Effects of *Mannheimia haemolytica* challenge with or without supplementation of *Saccharomyces cerevisiae boulardii* strain CNCM I-1079 on immune upregulation and behavior in beef steers¹

William Christian Kayser,[†] Gordon E. Carstens,^{†,2} Ira Loyd Parsons,[†] Thomas H. Welsh,[†]
Kevin E. Washburn,[‡] Sara D. Lawhon,^{||} William E. Pinchak,[§] John T. Richeson,[¶]
Eric Chevaux,[&] and Andrew L. Skidmore[&]

[†]Department of Animal Science, Texas A&M University, College Station, TX 77843; [‡]Department of Large Animal Clinical Sciences, Texas A&M University, College Station, TX 77843; ^{||}Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843; [§]Texas Agrilife Research and Extension Center, Vernon, TX 76385; [¶]Department of Agricultural Sciences, West Texas A&M University, Canyon, TX 79016; and [&]Lallemand Animal Nutrition, Milwaukee, WI 53218

ABSTRACT: Objectives of this experiment were to examine the effects of live yeast (LY) supplementation on immunological, physiological, and behavioral responses in steers experimentally challenged with *Mannheimia haemolytica* (MH). Thirty-six crossbred Angus steers (BW = 352 ± 23 kg) seronegative for MH were allocated within a 2 × 2 factorial arrangement: Factor 1 = roughage-based diet with LY (*Saccharomyces cerevisiae boulardii* CNCM I-1079, 25 g/per steer daily) or negative control (CON). Factor 2 = bronchoselective endoscopic inoculation with MH or phosphate buffer solution (PBS). Steers were fed their respective diets for 28 d prior to MH challenge on day 0. Reticulorumen temperature (RUT; ThermoBolus, Medria) was measured continuously at 5-min intervals and rectal temperature on days -4, 0, 1, 2, 3, 5, 7, 10, and 14 relative to MH inoculation. Compared with PBS-treated steers, the steers inoculated with MH had increased ($P < 0.05$) RUT from 2 to 24 h, reaching a zenith (>41 °C) 9 to 11 h post-MH challenge, whereas rectal temperature was increased ($P < 0.04$) in MH-inoculated steers on day 1 post-MH challenge. Supplementation with LY increased ($P < 0.05$) rectal temperature on days 0, 7, and 10, relative to CON steers. There

were inoculation × day interactions ($P < 0.01$) for lymphocyte, neutrophil, leukocyte, and haptoglobin concentrations. Steers challenged with MH had increased ($P < 0.05$) neutrophil concentration from days 1 to 3, leukocyte concentration on days 1 and 2 and haptoglobin concentration on days 1 to 5 post-MH challenge compared with PBS-treated steers. Steers supplemented with LY exhibited increased ($P < 0.02$) cortisol throughout the study compared with the CON treatment. Following inoculation, MH-challenged steers exhibited reduced ($P < 0.05$) DMI, eating rate, frequency, and duration of bunk visit (BV) events compared with PBS-treated steers. Results from this study demonstrate that the experimental challenge model effectively stimulated acute-immune responses and behavioral changes that are synonymous with naturally occurring bovine respiratory disease (BRD). However, supplementation with LY minimally altered the impact of the MH challenge on physiological and behavioral responses in this study. Continuously measured RUT was more sensitive at detecting febrile responses to MH challenge than rectal temperature. These results serve to guide future research on behavioral and physiological alterations exhibited during a BRD infection.

Key words: bovine respiratory disease, feeding behavior, *Mannheimia haemolytica*, *Saccharomyces cerevisiae boulardii*

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²Corresponding author: g-carstens@tamu.edu

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INTRODUCTION

There is a renewed sense of importance to the development of animal health strategies that reduce the use of antibiotics in livestock production, which has increased the emphasis placed on disease prevention and the discovery of antibiotic alternatives. In order for the beef industry to reduce the use of antibiotics, suitable alternatives are a prerequisite. Bovine respiratory disease (BRD) continues to cause economic losses and accounts for 67% to 87% of the morbidity events within feedyards (Edwards, 1996). One of the challenges associated with BRD is that current industry methods for detection of BRD rely on visual appraisal of clinical symptoms (Broom, 2006) that have limited diagnostic accuracy. In fact, Timsit et al. (2016) recently reported that these BRD detection methods have poor sensitivity (0.27), but high specificity (0.92). This low sensitivity of detection indicates that BRD cases often go untreated, which is one of the primary reasons that metaphylaxis therapy remains an effective strategy to mitigate BRD (Ives and Richeson, 2015).

Potential nonantibiotic alternatives include direct-fed microbials (DFM) that the FDA classifies as a source of naturally occurring live microorganisms (Yoon and Stern, 1995). Yeast products such as *Saccharomyces cerevisiae* are widely used in commercial ruminant production systems, specifically dairy operations as beneficial production responses (e.g., milk yield) have been demonstrated (Beauchemin et al., 2006). There is also some evidence to demonstrate that supplementation with *S. cerevisiae* is effective at reducing morbidity in stressed calves (Zinn et al., 1999). *Saccharomyces cerevisiae boulardii* (live yeast [LY]) is a subspecies of *S. cerevisiae* and is one of the most widely studied microorganisms due to its application in human patients to combat enteric diarrhea (Łukaszewicz, 2012). Supplementation with DFM could potentially serve as a nonantibiotic approach to improving health of newly received or stressed cattle (Duff and Galyean, 2007).

