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Dietary Trace Mineral Level and Source Affect Fecal Bacterial Mineral Incorporation and Mineral Leaching Potential of Equine Feces

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Abstract: Minerals excreted in feces have the potential to leach or runoff to water-ways, negatively impacting water quality. This study examined the effect of dietary trace mineral levels, and their source, on the leaching potential of minerals from equine feces. Nine horses were used in a replicated 3 × 3 Latin Square, with three dietary treatments provided as pellets: no added trace minerals (CON), added inorganic trace minerals (ING), and added organic trace minerals (ORG). Supplemental trace minerals included Co, Cu, Mn, and Zn. Horses were allowed ad libitum access to forage and fed their treatment pellets for 16 days prior to fecal sample collection. Estimated dietary mineral intake exceeded requirements for supplemented minerals. Regardless of the source, adding dietary trace minerals increased the fecal leaching potential of Cu, Zn, and P ($p < 0.05$). More Co leached from ORG compared to ING, while Zn leached in greater amounts from ING compared to ORG ($p < 0.05$). Fecal bacterial Zn content was greater ($p < 0.05$) for ORG compared to ING. Negative correlations were observed between bacterial mineral content and leaching for several minerals. Supplementing trace minerals in forms that increase microbial incorporation may provide a strategy to control fecal mineral leaching.

Keywords: bacterial biomass; leaching; trace mineral

1. Introduction

The excess excretion of minerals is a large concern for environmental soil and water quality. Phosphorus has received a great deal of attention as it can be excreted in relatively large amounts and is implicated in the eutrophication of water bodies. However, even though trace minerals are excreted in smaller quantities, these minerals can accumulate to environmentally concerning levels in the soil [1–3]. Additionally, the build-up of trace minerals in water bodies can be detrimental to aquatic organisms [4–6].

The practice of providing dietary minerals in excess of estimated requirements is commonly used in livestock diets to ensure that maximum health and production performance is maintained [7,8]. However, the limited trace mineral storing capacity of the body results in the excretion of minerals supplemented in excess of requirements [9]. Thus, overfeeding minerals is a practice that should be avoided in order to reduce the excretion of excess minerals into the environment [2]. However, similar to other livestock species [2,10], horses are often over-supplemented with minerals as well [11], leading to the unnecessary excretion of these excess minerals into the environment.

Trace minerals are typically added to the concentrate portion of equine diets either in an inorganic form (e.g., mineral sulfates, oxides) or an organic form (e.g., metal chelates or complexes) [12]. The most commonly available organic trace minerals used in domestic animal diets include Co, Cu, Mn, Fe, and Zn [13]. The value of replacing inorganic trace minerals with organic sources can include the increased bioavailability of the mineral to the animal, and also increased availability for uptake by intestinal microorganisms. An increase in the bioavailability of the mineral might result in less mineral being excreted, depending on the biological need for that mineral by the horse. Increased uptake by microbes is believed to reduce the solubility of the mineral and could thus have an impact on the leaching potential of that mineral, as shown in soil microbial communities and P leaching potential [14,15]. However, the effect of the trace mineral source on the leaching potential of equine manure has not been investigated.

The objective of this study was to examine the effect of trace mineral level and source (inorganic or organic) on fecal mineral excretion, bacterial mineral incorporation, and fecal mineral leaching potential. The hypothesis was that the supplementation of trace minerals to well above recommended amounts [12] would increase the fecal excretion of those minerals and that organic trace minerals would reduce leaching potential.

2. Materials and Methods

The animal part of this study was approved the Institutional Animal Care and Use Committee at the University of Kentucky.

Nine mature geldings (mean ± SD; 9.1 ± 2.1 years; 600.9 ± 50.6 kg initial body weight (BW)) were blocked by age into three groups. The groups were housed in pastures containing cool-season grasses and clovers. When the pasture supply was not sufficient due to changes in season, grass hay that was cut from the same farm was supplied in the form of round bales. Horses, therefore, had ad libitum access to forage and water at all times (Table A1).

The experiment was designed as a replicated 3 × 3 Latin Square. Three blocks of horses were randomly assigned within a block to one of three dietary treatments as described below. There was a total of three time periods of 21 days respectively, with each horse receiving each treatment in a random order. Therefore, upon completion of the study, each of the nine horses had received each dietary treatment.

