Review

Ruminant Trace Minerals Deficiency and the Potential Link Between the Source and Rumen Interaction. A Review

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Abstract: Dietary inclusion of trace minerals (TM), such as copper (Cu), manganese (Mn) and zinc (Zn) is of importance to cover the ever-evolving requirements for growth, production, and reproduction in ruminants. Various sources of TM are commercially available, such as inorganic (ITM), organic (OTM) or hydroxy (HTM) forms, however their bioavailability and efficiency to improve ruminant zootechnical parameters may be highly influenced by ruminal solubility and effects on the rumen environment. The objective of this review was to compile the most up-to-date information on the ruminal solubility of ITM, OTM and HTM, and their effects on fermentation parameters and rumen microbiota, aiming to support specialists from the animal feed industry when choosing TM products for ruminant supplementation. Some commonly used ITM sources, like sulfates, have a high ruminal solubility, while oxides are less soluble. The ruminal solubility of OTM is mostly found to be high, however data on these TM forms are still lacking. Regarding HTM, ruminal solubility is reported to be low, nevertheless results are inconsistent. Considering rumen fermentation, ITM show a negative effect, OTM might improve, while HTM do not affect parameters like dry matter degradability, volatile fatty acid production, pH, or microbial protein synthesis. As to rumen microbiota, ITM do not affect microbial populations; OTM could decrease abundance of some specific bacteria, like fibrolytic microorganisms, while studies with HTM are missing or inconclusive. Further research is necessary to better understand the ruminal solubility kinetics of TM sources and the different interactions with fermentation parameters and rumen microbiota to successfully apply precision TM supplementation of ruminants, tackling deficiency occurrences.

Keywords: ruminant; trace minerals; solubility; fermentation

1. Introduction

For most species the absorption site of trace minerals (TM) is located in the small intestine [1,2]. However, in ruminants and especially in the rumen, some interactions between microorganisms, minerals and other substances within the diet can occur resulting in reduced final mineral intestinal absorption [1]. These interactions could be influenced by factors such as solubility, hence the objective of this review was to compile an up-to-date reference on ruminal solubility of various TM sources currently used for supplementing ruminants and their effects on rumen fermentation parameters and microbial populations. The nutritional feeding systems for ruminants focus mainly on the global animal requirements [3,4], indicating dietary optimum levels of 10, 50 and 50 mg/kg DM, and regulatory maximum limits of 35, 150 and 120 mg/kg DM for Cu, Mn and Zn, respectively [5,6]. These TM are essential in animal feed, given their important physiological functions, such as participation in keratin, collagen and elastin synthesis; components or activators of enzymes; in addition to an important role for reproductive and immune systems [7–9]. In addition to the contribution to these metabolic functions, TM can also affect the ruminal environment and rumen function, given that rumen microorganisms require minerals for their growth (microbial protein synthesis) and fermentative activity [10–12]. Intestinal bioavailability of the dietary TM for ruminants is relatively quite low, reported levels are at 4-5%, 1-4% and 15-30% for Cu, Mn and Zn, respectively [3,13,14], while selecting the most optimal mineral source for supplementation is quite difficult. Furthermore, ruminal microbial uptake levels are not known. Insights on the ruminal solubility, microbial

 doi:10.20944/preprints202306.1246.v1

uptake, and effects on rumen environment of the various existing TM sources could support specialists from the animal feed industry when choosing TM products for dietary inclusion.

2. Trace mineral deficiency in ruminants

Trace mineral deficiency can often occur in ruminants in a marginal form (without any clinical signs)[15], especially in grazing and high producing animals [16]. One of the most widely spread TM deficiency is with Cu [17]. In a study assessing Cu deficiency in grazing sheep [18], it was found that serum and liver Cu content were not in the normal physiological range (7-24 μmol/L and 10-120 mg/kg fresh matter for serum and liver Cu concentration, respectively [19]), even though the Cu content of the grazed herbage (8.61 and 9.26 mg/kg DM for two analyzed pastures) was within the range to cover nutritional requirements for sheep (5-10 mg/kg DM) [5]. The low Cu absorption was mostly due to the high Mo content (>11 mg/kg DM) of the grass [15,18]. In a study assessing Cu deficiencies in grazing beef cattle herds [20], it was reported that approximately 39% (of a total of 2,007) of the reproduction cows and heifers, and 36% (of a total of 256) of the herds were marginally deficient. As for dairy cows, feeding a diet based on silage, pasture and hay (Cu content ranging from 6.5 to 9 mg/kg DM) without any Cu supplementation for a period of 161 days sharply decreased liver Cu from approximately 61 to 22 mg/kg DM [21].

The Mn content of ruminant diets is considered to supply sufficient quantities to cover the animals' requirements, hence very little research has been carried out on this mineral with ruminants [2]. Considering that the most frequent TM deficiencies in ruminants occur related to Cu and Zn, research is rarely focused on Mn. However, Mn deficiencies can occur with diets containing high proportions of silages (red clover silage, grass silage) due to a lower bioavailability of Mn in silages compared to hay [22].

