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Short communication

Analysis of selected rumen microbial populations in dairy heifers limit fed diets varying in trace mineral form and starch content

Kristina Kljak[a], Felipe Pino[b], Kevin J. Harvatine[b], Arlyn J. Heinrichs[b,*]

[a] Department of Animal Nutrition, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia
[b] Department of Animal Science, The Pennsylvania State University, 324 Henning Building, University Park, PA 16802, United States

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ABSTRACT

Eight rumen-cannulated Holstein heifers were used to explore the effect of trace mineral form and diet starch content on selected ruminal microbial populations under limit feeding conditions. Heifers were subjected to a split-plot, 4×4 Latin square design with 19-day periods. Trace mineral form [organic proteinates (OTM) or inorganic sulfates (ITM)] was the whole-plot factor, and starch content (3.5%, 12.9%, 22.3%, and 31.7% DM) was the sub-plot factor. Rumen samples were collected 3 h after feeding on day 18 of each period, and DNA was extracted. Relative abundances of 6 well-studied bacterial taxa, total anaerobic fungi, ciliate protozoa, methanogenic Archaea and bacteria were determined using validated primer sets by real-time quantitative PCR. Targeted populations had relative abundances comparable to those previously reported. Of the microbial populations measured, trace mineral form influenced only *Prevotella bryantii*, which was increased by OTM. Increasing dietary starch concentration linearly decreased methanogenic Archaea, total bacteria, *Prevotella* spp., and *Prevotella bryantii*, and tended to linearly decrease fungi and protozoa. In conclusion, contrary to the starch content, trace mineral form had limited impact on the abundance of selected microbial populations in limit fed heifers 3 h after feeding. The unexpected effect of starch content on bacterial populations and protozoa could be the result of different eating patterns of heifers fed diets varying in starch content.

1. Introduction

Fermentation in the rumen comprises a symbiotic relationship between the host animal and microorganisms within the rumen. Digestion of plant material through this complex relationship provides a large proportion of the nutrients needed to support productive activities of the host animal. It is generally assumed that the microbial population adapts to the substrate available for fermentation, and thus, that diet can cause changes in the microbial population within the rumen. This assumption was supported by previous research such as Tajima et al. (2001), who reported changes in the relative abundance of bacterial populations when high-hay diet was replaced with high-grain diet.

Trace minerals (TM) such as copper, manganese, zinc, and selenium are important components of enzyme systems of animals. They are also needed for proper functioning of the microorganisms participating in enzymatic processes in the rumen, which implies that fermentation may be impaired when a diet is deficient in TM (Durand and Kawashima, 1980). In the same manner, level or form of TM in the diet could modify the microbial population in the rumen. While many studies (Arelovich et al., 2000; Engle and Spears, 2000; Solaiman et al., 2007; Wang et al., 2009) have investigated the effects of supplemented TM on fermentation in the rumen, work regarding the effects of TM on microbial populations is scarce. In addition, supplementing an organic form of TM (OTM) instead of inorganic (ITM) could improve production, reproduction, and health (Rabiee et al., 2010), but the effects of OTM on populations of ruminal microorganisms has not yet been investigated.

Adequate nutrition of dairy heifers is essential to increase dairy industry profitability due to its effect on health and milk yield after calving. Limit feeding enables controlled growth of heifers while increasing feed efficiency and reducing manure production (Hoffman et al., 2007). Furthermore, a high energy diet, as one of the strategies of limit feeding, has no negative effect on rumen health (Lascano et al., 2009). However, the increased level of starch in such diets can affect rumen fermentation because the type of carbohydrate consumed modifies the microbial populations (Fernando et al., 2010). In addition, it is possible that TM supplementation could affect the microbial populations of heifers limit fed high energy diets (Kišidayová et al., 2001). Summers et al. (1957) observed that cellulose degradation

* Corresponding author.
E-mail address: ajh@psu.edu (A.J. Heinrichs).

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decreases at high levels of starch because of a TM deficiency caused by fast-growing, starch digesting bacteria to the detriment of slow-growing, cellulose digesting bacteria. Since changes in the rumen environment affect metabolic performance of heifers, the objective of the present study was to examine the influence of TM form and diet starch content on selected ruminal microbial populations of dairy heifers with restricted intakes. This study is a continuation of a study by Pino and Heinrichs (2016) where effects of TM form and starch content on total tract nutrient digestibility and rumen fermentation were evaluated. Briefly, results of that study showed that TM form did not affect dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose, or starch digestibility, while starch content affected only hemicellulose. Heifers fed OTM achieved lower mean and minimum ruminal pH with higher butyrate proportion in total volatile fatty acids (VFA) compared to ITM. The highest VFA production was achieved 4 h after feeding. Butyrate proportion linearly increased with increasing starch in diets. Due to the results of this previous study, amylolytic bacteria, including butyrate producing bacteria, were evaluated in our study.