Dietary treatments were provided using a soybean-meal based ration balancer pellet (Table 1). The trace minerals cobalt (Co), copper (Cu), manganese (Mn), and zinc (Zn) were incorporated in the pellets in either an organic (ORG) or inorganic (ING) form. As a third treatment (CON), no additional trace elements were added to the pellet, and the trace minerals present were therefore provided by the plant-based feed ingredients used to make the ration pellet and assumed to be organic [16]. Mineral intakes from the ORG and ING pellets alone exceeded dietary requirements for Co, Cu, and Zn [12]. Mineral intakes from the CON pellet alone exceed dietary requirements for Co [12]. However, if horses were assumed to consume 2% of their BW in hay dry matter (DM; Table A1), horses in all treatment groups would be exceeding their Co, Cu, Mn, and Zn requirements [12]. All three feeds consisted of the same base feed ingredients. The ORG and ING were formulated to contain the same amount of trace minerals, with the difference being the mineral source (organic or inorganic, respectively). For the ING pellet, the added trace mineral sources were cobalt carbonate, copper sulfate, manganous oxide, and zinc oxide. For the ORG pellet, the added trace mineral sources were cobalt proteinate, copper proteinate, manganese proteinate, and zinc proteinate (BioPlex®; Alltech, Nicholasville, KY, USA). The final feed analysis revealed that the ORG pellet had a lower concentration of the supplemented trace mineral than the ING pellet (Table 1). Thus, horses fed the ING and CON were fed the pellet at a rate of 0.2% of BW/day, while the horses receiving ORG were fed at a rate of 0.25% of BW/day to allow for an equal trace mineral intake between ORG and ING on a per kg BW basis.

Table 1. Analyzed chemical composition (DM basis) of control, inorganic, and organic pellets.

Item	CON	ING	ORG
DM, %	90.4	90.7	90.9
CP, %	33.1	32.4	37.5
NDF, %	13.8	13.1	13.3
ADF, %	10.2	9.5	8.7
Ca, %	3.97	3.78	3.89
P, %	2.24	2.12	2.24
Mg, %	0.29	0.41	0.38
K, %	1.96	1.96	1.96
Na, %	0.996	0.998	0.944
Fe, mg kg^{-1}	766	855	888
Zn, mg kg^{-1}	81	529	499
Cu, mg kg^{-1}	28	178	152
Mn, mg kg^{-1}	70	432	366
Mo, mg kg^{-1}	4.1	4.4	4.2
Co, mg kg^{-1}	0.93	1.88	2.00
Cr, mg kg^{-1}	13.2	10.4	13.4

All minerals except Cr were analyzed by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY, USA). Chromium was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) owned by a laboratory in the College of Agriculture, Food, and Environment at the University of Kentucky. Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; NDF, neutral detergent fiber; ADF, acid detergent fiber.

At the beginning of each period, there was a 5 day washout period during which horses only consumed forage and were not provided with any additional feed. After the washout period, horses were fed their assigned treatment pellet for a total of 16 days. Horses were fed the pelleted feed once a day in the morning (0800 h) via feed nosebags (Feedrite Feed Bag; Cashel Company, Granbery, TX, USA).

On day 16 of treatment, an 8 h total fecal collection was performed. Horses were brought into rubber-matted stalls, devoid of shavings, and were fed their treatment pellet. Hay pulled from the same round bales available in the fields were provided to the horses in the stalls using hay nets. Horses were closely monitored, and all feces excreted within 8 h (0800 to 1600 h) were collected from the floor immediately following defecation. Feces from each horse were composited into bags that were kept closed to avoid moisture loss. At the end of the collection, composited feces from each horse were thoroughly mixed, a wet weight was obtained, and approximately 1.75 kg of feces were saved and stored at −20 °C for later analysis.

Fecal samples from each horse were leached using an apparatus that simulates a rainfall event and allows for the capture of the leachate. We developed this simple method in our lab, and it is described below. After the DM content of the fecal samples was obtained, wet feces equating to 10 g of DM were weighed into filter cups attached to 50 ml centrifuge tubes (Tube Top Vacuum Filters, VWR International, LLC, Radnor, PA, USA). Coarse crepe filter paper with a 40 µm pore size was placed in the bottom of the filter cup to prevent large particles from leaching. Filter paper with a 20 µm pore size was placed on top of the feces to facilitate an even distribution of water. Distilled water was pumped to duplicate fecal samples through sprinkler heads attached via tubing to a multi-channel peristaltic pump. Samples were leached for 3 h 24 min with a flow rate of 0.45 ml/min, which was calculated to simulate an average rainfall event for Kentucky [17–20]. The leached fluid was collected in attached 50 ml centrifuge tubes. The centrifuge tubes were replaced with new tubes halfway through the rainfall event to prevent overflow. The pH of the collected fluid was measured in each of the two tubes collected for each duplicate sample. The leached feces were removed from the filter cup, dried at 55 °C for 48 h, and ground.