Objectives of this experiment were to examine the effects of LY supplementation on immunological, physiological, and behavioral responses in

steers experimentally challenged with *Mannheimia haemolytica* (MH).

MATERIALS AND METHODS

All animal care and use procedures were in accordance with the guidelines for use of Animals in Agricultural Teaching and Research as approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC # 2015-0379) as well as the Texas A&M University Institutional Biosafety Committee (IBC # 2015-068).

Animals

Thirty-six Angus crossbred steers (initial BW = 386 ± 25 kg), originating from 2 cattle populations located at the McGregor Research Center (McGregor, TX) and the Beef Cattle Systems Research Center (College Station, TX), were used in this study. The steers were approximately 11 mo of age at the start of the study. At approximately 4 to 5 mo of age and again 3 wk prior to weaning, steers were vaccinated for respiratory viral pathogens (Triangle 5; Boehringer Ingelheim Vetmedica, St. Joseph, MO; BHV-1, bovine viral diarrhea 1 and 2, bovine respiratory syncytial virus, and parainfluenza-3). The steers were also vaccinated for *Clostridial* diseases (Covexin 8; Merck, Madison, NJ) at 4 to 5 mo of age, but were not vaccinated for MH prior to enrollment in the study.

All animals were considered clinically healthy based upon daily observations for the 28 d prior to challenge. To qualify for the study, steers had to be seronegative for MH. To accomplish this, serum samples were collected from 84 steers to evaluate antibody titers against MH using the whole-cell agglutination test (Texas Veterinary Diagnostic Laboratory; TVMDL). The steers selected for the study had MH antibody titers that were less than 1:1 dilutions, which is the positive threshold established by TVMDL (ranged from 6 to 9 dilutions). Additionally, steers were confirmed to be negative for persistently infected bovine viral diarrhea virus (BVDV) through analysis of ear notch samples that were analyzed

using the BVD antigen-capture ELISA (BVD-Ag ELISA) method.

Experimental Design and Treatment Arrangements

Steers were stratified by herd origin, initial BW, MH titer dilution, exit velocity, and prestudy ADG, and randomly assigned to 1 of 4 treatments ($n = 9$) arranged in a 2×2 factorial array. Factor 1 being a roughage-based diet without (CON) or with added LY (*S. cerevisiae boulardii* strain CNCM I-1079 at 25 g-per steer daily; Proternative Advantage; Lallemand Animal Nutrition), and Factor 2 being bronchoselective endoscopic inoculation with MH or phosphate buffer solution (PBS). Therefore, the 4 treatments groups were ($n = 9$) as follows: 1) MH-CON, 2) MH-LY, 3) PBS-LY, and 4) PBS-CON.

This experiment lasted 18 d and was conducted within a larger 84-d growth and performance study. Throughout the study, all animals were housed in 4 pens equipped with electronic feed bunks (GrowSafe Systems Ltd., Airdrie, AB, Canada) at the Beef Cattle Systems Research Center (College Station, TX). Steers were segregated in pens by dietary treatment, with PBS-treated and MH-inoculated steers comingled within each pen. Therefore, steers were fed the CON diet in 2 pens, and the LY diet in the other 2 pens, with equal number of PBS-treated and MH-inoculated steers within pen. Steers were offered the diets *ad libitum* and were fed twice daily at 0700 and 1600 h. The diet (DM basis) contained 36.5% dry rolled corn, 24% corn dried distillers grains, 30% chopped alfalfa hay, 5% molasses, 2.5% dry mineral, and 2% premix, which was comprised of corn dry distillers grains and either the LY or an isocaloric isonitrogenous placebo.

The targeted intake of the LY product for this study was 25 g-per steer daily to maintain a level of colony forming units (CFU) of LY strain CNCM I-1079 at or above a prescribed level of 1×10^{10} per d. The proportions of the LY and placebo products in the premixes were adjusted weekly based on changes in dietary treatment feed intakes. The actual average intakes of LY and placebo were 24.3 and 24.9 g-per steer daily, respectively. Samples of the control and LY diets were collected and analyzed weekly to determine the presence of yeast colonies. Throughout the study, no CNCM I-1079 or wild yeast colonies were detected in the CON diet. Diets were delivered each day by hand, and the feed mixer flushed with chopped hay after mixing the LY diet.

Inoculation Preparation and Procedure

The *M. haemolytica* inoculum was prepared as described by Mosier et al. (1995). Briefly, *M. haemolytica* serotype A1 was grown on trypticase soy agar containing 5% sheep blood for 18 h at 37 °C in 7% CO₂. Colonies were inoculated into brain-heart infusion broth and incubated for 16 to 18 h at 37 °C with aeration. The bacteria were then centrifuged at $3,000 \times g$ for 15 min at 4 °C and washed with PBS twice. After the second wash, the bacteria were centrifuged as before and the pellet was resuspended in PBS at a final concentration of 1.2 to 1.4×10^9 CFU/10-mL dose. After preparation, the inoculum was placed on ice in a dark cooler and transported to the site of inoculation (approximately 17 km).

