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Ruminal Solubility and Bioavailability of Inorganic Trace Mineral Sources and Effects on Fermentation Activity Measured In Vitro

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Abstract: The aim of this study was to assess the effects of supplementation with inorganic sources of manganese (MnO, $MnSO_4$), zinc (ZnO, $ZnSO_4$) and copper ($CuSO_4$) at different levels (0.06%DM for Mn, 0.05%DM for Zn; 0.01 and 0.05%DM for Cu) on in vitro rumen fermentation, solubility and bioavailability. Fermentation activity was measured by total gas production (TGP) and dry matter degradability after 70 h of fermentation (dDM%). Trace mineral (TM) solubility was estimated via the TM concentration in the supernatant of the final fermentation medium (SOL) and TM bioavailability from the TM concentration in a bacterial-enriched fraction (BACT). Mn (regardless of source) and ZnO tended ($p < 0.10$) to decrease, while Cu showed no significant effect on TGP. The addition of inorganic Mn and of ZnO tended ($p < 0.10$) to decrease, $ZnSO_4$ tended to increase ($p < 0.10$), whilst Cu showed no effect on dDM%. Concerning solubility, Mn (MnO and $MnSO_4$), $ZnSO_4$ and $CuSO_4$ significantly ($p < 0.05$, $p < 0.001$ and $p < 0.01$) increased, while ZnO did not affect TM content in the SOL. These results indicate that $MnSO_4$, $ZnSO_4$ and $CuSO_4$ are highly soluble, MnO is quite soluble, while ZnO has a low solubility in the rumen. Based on the TM content in BACT, MnO, MnS_4 and $CuSO_4$ have high bioavailability, while ZnO is poorly assimilated by rumen bacteria. However, the lack of clear inhibition or improvement in fermentations suggests that the rumen microbiota have a low requirement for TM supplementation.

Keywords: ruminant; fermentation; solubility; trace minerals

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1. Introduction

Trace minerals, such as manganese (Mn), zinc (Zn) and copper (Cu), are essential minerals in animal feed as they have important physiological functions: components or activators of enzymes; participate in keratin, collagen and elastin synthesis (skin, appendages, bone and cartilage); as well as an important role for the immune and reproductive systems [1–3]. In ruminants, in addition to these metabolic functions focused on the animal, trace elements (Mn, Zn and Cu) may have some effects on the ruminal microflora, such as a positive effect on ruminal fermentations, by acting directly on microbial enzyme activity [4]. Moreover, previous in vitro studies have shown that the total exclusion or, on the contrary, a high dosage (100 µg/mL of in vitro medium) of Mn significantly lowers rumen cellulose digestion, while an addition of 5–30 µg/mL of in vitro medium of inorganic Mn increases cellulose digestion [5,6]. Furthermore, in vitro dry matter digestibility in rumen fluid is improved by the addition of 100 ppm of inorganic Mn [7]. Regarding Zn, early in vitro studies showed an increase in microbial protein synthesis after 5–7 ppm Zn supply [8]. In a more recent study, the rumen dry matter digestibility as well as total volatile fatty acid (VFA) production in ewes was increased with a supplementation of 30–40 mg/kg DM of inorganic Zn [9]. However, not all micro-organisms have the same sensitivity to Zn, which could also have negative effects. In an early study [10], it was found that protozoa tolerate a

dose of 25 µg/mL of in vitro medium of Zn, while the degradation of cellulose and urea by bacteria was greatly decreased. In general, a too-high concentration of Zn tends to decrease microbial activity, leading to a sharp reduction in ammonia concentrations [11]. In vitro studies with Cu showed that a high dosage of inorganic Cu (as $CuSO_4$) has a negative effect on rumen fermentation [12], and VFA production is inhibited [13]. However, the addition of 8 mg/kg DM of inorganic Cu (as $CuSO_4$) significantly improved the in vitro dry matter degradation and tended to increase the total microbial biomass [14]. Furthermore, the addition of 5, 7.5 and 10 mg/kg DM of Cu (as coated $CuSO_4$) to dairy cows' diet increased organic matter (OM) and neutral-detergent fiber (NDF) degradation, as well as the populations and activity of cellulolytic bacteria, such as *Rumminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* [15]. In ruminants, the recommended dose of dietary Mn, Zn and Cu, regarding the global animal needs, are approximately 50, 50 and 10 mg/kg DM [16], and the regulatory maximum limits are 150, 120 and 35 mg/kg DM for Mn, Zn and Cu, respectively [17]. Considering that dietary trace element absorption by ruminants is relatively low (1–4%, 15–30% and 4–5% for Mn, Zn and Cu, respectively) [18,19], selecting the most optimal source for supplementation becomes quite challenging. Moreover, the findings concerning different sources of inorganic trace minerals' solubility in the rumen and effects on rumen fermentation activity are still unclear, whilst little is known about their bioavailability to rumen bacteria. A better understanding of these potential ruminal effects, related to the dosage and the mineral form, would allow one to refine the recommended supply dosages and relate them to microorganism vs. animal needs.

This work contributes to the expansion of knowledge on the ruminal solubility and bioavailability of inorganic trace mineral sources (oxide and sulfate) and their effects on rumen fermentative activity. We hypothesized that the sulfate forms of trace minerals would be highly soluble in rumen fluid compared to oxide forms and, therefore, more bioavailable for rumen microorganisms. More precisely, the present study aimed to investigate the effects of inorganic sources of Mn (MnO and $MnSO_4$), Zn (ZnO and $ZnSO_4$) and Cu ($CuSO_4$) on in vitro rumen fermentation activity in conditions in which the trace mineral content in the substrate is below the recommended levels, and to identify the rumen fluid fraction(s) in which the different additional trace mineral sources are found after fermentation (big particle fraction (feed particles, insolubilized minerals, protozoa), bacteria-enriched fraction and final supernatant), to assess the ruminal solubility and bioavailability in each trace element.

2. Materials and Methods

The use of rumen-cannulated dairy cows in this study was approved by the Ethics Committee n°052 (Saint-Nolff, 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 AN (TRC, Saint-Nolff, France) to evaluate the rumen solubility and effects on fermentation activity of inorganic feed-grade Mn, Zn and Cu sources. The most commonly used inorganic trace mineral sources for ruminant supplementation were identified [20] and included in this study (supplied by SERMIX, Chierry, France): manganese oxide (MnO, 60%—Mn), manganese sulfate ($MnSO_4$, 32%—Mn), zinc oxide (ZnO, 72%—Zn), zinc sulfate ($ZnSO_4$, 35%—Zn) and copper sulfate ($CuSO_4$, 26%—Cu). Feed-grade copper oxide was not included in this study as it is considered unavailable for ruminants [20].