Mineral concentrations in fecal bacteria were also measured. Bacteria were separated from feces using a method modified from Bock, et al. [21]. Modifications include using 2 L 0.9% sodium chloride (NaCl) solution/kg wet feces, incubating NaCl/fecal mixture at 4 °C for 4 h before the first low-speed centrifugation step [21] to encourage particle-associated bacteria to dissociate [22], and centrifuging at 20,000× *g* for 25 min to sediment bacteria. After the last centrifugation step, the bacterial biomass was dried at 55 °C overnight and saved for analysis. Because the bacterial pellet was rinsed with NaCl, concentrations of Na in the bacterial biomass are not reported.

Leached feces, pre-leached feces, and the bacterial biomass were analyzed for mineral content using inductively coupled plasma (ICP) analysis following acidic closed-vessel microwave digestion. Two separate digestions were prepared: one for inductively coupled plasma mass spectrometry (ICP-MS) and one for inductively coupled plasma optical emission spectrometry (ICP-OES). ICP-MS was used to analyze for Cu, Mn, Zn, Co, chromium (Cr), and molybdenum (Mo), while ICP-OES was used to analyze for calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), Na, sulfur (S), and iron (Fe). For ICP-MS analysis, 0.25 g of dried and ground sample was weighed into Teflon digestion vessels. Ten milliliters of nitric acid (trace metal grade) was added to each vessel. Samples were microwave-digested (MARS 6; CEM Corporation, Matthews, NC) using the following protocol: ramp 20 to 25 min, hold 15 min at 200 °C, cool 15 min. One milliliter of ultrapure H_2O_2 was added to each vessel and the digestion repeated. Samples were then diluted as needed with distilled water before analysis with the ICP-MS. For ICP-OES analysis, the same procedure was followed except that 9 ml of nitric acid and 3 ml of hydrochloric acid were used for digestion. Digestion duplicates were included every ten samples. A standard reference material (peach leaves; NIST, Gaithersburg, MD, USA) was included in the digestion and analysis procedure for quality control.

The concentration of minerals in the pre-leached and in the leached feces was used to calculate the percentage of each mineral that leached. Data were analyzed as a replicated 3 × 3 Latin Square using an ANOVA (SAS Institute Inc., Cary, NC, USA). The statistical model included dietary treatment, period, block and horse within block. If the *p*-value for treatment was $p < 0.10$, means were separated using a Least Significant Difference (LSD) test. Simple correlations were made using Pearson's correlation coefficients (n = 54 when analyzed across all treatments; n = 18 when analyzed within treatment). Data are presented as means ± standard error of the mean (SEM). Significance was defined as $p < 0.05$ and a trend as $0.05 < p < 0.10$.

3. Results

All horses completed the study in good health, with no signs of mineral deficiencies or toxicities.

3.1. Pre-Leached Fecal Mineral Concentrations

Fecal mineral concentrations for each of the three dietary treatment groups are shown in Table 2. Horses fed the CON pellet had significantly lower concentrations of Cu and Zn ($p < 0.05$) and tended to have a lower fecal Co concentration ($p < 0.10$), which were three of the four trace minerals that were supplemented. The other supplemented mineral, Mn, was not different between treatments ($p > 0.10$), but was numerically lower in feces from the CON group. There were no differences between the ORG and ING treatments for any fecal minerals. There were also no differences among treatments in any other mineral measured (Table A2).

Table 2. Fecal mineral concentrations (DM basis), bacterial mineral concentrations (DM basis), amount and percentage of mineral that leached for Co, Cu, Mn, and Zn from horses fed control, inorganic, organic dietary treatments (n = 9).

