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

Antal Vigh *, Adriana Criste *, Kévin Gragnic, Léa Moquet, Christine Gerard *

Posted Date: 31 March 2023

doi: 10.20944/preprints202303.0548.v1

Keywords: ruminant; fermentation; solubility; trace-minerals

Article

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

Antal Vigh [1,2,*], Adriana Criste [2], Kévin Gragnic [1], Léa Moquet [1] and Christine Gerard [1,*]

[1] ADM Animal Nutrition, Talhouet Saint-Nolff, France
[2] University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
* Correspondence: antal-csaba.vigh@usamvcluj.ro (A.V.); christine.gerard@adm.com (C.G.)

Abstract: The aim of this study was to assess the effects of supplementation of 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; and 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 70hrs of fermentation (dDM%). Trace-mineral (TM) solubility was estimated by the TM concentration in the supernatant of the final fermentation medium (SOL) and TM bioavailability by 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 of 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 of BACT, MnO, MnS_4 and $CuSO_4$ have a high bioavailability, while ZnO is poorly assimilated by rumen bacteria. However, the lack of clear inhibition or improvement of fermentations suggests that the rumen microbiota has low requirement for TM supplementation.

Keywords: ruminant; fermentation; solubility; trace-minerals

1. Introduction

Trace-minerals like 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 important role for the immune and reproductive system [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 the rumen cellulose digestion, while an addition of 5-30 µg/ml of *in vitro* medium of inorganic Mn increases the cellulose digestion [5,6]. Furthermore, *in vitro* dry matter digestibility in rumen fluid is improved by an addition of 100 ppm of inorganic Mn [7]. Regarding Zn, early *in vitro* studies have shown 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 acids (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 the microbial activity, leading to a sharp reduction in ammonia concentrations [11]. *In*

 doi:10.20944/preprints202303.0548.v1

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, an 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, like *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 effect 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 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 the rumen fermentative activity. We hypothesized that the sulfate forms of trace-minerals would be highly soluble in rumen juice compared to oxide forms, and therefore more bioavailable for rumen microorganisms. More precisely, the present study, aimed to investigated 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 trace-mineral content of the substrate is below the recommended levels, and identify the rumen juice fraction(s) in which the different additional trace-mineral sources are found after fermentation (big particles fraction [feed particles, insolubilized minerals, protozoa], bacteria-enriched fraction, and final supernatant), to assess ruminal solubility and bioavailability of each trace element.

2. Materials and Methods

This study was carried out at the Talhouet Research Center of ADM AN (TRC, Saint-Nolff, France) to evaluate the rumen solubility and effect on fermentation activity of three feed-grade inorganic trace-minerals (supplied by SERMIX, Chierry, France): manganese (Mn), in oxide (MnO, 60% - Mn) and sulfate ($MnSO_4$, 32% - Mn) forms; zinc (Zn), in oxide (ZnO, 72% - Zn) and sulfate ($ZnSO_4$, 35% - Zn) forms; and copper (Cu), in sulfate ($CuSO_4$, 26% - Cu) form.

2.1. Experimental design and treatments

In vitro incubations, respecting the well-established recommendation regarding substrate, inoculum, and buffer [20], were conducted for 70 hours using the automatic system Gas Endeavour® (Bioprocess Control, Sweden). For each trace-mineral 3 consecutive incubations were realized, each treatment had 4 (for Mn and Zn treatments) or 6 (for Cu treatments) replicates. Fermentation activity was measured through continuous total gas production over the 70h of fermentation (TGP), and substrate dry-matter degradability (dDM%), pH and volatile fatty acid (VFA, mM) production at the end of the 70hrs 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 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 minerals. The correct separation of the different fractions by centrifugation was verified by a diaminopimelic acid analysis (DAPA) of the UNSOL and BACT [21,22]. For the incubations testing Mn sources, the substrate was incubated solely (CON), or with an addition of 0.06% DM of Mn (as MnO or $MnSO_4$), added at the start (MnO_0h and MnS_0h), after 24hrs (MnO_24h and MnS_24h) or 48hrs (MnO_48h and MnS_48h) of incubation. For the incubations testing Zn sources, the substrate was incubated solely (CON), or with an addition of

 doi:10.20944/preprints202303.0548.v1

0.05% DM of Zn (as ZnO or $ZnSO_4$), added at the start (ZnO_0h and ZnS_0h), after 24hrs (ZnO_24h and ZnS_24h) or 48hrs (ZnO_48h and ZnS_48h) of fermentation. For incubations testing Cu, the substrate was incubated solely (CON), or with an addition of 0.01% DM of Cu (as $CuSO_4$), added at the start (CuS_0.01_0h); or with an addition of 0.015% DM of Cu (as $CuSO_4$), added at the start (CuS_0.015_0h), after 24hrs (CuS_0.015_24h) or 48hrs (CuS_0.015_48h) of incubation. 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 and avoid a potential bias given by the S content of the $MnSO_4$ (18.6% S) and $ZnSO_4$ (19.9% S) [23,24].

