(SUMMARY OF THE PhD THESIS)

Ruminal solubility and bioavailability of different sulfur and trace mineral sources and effects on fermentation activity, degradation kinetics and mineral excretion

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(SUMMARY OF THE Ph.D. THESIS)

INTRODUCTION

Ruminants make up a significant proportion of domesticated animal species worldwide, and among the farmed livestock are the best adapted to use and degrade plant cell walls. Thanks to this ability, ruminants are the only livestock species that are not in competition with human food (Ripple et al. 2014; Cheng et al. 2022). The ruminants are able to degrade and use fibrous feed as a source of energy and nutrients thanks to the presence of a complex anaerobic microbiota in the rumen, composed mainly of bacteria, fungi, ciliate protozoa and methanogenic *Archaea* (Henderson et al. 2015). Ruminal microorganisms play different roles in feed digestion and act synergistically to ferment plant structural and nonstructural carbohydrates and proteins (Chaucheyras-Durand and Ossa 2014). Improvements in the ability of the rumen microbiota to degrade plant cell walls are generally highly desirable and usually lead to improved animal performances (Krause et al. 2003).

As with other nutrients, meeting the mineral requirements of the rumen microbiome is essential for a proper feeding of the ruminant: microorganisms require minerals for their growth (microbial protein synthesis) and activity (fibrolytic fermentations) (Martinez 1972). Dietary intake and supplementation with minerals should therefore be optimized to sustain proper rumen function. However, ruminant nutritional feeding systems (Noziere et al. 2018; Nutrient Requirements of Dairy Cattle 2021) mainly focus on the overall mineral requirements of animals for maintenance, growth and production, whereas the specific needs for the development and activity of the ruminal microbiota are poorly documented. For some elements, like sulfur (S) the rumen microbiota requirements (2.5 – 3.1 g/kg of degraded organic matter) are slightly higher than those established for the host animal (2.0 g/kg DM) (Komisarczuk Bony and Durand 1991; Meschy 2010). Considering trace minerals (TM), like manganese (Mn), zinc (Zn) and copper (Cu), which 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 (Hostetler et al. 2003; Hosnedlová et al. 2007; Sloup et al. 2017). 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 (Hilal et al. 2016). 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 (Chamberlain and Burroughs 1962; Martinez and Church 1970). Furthermore, *in vitro* dry matter digestibility (dDM%) in rumen fluid is improved by an addition of 100 ppm of inorganic Mn (Arelovich et al. 2000). Regarding Zn, early *in vitro* studies have shown an increase in microbial protein synthesis after 5-7 ppm of Zn supply (Martínez 1971). In a more recent study, the rumen dDM% as well as total volatile fatty acids (tVFA) production in ewes was increased with a supplementation of 30-40 mg/kg DM of inorganic Zn (Hosseini-Vardanjani et al. 2020). However, not all micro-organisms have the same sensitivity to Zn, which could also have negative effects. In an early study (Bonhomme et al. 1979), 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 (Hernández-Sánchez et al. 2019). *In vitro* studies with Cu showed that a high dosage of inorganic Cu (as $CuSO_4$) has a negative effect on rumen fermentation (Slyter and Wolin 1967), and VFA production is inhibited (Arce-Cordero et al. 2020). However, an addition of 8 mg/kg DM of inorganic Cu (as $CuSO_4$) significantly improved the *in vitro* dDM% and tended to increase the total microbial biomass (Vaswani et al. 2017). 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* (Wang et al. 2021). In ruminants, the recommended dose of dietary Mn, Zn and Cu, regarding the global animal requirements, are approximately 50, 50 and 10 mg/kg DM (Trumeau 2014), and the regulatory maximum limits are 150, 120 and 35 mg/kg DM for Mn, Zn and Cu, respectively (European Commission. Directorate General for Health and Food Safety. 2022). For most species, the absorption site of TM is located in the small intestine (Goff 2018; Byrne and Murphy 2022a). 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 (Goff 2018). Intestinal bioavailability of the dietary TM for ruminants is relatively low, as reported levels are at 4–5%, 1–4% and 15–30% for Cu, Mn and Zn, respectively (Spears 2003; Meschy 2007; Noziere et al. 2018), while selecting the most optimal mineral source for supplementation is quite difficult. Furthermore, ruminal microbial uptake levels are not known. From an environmental perspective, supplementation of farm animals with high levels of TM results in an accumulation of metals in the feces and urine, leading to increased emissions, affecting water supply chains, impairment of plant development, as well as an increased occurrence of antimicrobial resistance in livestock (Brugger and Windisch 2015). In the perspective of reducing heavy metal emissions from animal production, a better implementation of TM precision feeding is required (Lu et al. 2017). Dietary intake and supplementation with minerals should therefore be optimized in order to sustain proper rumen functions, including microbial biomass growth (microbial protein synthesis) and substrate degradation (cellulolytic activity). However, when selecting a mineral source for dietary inclusion in ruminants, ruminal solubility should be taken into consideration, as the overall bioavailability could be affected by higher rumen-soluble minerals (Byrne and Murphy 2022b). Furthermore, when estimating the rumen solubility of different mineral forms, the assessment method should be carefully considered, given that there is inconsistency in the previously published data, even when solubility was evaluated for the same mineral forms (Vigh et al. 2023). The refinement of mineral supplementation in ruminants is an important challenge that could improve zootechnical performances and a better use of resources. A better understanding of the effects on the ruminal environment of the various S and TM forms available on the market could support specialists from the animal feed industry when choosing mineral products for dietary inclusion.