Item	CON	ING	ORG	SEM	*p*-Value
Cobalt					
Fecal, mg kg^{-1}	0.735	0.917	0.938	0.0679	0.1023
Bacterial, mg kg^{-1}	1.69	2.11	2.20	0.18	0.1519
Leached, mg 10 g^{-1} DM	0.0021 ^{b}	0.0023 ^{b}	0.0034 ^{a}	0.0003	0.0058
Leached, %	27.63 $^{\alpha\beta}$	27.29 $^{\beta}$	35.40 $^{\alpha}$	2.75	0.0689
Copper					
Fecal, mg kg^{-1}	13.20 ^{b}	34.72 ^{a}	32.50 ^{a}	2.69	<0.0001
Bacterial, mg kg^{-1}	49.93 ^{b}	116.03 ^{a}	133.05 ^{a}	8.59	<0.0001
Leached, mg 10 g^{-1} DM	0.038 ^{b}	0.081 ^{a}	0.093 ^{a}	0.007	<0.0001
Leached, %	26.05	24.40	28.23	2.10	0.4402
Manganese					
Fecal, mg kg^{-1}	301.56	363.06	354.05	20.65	0.1106
Bacterial, mg kg^{-1}	1199.75 ^{b}	1460.64 ^{a}	1588.42 ^{a}	83.68	0.0163
Leached, mg 10 g^{-1} DM	0.72	0.63	0.37	0.09	0.7997
Leached, %	20.40	16.56	17.90	2.24	0.5004
Zinc					
Fecal, mg kg^{-1}	62.13 ^{b}	136.58 ^{a}	129.72 ^{a}	7.84	<0.0001
Bacterial, mg kg^{-1}	232.35 ^{c}	357.36 ^{b}	667.08 ^{a}	30.27	<0.0001
Leached, mg 10 g^{-1} DM	0.16 ^{c}	0.42 ^{a}	0.27 ^{b}	0.04	0.0002
Leached, %	21.89 $^{\alpha\beta}$	29.63 $^{\alpha}$	20.68 $^{\beta}$	2.84	0.0758

Within a row, treatment means followed by a similar letter (abc) do not differ ($p < 0.05$). Within a row, treatment means followed by a similar Greek letter (αβ) do not tend to differ ($0.05 < p < 0.10$). Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; SEM, standard error of the mean.

3.2. pH of Leachate

There was no effect of treatment on the pH of the leachate collected in the middle or at the end of the simulated rainfall event (Table A3). The pH values ranged from 6.76 to 6.93.

3.3. Percent Mineral That Leached

Zinc tended to leach the least from the ORG feces and the most from the ING feces (Table 2; $p = 0.0758$). Cobalt tended to leach the most from ORG feces compared to CON and ING (Table 2; $p = 0.0686$). There were no differences among treatments for Cu or Mn (Table 2; $p > 0.10$). Despite the three treatments having the same fecal concentration of P, S, Cr, and Mo, there were differences in the percent of each mineral that leached (Table 3). The supplemented groups (ORG and ING) had greater percentages of P that leached compared to CON ($p < 0.05$). Sulfur tended to leach the most from ING feces and the least from CON feces ($p = 0.0896$). Chromium leached more from ORG feces, with CON feces leaching the least and ING feces intermediate ($p < 0.05$). Molybdenum tended to leach the most from CON and the least from ING and ORG ($p = 0.0707$).

Table 3. Percentage of other minerals that leached from feces during a simulated rainfall event (n = 9).

Mineral	CON	ING	ORG	SEM	*p*-Value
Ca, %	20.86	26.16	24.98	2.22	0.2362
P, %	36.81 ^{b}	44.54 ^{a}	42.37 ^{a}	1.94	0.0214
Mg, %	32.70	35.52	33.95	1.41	0.3734
K, %	61.90	63.89	62.65	1.55	0.6598
Na, %	50.64	57.37	52.87	2.44	0.1514
S, %	22.43 $^{\beta}$	25.71 $^{\alpha}$	24.98 $^{\alpha\beta}$	1.07	0.0896
Fe, %	21.90	17.12	20.00	2.69	0.4689
Cr, %	8.81 ^{b}	17.08 ^{b}	30.57 ^{a}	4.16	0.0045
Mo, %	25.71 $^{\alpha}$	16.55 $^{\beta}$	16.68 $^{\beta}$	3.04	0.0707

Within a row, treatment means followed by a similar letter (ab) do not differ ($p < 0.05$). Within a row, treatment means followed by a similar Greek letter (αβ) do not tend to differ ($0.05 < p < 0.10$). Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; SEM, standard error of the mean.

Table 4 shows only the significant correlations ($p < 0.05$) between the amount (mg) of treatment minerals (i.e., Co, Co, Mn, and Zn) that leached and the amount (mg) of other minerals that leached from feces for each treatment group. Only correlations that had $r > 0.500$ are shown. Table A4 shows correlations between all minerals across treatments.

Table 4. Pearson correlations between the amount (mg) of treatment minerals and amount (mg) of other minerals that leached from fecal samples from each horse (in duplicate) for each treatment group (n = 18 per treatment).