2.1. Experimental Design and Treatments

In vitro incubations, respecting the well-established recommendation regarding substrate, inoculum and buffer [21], were conducted for 70 h using the automatic system Gas Endeavour® (Bioprocess Control, Lund, Sweden). The duration of the incubations (70 h) was established based on previous in vitro studies assessing the effects of high-trace-mineral concentrations on rumen fermentation [22] and to assess the solubility of sources (-oxides) considered have low rumen solubility. For each trace mineral, 3 consecutive

incubations were realized. The experimental design applied in this study (using simultaneously two Gas Endeavour® units) allowed for the use of 4 replicates for treatments with Mn and Zn (two sources, -oxide and -sulfate, for Mn and Zn), while for treatments with Cu, 6 replicates were established (as only one mineral source, Cu-sulfate was used, more gas production measuring cells were available). Fermentation activity was measured through continuous total gas production over the 70 h of fermentation (TGP), and substrate dry matter degradability (dDM%), pH and volatile fatty acid (VFA, mM) production at the end of the 70 h fermentations. Trace mineral (TM) solubility and bioavailability were assessed by measuring the trace element concentration in fractions obtained after successive centrifugations of the final fermentation medium, allowing one to separate the remaining big particle fractions (UNSOL; feed particles, insolubilized minerals, protozoa), a fraction enriched in ruminal bacteria (BACT) and a final supernatant (SOL) containing the solubilized minerals. The correct separation of the different fractions via centrifugation was verified through diaminopimelic acid analysis (DAPA) of the UNSOL and BACT [23,24]. For the incubations testing Mn sources, the substrate was incubated solely (CON) or with the addition of 0.06% DM of Mn (as MnO or $MnSO_4$), added at the start (MnO_0 h and MnS_0 h), after 24 h (MnO_24 h and MnS_24 h) or 48 h (MnO_48 h and MnS_48 h) of incubation. For the incubations testing Zn sources, the substrate was incubated solely (CON) or with the addition of 0.05% DM of Zn (as ZnO or $ZnSO_4$), added at the start (ZnO_0 h and ZnS_0 h), after 24 h (ZnO_24 h and ZnS_24 h) or 48 h (ZnO_48 h and ZnS_48 h) of fermentation. For incubations testing Cu, the substrate was incubated solely (CON) or with the addition of 0.01% DM of Cu (as $CuSO_4$), added at the start (CuS_0.01_0 h), or with an addition of 0.015% DM of Cu (as $CuSO_4$), added at the start (CuS_0.015_0 h), after 24 h (CuS_0.015_24 h) or 48 h (CuS_0.015_48 h) of incubation. The high mineral dosages applied in this study (0.06, 0.05% DM for Mn and Zn, respectively; 0.01 and 0.015% DM for Cu) were based on previous in vitro studies, assessing the effects of high trace element supplementation levels on rumen fermentation and solubility [7,22,25]. Furthermore, the addition of minerals after 24 or 48 h of fermentation aimed to assess the effects of trace element supplementation of a highly mineral-depleted medium. Supplying the trace minerals after the start of the fermentations (24 or 48 h) could allow rumen microbes to assimilate the soluble or available minerals provided by the substrate and the rumen fluid before supplementation. To take into account the addition of sulfur (S) in the MnS and ZnS treatments, in replicates containing the CON, MnO and ZnO treatments, sodium sulfate ($NaSO_4$; 22.6% S) was also added in order to ensure the same supply of fermentable S (potential limiting factor of fermentation activity under all conditions to avoid a potential bias given by the S content of the $MnSO_4$ (18.6% S) and $ZnSO_4$ (19.9% S)) [26,27].

2.2. Substrate

The substrates used for this study were selected based on their low content of the targeted mineral (Table 1), in order to mimic a low-TM ration. For incubations testing Mn sources, the substrate was composed of (%DM): 99.96% of maize silage and 0.04% urea. The Mn content of the maize silage was 15.0 mg/kg DM, below the INRA 2018 recommendations for cattle diets (50 mg/kg DM). For incubations testing Zn and Cu, the substrate was composed of (%DM): 99.96% hay and 0.04% urea. The Zn and Cu contents of the hay were 13.0 and <5.0 mg/kg DM, below the INRA 2018 recommendations for cattle diets (50 and 10 mg/kg DM for Zn and Cu, respectively). The hay substrate for the Cu incubations was also selected based on the low content of sulfur (S), the main rumen antagonist of Cu [20,28]. The maize silage and hay used as substrates for the in vitro incubations were the same forages that were distributed in the ration of the rumen fluid donor cows. Urea was added in order to provide a source of fermentable nitrogen (N) for the bacteria, preventing N from being the limiting factor in the fermentations [27].

Table 1. Mineral content in substrates used for the in vitro fermentations.

Item	Maize Silage	Hay
DM (%)	37.50	88.90
Total ashes (%DM)	2.90	4.20
Ca (g/kg DM)	2.60	1.95
P (g/kg DM)	1.94	1.70
S (g/kg)	1.22	0.25
Cu [1] (mg/kg DM)	<5.00	<5.00
Mn (mg/kg DM)	15.00	224.00
Zn (mg/kg DM)	20.0	13.00

[1] Quantification limit for Cu in forages was 5.0 mg/kg DM (UpScience Laboratory, Saint-Nolff, France).

2.3. 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 2) in two equal meals per day for at least 21 consecutive days before sampling the rumen fluid; the ruminal flora of the donor animals was considered to be stabilized. The cows were housed at the TRC site in a free-stall barn, with rubber matrasses and permanent access to water distributed through automatic drinkers. The TM content of the diet of the donor cows was 57.0, 33.9 and 7.2 mg/kg DM for Mn, Zn and Cu, respectively. The Mn content in the diet was above while Zn and Cu were below the recommendations for cattle (50.0, 50.0 and 10.0 mg/kg DM for Mn, Zn and Cu, respectively) [19].

Table 2. Composition and chemical analysis of donor animal diet.

Diet Composition	Intake (Kg DM/Head/Day)
Corn silage	3.54
Hay	2.00
Complete feed [1]	3.00
Diet nutritional values	**Concentration**
CP (%DM)	14.20
Starch (%DM)	19.80
Total fiber (%DM)	22.50
Mn (mg/kg DM)	57.00
Zn (mg/kg DM)	33.90
Cu (mg/kg DM)	7.20

[1] Complete feed content: rapeseed meal—39.0%, wheat bran—25.0%, soybean meal—18.1%, sunflower meal—10.0%, corn—4.1%, urea—1.5%, salt—1.0%, 0.96% $CaCO_3$ and 0.2% premix (included Mn as MnO; Zn as ZnO; Cu as CuS).

2.4. Buffer

The buffer used in this study was prepared based on the buffer proposed by [29], described in [27]. The buffer contained no S source to limit antagonism with trace minerals [20,28]. The buffer to inoculum ratio used in this study was 4:1.

2.5. Preparation and Launch of Incubations

The substrates were prepared as follows: the maize silage was first oven-dried (72 h at 60 °C) then ground (3 mm sleeve), and the residual DM content was measured (4 h at 103 °C). The hay was directly ground (3 mm sleeve) and the residual DM content was measured (4 h at 103 °C). The DM of the urea was considered 100%. The day before the incubations were launched, nylon bags (10.0 cm L × 5.0 cm L, 50 µm pores Ankom bags, Humeau Laboratories, France) containing the substrate (3.7 g DM) and, if applicable, additional TMs (Mn_0 h, Zn_0 h and Cu_0 h) were introduced into the incubation flasks (500 mL). The buffer was then added (385 mL) using an automatic dispenser in order to respect the minimum of 2 h of pre-hydration necessary for a good start with the fermen-

tations [21]. The hydrated bottles were hermetically sealed and refrigerated (4 °C) until the next morning. Prior to each incubation (maximum 1 h), the inoculum was prepared from the ruminal content sampled from the 3 rumen fluid donor cows [21]. The sampling was carried out in the morning, before feeding to limit the variability in 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 a 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_2 (235 mL) was used to flush the residual O_2 from the system and promote the proper start of anaerobic fermentations [21]. The measuring devices were launched as soon as the saturation with N_2 was finished. The Gas Endeavour® devices were configured to record data normalized by temperature and humidity. For the addition of trace minerals after the start of the incubations (Mn_24 h, Mn_48 h, Zn_24 h, Zn_48 h, Cu_24 and Cu_48 h, respectively), the quantities of minerals were directly introduced after 24 or 48 h by pausing the in vitro system (gas production registration and stirring) and opening the flasks as little as possible to limit the O_2 contamination.