On day 0, all animals were inoculated with either MH or PBS. To avoid the chance that PBS-treated steers would receive MH via contamination of the instruments used for inoculation, the PBS-treatment groups were inoculated prior to the MH-treatment groups. The inoculations were performed with an endoscope as described by Theurer et al. (2013). Steers were restrained in a standard squeeze chute that allowed the head to be restrained. An endoscope 1 m in length was inserted into the ventral meatus of one nostril and passed into the trachea to the level of the right apical lung lobe bronchi allowing visualization of the opening. A sterile bronchoalveolar lavage tube was inserted into the endoscope portal and passed until the tip of the lavage tube was visible emerging from the endoscope. Thereafter, the lavage tube was advanced another 1 to 2 cm into the opening of the right apical lung lobe bronchi. Once in place, the PBS-treated steers were administered 10 mL of PBS followed by a 60-mL flush of PBS (total PBS = 70 mL). Following the inoculation of the PBS-treated steers, the endoscope was disinfected with chlorhexidine solution and rinsed with saline. Subsequently, the MH-inoculated steers were challenged with 10 mL of *M. haemolytica* serotype A1 at 1.2 to 1.4×10^9 CFU/mL followed by 60 mL of PBS for a total volume of 70 mL.

Data Collection

Temperature monitoring and clinical illness scoring. Rectal temperatures were recorded using a digital thermometer (Cooper TM99A, Cooper-Atkins Corporation, Middlefield, CT) on days -4, 0, 1, 2, 3, 5, 7, 10, and 14, with BW also recorded on these days. In addition, radiofrequency biothermal boluses (ThermoBolus, Medria, Châteaubourg,

France) were inserted into the rumen of all steers prior to initiation of the study. The ThermoBolos continuously recorded reticulo-rumen temperature (RUT) at 5-min intervals. A proprietary algorithm was used to remove variation in RUT due to drinking events. Summary statistics of RUT were computed on a daily basis (beginning at 00:00) for the duration of the experiment and an hourly basis from 24 h preinoculation to 48 h postinoculation. All steers were monitored by 2 experienced evaluators twice daily throughout the experiment for clinical signs of BRD. The visual evaluation method used in the experiment has been described in detail by [Step et al. \(2008\)](#). The criteria included signs of depression, inappetence, and respiratory distress, with evaluators assigning scores of 1 to 4 (1 = mild, 2 = moderate, 3 = severe, and 4 = moribund) for each criteria. Steers receiving a score of 3 or greater were pulled from the pen and subjected to a medical evaluation. Antimicrobial therapy was administered if rectal temperature exceeded 40.5 °C, and steers were returned to their home pen after treatment. Rectal temperature and BW were recorded for each animal that was examined for clinical signs consistent with BRD. The first antimicrobial treatment for BRD cases consisted of tulathromycin (Draxxin, Zoetis, Parsippany, NJ) at 2.5 mg/kg of BW. If the initial antimicrobial treatment was deemed ineffective after 7 d, steers were then administered ceftiofur hydrochloride (Excenel, Zoetis, Parsippany, NJ) at 2.2 mg/kg of BW.

Serum haptoglobin and cortisol. Blood samples (10 mL; Vacutainer with no additive, Becton, Dickson and Company, Franklin Lakes, NJ) were collected via jugular venipuncture with an 18-gauge needle on days -4, 0, 1, 2, 3, 5, 7, 10, and 14 relative to MH inoculation. After collection samples were immediately placed in a cooler on ice, blood samples were centrifuged at 3,000 x g for 20 min at 20 °C, and duplicate serum aliquots stored at -20 °C until subsequent analysis. Haptoglobin concentration was determined at the West Texas A&M University Animal Health Laboratory (Canyon, TX) with a commercial, bovine-specific sandwich ELISA kit (Immunology Consultants Laboratory, Inc., Portland, OR). The interassay CV for the haptoglobin assay was 16.1%. Serum concentrations of cortisol were determined as described by [Littlejohn et al. \(2016\)](#). A solid phase radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX) using antiserum-coated tubes was prepared according to the manufacturer's directions. Serum cortisol concentrations were determined as the average of

duplicate samples compared with a standard curve generated from known concentrations of cortisol using Assay Zap software (Biosoft, Cambridge, UK). The minimum detectable cortisol concentration for the assay was 1.2 ng/mL, and the interassay CV was 8.8%.

Hemogram. Blood samples (7 mL; EDTA, Becton, Dickson and Company, Franklin Lakes, NJ) were collected via jugular venipuncture with an 18-gauge needle on days -4, 0, 1, 2, 3, 5, 7, 10, and 14 relative to MH inoculation. Samples were immediately submitted to a commercial lab (Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX) for total and differential leukocyte determination, total erythrocytes, hematocrit, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelets. Blood counts were performed with an automated hemocytometer (ADVIA 120, Siemens Healthcare Diagnostics, Tarrytown, NY) using the factory-installed cattle setting (ADVIA 120 Multispecies System Software, Version 2.206 MS, Siemens Healthcare Diagnostics). The hemocytometer counts leukocytes, erythrocytes, and platelets by optical scatter and fluorescence. Hemoglobin concentration was determined by the cyano-methemoglobin technique. Differential leukocyte percentages were determined by counting cells on modified blood smears and absolute counts calculated using the total leukocyte count from the hemocytometer.