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 mime 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 content of the hay were 13.0 and <5.0 mg/kg DM, below the INRA 2018 recommendations for cattle diets (50 and 10 mg/kgDM 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 [25,26]. 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 juice 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 of the fermentations [24].

Table 1. Mineral content of 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 juice donor cows

The 3 rumen juice 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 of rumen juice; 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 of 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; trace-mineral content of rumen juice.

Diet composition	Intake (kg DM/head/day)
Corn silage	3.54
Hay	2.00

 doi:10.20944/preprints202303.0548.v1

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 [27], described in [24]. The buffer contained no S source to limit antagonism with trace-minerals [25,26]. 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 (72hrs at 60°C) then grinded (3 mm sleeve) and the residual DM content was measured (4h at 103°C). The hay was directly grinded (3 mm sleeve) and the residual DM content was measured (4h at 103°C). The DM of the urea was considered 100%. The day before the incubations were launched, the 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 TMs (Mn_0h, Zn_0h and Cu_0h), 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 hours of pre-hydration necessary for the good start of the fermentations [20]. 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 juice donor cows [20]. 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 hour 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 [20]. 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_24h, Mn_48h, Zn_24h, Zn_48h, Cu_24 and Cu_48h, respectively) the quantities of minerals were directly introduced after 24 or 48 hours 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 70hrs and recorded using the software provided by Bioprocess Control (version ge_2.1[v1.2948]). Treatments which were supplemented with Mn, Zn or Cu after 24 or 48hrs were considered as CON before the addition of the TMs (MnO_24h, MnS_24h, MnO_48h, MnS_48h, ZnO_24h, ZnS_24h, ZnO_48h, ZnS_48h, CuS_0.015_24h and CuS_0.015_48h, respectively). The cumulated values of TGP after 24, 48 and 72hrs were used for the comparisons of the treatments.

At the end of the incubations,

 doi:10.20944/preprints202303.0548.v1

- 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 hours. After defrosting, the nylon bags were washed a second time in cold water for 2 minutes in the washing machine and oven dried for 48 hours at 60°C. The nylon bags containing the dry matter (DM) residues were weighed, and the dDM% was calculated [28].
- Replicates of the same treatment of 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) by gas chromatography (Upscience, Saint-Nolff, France).
- The pooled final fermentation medium was centrifuged to separate 3 different fractions, based on the method described by [22]: UNSOL (containing undegraded feed particles, protozoa and insolubilized minerals), BACT and SOL. The pooled fermentation medium was first refrigerated (4°C) for 6 hours [29], then agitated with a magnetic stirrer (400 rpm) for 45 seconds to detach the bacteria bound to fiber particles. 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 18500 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 TM (UT2A, Pau, France) analysis. The SOL samples were analyzed only for TM content (UT2A, Pau, France), as previous studies done 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 of the fractions [30].
- Total DAPA was used as rumen bacterial synthesis marker and calculated based on DAPA concentration of UNSOL and BACT [31].The DAPA concentration of BACT was also used to confirm the enrichment with bacteria.
- 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, 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

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 24hrs (p=0.49), 48hrs (p=0.18) or after 70hrs (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 48hrs compared to CON (86.5, 86.6 and 88.6 % dDM for MnO_24h, MnS_48h 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 70hrs incubation. 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 70hrs of fermentation, did not differ (p=0.35) between the treatments. However, when looking at the effect of Mn regardless of source added form the start of the incubation (MnO_0h + MnS_0h), the total DAPA tended (p=0.06) to be lower compared to CON (1.56, 1.52 and 1.93 mg DAPA for MnO_0h, MnS_0h and CON, respectively).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2023 doi:10.20944/preprints202303.0548.v1

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

Item	CON	MnO_0h	MnO_24h	MnO_48h	MnS_0h	MnS_24h	MnS_48h	SEM	*p* Value
TGP[1] at 24h (ml/gDM)	143	149	-	-	141	-	-	5.19	0.49
TGP at 48h (ml/gDM)	218	211	213	-	204	219	-	5.23	0.18
TGP at 70h (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 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).