2.6. Measurements, Samplings and Analysis

- The TGP was continuously registered for 70 h and recorded using the software provided by Bioprocess Control (version ge_2.1[v1.2948]). Treatments supplemented with Mn, Zn or Cu after 24 or 48 h were considered as CON before the addition of the TMs (MnO_24 h, MnS_24 h, MnO_48 h, MnS_48 h, ZnO_24 h, ZnS_24 h, ZnO_48 h, ZnS_48 h, CuS_0.015_24 h and CuS_0.015_48 h, respectively). The cumulated values of TGP after 24, 48 and 72 h were used for the comparisons of the treatments.
- 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).
- The nylon bags containing the undegraded substrate were removed from the flasks, rinsed briefly with cold water, then frozen (−18 °C) for 24 h (adaptation of the in situ model for rumen degradation of DM) [30]. Freezing of the nylon bags aimed to halt the fermentation and substrate degradation as fast as possible, and to detach (at least partially) the bacteria from feed particles. After defrosting, the nylon bags were washed a second time in cold water for 2 min in the washing machine and oven-dried for 48 h at 60 °C. The nylon bags containing the dry matter (DM) residues were weighed, and the dDM% was calculated [31].
- Replicates of the same treatment in the final fermentation medium were pooled and sampled (12 mL/pool). The samples were frozen (−18 °C) before being sent for VFA analysis (total concentration and individual profile) using gas chromatography (Upscience, Saint-Nolff, France).
- The pooled final fermentation medium was centrifuged to separate 3 different fractions, based on the method described by [24]: UNSOL (containing undegraded feed particles, protozoa and insolubilized minerals), BACT and SOL. The pooled fermentation medium was first refrigerated (4 °C) for 6 h [32] and then agitated with a magnetic stirrer (400 rpm) for 45 s to detach the bacteria bound to fiber particles. Next, the fermentation medium was centrifuged (Haraeus Multifuge X3R, Thermo Fisher Scientific, Strasbourg, France) at 100× *g* for 5 min at 4 °C; the total quantity of obtained pellet (UNSOL) was then recovered and frozen at −80 °C before freeze-drying (CHRIST BETA 1-8 LSC PLUS, Martin Christ, Osterode am Harz, Germany). The obtained supernatant was centrifuged at 18,500× *g* for 20 min 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 TM (UT2A, Pau, France) analysis. The SOL samples were

analyzed only for TM content (UT2A, Pau, France), as previous studies in the lab had consistently shown that this fraction contained no DAPA.

- TM (total TM and % of total TM) in each fraction (UNSOL, BACT and SOL) of the final fermentation medium was calculated based on TM content in the fractions [33].
- Total DAPA was used as a rumen bacterial synthesis marker and calculated based on DAPA concentration of UNSOL and BACT [34]. The DAPA concentration of BACT was also used to confirm the enrichment with bacteria.
- Data were statistically analyzed via analysis of variance and Tukey test with R software (version 4.1.3), with the treatment as a fixed factor, the replicates (incubation flasks) or the incubations (for TM analysis in the fractions) as a random factor.

3. Results

3.1. Manganese

3.1.1. Fermentation Parameters

The results of inorganic Mn effects on TGP, dDM% and final fermentation products (pH, VFA, DAPA) are presented in Table 3. TGP did not differ between the treatments in the first 24 h ($p = 0.49$), 48 h ($p = 0.18$) or after 70 h ($p = 0.10$) of fermentation. Mn supplementation tended ($p = 0.09$) to have a negative effect on dDM%; the lowest degradability was observed when Mn was added after 24 or 48 h compared to CON (86.5, 86.6 and 88.6% dDM for MnO_24 h, MnS_48 h and CON, respectively). There were no significant differences ($p = 0.94$) between the treatments regarding total VFA nor regarding acetate ($p = 0.64$), propionate ($p = 0.30$) and butyrate ($p = 0.06$) relative to the total VFA measured at the end of the 70 h incubation.

Table 3. Influence of inorganic Mn source and addition time on fermentation parameters.

Item	CON	MnO_0 h	MnO_24 h	MnO_48 h	MnS_0 h	MnS_24 h	MnS_48 h	SEM	*p* Value
TGP [1] at 24 h (mL/gDM)	143	149	-	-	141	-	-	5.2	0.49
TGP at 48 h (mL/gDM)	218	211	213	-	204	219	-	5.2	0.18
TGP at 70 h (mL/gDM)	235	226	224	244	222	235	237	5.4	0.10
dDM [2] (%)	88.6	88.0	86.5	87.3	87.1	87.4	86.6	0.56	0.09
Total VFA [3] (mM)	89.8	85.3	88.6	87.6	88.8	85.4	86.4	3.40	0.94
Acetate (%)	58.0	58.0	58.1	58.8	58.0	58.7	58.0	0.40	0.64
Propionate (%)	21.8	22.3	21.4	21.1	22.6	21.6	22.0	0.45	0.30
Butyrate (%)	14.7	14.4	14.9	14.6	13.9	14.4	14.7	0.21	0.06
Acetate:Propionate	2.67	2.61	2.71	2.79	2.57	2.72	2.64	0.068	0.36
Final pH	6.51	6.53	6.52	6.53	6.52	6.55	6.54	0.029	0.90
Total DAPA [4] (mg)	1.93	1.56	1.76	1.98	1.52	1.79	1.63	0.163	0.35
UNSOL [5]:BACT [6]-DAPA ratio	2.6	2.7	2.9	2.9	2.9	2.8	3.0	0.27	0.94

SEM = Standard error of the mean; [1] Total gas production (TGP); [2] Dry matter degradability (dDM%); [3] Volatile fatty acids (VFA); [4] Diaminopimelic acid (DAPA); [5] Big particles fraction (UNSOL); [6] Bacteria rich fraction (BACT).

The pH was unaffected by the addition of Mn and averaged >6.50 across all treatments. The bacterial synthesis, based on total DAPA (mg) analysis after 70 h of fermentation, did not differ ($p = 0.35$) between the treatments. However, when looking at the effect of Mn, regardless of the source added from the start of the incubation (MnO_0 h + MnS_0 h), the total DAPA tended ($p = 0.06$) to be lower compared to CON (1.56, 1.52 and 1.93 mg DAPA for MnO_0 h, MnS_0 h and CON, respectively).

3.1.2. Ruminal Mineral Solubility and Bioavailability

The results of Mn concentration and proportions recovered in the different centrifugation fractions at the end of the 70 h in vitro fermentations are presented in Table 4. 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 (Table 3). As expected, the

Mn concentration was greater in all centrifugation fractions in all experimental treatments compared to CON ($p < 0.01$, $p < 0.001$ and $p < 0.05$ for UNSOL, BACT and SOL, respectively). The Mn concentration of the UNSOL was significantly higher when MnO was added after 24 or 48 h of fermentation compared with the addition from the start of the incubation ($p < 0.01$; 458, 457 and 252 mg/kg DM for MnO_24 h, MnO_48 h and MnO_0 h, respectively). As for treatments with $MnSO_4$, the Mn concentration of the UNSOL tended to be higher with the addition of Mn after 24 or 48 h compared to when it was added from the start of the incubation (373, 406 and 306 mg/kg DM for MnS_24 h, MnS_48 h and MnS_0 h, respectively). There were no significant differences in the Mn concentration of the UNSOL between the MnO_0 h and MnS_0 h treatments.

Table 4. Influence of inorganic Mn source and addition time on solubility/bioavailability parameters.