DMI and feeding behavior. Pens were equipped with electronic feed bunks (GrowSafe Systems Ltd., Airdrie, AB, Canada) to facilitate collection of feed intake and feeding behavior data on an individual-animal basis. The GrowSafe system consisted of feed bunks equipped with load bars to measure feed disappearance, and an antenna located within each feed bunk to record animal presence via detection of EID tags. Assigned feed disappearance (AFD) rates were computed daily for each feed bunk to assess the quality of feed intake data. The AFD averaged 98% throughout the 84-d study; thus, no data were deleted from analysis. Feeding behavior traits were based on frequency and duration of bunk visit (BV) events. A BV event commenced when the EID of an animal was first detected, and ended when the time between consecutive EID recordings exceeded 100 s, was detected at another feed bunk, or when the EID of another animal was detected at the same feed bunk ([Mendes et al.,](#)

2011). BV frequency was defined as the number of independent events recorded regardless of whether feed was consumed, and BV duration as the sum of the lengths of all BV events recorded during a 24-h period. Feed intake was allocated to individual animals based on continuous recordings of feed disappearance during each BV event. A subroutine of the GrowSafe 6000E software (Process Feed Intakes) was used to compute daily feed intake. For this study, eating rate was computed as the ratio of daily DMI to daily BV duration.

Statistical Analysis

This experiment was designed as a randomized complete block with a 2×2 factorial treatment arrangement with animal serving as the experimental unit. Temperature, feeding behavior traits, haptoglobin, cortisol, and hemogram data were analyzed using the MIXED procedure of SAS (SAS 9.4, SAS Institute Inc., Cary, NC), with an autoregressive covariance structure. The model included the fixed effects of diet, inoculation, time (day or hour), and all possible interactions. Initial exit velocity, which is a measurement of animal temperament, was used as a covariate for all analyses. Although steers were randomized into treatments based on prestudy exit velocity, preliminary analysis revealed treatment differences in exit velocity on days -28 and -14 of the study. Therefore, initial exit velocity was included as covariate term in the model described above. When a fixed effect $\times$ time interaction ($P \leq 0.05$) was detected, the SLICE output option was used to compare treatment means at each time point. In the event, a diet $\times$ inoculation or a diet $\times$ inoculation $\times$ day interaction was detected ($P \leq 0.05$), and treatment means were compared using the PDIF option of SAS. Growth rates for individual steers were modeled by linear regression of BW measurements on study day using PROC GLM (SAS, 9.4), and regression coefficients used to compute ADG, initial, and final BW.

RESULTS

There were no diet $\times$ inoculation interactions detected for initial BW ($P = 0.95$), ADG ($P = 0.22$), or final BW ($P = 0.63$), and there were no differences between the inoculation ($P = 0.62$) and dietary ($P = 0.64$) treatments for initial BW. Steers inoculated with MH had reduced ($P < 0.03$) ADG (0.67 vs. 1.21, kg/d) compared with the PBS-treated steers. There was a tendency ($P < 0.08$) for LY supplementation to improve ADG (1.15 vs. 0.74, kg/d)

relative to the CON steers, but caution should be taken when interpreting the results as the length of the experimental period was only 18 d. Final BW was not affected by dietary or inoculation treatments; however, final BW of MH-challenged steers was numerically 14 kg lighter ($P = 0.17$) compared with PBS-treated steers.

Throughout the study, only 1 steer (MH-CON treatment) received a clinical illness score of ≥ 3 , which was subsequently administered antimicrobial therapy as rectal temperature exceeded 40.5 °C. There were no retreatments or mortalities throughout the study. Clinical illness scores were not affected by the inoculation (MH = 0.25, PBS = 0.21; $P = 0.65$) or dietary (LY = 0.20, CON = 0.26; $P = 0.72$) treatments. The evaluators were not blinded to the treatment assignments; however, animals were comingled within pen and there was no visual identifier of inoculation treatment. Day affected ($P < 0.01$) clinical illness scores, with steers having higher ($P < 0.05$) clinical illness scores on days 4 and 5 postinoculation (0.63) than the remaining 8 d postinoculation (average = 0.10). During the first 10 d following MH inoculation, there was a heat event during which the average maximal temperature and relative humidity were 35 °C and 96%, respectively. The PBS-treated steers that received clinical illness scores of >0 were likely due to observed symptoms of heat stress that share commonality with those associated with mild BRD. At harvest, the lungs of all animals were inspected for lesions or abnormalities. The MH-CON steer that received antimicrobial therapy had a small consolidation in the cranioventral region of the right lung lobe. However, the lungs of the remaining steers were all observed to be grossly normal.

The effects of inoculation treatment on RUT from 24 h prior to 48 h post-MH challenge are shown in Figure 1. There was an inoculation treatment $\times$ hour interaction for RUT. Compared with the PBS-treated steers, the steers challenged with MH exhibited rapid increases in RUT from 2 to 24 h post-MH challenge (Figure 1), reaching a zenith (>41 °C) from 9 to 11 h post-MH challenge. Differences in RUT due to inoculation treatment were not detected by 18 h post-MH challenge, and dietary treatment did not affect RUT during the post-MH-challenge period.