When it comes to Zn supplementation in ruminants, dietary inclusion levels need to be carefully evaluated given that the percentage of absorbed Zn decreases as dietary Zn increases [14,23]. Severe Zn deficiency in ruminants is rare under normal farming conditions, and it is easily diagnosed (serum Zn concentration < 0.5 mg/L) in comparison to marginal Zn deficiency, for which reliable indicators are lacking, apart from a positive response in production (growth, reproduction, or milk production) [15]. In a study with lactating Holstein dairy cows, a basal diet containing 31.2 mg/kg DM of Zn was supplemented with increasing levels (10, 20 or 30 mg/kg DM) of inorganic Zn (as coated $ZnSO_4$). Results showed a linear increase of milk production, as well as total milk solids (fat and protein) in the Zn supplemented groups [24]. Supplementing Zn at levels of 40 or 80 mg/kg DM (as $ZnSO_4$ or Zn hydroxychloride) in pre-ruminant crossbred calves (starting age 10 days) significantly improved growth performance (body weight, average daily gain and body length) when compared to non-supplemented animals after a 90 days period [25]. When considering Zn effect on reproduction, in a study with lactating dairy cows results showed that supplementing 80 or 120 mg/kg DM of Zn (as $ZnSO_4$) for 90 days (45 days before and 45 days after calving) significantly improved reproductive efficiency (reduction in postpartum estrus interval, days to first insemination, service period, service per conception and increased conception rate) in both supplemented groups compared to no supplementation [26].

Trace mineral deficiencies affect the reproductive system and fertility in ruminants [27]. Blood concentrations of Cu and Zn were associated with luteal activity in Holstein dairy cows, showing improved reproduction parameters with higher blood TM concentrations [28]. In dairy goats, Cu deficiency mainly affected the reproductive system showing prolonged anestrus, embryonic resorption, high indices of retained placenta, small ovaries without viable follicles; while kids born from Cu-deficient goats were week, presenting neonatal and late ataxia [29].

The assessment of TM status of ruminants is often quite challenging, especially when clinical signs of deficiency are lacking [15]. Furthermore, the TM content of pasture herbage and forages are highly variable and influenced by factors such as region, season, harvest, or farming and feeding practices [30–35]; and have a strong effect on the TM status of ruminants [36–39]. The low level of TM in forages coupled with rumen antagonisms [17,40] can result in an epidemiology of TM deficiencies in ruminants [41]. Reducing TM deficiencies (especially marginal deficiencies) in ruminants can be limited trough TM supplementation of the cropping lands (ex. manure application) [42], or by dietary inclusion of TM in ruminant diets [41]. Given that the TM requirements in ruminants are not static [5,13], evolving with the selection for more feed-efficient and higher producing animals [16], TM physiological status could be affected by the ruminal solubility and bioavailability of different supplemental sources, like inorganic, organic or hydroxy TM. Considering this, choosing the optimal TM sources for ruminant supplementation can be quite challenging: a more rumen-soluble TM might have a higher bioavailability, however the occurrence of complexation with other minerals could also increase, making it less bioavailable. The optimal TM sources would be characterized by a high ruminal

 doi:10.20944/preprints202306.1246.v1

solubility coupled with a low reactivity with other elements in the rumen environment and the lack of negative effects on rumen fermentation parameters.

3. Available trace mineral forms for dietary inclusion

Trace mineral sources for supplementing ruminants are numerous and include inorganic (ITM), organic (OTM) or hydroxy (HTM) mineral forms [2,6,43,44]. The ITM salts such as carbonates, chlorides, oxides and sulfates are characterized as a specific metal (Cu, Mn, Zn) bound to a non-carbon-containing ligand [45–49], and are widely available at a low cost. These sources are traditionally used for livestock supplementation [50]. The OTM such as glycinates, amino acid-complexes, amino acid chelates or different proteinates are formed through specific processes, binding the metal component (Cu, Mn, Zn) to a carbon-containing ligand [51]. The HTM are defined as a specific metal (Cu, Mn, Zn) bound via a coordinated covalent bound with a hydroxyl ligand and are considered inorganic [14]. However, the covalent bound to an OH group instead of carbon containing ligands, make HTM seem like OTM [52]. In ruminant feed HTM (Cu, Mn and Zn) are provided in the form of copper hydroxy chloride (Cu-Hyd) [53], manganese hydroxy chloride (Mn-Hyd) [54] and zinc hydroxy chloride (Zn-Hyd) [55]. Other TM forms and sources are studied for ruminant supplementation, including nano minerals [56], or even different seaweeds [57,58], however little research is available on these last forms considering rumen solubility.