RESEARCH PURPOSE AND OBJECTIVES

Based on the above, this doctoral research project aimed to assess the modifications induced by different S and TM sources in the ruminal environment, nutrient digestibility, and mineral excretion. The research was focused on S and trace elements (Zn, Cu and Mn). Calcium (Ca) and phosphorus (P) were not included, as Ca is usually supplied in excess and P has already been studied in details during another doctoral program (Bravo et al. 2003).

To fulfill the purpose of the thesis research, several objectives have been defined:

O1 Development of an *in vitro* experimental model to assess the rumen solubility and bioavailability of sulfur and trace minerals, and the effects on rumen function.

O2 Evaluate and better understand the ruminal solubility, bioavailability, effect on fermentation activity and rumen microbiota of different inorganic sulfur sources.

O3 Assess the effects of supplementation of various sources of trace minerals on rumen fermentation, solubility, and bioavailability.

O4 Propose an adjustment of the sulfur and trace mineral supplementation of ruminant animals considering the source and effect on rumen function of minerals.

The literature analysis included in the first part of this thesis was published as a systematic review article in Agriculture journal (ISI indexed with IF 3.6), while the results were published in three original articles (one in Agriculture, an ISI indexed journal with IF 3.6 and two in Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, a BDI indexed journal). Furthermore, the outcomes of the studies conducted during this doctoral research were presented at several international scientific conferences in the form of oral or poster presentations (Annual Meeting of the European Association of Animal Production, 2022 – Porto, Portugal and 2023 – Lyon, France; International Symposium on the Nutrition of Herbivores, 2023 – Florianopolis, Brazil; International Conference "Life Sciences for Sustainable Development", 2022 and 2023 – Cluj-Napoca, Romania).

The thesis is structured in two parts, with the first part including the "INTRODUCTION" and "LITERATURE REVIEW", describing the most common practices for dietary inclusion of sulfur and trace minerals in ruminants. The literature review describes the importance of supplementing ruminants with sulfur (S) and trace minerals (TM), including copper (Cu), manganese (Mn) and zinc (Zn) to cover the ever-evolving requirements for growth, production, and reproduction. Furthermore, the commercially available mineral forms, such as inorganic (ITM), organic (OTM) or hydroxy (HTM) TM are reviewed in terms of ruminal solubility and effects on the ruminal environment (fermentation, nutrient degradability, bioavailability, and microbial populations) focusing on the potential link between the mineral source and rumen function. In this part, the existing *in vitro* and *in vivo* methods to assess the ruminal solubility of minerals are also discussed. The second part of the thesis is composed of seven chapters, "Chapter II, Chapter III, Chapter IV, Chapter V, Chapter VI, Chapter VII and Chapter VIII", which present and discuss the research results obtained over the course of the doctoral study.

