Rumen Microbial Changes Associated With High Sulfur – A Basis for Developing Treatments for Ruminant Livestock in High Sulfur Water Regions

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FINAL REPORT

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Abstract: Reliable drinking water sources that meet minimum quality standards are essential for successful livestock production. Recent surveys have shown that many water sources, especially throughout the semi-arid rangelands of the U.S., are not of sufficient quality to support optimum herd/flock health and performance, in particular because of high concentrations of sulfur (S) and S-compounds present in the water. High S concentrations in water sources can arise from several factors. First, water sources can be naturally high in S. Second, drought conditions can cause S to be concentrated within the water source. Third, conventional oil and gas production can also increase S content within the water source. Combinations of these conditions can further exacerbate S levels in the water. Many of these water sources are used for livestock production systems, especially throughout the western states. However, high-S water is associated with poor performance and health in ruminant livestock, and is a primary cause of polioencephalomalacia (PEM), a disease state that can cause 25% morbidity and 25-50% mortality in affected populations. Producers are typically limited in available water resources and cannot avoid high S water situations; there are also no practical means of treating high S water. Although no effective treatments are currently available for animals suffering from the effects of high dietary S, it has been noted that animals vary in their response to elevated levels of S. While some animals consuming high S water exhibit reduced performance and/or poor health, others appear unaffected. We hypothesize that differences in rumen microbial populations, which are responsible for the breakdown of S and S-compounds, are associated with the variation in animal response to high S. Therefore, in this study we aim to determine 1) how rumen microbial populations change in response to high S water; 2) if the extent of those changes are associated with host ability to better tolerate high S; and 3) the functionality of the rumen microbial changes through a more global analysis of the rumen microbiome. A better understanding of the rumen microbial response to high S will lead to development of treatments for affected animals.

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Statement of Critical Regional or State Water Problem: *Need for Project.* Ruminant livestock consuming water high is sulfur (S) and S-compounds (e.g., sulfate) are prone to poor performance and health. High S can also cause polioencephalamalcia (PEM), a disease state in that can cause 25% morbidity and 25-50% mortality in affected population. Ruminants are especially susceptible to S toxicity because of S metabolism in the rumen by sulfate-reducing bacteria (SRB). High S triggers metabolism by SRB to sulfide, which ultimately increases hydrogen sulfide (H_2S) production in the rumen. It is this increase in H_2S production that is thought to be causal to the poor performance and health (including PEM) of ruminant animals.

Unfortunately, many livestock water sources, especially throughout the semi-arid rangelands of the U.S. and Wyoming, are high in S and S-compounds because of underlying soil conditions or man-made contaminants (e.g., conventional gas and oil water), and are exacerbated by evaporation concentrating S during persistent drought conditions. Producers are typically limited in available water resources, and there are no practical means of treating high S water, especially in range conditions, nor animals suffering the effects of high S water.

The literature is replete with studies aimed at identifying treatments for animals affected by high S. Why, then, have no effective, consistent treatments been discovered yet? Most studies have identified potential treatments *in vitro*, or in laboratory studies using rumen bacterial cultures. However, once these treatments were tested *in vivo*, or in the live animal, limited effects on animal health or performance were typically observed. It is apparent from these studies that *in vivo* and *in vitro* conditions respond differently to high S, indicating the need for a whole-animal approach. A better understanding of how rumen microbial populations (e.g., bacteria) *in vivo* change in response to high S is a critical first step to whole animal studies aimed at targeted treatment development.

Who Would Benefit and Why. Many water sources high in S are still used for livestock production due to lack of alternative available sources, especially in range production settings. Additionally, in many of these areas it is neither feasible nor practical to haul in water low in S. Therefore, identification of an effective treatment for either 1) high S water sources or 2) animals suffering from high S would benefit livestock producers by 1) preventing the health and performance problems associated with livestock consuming high S water, and 2) allowing them to use available water resources despite high S concentrations.

Statement of Results or Benefits: *Information Gained.* Past studies of changes in rumen microbial population in response to high S have utilized technologies either limited to determining the presence/absence of microbial species, or have utilized *in vitro* culture methods capable of altering bacterial metabolism so that *in vivo* conditions are not truly reflected [1]. We used DNA sequencing techniques [2] to better identify and quantify changes in rumen microbial populations in response to high S water.

