Zinc AA supplementation alters yearling ram rumen bacterial communities but zinc sulfate supplementation does not¹

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ABSTRACT: Despite the body of research into Zn for human and animal health and productivity, very little work has been done to discern whether this benefit is exerted solely on the host organism, or whether there is some effect of dietary Zn upon the gastrointestinal microbiota, particularly in ruminants. We hypothesized that (i) supplementation with Zn would alter the rumen bacterial community in yearling rams, but that (ii) supplementation with either inorganically sourced ZnSO₄, or a chelated Zn AA complex, which was more bioavailable, would affect the rumen bacterial community differently. Sixteen purebred Targhee yearling rams were utilized in an 84-d completely randomized design, and allocated to one of three pelleted dietary treatments: control diet without fortified Zn (~1 × NRC), a diet fortified with a Zn AA complex (~2 × NRC), and a diet fortified with ZnSO₄ (~2 × NRC). Rumen bacterial community was assessed using Illumina

MiSeq of the V4 to V6 region of the 16S rRNA gene. One hundred and eleven OTUs were found with > 1% abundance across all samples. The genera *Prevotella*, *Solobacterium*, *Ruminococcus*, *Butyrivibrio*, *Olsenella*, *Atopobium*, and the candidate genus *Saccharimonas* were abundant in all samples. Total rumen bacterial evenness and diversity in rams were reduced by supplementation with a Zn AA complex, but not in rams supplemented with an equal concentration of ZnSO₄, likely due to differences in bioavailability between organic and inorganically sourced supplement formulations. A number of bacterial genera were altered by Zn supplementation, but only the phylum Tenericutes was significantly reduced by ZnSO₄ supplementation, suggesting that either Zn supplementation formulation could be utilized without causing a high-level shift in the rumen bacterial community which could have negative consequences for digestion and animal health.

Keywords: 16S rRNA gene, bacteriome, Tenericutes, zinc supplementation

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INTRODUCTION

The benefits of dietary Zn to mammalian health has been well established, including for disease resistance (Engle, 2001), as well as for the

function of over 300 enzymes, particularly those involved in DNA synthesis, cell growth, and cell division (MacDonald, 2000; Dibley, 2001). Yet, like all trace minerals, the benefits of dietary Zn exist within a “Goldilock’s Zone”, outside of which, deficiency and toxicity can cause harmful side effects (reviewed in López-Alonso, 2012). Importantly, the type and magnitude of these negative side effects are different between monogastrics and ruminants. Unlike in monogastrics, subclinical Zn deficiencies in ruminants do not

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reduce antibody production or disease resistance, but clinical Zn deficiency alters the profile of serum leukocytes (reviewed in [Spears, 2003](#)).

Moreover, microorganisms have their own requirements for trace minerals. High levels of Zn outcompetes manganese for binding sites in *Streptococcus pneumoniae*, causing cell death ([McDevitt et al., 2011](#)). On the other hand, high Zn increased multi-drug-resistant *Escherichia coli* CFUs isolated from swine feces, digesta, and mucosal-associated communities ([Ciesinski et al., 2018](#)). In high-biomass and high-diversity host-associated ecosystems, such as the GI tract, competition over trace minerals can be fierce, and one mark of pathogenicity is a high binding affinity for these resources ([Patzner and Hantke, 1998](#); [Campoy et al., 2002](#); [Gielda and DiRita, 2012](#); [Porcheron et al., 2013](#); [Ma et al., 2015](#)).

Zn absorption and utilization by ruminants is improved when it is from an organic, rather than synthetic, source, and when it is coupled to an AA complex ([Spears, 2003](#); [Nayeri et al., 2014](#)). Zn supplementation altered the digestion and passage of both the dietary and microbial AAs, but it was speculated that the effect on microbial populations was due to an interaction with crude protein, rather than the Zn supplementation itself ([Miller, 1970](#); [Froetschel et al., 1990](#)). Despite the body of research into Zn for human and animal health and productivity, very little work has been done to discern whether this benefit is exerted solely on the host organism, or whether there is some benefit of dietary Zn upon the gastrointestinal microbiota (reviewed in [Hilal et al., 2016](#)), particularly in ruminants. We hypothesized that (i) supplementation with Zn would alter the rumen bacterial community in yearling rams, but that (ii) supplementation with either inorganically sourced ZnSO_4 or a chelated Zn AA complex, which was more bioavailable, would affect the rumen bacterial community differently.