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 70hrs *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 48hrs of fermentation compared with the addition from the start of the incubation ($p<0.01$; 458, 457 and 252 mg/kgDM for MnO_24h, MnO_48h and MnO_0h, 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 48hrs compared to when it was added from the start of the incubation (373, 406 and 306 mg/kgDM for MnS_24h, MnS_48h and MnS_0h, respectively). There were no significant differences in the Mn concentration of the UNSOL between the MnO_0h and MnS_0h treatments. The Mn concentration of the BACT was significantly higher when MnO was added after 24 or 48hrs compared with the addition from the start of the incubation ($p<0.05$; 330, 327 and 238 mg/kgDM for MnO_24h, MnO_48h and MnO_0h, respectively). No significant differences were observed in the Mn concentration of the BACT between treatments MnS_0h, MnS_24h and MnS_48h, respectively. The Mn concentration of the SOL tended to be higher with MnS_48h when compared to MnO_48h ($p=0.08$). 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_0h and MnS_0h a high percentage (>90%) of total Mn was in the SOL (93.0 and 93.4 %, respectively). Moreover, the Mn % of the MnO_0h was significantly higher compared to MnO_48h treatment ($p<0.05$). The Mn % in the SOL fraction of the MnS_48h was significantly higher compared to MnO_48h treatment (84.2 % vs 93.6%, $p<0.05$), showing a source-related solubility.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2023 doi:10.20944/preprints202303.0548.v1

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

Item	CON	MnO_0h	MnO_24h	MnO_48h	MnS_0h	MnS_24h	MnS_48h	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]	0.49	<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 mean; [1] Big particles 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 ($p \leq 0.05$).

3.2. Zinc

3.2.1. Fermentation parameters

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 24hrs of incubation (p=0.14), while it was significantly different between the treatments after 48hrs (p<0.01) and 70hrs (p<0.05) of fermentation. After 48hrs of fermentation the TGP of ZnO_0h was significantly (p<0.01) lower, while ZnS_0h was not different when compared to CON (126, 139 and 142 ml/g DM for ZnO_0h, ZnS_0h and CON, respectively). By the end of the 70hrs incubation, the lowest TGS was observed with ZnO_0h, however this only tended (p=0.054) to be different, while ZnS_0h showed no significant effect when compared to CON (147, 159 and 168 ml/g DM for ZnO_0h, ZnS_0h and CON, respectively). Furthermore, the addition of Zn after 24 or 48hrs of fermentation (ZnO_24h, ZnS_24h, ZnO_48h and ZnS_48h) did not affect TGP when compared to CON. A significantly (p<0.05) higher TGP was registered with ZnO_24h when compared to ZnO_0h. Concerning dDM%, there was a significant difference (p<0.05) between the treatments at the end of the 70hrs incubation. The lowest dDM% was observed with ZnO_0h and tended (p=0.06) to be lower, while ZnS_0h was not different compared to CON (67.5, 70.3 and 71.8 % dMD for ZnO_0h, ZnS_0h and CON, respectively). Significantly higher dDM% was observed with ZnO_24h and ZnS_48h when compared to ZnO_0h (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 70hrs 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 70hrs of fermentation, was significantly (p<0.05) different between the treatments. Significantly (p<0.01) lower DAPA was observed when Zn was supplemented after 48hrs of fermentation (ZnO_48h+ZnS_48h) compared to CON (2.02, 2.05 and 2.71 mg DAPA for ZnO_48h, ZnS_48h and CON, respectively).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2023 doi:10.20944/preprints202303.0548.v1

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

Item	CON	ZnO_0h	ZnO_24h	ZnO_48h	ZnS_0h	ZnS_24h	ZnS_48h	SEM	*p* Value
TGP[1] at 24h (ml/gDM)	89	83	-	-	86	-	-	2.9	0.14
TGP at 48h (ml/gDM)	142^{b}	126^{a}	146^{b}	-	139^{ab}	139^{ab}	-	3.6	<0.01
TGP at 70h (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 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 70hrs *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 48hrs of fermentation (ZnO_24h, ZnS_24h, ZnO_48h and ZnS_48h, respectively). When Zn was added from the start of the fermentation, the Zn concentration of UNSOL was numerically higher with ZnO_0h and tended (p=0.06) to be higher with ZnS_0h compared to CON (357, 611 and 223 mg/kg DM for ZnO_0h, ZnS_0h and CON, respectively). The Zn concentration of the BACT was significantly (p<0.001) higher when $ZnSO_4$ was added after 24 and 48hrs 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_0h, ZnO_24h, ZnO_48h and ZnS_0h 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_0h, ZnS_24h, ZnS_48h and CON, respectively). The Zn concentration of the SOL when ZnO (ZnO_0h, ZnO_24h and ZnO_48h) 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_0h, ZnO_24h, ZnO_48h, ZnS_0h, ZnS_24h, ZnS_48h 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 addition time (25.4, 9.0,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2023 doi:10.20944/preprints202303.0548.v1

10.2, 32.4, 16.5 and 19.0 % for ZnO_0h, ZnO_24h, ZnO_48hm ZnS_0h, ZnS_24h and ZnS_48h, respectively).

