



# Effects of Sulfur Sources on Ruminal S Bioavailability, Fermentation Activity and Microbial Populations Measured *In Vitro*

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## RESEARCH ARTICLE

### Abstract

The aim of this study was to better understand the ruminal solubility, bioavailability, effect on fermentation and microbiota of different sulfur (S) forms. *In vitro* fermentations with rumen fluid were conducted for 70hrs. Hay was incubated solely (CON), or with 0.5%DM of S, as: elemental sulfur (ES), Na<sub>2</sub>SO<sub>4</sub> (NaS), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (NHS) and MgSO<sub>4</sub> (MgS). Fermentation was assessed by gas production (TGP) and dry-matter degradability (dDM%). Solubility and bioavailability were estimated by S concentration of the supernatant (SOL) and of bacteria (BACT). Microbial community was assessed by amplification of the 16SrRNA gene. NHS decreased ( $p<0.05$ ), ES and NaS didn't affect, while MgS increased ( $p<0.01$ ) TGP. MgS increased ( $p<0.001$ ), whilst ES, NaS and NHS showed no effect on dDM%. Sulfate sources increased ( $p<0.001$ ), while ES did not affect the S content of SOL. S content of BACT suggests that sulfates have a high bioavailability, while ES is poorly assimilated by bacteria. Some variations of the microbial community were observed, including a lower abundance of methanogens with all S sources and a higher abundance of *Desulfovibrio* with MgS. These findings suggest that ES has limited solubility, whereas sulfates, particularly MgS, exhibit a high level of solubility in the rumen, resulting in a more effective utilization by bacteria.

**Keywords:** Ruminant; fermentation; solubility; sulfur.

## INTRODUCTION

Rumen microorganisms require minerals for their growth (microbial protein synthesis) and activity (fibrolytic fermentations) (Martinez, 1972). However, ruminant nutritional feeding systems (2018, bk. INRA; Nutrient Requirements of Dairy Cattle, 2021) mainly focus on the overall mineral requirements of animals for maintenance, growth and production, whereas the specific needs for the development and activity of the ruminal microbiota are poorly documented. For some elements, as sulfur (S), it seems that rumen microbiota requirements (2.5 – 3.1 g/kg of degraded organic matter) are slightly higher than those established for the host animal (2.0 g/kg DM) (Komisarczuk Bony and Durand, 1991; Meschy, 2010). In an *in vitro* study with rumen fluid by Stevani et al. (1992), it was demonstrated that an addition of S (as NaSO<sub>4</sub>) to a straw substrate significantly improves the microbial activity, increasing the organic matter (OM) digestibility

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and the total volatile fatty acids (tVFA) production. Sulfur supplementation improves the fermentative activity in the rumen, especially when diets containing a high amount of roughages are fed to the animals, by having a positive effect on specific bacterial populations. McSweeney and Denman (2007) demonstrated that S (as NaSO<sub>4</sub>) supplementation of a low-S diet (100% Angleton grass hay) significantly increases the growth of fibrolytic rumen bacteria (*Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) populations. The positive effect of S on improvement of rumen fermentative activity is not always reached. In an *in vivo* study by Silva et al. (2014), crossbreed steers consuming a roughage-only (*Brachiaria dictynoeura* hay) diet showed no improvement of nutrient (dry matter, fiber, or crude protein) apparent ruminal digestibility when different sources of S (ES – 70% S, ES – 90% S, CaSO<sub>4</sub>, or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) were supplemented. Ososanya et al. (2016) showed that supplementing 0.25% DM of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to *in vitro* fermentations using rumen fluid collected from ewes reduces methane production, while fermentation parameters like OM degradation or tVFA production are not affected. Establishing the right amount of S supplementation for ruminants is important, due to the competition for H<sup>+</sup> between methanogens and sulphate-reducing bacteria (Shah et al., 2020). Considering this, Wu et al. (2021) demonstrated that an increased amount of S (0.40% - 1.20% DM, as NaSO<sub>4</sub>) not only affects the fermentation parameters (tVFA, NH<sub>3</sub>-N), but also increases the population of *Desulfovibrio* without significant effect on the whole rumen microbiota. Several sources of S are available for use in ruminant supplementation, but still little is known of their effectiveness of use by rumen microbiota, rendering thus the selection of the right supplemental S source challenging. A better understanding of the ruminal bioavailability of different forms of S, the proportions assimilated by microbes and their effect on ruminal growth and activity will allow the refinement of the recommendations concerning the supplementation of ruminants.

This work contributes to the expansion of knowledge on the solubility of different sources of inorganic S and their effects on rumen fermentative activity and bacterial community composition. We hypothesized that some sulfates (-SO<sub>4</sub>) with high S content (>20%) would be highly soluble in rumen fluid compared to elemental sulfur (ES), and therefore, more bioavailable for rumen microorganisms. The present study aimed (1) to investigate the effects of ES, sodium sulfate (NaSO<sub>4</sub>), ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and magnesium sulfate (MgSO<sub>4</sub>) on rumen fermentation activity measured *in vitro*, in conditions in which S content of the substrate is below the recommended levels; (2) identify the rumen fluid fraction(s) in which the additional S is found after fermentation (big particles fraction [feed particles, protozoa, insolubilized minerals], bacteria-enriched fraction or final supernatant); and (3) observe the shifts in the microbial community to assess ruminal bioavailability.

## MATERIALS AND METHODS

The use of rumen-cannulated dairy cows in this study was approved by the Ethics Committee n°052 (France) and the study protocol was registered under the number APAFIS#28768-2020122108098663 v3 by the French Ministry of Scientific Research.

This study was carried out at the Talhouet Research Center of ADM Animal Nutrition (TRC, Saint-Nolff, France) to evaluate the rumen solubility and effect on fermentation activity and microbial community composition of four feed-grade inorganic S sources (supplied by SERMIX, Chierry, France): elemental sulfur (ES; >97% S), sodium sulfate (NaS; NaSO<sub>4</sub>; 22% S), ammonium sulfate (NHS; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 24% S) and magnesium sulfate (MgS; MgSO<sub>4</sub>; 26% S).

### Experimental design and treatments

*In vitro* incubations, respecting the established recommendations regarding substrate, inoculum, and buffer (Yáñez-Ruiz et al., 2016), were conducted for 70 hrs using the automatic system Gas Endeavour® (Bioprocess Control, Lund, Sweden). Each sulfate source of S (NaS, NHS and MgS) was tested against ES along 3 incubation blocks, each incubation block regrouped 3 consecutive incubations, while each incubation had 6 replicates per treatment. Fermentation activity was measured through total gas production (TGP; ml/g DM; continuous measurement for 70 hrs), gas production rate (GP rate; ml/h), substrate dry-matter degradability (dDM%), pH and volatile fatty acid (VFA, mM) production (total concentration [tVFA] and individual profile) at the end of the 70 hrs fermentations. The microbial community composition was assessed by DNA extraction and 16S rRNA gene sequencing (Henderson et al., 2015). S solubility was assessed by measuring the concentration in fractions obtained after successive centrifugations of the final fermentation medium, allowing to separate the remaining big particles fraction (UNSOL; feed particles, insolubilized minerals, protozoa), a fraction enriched in ruminal bacteria (BACT), and a final supernatant (SOL), containing the solubilized S. The correct separation of the different fractions by centrifugation was verified by a diaminopimelic acid analysis (DAPA) of the UNSOL and BACT (Krawielitzki et al., 1978, 1989). For the first incubation block (3 incubations testing NaS), the substrate was incubated solely (CON), or with an addition of 0.50% DM of S, as ES or NaS, added at the start (ES\_0h and NaS\_0h) or after 48 hrs (ES\_48h and NaS\_48h) of incubation. For the second incubation block (3 incubations testing NHS), the substrate was incubated solely (CON, CON\_48h), or with an addition of 0.50% DM of S, as ES or NHS, added at the start (ES\_0h and NHS\_0h) or after 48 hrs (ES\_48h and NHS\_48h) of incubation. To account for the nitrogen (N) input brought by the

NHS treatment, for replicates of CON, CON\_48h, ES\_0h and ES\_48h treatments, an equivalent amount of N (as urea; 46% N) was also added in order to ensure the same supply of fermentable N (potential limiting factor of fermentation activity), and avoid a potential bias given by the N content of the NHS (21% N) (Mould et al., 2005). For the third incubation block (testing MgS), the substrate was incubated solely (CON), or with an addition of 0.50% DM of S, as ES or MgS, added at the start (ES\_0h and MgS\_0h) or after 48 hrs (ES\_48h and MgS\_48h) of incubation. ES (ES\_0h and ES\_48h) was added as positive control in all incubations.