METHODS

Animals and Diets

The experimental protocol used in this study was approved by the Agricultural Animal care and Use Committee at Montana State University (#2016-AA09). All animals used in this study were provided by the Montana Agricultural Experiment Station, and the study was conducted at the Fort Ellis Research Station at Montana State University (Bozeman, MT). Forty-four purebred yearling Targhee rams (14 mo; 68 ± 18 kg BW), of which 16 were utilized for microbial analysis presented here, were used in an 84-d completely randomized design. Rams were ranked and randomly assigned by BW and initial serum Zn concentration, then allocated to one of three pelleted dietary treatments: (i) control diet without fortified Zn (CON; $n = 15$; [Table 1](#)); (ii) a diet fortified with a Zn AA complex (ZnAA, Zinpro Corp; $n = 14$); and (iii) a diet fortified with ZnSO_4 (ZnSO_4 ; $n = 15$). Thus, treatment diets were designed to provide recommended dietary Zn requirements in the CON group and $2 \times$ NRC recommended Zn levels in the ZnAA and ZnSO_4 treatment groups. Ram treatment groups were randomly assigned to drylot pens (6.4×21.9 m) with approximately one-third of the pen covered by a three-sided barn. Rams were fitted with an electronic identification tag and each pen was equipped with two GrowSafe bunks (GrowSafe Systems Ltd., Airdrie, AB, Canada) to monitor individual intake. Rams had ad libitum access to NaCl, feed, and water. Rams were denied access to a complete free choice granulated mineral premix for 50 d prior to the start of the study to normalize serum Zn concentrations. Rams were fed a dry second cutting of timothy grass hay (14 mg/kg Zn) diet from -16 to 0 d before the trial to deplete circulating Zn concentration in the blood. After the completion of the study on day 87, rumen samples were extracted via

Table 1. Bacterial diversity indices in rumen digesta from sheep receiving Zn from foliage (control), organic Zn AA complex (ZnAA), or inorganic ZnSO_4 sources

Treatment	CHAO	OTUs	Good's coverage (%)	Inverse Simpson	Shannon–Weiner diversity	Shannon evenness
Control ($n = 4$)	317 ^a	240 ^a	98.80 ^a	11.79 ^a	3.36 ^a	0.61 ^a
ZnAA ($n = 6$)	292 ^a	201 ^a	98.71 ^a	7.18 ^b	2.69 ^b	0.51 ^b
ZnSO_4 ($n = 6$)	331 ^a	245 ^a	98.56 ^a	10.43 ^{ab}	3.07 ^{ab}	0.56 ^{ab}

Significance ($P < 0.05$) was tested by Student's *T*-test.

Superscripts indicate significant differences among means within a column.

oral lavage from six randomly selected rams from each treatment group. Additional information and results on the effect of Zn on serum Zn status, wool growth, and animal performance can be found elsewhere (Page et al., 2017).

DNA Extraction and Sequencing

Protocols used are as previously described (Ishaq et al., 2017) and reprinted here briefly. Nucleic acids were extracted from 1 g of whole rumen digesta using the PowerSoil 96-well Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following kit instructions specific to “centrifugation exclusively”. PCR was performed to amplify the V3 to V4 region of the 16S rRNA gene using the KAPA HotStart PCR Kit (Kapa Biosystems, Wilmington, MA): 341F (5'-CCTACGGGAGGCAGCAG-3') (Muyzer et al., 1993) and 806R (5'-GGACTACHVGG-GTWTCTAAT-3') (Caporaso et al., 2011). PhiX was used as a positive control at a 10% spike-in, and molecular-grade sterilized water was used as a negative control. High-throughput sequencing was performed using an Illumina MiSeq (Illumina, San Diego, CA) and a 500-cycle V2 kit. Output data included demultiplexed fastq files, with the primer and index sequences removed. Sequencing data can be found in the Sequence Read Archive at NCBI under BioProject PRJNA472838.