4. Trace mineral rumen solubility and effects on the ruminal environment

There are numerous studies addressing the overall effects of TM in ruminants, however still little is known about their ruminal solubility and effects on the rumen microbial populations. Ruminal solubility is to be considered when choosing a TM sources for dietary inclusion, given that it was identified as one of the factors closely related to TM relative bioavailability in ruminants [2,59]. Solubility of ITM in rumen fluid was found to be in close relation with their mineral presentation form: sulfates are considered highly soluble compared to oxides [60]. Even though it was demonstrated that OTM like glycinates, amino acid-complexes, amino acid chelates or different proteinates have a high stability in the 6.0 – 7.0 pH range (similar to the one found in the rumen environment [61]), showing minimum effect of enzymatic hydrolysis [51], solubility of OTM in rumen fluid, exposed to bacterial fermentative activity, could be significantly affected [60]. When considering ruminal solubility of HTM, they appear to be relatively insoluble, however results are not equivocal for the hydroxy form of Cu, Mn and Zn [62]. In the following, an overview of different solubility evaluation methods as well as the ruminal solubility of various sources (inorganic, organic and hydroxy) of Cu, Mn and Zn are presented while also considering effects on rumen fermentation parameters and microbial population changes.

4.1. Ruminal solubility evaluation methods

For an overview of TM solubility, the assessment can be performed through *in vitro* models with deionized water as a solvent [63]. However, even though TM solubilization in deionized water may be a good indicator of overall solubility of a specific TM, the ruminal solubility of TM may be affected by the rumen environment, and it was shown to be significantly lower when compared to solubility in deionized water [60]. Considering this, when assessing ruminal solubility of TM via *in vitro* studies, for a more factual representation of the various interactions in the ruminal environment, rumen fluid-based models are to be privileged. Key aspects, like donor animals, rumen fluid processing as inoculum, incubation substrate and buffer choice for *in vitro* fermentation techniques are well established for the assessment of rumen function (fermentation activity, gas production, nutrient degradation) [64], however for TM ruminal solubility, the techniques are not yet harmonized. One of the applied methods for the assessment of ruminal solubility of TM following *in vitro* fermentations (of 24, 48 hours or continuous fermentations) is the analysis of TM concentration in a centrifugation supernatant [65,66]. Based on this method, the final fermentation medium (mix of rumen fluid, buffer, substrate and various TM) is centrifuged (12,000 – 18,000 x *g* at 23°C for 15 minutes) to separate the particulate matter (feed particles, protozoa, bacteria and insolubilized TM) obtaining a supernatant (containing the solubilized minerals), which is analyzed for TM concentration. Next, the ruminal solubility of a specific TM may be expressed as an absolute value (based on the TM concentration of the supernatant) or a relative value (related to a sulfate TM, considered as 100 % rumen soluble). In recent studies [67,68], the ruminal solubility of various minerals was assessed based on a separation of the final fermentation medium (after 70 hours of fermentation) by multiple centrifugations: at 100 *x g* (5 minutes

 doi:10.20944/preprints202306.1246.v1

at 4^{0}C), to separate an insoluble fraction (containing feed particles, protozoa and insolubilized minerals); the obtained supernatant is then further centrifuged at 18,500 *x* g (20 minutes at 4^{o}C) to separate a bacteria enriched fraction and a final supernatant, containing only solubilized minerals; the mineral concentration of each centrifugation fraction is then analyzed. Next, the ruminal solubility of TM can be expressed as a percentage of solubilized mineral in the final supernatant (based on the total mineral analyzed in the different centrifugation fractions.

The ruminal solubility of TM can also be determined using *in vivo* models. In a study by Arelovich et al. [69], the ruminal fluid (100 mL) sampled from rumen cannulated heifers (supplemented with different levels of TM), was first filtered with a cheesecloth, acidified (addition of 2 mL of 20% sulfuric acid solution) and centrifuged (16,000 *x g*) to obtain a supernatant (containing the solubilized minerals). Ruminal solubility of TM was expressed in absolute values based on the TM concentration of the supernatant. In other *in vivo* studies [62,70], the ruminal solubility of different TM sources was assessed following a supplementation with different levels of TM and analysis of the mineral concentration of the rumen content. Samples of rumen fluid were separated by ultracentrifugation (28,000 *x g* for 30 minutes at 4^{o}C) in solids pellets (containing the insolubilized minerals) and a supernatant, considered to contain the rumen soluble minerals. The ruminal solubility of TM was expressed as an absolute value (based on the mineral concentration of the supernatant), or a relative value (as a percentage of whole ruminal mineral, calculated based on the total amount of rumen content samples and mineral concentration of the centrifugation fractions).

4.2. Copper ruminal solubility

One of the most commonly used inorganic Cu source for ruminant supplementation is $CuSO_4$ [14], often used as a comparison basis for rumen solubility with other Cu sources. In a study by Deters et al. [63], the solubility of $CuSO_4$ and glycinate bound Cu (Cu-Gly) in deionized water (at 5.2 pH) was 100% and 68.9%, respectively. In a similar study by Clarkson et al. [60], the solubility of a range of Cu sources ($CuSO_4$, $CuCl_2$, CuO, $CuCO_3$, Cu-Hyd, Cu EDTA, Cu proteinate and Cu acetate) was estimated in either deionized water or rumen fluid. The results showed that solubility across all Cu sources was lower in rumen fluid than in deionized water (mean relative solubility across all Cu sources was 33% and 64% in rumen fluid and deionized water, respectively). These findings suggest that the rumen environment has a significant effect on the solubility of different TM sources. Table 1 summarizes the available literature data on ruminal solubility of various Cu sources.