Item	CON	MnO_0 h	MnO_24 h	MnO_48 h	MnS_0 h	MnS_24 h	MnS_48 h	SEM	*p* Value
UNSOL [1]—Mn (mg/kg DM)	110 ^{a}	252 ^{ab}	458 ^{d}	457 ^{d}	306 ^{bc}	373 ^{bcd}	406 ^{cd}	30.2	<0.01
BACT [2]—Mn (mg/kg DM)	76 ^{a}	238 ^{b}	330 ^{c}	327 ^{c}	291 ^{bc}	326 ^{c}	315 ^{bc}	17.2	<0.001
SOL [3]—Mn (mg/kg)	0.81 ^{a}	3.13 ^{b}	3.17 ^{b}	3.02 ^{b}	4.40 ^{b}	3.67 ^{b}	4.63 ^{b}	0.370	<0.05
Total [4] Mn (mg)	1.02 ^{a}	3.76 ^{b}	4.00 ^{b}	3.91 ^{b}	4.73 ^{b}	4.51 ^{b}	5.60 ^{b}	0.490	<0.001
UNSOL—Mn (% of total Mn)	7.6 ^{ab}	3.8 ^{a}	8.3 ^{ab}	11.5 ^{b}	3.9 ^{a}	5.9 ^{ab}	3.7 ^{a}	1.49	<0.001
BACT—Mn (% of total Mn)	3.6	3.2	4.1	4.2	2.7	3.3	2.8	0.49	0.24
SOL—Mn (% of total Mn)	88.8 ^{ab}	93.0 ^{b}	87.6 ^{ab}	84.2 ^{a}	93.4 ^{b}	90.8 ^{b}	93.6 ^{b}	1.59	<0.01

SEM = Standard error of the mean; [1] big particle fraction (UNSOL; freeze-dried fraction); [2] bacteria-rich fraction (BACT; freeze-dried fraction); [3] final supernatant (SOL; liquid fraction); [4] total Mn = Mn_{UNSOL} + Mn_{BACT} + Mn_{SOL}; $^{a–d}$ means in the same row with different superscripts differ ($p \leq 0.05$).

The Mn concentration of the BACT was significantly higher when MnO was added after 24 or 48 h compared with the addition from the start of the incubation ($p < 0.05$; 330, 327 and 238 mg/kg DM for MnO_24 h, MnO_48 h and MnO_0 h, respectively). No significant differences were observed in the Mn concentration of the BACT between treatments MnS_0 h, MnS_24 h and MnS_48 h, respectively. The Mn concentration of the SOL tended to be higher with MnS_48 h when compared to MnO_48 h ($p = 0.08$). When analyzing the dispersion of Mn relative to the total amount (% of total Mn) in the different fractions, it was observed that with treatments MnO_0 h and MnS_0 h, a high percentage (>90%) of total Mn was in the SOL (93.0 and 93.4%, respectively). Moreover, the Mn% in the MnO_0 h was significantly higher compared to the MnO_48 h treatment ($p < 0.05$). The Mn% in the SOL fraction of the MnS_48 h was significantly higher compared to the MnO_48 h treatment (84.2% vs. 93.6%, $p < 0.05$), showing source-related solubility.

3.2. Zinc

3.2.1. Fermentation Parameters

The results of inorganic Zn effects on TGP, dDM% and final fermentation products (pH, VFA, DAPA) are presented in Table 5. TGP did not differ in the first 24 h of incubation ($p = 0.14$), while it was significantly different between the treatments after 48 h ($p < 0.01$) and 70 h ($p < 0.05$) of fermentation. After 48 h of fermentation, the TGP of ZnO_0 h was significantly ($p < 0.01$) lower, while ZnS_0 h was not different when compared to CON (126, 139 and 142 mL/g DM for ZnO_0 h, ZnS_0 h and CON, respectively). By the end of the 70 h incubation, the lowest TGS was observed with ZnO_0 h; however, this only tended ($p = 0.054$) to be different, while ZnS_0 h showed no significant effect when compared to CON (147, 159 and 168 mL/g DM for ZnO_0 h, ZnS_0 h and CON, respectively). Furthermore, the addition of Zn after 24 or 48 h of fermentation (ZnO_24 h, ZnS_24 h, ZnO_48 h and ZnS_48 h) did not affect TGP when compared to CON. A significantly

($p < 0.05$) higher TGP was registered with ZnO_24 h when compared to ZnO_0 h. Concerning dDM%, there was a significant difference ($p < 0.05$) between the treatments at the end of the 70 h incubation. The lowest dDM% was observed with ZnO_0 h and tended ($p = 0.06$) to be lower, while ZnS_0 h was not different compared to CON (67.5, 70.3 and 71.8% dMD for ZnO_0 h, ZnS_0 h and CON, respectively). Significantly higher dDM% was observed with ZnO_24 h and ZnS_48 h when compared to ZnO_0 h ($p < 0.05$). There were no significant differences ($p = 0.64$) between the treatments regarding total VFA nor regarding acetate ($p = 0.96$), propionate ($p = 0.93$) and butyrate ($p = 0.65$) relative to the total VFA measured at the end of the 70 h incubations. The pH was unaffected by the addition of Zn and averaged >6.50 across all treatments. The microbial synthesis, based on total DAPA (mg) analysis after 70 h of fermentation, was significantly ($p < 0.05$) different between the treatments. Significantly ($p < 0.01$) lower DAPA was observed when Zn was supplemented after 48 h of fermentation (ZnO_48 h + ZnS_48 h) compared to CON (2.02, 2.05 and 2.71 mg DAPA for ZnO_48 h, ZnS_48 h and CON, respectively).

Table 5. Influence of inorganic Zn source and addition time on fermentation parameters.

Item	CON	ZnO_0 h	ZnO_24 h	ZnO_48 h	ZnS_0 h	ZnS_24 h	ZnS_48 h	SEM	*p* Value
TGP [1] at 24 h (mL/gDM)	89	83	-	-	86	-	-	2.9	0.14
TGP at 48 h (mL/gDM)	142 [b]	126 [a]	146 [b]	-	139 [ab]	139 [ab]	-	3.6	<0.01
TGP at 70 h (mL/gDM)	168 [ab]	147 [a]	169 [b]	159 [ab]	159 [ab]	158 [ab]	167 [ab]	4.4	<0.05
dDM [2] (%)	71.8 [ab]	67.5 [a]	72.5 [b]	71.8 [ab]	70.3 [ab]	70.9 [ab]	72.8 [b]	1.10	<0.05
Total VFA [3] (mM)	70.9	65.0	75.8	70.7	72.3	74.6	71.6	4.06	0.64
Acetate (%)	65.1	64.6	65.3	65.3	65.2	65.5	65.4	0.59	0.96
Propionate (%)	20.7	20.8	20.7	20.7	20.2	20.4	20.5	0.37	0.93
Butyrate (%)	9.0	9.2	9.0	8.7	9.5	9.2	8.9	0.24	0.65
Acetate:Propionate	3.15	3.11	3.15	3.16	3.22	3.22	3.19	0.074	0.93
Final pH	6.55	6.58	6.55	6.57	6.56	6.56	6.56	0.006	0.50
Total DAPA [4] (mg)	2.71 [b]	2.61 [b]	2.57 [b]	2.02 [a]	2.46 [b]	2.44 [b]	2.05 [a]	0.148	<0.05
UNSOL [5]:BACT [6]-DAPA ratio	1.8	2.0	2.0	1.9	2.1	2.0	1.9	0.10	0.58

SEM = Standard error of the mean; [1] Total gas production (TGP); [2] Dry matter degradability (dDM%); [3] Volatile fatty acids (VFA); [4] Diaminopimelic acid (DAPA); [5] Big particles fraction (UNSOL); [6] Bacteria rich fraction (BACT); [a–b] means in the same row with different superscripts differ ($p \leq 0.05$).