Mineral	CON	ING	ORG
Co	Cr (0.791), Mn (0.846), Cu (0.793), Zn (0.838)	Mn (0.909), Fe (0.737), Cu (0.849), Zn (0.834), Ca (0.666), P (0.756)	Cr (0.858), Mn (0.806), Cu (0.785), Zn (0.734), Mo (0.616), Mg (0.503), P (0.712)
Cu	Mn (0.911), Co (0.793), Zn (0.793)	Mn (0.807), Fe (0.596), Co (0.849), Zn (0.812), Ca (0.674), Mg (0.491), Na (0.475), P (0.800)	Cr (0.681), Mn (0.752), Co (0.785), Zn (0.939), Mg (0.652), Na (0.719), P (0.857)
Mn	Co (0.846), Cu (0.911), Zn (0.641)	Fe (0.650), Co (0.909), Cu (0.807), Zn (0.916), Ca (0.654), Na (0.538), P (0.725)	Cr (0.832), Co (0.806), Cu (0.752), Zn (0.805), Mg (0.609), Na (0.633), P (0.655)
Zn	Cr (0.755), Mn (0.641), Co (0.838), Cu (0.793), K (−0.528)	Mn (0.916), Fe (0.654), Co (0.934), Cu (0.812), Ca (0.896), Mg (0.726), Na (0.527), P (0.899), S (0.694)	Cr (0.737), Mn (0.805), Co (0.734), Cu (0.939), Mg (0.695), Na (0.638), P (0.780)

Only correlations with $r > 0.500$ are shown ($p < 0.05$). Results expressed as: mineral (r value). Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment.

In general, there were more correlations between the treatment minerals and other minerals within the ING group compared with CON and ORG. Additionally, some unique correlations existed only within specific treatments. Within the ING group, but not the ORG group, these unique correlations include relationships between Co and Fe, Ca; between Cu and Fe, Ca; between Mn and Fe, Ca; and between Zn and Fe, Ca, S. Within the ORG group, but not the ING group, these unique correlations include those between Co and Cr, Mo, Mg; between Cu and Cr; between Mn and Cr, Mg; and between Zn and Cr. In the CON group, the only unique correlation was between Zn and K. This correlation was also the only negative correlation observed ($r = -0.528$).

3.4. Bacterial Mineral Concentration

Fecal bacterial mineral concentrations were also examined to determine if bacterial mineral incorporation affected mineral mobility. Copper and Mn concentrations were greatest in the ORG and ING compared to CON (Table 2; $p < 0.05$). Zinc was greatest in the ORG treatment, intermediate in ING, and lowest in CON (Table 2; $p < 0.05$). Sulfur concentration in bacterial biomass was greatest in the ORG group and lowest in CON, with ING not different from either (Table 5; $p < 0.05$).

Table 5. Other mineral concentrations in fecal bacterial biomass.

Mineral	CON	ING	ORG	SEM	*p*-Value
Ca, %	2.15	2.08	2.11	0.11	0.8975
P, %	1.44	1.41	1.43	0.07	0.9396
Mg, %	0.38	0.38	0.38	0.02	0.9792
K, %	0.19	0.18	0.19	0.01	0.8263
S, %	0.36^{b}	0.37^{ab}	0.38^{a}	0.004	0.0156
Fe, mg kg^{-1}	4670	4669	4663	403	0.9999
Cr, mg kg^{-1}	8.75	9.35	9.81	0.74	0.6098
Mo, mg kg^{-1}	4.78	4.90	4.94	0.55	0.9775

Within a row, treatment means followed by a similar letter (ab) do not differ ($p < 0.05$). Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; SEM, standard error of the mean.

Correlations were made between the fecal bacterial mineral concentration and percentage of mineral that leached (Table 6). There were significant negative correlations for Zn, P, Mg, and Mo ($p < 0.05$) suggesting that greater accumulation of these minerals in bacteria resulted in decreased solubilization from feces.

Table 6. Pearson correlations between fecal bacterial mineral concentration and percentage of mineral that leached from fecal samples from each horse (duplicate) for all treatments (n = 54).