In the third chapter (Chapter III.) some insights are given on the development of an *in vitro* model for the evaluation of the rumen solubility and bioavailability of minerals and their effects on rumen fermentation parameters. Chapter III. consists of another five subchapters covering a section of introduction (3.1.), material and methods (3.2.), results (3.3.), discussions (3.4.) and conclusions (3.5.). Subchapter material and methods (3.2.) is divided into another six sections (from 3.2.1. to 3.2.6.), while the results (3.3.) subchapter is presented in two distinguished sections (3.3.1 and 3.3.2.). The focus during the methodological trials presented in Chapter III. was on the establishment of *in vitro* fermentation conditions based on the incubation of a S-deficient substrate in a buffered rumen fluid in which the activity of the ruminal flora is sensitive to the supplementation with S (the reference S source was elemental sulfur with a S content of >97%, added in increased doses [0.25 and 0.50 % DM] at the start, after 24 hours and 48 hours of incubation), and the identification of the fractions (big particles [UNSOL], containing feed particles, protozoa and insolubilized minerals; bacteria enriched [BACT] fraction, containing mainly rumen bacteria; and a final supernatant [SOL], containing the solubilized S) in which the additional S would be found after the 70 hours fermentations. Fermentation activity was measured through total gas production (TGP, ml/g DM), gas production rate (GP rate, ml/h), substrate dry matter degradability (dDM%), pH, volatile fatty acid (VFA, mM/kg) production, ammonia-nitrogen (NH_3-N, mg/dL), and microbial protein synthesis assess by a diaminopimelic acid (DAPA) analysis. The mineral solubility was assessed by analyzing the S concentration in the fractions (UNSOL, BACT and SOL) obtained after successive centrifugations of the final fermentation medium. According to the results registered during the 3 consecutive incubations realized in this study, the addition of 0.50% DM of S after 48 hours of incubation seemed to be the most interesting in the aim to study the response of *in vitro* fermentation activity of microorganisms with S supplementation. Also, the addition of 0.5% DM of S at the start of incubation could be interesting to keep as a working treatment as it allowed to compare the time of supplementation. In addition, the *in vitro* model developed during this study can be used for assessing the ruminal solubility of other minerals, including Mn, Zn and Cu, with taking into consideration the following procedures: separation of the final fermentation medium (after 70 hours of fermentation) by multiple centrifugations: at 100× *g* (5 min at 4 °C), to separate an insoluble fraction (UNSOL, containing feed particles, protozoa and insolubilized minerals); the obtained supernatant is then further centrifuged at 18,500× *g* (20 min at 4 °C) to separate a bacteria-enriched fraction (BACT) and a final supernatant (SOL), containing only solubilized minerals;

and the mineral concentration of each centrifugation fraction is then analyzed. Next, the ruminal solubility of minerals can be expressed as a percentage of the solubilized mineral in the final supernatant (based on the total mineral analyzed in the different centrifugation fractions).

The following chapter (Chapter IV.) describes the effects of various S sources on ruminal S bioavailability, fermentation activity and microbial populations measured using the *in vitro* model described in Chapter III. Like the previous chapter, Chapter IV. is divided into another five subchapters covering an introduction (4.1.), material and methods (4.2.), results (4.3.), discussions (4.4.), and conclusions (4.5.). Subchapter material and methods (4.2.) is divided into further six sections (from 4.2.1. to 4.2.6.), while the results (4.3.) subchapter is presented in three distinguished sections (from 4.3.1 to 4.3.3.). The aim of this study (published as an original article) was to better understand the ruminal solubility, bioavailability, effects on fermentation and microbiota of different S sources. *In vitro* fermentations with rumen fluid were conducted for 70 hours. Hay (S-deficient substrate) was incubated solely (CON), or with 0.5%DM of S, as: elemental sulfur (ES), Na_2SO_4 (NaS), $(NH_4)_2SO_4$ (NHS) and $MgSO_4$ (MgS). Fermentation activity was assessed by total gas production (TGP, continuous measurement) and dry-matter degradability (dDM%). Solubility and bioavailability were estimated by S concentration of the supernatant (SOL) and of bacteria (BACT). The microbial community was assessed by amplification of the 16SrRNA gene. NHS decreased ($p<0.05$), ES and NaS didn't affect, while MgS increased ($p<0.01$) TGP. MgS increased ($p<0.001$), whilst ES, NaS and NHS showed no effect on dDM%. Sulfate sources (NaS, NHS, and MgS) increased ($p<0.001$), while ES did not affect the S content of SOL. The S content of BACT suggests that sulfates have a high bioavailability, while ES is poorly assimilated by bacteria. Some variations of the microbial community were observed, including a lower abundance of methanogens with all S sources and a higher abundance of *Desulfovibrio* with MgS. The results of this study indicate that $NaSO_4$, $(NH_4)_2SO_4$ and $MgSO_4$ are highly soluble in the rumen, while elemental sulfur has a low solubility. Furthermore, the more soluble sulfate sources of S seem to be better assimilated by rumen bacteria compared to ES, hence a higher bioavailability. Regarding the rumen function, $(NH_4)_2SO_4$ seems to have a negative effect on rumen fermentation, ES has no significant effect, while $NaSO_4$ and $MgSO_4$ can improve specific fermentation parameters. The microbial population analysis indicates that S could have an impact on some specific bacteria abundance, however the 70 hours incubations used in this study are probably not the optimal recommended conditions for microbial diversity analysis.