3.2.2. Ruminal Mineral Solubility and Bioavailability

The results of Zn concentration and proportions recovered in the different centrifugation fractions at the end of the 70 h in vitro fermentations are presented in Table 6. The DAPA concentration ratio of the UNSOL:BACT averaged 1:2.0 across treatments (Table 5). As expected, there were significant differences in the Zn concentration between all 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 Zn concentration of the UNSOL was significantly higher when Zn was supplemented after 24 and 48 h of fermentation (ZnO_24 h, ZnS_24 h, ZnO_48 h and ZnS_48 h, respectively). When Zn was added from the start of the fermentation, the Zn concentration of UNSOL was numerically higher with ZnO_0 h and tended ($p = 0.06$) to be higher with ZnS_0 h compared to CON (357, 611 and 223 mg/kg DM for ZnO_0 h, ZnS_0 h and CON, respectively).

Table 6. Influence of inorganic Zn source and addition time on solubility parameters.

Item	CON	ZnO_0 h	ZnO_24 h	ZnO_48 h	ZnS_0 h	ZnS_24 h	ZnS_48 h	SEM	*p* Value
UNSOL [1]—Zn (mg/kg DM)	223 ^{a}	357 ^{a}	970 ^{bcd}	857 ^{bc}	611 ^{ab}	1287 ^{d}	1137 ^{cd}	81.8	<0.001
BACT [2]—Zn (mg/kg DM)	153 ^{a}	249 ^{a}	363 ^{a}	348 ^{a}	547 ^{a}	1200 ^{b}	1147 ^{b}	98.1	<0.001
SOL [3]—Zn (mg/kg)	0.085 ^{a}	0.121 ^{a}	0.082 ^{a}	0.079 ^{a}	0.271 ^{b}	0.252 ^{b}	0.276 ^{b}	0.0226	<0.001
Total [4] Zn (mg)	0.31 ^{a}	0.53 ^{ab}	1.04 ^{cd}	0.68 ^{abc}	0.94 ^{bcd}	1.72 ^{e}	1.25 ^{de}	0.102	<0.001
UNSOL—Zn (% of total Zn)	48.3	50.7	71.8	68.8	40.7	49.7	42.7	7.27	0.051
BACT—Zn (% of total Zn)	20.8	23.9	19.2	21.0	27.0	33.8	38.3	7.06	0.43
SOL—Zn (% of total Zn)	30.9 ^{d}	25.4 ^{cd}	9.0 ^{a}	10.2 ^{a}	32.4 ^{d}	16.5 ^{ab}	19.0 ^{bc}	1.75	<0.001

SEM = Standard error of the mean; [1] big particle fraction (UNSOL); [2] bacteria-rich fraction (BACT); [3] final supernatant (SOL); [4] total Zn = Zn $_{UNSOL}$ + Zn $_{BACT}$ + Zn $_{SOL}$; $^{a–e}$ means in the same row with different superscripts differ ($p \leq 0.05$).

The Zn concentration of the BACT was significantly ($p < 0.001$) higher when $ZnSO_4$ was added after 24 and 48 h of fermentation compared to CON (1200, 1147 and 153 mg/kg DM for ZnS_24, ZnS_48 and CON, respectively). The BACT Zn concentration was only numerically higher with ZnO_0 h, ZnO_24 h, ZnO_48 h and ZnS_0 h compared to CON. The highest Zn concentrations of the SOL were observed with the sulfate form, significantly ($p < 0.001$) higher compared to CON (0.217, 0.252, 0.276 and 0.085 mg/kg for ZnS_0 h, ZnS_24 h, ZnS_48 h and CON, respectively). The Zn concentration of the SOL when ZnO (ZnO_0 h, ZnO_24 h and ZnO_48 h) was added showed no difference when compared to CON. When analyzing the dispersion of Zn relative to the total amount (% of total Zn) in the different fractions, it was observed that a high percentage of Zn was in the UNSOL. Firstly, considering the total amount of analyzed Zn (0.31 mg) in the CON (no additional Zn), 48.3% was in the UNSOL, 20.8% in the BACT and 30.9% was in the SOL. When considering the Zn (% of total Zn) analyzed in the UNSOL, there were no significant differences ($p = 0.051$) between the treatments. However, all the treatments with ZnO were numerically higher, while the treatments with $ZnSO_4$ showed only slight differences when compared to CON (50.7, 71.8, 68.8, 40.7, 49.7, 42.7 and 48.3% for ZnO_0 h, ZnO_24 h, ZnO_48 h, ZnS_0 h, ZnS_24 h, ZnS_48 h and CON, respectively). When considering the Zn content (% of total Zn) analyzed in the BACT, there were no significant differences ($p = 0.43$) between the CON and the treatments with ZnO or $ZnSO_4$. Regarding the Zn content (% of total Zn) of the SOL, all the treatments with ZnO were significantly lower when compared to treatments with $ZnSO_4$, regardless of the addition time (25.4, 9.0, 10.2, 32.4, 16.5 and 19.0% for ZnO_0 h, ZnO_24 h, ZnO_48 hm ZnS_0 h, ZnS_24 h and ZnS_48 h, respectively).

3.3. Copper

3.3.1. Fermentation Parameters

The results of inorganic Cu effects on TGP, dDM% and final fermentation products (pH, VFA, DAPA) are presented in Table 7. TGP did not vary significantly between treatments in the first 24 h ($p = 0.26$), 48 h ($p = 0.32$) or after 70 h ($p = 0.44$) of fermentation. Cu supplementation showed no significant ($p = 0.15$) effect on dDM%. There were no significant differences ($p = 0.81$) between treatments regarding total VFA nor regarding acetate ($p = 0.79$), propionate ($p = 0.46$) and butyrate ($p = 0.93$) relative to the total VFA measured at the end of the 70 h incubations. The pH was unaffected by the addition of Cu and averaged >6.50 across all treatments. The bacterial synthesis, based on total DAPA analysis after 70 h of fermentation, did not differ ($p = 0.89$) between the treatments.

Table 7. Influence of $CuSO_4$ dose and addition time on fermentation parameters.

Item	CON	CuS_0.01_0 h	CuS_0.015_0 h	CuS_0.015_24 h	CuS_0.015_48 h	SEM	*p* Value
TGP [1] at 24 h (mL/gDM)	85	88	80	-	-	3.1	0.26
TGP at 48 h (mL/gDM)	130	138	129	135	-	4.1	0.32
TGP at 70 h (mL/gDM)	159	162	161	164	156	3.2	0.44
dDM [2] (%)	66.9	68.2	70.0	67.6	67.4	0.90	0.15
Total VFA [3] (mM)	76.1	74.5	77.1	73.7	71.5	3.51	0.81
Acetate (%)	66.0	66.0	66.4	66.8	64.5	1.34	0.79
Propionate (%)	20.3	20.7	20.0	19.8	21.7	0.71	0.46
Butyrate (%)	8.1	8.2	8.0	8.3	8.6	0.56	0.93
Acetate:Propionate	3.26	3.19	3.32	3.39	2.98	0.178	0.58
Final pH	6.60	6.59	6.60	6.59	6.61	0.007	0.58
Total DAPA [4] (mg)	2.07	1.87	1.87	1.78	1.69	0.280	0.89
UNSOL [5]:BACT [6]-DAPA ratio	2.3	2.4	2.8	2.4	2.5	0.15	0.37

SEM = Standard error of the mean; [1] total gas production (TGP); [2] dry matter degradability (dDM%); [3] volatile fatty acids (VFAs); [4] diaminopimelic acid (DAPA); [5] big particle fraction (UNSOL); [6] bacteria-rich fraction (BACT).

3.3.2. Ruminal Mineral Solubility and Bioavailability

The results of $CuSO_4$ concentration and proportions recovered in the different centrifugation fractions at the end of the 70 h in vitro fermentations are presented in Table 8. The correct application of the centrifugation protocol was confirmed by the DAPA concentration ratio of the UNSOL:SOL, which was >1:2.3 across all treatments (Table 7).

Table 8. Influence of $CuSO_4$ dose and addition time on solubility parameters.