DNA Analysis

Paired-end 16S rRNA sequences for each sample were assembled into contiguous segments using PANDAsseq (Masella et al., 2012), and default quality parameters along the overlap. Mothur ver. 1.38 (Schloss et al., 2009; Kozich et al., 2013) was then used to cull sequences which were <300 b or >580 b, contained homopolymers >8 b, or which contained any ambiguous bases (*N*). The Silva v119 reference database, which contains bacterial and archaeal 16S rRNA sequences, was used to align sequences using the Wang algorithm (Wang et al., 2007), as a reference for identifying chimeras using the mothur-integrated version of UCHIME (Edgar et al., 2011), and for sequence identification with the mothur-implemented RDP classifier at 80% confidence cutoff. Any sequences that would not classify to phylum were removed. Sequences were preclustered to consolidate sequences with <2 base differences (Huse et al., 2010). Pairwise comparisons of taxonomic diversity were made using a Student's *t*-test, with significance determined as $P < 0.05$.

Sequences were clustered into operational taxonomic units (OTUs) based on a 3% genetic distance cutoff using the nearest-neighbor algorithm. Singleton OTUs were removed. The diversity measures, CHAO (Chao and Shen, 2010), Inverse Simpson (Jost, 2006), Good's Coverage (Good, 1953), Shannon–Weiner diversity (Shannon and Weaver, 1949), and Shannon evenness values, are presented as group means, and pairwise comparisons were made using Student's *t*-test. linear discriminant analysis was run using mothur-integrated LefSe (Segata et al., 2011) to detect discriminatory OTUs, using Wilcoxon rank test to determine significance. R statistical packages (RCoreTeam, 2015) were used to calculate redundancy analysis, vegan; calculate Pearson's correlations, corrrplot; compare community distance with PERMANOVA using both weighted (Bray–Curtis) and unweighted (Jaccard) metrics, adonis, RVAideMemoire; visualize abundance with heatmaps, phyloseq; and plot figures, ggplot, ggarrange. One control sheep (Flock ID T5082) was found to have a dramatically different bacterial community, and half the number of OTUs, than other sheep in the control group, and was removed from the analysis, but is included in the Supplemental Figures 1 and 2 for reference.

RESULTS

Receiving an organic ZnAA significantly altered the rumen bacterial community as compared to receiving Zn from basal dietary Zn concentrations (control) (Figure 1). This was driven by decreases in Shannon diversity and evenness as compared to control, with no significant change in total bacterial richness despite a numerical reduction (Table 1). Overall, both the community membership structure (PERMANOVA, unweighted Jaccard Similarity, $F = 1.70$, $R^2 = 0.21$, $P = 0.036$) and relative abundance of that structure (PERMANOVA, Bray–Curtis, $F = 1.34$, $R^2 = 0.17$, $P = 0.04$) were significant by treatment. However, this was driven by a significant interaction only between the ZnAA and control groups, for both bacterial community structure (pairwise PERMANOVA, BC, $P = 0.046$, Bonferroni correction) and weighted structure (pairwise PERMANOVA, uJ, $P = 0.048$, BF). ZnSO₄ treatment was not different from either control or ZnAA for diversity indices (Table 1), nor by community structure.

The most abundant phyla across all samples were Bacteroidetes, Firmicutes, the candidate phylum TM7, and Actinobacteria (Supplemental Figure 2). However, the Tenericutes phylum was

Figure 1. Clustering of bacterial communities in rumen digesta from sheep receiving Zn from basal dietary components (control), organic Zn AA complex (**ZnAA**), or inorganic ZnSO_4 sources. (A) Redundancy-based analysis (**RDA**) of bacterial communities from sheep rumen digesta under different diet treatments, using Bray–Curtis dissimilarity. (B) RDA of Hellinger-transformed communities showing significant factors driving community distance.

the only phylum significantly affected by Zn source: Tenericutes was increased in ZnSO_4 compared to Control (1.7% and 1.01% mean abundance, $P < 0.05$), and decreased in ZnAA compared to ZnSO_4 (0.05% and 1.7% mean abundance, $P < 0.05$). These sequences belonged to an unclassified genus in the class Mollicutes. Additionally, bacteria which were unclassified at the phylum level were lower in ZnSO_4 compared to control (3.9% and 7.9% mean abundance, $P < 0.05$), and compared to the ZnAA group (6.4% mean abundance, $P < 0.05$) (Supplemental Figure 2).