2. Materials and methods

2.1. Experimental diets and animals

All procedures utilizing animals were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (PSI # 42881). Eight Holstein heifers fitted with a 10-cm rumen cannula (Bar Diamond, Pharm, ID) were housed in individual tie-stalls in a mechanically ventilated barn with free access to water. After a pre-trial adaptation period, heifers were randomly assigned to a split-plot 4×4 Latin square experimental design. Each period lasted 19 days and included 15 days of adaptation and 4 days of sampling. A detailed description of the experimental design and treatments was previously reported by Pino and Heinrichs (2016). Briefly, the whole-plot factor was the type of trace minerals supplemented (ITM as sulfates and OTM as proteinates; Bioplex, Alltech Inc., Nicholasville, KY), and the sub-plot was the level of starch in the diet (3.94%, 12.95%, 22.25%, and 31.73% DM; Supplemental Table 1). Rations were formulated to provide a forage-to-concentrate ratio of 55:45, while the increasing starch level of treatments was achieved by increasing the content of ground corn and decreasing the content of beet and citrus pulp.

2.2. Ruminal sample collection

Rumen digesta samples were collected on day 18 of each experimental period 3 h after feeding; according to Weimer et al. (1999) this time is near the midpoint of the upper and lower pH values of ruminal contents within the feeding cycle. Representative samples (400–500 g) were taken during complete rumen evacuation of the cannulated heifers; after transfer into large plastic containers, rumen contents were mixed and sampled in a manner to maintain solid-to-liquid ratio as in the rumen environment. All procedures were done in the shortest possible time and in a manner to minimize oxygen exposure. Digesta was then immediately returned to the rumen of each heifer, while rumen samples were stored at −20 °C until analyses. Samples were freeze-dried (Ultra 35-XL; Virtis Co. Inc., Gardiner, NY) and ground (Wiley mill, Arthur H. Thomas, Philadelphia, PA) just prior to the DNA extraction.

2.3. DNA extraction and real-time PCR analysis

Microbial DNA was isolated from freeze-dried rumen digesta using a commercial kit (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek, Inc., Norcross, GA) employing bead-beating processing and purification by column extraction. Extraction was performed according to manufacturer recommendations except for modification of sample weight and volume of SLB buffer (100 mg and 1.0 mL, respectively). Isolated DNA was quantified by spectroscopy (NanoDrop ND-1000 Spectrophotometer, Nanodrop technologies, Wilmington, DE), and sample volumes were adjusted to achieve uniform DNA concentrations (100 ng/μL) across all samples.

Quantitative real-time PCR was used to determine the relative abundance of 6 bacterial populations [*Prevotella* spp. (genus level primer with *Prevotella bryantii* GA33 as test strain), *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio*, *Fibrobacter succinogenes* (strain S85), *Prevotella bryantii* (strain B_14), *Megasphaera elsdenii* (strain YE34), and *Selenomonas ruminatum* (strain D)], total bacteria, anaerobic fungi, ciliate protozoa and methanogenic Archaea. Six bacterial populations were chosen based on volatile fatty acid changes from the previous study (Felipe and Pino, 2016), with a special focus on butyric acid producing populations of *B. fibrisolvens* and *M. elsdenii*. Previously validated primers (final concentration 400 nM) and conditions were used (Supplemental Table 2). Quantitative real-time PCR was performed in triplicate using a commercial mix (PerfeCTa SYBR Green SuperMix, with ROX, Quanta Biosciences, Gaithersburg, MD) and an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY). Primer specificity was evaluated by melting curve analysis, and no evidence of off-target amplification was observed. The number of cycles required to reach threshold adjusted for each taxa (Ct) was recorded for each sample. Reaction efficiencies were estimated using a 7-point sample dilution curve for each primer on each plate and calculated according to the equation $E=10^{-1/slope}$ where slope is derived from regressing Ct values against their log-transformed dilution coefficients (Supplemental Table 2).

2.4. Calculation and statistical analysis

Six bacterial populations were quantified as a percent of total bacteria using the equation $E_{total\ bacteria}^{Ct(total\ bacteria)}/E_{target}^{Ct(target)}$ where E represents the efficiency of the reaction for the universal bacteria primer set ($E_{total\ bacteria}$) or the target gene (E_{target}), and Ct of both total bacteria and target primer sets is the average value for each sample. Abundance of total bacteria, anaerobic fungi, ciliate protozoa, and methanogenic Archaea are reported relative to the diet with ITM supplementation and 3.54% starch. This diet was chosen as a control because ITM is the conventional supplementation method and utilizing the lowest starch content facilitates data presentation in ascending order of starch content.

All statistical analyses were conducted in SAS (release 9.4, SAS Institute Inc., Cary, NC) using the MIXED procedure as previously described Pino and Heinrichs (2016). The 4×4 Latin square model included TM, level of starch treatment, and their interaction as a fixed effects, and heifer within TM treatment as a random effect. Residual variances were assumed to be normally distributed, and all data is presented as least square means. Differences were considered significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$ for main effects.

3. Results

Relative abundances of the taxa investigated are presented in Table 1. Total bacteria, fungi, ciliate protozoa, and methanogenic Archaea populations were not affected by TM form, however, their abundance decreased with increasing starch content in heifers' diets (Table 1). Changes due to dietary starch were significant for populations of methanogenic Archaea and total bacterial species ($P=0.012$ and 0.047, respectively), while populations of fungi and protozoa showed tendencies ($P=0.062$ and 0.075, respectively).

With a range from 13% to 23%, *Prevotella* spp. comprised the largest proportion of total bacteria from the selected taxa. Abundance of remaining bacterial populations was less than 1% except for *S. ruminatum*, which ranged from 2.2% to 2.8%. *P. bryantii* was the only investigated taxon that showed a response to dietary TM form. Heifers