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

Item	CON	ZnO_0h	ZnO_24h	ZnO_48h	ZnS_0h	ZnS_24h	ZnS_48h	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 mean; [1] Big particles 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 ($p \leq 0.05$).

3.3. Copper

3.3.1. Fermentation parameters

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 24hrs (p=0.26), 48hrs (p=0.32) or after 70hrs (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 70hrs 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 70hrs of fermentation, did not differ (p=0.89) between the treatments.

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 70hrs *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 7. Influence of $CuSO_4$ dose and addition time on fermentation parameters.

Item	CON	CuS_0.01_0h	CuS_0.015_0h	CuS_0.015_24h	CuS_0.015_48h	SEM	*p* Value
TGP[1] at 24h (ml/gDM)	85	88	80	-	-	3.1	0.26
TGP at 48h (ml/gDM)	130	138	129	135	-	4.1	0.32
TGP at 70h (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

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2023 doi:10.20944/preprints202303.0548.v1

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[4]:BACT[5]-DAPA ratio	2.3	2.4	2.8	2.4	2.5	0.15	0.37

SEM = Standard error of 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).

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 of the UNSOL was significantly (p<0.05) higher when Cu was supplemented at the 0.015% DM dosage (regardless of addition time) compared to the 0.01% DM dosage and CON.

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

Item	CON	CuS_0.01_0h	CuS_0.015_0h	CuS_0.015_24h	CuS_0.015_48h	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 mean; [1] Big particles 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 ($p \leq 0.05$).

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_0h, CuS_0.015_24h, CuS_0.015_48h, CuS_0.01_0h 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_0h and CuS_0.015_0h). Moreover, the Cu % with the CuS_0.015_0h was numerically higher compared to CuS_0.015_24h and CuS_0.015_48h treatments, suggesting a time-related solubility.

 doi:10.20944/preprints202303.0548.v1

4. Discussion

Trace-minerals are important micronutrients for favorable growth and health of animals, especially ruminants [32]. 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 precis TM supplementation of 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 juice, 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. [33], it was found that supplementing 15 or 150 mg/kg DM of Mn in an *in vitro* incubation with rumen juice, using as substrate fungi-treated wheat straw, fermentation activity and gas production is 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 70hrs incubation (MnO_0h and MnS_0h) compared to treatments supplemented only after 48hrs of fermentation (MnO_48h and MnS_48h) might express a decrease of 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. [34], it was demonstrated that lambs receiving a high-Mn diet (184 mg/kgDM; 3.7x 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 [30], 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, deducing 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 closed 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 in relation with the decrease of enzymatic activities of rumen microorganisms when Mn is supplemented, demonstrated by Kišidayová et al. [34]. The pH values obtained in this study were in the normal range of microbial growth and nutrients degradation [35]. Mn did not affect pH, which is consistent with the findings of Genther and Hansen [30], 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_48h) compared to CON, hence a tendency for lower microbial synthesis might be induced by inorganic Mn. The lower total DAPA, especially for MnO_0h and MnS_0h compared to CON, can be directly correlated with the overall lower VFA production. This correlation was demonstrated in a study by Maskaľová et al. [36], 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, hence on the 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) [37], suggesting that the currently recommended intake levels have been defined rather 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 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 [22]. Based on the chemistry of the inorganic Mn sources [38,39], 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 juice. Even though the UNSOL of all the treatments had a higher Mn