How Information Was/Will Be Used. Identification of rumen microbial species important to the response to high S water is a critical steps towards development of effective prevention and treatment strategies. The results from study enabled us to determine microbial species that responded (via changes in abundance – either lesser or greater abundance) to the high S drinking water, and furthermore determine those species that appear to 'adapt' to the high S challenge by returning to pre-challenge abundance. Microbial species that are particularly responsive to high S water may help to identify treatments for high S water, such as promoting microbial species that help process and eliminate the excess S or limiting growth of species associated with the H_2S generation that leads to impaired animal health and performance. Our *next step* will be to closely study the confirmed microbial species to determine functional properties that convey this responsiveness to high dietary S, and then determine means to optimize abundances to improve overall animal response.

Nature, Scope, and Objectives of the Project: The basic nature of the proposed research was to determine changes in rumen microbial populations in response to high S water. Our objective was to use DNA sequencing to quantify and characterize rumen microbial populations in sheep (our model ruminant) consuming high S water; this approach allowed us to more accurately determine important rumen microbial changes in response to high S. We hypothesized that differences in rumen microbial populations, which are responsible for the breakdown of S and S-compounds, were associated with the host animal response to high S. Therefore, our objectives were to 1) determine how rumen microbial populations change in response to high S water, 2) determine if the extent of those changes were associated with variation in host animal response, and 3) determine functionality of the rumen microbial changes through a more global analysis of the rumen microbiome.

Timeline of Research Activities: ***Year 1*** – Completed animal trial, serum mineral analyses, production data analyses, and DNA extractions and preparations. ***Year 2*** – DNA sequencing (conducted at the DNA Core Laboratory in Columbia, Missouri) completed. Bioinformatic analyses were conducted to identify specific rumen microbial species that change (in abundance) in response to high S water, and importantly identify if such changes infer a "tolerance" to high S. Analysis of ruminal volatile fatty acids (VFAs) was also completed to determine shifts in rumen function. M.S. student Cara Clarkson completed her M.S. thesis in the summer of 2014. ***Year 3*** – Project was extended to include a second animal trial to confirm responsive microbial species and perform a functional analysis to better understand the biology underlying host response to high S water. M.S. student Amy Abrams is on-track to complete her M.S. thesis in summer of 2016.

Methods, Procedures, and Results: The initial animal study used Hampshire wether lambs (n = 40; 6 months of age) maintained at the UW Stock Farm. Lambs were administered a high S water treatment (~3,000 mg/L) over a 28 d trial period; they were individually penned to enable collection of individual feed and water intake. This level of S was chosen based on previous studies and because sheep have a higher tolerance to S because of S requirements for wool [3]. A 28 d trial period was chosen as signs of S toxicity would be more easily observed with the collection of individual feed and water intake; a common sign of S toxicity is decreased feed and water intake. Individual water and feed intake were estimated, along with average daily gain and feed efficiency measures. Blood and rumen fluid samples were collected on d 0 (baseline), d 7, and d 28.

Blood samples were analyzed for S, Cu, and Mo content, and no differences between individual animals were detected. Lambs were selected as highly tolerant (n = 4) and lowly tolerant (n = 4) to high dietary S based on individual water and feed intake, average daily gain and feed efficiency measures, and daily behavioral responses (recorded on a scale of 1 to 5). Rumen fluid samples collected on d 0, d 7, and d 28 (n = 24 samples in total were used for both VFA and DNA analyses. The VFA analysis indicated that intolerant lambs had greater concentrations of isobutyrate and isovalerate, but a lower concentration of valerate. The response in valerate has been observed in other high-S studies, and may indicate a potential adaptation by the rumen microbial population in response to high S. Also, initial concentrations of propionate were greater in tolerant lambs. Propionate is an end-product of starch and sugar fermentation, and considered a more efficient energy source for fermentation. The greater initial concentrations of propionate in tolerant animals may have given rise to greater ability to metabolize high levels of S.

The DNA analysis was used to generate rumen microbial profiles associated with response to high S water. In total, 145 microbial taxa (assumed to be single microbial species) were identified in the rumen fluid, with 29 affected by the tolerance class (tolerant or intolerant) by sampling day interaction; 39 affected by tolerance classification; and 26 affected by sampling day. Species in the *Prevotella* genus were highly detected as would be expected, but there were also numerous specie differences that may be potential indicators of tolerance to high S water. Also, some species responded initially to the high-S water challenge, but then returned to more 'normal' abundances, indicating that certain microbial species are capable of, and important for, adapting to a high S water challenge.