The next chapter (Chapter V.) presents the evaluation of inorganic sulfur sources effects on ruminal degradation of forages and rumen microbiota measured by an *in-situ* model. Chapter V. is divided into five subchapters covering an introduction (5.1.), material and methods (5.2.), results (5.3.), discussions (5.4.), and conclusions (5.5.). Subchapter material and methods (5.2.) is divided into further five sections (from 5.2.1. to 5.2.5.), while the results (5.3.) subchapter is presented in two distinguished sections (5.3.1 and 5.3.2.). This *in vivo* study aimed to assess the effects of the dietary inclusion of inorganic S sources (elemental sulfur and $MgSO_4$) in ruminants on rumen degradation kinetics of forages (Bermuda grass hay and maize silage), as well as changes in the rumen microbial populations. Following a Latin square design, six Nellore rumen cannulated steers (averaging 943±96 kg live body weight) were divided to three experimental treatments: no S supplementation (CON) and supplementation with 0.05 % DM intake of S/day with either elemental sulfur (ES) or magnesium sulfate ($MgSO_4$). Experimental periods included 2 weeks for treatment adjustment followed by 2 weeks for substrate incubation. Forage DM and NDF degradation kinetics were assessed using the standard nylon bag technique, while the rumen microbial community composition was assessed by DNA extraction and 16S rRNA gene sequencing. Hay DM degradation was significantly increased with $MgSO_4$ when compared to CON and ES ($p<0.05$), but no significant effect was observed on maize silage DM degradation. $MgSO_4$ significantly increased hay NDF degradation when compared to CON and ES ($p<0.05$), while ES decreased the NDF degradation of maize silage ($P<0.05$). Regarding the changes in the microbial populations, no significant differences were registered between the treatments on alpha- and beta-diversity. Nevertheless, the S supplementation showed significant effects on some specific microbial communities, such as the absolute abundance of the methanogenic *Archaea* was significantly ($p<0.01$) lower in the S supplemented groups when compared to CON, regardless of the S source. Furthermore, the abundance of *Desulfovibrio* was significantly increased when $MgSO_4$ was supplemented while ES showed no significant effect when compared to CON. Regarding the fibrolytic bacteria, the population of *Fibrobacter* genus was significantly higher ($p<0.05$) for S-supplemented animals, While the *Ruminococcus* did not differ when compared to CON. The results of this study indicate that supplementation of roughage -based diets in ruminants with up to 0.30% DM of S (an increase of 0.05 – 0.10 % DM above the recommended dosage of 0.20 % DM), using a highly rumen-soluble S source may improve substrate degradation and ruminal fermentation efficacy. Furthermore, the dietary inclusion of a bioavailable S source could have a beneficial impact not only on specific rumen functions like fibrolytic bacteria activity, but also in modulating methanogenic populations with the potential to reduce enteric methane production.