### Substrate

The substrate used for this study was composed of (% DM): 99.96% hay and 0.04% urea. The hay substrate for the *in vitro* incubations was selected based on the low content of S. Indeed, the S content of the hay was 1.51 g/kg DM, below the INRA (2018) recommendations for cattle diets (2.00 g/kg DM). The hay used as substrate was the same as the one distributed in the ration of the rumen fluid donor cows.

### Rumen fluid donor cows

The 3 rumen fluid donor cows used in this trial were dry, hysterectomized and rumen-cannulated Holstein cows from the TRC. The cows received a ration (Table 1), in two equal meals per day for at least 21 consecutive days before sampling of rumen fluid; their ruminal flora was considered to be stabilized. The cows were housed at the TRC site in a free-stall barn, with rubber mattresses and permanent access to water distributed through automatic drinkers. The S content of the diet of the donor cows was 2.83 g/kg DM, above the recommendations for cattle (2.00 g/kg DM) (Noziere et al., 2018).

**Table 1.** Composition and chemical analysis of rumen fluid donor animals' diet

Composition	Intake (kg DM/head/day)/Content
Corn silage	3.68
Hay	1.32
Complete feed†	2.80
Crude Protein (% DM)	16.40
Starch (% DM)	17.40
Total fiber (% DM)	19.00
S (g/kg DM)	2.83

†Complete feed composition: rapeseed meal – 39.0%, wheat bran – 25.0%, soybean meal – 18.1%, sunflower meal – 10.0%, corn distillers' grain – 4.1%, urea – 1.5%, salt – 1.0%, 0.96% CaCO<sub>3</sub> and 0.2% premix (contained no additional S); 32.9 Crude protein (%DM), 7.3 Starch (%DM), 11.5 Total fiber (% DM) and 4.54 g/kg DM of S.

### Buffer

The buffer used in this study was prepared based on the buffer proposed by Tilley and Terry (1963), described in Mould et al. (2005). The buffer contained no S, to exclude the additional S source. The buffer to inoculum ration used in this study was 4:1.

### Preparation and launch of incubations

The hay substrate was directly grinded (3 mm sleeve) and the residual DM content was measured (4 hrs at 103 °C). The DM of the urea was considered 100%. The day before the incubations were launched, nylon bags (10.0 cm L x 5.0 cm l, 50µm pores Ankom bags, Humeau Laboratories, France) containing the substrate (3.7 g DM) and, if applicable, additional S (ES\_0h, NaS\_0h, NHS\_0h and MgS\_0h), were introduced into the incubation flasks (500 ml).

The buffer was added (385 ml) using an automatic dispenser in order to respect the minimum of 2 hrs of pre-hydration necessary for the good start of the fermentations (Yáñez-Ruiz et al., 2016). The hydrated bottles were hermetically sealed and refrigerated (4 °C) until the next morning. Previous to each incubation (maximum 1h), the inoculum was prepared from the ruminal content sampled from the 3 rumen fluid donor cows (Yáñez-Ruiz et al., 2016; Yacout et al., 2021). The sampling was carried out in the morning, before feeding to limit the variability of the inoculum in relation to postprandial kinetics. On the morning of the incubations, the flasks were randomly allocated to two Gas Endeavour® devices and warmed in the water bath at 39 °C approximately 1 h before the beginning of the inoculation. The flasks were connected to the measuring devices with Tygon tubing before adding the inoculum (96 ml) through a 2-way valve using an automatic dispenser. After the inoculation, N<sub>2</sub> (235 ml) was used to flush the residual O<sub>2</sub> from the system and promote the proper start of anaerobic fermentations (Yáñez-Ruiz et al., 2016).

The measuring devices were launched as soon as the saturation with N<sub>2</sub> was finished. The Gas Endeavour® devices were configured to record data normalized by temperature and humidity. For the addition of S after the start of the incubations (ES\_48h, NaS\_48h, NHS\_48h and MgS\_48h, respectively) the quantities of minerals were directly introduced after 48 hrs by pausing the *in vitro* system (gas production registration and stirring) and opening the flasks as little as possible to limit the O<sub>2</sub> contamination.

### Measurements, samplings, and analysis

- The TGP was continuously registered for 70 hrs and recorded using the software provided by Bioprocess Control (version ge\_2.1[v1.2948]).
- At the end of the incubations, the flasks were opened consecutively, and the pH of the medium was measured immediately (flasks maintained in the water bath).
- At the end of the 70 hrs incubations, the nylon bags containing the remaining substrate were removed from the flasks, rinsed briefly with cold water, then frozen (-18 °C) for 24 hrs (adaptation of the *in situ* model for rumen degradation) (Dulphy et al., 1999). After defrosting, the nylon bags were washed a second time in cold water for 2 minutes and oven dried for 48 hrs at 60 °C. The nylon bags containing the dried residues were weighed, and the dry matter degradability (dDM%) was calculated (Valentin et al., 1999).
- A partitioning factor (PF) was calculated as the ratio of total degraded DM (mg) to TGP (ml) at the end of the 70 hrs fermentation (Blümmel et al., 1997).
- Replicates of the same treatment of the final fermentation medium were pooled and sampled. The samples (12 ml/pool) were frozen (-18 °C) before being sent for VFA and NH<sub>3</sub>-N analysis (Upscience, Saint-Nolff, France). Another set of pooled samples (50 ml/pool) were frozen at -80 °C before freeze-drying (CHRIST BETA 1-8 LSC PLUS, Martin Christ, Germany) and DAPA analysis (Upscience, Saint-Nolff, France). A third set of pooled samples (9 ml/pool) were frozen at -80 °C and sent for DNA extraction and 16S rRNA gene sequencing to compare the microbial community composition between treatments (Agri-Food and Biosciences Institute, Belfast, Northern Ireland).
- The total quantity of pooled final fermentation medium was centrifuged to separate 3 different fractions, based on the method described by Krawielitzki et al. (1989): UNSOL, BACT and SOL. The pooled fermentation medium was first refrigerated (4 °C) for 6 hrs, then agitated with a magnetic stirrer (400 rpm) for 45 seconds to detach the bacteria bound to fiber particles (Dehority and Grubb, 1980). Next, the fermentation medium was centrifuged (Haraeus Multifuge X3R, Thermo Fisher Scientific) at 100 x g for 5 minutes at 4 °C, the total quantity of obtained pellet (UNSOL) was recovered and frozen at -80 °C before freeze-drying (CHRIST BETA 1-8 LSC PLUS, Martin Christ, Germany). The obtained supernatant was centrifuged at 18,500 x g for 20 minutes at 4 °C. The total quantity of the SOL was registered and then sampled (10 ml) and frozen at -80°C; the total quantity of obtained pellet (BACT) was recovered and frozen at -80 °C before freeze-drying. Following the freeze-drying, the UNSOL and BACT were sent for DAPA (Upscience, Saint-Nolff, France) and mineral (UT2A, Pau, France) analysis. The SOL samples were analyzed only for mineral content (UT2A, Pau, France), as previous studies done in the lab had consistently shown that this fraction contained no DAPA.
- The S (total S and % of total S) in each fraction (UNSOL, BACT and SOL) of the final fermentation medium was calculated based on S content of the fractions (Vigh et al., 2023).
- The DAPA concentration of the final fermentation medium was used as rumen bacterial synthesis marker (Dufva et al., 1982).
- Data were statistically analyzed by Analysis of Variance and Tukey-test with R software (version 4.1.3), with the treatment as a fixed factor and the replicates (incubation flasks) or the incubations (for S analysis in the fractions) as a random factor. Treatments which were supplemented with S after 48 hrs were considered as CON before the addition of the S source (ES\_48h, NaS\_48h, NHS\_48h, and MgS\_48h, respectively).