 doi:10.20944/preprints202303.0548.v1

concentration compared to the CON (suggesting that part of the added Mn was not solubilized), the Mn concentration of the SOL of all treatments was significantly higher (p<0.01) compared to the CON treatment. These results are similar with the ones found in *in vivo* studies [30,40], 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 a progressive solubilization: the Mn concentration of the UNSOL was lower when Mn was supplemented from the start of the incubation (MnO_0h and MnS_0h) compared to when it was added after 24hrs (MnO_24h and MnS_24h) or 48hrs (MnO_48h and MnS_48h). 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 70hrs fermentation and are similar to the results expressed by Genther and Hansen [30], showing that close to 66% of Mn content of rumen fluid was in the supernatant obtained after ultracentrifugation of the rumen juice. 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 the interval of 1-4% [19,41,42]. Regarding the differences between MnO and $MnSO_4$ solubility, after 22hrs of exposure to Mn supply (MnO_48h and MnS_48h 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 a higher solubility of the sulfate form. After 48hrs (treatments MnO_24h and MnS_24h), the difference was lower (91% for the sulfate, 88% for the oxide; p=0.78), and after 70hrs of incubation (treatments MnO_0h and MnS_0h), no more existing (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 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_0h) significantly (p<0.05) lowered the TGP in the first 48hrs of fermentations and tended (p=0.054) to be lower by the end of the 70hrs incubation compared to CON. These findings are not consistent with Riazi et al. [43], who in an in vitro study with rumen juice collected from adult sheep, observed a significant increase of 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. [44], a significant decrease of gas production was observed during an in vitro fermentation supplementing 25 mg of Zn (as organic Zn) in 250 mg substrate (rumen juice 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_0h) 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 [30], 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 of DM degradation were observed when supplementing 30 and 40 mg/kg DM of Zn (as ZnO) to ewes consuming a low-Zn content 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% were observed when Zn was added after 24hrs (ZnO_24h) or 48hrs (ZnS_48h) 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

 doi:10.20944/preprints202303.0548.v1

fermentation. In this study, the supplemented Zn (as ZnO and $ZnSO_4$) was 10x the zootechnical recommendations, which could explain the negative effects on fermentations, when added from the start of the incubations, and the slight increase of dDM%, when supplementation occurred after 24 or 48hrs. A well-balanced Zn supplementation was presented in a study by Wang et al. [45]: 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 of 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. [46]. However, numerically higher total VFA concentrations were observed when ZnO was added after 24hrs of fermentation (ZnO_24h), consistent with the highest dDM% also observed for this treatment, or when Zn was supplemented as sulfate (ZnS_0h, ZnS_24h and ZnS_48h, 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 nutrients degradation [35]. 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. [45], 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 48hrs of fermentation (ZnO_48h + ZnS_48h) compared to CON. Furthermore, the total DAPA of all the other treatments with additional Zn (ZnO_0h, ZnS_0h, ZnO_24h and ZnS24h, 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) [37], suggesting that the currently recommended intake levels have been defined rather 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 [22]. Based on the chemistry of the inorganic Zn sources [47,48], 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 juice 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 [30], who reported a significantly higher Zn concentration of 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_24h and ZnS_48h) were 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 bacteria 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_24h and ZnS_48h). The initial hypothesis would be that the sulfate form of Zn also has a low solubility in rumen juice (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 of the UNSOL with $ZnSO_4$ treatments compared to ZnO treatment could be explained by the

 doi:10.20944/preprints202303.0548.v1

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 70hrs 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 the interval of 15-30% [49]. Regarding the differences between ZnO and $ZnSO_4$ solubility, after 22hrs of Zn exposure (ZnO_48h and ZnS_48h 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 a higher solubility of the sulfate form. After 48hrs of Zn exposure (treatments ZnO_24h and ZnS_24h), the difference was lower (17 % for the sulfate, 9 % for the oxide; $p=0.09$), and after 70hrs of incubation (treatments ZnO_0h and ZnS_0h), still existing (32 % for the sulfate and 25 % for the oxide; $p=0.14$).

4.3. Copper

In early [12], as well as more recent *in vitro* studies [50], Cu was identified as a TM with complex responses on fermentation. In the present study, the results registered during the 70hrs incubations with rumen juice 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 juice collected from 3 freshly slaughtered cattle, induced no significant variation in the gas production [14]. However, some numerically higher TGP was observed when Cu was added at the start or after 24hrs of fermentation (CuS_0.01_0h, CuS_0.015_0h and CuS_0.015_24h, respectively), suggesting a slight improvement of fermentations. These finding are consistent with Wilk et al. [50], 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 of 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 equivalent level to $CuSO_4$, induced no detectable changes in the substrate degradation. The numerical increase of the dDM% in all treatments with additional Cu observed in this study is consistent with Vaswani et al. [14] and Zhang et al. [51], 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, persistent with the lack of increased TGP. These findings are consistent with the ones 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 [52], 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 [35], and not affected by additional Cu. Contrary to this study, a significant decrease of 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 [13,30,50,52]. 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 [53].