Table 1. Ruminal solubility of different sources of copper

Experimental model	Cu dosage	Cu source	Solubilization time / supplementation period	Ruminal solubility*	Ref.
in vivo	5 and 25 mg/kg DM	$CuSO_4$ Cu-Hyd	12 days	↑; ~ 17 and 14 % ↓; ~ 15 and 9 %	[62]
in vivo	20 mg/kg DM	$CuSO_4$ Cu-Hyd	24 hours	↑; ~ 68, 30 and 12% after 4, 12 and 24 hours following ruminal administration ↓; ~ 15, 20 and 15% after 4, 12 and 24 hours following ruminal administration	[70]
in vitro	4, 12, 96 mg/kg DM (with 0.1% DM of S or 2% DM of urea)	Cu-Lys $CuSO_4$	24 hours	↑ (with urea) ↓ (with S) ↓ (with S)	[59]
in vitro	4 mM solution	$CuSO_4$ $CuCl_2$ CuO	25 hours	↑; ~ 49% ↑; ~ 49% ↓; ~ 9%	[60]

 doi:10.20944/preprints202306.1246.v1

		$CuCO_3$		↓; ~ 19%	
		Cu-Hyd		↓; ~ 17%	
		Cu EDTA		↑; ~ 49%	
		Cu-Prot		↑; ~ 49%	
		Cu-Acet		↓; ~ 33%	
in vitro	100 and 500 mg/kg DM	$CuSO_4$	22, 46 and 70 hours	↑; ~ 42, 37 and 57 %	[67]
in vitro	12 mg/kg fresh matter	$CuSO_4$ Enc. Cu	24 hours	↑ ↓	[66]

Cu: copper; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; Cu-Lys: copper lysine; $CuSO_4$: copper sulfate; $CuCl_2$: copper chloride; CuO: copper oxide; $CuCO_3$sulfate. carbonate; Cu-Hyd: copper hydroxychloride; Cu-EDTA: copper disodium edetate; Cu-Prot: copper proteinate; Cu-Acet: copper acetate; Cu-Gly: copper glycinate; Lip.Enc-$CuSO_4$: lipid encapsulated copper sulfate; Enc. Cu: mix of tribasic copper chloride and copper sulfate within a polysaccharide polymer coating; *Ruminal solubility: where available, values of relative solubility were given.

In an *in vivo* study [70], assessing the ruminal solubility of two sources of Cu (as $CuSO_4$ and Cu-Hyd, respectively) it was found that the Cu concentration of the rumen fluid supernatant was higher with $CuSO_4$ when compared to Cu-Hyd (approximately 0.65 and 0.20 mg/L for $CuSO_4$ and Cu-Hyd, respectively), concluding that $CuSO_4$ has a high, while Cu-Hyd has a low ruminal solubility. In a similar study by Genther and Hansen [62], at an inclusion level of 25 mg/kg DM of Cu, the ruminal solubility of $CuSO_4$ was higher compared to Cu-Hyd (approximately 14 and 9 %, respectively). These results indicate well the rumen solubility of the two Cu sources, however the solid fraction, beside undegraded feed fractions and insoluble minerals, also contains rumen microorganisms (protozoa, bacteria), which could assimilate solubilized Cu. In an *in vitro* study, the solubility of $CuSO_4$ in rumen fluid was found to be high, given that >50% of the total additional Cu was found in a final supernatant obtained after centrifugation of the final fermentation medium. Furthermore, approximately 18 to 27% of the supplemented Cu was analyzed in a bacteria enriched fraction, indicating that Cu might be assimilated by rumen bacteria [67].

Given the rumen antagonism of Cu with other minerals (mainly sulfur and molybdenum)[17,60], highly rumen soluble Cu sources (like $CuSO_4$) often present a decreased bioavailability for ruminants, in relation with antagonist minerals present in the rumen content [43]. In order to limit the effect of the rumen environment on mineral additives, various methods, like lipid encapsulation or polymer coating were developed [71]. When comparing the rumen solubility of $CuSO_4$ and an encapsulated mixture of different Cu sources (Enc. Cu; mix of tribasic copper chloride and $CuSO_4$ with a polysaccharide polymer coating), Wilk et al. [66] found that Enc. Cu had a lower solubility when compared to $CuSO_4$ (rumen fluid supernatant Cu concentration was 0.38 and 0.70 mg/kg for Enc. Cu and $CuSO_4$, respectively).