Item	CON	CuS_0.01_0 h	CuS_0.015_0 h	CuS_0.015_24 h	CuS_0.015_48 h	SEM	*p* Value
UNSOL [1]—Cu (mg/kg DM)	32 [a]	118 [a]	485 [b]	599 [b]	697 [b]	55.0	<0.01
BACT [2]—Cu (mg/kg DM)	27 [a]	95	418 [b]	660 [b]	656 [b]	46.4	<0.001
SOL [3]—Cu (mg/kg)	0.044 [a]	0.114 [a]	0.415 [b]	0.438 [b]	0.495 [b]	0.0473	<0.01
Total [4] Cu (mg)	0.086 [a]	0.242 [a]	0.815 [b]	1.290 [c]	1.304 [c]	0.0835	<0.001
UNSOL—Cu (% of total Cu)	30.9	29.3	31.3	39.6	38.9	4.46	0.45
BACT—Cu (% of total Cu)	12.1	17.5	26.5	23.2	18.7	3.82	0.22
SOL—Cu (% of total Cu)	57.0	53.2	57.8	37.2	42.4	5.39	0.13

SEM = Standard error of the mean; [1] big particle fraction (UNSOL); [2] bacteria-rich fraction (BACT); [3] final supernatant (SOL); [4] total Cu = Cu_{UNSOL} + Cu_{BACT} + Cu_{SOL}; [a–c] means in the same row with different superscripts differ ($p \leq 0.05$).

As expected, there were significant differences in the Cu concentration between the treatments in all centrifugation fractions ($p < 0.01$, $p < 0.001$ and $p < 0.01$ for UNSOL, BACT and SOL, respectively). Compared to CON, the Cu concentration in the UNSOL was significantly ($p < 0.05$) higher when Cu was supplemented at a 0.015% DM dosage (regardless of addition time) compared to the 0.01% DM dosage and CON. The BACT Cu concentration was significantly ($p < 0.05$) higher at the 0.015% DM supplementation level (regardless of addition time) compared to the 0.01% DM dosage and CON. Significantly ($p < 0.05$) higher Cu concentrations of the SOL were observed with the addition of 0.015% DM of Cu compared to the 0.01% DM dosage and CON (0.415, 0.438, 0.495, 0.144 and 0.044 mg/kg for CuS_0.015_0 h, CuS_0.015_24 h, CuS_0.015_48 h, CuS_0.01_0 h and CON,

respectively). When analyzing the dispersion of Cu relative to the total amount (% of total Cu) in the different fractions, it was observed that a high percentage (>50%) of total Cu was in the SOL, when supplementation was made at the start of the incubations (CuS_0.010_0 h and CuS_0.015_0 h). Moreover, the Cu% with the CuS_0.015_0 h was numerically higher compared to CuS_0.015_24 h and CuS_0.015_48 h treatments, suggesting time-related solubility.

4. Discussion

Trace minerals are important micronutrients for the favorable growth and health of animals, especially ruminants [35]. Even so, studies tackling their fate and effects in the rumen are still scarce. A better understanding of ruminal metabolism is necessary for a more precise TM supplementation in ruminants.

4.1. Manganese

Based on the TGP production observed in this study, a trend seems to emerge for a negative effect of Mn on in vitro fermentations with rumen fluid, when supplemented at a high dosage (600 mg/kg DM; 12x the recommended dosage for ruminants). In a similar study by van Kuijk et al. [36], it was found that supplementing 15 or 150 mg/kg DM of Mn in an in vitro incubation with rumen fluid, using as substrate of fungi-treated wheat straw, fermentation activity and gas production decreased. The negative effect of Mn on the TGP is highlighted by the effect of supplementation duration. The significantly lower TGP induced by treatments receiving Mn from the start of the 70 h incubation (MnO_0 h and MnS_0 h) compared to treatments supplemented only after 48 h of fermentation (MnO_48 h and MnS_48 h) might express a decrease in the fermentative activity of the rumen microbiota due to the long exposure to Mn. Relevant to these results, in a study by Kišidayová et al. [37], it was demonstrated that lambs receiving a high-Mn diet (184 mg/kgDM; 3.7× the recommended dosage for ruminants) over a 16-week period had lower rumen fermentation, as some specific activities were decreasing (cellulolytic and amylolytic), even if the number of bacteria was not affected. The potential negative effects of Mn on fermentation can also be observed based on the substrate disappearance. The dDM% showed lower values in all treatments with Mn supplementation compared to CON, which is consistent with the findings of Genther and Hansen [33], showing a tendency for lower overall dry matter degradability when steers were supplemented with inorganic Mn (as $MnSO_4$) at a level of 60 mg/kg DM. No significant differences were found between the treatments when VFAs were analyzed, suggesting that neither the MnO nor the $MnSO_4$ affect the short chain fatty acids' production, at least not enough to be detected in our experimental model (concentration values close to the LOQ—limit of quantification— of the analysis method). However, the numerically lower VFA production in all treatments receiving Mn compared to CON could be related to the decrease in enzymatic activities of rumen microorganisms when Mn is supplemented, demonstrated by Kišidayová et al. [37]. The pH values obtained in this study were in the normal range of microbial growth and nutrient degradation [38]. Mn did not affect pH, which is consistent with the findings of Genther and Hansen [33], showing an average ruminal pH of 6.4 in steers supplemented with a high dosage of $MnSO_4$. The total DAPA (mg; total DAPA in UNSOL + total DAPA in BACT) was numerically lower in most treatments (except for MnO_48 h) compared to CON; hence, a tendency for lower microbial synthesis might be induced by inorganic Mn. The lower total DAPA, especially for MnO_0 h and MnS_0 h compared to CON, may be directly correlated with the overall lower VFA production. This correlation was demonstrated in a study by Maskal'ová et al. [39], showing a high regression coefficient between DAPA and VFA ($r = 0.813$). These two indicators (total VFA and DAPA) suggest a negative effect of Mn on the fermentation activity and, hence, on bacterial protein synthesis. The Mn concentration in this study was 15 mg/kg DM for the substrate, below the recommended level for ruminants (50 mg/kg DM), and 600 mg/kg DM for the supplementation. Even so, the total Mn concentration in the treatments (615 mg/kg DM) was below the toxicity

level for ruminants (2000 mg/kg DM) [40], suggesting that the currently recommended intake levels are defined to cover the needs of the animal and do not seem related to the requirements of microorganisms.

Based on the DAPA analysis, it can be confirmed that the centrifugation method was properly applied, allowing one to obtain a bacterial-enriched fraction (BACT), given that the DAPA concentration ratio of the UNSOL/BACT in this study (1:2.8) respected the one mentioned in the literature [24]. Based on the chemistry of the inorganic Mn sources [41,42], we hypothesized that MnO would be less soluble than $MnSO_4$. However, the results in this study show that both inorganic sources of Mn are quite soluble in rumen fluid. Even though the UNSOL of all the treatments had a higher Mn concentration compared to the CON (suggesting that part of the added Mn was not solubilized), the Mn concentration of the SOL in all treatments was significantly higher ($p < 0.01$) compared to the CON treatment. These results are similar to those found in in vivo studies [33,43], showing that two sources of Mn ($MnSO_4$ and hydroxy-Mn) supplemented at a level of 60 mg/kg DM are equally rumen soluble. Moreover, a time response was also observed, suggesting progressive solubilization; the Mn concentration of the UNSOL was lower when Mn was supplemented from the start of the incubation (MnO_0 h and MnS_0 h) compared to when it was added after 24 h (MnO_24 h and MnS_24 h) or 48 h (MnO_48 h and MnS_48 h). When analyzing the dispersion of Mn relative to the total amount (% of total Mn) analyzed in the different fractions, 84 to 93% was found in the SOL, 4 to 11% in the UNSOL and only 2 to 4% in the BACT. These findings indicate that there was a high amount of Mn solubilized during the 70 h fermentation and are similar to the results expressed by Genther and Hansen [33], showing that close to 66% of Mn content in rumen fluid was in the supernatant obtained after ultracentrifugation of the rumen fluid. A small part of the solubilized Mn was assimilated by the rumen bacteria (2–4% of total Mn), suggested in this study by the significantly higher Mn concentration of the BACT with all treatments when compared to CON ($p < 0.001$). The percentage of the assimilated Mn by the rumen bacteria found in this study (2–4% of total Mn) was quite close to the intestinal apparent absorption of Mn in ruminants, in an interval of 1–4% [19,44,45]. Regarding the differences between MnO and $MnSO_4$ solubility, after 22 h of exposure to Mn supply (MnO_48 h and MnS_48 h treatments), 94% of the Mn was recovered in the supernatant with the sulfate source while only 84% with the oxide source ($p < 0.05$), suggesting higher solubility in the sulfate form. After 46 h (treatments MnO_24 h and MnS_24 h), the difference was lower (91% for the sulfate, 88% for the oxide; $p = 0.78$), and after 70 h of incubation (treatments MnO_0 h and MnS_0 h), no more existed (93% for both sources).