The results on solubility parameters observed in this study confirm the expected high solubility of $CuSO_4$ in rumen juice. The high dosage of Cu used in this study, regardless of addition time (at the start, after 24 or 48hrs of fermentation) was well solubilized, as indicated by the Cu concentration in the SOL. In a previous study by Genther and Hansen [30], 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). This same study also reported a dosage effect [30]: 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 [22]. 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 [30], who observed only 14 to 17 % of total Cu in the supernatant of rumen fluid. However, in a recent study comparing solubility of different sources of Cu [54], 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 10x and 50x of the recommended supplementation level for ruminants [55], 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 a low solubility. Additionally, the more soluble sulfate sources of TM seem to be better assimilated by rumen bacteria compared to the oxide TM, hence 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).

Author Contributions: Conceptualization, A.V.; methodology, A.V. and C.G.; software, A.V.; validation, C.G.; investigation, A.V.; resources, K.G.; data curation, A.V. and L.M.; writing—original draft preparation, A.V.; writing—review and editing, A.V., A.C. and C.G.; supervision, C.G.; project administration, A.V.; funding acquisition, A.V. and C.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by ADM ANIMAL NUTRITION, SAINT-NOLFF, FRANCE

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee n°052, France and registered under the number APAFIS#28768-2020122108098663 v3, February 11, 2021, by the French Scientific Research Ministry.

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

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hosnedlová B, Travnicek J, Šoch M. Current view of the significance of zinc for ruminants: A review. Agricultura tropica et subtropica. 2007;40:57–64.
2. Sloup V, Jankovská I, Nechybová S, Peřinková P, Langrova I. Zinc in the Animal Organism: A Review. Scientia Agriculturae Bohemica. 2017;48.
3. Hostetler CE, Kincaid RL, Mirando MA. The role of essential trace elements in embryonic and fetal development in livestock. The Veterinary Journal. 2003;166:125–39.
4. Hilal E, Elkhairey M, Osman A. The Role of Zinc, Manganse and Copper in Rumen Metabolism and Immune Function: A Review Article. Open Journal of Animal Sciences. 2016;06:304–24.
5. Chamberlain CC, Burroughs W. Effect of Fluoride, Magnesium and Manganese Ions on in Vitro Cellulose Digestion by Rumen Microorganisms1. Journal of Animal Science. 1962;21:428–32.