4.3. Manganese ruminal solubility

Given that Mn is poorly absorbed by ruminants [72,73], and that it is considered that Mn content of the basal diet might cover the rumen microbial requirements [74], little research is focused on ruminal solubility of different Mn sources. In a study by Caldera et al. [70], the ruminal solubility of two Mn sources ($MnSO_4$ and Mn-Hyd) was compared by administrating a pulse dose of 40 mg/kg DM of Mn to rumen canulated steers consuming a basal diet with a Mn content of 19.2 mg/kg DM. The ruminal solubility of the two Mn sources was assessed based on the Mn concentration of the rumen fluid supernatant (considered to contain the soluble Mn) and solid fraction (containing the insoluble Mn). After only 4 hours from the pulse dose administration, the mineral analysis of the two fractions showed a Mn content of approximately 0.7 mg/L and 9.0 mg/kg DM with the $MnSO_4$; and 0.5 mg/L and 14.0 mg/kg DM with the Mn-Hyd in the supernatant and solid fraction, respectively. However, after 24 hours, the Mn content of the supernatant was approximately 0.3 and 0.5 mg/L, while of the solid fraction approximately 14.0 and 9.0 mg/kgDM with $MnSO_4$ and Mn-Hyd, respectively. Furthermore, the reported relative solubility after 4 hours was about 20 and 10 %; and after 24 hours about 6 and 15 % for $MnSO_4$ and Mn-Hyd, respectively. These findings indicate that both Mn sources ($MnSO_4$ and Mn-Hyd) are quite soluble in the rumen. In a similar study, supplementing rumen cannulated steers with 60 mg/kg DM of

 doi:10.20944/preprints202306.1246.v1

Mn (as $MnSO_4$ or Mn-Hyd), the ruminal solubility of $MnSO_4$ showed no difference when compared to Mn-Hyd (relative solubility of approximately 65 and 66 % for $MnSO_4$ and Mn-Hyd, respectively) [62]. In an *in vitro* study, the ruminal solubility of two inorganic Mn sources ($MnSO_4$ and MnO) was assessed [67]. Results showed that $MnSO_4$ and MnO are equally soluble in the rumen fluid after 70 hours of fermentation. However, when solubility was assessed at a shorter period (22 hours), $MnSO_4$ showed a higher solubility when compared to MnO. The ruminal solubility of various Mn sources is summarized in Table 2.

Table 2. Ruminal solubility of different sources of manganese

Experimental model	Mn dosage	Mn source	Solubilization time / supplementation period	Ruminal solubility*	Ref.
in vivo	40 mg/kg DM	$MnSO_4$	24 hours	↑; ~ 20, 12 and 6 % after 4, 12 and 24 hours following ruminal administration	[70]
		Mn-Hyd		↓; ~ 10, 7 and 15 % after 4, 12 and 24 hours following ruminal administration	
in vivo	40 mg/kg DM	$MnCl_2$	16 days	↓	[69]
in vivo	15 and 60 mg/kg DM	$MnSO_4$ Mn-Hyd	12 days	↑; ~ 62 and 65 % ↓; ~ 50 and 66 %	[62]
in vitro	600 mg/kg DM	$MnSO_4$ MnO	22, 46 and 70 hours	↑; ~ 94, 91 and 93 % ↑; ~ 84, 88 and 93 %	[67]

Mn: Manganese; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; $MnSO_4$: manganese sulfate; Mn-Hyd: manganese hydroxychloride; $MnCl_2$: manganese chloride; MnO: manganese oxide; *Ruminal solubility: where available, values of relative solubility were given.

4.4. Zinc ruminal solubility

In a recent *in vitro* study [67], the ruminal solubility of $ZnSO_4$ was significantly higher when compared to ZnO (32 and 25 %; $p < 0.05$). Furthermore, of the total analyzed Zn (sum of total Zn in different fraction of the centrifuged rumen fluid), 27 to 38% and 19 to 24% for $ZnSO_4$ and ZnO, respectively, was found in the bacteria enriched fraction, indicating not only that Zn might be assimilated by rumen bacteria, but also a higher ruminal bioavailability of $ZnSO_4$ when compared to ZnO. In a similar study, Fellner et al. [65] analyzed the ruminal solubility of ZnO and HiZnox (a greater purity potentiated ZnO) using continuous *in vitro* fermentations. The results showed a lower solubility of ZnO when compared to HiZnox, based on the Zn concentration of the rumen fluid supernatant (0.4 and 0.5 mg/kg for ZnO and HiZnox, respectively).

The ruminal solubility of some Zn sources was also assessed using *in vivo* models. Following a supplementation with 60 mg/kg DM of Zn (as $ZnSO_4$ or Zn-Hyd) to rumen canulated steers, the ruminal solubility after 24 hours was 15 and 10 % for $ZnSO_4$ and Zn-Hyd, respectively [70]. In a similar study by Genther and Hansen [62], a lower ruminal solubility was reported for $ZnSO_4$ when compared to Zn-Hyd (7 and 11 %, respectively) when animals were supplemented with 120 mg/kg DM of Zn. Another study on ruminal solubility of TM reported a high ruminal solubility when supplementing steers with 30, 250 and 470 mg/kg DM of Zn as $ZnCl_2$ [69]. The solubility of Zn might also be affected by clay minerals ingested with different feeds, as shown in an *in vitro* study by Schlattl et al. [75]: the addition of a clay mixture (90% bentonite and 10% kaolinite) reduced Zn (as nitrous Zn) solubility by 42, 49 and 52% under ruminal (pH 7.02), abomasal (pH 2.00) and duodenal (pH 3.58) conditions. Table 3 summarizes the ruminal solubility of various Zn sources.