4.2. Zinc

In early in vitro studies on rumen fermentation, it was demonstrated that a high dosage of Zn (as $ZnSO_4$) has a strong inhibitory effect, especially on cellulolytic activity [10]. When looking at the Zn effect on rumen fermentations, based on the TGP results registered in this study, the first impressions reveal a negative effect on fermentation of treatments with ZnO, while treatments with $ZnSO_4$ induced no significant variations. More specifically, the addition of ZnO from the start of the incubation (ZnO_0 h) significantly ($p < 0.05$) lowered the TGP in the first 48 h of fermentations and tended ($p = 0.054$) to be lower by the end of the 70 h incubation compared to CON. These findings are not consistent with Riazi et al. [46], who, in an in vitro study with rumen fluid collected from adult sheep, observed a significant increase in gas production when supplementing 20, 40 or 60 mg/kg DM of Zn (as ZnO) to a substrate already containing 25 mg/kg DM of Zn. However, the level of Zn supplementation used in this study was 500 mg/kg DM, which could explain the negative effect on TGP. Similar to the results registered during our experiment, in a more recent study by Petrič et al. [25], a significant decrease in gas production was observed during an in vitro fermentation supplementing 25 mg of Zn (as organic Zn) in 250 mg substrate (rumen fluid collected from lambs fed a diet containing 70 mg/kg DM of Zn). Some negative effects of ZnO on rumen fermentation are also shown by the

substrate degradation. In this study, the addition of ZnO from the start of the incubation (ZnO_0 h) tended (p = 0.06) to decrease the substrate degradation by a mean −4.3% dDM compared to CON. This finding is similar to the results presented by Genther and Hansen [33], showing a tendency for lower DM disappearance when high levels of Zn (120 mg/kg DM, as $ZnSO_4$) were supplemented to steers. Opposing results, presenting a significant increase in DM degradation, were observed when supplementing 30 and 40 mg/kg DM of Zn (as ZnO) to ewes consuming a low-Zn diet (22 mg/kg DM) [9], below the recommendations for ruminants (50 mg/kg DM) [19]. Comparable to the results of Hosseini-Vardanjani et al. [9], in this study, the highest dDM% was observed when Zn was added after 24 h (ZnO_24 h) or 48 h (ZnS_48 h) of fermentation. This finding could reveal a requirement for Zn of the rumen microbiota when the substrate is strongly depleted (Zn content of the substrate was 13 mg/kg DM, below the recommended 50 mg/kg DM). However, the Zn source and inclusion levels need to be carefully evaluated to avoid negative effects on rumen fermentation. In this study, the supplemented Zn (as ZnO and $ZnSO_4$) was 10× the zootechnical recommendations, which could explain the negative effects on fermentations when added from the start of the incubations, and the slight increase in dDM% when supplementation occurred after 24 or 48 h. A well-balanced Zn supplementation was presented in a study by Wang et al. [47]: dairy cows consuming a mixed diet (forages and concentrated feed) containing 31.2 mg/kg DM of Zn were supplemented with 20 mg/kg DM of Zn (as coated $ZnSO_4$). A significant improvement in rumen fermentation was observed (increased degradability of DM, crude protein and fiber), as well as an increase in the cellulolytic activity of rumen microbial populations. Regarding VFA production, there were no significant variations between the treatments, which is consistent with Felner et al. [48]. However, numerically higher total VFA concentrations were observed when ZnO was added after 24 h of fermentation (ZnO_24 h), consistent with the highest dDM% also observed for this treatment, or when Zn was supplemented as sulfate (ZnS_0 h, ZnS_24 h and ZnS_48 h, respectively) compared to CON. These results are similar to Hosseini-Vardanjani et al. [9], who found significantly higher total VFA concentrations when evaluating the effects on rumen fermentation of additional ZnO and $ZnSO_4$, respectively. The pH values registered during these in vitro incubations with different inorganic Zn sources were in the normal range of microbial growth and nutrient degradation [38]. Furthermore, pH did not vary significantly when either of the inorganic Zn was supplemented. These results are consistent with the findings of Wang et al. [47], who noted no variation in pH following a supplementation with ZnO (mean pH 6.5) or $ZnSO_4$ (mean pH 6.4) of in vitro fermentation substrates. The amount of total DAPA (mg/kg DM; total DAPA in UNSOL + DAPA in BACT) was significantly ($p < 0.01$) lower when Zn was supplemented only after 48 h of fermentation (ZnO_48 h + ZnS_48 h) compared to CON. Furthermore, the total DAPA in all the other treatments with additional Zn (ZnO_0 h, ZnS_0 h, ZnO_24 h and ZnS24 h, respectively) were numerically lower compared to CON, suggesting a negative effect of Zn on the bacterial protein synthesis. The Zn concentration in this study was 13 mg/kg DM for the substrate, below the recommended level for ruminants (50 mg/kg DM) and 500 mg/kg DM for the supplementation. Even so, the total Zn concentration in the treatments (513 mg/kg DM) was below the toxicity level for ruminants (2500 mg/kg DM) [40], suggesting that the currently recommended intake levels are defined to cover the needs of the animal and do not seem related to the requirements of microorganisms.

Based on the DAPA analysis, it can be confirmed that the centrifugation method was properly applied following incubations with supplemental Zn, given that the DAPA concentration ratio of the UNSOL/BACT in this trial (1:2.0) was consistent with the one mentioned in the literature [24]. Based on the chemistry of the inorganic Zn sources [49,50], we hypothesized that ZnO would be less rumen soluble than $ZnSO_4$. The results obtained in this study confirm that $ZnSO_4$ is highly soluble in rumen fluid when compared to ZnO. Firstly, the Zn concentration of the SOL content was significantly ($p < 0.001$) higher when supplementing $ZnSO_4$ (regardless of addition time) compared to ZnO and CON.