The second animal trial used Hampshire-cross lambs (n = 12; 6 months of age) also maintained at the UW Stock Farm. Similar to the first animal trial, these lambs were administered a high S water treatment (~3,000 mg/L) over a 28 d trial period; they were individually penned to enable collection of individual feed and water intake. Lambs remained individually penned for another 7 d after the trial period to allow for individual data collection to monitor recovery after removal of the high S water treatment. Individual water and feed intake were estimated, along with average daily gain and feed efficiency measures. Blood and rumen fluid samples were collected on d 0 (baseline), d 7, d 28, and d 35.

The DNA analysis revealed a total of 287 taxa in the samples from the second trial; abundance of 39 of those taxa differed with sampling day. When looking at abundance changes in association with the high S water treatment, several of the microbial species were in common between the two animal trials. While some of these were not in agreement across the two trials, several were and may be candidates for future treatment development. Two species of particular interest, due to their functions and agreement between the two trials, include *Prevotella nigrescens* and *Butyrivibrio fibrisolvens.* The functional analysis, based on sequence data from the second trial, is near completion and is expected to add new insights into host response to high dietary S.

Significance. Results overall confirm that the rumen microbiome does respond to high S water, and suggests that certain microbial species are particularly important to the host animal response to high S. Future research efforts will be aimed at using these results to develop treatments to improve animal response to high S water.

Student Training. This research served as two M.S. thesis projects – one for Ms. Cara Clarkson (M.S. – Summer 2014) and one for Ms. Amy Abrams (M.S. – Summer 2016). Both students were trained the areas of animal production, toxicity, genomics, water quality, and laboratory analyses (DNA sequencing, VFAs, etc.) and were responsible for carrying out all aspects of this research project, including both the animal and laboratory components. Ms.

Clarkson's portion of the research generated two research abstracts and two meeting proceedings; one manuscript has been prepared and is in the process of peer-review prior to journal submission (*Small Ruminant Research*). Ms. Clarkson is currently employed in management in the animal industry (Assistant Ranch Manager; Cheley Colorado Camps; Estes Park), with plans of seeking a Ph.D. program in the near future. Ms. Abrams' portion has generated two research abstracts and one meeting proceeding to-date; we anticipate an additional manuscript from this portion of the work shortly. Ms. Abrams was recently honored as an Outstanding Young Scholar by the Western Section of the American Society of Animal Science and was also selected as the Outstanding M.S. Student by the University of Wyoming's Chapter of Gamma Sigma Delta. She will be starting a Ph.D. program in Fall of 2016 at South Dakota State University. Finally, many undergraduates have assisted with these projects, further adding to the student training portion of this grant.

Publications To-Date:

- Abrams, A.N., C.J. Clarkson, K.J. Austin, M.J. Ellison, H.C. Cunningham, G. Conant, W.R. Lamberson, T. Taxis, and K.M. Cammack. 2016. Altered rumen microbial populations in response to high sulfate water in lambs. Proc. Western Sec. Young Scholar. *Accepted*.
- Powell, S., A.N. Abrams, K.J. Austin, D.C. Rule, E.A. Van Kirk, M.J. Ellison, H.C. Cunningham, G. Conant, W.R. Lamberson, T. Taxis, and K.M. Cammack. 2016. High sulfate water affects volatile fatty acid profiles in lambs. Plant & Animal Genomes XXIV Conference. P0608.
- Abrams, A.N., K.J. Austin, M.J. Ellison, H.C. Cunningham, G. Conant, T. Taxis, W.R. Lamberson, and K.M. Cammack. 2016. Effect of high sulfate water on rumen microbial populations in lambs. Plant & Animal Genomes XXIV Conference. P0607.
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- Clarkson, C.J., H.C. Cunningham, L.E. Speiser, M.J. Ellison, K.J. Austin, and K.M. Cammack. 2014. Effect of high sulfur water on behavior, performance, and volatile fatty acid production in lambs. Western Sec. ASAS. *In press*. (Abstract and Meeting Proceeding)
- Abrams, A. N., K. J. Austin, M. J. Ellison, H. C. Cunningham, G. Conant, W. R. Lamberson, T. Taxis, and K. M. Cammack. 2015. High sulfate water affects rumen microbial populations in lambs. (Abstract.) High Plains Nutrition and Management Roundtable, Lingle, Wyoming. September 10, 2015.