## RESULTS AND DISCUSSIONS

### Fermentation parameters

Results of different S sources effects on TGP and GP rate are presented in Table 2. The treatment x incubation interaction effect on TGP, tested for the CON, ES\_0h and ES\_48h, was not significant ( $p=0.60$ ), enabling thus the comparison of treatments between all incubations. The TGP was significantly different between the treatments throughout the 70 hrs incubation. After 1 h of fermentation, the highest TGP production was observed with MgS\_0h, the lowest with NHS\_0h, while ES\_0h and NaS\_0h showed no significant differences when compared to CON ( $p<0.01$ ;  $11.4\pm0.82$ ,  $7.5\pm0.90$ ,  $9.4\pm0.45$ ,  $8.5\pm0.72$  and  $8.9\pm0.26$  ml/g DM for MgS\_0h, NHS\_0h, ES\_0h, NaS\_0h and CON, respectively). After the first 24 hrs of fermentation, the TGP production was the highest with MgS\_0h, the lowest with NHS\_0h, while ES\_0h and NaS\_0h did not vary significantly when compared to CON. After 48 hrs of fermentation, the TGP with NHS\_0 was significantly ( $p<0.01$ ) lower, whilst ES\_0h, NaS\_0h and MgS\_0h was not different when compared to CON. At the end of the 70 hrs fermentation the TGP was significantly ( $p<0.01$ ) higher with MgS\_48h, significantly

( $p < 0.05$ ) lower with NHS\_0h, while ES\_0h, ES\_48h, NaS\_0h, NaS\_48h, NHS\_48 and MgS\_0h showed little variations when compared to CON. Regarding the GP rate, significant variations were observed during the 70 hrs incubation. In the 0-1 h interval, the GP rate with MgS\_0h was significantly ( $p < 0.05$ ) higher, with NHS\_0h tended ( $p \leq 0.10$ ) to be lower, while ES\_0h and NaS\_0h showed no significant variations when compared to CON ( $42.2 \pm 3.02$ ,  $27.9 \pm 3.32$ ,  $34.7 \pm 1.66$ ,  $31.3 \pm 2.67$  and  $32.9 \pm 0.96$  ml/h for MgS\_0h, NHS\_0h, ES\_0h, NaS\_0h and CON, respectively). Considering the GP rate in the 1-2 hrs interval, no significant variations were observed between the treatments, while in the 2-4 hrs interval the highest GP rate was registered with NaS\_0h. In the 24-48 hrs interval, the GP rate was significantly ( $p < 0.01$ ) lower with NHS\_0h, tended ( $p \leq 0.10$ ) to be lower with MgS\_0 and NaS\_0h, whilst with ES\_0h showed no difference when compared to CON. Following the S supplementation after 48 hrs of fermentation, the GP rate was significantly ( $p < 0.001$ ) increased with all the treatments when compared to CON ( $3.9 \pm 0.072$ ,  $4.0 \pm 0.11$ ,  $4.0 \pm 0.12$ ,  $3.9 \pm 0.12$  and  $3.4 \pm 0.06$  for ES\_48h, NaS\_48h, NHS\_48h, MgS\_48h and CON, respectively).

**Table 2.** The effect of S sources on TGP (LSMEAN $\pm$ SEM in ml/gDM) and GP rate (mean  $\pm$  S.E. in ml/h) during 70 hrs of fermentation

TGP† (ml/gDM)	CON	ES_0h	ES_48h	NaS_0h	NaS_48h	NHS_0h	NHS_48h	MgS_0h	MgS_48h	P Value
1h	8.9 $\pm$ 0.26 <sup>ab</sup>	9.4 $\pm$ 0.45 <sup>ab</sup>	-	8.5 $\pm$ 0.72 <sup>ab</sup>	-	7.5 $\pm$ 0.90 <sup>a</sup>	-	11.4 $\pm$ 0.82 <sup>b</sup>	-	<0.01
2h	13.9 $\pm$ 0.29 <sup>ab</sup>	14.7 $\pm$ 0.52 <sup>ab</sup>	-	13.7 $\pm$ 0.87 <sup>ab</sup>	-	12.2 $\pm$ 1.07 <sup>a</sup>	-	16.7 $\pm$ 0.97 <sup>b</sup>	-	<0.05
4h	20.8 $\pm$ 0.31 <sup>ab</sup>	21.9 $\pm$ 0.58 <sup>ab</sup>	-	21.1 $\pm$ 0.97 <sup>ab</sup>	-	18.3 $\pm$ 1.18 <sup>a</sup>	-	23.5 $\pm$ 1.08 <sup>b</sup>	-	<0.01
18h	61.0 $\pm$ 0.83 <sup>ab</sup>	63.5 $\pm$ 1.54 <sup>ab</sup>	-	63.7 $\pm$ 2.61 <sup>ab</sup>	-	52.6 $\pm$ 3.16 <sup>a</sup>	-	65.9 $\pm$ 2.90 <sup>b</sup>	-	<0.05
24h	79.2 $\pm$ 1.06 <sup>ab</sup>	82.2 $\pm$ 1.97 <sup>ab</sup>	-	82.5 $\pm$ 3.32 <sup>ab</sup>	-	68.6 $\pm$ 4.02 <sup>a</sup>	-	85.0 $\pm$ 3.70 <sup>b</sup>	-	<0.05
48h	128 $\pm$ 1.4 <sup>b</sup>	132 $\pm$ 2.5 <sup>b</sup>	-	129 $\pm$ 4.3 <sup>b</sup>	-	112 $\pm$ 5.2 <sup>a</sup>	-	134 $\pm$ 4.7 <sup>b</sup>	-	<0.01
70h	148 $\pm$ 2.1 <sup>b</sup>	153 $\pm$ 2.4 <sup>bc</sup>	151 $\pm$ 2.4 <sup>bc</sup>	146 $\pm$ 4.1 <sup>ab</sup>	150 $\pm$ 3.8 <sup>bc</sup>	129 $\pm$ 4.9 <sup>a</sup>	144 $\pm$ 4.4 <sup>ab</sup>	151 $\pm$ 4.5 <sup>bc</sup>	167 $\pm$ 4.2 <sup>c</sup>	<0.001
GP rate‡ (ml/h)										
0-1h	32.9 $\pm$ 0.96 <sup>ab</sup>	34.7 $\pm$ 1.66 <sup>ab</sup>	-	31.3 $\pm$ 2.67 <sup>ab</sup>	-	27.9 $\pm$ 3.32 <sup>a</sup>	-	42.2 $\pm$ 3.02 <sup>b</sup>	-	<0.01
1-2h	18.7 $\pm$ 0.29	19.4 $\pm$ 0.55	-	19.4 $\pm$ 0.92	-	17.5 $\pm$ 1.12	-	20.1 $\pm$ 1.03	-	0.32
2-4h	12.7 $\pm$ 0.17 <sup>ab</sup>	13.2 $\pm$ 0.32 <sup>ab</sup>	-	13.8 $\pm$ 0.54 <sup>b</sup>	-	11.4 $\pm$ 0.66 <sup>a</sup>	-	12.7 $\pm$ 0.61 <sup>ab</sup>	-	<0.05
4-18h	14.6 $\pm$ 0.20 <sup>ab</sup>	15.2 $\pm$ 0.38 <sup>b</sup>	-	15.3 $\pm$ 0.63 <sup>ab</sup>	-	12.6 $\pm$ 0.77 <sup>a</sup>	-	15.7 $\pm$ 0.71 <sup>b</sup>	-	<0.05
18-24h	11.2 $\pm$ 0.17	11.5 $\pm$ 0.31	-	11.6 $\pm$ 0.52	-	9.8 $\pm$ 0.63	-	11.8 $\pm$ 0.58	-	0.13
24-48h	7.6 $\pm$ 0.07 <sup>b</sup>	7.7 $\pm$ 0.12 <sup>b</sup>	-	7.1 $\pm$ 0.20 <sup>ab</sup>	-	6.7 $\pm$ 0.25 <sup>a</sup>	-	7.5 $\pm$ 0.23 <sup>ab</sup>	-	<0.001
48-70h	3.4 $\pm$ 0.06 <sup>b</sup>	3.5 $\pm$ 0.07 <sup>bc</sup>	3.9 $\pm$ 0.07 <sup>d</sup>	2.8 $\pm$ 0.11 <sup>a</sup>	4.0 $\pm$ 0.11 <sup>d</sup>	2.9 $\pm$ 0.13 <sup>a</sup>	4.0 $\pm$ 0.12 <sup>d</sup>	2.9 $\pm$ 0.13 <sup>a</sup>	3.9 $\pm$ 0.12 <sup>cd</sup>	<0.001