There were 871 genus-level OTUs, represented by 137 genera genus-placeholders, which contained at least two sequences per OTU (Figure 2). One hundred and eleven OTUs were found with $>1\%$ abundance across all samples. The genera *Prevotella*, *Solobacterium*, *Ruminococcus*, *Butyrivibrio*, *Olsenella*, *Atopobium*, and the candidate genus *Saccharimonas* were abundant in all samples. There were only eight OTUs which were found in all sheep rumen, being among the most abundant OTUs, and none were significantly different by treatment: Otu00002, unclassified; Otu00003, *Solobacterium* (100% confidence); Otu00006, *Solobacterium* (100% confidence); Otu00010, Candidatus *Saccharimonas* (100% confidence); Otu00013, *Atopobium* (80% confidence); Otu00015, *Olsenella* (100% confidence); and Otu00024, *Butyrivibrio* (99% confidence).

Twenty-seven genus-level OTUs were discriminant to a particular treatment, 15 of which were more abundant in the ZnAA group, 10 of which were more abundant in the ZnSO_4 group, and two of which were higher in the control group (Figure 3). These two OTUs identified as *Faecalibacterium* and *Comamonas*. The *Comamonas* OTU was the only one identified as such, and one Control sample had markedly higher abundance than any other sample; however, this comparison was significant even when the sample was excluded. The enriched taxa in the Zn treatments were OTUs from genera which are typically associated with improved digestion in ruminants; such as *Prevotella*, *Ruminococcus*, the BS-11 gut group, *Roseburia*, and an unclassified genus within the Lachnospiraceae family, as well as several other genera (Figure 4).

DISCUSSION

Mineral supplementation can improve the dietary value of mineral-deficient diets, and can allow for the use of lower-quality forages and bulk feeds. While slight Zn deficiency may not result in clinical manifestation in ruminants, a severe Zn deficiency has implications with the immune system, which have the potential to affect host–microbial interactions. Moreover, to our knowledge, few studies have investigated the effect of Zn supplementation

Figure 2. Genus-level-OTU diversity of bacteria in rumen digesta from sheep receiving Zn from basal dietary components (control), organic Zn AA complex (ZnAA), or inorganic ZnSO₄ sources. A total of 137 out of 871 OTUs had unique genus-level taxonomy or designations. One control sheep was determined to be an outlier and removed from this analysis.

on the rumen microbiota (reviewed in Hilal et al., 2016).

Current dietary Zn recommendations in sheep range from 20 to 51 mg/kg dependent on physiological status of the animal, while recommendations in cattle are less defined, at 30 mg/kg independent of physiological state of the animal (NRC, 2007, 2015). Appropriate concentrations of dietary Zn have shown to be beneficial in several economic aspects of sheep production, including but not limited to growth, reproduction, and wool characteristics (Underwood and Somers, 1969; White et al., 1994; Jafarpour et al., 2015).

Zn status of the animal is also dependent on the inherent bioavailability of the Zn source (Cao et al., 2000). Cao and others (2000) reported that organic sources (Zn proteinate, ZnAA, and Zn methionine) were more bioavailable than ZnSO₄ and ZnO, the more commonly used inorganic sources of Zn in ruminant mineral supplementation. However, proponents of chelated or “organic” Zn sources in

trace mineral supplementation discuss their ability to avoid mineral antagonism in the rumen environment resulting in enhanced absorption compared to inorganic Zn oxide and sulfate sources. The Zn source in the ZnAA group in theory would be less available to rumen degradation due to a stronger chelated ligand of soluble Zn salt to a partially hydrolyzed polypeptide (AAFCO, 2000). Thus, the decreased solubility found in the Zn AA complex of the ZnAA group would likely result in a markedly decreased soluble Zn pool in the rumen compared to that observed in the CON and ZnSO₄ groups. The overall lower reactivity with other dietary components in the forestomach with the ZnAA compared to those in CON and ZnSO₄ likely contributes to the decreased Shannon diversity and evenness.