The following chapter (Chapter VI.) describes the ruminal solubility and bioavailability of inorganic trace mineral sources and effects on fermentation activity measured using the *in vitro* model described in Chapter III. Chapter VI. is divided into another five subchapters covering an introduction (6.1.), material and methods (6.2.), results (6.3.), discussions (6.4.), and conclusions (6.5.). Subchapter material and methods (6.2.) is divided into further six sections (from 6.2.1. to 6.2.6.), the results (6.3.) subchapter in three sections (from 6.3.1 to 6.3.3.), while the discussions (6.4) subchapter in another three sections (from 6.4.1. to 6.4.3.). The aim of this study (published as an original article) was to assess the effects of supplementation with inorganic sources of manganese (MnO, $MnSO_4$), zinc (ZnO, $ZnSO_4$) and copper ($CuSO_4$) at different levels (0.06%DM for Mn, 0.05%DM for Zn; 0.01 and 0.05%DM for Cu) on *in vitro* rumen fermentation, solubility, and bioavailability. Fermentation activity was measured by total gas production (TGP) and dry matter degradability after 70 hours of fermentation (dDM%). Trace mineral (TM) solubility was estimated via the TM concentration in the supernatant of the final fermentation medium (SOL) and TM bioavailability from the TM concentration in a bacterial-enriched fraction (BACT). Mn (regardless of source) and ZnO tended ($p<0.10$) to decrease, while Cu showed no significant effect on TGP. The addition of inorganic Mn and of ZnO tended ($p<0.10$) to decrease, $ZnSO_4$ tended to increase ($p<0.10$), whilst Cu showed no effect on dDM%. Concerning solubility, Mn (MnO and $MnSO_4$), $ZnSO_4$ and $CuSO_4$ significantly ($p<0.05$, $p<0.001$ and $p<0.01$) increased, while ZnO did not affect TM content in the SOL. These results indicate that $MnSO_4$, $ZnSO_4$ and $CuSO_4$ are highly soluble, MnO is quite soluble, while ZnO has a low solubility in the rumen. Based on the TM content in BACT, MnO, MnS_4 and $CuSO_4$ have high bioavailability, while ZnO is poorly assimilated by rumen bacteria. However, the lack of clear inhibition or improvement in fermentations suggests that the rumen microbiota have a low requirement for TM supplementation.

In the last chapter (Chapter VII.), a description is made on the effects of low-dose organic trace minerals supplementation on the mineral excretion and physiological mineral status in small ruminants. Chapter VII. is divided into another five subchapters covering an introduction (7.1.), material and methods (7.2.), results (7.3.), discussions (7.4.), and conclusions (7.5.). Subchapter material and methods (7.2.) is divided into further three sections (from 7.2.1. to 7.2.3.), while the results (7.3.) subchapter in four sections (from 7.3.1 to 7.3.4.). The aim of this study (published as an original article) was to investigate on the effects of replacing inorganic trace mineral (ITM) supplementations of Mn, Zn and Cu by iso or lower doses of organic TM (OTM) on mineral status and mineral excretion of sheep. Following a Latin square design, nine castrated rams were divided into three experimental treatments: supplementation either with ITM (INORG) or OTM (ORG) following the recommended levels, and OTM at a reduced dosage (ORGLow). After an adaptation period, samples of feces, urine and blood were collected for 12 days in metabolic stalls. Serum mineral content showed differences only for Cu, which was significantly higher ($p<0.01$) with ORGLow when compared to INORG and ORG. Total daily feces mineral excretions were downregulated with ORGLow compared to the INORG or ORG. The results of this study suggest that supplementing sheep with a low dosage of OTM significantly reduces mineral excretion without a negative effect on the physiological mineral status of the animals. Nevertheless, further long-term studies are necessary to assess the mineral mobilization from body storages during supplementation with low dosages of OTM.

GENERAL CONCLUSIONS

When considering mineral supplementation in ruminants, especially when it comes to elements like S, Mn, Zn and Cu a special attention is required in selecting the right mineral source given that their solubility in the rumen is closely related to the rumen function (fermentation, substrate degradation, interactions between microorganisms), mineral intestinal absorption and fecal mineral excretion.