6. Martinez A, Church DC. Effect of various mineral elements on in vitro rumen cellulose digestion. J Anim Sci. 1970;31:982–90.
7. Arelovich HM, Owens FN, Horn GW, Vizcarra JA. Effects of supplemental zinc and manganese on ruminal fermentation, forage intake, and digestion by cattle fed prairie hay and urea. J Anim Sci. 2000;78:2972–9.
8. Martínez A. Effect of some major and trace element interactions upon in vitro rumen cellulose digestion. 1971 [cited 2023 Feb 26]. Available from: https://www.semanticscholar.org/paper/Effect-of-some-major-and-trace-element-interactions-Mart%C3%ADnez/d4c7456f215fd8fbff26845bc944c5b713ae9c9b
9. Hosseini-Vardanjani SF, Rezaei J, Karimi-Dehkordi S, Rouzbehan Y. Effect of feeding nano-ZnO on performance, rumen fermentation, leukocytes, antioxidant capacity, blood serum enzymes and minerals of ewes. Small Ruminant Research. 2020;191:106170.
10. Bonhomme A, Durand M, Dumay C, Beaumatin P. Etude in vitro du comportement des populations microbiennes du rumen en présence de zinc sous forme de sulfate. Ann Biol anim Bioch Biophys. 1979;19:937–42.
11. Hernández-Sánchez D, Cervantes-Gómez D, Ramírez-Bribiesca JE, Cobos-Peralta M, Pinto-Ruiz R, Astigarraga L, et al. The influence of copper levels on in vitro ruminal fermentation, bacterial growth and methane production. J Sci Food Agric. 2019;99:1073–7.
12. Slyter LL, Wolin MJ. Copper sulfate-induced fermentation changes in continuous cultures of the rumen microbial ecosystem. Appl Microbiol. 1967;15:1160–4.
13. Arce-Cordero JA, Monteiro HF, Lelis AL, Lima LR, Restelatto R, Brandao VLN, et al. Copper sulfate and sodium selenite lipid-microencapsulation modifies ruminal microbial fermentation in a dual-flow continuous-culture system. J Dairy Sci. 2020;103:7068–80.
14. Vaswani S, Sidhu VK, Roy D, Kumar M, Kushwaha R. Effect of Copper Supplementation on In -vitro Rumen Fermentation Characteristics. International Journal of Livestock Research. 2017;1.
15. Wang C, Han L, Zhang GW, Du HS, Wu ZZ, Liu Q, et al. Effects of copper sulphate and coated copper sulphate addition on lactation performance, nutrient digestibility, ruminal fermentation and blood metabolites in dairy cows. British Journal of Nutrition. Cambridge University Press; 2021;125:251–9.
16. Trumeau D. Les oligo-éléments en élevage bovin. Analyse descriptive des profils métaboliques en oligo-éléments établis en laboratoire d'analyse et liens avec les aspects cliniques. ONIRIS, Nantes, France; 2014.
17. European Commission. Directorate General for Health and Food Safety. European Union register of feed additives pursuant to Regulation (EC) No 1831/2003.Annex I, List of additives (Released date 06.12.2022) . [Internet]. LU: Publications Office; 2022 [cited 2023 Feb 26]. Available from: https://data.europa.eu/doi/10.2875/110483
18. Meschy F. Alimentation minérale et vitaminique des ruminants : actualisation des connaissances. INRAE Productions Animales. 2007;20:119–28.
19. Inra, Noziere P, Sauvant D, Delaby L. Inra, 2018. Alimentation des ruminants [Internet]. Editions Quae; 2018 [cited 2023 Feb 26]. p. 728 p. Available from: https://hal.inrae.fr/hal-02789908
20. Yáñez-Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi DP, O'Kiely P, et al. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—a review. Animal Feed Science and Technology. 2016;216:1–18.
21. Krawielitzki R, Piatkowski B, Kreienbring F. Untersuchungen zum Gehalt an 2,6-Diaminopimelinsäure (DAP) und das DAP: N-Verhältnis in Pansenbakterien in Abhängigkeit von der Zeit nach der Fütterung. Archiv für Tierernaehrung. 1978;28:701–8.
22. Krawielitzki R, Voigt J, Piatkowski B. Der Einfluß unterschiedlicher Zentrifugationsbedingungen bei der Isolierung gemischter Pansenbakterien auf deren Gehalt an Stickstoff und Diaminopimelinsäure: Verwendung von Duodenalchymus als Ausgangsmaterial. Archiv für Tierernaehrung. 1989;39:813–21.
23. McSweeney CS, Denman SE. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. J Appl Microbiol. 2007;103:1757–65.
24. Mould FL, Morgan R, Kliem KE, Krystallidou E. A review and simplification of the in vitro incubation medium. Animal Feed Science and Technology. 2005;123–124:155–72.
25. Spears JW. Trace mineral bioavailability in ruminants. J Nutr. 2003;133:1506S-9S.
26. Ledoux D, Shannon M. Bioavailability and Antagonists of Trace Minerals in Ruminant Metabolism. 2005;
27. Tilley JMA, Terry RA. A TWO-STAGE TECHNIQUE FOR THE IN VITRO DIGESTION OF FORAGE CROPS. Grass and Forage Sci. 1963;18:104–11.