Mineral	*r*	*p*-Value
Co	−0.1684	0.2281
Cu	−0.0180	0.8980
Mn	−0.1397	0.3333
Zn	−0.4242	0.0019
Ca	−0.1492	0.2863
P	−0.3568	0.0081
Mg	−0.4196	0.0016
K	−0.2132	0.1217
S	−0.2103	0.1270
Fe	−0.1140	0.4165
Cr	−0.2715	0.1092
Mo	−0.5307	<0.0001

4. Discussion

With the exception of Co, adding trace minerals to the diet increased the fecal concentration of those minerals (Cu, Mn, and Zn), regardless of organic or inorganic source. The fecal concentration of other minerals was not affected by supplementing Co, Cu, Mn, and Zn. The accumulation of excreted minerals in the soil, particularly Zn and Cu, can build up to levels which can cause environmental concern [1]. Thus, the supplementation of these minerals should be restricted to the animals' requirement in order to reduce excessive excretion. There is some literature regarding horses that suggests that organic minerals may be better absorbed than inorganic minerals [23,24] and would therefore be able to be fed at a lower rate than inorganic minerals, further reducing potential fecal excretion. However, other equine studies have found no difference in digestibility between inorganic and organic trace minerals [23,25,26] or have reported improved digestibility for inorganic trace minerals [27]. It is important to note that many of these studies, including the current study, have fed above the current trace mineral requirement [12], which would mask any effect of mineral source on digestibility. More studies comparing trace mineral source on digestibility need to be performed in order to elucidate trace mineral requirements for horses, provide more precise supplementation, and reduce excessive excretion.

The effect of trace mineral levels and source on leaching potential varied by mineral. Compared to CON, adding trace minerals increased the amount of Cu and Zn that leached from the feces, regardless of source. Zinc from ING leached more than Zn from ORG, and Co from ORG leached more than ING and CON. In order to explain differences in leaching among treatments, correlations were made between fecal leaching potential, fecal bacterial concentration, and other minerals that leached.

A greater amount of Cu leached when Cu was added to the diets, regardless of source. However, when the amount of Cu leached was expressed as a percentage of pre-leached fecal Cu concentration, there were no longer significant differences among treatments. Despite fecal bacteria accumulating more Cu in supplemented groups compared to CON, fecal bacterial Cu concentration was not correlated with Cu leaching. When examining the correlations between the amount of Cu that leached and other minerals that leached, inorganic Cu had the greatest number of correlations with other minerals (eight other minerals). Inorganic Cu leached with Fe, while organic Cu did not. The protected form of the organic chelated Cu prevented Cu from interacting with other elements and reduced the likelihood that they would leach together [28]. However, organic Cu leached the same amount as inorganic Cu, suggesting that Cu leaching is driven mostly by the amount excreted in the feces and is not influenced by source. In pigs fed Cu as $CuSO_4$, more than half of the Cu in the resulting manure was organically bound, and less than 10% was soluble in water [29]. Inorganic Cu may bind organic material in the manure and thus reduce water solubility to a status similar to organic Cu.

Greater amounts of Co leached from fecal material obtained from the ORG treatment group than from the CON and ING treatments. There was no effect of treatment on the bacterial accumulation of Co, and fecal bacterial concentration was not correlated with Co leaching. However, there were significant correlations between the amount of Co that leached and other minerals that leached from the feces. When considering ING alone, leached Co had significant correlations with six different minerals. When examining ORG alone, Co leached with seven minerals, but organic cobalt leached with Cr, Mo, and Mg, whereas inorganic Co did not. Possibly, Cr, Mo, and Mg influence organic Co's mobility more than inorganic Co's mobility.

Manganese mobility was not influenced by dietary treatment, nor was there a correlation between Mn bacterial incorporation and Mn leaching. Inorganic Mn appears to have leached with Fe and Ca, while organic Mn did not. Organic Mn leached with Cr and Mg, while inorganic did not. The different forms of the organic and inorganic forms appear to result in different interactions between minerals in the feces, leading to differences in mobility in water.

The amount of Zn that leached from feces, as a percentage of pre-leached fecal Zn concentration, tended be highest from the ING treatment compared to ORG, with CON not different from either. However, when expressed as an amount (mg), significantly more Zn leached from ING compared to ORG, and CON leached the least amount of Zn. To put these values in perspective, a 500 kg horse fed the CON diet (excreting 5 kg fecal DM/day) would leach 29.2 g of Zn/yr, which is equal to 73 days' worth of the horse's Zn dietary requirement. The same horse fed the ING diet would leach 77 g of Zn/yr (191 days' worth of dietary Zn requirement) and, when fed the ORG diet, this horse's feces would leach 49 g Zn/yr (123 days' worth of dietary Zn requirement).