Current findings agree with studies that reported greater growth performance from ruminant animals supplemented with organic sources of Zn compared to those supplemented with inorganic sources (Spears, 1989; Rojas et al., 1995; Gunter

Figure 3. OTU abundance of significantly different bacterial genera in rumen digesta from sheep receiving Zn from basal dietary components (control), organic Zn AA complex (ZnAA), or inorganic ZnSO₄ sources. Linear discriminant analysis identified OTUs, and significance was determined by Wilcoxon rank test ($P < 0.05$). OTUs are labelled by lowest rank of taxonomic classification, OTU number (ranked by sequence abundance), and which group it was discriminatory to.

et al., 2001). However, Spears (1989) reported similar Zn absorption between Zn methionine and ZnO, but retention was greater in lambs consuming Zn methionine, indicating that metabolism postabsorption or tissue retention differences exist between sources. Rams in the current study supplemented with ZnAA had greater ADG and tended to have increased feed efficiency compared to the control and ZnSO₄-treated rams (Page et al., 2017).

Previous studies in ruminants have highlighted the importance of diet (Henderson et al., 2015), feed efficiency (Jami et al., 2014; Myer et al., 2015; Shabat et al., 2016; Perea et al., 2017), and DMI (Hernandez-Sanabria et al., 2012; Myer et al., 2015; Ishaq et al., 2017) in selecting for rumen bacterial communities. Zn supplementation has been shown to improve feed intake, feed efficiency, and ADG at optimal concentrations (Ott et al., 1966; Fadayifar et al., 2012; Jafarpour et al., 2015; Page et al., 2017) and rumen bacteria are often correlated

with ADG (Hernandez-Sanabria et al., 2012; Myer et al., 2015; Ishaq et al., 2017). Zinc homeostasis is tightly regulated (Kendall et al., 2000) resulting in a relatively narrow range in serum Zn concentrations in response to changing dietary concentrations (Cousins, 1985). In the current study, rams in the ZnSO₄ group had greater serum Zn concentrations than CON rams, but not different than ZnAA (ZnSO₄ 0.71 µg/mL, CON 0.63 µg/mL, and ZnAA 0.66 µg/mL). Increasing dietary Zn in ruminants can actually lower their serum concentration of Zn in some instances (reviewed in Miller, 1970), indicating that the additional supplementation of soluble Zn sources may be redirected towards GI tract microorganisms. While total microbial protein was increased (reviewed in Miller, 1970), the benefit of dietary Zn was only noted in protozoa, as cellulolytic bacteria were adversely affected (Bonhomme et al., 1979). Interestingly, when Zn supplementation was fed to sheep in conjunction with a probiotic

Figure 4. Significant Pearson's correlations between factors and selected bacterial OTUs in rumen digesta from sheep receiving Zn from basal dietary components (control), organic Zn AA complex (ZnAA), or inorganic ZnSO₄ sources. Selected OTUs included OTUs which were discriminant by linear discriminant analysis labelled by OTU number and discriminatory treatment group, as well as the top 2 to 10 most abundant OTUs. Selected factors were chosen using RDA. The first checkpoint for BW was an averaged weight from 05/31/16 to 06/01/16, the second from 06/18/16 to 06/19/16, and the third from 07/26/16 to 07/27/16. One control sheep was determined to be an outlier and removed from this analysis.

of *Enterobacter* sp., *Bacillus* sp., *Lactobacillus* sp., and *Saccharomyces* sp., Zn absorption by the host was increased (Kwak et al., 2016).