28. Valentin SF, Williams PEV, Forbes JM, Sauvant D. Comparison of the in vitro gas production technique and the nylon bag degradability technique to measure short- and long-term processes of degradation of maize silage in dairy cows. Animal Feed Science and Technology. 1999;78:81–99.
29. Dehority BA, Grubb JA. Effect of short-term chilling of rumen contents on viable bacterial numbers. Appl Environ Microbiol. 1980;39:376–81.
30. Genther ON, Hansen SL. The effect of trace mineral source and concentration on ruminal digestion and mineral solubility. Journal of Dairy Science. 2015;98:566–73.
31. Dufva GS, Bartley EE, Arambel MJ, Nagaraja TG, Dennis SM, Galitzer SJ, et al. Diaminopimelic Acid Content of Feeds and Rumen Bacteria and Its Usefulness as a Rumen Bacterial Marker1. Journal of Dairy Science. 1982;65:1754–9.
32. Overton TR, Yasui T. Practical applications of trace minerals for dairy cattle1,Journal of Animal Science. 2014;92:416–26.
33. 33. van Kuijk SJA, Sonnenberg ASM, Baars JJP, Hendriks WH, Cone JW. The effect of adding urea, manganese and linoleic acid to wheat straw and wood chips on lignin degradation by fungi and subsequent in vitro rumen degradation. Animal Feed Science and Technology. 2016;213:22–8.
34. Kišidayová S, Pristaš P, Zimovčáková M, Blanár Wencelová M, Homol'ová L, Mihaliková K, et al. The effects of high dose of two manganese supplements (organic and inorganic) on the rumen microbial ecosystem. PLoS One. 2018;13:e0191158.
35. Weimer PJ. Manipulating ruminal fermentation: a microbial ecological perspective. Journal of Animal Science. 1998;76:3114–22.
36. Maskaľová I, Vajda V, Bujnak L. 2,6-Diaminopimelic acid as a biological marker of rumen synthesis and fermentation capacities in the transition period and early lactation of dairy cows. Acta Veterinaria Brno. 2014;83:355–61.
37. Reis L, Pardo P, Camargos A, Oba E. Mineral element and heavy metal poisoning in animals. African Journal of Medicine and Medical Sciences. 2010;1.
38. PubChem. Manganese(II) oxide [Internet]. [cited 2023 Feb 26]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/14940
39. PubChem. Manganese sulfate monohydrate [Internet]. [cited 2023 Feb 26]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/177577
40. Guimaraes O, Wagner JJ, Spears JW, Brandao VLN, Engle TE. Trace mineral source influences digestion, ruminal fermentation, and ruminal copper, zinc, and manganese distribution in steers fed a diet suitable for lactating dairy cows. Animal. 2022;16:100500.
41. Jongbloed AW, Kemme PA, De Grotte G, Lippens M, Meschy F. Bioavailability of major trace minerals. Brussels, Belgium: EMFEMA, International Association of the European Manufacturers of Major Trace and Specific Feed Mineral Materials; 2002.
42. Bielik V, Kolisek M. Bioaccessibility and Bioavailability of Minerals in Relation to a Healthy Gut Microbiome. Int J Mol Sci. 2021;22:6803.
43. RIAZI H, REZAEI J, ROUZBEHAN Y. Effects of supplementary nano-ZnO on in vitro ruminal fermentation, methane release, antioxidants, and microbial biomass. Turkish Journal of Veterinary & Animal Sciences. 2019;43:737–46.
44. Petrič D, Mravčáková D, Kucková K, Kišidayová S, Cieslak A, Szumacher-Strabel M, et al. Impact of Zinc and/or Herbal Mixture on Ruminal Fermentation, Microbiota, and Histopathology in Lambs. Front Vet Sci. 2021;8:630971.
45. Wang C, Xu YZ, Han L, Liu Q, Guo G, Huo WJ, et al. Effects of zinc sulfate and coated zinc sulfate on lactation performance, nutrient digestion and rumen fermentation in Holstein dairy cows. Livestock Science. 2021;251:104673.
46. Fellner V, Durosoy S, Kromm V, Spears JW. Effects of supplemental zinc on ruminal fermentation in continuous cultures**Use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service or criticism of similar products not mentioned. Applied Animal Science. 2021;37:27–32.
47. PubChem. Zinc Oxide [Internet]. [cited 2023 Feb 26]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/14806
48. PubChem. Zinc Sulfate [Internet]. [cited 2023 Feb 26]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/24424

49. Hattori R, Torii S, Funaba M, Matsui T. Determination of true absorption and fecal endogenous loss of zinc in goats. Animal Science Journal. John Wiley & Sons, Ltd; 2010;81:564–8.
50. Wilk M, Pecka-Kiełb E, Pastuszak J, Asghar MU, Mól L. Effects of Copper Sulfate and Encapsulated Copper Addition on In Vitro Rumen Fermentation and Methane Production. Agriculture. Multidisciplinary Digital Publishing Institute; 2022;12:1943.
51. Zhang W, Wang R, Zhu X, Kleemann DO, Yue C, Jia Z. Effects of Dietary Copper on Ruminal Fermentation, Nutrient Digestibility and Fibre Characteristics in Cashmere Goats. Asian-Australasian Journal of Animal Sciences. Asian-Australasian Association of Animal Production Societies; 2007;20:1843–8.
52. Engle TE, Spears JW. Dietary copper effects on lipid metabolism, performance, and ruminal fermentation in finishing steers. Journal of Animal Science. 2000;78:2452–8.
53. Jr K, Lenártová V, Holovská K, Sobeková A, Javorský P. Heavy metals and the oxidative stress in some rumen bacteria. Biologia - Section Cellular and Molecular Biology. 2005;60:649–53.
54. Clarkson AH, Paine SW, Kendall NR. Evaluation of the solubility of a range of copper sources and the effects of iron & sulphur on copper solubility under rumen simulated conditions. J Trace Elem Med Biol. 2021;68:126815.
55. Spears JW, Brandao VLN, Heldt J. Invited Review: Assessing trace mineral status in ruminants, and factors that affect measurements of trace mineral status. Applied Animal Science. 2022;38:252–67.