Note: Different letters between TGP and GP rate denote significant differences (Tukey test for comparing a family of 9 estimates,  $p < 0.05$ ). †Total gas production (TGP), ‡Gas production rate (GP rate).

The effects of different S sources on fermentation end products (pH, VFA, dDM%, PF, NH<sub>3</sub>-N and DAPA) are presented in Table 3.

**Table 3.** The effect of S sources on fermentation end products after 70 hrs of fermentation (LSMEAN $\pm$ SEM)

Item	CON	ES_0h	ES_48h	NaS_0h	NaS_48h	NHS_0h	NHS_48h	MgS_0h	MgS_48h	P Value
Final pH	6.57 $\pm$ 0.012	6.58 $\pm$ 0.015	6.60 $\pm$ 0.015	6.58 $\pm$ 0.024	6.58 $\pm$ 0.022	6.56 $\pm$ 0.029	6.60 $\pm$ 0.026	6.60 $\pm$ 0.026	6.59 $\pm$ 0.025	0.88
tVFA (mM/kg)	69.7 $\pm$ 2.57	70.3 $\pm$ 2.97	72.4 $\pm$ 2.97	72.7 $\pm$ 5.15	69.2 $\pm$ 5.15	68.6 $\pm$ 5.15	64.2 $\pm$ 5.15	73.2 $\pm$ 5.15	74.8 $\pm$ 5.15	0.90
Acetate (% tVFA)	63.9 $\pm$ 0.38	64.1 $\pm$ 0.44	63.7 $\pm$ 0.44	63.4 $\pm$ 0.77	63.8 $\pm$ 0.77	63.4 $\pm$ 0.77	64.8 $\pm$ 0.77	64.8 $\pm$ 0.77	64.4 $\pm$ 0.77	0.82
Propionate (% tVFA)	20.6 $\pm$ 0.28	20.5 $\pm$ 0.32	20.7 $\pm$ 0.32	20.9 $\pm$ 0.56	20.4 $\pm$ 0.56	21.3 $\pm$ 0.56	21.1 $\pm$ 0.56	20.7 $\pm$ 0.56	21.0 $\pm$ 0.56	0.93
Butyrate (% tVFA)	9.5 $\pm$ 0.17	9.5 $\pm$ 0.20	9.7 $\pm$ 0.20	10.1 $\pm$ 0.34	9.9 $\pm$ 0.34	9.3 $\pm$ 0.34	9.3 $\pm$ 0.34	9.3 $\pm$ 0.34	9.2 $\pm$ 0.34	0.58
C2:C3† ratio	3.11 $\pm$ 0.055	3.13 $\pm$ 0.063	3.08 $\pm$ 0.063	3.04 $\pm$ 0.109	3.13 $\pm$ 0.109	2.97 $\pm$ 0.109	3.08 $\pm$ 0.109	3.14 $\pm$ 0.109	3.07 $\pm$ 0.109	0.96
dDM (%)	67.1 $\pm$ 0.36 <sup>a</sup>	68.3 $\pm$ 0.42 <sup>a</sup>	66.8 $\pm$ 0.43 <sup>a</sup>	68.6 $\pm$ 0.71 <sup>ab</sup>	66.2 $\pm$ 0.67 <sup>a</sup>	67.3 $\pm$ 0.91 <sup>a</sup>	65.9 $\pm$ 0.77 <sup>a</sup>	71.4 $\pm$ 0.79 <sup>b</sup>	69.0 $\pm$ 0.74 <sup>ab</sup>	<0.001
PF‡	5.0 $\pm$ 0.07 <sup>ab</sup>	4.9 $\pm$ 0.08 <sup>ab</sup>	4.9 $\pm$ 0.08 <sup>ab</sup>	5.2 $\pm$ 0.13 <sup>abc</sup>	4.8 $\pm$ 0.12 <sup>ab</sup>	5.8 $\pm$ 0.17 <sup>c</sup>	5.1 $\pm$ 0.14 <sup>ab</sup>	5.2 $\pm$ 0.15 <sup>bc</sup>	4.6 $\pm$ 0.14 <sup>a</sup>	<0.001
NH <sub>3</sub> -N (mg/dL)	52.1 $\pm$ 1.30	52.2 $\pm$ 1.50	52.0 $\pm$ 1.50	54.2 $\pm$ 2.60	51.1 $\pm$ 2.60	55.1 $\pm$ 2.60	56.5 $\pm$ 2.60	49.3 $\pm$ 2.60	48.0 $\pm$ 2.60	0.40
DAPA (mg/kg DM)	374 $\pm$ 25.6	336 $\pm$ 29.6	311 $\pm$ 29.6	335 $\pm$ 51.2	343 $\pm$ 51.2	394 $\pm$ 51.2	411 $\pm$ 51.2	262 $\pm$ 51.2	261 $\pm$ 51.2	0.26
UNSOL: BACT DAPA ratio	3.0 $\pm$ 0.11	2.9 $\pm$ 0.13	3.0 $\pm$ 0.13	2.7 $\pm$ 0.20	2.4 $\pm$ 0.20	2.9 $\pm$ 0.20	3.1 $\pm$ 0.20	3.1 $\pm$ 0.20	3.1 $\pm$ 0.20	0.26

Note: Different letters between dDM (%) and PF denote significant differences (Tukey test for comparing a family of 9 estimates,  $p < 0.05$ ). †Acetate/Propionate ratio (C2:C3), ‡Partitioning factor (PF).

The pH was unaffected by the addition of S and averaged >6.50 across all treatments. There were no significant differences ( $p=0.90$ ) between the treatments regarding tVFA (mM), nor regarding acetate ( $p=0.82$ ), propionate ( $p=0.93$ ) and butyrate ( $p=0.58$ ) relative to the tVFA measured at the end of the 70 hrs incubations. The dDM% was significantly ( $p<0.001$ ) increased with MgS\_0h when compared to CON ( $71.4\pm0.79$  and  $67.1\pm0.36$  dDM % for MgS\_0h and CON, respectively), while the other treatments showed no denoting effects. The PF was significantly ( $p<0.001$ ) higher with NHS\_0h, while only numerical variations were observed with the other treatments when compared to CON. No significant ( $p=0.40$ ) differences were registered between the treatments regarding NH<sub>3</sub>-N concentration. The bacterial synthesis, based on DAPA (mg/kg DM of freeze-dried substrate) concentration of the final fermentation medium did not differ ( $p=0.26$ ) between the treatments. The correct application of the centrifugation protocol was confirmed by the DAPA concentration ratio of the UNSOL:BACT, which was >1:2.3 across all treatments.