Table 3. Ruminal solubility of different sources of zinc

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Experimental model	Zn dosage	Zn source	Solubilization time / supplementation period	Ruminal solubility*	Ref.
in vivo	60 mg/kg DM	$ZnSO_4$ Zn-Hyd	24 hours	↑; ~ 39, 15 and 15 % after 4, 12 and 24 hours following ruminal administration ↓; ~ 10, 5 and 10 % after 4, 12 and 24 hours following ruminal administration	[70]
in vivo	30, 250 and 470 mg/kg DM	$ZnCl_2$	16 days	↑; linear increase of Zn concentration in the rumen fluid (2.26, 7.6 and 11.6 mg/L)	[69]
in vivo	30 and 120 mg/kg DM	$ZnSO_4$ Zn-Hyd	12 days	↓; ~ 12 and 7 % ↑; ~ 14 and 11 %	[62]
in vitro		Nitrous-Zn		↓ (with clay)	[75]
in vitro	500 mg/kg DM	$ZnSO_4$ ZnO	22, 46 and 70 hours	↑; ~ 19, 17 and 32 % ↓; ~ 10, 9 and 25 %	[67]
in vitro	30 and 120 mg/kg DM	ZnO HiZox	8 days continuous culture fermentation	↓ ↑	[65]

Zn: Zinc; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; Zn-Gly: zinc glycinate; Zn-AA: zinc amino acid complex; $ZnSO_4$: zinc sulfate; Zn-Hyd: zinc hydroxychloride; $ZnCl_2$: zinc chloride; ZnO: zinc oxide; HiZox: high purity potentiated zinc oxide; Coat-$ZnSO_4$: coated zinc sulfate; *Ruminal solubility: where available, values of relative solubility were given.

4.5. Trace mineral effects on rumen fermentation parameters

When considering rumen fermentation, Cu was identified as a TM with complex responses. In an *in vivo* study with rumen cannulated steers [70], supplementing Cu at levels of 10 mg/kg DM (as $CuSO_4$ or Cu-Hyd) did not affect apparent dry matter (DM) digestibility, while apparent neutral detergent fiber (NDF) digestibility tended ($p < 0.10$) to be lower with $CuSO_4$ when compared to Cu-Hyd (37.8 and 41.2 %, respectively). In a similar study [62], supplementing a high dosage of Cu (25 mg/kg DM) as $CuSO_4$ decreased, while supplementing with Cu-Hyd did not affect rumen DM disappearance when compared with a control (CON, no Cu supplementation) diet ($p < 0.03$; 63.5, 64.4 and 65.6 % for $CuSO_4$, Cu-Hyd and CON, respectively). In this study, the two Cu sources did not affect NDF degradation. In an *in* vitro study by Wilk et al. [66], it was found that Cu sources (Enc. Cu and $CuSO_4$, respectively) did not affect DM degradability, while $CuSO_4$ increased *in vitro* gas production, propionate concentration and decreased acetate to propionate ratio when compared to Enc. Cu. The two Cu sources did not affect *in vitro* methanogenesis. In a similar study [76], lipid encapsulated $CuSO_4$ decreased acetate, increased butyrate molar proportion, and tended to decrease acetate to propionate proportion, but did not affect nutrient (DM, organic matter, crude protein and NDF) digestibility when compared to $CuSO_4$. Table 4 presents a compilation of various Cu sources effects on rumen fermentation parameters.

Table 4. Effects of different sources of Cu on rumen fermentation

Cu source	GP	CH_4	VFA	A:P	pH	DMd	NH_3-N	MPS	Ref.
Cu-Lys	ND	ND	ND	ND	ND	-	ND	ND	[59]
$CuSO_4$	ND	ND	ND	ND	ND	-	ND	ND	
Cu-Gly	ND	=	=	=	=	ND	+	ND	[77]
Lip.Enc. $CuSO_4$	ND	ND	- +	-	=	=	=	=	[76]
$CuSO_4$	ND	ND	ND	ND	=	=	ND	ND	[70]

 doi:10.20944/preprints202306.1246.v1

Cu-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$CuSO_4$	ND	ND	ND	ND	=	-	ND	ND	[62]
Cu-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$CuSO_4$	=	ND	=	=	=	=	ND	=	[67]
$CuSO_4$	+	=	=	-	=	=	ND	ND	[66]
Enc. Cu	=	=	=	=	=	=	ND	ND	
$CuSO_4$	ND	ND	+	+	-	+	-	ND	[78]
Coat. $CuSO_4$	ND	ND	+	+	-	+	-	ND	

Cu: copper; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; Cu-Lys: copper lysine; $CuSO_4$: copper sulfate; $CuCl_2$: copper chloride; CuO: copper oxide; $CuCO_3$sulfate. carbonate; Cu-Hyd: copper hydroxychloride; Cu-EDTA: copper disodium edetate; Cu-Prot: copper proteinate; Cu-Acet: copper acetate; Cu-Gly: copper glycinate; Lip.Enc-$CuSO_4$: lipid encapsulated copper sulfate; Enc. Cu: mix of tribasic copper chloride and copper sulfate within a polysaccharide polymer coating; Coat. $CuSO_4$: coated (with hydrogenated fat) copper sulfate.

