF content of the initial feed substrate and the residues were analyzed using method described by Van Soest (1991), and then NDF disappearance was calculated.

DNA extraction and quantitative PCR (qPCR)

The repeated [bead beating plus](#) column purification (RBB+C) method (Yu and Morrison, 2004) was used for extracting DNA from each culture sample. The quality of the DNA extracts was examined using agarose gel (1%) electrophoresis, and the DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Fisher Scientific, Suwanee, GA, USA). The DNA samples were stored at -20°C until analysis. The population of total bacteria, SRB, and domain *Archaea* was quantified using specific [qPCR](#) assays against respective qPCR standards prepared in this study. For quantification of SRB in the culture samples, two genes, the 16S rRNA gene of the genus *Desulfovibrio* and the gene coding for the alpha subunit of the adenosine-5'-phosphosulfate reductase gene (*apsA*) were targeted. One sample-derived qPCR standard was prepared for each of the select groups of target using respective specific primers and a composite metagenomic DNA sample that consisted of an equal amount of DNA from all the DNA samples to be quantified (Chen et al., 2007). Then, each of the standards was purified using a QIAquick PCR purification kit (QIAGEN, Inc, Valencia, CA) and quantified using a Quant-itTM dsDNA Broad Range Kit (Invitrogen). For each standard, copy number concentration was calculated based on its length (bp) and the mass concentration (Yu et al., 2005). Tenfold serial dilutions were prepared in Tris-EDTA (TE) buffer prior to qPCR assays.

All the qPCR assays were performed in three replicates essentially as described previously (Wu et al., 2015).

Statistical analysis

The data were analyzed using the mixed model procedure of SAS (SAS Institute, Cary, NC) in a 2 (ferric iron types) $\times$ 6 ([concentration](#)) factorial design. [Each concentration of each of the two types of ferric compounds was considered as one experimental unit with three replicates per experimental unit \(\$n=3\$ \)](#). Orthogonal polynomial contrasts were used to examine the linear, quadratic, or cubic effect of the increasing [concentrations](#) of ferric compounds. Statistical significance was declared at $P \leq 0.05$.

Results

Effects of ferric iron on *in vitro* fermentation and [production of sulfide and methane](#).

The addition of both ferric oxide and ferric citrate decreased ($P < 0.01$) [production of both](#) H₂S in the headspace and aqueous sulfide [in the](#) *in vitro* rumen culture without affecting ($P > 0.05$) total gas production, pH, or disappearance of DM and DNF (Tables 3, 4). However, [ferric citrate decreased](#) sulfide production [to greater magnitudes than ferric oxide](#). [Besides, increasing concentrations](#) of ferric oxides resulted in cubic decrease in H₂S gas in the headspace and quadratic decrease in aqueous sulfide ($P < 0.05$), while ferric citrate resulted in cubic decrease in both headspace H₂S gas and aqueous sulfide ($P < 0.05$). Neither of the two forms of ferric iron affected methane production at their tested [concentration](#), but increasing [concentrations](#) of ferric citrate tended ($P = 0.08$) to lower methane production quadratically.

Effects of ferric iron on VFA production. Total VFA concentration was not affected by either [of the two](#) ferric iron forms at the [concentrations](#) tested ($P>0.05$) (Tables 5, 6). The addition of ferric oxide had no significant effect on VFA profile (Table 5). However, molar proportion of acetate and acetate:propionate (A:P) ratio were linearly increased with increasing [concentration](#) of ferric citrate ($P<0.05$), while butyrate and valerate were linearly decreased ($P<0.05$) (Table 6).

Effects of ferric iron on microbial population. The abundance of total bacteria, total archaea, total SRB, and the genus *Desulfovibrio* in all the *in vitro* rumen culture samples [was](#) quantified using respective qPCR. Except for 25 mg/L of ferric citrate, which resulted in decrease in the total bacterial population by 24.1%, the population of total bacteria in the *in vitro* rumen cultures [was not significantly affected by either ferric iron at the tested concentrations](#) (Figure 1). Ferric citrate significantly increased the population of total *Archaea* but to different magnitudes at the different [concentrations](#) tested, with 150 and 200 mg/L resulting in greater increase than the other three [concentrations](#). With respect to ferric oxide, 50 and 200 mg/L resulted in significant increase in total *Archaea* population. The abundance of *apsA* gene was significantly decreased by ferric citrate but increased [slightly](#) by ferric oxide. The population of *Desulfovibrio* was increased substantially in a [concentration](#)-dependent manner by ferric citrate, but it was not affected by ferric oxide [at any of the concentrations](#).