### Mineral solubility

The results concerning the solubility of different S sources at the end of the 70 hrs *in vitro* fermentations are presented in Table 4. As expected, there were significant differences in the S concentration between the treatments in all centrifugation fractions ( $p<0.001$ ,  $p<0.001$  and  $p<0.001$  for UNSOL, BACT and SOL, respectively). Compared to CON, the highest S concentration of the UNSOL was registered with the ES treatments ( $p<0.001$ ;  $3.8\pm0.14$ ,  $5.9\pm0.16$  and  $8.7\pm0.16$  g/kg DM for CON, ES\_0h and ES\_48h, respectively). The S concentration of the BACT was significantly ( $p<0.001$ ) higher with the sulfate sources of S (regardless of addition time), while with ES it was not different when compared to CON ( $10.1\pm0.4$ ,  $7.8\pm0.4$ ,  $10.4\pm0.4$ ,  $8.1\pm0.4$ ,  $11.1\pm0.4$ ,  $7.7\pm0.4$ ,  $7.2\pm0.2$ ,  $6.5\pm0.2$  and  $6.3\pm0.2$  g/kg DM for NaS\_0h, NaS\_48h, NHS\_0h, NHS\_48h, MgS\_0h, MgS\_48h, ES\_0h, ES\_48h and CON, respectively).

**Table 4.** Solubility of S sources after 70 hrs of fermentation (LSMEAN $\pm$ SEM)

Item	CON	ES_0h	ES_48h	NaS_0h	NaS_48h	NHS_0h	NHS_48h	MgS_0h	MgS_48h	<i>P</i> Value
UNSOL - S (g/kg DM)	3.8 $\pm$ 0.14 <sup>a</sup>	5.9 $\pm$ 0.16 <sup>c</sup>	8.7 $\pm$ 0.16 <sup>d</sup>	4.7 $\pm$ 0.31 <sup>ab</sup>	4.7 $\pm$ 0.31 <sup>ab</sup>	4.6 $\pm$ 0.31 <sup>ab</sup>	5.0 $\pm$ 0.31 <sup>bc</sup>	5.1 $\pm$ 0.31 <sup>bc</sup>	5.5 $\pm$ 0.31 <sup>bc</sup>	<0.001
BACT - S (g/kg DM)	6.3 $\pm$ 0.19 <sup>a</sup>	7.2 $\pm$ 0.22 <sup>abc</sup>	6.5 $\pm$ 0.22 <sup>ab</sup>	10.1 $\pm$ 0.38 <sup>d</sup>	7.8 $\pm$ 0.38 <sup>bc</sup>	10.4 $\pm$ 0.38 <sup>d</sup>	8.1 $\pm$ 0.38 <sup>c</sup>	11.1 $\pm$ 0.38 <sup>d</sup>	7.7 $\pm$ 0.38 <sup>bc</sup>	<0.001
SOL - S (mg/kg)	8.3 $\pm$ 0.51 <sup>a</sup>	9.2 $\pm$ 0.59 <sup>a</sup>	8.5 $\pm$ 0.59 <sup>a</sup>	21.3 $\pm$ 1.03 <sup>b</sup>	49.0 $\pm$ 1.03 <sup>d</sup>	22.7 $\pm$ 1.03 <sup>b</sup>	40.3 $\pm$ 1.03 <sup>c</sup>	18.2 $\pm$ 1.03 <sup>b</sup>	41.3 $\pm$ 1.03 <sup>c</sup>	<0.001
UNSOL - S (% of total S)	24.4 $\pm$ 1.74 <sup>bc</sup>	30.9 $\pm$ 2.00 <sup>cd</sup>	38.1 $\pm$ 2.00 <sup>d</sup>	15.4 $\pm$ 3.5 <sup>ab</sup>	7.7 $\pm$ 3.47 <sup>a</sup>	13.5 $\pm$ 3.5 <sup>ab</sup>	8.2 $\pm$ 3.5 <sup>a</sup>	12.7 $\pm$ 3.5 <sup>ab</sup>	8.4 $\pm$ 3.5 <sup>a</sup>	<0.001
BACT - S (% of total S)	17.9 $\pm$ 0.87 <sup>d</sup>	17.1 $\pm$ 1.00 <sup>cd</sup>	12.8 $\pm$ 1.00 <sup>bc</sup>	11.3 $\pm$ 1.73 <sup>abc</sup>	3.9 $\pm$ 1.73 <sup>a</sup>	15.6 $\pm$ 1.73 <sup>cd</sup>	6.5 $\pm$ 1.73 <sup>ab</sup>	18.4 $\pm$ 1.73 <sup>cd</sup>	7.1 $\pm$ 1.73 <sup>ab</sup>	<0.001
SOL - S (% of total S)	57.7 $\pm$ 1.38 <sup>b</sup>	52.0 $\pm$ 1.59 <sup>ab</sup>	49.0 $\pm$ 1.59 <sup>a</sup>	73.4 $\pm$ 2.75 <sup>cd</sup>	88.4 $\pm$ 2.75 <sup>e</sup>	70.6 $\pm$ 2.75 <sup>c</sup>	85.4 $\pm$ 2.75 <sup>de</sup>	68.9 $\pm$ 2.75 <sup>c</sup>	84.5 $\pm$ 2.75 <sup>de</sup>	<0.001

Note: Different letters between UNSOL, BACT and SOL denote significant differences (Tukey test for comparing a family of 9 estimates,  $p < 0.05$ ).

Regarding the S concentration in the SOL, significantly ( $p<0.001$ ) higher values were observed when supplementation was made with the sulfate forms (NaS, NHS or MgS added at the start or after 48 hrs of fermentation), whilst treatments with ES showed no differences when compared to CON ( $21.3\pm1.0$ ,  $49.0\pm1.0$ ,  $22.7\pm1.0$ ,  $40.3\pm1.0$ ,  $18.2\pm1.0$ ,  $41.3\pm1.0$ ,  $9.2\pm0.6$ ,  $8.5\pm0.6$  and  $8.3\pm0.5$  mg/kg for NaS\_0h, NaS\_48h, NHS\_0, NHS\_48h, MgS\_0h, MgS\_48h, ES\_0h, ES\_48h and CON, respectively). When analyzing the dispersion of S relative to the total amount (% of total S) in the different fractions, significant variations were observed between the treatments. Firstly, when considering the dispersion of S in the different fractions for the CON (no additional S), 24.4 % was in the UNSOL, 17.9% in the BACT and 57.7% was in the SOL. When considering the UNSOL of the treatments with additional S, the % of S with ES (added at the start or after 48 hrs of fermentation) was significantly ( $p<0.001$ ) higher when compared to treatments with sulfate sources ( $30.9\pm2.0$ ,  $38.1\pm2.0$ ,  $15.4\pm3.5$ ,  $7.7\pm3.5$ ,  $13.5\pm3.5$ ,  $8.2\pm3.5$ ,  $12.7\pm3.5$  and  $8.4\pm3.5$  % for ES\_0h, ES\_48h, NaS\_0h, NaS\_48h, NHS\_0h, NHS\_48h, MgS\_0h and MgS\_48h, respectively). Regarding the S content (% of total S) of the SOL, all treatments with sulfate sources (NaS, NHS or MgS, regardless of addition time) were significantly ( $p<0.001$ ) higher when compared to treatments with ES ( $73.4\pm2.7$ ,  $88.4\pm2.7$ ,  $70.6\pm2.7$ ,  $85.4\pm2.7$ ,  $68.9\pm2.7$ ,  $84.5\pm2.7$ ,  $52.0\pm1.6$  and  $49.0\pm1.6$  % for NaS\_0h, NaS\_48h, NHS\_0h, NHS\_48, MgS\_0h, MgS\_48h, ES\_0h and ES\_48h, respectively).