When it comes to Mn effect on rumen fermentation, results of *in vivo* studies assessing the effects of supplementing different sources of Mn to rumen cannulated steers are various. A significant decrease of DM disappearance was shown with $MnSO_4$, while Mn-Hyd did not affect DM disappearance (supplementation levels of 15 or 60 mg/kg DM of Mn); the two sources of Mn did not affect NDF degradability [62]. In another study on the effects of Mn supplementation (40 mg/kg DM as $MnSO_4$ or Mn-Hyd), the DM digestibility was not affected by the treatments, while NDF digestibility tended to be lower with $MnSO_4$ when compared to Mn-Hyd [70]. Varied results are equally reported when analyzing Mn effect on fermentation during i*n vitro* studies with rumen fluid. In a study by Areolovich et al. [69], the addition of 100 mg/DM of Mn (as $MnCl_2$) significantly increased DM degradation, without affecting volatile fatty acids (VFA) concentration. In a more recent study [67], an overall negative effect of Mn (600 mg/kg DM as MnO or $MnSO_4$) was registered: decrease of total gas production, DM degradability and butyrate (% of total VFA) concentration. Table 5 presents the effects on rumen fermentation of some Mn sources.

Table 5. Effects of different sources of Mn on rumen fermentation

Mn source	GP	CH_4	VFA	A:P	pH	DMd	NH_3-N	MPS	Ref.
$MnSO_4$	ND	ND	ND	ND	=	=	ND	ND	[70]
Mn-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$MnCl_2$	ND	ND	=	=	=	+	=	ND	[69]
$MnSO_4$	ND	ND	ND	ND	=	-	ND	ND	[62]
Mn-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$MnSO_4$	-	ND	=	=	=	-	ND	=	[67]
MnO	-	ND	=	=	=	-	ND	=	

Mn: manganese; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; $MnSO_4$: manganese sulfate; Mn-Hyd: manganese hydroxychloride; $MnCl_2$: manganese chloride; MnO: manganese oxide.

Regarding Zn effect on rumen fermentation, results of early *in vitro* studies have shown that a high dosage of Zn (as $ZnSO_4$) has a strong negative effect [79]. In more recent *in vitro* studies, the negative effects of Zn on rumen fermentation vary according to different sources of Zn. In a study by Fellner et al. [65], it was shown that an addition of 30 or 120 mg/kg DM of Zn as ZnO or HiZnox (a greater purity potentiated ZnO) affect differently the fermentation parameters: HiZnox reduced apparent DM disappearance, increased acetate to propionate ratio; ZnO reduced acetate, NH_3-N and CH_4 concentration, while both Zn sources increased culture pH. In another *in vitro* study [67], the total gas production and DM degradability was significantly reduced by ZnO, while $ZnSO_4$ did not affect the fermentation parameters. Both inorganic Zn sources decreased the microbial protein synthesis. In a study assessing the effect of an organic source

 doi:10.20944/preprints202306.1246.v1

of Zn on rumen fermentation, it was found that the addition of 25 mg/kg DM of Zn as zinc proteinate (Zn-Prot) increased DM and organic matter digestibility, but did not affect acetate to propionate ration, nor CH_4 concentration [80]. Table 6 compiles the effects on fermentation parameters of various inorganic and organic Zn sources.

Table 6. Effects of different sources of Zn on rumen fermentation

Zn source	GP	CH_4	VFA	A:P	pH	DMd	NH_3-N	MPS	Ref.
Zn-Gly	ND	=	=	=	=	ND	+	ND	[77]
$ZnSO_4$	ND	ND	ND	ND	=	=	ND	ND	[70]
Zn-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$ZnCl_2$	ND	ND	-	-	- +	-	-	ND	[69]
$ZnSO_4$	ND	ND	ND	ND	=	-	ND	ND	[62]
Zn-Hyd	ND	ND	ND	ND	=	=	ND	ND	
$ZnSO_4$	-	ND	=	=	=	=	ND	-	[67]
ZnO	-	ND	=	=	=	-	ND	-	
ZnO	ND	-	=	-	+	=	-	ND	[65]
HiZox	ND	=	=	+	+	-	=	ND	
Zn-Prot	ND	=	=	=	ND	+	ND	ND	[80]
Zn-AA	-	-	=	ND	=	=	=	ND	[81]
$ZnSO_4$	ND	ND	+	+	=	+	-	ND	[24]
Coat. $ZnSO_4$	ND	ND	+	+	=	+	-	ND	

Zn: zinc; GP: Gas production; VFA: Volatile fatty acids; A:P: Acetate to propionate ration; DMd: Dry matter degradability; MPS: Microbial protein synthesis; ↑: high; ↓: low; +: increase; -: decrease; =: no effect; ND: not determined; Zn-Gly: zinc glycinate; Zn-AA: zinc amino acid complex; $ZnSO_4$: zinc sulfate; Zn-Hyd: zinc hydroxychloride; $ZnCl_2$: zinc chloride; ZnO: zinc oxide; HiZox: high purity potentiated zinc oxide; Coat-$ZnSO_4$: coated (with hydrogenated fat) zinc sulfate.