Discussion

In the past [decade](#), bioethanol byproducts such as DDGS has become abundantly available and cost competitive as a feed ingredient because of the expansion of bioethanol industry in the United States and beyond. [Because](#) the high content of sulfate in DDGS can [increase](#) the

potential risk of sulfur-associated PEM [in cattle](#) (Felix et al., 2012), [intervention is needed to allow DGGs to be included at relatively high percentage. Redirection of reducing equivalent away from sulfate reduction to respiration by other microbes is a potential strategy. In the rumen, methanogens are primarily responsible for](#) removal of [hydrogen](#), stimulating fermentation [and increasing feed digestion and microbial growth \(Russell and Wallace, 1997\).](#) However, dissimilatory disposal of [hydrogen](#) through methanogenesis leads to methane emission. [In addition, methanogenesis is less thermodynamically favorable than sulfate reduction by SRB. Iron is a required element by cattle and thus ferric iron is a potentially strategy to inhibit sulfide production and lower H₂S concentration in the gas belched out from cattle.](#)

As hypothesized, both ferric citrate and ferric oxide decreased H₂S gas in the headspace and aqueous sulfide in the *in vitro* rumen culture. Because both H₂S and aqueous [sulfide](#) were decreased, microbial reduction of sulfate was evidently inhibited. The form of ferric iron has been suggested to be a critical factor affecting its ability to compete with sulfate for electron donors (Lovely and Phillips, 1987). In the present study, ferric oxide, which is less soluble but inexpensive, and ferric citrate, which is soluble but expensive, were evaluated in a comparative manner. Not surprisingly, ferric oxide was less inhibitory to sulfidogenesis than ferric citrate. Ferrous iron forms from ferric reduction, and ferrous iron can combine with sulfide forming [insoluble black](#) iron sulfide. Indeed, the *in vitro* cultures that received ferric oxide and ferric citrate appeared to be black, while the control cultures did not, suggesting formation of iron sulfide. Thus, precipitation of sulfide as iron sulfide has probably also contributed to the observed decrease in H₂S concentration in the headspace. [Because it is insoluble \(solubility product constant at 25°C, \$pK_{sp} = 6 \times 10^{-19}\$ \), iron sulfide is not bioavailable or toxic. Indeed, iron sulfide injected at 100 mg/kg body weight did not cause apparent toxicity in mice \(Yang et al.,](#)

2016). It should be point out that although iron sulfide increases its solubility at pH below seven, the typical pH of the rumen is above 5.6 and at this pH much of the iron sulfide remains insoluble.

It has been [demonstrated](#) that molybdate (MoO_4^{2-}) can inhibit sulfate reduction by SRB in various environments, including rumen fluid (Gawthorne and Nader, 1976; Kung et al., 2000). Kung et al. (2000) reported that adding 10 and 25 [mg/L](#) of [molybdate](#) to high-sulfur diet decreased H_2S production by 12 and 77%, respectively. Different from ferric iron, molybdate, as an analog of sulfate, decreased sulfide production by blocking the activation step of sulfate reduction that is catalyzed by ATP sulfurylase (Oremland and [Capone](#), 1988). The same author also showed that 9,10-anthraquinone at 10 [mg/L](#) decreased sulfide production by 71% in rumen fluid. It was suggested that 9,10-anthraquinone inhibits sulfide production by uncoupling electron transport from ATP synthesis in SRB (Cooling et al., 1996). However, it is probably not practical to decrease sulfur-associated PEM in cattle using molybdate or 9,10-anthraquinone because both are expensive chemicals.

Neither ferric citrate nor ferric oxide affected most of the fermentation parameters, but the VFA profile was affected by ferric citrate. Molar proportion of acetate and A:P ratio were increased, while molar proportion of butyrate and valerate were decreased by ferric citrate. The increased A:P ratio was due to increasing proportion of acetate rather than decreasing propionate production. In general, increased A:P ratio in the rumen accompanies decrease in feed efficiency because acetate is not gluconeogenic in animal tissues and much of it is oxidized or used for lipogenesis. Furthermore, acetic acid production can results in heat production during feed digestion in the rumen, which is dissipated ([McClymont G. L., 1952](#)). However, as dietary

sulfate content increases, A:P ration decreases (Drewnoski et al, 2012). Thus, the A:P depressing effect of ferric iron may be offset to some extent by increase in dietary sulfur content.