Changes in the microbial populations

Based on the quality of extracted DNA from the final fermentation medium samples, gene-sequencing data could be generated only for part of the samples (16 out of 36), for treatments CON, ES, MgS and NaS. According to the phylum level comparison of the bacterial community groups in the final fermentation medium (Figure 1), *Firmicutes* and *Bacteroidetes* constituted the major phyla, accounting for about 60% and 20% of the total bacterial population.

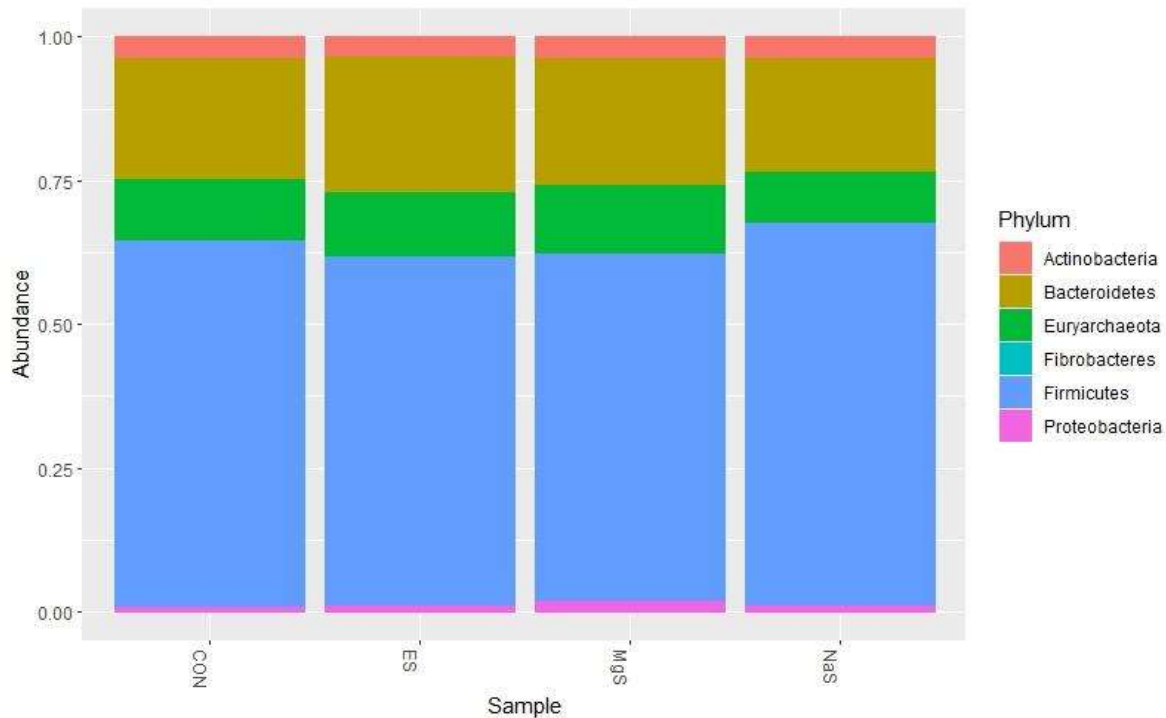
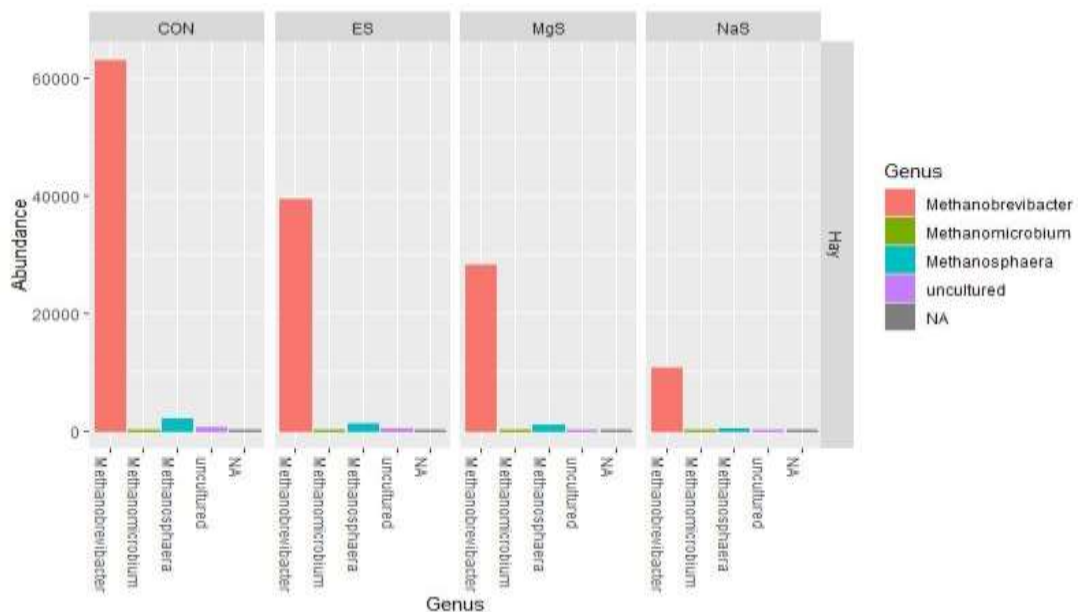


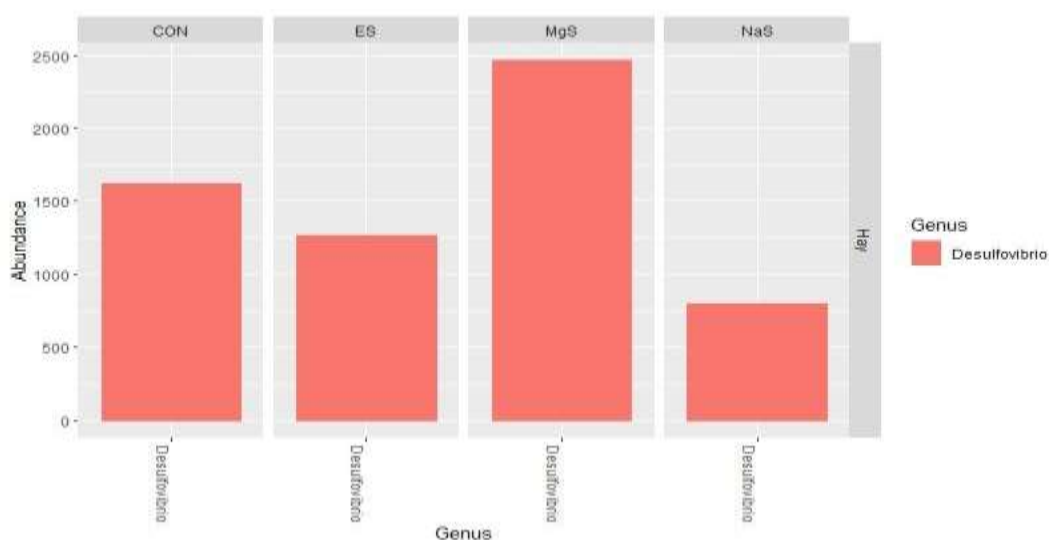
Figure 1. Sulfur source (ES, MgS or NaS) effect after 70 hrs of fermentation on relative abundance of major bacterial communities at the phylum level.