The interplay of ruminant host—diet—microbiome complicates our understanding of the mechanisms of ruminant digestion and animal production. While a diverse rumen microbiome, having both high richness and species evenness, is widely associated with healthy animals lacking dysbiosis

(Petri et al., 2013a,b; Ishaq et al., 2017), many dietary studies nevertheless report better animal production, namely ADG and RFI, on starch-based diets despite low-diversity rumen bacterial communities diets (Wu et al., 2011; Belanche et al., 2012; Li et al., 2013; Shabat et al., 2016). Notably, Shabat et al. (2016) indicated that high feed efficiency was found in low-diversity dairy cattle rumen, although others have not (Jewell et al., 2015; Perea et al.,

2017), thus this effect may be specific to host species or physiological state.

In the present study, rams receiving a ZnAA-complex supplement had a lower rumen bacterial diversity than control rams, although richness was not significantly lower—possibly due to small sample size presented here but large individual variability inherent to host-associated microbiomes. Yet, the rams receiving the ZnAA treatment had higher ADG and tended to be more feed efficient than either control or ZnSO₄ groups (Page et al., 2017). This begs several questions. Does having high bacterial diversity in the rumen, and a resulting competition between host and microbiome for nutrients, preclude better animal performance? Which species can better compete and thrive in a low-diversity rumen, when the animal is in a healthy state, and is this a healthy steady state? If these species are beneficial to host digestion, is their emergence a sign that the functional rumen microbiome is able to be more focused on fermentation and less on microbial competition? Previous studies which showed a reduction in bacteria but increased in protozoa would seem to support this last idea (Bonhomme et al., 1979; Hilal et al., 2016).

Members of the phylum Tenericutes, the only phylum to be significantly altered in the present study, were increased in the ZnSO₄ group, which had numerically lower bacterial richness as well. Tenericutes are common rumen inhabitants (Mao et al., 2015) which lack cell walls, and in ruminants are commonly identified as pathogenic. Yet they are also associated with fiber particles in healthy cattle (AlZahal et al., 2017), with older ruminants (Jami et al., 2013), and positively correlated with residual feed intake and average milk fat (Jami et al., 2014).

With the exception of one OTU identified as *Prevotella* sp., most of the discriminatory OTUs were increased in one of the Zn treatment groups over Control. As total diversity was statistically reduced in the ZnAA, and only numerically reduced in the ZnSO₄ group compared to control, this again implies that certain taxa were enriched by Zn treatments at the expense of total richness. However, these enriched taxa were most likely to be beneficially associated with digestive function; including a number of OTUs identified as *Prevotella*, *Ruminococcus*, the BS-11 gut group, *Roseburia*, and unclassified genera within the Lachnospiraceae family (Scott et al., 2008; Bekele et al., 2010; Christopherson et al., 2014). It should be noted that Jewell et al. (2015) found a number of OTUs identified as the same genus associated with high or low feed efficiency, including many *Prevotella*.

While the abundance of the genus *Prevotella* was not changed in the present study, nevertheless, the particular *Prevotella* OTUs associated with Zn supplementation over Control was dramatically shifted.

Other indicator taxa appear to simply be along for the ride, such as those in the candidate-genus *Saccharimonas*; an obligate parasite of the oral bacteria *Actinomyces odontolyticus* (Bor et al., 2016). In the present study, *Actinomyces* was found in one control and one ZnAA sample, with a number of other unclassified Actinobacteria found throughout. Others are known to be affected by diet, including *Solobacterium*, which has been isolated from the human GI tract (Kageyama and Benno, 2000) and is particularly affected by lauric acid (Hristov et al., 2012; Petri et al., 2013a).

CONCLUSION

Total rumen bacterial evenness and diversity in yearling rams were reduced by supplementation with a Zn AA complex, but not in rams supplemented with an equal concentration of ZnSO₄. This is likely due to differences in bioavailability between organic and inorganically sourced supplement formulations. The only phylum to be significantly changed by ZnSO₄ supplementation was Tenericutes, suggesting that either Zn supplementation formulation could be utilized without causing a high-level shift in the rumen bacterial community which could have negative consequences for digestion and animal health.

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