4..6. Trace mineral effects on rumen microbiota

Studies addressing TM effects on rumen microbial community composition are still scarce, and the reported results do not always agree. Several studies with different Zn sources show that Zn can affect differently the rumen bacterial community. As shown by Ishaq et al. [82], Zn amino acid complex (Zn-AA) reduces rumen bacterial diversity, while $ZnSO_4$ has no significant effect. Consistent with these results, total rumen bacterial populations decreased in lambs supplemented with 70 mg/kg DM of Zn (as Zn-AA) [81]. Furthermore, significant shifts in the relative abundance of fibrolytic bacteria populations, such as an increase of *Ruminococcus albus* and a decrease of *Ruminococcus flavefaciens*; or an increase of lactic acid producing bacteria (*Streptococcus bovis*) were noted; while the methanogenic *Archaea* abundance was not affected by Zn-AA supplementation [81]. In another study, supplementation of lactating Holstein dairy cows with increasing levels of Zn (10, 20 or 30 mg/kg DM as coated $ZnSO_4$) significantly increased total bacteria and the main fibrolytic rumen microbial populations of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* [24].

Regarding Cu effect on rumen microbiota, supplementing a basal lactating dairy cows' diet (Cu content of 8.51 mg/kg DM) with 7.5 mg/kg DM of inorganic Cu (as $CuSO_4$ or coated $CuSO_4$) significantly increased total bacteria populations and specific fibrolytic bacteria, such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens*, as well as some microbial enzyme activity (cellobiase, xylanase and pectinase), but decreased the populations of *Prevotella ruminicola*, *Ruminobacter amylophilus* and α-amylase activity [78].

The effects of inorganic and organic Mn on the rumen microbial ecosystem were observed in a study with ewe lambs [83]. The animals were fed a basal diet with a Mn level of 34.3 mg/kg DM and supplemented during 16 weeks with 182.7 and 184 mg/kg DM of Mn as inorganic ($MnSO_4$) and organic (glycinate chelate) Mn, respectively. Inorganic Mn did not affect, while organic Mn decreased the variability (lower Shannon-Wiener diversity index) of eubacterial populations. Regarding enzymatic activity, α-amylase activity decreased with $MnSO_4$, while carboxymethyl-cellulase activity increased with both Mn sources.

When comparing different levels of TM dietary inclusion, no noticeable alterations were observed in α and β-diversity of ruminal microbiota in beef heifers (virgin vs. pregnant) fed a control (no TM supplementation) diet (total

intake of 13.7, 103.9 and 130.2 for Cu, Mn and Zn, respectively) or a high TM supplemented diet (total intake of 285.8, 953.4 and 1,051.8 mg/kg DM of Cu, Mn and Zn, respectively) [84,85]. Following a supplementation of lactating yaks with inorganic TM via slow release rumen boluses, some variations in the ruminal bacterial communities were observed, however the specific TM responsible for the changes was not clearly identified [86]. In a recent study with heat-stressed dairy steer [77], supplementing high levels of organic TM (28 and 350 mg/kg DM of Cu and Zn as Cu- and Zn-glycinate, respectively), no effects were registered on enteric CH_4 production or rumen methanogenic microbial populations [77].

5. Conclusions

Results considering TM effects on rumen fermentation parameters, such as DM and NDF disappearance, VFA production, microbial synthesis are inconsistent, even when supplementation occurs with the same TM forms. These differences in effects on fermentation parameters might be influenced by the experimental design (*in vitro* or *in vivo* models), the supplemental TM dosages as well as the presence of other minerals (antagonists) in the ruminal environment. Furthermore, when selecting a TM source for ruminant supplementation, ruminal solubility should be taken into consideration, as the overall bioavailability of TM could be affected by higher rumen soluble minerals: a higher rumen solubility of a specific TM might improve its bioavailability, but at the same time a greater sensitivity to negative interactions (antagonism) with other minerals could be present, decreasing bioavailability. Based on this hypothesis, the observed TM deficiencies are not linked to dietary intakes, which may be sufficient, but to negative interactions with other minerals, a phenomenon dependent on the ruminal solubility of TM. Some ITM show a negative effect on ruminal fermentation parameters, but do not affect rumen microbiota. On the other hand, OTM might improve fermentative activity, while having negative effects on specific bacteria populations (ex. fibrolytic bacteria). However, there are a low number of studies with OTM and HTM, hence drawing any conclusions remains challenging. Further studies are necessary for the assessment of rumen solubility, bioavailability and effects on rumen fermentation and microbiota of different TM sources. A better understanding of the ITM, OTM and HTM solubility kinetics in the ruminal environment might help better understand and effectively address deficiencies by implementing precision feeding and TM supplementation of ruminant animals.

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

Funding: This research received no external funding.

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

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