Regarding the microbial community composition, there was no significant difference between the treatments. Even so, some taxonomy shifts were observed, including a numerically lower abundance of methanogenic Archaea with additional S (regardless of source), and a numerically higher abundance of the *Desulfovibrio* genus with MgS when compared to CON (Figure 2).

(a)

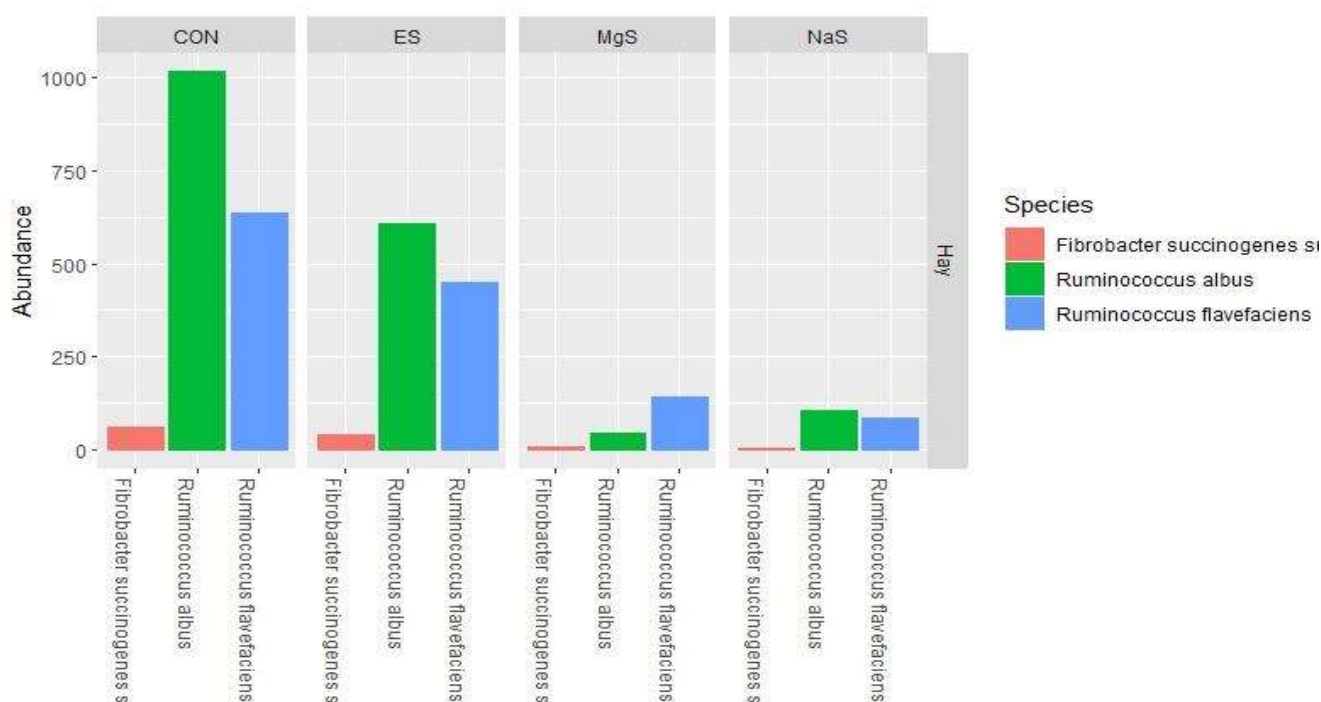


(b)



**Figure 2.** Sulfur source (ES, MgS or NaS) effect after 70 hrs of fermentation on absolute abundance count of: (a) Methanogenic *Archaea*, including *Methanobrevibacter*, *Methanomicrobium* and *Methanospheara* genus; (b) *Desulfovibrio* genus.

Furthermore, the abundance of fibrolytic bacteria like *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* species was numerically lower with ES, MgS and NaS treatments when compared to CON (Figure 3).



**Figure 3.** Sulfur source (ES, MgS or NaS) effect after 70 hrs of fermentation on absolute abundance count of fibrolytic bacteria species: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*.

In early studies on rumen fermentation, it was demonstrated that S can improve the fermentative activity of rumen microbiota (Bird, 1973). In the present study, the results observed on TGP indicate a positive effect of S on *in vitro* fermentations with rumen fluid. However, not all S sources affect the rumen fermentation in the same manner. In this study, the supplementation of 0.50% DM of S as ES had no significant effect on TGP when added from the start (ES\_0h) or after 48 hrs (ES\_48h) of fermentation. These findings are consistent with the results of



Promkot et al. (2007), who observed no increase in gas production during 48 hrs *in vitro* incubations with rumen fluid, supplementing a roughage substrate (cassava hay) with S (as ES) at 0.2, 0.5 or 1.0 % DM. Regarding the effects of sulfate sources, the lowest TGP was observed with NHS treatments (NHS\_0h, NHS\_48h), indicating a negative effect on fermentations. These results are consistent with Ososanya et al. (2016), who observed a decreased gas production when 0.75% DM of ammonium sulfate was added to *in vitro* incubations with rumen fluid. The negative effect on fermentation could be partially explained by the findings of Lu et al. (2014), who observed a negative effect of ammonium ( $\text{NH}_4^+$ ) on the nutrient uptake by rumen epithelial cells. Starting with the 1<sup>st</sup> h of incubation, and throughout the 70 hrs fermentation, the highest TGP in this study was observed with MgS treatments. In the same manner, the addition of MgS or NaS after 48 hrs of fermentation, significantly ( $p < 0.001$ ) increased the GP rate between 48 and 70 hrs. Consistent with these results, in a study by Wu et al. (2021) it was demonstrated that an addition of 0.5% DM of S (as  $\text{NaSO}_4$ ) increases gas production during *in vitro* incubations with rumen fluid.

The pH values observed in this study were in the normal range of microbial growth and nutrient degradation (Weimer, 1998). The addition of 0.5% DM of S did not affect pH, which is consistent with the findings of Silva et al. (2014), showing an average ruminal pH of 6.7 in steers supplemented with elemental sulfur (70% S or 98% S),  $\text{CaSO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$ , respectively. Regarding total VFA production and individual VFAs (acetic, propionic and butyric; as % of total VFA), no significant differences were found between the treatments. These observations are consistent with the findings of Wu et al. (2015), who registered no effect on total VFA production or individual VFA profile when 0.32 % DM of S (as  $\text{H}_2\text{SO}_4$  or  $\text{NaSO}_4$ ) was added during *in vitro* incubations with rumen fluid and compared with a CON (no additional S). In this study, the lowest total VFA values were observed with the addition of NHS as S source (NHS\_0h and NHS\_48h), which could be, in addition to the results obtained on TGP, another indicator of the negative effect of NHS on rumen fermentations. These observations are similar with the results of Ososanya et al. (2016), who reported that total VFA production tended to be lower during *in vitro* incubations with rumen fluid sampled from ewes supplemented with 0.50 or 0.75% DM of S (as  $(\text{NH}_4)_2\text{SO}_4$ ). Regarding dDM% recorded in this study, the addition of 0.50% DM of S as  $\text{MgSO}_4$  (MgS\_0h) significantly increased the substrate degradability, indicating together with the higher TGP an improved rumen fermentation. Furthermore, the addition of  $\text{MgSO}_4$  after 48 hrs of incubation (MgS\_48h), improved fermentation (based on the higher GP rate between 48 and 70 hrs), however only a numerical increase was registered for the degraded dry matter, implying that the positive effect on this parameter may take more than 24 hrs. These results are similar to Wu et al. (2015), reporting an increased DM degradation when 0.32% DM of S (as  $\text{NaSO}_4$ ) was supplemented during *in vitro* incubations. Supplementation with ES from the start (ES\_0h) or after 48 hrs of fermentation (ES\_48h) showed no significant improvement of dDM%, which is consistent with Promkot et al. (2007), who during *in vitro* incubation (cassava hay as substrate) observed no differences in DM degradation between treatments with additional S (0.2, 0.5 or 1.0 % DM) as elemental sulfur. Considering  $\text{NH}_3\text{-N}$ , in this study the S supplementation showed no significant effect, which is consistent with the observation of Silva et al. (2014) and Wu et al. (2021). The microbial protein synthesis was not affected by the additional S, as also indicated by the DAPA concentration of the final fermentation medium. These findings are similar to the results reported by Promkot et al. (2007), who observed no significant effect on total microbial mass when 0.2, 0.5 or 1.0 % DM of S was supplemented during *in vitro* fermentations with rumen fluid. The PF, which is the ratio between the total degraded DM (mg) and the TGP (ml) after 70 hrs of fermentation (Blümmel et al., 1997), was significantly affected by the S supplementation. NHS significantly ( $p < 0.001$ ) increased the PF, while the lowest values were observed with MgS. This observation could indicate that when NHS was supplemented, the energy resulting from the degraded DM was directed towards microbial protein synthesis, while in the case of MgS supplementation, the energy was directed towards VFA production. This assumption can be confirmed, considering that the highest DAPA values were observed with NHS treatments, while the lowest with MgS treatments. In addition, the lowest total VFA production was registered with NHS treatments and the highest with MgS treatments. Considering that N supplied by the  $(\text{NH}_4)_2\text{SO}_4$  might have induced a higher microbial synthesis, all other treatments (CON, ES) were supplemented with urea, as a soluble N source. Based on the higher microbial synthesis with the NHS treatments, it could be hypothesized that N as  $(\text{NH}_4)_2\text{SO}_4$  is more available as urea-N to increase microbial biomass yield.