Livestock contributes about 18% to the global anthropogenic greenhouse gas (GHG) emissions, accounting for about 37% of the total anthropogenic methane emission (FAO, 2006). Different rumen modifiers/inhibitors, such as condensed tannins, saponins, nitrate, sulfate, and essential oil, have been evaluated to lower [methane](#) production both *in vitro* and *in vivo* (Patra et al., 2014; Wu et al., 2015). To the best of our knowledge, this is the first study that evaluated ferric iron as an anti-methane inhibitor [in the rumen](#). Contrary to what we hypothesized, neither form of the ferric iron significantly lowered [methane](#) production in the *in vitro* cultures, and only numerical decrease in methane production was noted in the cultures that received ferric citrate. This is surprising and contradictory to the substantial decrease in methane production observed in the constructed wetland (Pangala et al., 2010) and to the prediction from thermodynamics of sulfate reduction. [One plausible explanation is that the ferric concentrations](#) used in the present study might be below the effective concentration. Future research is warranted to further evaluate ferric iron as anti-methane inhibitors to lower methane production by rumen microbiome under different dietary conditions, including diets that contain normal sulfur content or containing DDGS.

No study was found in the literature that reported the effect of ferric iron on rumen microbes. To understand how ferric iron affects rumen fermentation, the populations of total bacteria, total archaea, total SRB (represented by *apsA* as a proxy), and the genus *Desulfovibrio* were quantified. The abundance of total bacteria was not affected by either form of the ferric iron, except for ferric citrate at 25 mg/mL, which decreased the abundance of total bacteria. The lack of overall effect on total bacterial abundance is consistent with the fermentation parameters,

including total gas production, DM and NDF disappearance, and total VFA concentration. These results suggest that ferric iron at the tested concentrations does not adversely affect feed digestion or fermentation, [which is consistent with the finding reported by Pogge et al. \(2014\)](#). Unexpectedly, the abundance of total *Archaea* was increased by ferric iron, especially by ferric citrate. However, methane production was not increased. These results further corroborate the uncoupling of methane production from abundance of total archaea in short-term *in vitro* cultures of rumen microbiome (Patra and Yu, 2014). The long-term effects of ferric iron on abundance of rumen archaea need to be determined in future studies.

The first strain of SRB of rumen origin was isolated from the rumen of hay-fed sheep, and it was later classified as *Desulfotomaculum ruminis* (Coleman, 1960). The second strain of rumen SRB, also isolated from sheep but fed a diet containing sulfate, was classified to the genus *Desulfovibrio* (Huisinigh et al., 1974). In the literature, *Desulfovibrio* spp. are reported as the prevalent SRB in the rumen, and some of them can reduce ferric iron (Huisinigh et al., 1974). Thus, both the abundance of *Desulfovibrio* and total [apsA gene](#) [were](#) quantified to evaluate the effect of ferric iron on SRB. Consistent with the sulfide [production](#) results and as expected, the populations of total SRB were decreased by ferric citrate. [However, the ferric oxide did not decrease total SRB](#). The greater inhibition [to total](#) SRB by ferric citrate is probably attributed to [its](#) greater solubility, and thus biological availability. [Interestingly, the population of Desulfovibrio was increased by ferric citrate. The stimulatory effect of ferric citrate on Desulfovibrio may be attributed to the ability of some species of this genus, such as D. vulgaris, to use ferric iron as an terminal electron acceptor also \(Park et al., 2008\). It should be noted that citrate was added to the cultures that received ferric citrate. Thus, citrate might be another factor that affected the populations of Desulfovibrio.](#) Future studies [including citric acid as a control](#) are

needed to [assess the effect of citrate on *Desulfovibrio* and to](#) further confirm the sensitivity of different species of rumen SRB to ferric iron.

Conclusions

Sulfidogenesis from sulfate present in high-sulfur diet, such as DDGS, can be suppressed competitively by adding ferric iron. Chemical formation of insoluble iron sulfide also decrease eructation of hydrogen sulfide, further lowering the risk of sulfur-associated polioencephalomalacia that is often observed in cattle fed large amounts of DDGS. Ferric iron probably does not adversely affect feed digestion or fermentation though it does not decrease methane production. This is the first study [to examine how](#) ferric iron [affects sulfate metabolism and SRB in the](#) rumen. [Future](#) research, both *in vitro* and *in vivo*, is warranted to systematically evaluate ferric iron for its effect on all aspects of rumen function [and](#) rumen microbiome.