There was an inoculation treatment $\times$ day interaction ($P < 0.01$) for rectal temperature; thus, daily main-effect means are presented in Table 1. Steers challenged with MH had greater ($P < 0.05$) rectal temperature on day 1 compared with PBS-treated steers. Additionally, the PBS-treated steers had

greater ($P < 0.05$) greater rectal temperature on day -4 than MH-inoculated steers, which is unexplainable given that inoculation treatment did not affect RUT on day -4. Overall, steers supplemented with

Figure 1. Model-adjusted least squares mean rumen temperature (RUT) by hour relative to experimental inoculation with *Mannheimia haemolytica* (MH) or phosphate buffer solution (PBS; negative control). The model included effects for study hour and repeated measures on individual steers with initial exit velocity as a covariate. ♦ Significant differences ($P < 0.05$) between treatment groups within study hour. The interaction between trial hour and treatment group was significant ($P < 0.001$).

LY exhibited greater ($P < 0.02$) rectal temperature (39.64 vs. 39.46 °C) compared with the CON steers. Furthermore, there was a dietary treatment x day interaction ($P < 0.02$) for rectal temperature, with LY steers having greater rectal temperature on days 0, 7, and 10 than CON steers. There was no significant dietary x inoculation x day interaction detected for rectal temperature.

Overall, the MH-challenged steers had increased RUT ($P < 0.03$) compared with PBS-treated steers, although there was a tendency ($P < 0.07$) for an inoculation treatment x day interaction. Steers challenged with MH had greater ($P < 0.01$) RUT on day 0 and a tendency ($P < 0.09$) for increased RUT on days 1 and 2. Dietary treatment did not affect overall RUT during the 14-d postchallenge period, nor was there a significant dietary x inoculation x day interaction detected for RUT.

Total leukocyte, differential leukocyte count, haptoglobin, and cortisol data are presented in Table 2. An inoculation x diet x day interaction ($P < 0.01$) was detected for MCHC (Table 3). The MH-LY steers had decreased ($P < 0.05$) MCHC on days 1, 2, and 3 compared with MH-CON steers,

Table 1. Main effects of live-yeast (LY) treatment and inoculation type on rectal and rumen temperature prechallenge and postchallenge with *Mannheimia haemolytica* (MH)

Item	Diet ¹				Inoculation ²				Interaction ³ P-values			
	CON	LY	SE	P-value	MH	PBS	SE	P value	In*Diet	Diet*D	In*D	In*Diet*D
No. of steers	18	18	—	—	18	18	—	—				
Rectal temperature °C												
All days	39.46	39.64	0.04	0.02	39.54	39.55	0.04	0.82	0.76	0.02	0.01	0.51
Day -4	39.80	39.86	0.09	0.68	39.70	39.96	0.09	0.05	0.55	—	—	—
Day 0	39.55	39.80	0.09	0.04	39.63	39.72	0.09	0.49	0.98	—	—	—
Day 1	39.68	39.80	0.14	0.53	39.95	39.54	0.14	0.04	0.67	—	—	—
Day 2	39.63	39.67	0.07	0.68	39.69	39.62	0.07	0.52	0.10	—	—	—
Day 3	39.35	39.48	0.10	0.22	39.36	39.46	0.10	0.35	0.38	—	—	—
Day 5	39.44	39.60	0.11	0.17	39.53	39.51	0.11	0.85	0.37	—	—	—
Day 7	39.50	39.86	0.10	0.01	39.64	39.73	0.10	0.33	0.87	—	—	—
Day 10	39.37	39.63	0.12	0.03	39.50	39.50	0.12	0.96	0.30	—	—	—
Day 14	39.57	39.75	0.11	0.12	39.66	39.67	0.11	0.96	0.96	—	—	—
Reticulo-rumen temperature °C												
All days	39.51	39.54	0.04	0.64	39.59	39.46	0.04	0.03	0.97	0.45	0.07	0.91
Day -4	39.40	39.43	0.09	0.76	39.41	39.41	0.09	0.99	0.99	—	—	—
Day 0	39.83	39.86	0.13	0.84	40.09	39.61	0.13	0.01	0.77	—	—	—
Day 1	39.72	39.68	0.14	0.79	39.84	39.57	0.14	0.07	0.52	—	—	—
Day 2	39.73	39.64	0.09	0.47	39.80	39.57	0.09	0.09	0.52	—	—	—
Day 3	39.52	39.47	0.09	0.59	39.53	39.46	0.09	0.42	0.74	—	—	—
Day 5	39.55	39.55	0.09	0.99	39.61	39.48	0.09	0.15	0.92	—	—	—
Day 7	39.37	39.54	0.10	0.09	39.51	39.40	0.10	0.27	0.82	—	—	—
Day 10	39.45	39.51	0.07	0.57	39.48	39.47	0.07	0.92	0.85	—	—	—
Day 14	39.63	39.63	0.09	0.98	39.64	39.61	0.09	0.77	0.93	—	—	—

¹Diet: CON = control (negative control); LY = supplementation with *Saccharomyces cerevisiae boulardii* at 25 g-per steer daily.

²Inoculation: MH= *Mannheimia haemolytica* endobronchial challenge; PBS = phosphate buffer solution (negative control).

³Interactions: In*Diet = Inoculation x Diet; In*D = Inoculation x Day; Diet*D = Diet x Day; In*Diet*D = Inoculation x Diet x Day.