Related Research: The current NRC recommendation for dietary sulfur is < 0.3% dry matter (DM), with the maximum tolerable concentration estimated at 0.4% DM [4]. Sulfur content in water, however, is typically reported in parts per million (ppm), and the most common form of S in water is SO_4^{2}. Polioencephalmalacia is associated with water SO_4^{2-} concentrations of ≥ 2,000 mg/L, which when combined with a typical 0.2% DM S feedstuff results in 0.53% DM total dietary S [5]. Therefore, when S or SO_4^{2-} content of water is included in the estimation of dietary S, the total dietary S is often much higher than anticipated.

Mechanism in the Rumen: In ruminants, production of toxic metabolites from S occurs in the rumen. Two classes of bacteria, assimilatory and dissimilatory, are present in the rumen capable of reducing SO_4^{2-}. Sulfide is produced by assimilatory S-reducing bacteria (SRB) and is used immediately for incorporation into metabolic processes [6]. The assimilatory SRB also reduce SO_4^{2-} to create amino acids. Dissimilatory SRB use SO_4^{2-} in respiration pathways and for energy to fuel growth and metabolism. However, in the respiratory pathways excess S^{2-} and H_2S are produced [7]. It is the dissimilatory class of SRB that cause the overproduction of toxic S products leading to cell damage, secondary infections, and the development of sPEM. These bacteria can also produce high amounts of S^{2-} [7], causing H_2S levels to increase rapidly.

Rumen Microbes. The rumen ecosystem is incredibly complex, and limitations in past technology have made it difficult to accurately classify and characterize rumen microbes. Traditional methods for determining microbial composition have relied on culture techniques. However, not all microbes can be cultivated with conventional laboratory procedures; therefore, the microbes that are cultured do not accurately represent the microbial communities [2]. These traditional methods have also been cited for lack of sensitivity [1]. DNA sequencing is a more sensitive technique, allowing for accurate identification of known and previously unknown microbes [2]. Recently other molecular techniques, such as PCR, have been used to successfully detect changes in SRB in response to S [8]. However, PCR techniques are dependent upon the probe sets chosen for specific bacteria; DNA sequencing quantifies each strain present and is not limited to detecting differences in only those bacteria queried.

Sources of High-S Water: Survey and field data have consistently shown surface and subsurface water can be high in SO_4^{2-}, particularly throughout the western regions of the U.S. The Water Quality for Wyoming Livestock & Wildlife review [9] reported that of > 450 forage and water collection sites located throughout the U.S., 11.5% exceeded the dietary S concentrations considered safe for livestock. Of those sites, 37% were located in the western U.S., including Wyoming. Drought further exacerbates the high SO_4^{2-} problem, as SO_4^{2-} is concentrated in the water due to greater evaporation and reduced moisture recharge [10]. Conventional gas and oil produced water discharge can also be high in SO_4^{2-}, particularly in arid regions such as the Big Horn Basin (John Wagner, personal communication). Of five water discharge sites sampled in the Big Horn Basin, two exceeded 2,000 mg SO_4^{2-}/L [11], well above the limit for livestock consumption. Although many CBM water sources are low in SO_4^{2-}, including those in the Powder River Basin, there have been reports of high and variable SO_4^{2-} concentrations (hundreds to thousands of mg/L) in CBM waters from the Fort Union Formation in Campbell County [11]. Because of the limited availability of water resources in those regions, many of those sources high in SO_4^{2-} are still the only option for livestock production, and there are no feasible methods for removing SO_4^{2-} from water (especially in a range situation).

High-S Water Effects on Livestock: Several experimental and field studies have reported reductions in performance of animals exposed to high S drinking water sources. Declines in average daily gain in cattle consuming high SO_4^{2-}water have been reported in both grazing and confined environments [12]. In addition, decreases in feed consumption and overall body weight gain are consistently reported. Polioencephalomalacia is characterized by necrosis of the cerebral cortex and remains one of the most prevalent central nervous system diseases in cattle and sheep [4]. Clinical signs of PEM include head pressing, blindness, incoordination, and recumbency accompanied by seizures, with young ruminants more commonly affected. The limited amount and availability of quality water is problematic for producers, especially when livestock consuming SO_4^{2-} contaminated water are also exposed to forages with elevated S levels.

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