The reason why CON leached the least amount of Zn could be related to the lower fecal Zn concentration and therefore the smaller amount of Zn available to leach. Despite similar fecal concentrations between ING and ORG, more Zn leached from ING compared to ORG, potentially due to the bacterial incorporation of Zn. Organic Zn was incorporated into bacterial biomass to a greater extent than inorganic Zn. Similarly, organic Zn (Zn–polysaccharide) was more available to rumen bacteria compared to inorganic Zn oxide [30]. These studies suggested that the organic form of Zn is more soluble than its inorganic counterpart and thus is more available for bacterial uptake [30]. There was an overall negative correlation between bacterial Zn concentration and the amount of Zn that leached, meaning that the more Zn was incorporated into bacteria, the less Zn leached. This suggests that the incorporation of this mineral into the bacterial biomass decreases its solubility during the leaching process. While there are limited data regarding the mechanism behind specific trace mineral incorporation into fecal bacteria reducing solubility, there is information related to soil microbial communities and P. In soil, the P associated with the microbial biomass is insoluble in the soil solution and unavailable for plant uptake [15]. When microbes release P, water-soluble P increases [14], further suggesting that P incorporated into microbes reduces the potential for leaching. While these studies were performed with P and soil bacteria, which may differ from trace minerals and equine fecal bacteria, it is possible that similar mechanisms occurred in the current study. These data support the idea that the incorporation of Zn into the fecal bacterial biomass could reduce leaching of Zn.

Interactions between minerals influence solubility and mobility in water [31]. This research suggests that the form of the mineral in the manure plays a large role in the interactions that occur between minerals. When looking at correlations between leached minerals separated by treatment, there are more correlations for ING minerals compared to ORG minerals or minerals in the CON group. Fecal minerals in the CON group are also assumed to be organic, as no added inorganic minerals were fed to these animals. The protected nature of organic, or chelated, minerals prevents interactions with other minerals and reduces the likelihood that organic minerals would leach with other elements [13].

However, there were some significant correlations for organic minerals. Notable correlations that are only present in the ORG treatment groups, but not in the ING group, include Cr, Mo, and Mg. Chromium leaching was significantly correlated with all of the supplemented organic minerals (Co, Cu, Mn, and Zn), whereas Mo was only correlated with Co. Magnesium was only correlated with Co and Mn. In fact, Cr leached significantly more from ORG feces compared with ING or CON feces. The same pattern was not observed for Mo or for Mg. The association of Cr with the organic minerals can potentially be explained by the interaction between Cr and organic ligands. Chromium forms stable complexes with protein ligands; for example, transferrin and chromodulin [32]. The specific form of the organic minerals added to the ORG treatment diet was mineral proteinates. It is possible that Cr in the feces preferred to bind to the proteinates present in the ORG feces and thus was more likely to leach with the organic minerals.

Correlations that were present for ING minerals, but not for the ORG treatment group, include those involving Fe, Ca, and S. There were tendencies for S to leach more from ING feces compared to CON, but no significant differences between treatments were observed for Fe or Ca. The increase in S leaching from the ING treatment may be because Cu was added to the ING treatment diet as copper sulfate, and sulfate ions may be more soluble than organically complexed S.

Regardless of source, adding trace minerals to the diet increased the percentage of P that leached from the feces despite similar initial fecal P concentrations. Additionally, there were significant correlations between the amount of P that leached and the trace minerals Co, Cu, Mn, and Zn for the ING and ORG groups. The reason why P leached more from ING and ORG feces is unclear. Phytate—a form of P found commonly in grains—is known to bind Zn and Cu [33], but the diets fed to the horses should have minimal amounts of phytate, as they were fed forage and a soybean-meal based pellet. Additionally, the only difference between the diets was the amount and source of trace elements; thus, the concentration of all other dietary components, including phytate, should be similar. Manganese deficiencies have been reported in plants grown in high P soils, but the effect has been attributed to competition for plant uptake, as Mn–P precipitation in a hydroponic growing system was not observed [34].

The increase of P leaching with trace mineral supplementation is important from an environmental management standpoint. Reducing total fecal P excretion by animals by reducing P supplementation of the diet has been the main way to reduce P runoff and eutrophication of water bodies. However, in this study, even at similar fecal P concentrations, the mobility of P in water can be influenced by trace mineral supplementation. This research suggests that care should be taken when adding trace minerals in excess of requirements to equine diets, due to the potential to increase the leaching potential of P.