Table 2. Main effects of diet and inoculation treatments on complete blood cell count and haptoglobin concentrations

Item	Diet ¹				Inoculation ²				Interaction ³ <i>P</i> -values			
	CON	LY	SE	<i>P</i> value	MH	PBS	SE	<i>P</i> value	In*Diet	Diet*D	In*D	In*Diet*D
<i>N</i>	162	162	—	—	162	162	—	—	—	—	—	—
Eosinophil, K/ μ L	0.31	0.23	0.05	0.14	0.25	0.29	0.05	0.43	0.69	0.85	0.19	0.28
Basophils, K/ μ L	0.05	0.05	0.01	0.75	0.05	0.06	0.01	0.20	0.11	0.04	0.11	0.17
Lymphocytes, K/ μ L	6.61	6.65	0.28	0.92	6.47	6.80	0.28	0.41	0.76	0.76	0.01	0.16
Neutrophils, K/ μ L	3.62	3.59	0.19	0.90	4.45	2.76	0.19	0.01	0.40	0.15	0.01	0.60
Monocytes, K/ μ L	0.50	0.41	0.04	0.06	0.50	0.41	0.04	0.06	0.71	0.67	0.54	0.80
Hematocrit, %	33.9	34.2	0.52	0.63	33.8	34.2	0.52	0.55	0.11	0.65	0.41	0.44
Hemoglobin, g/dL	12.7	12.7	0.25	0.96	12.5	12.8	0.25	0.27	0.11	0.24	0.23	0.70
MCH ⁴ , pg	15.7	15.9	0.21	0.63	15.7	15.8	0.21	0.76	0.25	0.09	0.98	0.48
MCV ⁵ , fL	42.0	42.5	0.54	0.56	42.2	42.3	0.54	0.89	0.22	0.37	0.08	0.09
MCHC ⁶ , g/dL	37.5	37.4	0.12	0.36	37.4	37.5	0.12	0.35	0.78	0.14	0.07	0.01
Erythrocytes, M/ μ L	8.06	8.05	0.17	0.94	8.03	8.09	0.17	0.70	0.81	0.07	0.09	0.67
Leukocytes, K/ μ L	11.1	10.9	0.38	0.79	11.7	10.3	0.38	0.02	0.42	0.16	0.01	0.66
Platelets, K/ μ L	523	537	29.0	0.63	535	526	29.0	0.76	0.21	0.69	0.01	0.07
Haptoglobin, mg/dL	17.9	17.8	6.29	0.99	33.6	2.10	6.29	0.01	0.57	0.5	0.01	0.52
Cortisol, ng/mL	26.1	34.4	3.20	0.02	28.5	32.0	3.20	0.27	0.46	0.3	0.09	0.87

¹Diet: CON = control (negative control); LY = supplementation with *Saccharomyces cerevisiae boulardii* at 25 g-per steer daily.

²Inoculation: MH= *Mannheimia haemolytica* endobronchial challenge; PBS = phosphate buffer solution (negative control).

³Interactions: In*Diet = Inoculation x Diet; In*D = Inoculation x Day; Diet*D = Diet x Day; In*Diet*D = Inoculation x Diet x Day.

⁴MCH = mean corpuscular hemoglobin.

⁵MCV = mean cell volume.

⁶MCHC = mean corpuscular hemoglobin concentration.

Table 3. Mean corpuscular hemoglobin concentration (MCHC)¹ by day

Treatment ²	−4	0	1	2	3	5	7	10	14
MH-CON	39.2	39.0	39.4 ^a	39.1 ^a	39.0 ^a	35.0 ^a	38.8	33.1 ^a	34.9
MH-LY	38.8	38.6	38.7 ^b	38.2 ^b	38.2 ^b	35.6 ^{ab}	38.5	33.7 ^{ab}	35.8
PBS-CON	38.7	38.8	39.0 ^{ab}	38.6 ^{ab}	38.5 ^{ab}	36.5 ^b	38.7	34.1 ^b	35.1
PBS-LY	38.9	39.0	38.9 ^{ab}	38.5 ^b	38.5 ^{ab}	35.2 ^{ab}	38.8	34.2 ^b	35.5

^{a-c}Within column, rows with different subscripts differ ($P < 0.05$).

¹Presented means are in g/dL.

²Treatment: MH-CON = endobronchial *Mannheimia haemolytica* challenge and control diet (negative control); MH-LY = endobronchial *Mannheimia haemolytica* challenge and supplementation with *Saccharomyces cerevisiae boulardii* a 25 g-per steer daily (LY); PBS-CON = phosphate buffer challenge (negative control) and control diet; PBS-LY = phosphate buffer challenge and supplementation with LY.

Interaction of diet x inoculation x day was significant at $P < 0.01$.

with the PBS-CON and PBS-LY steers being intermediate. Furthermore, on day 5 the PBS-CON steers had increased ($P < 0.05$) MCHC relative to MH-CON steers, with MH-LY and PBS-LY steers being intermediate. On day 10, MCHC for MH-CON steers was lower ($P < 0.05$) compared with PBS-CON and PBS-LY steers, with MCHC of MH-LY steers being intermediate.

Dietary treatment did not affect the hemogram (Table 2), although there was a dietary treatment x day interaction ($P < 0.04$) for basophil concentration. Steers fed the CON diet had increased ($P < 0.05$) basophil concentrations only on day 2 compared with steers fed the LY diet (data not shown), although the biological relevance of this dietary

treatment response is questionable given the relatively large SE for basophil concentrations.