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1 Table 1 Composition of the diet fed to the cows from which rumen fluid was collected as
2 inoculum

Ingredients	% DM
Corn silage	39.0
Alfalfa hay	35.0
Ground corn	9.3
Soybean meal	6.5
DDGS	2.8
Wheat middling	2.4
Megalac ¹	0.8
Aminoplus ²	4.2

3 ¹ Rumen protected fat, combined natural plant oil (palm fatty acids) with calcium.
4 ² Rumen undegradable protein (EUP), containing 51.1% crude protein (based on DM), of which 72.0% is RUP.

5 Table 2 Specific PCR primers used, target genes, annealing temperature, and amplicon length

Primer	Sequence (5'→3')	Target gene	Annealing (°C)	Amplicon length (bp)	Reference
Eub358f	TCCTACGGGAGGCAGCAGT	16S rRNA gene of bacteria	60	467	Nadkarni et al. 2002
Eub806r	GGACTACCAGGGTATCTAATCCTGTT				
APS3f	TGGCAGATCATGWTYAAAYGG	<i>apsA</i> * of SBR	58	396	Christophersen et al., 2011
APS2r	GGGCCGTAACCRTCYTTRAA				
DSV691f	CCGTAGATATCTGGAGGAACATCAG	16S rRNA gene of	62	135	Fite et al., 2004
DSV826r	ACATCTAGCATCCATCGTTTACAGC	<i>Desulfovibrio</i>			
ARC787f	ATTAGATACCCSBGTAGTCC	16S rRNA gene of archaea	56	273	Yu et al., 2005
ARC1059r	GCCATGCACCWCCTCT				

6
7 **apsA*, the gene coding for the alpha subunit of adenosine 5'-phosphosulfate reductase.

8

Table 3 Effects of addition of ferric oxide (Fe₂O₃) on *in vitro* fermentation and sulfide production

Items	Ferric iron concentration (mg/mL)						SEM	Concentration ¹			
	0	25	50	100	150	200		P value	L	Q	C
Fermentation characteristics											
pH	6.93	6.95	6.93	6.93	6.97	6.91	0.017	0.335	0.745	0.740	0.970
Gas production (mL)	58.97	61.98	61.94	62.48	55.48	59.10	2.677	0.454	0.783	0.782	0.118
DM disappearance (%)	70.87	68.70	71.45	68.59	69.23	68.57	1.273	0.493	0.113	0.784	0.557
NDF disappearance (%)	57.02	56.00	59.02	55.02	57.38	54.38	2.301	0.780	0.366	0.884	0.563
Methane (mL)	7.77	7.70	7.80	8.23	6.93	7.47	0.584	0.739	0.812	0.587	0.303
Sulfide production											
H ₂ S gas (mg) ²	0.32 ^a	0.30 ^a	0.28 ^a	0.23 ^b	0.20 ^{bc}	0.19 ^c	0.056	<0.001	<0.001	0.203	0.001
Aq. sulfide (mg) ³	3.64 ^a	2.83 ^b	2.73 ^b	2.24 ^{bc}	2.04 ^c	1.49 ^d	0.414	<0.001	<0.001	0.013	0.083

¹ L=linear; Q=quadratic; C=cubic.

² H₂S produced in the headspace of the *in vitro* cultures.

³ Aqueous sulfide produced in the *in vitro* cultures.

Means followed by different superscripts are significantly different ($P<0.05$).

16

Table 4 Effects of addition of ferric citrate ($C_6H_5FeO_7$) on *in vitro* fermentation and sulfide production

Items	Ferric iron concentration (mg/mL)						SEM	Concentration ¹			
	0	25	50	100	150	200		P value	L	Q	C
Fermentation characteristics											
pH	6.93	6.96	6.94	6.93	6.89	6.93	0.016	0.190	0.226	0.245	0.203
Gas production (mL)	58.97	53.71	52.78	56.83	59.34	56.01	2.476	0.380	0.686	0.111	0.170
DM disappearance (%)	70.87	70.86	72.21	69.30	68.34	68.97	1.019	0.144	0.250	0.467	0.104
NDF disappearance (%)	57.02	60.71	59.46	63.33	65.63	64.79	2.293	0.159	0.130	0.627	0.448
Methane (mL)	7.77	6.74	6.57	7.11	7.51	7.02	0.392	0.309	0.841	0.082	0.137
Sulfide production											
H ₂ S gas (mg) ³	0.32 ^a	0.23 ^b	0.22 ^b	0.16 ^c	0.11 ^d	0.09 ^d	0.008	<0.001	<0.001	<0.001	0.001
Aq. sulfide (mg) ⁴	3.64 ^a	3.05 ^b	2.71 ^c	1.59 ^d	0.91 ^e	0.70 ^e	0.093	<0.001	<0.001	0.040	<0.001