5. Conclusions

The addition of the trace minerals Co, Cu, Mn, and Zn to the diet, regardless of source, increased the leaching of Cu, Zn, and P from the feces compared to a control diet, which can contribute to issues related to water quality. Different rainfall events may affect the amount and rate of leaching, and so future studies should examine additional rainfall scenarios. Additionally, negative relationships between fecal bacterial mineral concentrations and mineral leaching potential suggest that bacterial incorporation of minerals may reduce leaching potential. Organic trace minerals added to the diet increased the fecal leaching of Co and Cr compared to inorganic trace mineral addition, and inorganic trace mineral supplementation increased the fecal leaching of Zn compared to organic trace mineral addition. Interactions between minerals of different forms in the feces appear to influence leaching potential. Dietary supplementation of organic Zn increased fecal bacterial concentrations of Zn. This research suggests that nutritional practices that enhance Zn uptake by intestinal bacteria may be useful in decreasing the impacts of leaching into water systems.

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Appendix A

Table A1. Analyzed chemical composition (DM basis) of grass hay and pasture that was available ad libitum to horses throughout the study.

Item	Grass Hay	Pasture
DM, %	92.5	88.5
CP, %	10.9	23.1
NDF, %	68.9	50.4
ADF, %	40.2	30.8
Ca, %	0.48	0.56
P, %	0.43	0.58
Mg, %	0.21	0.28
K, %	1.3	3.86
Na, %	0.086	0.01
Fe, mg kg^{-1}	724	246
Zn, mg kg^{-1}	31	37
Cu, mg kg^{-1}	12	9
Mn, mg kg^{-1}	171	93
Mo, mg kg^{-1}	1	1
Co, mg kg^{-1}	0.3	0.18
Cr, mg kg^{-1}	N.D.	N.D.

All minerals except Cr were analyzed by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY, USA). Chromium was analyzed using ICP-MS owned by a laboratory in the College of Agriculture, Food, and Environment at the University of Kentucky. N.D. = non-detect; NDF = neutral detergent fiber; ADF = acid detergent fiber

Table A2. Other fecal mineral concentration for horses fed control, inorganic, and organic dietary treatments (DM basis).

Mineral	CON	ING	ORG	SEM	*p*-Value
Ca, %	1.13	1.13	1.03	0.09	0.6423
P, %	0.83	0.86	0.83	0.03	0.7653
Mg, %	0.28	0.30	0.28	0.01	0.3461
K, %	1.00	0.99	1.00	0.05	0.9758
Na, %	0.16	0.15	0.15	0.017	0.9294
S, %	0.15	0.16	0.15	0.005	0.7994
Fe, mg kg^{-1}	1587	1619	1500	158	0.8613
Cr, mg kg^{-1}	8.11	7.97	9.70	1.05	0.4505
Mo, mg kg^{-1}	1.48	1.38	1.38	0.24	0.9411

Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; SEM, standard error of the mean.

Table A3. pH of leachate collected in the middle (pH 1) and at the end (pH 2) of a simulated rainfall event from feces of horses fed control, inorganic, and organic dietary treatments.

Item	CON	ING	ORG	SEM	*p*-Value
pH 1	6.81	6.76	6.81	0.019	0.1101
pH 2	6.93	6.88	6.91	0.020	0.2739

Abbreviations: CON, control treatment; ING, inorganic treatment; ORG, organic treatment; SEM, standard error of the mean.

Table A4. Pearson correlations between amounts (mg) of minerals that leached from fecal samples from each horse (duplicate) for all treatments (n = 54).

Mineral	Correlated Mineral (*r* Value)
Ca	P (0.790), Mg (0.667), Fe (0.719), Zn (0.637)
P	Ca (0.790), P (0.641), Cu (0.653), Fe (0.557), Zn (0.609)
Mg	Ca (0.667), P (0.641), S (0.746), Cu (0.521), Fe (0.628), Zn (0.649)
K	–
Na	–
S	Mg (0.746)
Co	Cu (0.711), Mn (0.799), Zn (0.616), Cr (0.778), Ni (0.700), Pb (0.883)
Cu	P (0.653), Mg (0.521), Mn (0.590), Co (0.711), Pb (0.506)
Mn	Cu (0.590), Zn (0.695), Co (0.799), Cr (0.516), Ni (0.674), Cd (0.577), Pb (0.893)
Zn	Ca (0.637), P (0.609), Mg (0.649), Fe (0.539), Mn (0.695), Co (0.616), Cd (0.575), Pb (0.591)
Fe	Ca (0.719), P (0.557), Mg (0.628), Zn (0.539)
Cr	Mn (0.516), Co (0.778), Pb (0.742)
Mo	–

Only correlations with $r > 0.500$ are shown ($p < 0.05$). Results expressed as: mineral (r value).

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