Significant inoculation treatment x day interactions ($P < 0.01$) were detected for serum concentrations of lymphocytes, neutrophils, leukocytes, platelets, and haptoglobin (Table 2). Although an inoculation treatment x day interaction ($P < 0.01$) was detected for concentration of lymphocytes (Figure 2A), the inoculation-treatment means did not differ ($P > 0.05$) on either day during the post-MH-challenge period. Serum neutrophil concentrations increased rapidly following MH inoculation reaching a zenith on day 1 and remained elevated compared with PBS-treated steers on days 2, 3, 7, and 10 post-MH challenge (Figure 2B). In

addition, the MH challenge induced a rapid increase ($P < 0.05$) in leukocyte concentrations (Figure 2C), with MH-inoculated steers having higher leukocyte concentrations than PBS-treated steers on days 1 and 2 post-MH challenge. Although overall platelet concentrations were similar for PBS-treated and MH-inoculated steers, the inoculation x day interaction ($P < 0.01$; data not shown) was detected as PBS-treated steers had higher platelet concentrations than MH-inoculated steers on day 5.

During the 14-d post-MH-challenge period, overall serum haptoglobin concentrations were 15-fold higher ($P < 0.01$; 33.6 vs. 2.1 ng/mL) in MH-inoculated compared with PBS-treated steers (Figure 2D). The MH-inoculated steers had greater haptoglobin concentrations on days 1, 2, 3, and 5 compared with PBS-treated steers. Serum cortisol concentrations were not affected by the inoculation treatment, nor were any interactions detected. However, steers supplemented with LY had greater ($P < 0.02$) cortisol concentration (34.4 vs. 26.1 ng/mL; Table 2) throughout the 14-d post challenge compared with CON-fed steers.

Significant inoculation x day interactions ($P < 0.01$) were detected for DMI, BV frequency and duration, and eating rate (Figure 3). Compared with PBS-treated steers, the MH-inoculated steers exhibited a sharp decline ($P < 0.05$) in DMI on the day of MH inoculation, with the reduction in DMI continuing until day 3 post-MH inoculation. Likewise, the frequency and duration of BV events

were reduced ($P < 0.05$) in MH-inoculated steers on the day of MH inoculation, but thereafter did not differ from PBS-treated steers. In MH-inoculated steers, BV duration was numerically higher during days 9 to 12 post-MH challenge, with statistical difference detected ($P < 0.05$) on day 11 compared with PBS-treated steers. The inoculation treatment also affected eating rate, but the reduction in eating rate due to MH challenge was more delayed compared with changes in DMI and the feeding behavior traits. The MH-challenged steers had reduced ($P < 0.05$) eating rate on days 5, 7 to 12, and 14 compared with the PBS-treated steers.

DISCUSSION

The results of this study demonstrate that the MH-challenge model successfully initiated immunological signs of disease, despite the fact that there were few observed clinical signs of disease detected following the MH challenge. Gross clinical signs of disease resulting from the challenge model were not expected as previous studies using a similar MH strain with intratracheal delivery reported that MH-challenged animals appeared clinically normal or displayed mild increases in clinical illness scores that were not different from clinically normal (Corrigan et al., 2007; Capik et al., 2015).

Body temperature is a key measurement used to inform decision paradigms on the health status

Figure 2. Model-adjusted least squares mean lymphocytes (A), neutrophils (B), leukocytes (C), and haptoglobin (D) by day relative to experimental inoculation with *Mannheimia haemolytica* (MH) or phosphate buffer solution (PBS; negative control). The model included effects for study day and repeated measures on individual steers with initial exit velocity as a covariate. ♦ Significant differences ($P < 0.05$) between treatments groups within trial day. The inoculation x day interaction was significant ($P < 0.01$).

Figure 3. Model-adjusted least squares mean DMI (A), bunk visit (BV) frequency (events/d; B), BV duration (C), and eating rate (D) by day relative to experimental inoculation with *Mannheimia haemolytica* (MH) or phosphate buffer solution (PBS; negative control). The model included effects for study day and repeated measures on individual steers with initial exit velocity as a covariate. ♦ Significant differences ($P < 0.05$) between treatments groups within trial day. The inoculation $\times$ day interaction was significant ($P < 0.01$).

of livestock. Temperature is the most common and often the only objective measurement in determination of BRD. However, animals in a febrile state may not always display clinical signs of disease. Capik et al. (2015) examined the transmission dynamics among beef calves experimentally challenged with MH and reported a weak association between clinical illness signs and rectal temperature. Timsit et al. (2011) monitored RUT in young bulls after feedyard arrival to assess its value for preclinical detection of BRD. Reticulo-rumen hyperthermia exhibited a positive predictive value of 73% for detection of BRD, and of the bulls that were correctly detected, the hyperthermia alert was detected 1 to 3 d prior to observed clinical symptoms. In the current study, inoculation with MH created a rapid increase in RUT that returned to normal within 24 h of inoculation, which likely was associated with an endotoxin release from the MH. Rose-Dye et al. (2011) reported similar RUT patterns in steers that were intratracheally challenged with MH. Although RUT was only statistically greater during the 24 h following post-MH inoculation, there was a tendency for increased RUT on days 2 and 3 following MH inoculation. These differences resulted in an overall main effect increase in RUT do to MH inoculation treatment during the 14-d post-MH inoculation period.