Based on the DAPA analysis, it can be confirmed that the centrifugation method was properly applied in this study, allowing to obtain a bacterial enriched fraction (BACT), given that the DAPA concentration ratio of the UNSOL/BACT averaged 1:2.9 in this study, respecting the one mentioned by Kravietzki et al. (1989). Based on the chemistry of the inorganic S sources (PubChem), we hypothesized that ES would be less soluble than sulfate sources (NaS, NHS and MgS). The results obtained in this study confirm that  $\text{NaSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{MgSO}_4$  are highly soluble in rumen fluid when compared to elemental sulfur. Firstly, the S concentration of the SOL was significantly higher ( $p < 0.001$ ) when supplementing sulfates (regardless of addition time) compared to ES and CON. Furthermore, the S concentration of the UNSOL was significantly higher ( $p < 0.001$ ) with ES compared to sulfate treatments and CON. Moreover, the S concentration of the BACT of treatments with sulfate sources were significantly ( $p < 0.001$ ) higher compared to ES and CON, suggesting not only a higher solubility, but also a higher assimilation by rumen bacteria. The observations on S concentration of these three parameters (UNSOL, SOL and BACT) indicate the higher

bioavailability of sulfate sources (NaS, NHS and MgS) compared to ES. These findings are in close relation with the higher true digestibility (77.8% and 36.0%) and true retention (56.0% and 26.8%) of S in NaSO<sub>4</sub> form compared to elemental sulfur reported by Johnson et al. (1971) and Kandyliis (1981). In addition, the significant increase of S concentration of rumen bacteria (BACT) observed in this study when supplementation was made with sulfate sources, is consistent with the observations made by Whanger et al. (1978) and Dewhurst et al. (2007), showing that sulfates are more rapidly transformed in different sulfur compounds (S<sup>2-</sup>, HS<sup>-</sup>, H<sub>2</sub>S) in the rumen compared to elemental sulfur, facilitating the S utilization by rumen microorganisms. When analyzing the dispersion of S relative to the total amount (% of total S) in the different fractions, depending on S sources, 49 to 52 % (for ES) and 69 to 88 % (for -SO<sub>4</sub>) was found in the SOL; 31 to 38 % (for ES) and 8 to 15 % (for -SO<sub>4</sub>) in the UNSOL; and 13 to 17 % (for ES) and 4 to 18 % (for -SO<sub>4</sub>) in the BACT. These findings indicate that only a small part of the added ES solubilized during the 70 hrs fermentation, while with additional sulfates (NaS, NHS, MgS) a high amount of S was solubilized in the rumen fluid. Regarding the differences between ES and -SO<sub>4</sub> solubility, after 22 hrs of S exposure (ES\_48h, NaS\_48h, NHS\_48h and MgS\_48h treatments), 85, 85 and 88 % was recovered in the SOL with NaS, NHS and MgS, respectively, while only 49 % with the ES ( $p < 0.001$ ), indicating a higher short-term solubility of the sulfate forms. After 70 hrs of S exposure, the difference was lower but still significant ( $p < 0.001$ ; 52, 73, 71 and 69 % for ES, NaS, NHS and MgS, respectively).

Regarding the rumen microbiota, the major bacteria at phylum level in this study were *Firmicutes* and *Bacteroidetes*. These two phyla account for the highest proportions of rumen bacteria, they participate to fiber and cellulose degradation (*Firmicutes*); and carbohydrates and proteins breakdown (*Bacteroidetes*) (Cholewińska et al., 2020). Given the quality of extracted DNA and the low number of successfully analyzed samples (16 out of 36), no significant differences could be highlighted in this study on microbial community composition. Nevertheless, based on the absolute abundance, the methanogens were numerically lower with additional S compared to CON. This observation is consistent with Kim et al. (2020), who observed no significant difference, but numerically lower methanogens when S (as detoxified S powder, 54.2% S) was added at levels 0.2, 0.4, 0.6, 0.8 or 1.0 % DM during 12 hrs *in vitro* fermentations with rumen fluid. Based on the absolute abundance at genus level, the *Desulfovibrio* was numerically higher with the MgS treatments compared to CON and the other treatments, which could be used as an indicator of high rumen solubility of MgSO<sub>4</sub>. *Desulfovibrio* was identified as a sulphate-reducing bacteria (Huisinigh et al. 1974), and its abundance is strongly influenced by the rumen-soluble S. Similar to our observations, Wu et al. (2021) reported significantly higher abundance of the *Desulfovibrio* population when 0.5 to 1.2% DM of S (as NaSO<sub>4</sub>) was added to *in vitro* incubations with rumen fluid. Based on the observation on microbial communities, the absolute abundance of *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* species was numerically lower in treatments with additional S compared to CON. These three species belong to the *Fibrobacter* phylum and are part of the cellulolytic bacteria group (Koike and Kobayashi, 2001; Cholewińska et al., 2020). The observations made in this study are not consistent with McSweeney and Denman (2007), who reported a significant increase of fibrolytic rumen bacteria growth following a S (as NaSO<sub>4</sub>) supplementation of cattle fed a low S-containing diet. Based on the findings of Soto et al. (2013), who reported a sharp decline of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* during *in vitro* incubations longer than 24 hrs, the lower abundance of fibrolytic bacteria observed in this study (70 hrs incubation), might be explained by the exhaustion of fermentable substrate and the saturation with fermentation end products of the *in vitro* medium. For future *in vitro* studies investigating the effect of different mineral sources on rumen microbial composition, the use of 24 or 48 hrs fermentations is recommended.

## CONCLUSIONS

The results of this study indicate that NaSO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub> are highly soluble in the rumen, while elemental sulfur has a low solubility. Furthermore, the more soluble sulfate sources of S seem to be better assimilated by rumen bacteria compared to ES, hence a higher bioavailability. Regarding the rumen function, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> seems to have a negative effect on rumen fermentation, ES has no significant effect, while NaSO<sub>4</sub> and MgSO<sub>4</sub> can improve specific fermentation parameters. The microbial population analysis indicates that S could have an impact on some specific bacteria abundance, however the 70 hrs incubations used in this study are probably not the optimal recommended conditions for microbial diversity analysis.

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### Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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