The first step of this doctoral research was the development of an *in vitro* model to assess the rumen solubility of various minerals. Based on the results obtained after numerous incubations using rumen fluid as an inoculum, it was concluded that one of the most relevant methods to assess the ruminal solubility of S, Mn, Zn and Cu is based on the separation of the final fermentation medium (after 70 hours of fermentation) by multiple centrifugations. The first centrifugation should be performed at 100× *g* (5 min at 4 °C), to separate an insoluble fraction, containing feed particles, protozoa, and insolubilized minerals). The obtained supernatant is then further centrifuged at 18,500× *g* (20 min at 4 °C) to separate a bacteria-enriched fraction and a final supernatant, containing only solubilized minerals. The mineral concentration of each centrifugation fraction should then be analyzed. Next, the ruminal solubility of S, Mn, Zn and Cu can be expressed as a percentage of the solubilized mineral in the final supernatant, based on the total mineral content analyzed in the different centrifugation fractions. Furthermore, the TM concentration of the bacteria-enriched fraction could be an indicator of the overall bioavailability, as the TM (% of total TM) assimilated by rumen bacteria seemed to be related to the intestinal apparent absorption of TM in ruminants.

Using the above-mentioned *in vitro* model the ruminal solubility of several inorganic S sources was assessed. The results of this study indicate that $NaSO_4$, $(NH_4)_2SO_4$ and $MgSO_4$ are highly soluble in the rumen, while ES has a low solubility. Given their high solubility, the sulfate ($-SO_4$) sources of S seem to be better assimilated by rumen bacteria compared to ES, hinting for a higher rumen bioavailability. Based on the fermentation parameters measured *in vitro* (TGP, dDM, PF), it can be highlighted that $(NH_4)_2SO_4$ has a negative effect on rumen function, ES has no significant effect, while $NaSO_4$ and $MgSO_4$ can improve specific fermentation parameters. The additional *in vivo* study with rumen cannulated steers, with a dietary inclusion of either $MgSO_4$ or ES above the recommended levels confirmed the positive effect of a highly rumen-soluble S source ($MgSO_4$) on rumen function, promoting not only a higher substrate (DM and NDF) degradation, but also a beneficial impact on specific microbial populations, like fibrolytic bacteria. Furthermore, the analysis of the rumen microbiota suggested that S, regardless of source (ES or $-SO_4$) might have a positive role in modulating methanogenic populations with the potential to reduce enteric methane production.

Based on the *in vitro* studies assessing the ruminal solubility of inorganic Mn, Zn and Cu sources, it was observed 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 $-SO_4$ sources of TM seem to be better assimilated by rumen bacteria compared to the oxide TM, hence a potentially higher bioavailability. Nevertheless, based on the *in vitro* studies results, high dosages of inorganic TMs, regardless of source (oxides or sulfates), seem to have a negative effect on rumen fermentation activity, like total gas production, dry matter degradability and microbial protein synthesis.

The results highlighted in this doctoral study suggest that ruminants could be supplemented with a reduced dosage of organic TMs compared to inorganic TMs at nutritional systems' recommended levels, presenting a viable strategy in the mitigation of mineral excretion to the environment. Furthermore, the reduced TM supplemental levels presented no negative effects on nutrient digestibility and did not compromise the physiological mineral status of the animals.

RECOMMENDATIONS

Considering the outcomes of this doctoral research, the rumen fluid based *in vitro* models are relevant for the assessment of minerals' ruminal solubility, but not well suited for observations of the rumen microbiota as influenced by sulfur and trace minerals. For the latter, *in vivo* models are better suited.

The results of this study indicate that supplementation of roughage-based diets in ruminants with up to 0.30% DM of S (above the nutritional systems' recommended levels of 0.20% DM), using a highly rumen-soluble S source may improve substrate degradation and ruminal fermentation efficacy. Nevertheless, additional studies are recommended in ruminants supplemented with highly rumen-soluble sulfur sources covering the rumen microbiota requirements in S (higher than the host animals' requirements) and the effects on the various trace minerals (inorganic and/or organic at recommended and reduced supplemental dosages) bioavailability, given that S is one of the main antagonists for Cu, Mn, and Zn intestinal absorption. In addition, further long-term studies are necessary to assess the mineral mobilization from body storages during supplementation with low dosage of organic trace minerals in ruminants.

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