17

¹ L=linear; Q=quadratic; C=cubic.

18

² H₂S produced in the headspace of the *in vitro* cultures.

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³ Aqueous sulfide produced in the *in vitro* cultures.

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Means followed by different superscripts are significantly different ($P<0.05$).

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23 Table 5 Effects of addition of ferric oxides (Fe₂O₃) on total VFA and VFA profile

Items	Ferric iron concentration (mg/mL)						SEM	Concentration ¹			
	0	25	50	100	150	200		P value	L	Q	C
Total VFA (mM)	85.55	87.68	77.82	90.88	78.58	80.50	5.750	0.527	0.664	0.388	0.684
Individual VFAs (% mM)											
Acetate (A)	61.54	61.78	61.46	61.93	63.06	62.31	0.646	0.549	0.219	0.935	0.316
Propionate (P)	19.16	18.52	20.09	18.69	18.62	18.41	0.626	0.460	0.231	0.982	0.218
A:P ratio	3.22	3.34	3.08	3.32	3.39	3.39	0.109	0.388	0.149	0.882	0.171
Isobutyrate	2.71	2.37	1.99	2.62	2.14	2.38	0.353	0.704	0.996	0.264	0.669
Butyrate	10.08	10.85	10.60	10.44	10.24	10.55	0.365	0.662	0.903	0.199	0.541
Isovalerate	4.08	4.10	3.63	4.01	3.75	4.03	0.236	0.612	0.966	0.739	0.468
Valerate	2.44	2.38	2.56	2.33	2.21	2.34	0.127	0.806	0.520	0.728	0.762

24 ¹ L=linear; Q=quadratic; C=cubic.

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Table 6 Effects of addition of ferric citrate ($C_6H_5FeO_7$) on total VFA and VFA profile

Items	Ferric iron concentration (mg/mL)						SEM	Concentration ¹			
	0	25	50	100	150	200		P value	L	Q	C
Total VFA (mM)	85.55	74.79	74.45	73.82	69.32	72.91	4.409	0.291	0.078	0.226	0.757
Individual VFAs (% mM)											
Acetate (A)	61.54 ^c	63.15 ^{bc}	64.40 ^{ab}	65.55 ^a	65.15 ^{ab}	66.15 ^a	0.517	0.003	0.006	0.194	0.631
Propionate (P)	19.16	18.84	19.01	17.78	19.24	17.73	0.461	0.158	0.099	0.811	0.918
A:P ratio	3.22 ^b	3.35 ^{ab}	3.40 ^{ab}	3.69 ^a	3.39 ^{ab}	3.74 ^a	0.090	0.025	0.009	0.848	0.824
Isobutyrate	2.71	2.25	2.01	1.94	1.92	2.08	0.241	0.291	0.070	0.452	0.349
Butyrate	10.08 ^a	9.79 ^{ab}	9.24 ^{ab}	9.52 ^{ab}	8.63 ^b	8.98 ^{ab}	0.274	0.049	0.049	0.219	0.477
Isovalerate	4.08	3.72	3.33	3.31	3.13	3.22	0.195	0.061	0.019	0.313	0.577
Valerate	2.44 ^a	2.26 ^{ab}	2.02 ^b	1.91 ^b	1.94 ^b	1.85 ^b	0.090	0.012	0.003	0.394	0.542

29

30 ¹ L=linear; Q=quadratic; C=cubic.31 Means followed by different superscripts are significantly different ($P<0.05$).

FIGURE

Fig. 1 Abundance of microbial populations in the *in vitro* rumen cultures with different ferric iron addition. Error bars indicate the standard error of the means (n=3), and asterisks denote significant difference ($P<0.05$). 0, 25, 50, 100, 150, and 200 represent 0, 25, 50, 100, 150, and 200 mg/mL of ferric iron (Fe^{3+}).