These results are consistent with Genther and Hansen [33], who reported a significantly higher Zn concentration in the supernatant of ultracentrifuged rumen fluid when steers were supplemented with high levels (120 mg/kg DM) of Zn (as $ZnSO_4$ and hydroxy-Zn). Furthermore, the Zn concentration of the BACT of treatments with $ZnSO_4$ (ZnS_24 h and ZnS_48 h) was significantly ($p < 0.001$) higher compared to treatments with ZnO, suggesting a higher assimilation by rumen bacteria. However, the hypothesis that bacteria incorporate solubilized Zn needs further investigation, considering that Bonhomme et al. [10], in an in vitro rumen fermentation study with $ZnSO_4$, suggested that Zn could stay attached to the bacteria cell wall rather than being absorbed by bacteria. The negative effect of Zn on rumen fermentation could be related to being attached to the bacterial cell wall, damaging their integrity and, thus, their fermentative activity. Even though the UNSOL of all the treatments had a higher Zn concentration compared to CON (indication that part of the added Zn was not solubilized), the highest UNSOL Zn concentrations were observed with $ZnSO_4$ treatments (ZnS_24 h and ZnS_48 h). The initial hypothesis would be that the sulfate form of Zn also has low solubility in rumen fluid (not consistent with Zn concentration of the SOL). However, rumen protozoa can assimilate rumen-soluble Zn [10]. Thus, in our study, the higher Zn content in the UNSOL with $ZnSO_4$ treatments compared to the ZnO treatment could be explained by the uptake of highly soluble Zn by the protozoa (also recovered in the UNSOL). When analyzing the dispersion of Zn relative to the total amount (% of total Zn) in the different fractions, depending on Zn source, 9 to 25% (for ZnO) and 17 to 32% (for $ZnSO_4$) was found in the SOL; 51 to 72% (for ZnO) and 41 to 50% (for $ZnSO_4$) in the UNSOL; and 19 to 24% (for ZnO) and 27 to 38% (for $ZnSO_4$) in the BACT. This finding indicates that only a small part of the added Zn solubilized during the 70 h fermentation, and if solubilized, Zn could be assimilated by the rumen bacteria (19–38% of total Zn). The percentage of the total Zn in the BACT found in this study (19–38% of total Zn) was quite close to the intestinal apparent absorption of Zn in ruminants, in an interval of 15–30% [51]. Regarding the differences between ZnO and $ZnSO_4$ solubility, after 22 h of Zn exposure (ZnO_48 h and ZnS_48 h treatments), 19% of the Zn was recovered in the SOL with the sulfate source, while only 10% with the oxide source ($p < 0.05$), indicating higher solubility in the sulfate form. After 46 h of Zn exposure (treatments ZnO_24 h and ZnS_24 h), the difference was lower (17% for the sulfate, 9% for the oxide; $p = 0.09$), and after 70 h of incubation (treatments ZnO_0 h and ZnS_0 h), it still existed (32% for the sulfate and 25% for the oxide; $p = 0.14$).

4.3. Copper

In [12], as well as more recent in vitro studies [52], Cu was identified as a TM with complex responses to fermentation. In the present study, the results registered during the 70 h incubations with rumen fluid suggest that $CuSO_4$ has no significant effect on TGP. Similar to this, it was found that supplementing inorganic Cu (as $CuSO_4$) at different rates (4, 8 or 16 mg/kg DM) to in vitro incubations with rumen fluid collected from three freshly slaughtered cattle induced no significant variation in gas production [14]. However, some numerically higher TGP was observed when Cu was added at the start or after 24 h of fermentation (CuS_0.01_0 h, CuS_0.015_0 h and CuS_0.015_24 h, respectively), suggesting a slight improvement in fermentations. These findings are consistent with Wilk et al. [52], who recorded significantly higher in vitro gas production when $CuSO_4$ was added to a low-Cu total mixed ration (5.46 mg/kg DM). Regarding dDM%, some numerical variations were observed in favor of treatments with additional Cu. Firstly, the increase in degradability was not due to the SO_4^{-2}. As shown in a study by Slyter et al. [12], the addition of SO_4^{-2} from sodium salt ($NaSO_4$) at an equivalent level to $CuSO_4$ induced no detectable changes in substrate degradation. The numerical increase in the dDM% in all treatments with additional Cu observed in this study is consistent with Vaswani et al. [14] and Zhang et al. [53], who noted increased in vitro degradability of dry matter when inorganic Cu was supplemented at levels that varied between 4 and 30 mg/kg DM. No significant effect of Cu supplementation was observed regarding total VFA production, consistent with

the lack of increased TGP. These findings are consistent with those presented by Slyter et al. [12], who observed no change in the VFA profile following the addition of 15–35 mg of Cu to substrates during in vitro incubations and with the results obtained by Engle and Spears [54], who noted no effect on ruminal VFA molar proportions in steers supplemented with 10 or 20 mg/kg DM of Cu (as $CuSO_4$). The pH values observed in this study were in the normal range of ruminal fermentations [38], and they were not affected by additional Cu. Contrary to this study, a significant decrease in pH during in vitro rumen fermentations was observed by Vaswani et al. [14], following a supplementation of 8 mg/kg DM of Cu (as $CuSO_4$). However, numerous studies registered no effect of Cu on ruminal pH when supplementing steers with a high dosage (25 mg/kg DM) of inorganic Cu [13,33,52,54]. Based on the numerically lower total DAPA (mg) in all treatments with supplemental Cu, a negative effect of $CuSO_4$ on rumen microbial synthesis could be implied [55].

The results on solubility parameters observed in this study confirm the expected high solubility of $CuSO_4$ in rumen fluid. The high dosage of Cu used in this study, regardless of addition time (at the start, after 24 or 48 h of fermentation), was well solubilized, as indicated by the Cu concentration in the SOL. In a previous study by Genther and Hansen [33], a significantly higher Cu concentration (0.30 mg/L) was observed in the supernatant obtained after ultracentrifugation of rumen fluid sampled from steers with supplemental Cu (25 mg/kg DM as $CuSO_4$) compared to a control group (0.11 mg/L). The same study also reported a dosage effect [33]: a supplementation with a high dosage of Cu (25 mg/kg DM of Cu) resulted in a significantly higher Cu concentration in the rumen fluid supernatant when compared to a low dosage (5 mg/kg DM) of additional Cu (0.30 mg/L and 0.19 mg/L for high and low Cu, respectively). The dosage effect was equally observed in this study, as an addition of 500 mg/kg DM of inorganic Cu significantly increased the Cu concentration of the SOL compared to 100 mg/kg DM of additional Cu.

The DAPA analysis of the UNSOL and BACT confirms that the centrifugation method was properly applied, and the UNSOL:BACT DAPA concentration ratio was according to the one (1:2.3) previously mentioned [24]. Regarding the BACT, the significantly higher concentration of Cu in the treatments indicates that the bacteria could assimilate copper in a dose-dependent manner. When analyzing the dispersion of Cu relative to the total amount (% of total Cu) analyzed in the different fractions, around 37 to 58% was found in the SOL, 29 to 40% in the UNSOL and 12 to 27% in the BACT. These results are not consistent with Genther and Hansen [33], who observed only 14 to 17% of total Cu in the supernatant of rumen fluid. However, in a recent study comparing the solubility of different sources of Cu [56], it was demonstrated that $CuSO_4$ has a mean solubility range of 33 to 49% in rumen fluid, similar to the one assessed in our study in the SOL. Furthermore, as already shown, $CuSO_4$ solubility is dose-dependent and, in this study, the additional Cu was 10× and 50× of the recommended supplementation level for ruminants [57], which could explain the higher percentage of Cu in the SOL.

5. Conclusions

The results of this study indicate that $MnSO_4$, $ZnSO_4$ and $CuSO_4$ are highly soluble in the rumen, MnO is quite soluble, while ZnO has low solubility. Additionally, the more soluble sulfate sources of TM seem to be better assimilated by rumen bacteria compared to the oxide TM and, hence, have a higher bioavailability. The TM (% of total TM) assimilated by the rumen bacteria seems to be related to the intestinal apparent absorption of TM in ruminants. Regarding rumen function, Mn seems to have a negative effect on fermentation, while Zn and Cu, even if they present no special requirements for microbial protein synthesis, could improve specific fermentation parameters. Further investigations are necessary, including not only inorganic sources of TM but also different complexes (organic TM, hydroxy-TM).

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