Results from RUT are easier to interpret than rectal temperature, and are in agreement with other biological markers of inflammation. Steers challenged with MH exhibited an increase in rectal temperature the day after challenge. Previous

reports have shown similar transient increases in rectal temperature due to MH challenge. Corrigan et al. (2007) and Hanzlicek et al. (2010) reported increases in rectal temperature the day of challenge, although animals returned to prechallenge levels within 24 h. Theurer et al. (2013) and Burciaga-Robles et al. (2010) both reported increases in rectal temperature that initiated <6 h after inoculation and persisted for 24 h. The current study measured rectal temperature once per day, whereas the other aforementioned studies measured rectal temperature on varying serial hour schedules throughout the initial 24 h. Measuring rectal temperature only once per day may have introduced more variance into the measurement resulting in the differences observed between the dietary treatments, and the challenge treatments prior to challenge administration. Hanzlicek et al. (2010) measured rectal temperature 3 times per day in beef steers experimentally challenged with MH, and reported that rectal temperature was lower in the morning (39.6 °C) and at noon (39.4 °C) then in the evening (40.2 °C).

It is counter-intuitive that supplementation with LY would increase body temperature and there is no evidence in the literature to support these findings. In fact, Zhu et al. (2016) reported that there was a tendency for rectal temperature to be reduced in a linear manner as dietary concentration of *S. cerevisiae* increased in lactating dairy cattle subjected to heat stress. Furthermore, there were no differences in RUT between the CON and LY steers in this study. In the current study, these dietary treatment differences in rectal temperature may have been

associated with environmental effects. Hanzlicek et al. (2010) commented that typical clinical measurements for detecting disease such as rectal temperature, heart rate, and respiratory rate yielded conflicting results and varied throughout the day in MH-challenged animals. Furthermore, rectal temperature measurements in their study exceeded their upper reference (39.5 °C) limit throughout the study, which was attributed to high environmental temperatures and the restraint stress associated with the physical examination. Results from this study and others suggest that RUT may be a more accurate measurement for identifying febrile animals, as the data are measured throughout the day and are not influenced by the stress of animal handling.

Mean corpuscular hemoglobin concentration is the cellular hemoglobin concentration per average erythrocyte and is calculated as the hemoglobin to hematocrit ratio (Smith et al., 2015). The most common cause of a reduction in MCHC is a regenerative anemia (Jones and Allison, 2007). *Mannheimia haemolytica* possess multiple virulence factors that contribute to pathogenesis, which include a lipopolysaccharide complex that causes hemorrhage, edema, acute inflammation, and a leukotoxin that leads to lysis of leukocytes and platelets (Griffin et al., 2010). These virulence factors can induce mild anemic symptoms associated with an acute infection (Smith et al., 2015). Hanzlicek et al. (2010) reported a reduction in MCHC in beef steers on day 3 following a MH challenge. Although Burciaga-Robles et al. (2010) did not report MCHC values, they found reductions in MCH (ratio of hemoglobin to erythrocytes) due to MH challenge which is similarly reduced by a regenerative anemia (Smith et al., 2015). Contrary to these studies, Corrigan et al. (2007) observed no change in MCHC in response to a MH challenge in steers. In the current study, there was a diet x inoculation x day interaction detected for MCHC. The impact of the MH challenge was not detected until days 5 and 10, where the PBS-CON steers had greater ($P < 0.05$) MCHC than the MH-CON steers, and the PBS-LY and MH-LY steers were intermediate. LY supplementation reduced ($P < 0.05$) MCHC in the MH-LY steers on days 1 to 3 post-MH challenge relative to the MH-CON steers and the PBS-LY and PBS-CON steers were intermediate. Although these differences were significant, the magnitude of these differences was small and the biological relevance of this response remains to be determined.

Steers challenged with MH exhibited altered leukograms synonymous with a bacterial infection

(Jones and Allison, 2007). Although there were no differences between inoculation treatments on any given day, lymphocytes for MH-challenge steers were numerically lower 24 h post-inoculation. Burciaga-Robles et al. (2010) and Gånheim et al. (2003) both reported decreases in lymphocytes resulting from intratracheal inoculation with MH. In contrast, Corrigan et al. (2007) and Hanzlicek et al. (2010) reported no differences in lymphocytes associated with MH challenge. Lymphopenia is associated with stress, endotoxemia, and acute viral and bacterial infections (Jones and Allison, 2007). There were considerable differences in lymphocyte counts across the aforementioned studies, and when differences were observed due to MH challenge, the animals had greater lymphocytes recorded. Therefore, the conflicting results across studies may be due to a host-bacterial interaction or dependent upon the lymphocyte count prior to challenge.

Neutrophils are the predominant leukocyte in mammals and can migrate from storage in bone marrow into circulation within 2 h of insult (Tizard, 2013; Smith, 2015). Neutrophilia occurs in response to infectious processes, tissue injury, neoplastic diseases, and noninflammatory conditions (Jones and Allison, 2007). Steers challenged with MH exhibited nearly a 3-fold increase in neutrophils within 24 h of inoculation. The magnitude of the neutrophilia response to MH challenge in this study was similar to previous studies (Gånheim et al., 2003; Corrigan et al., 2007; Burciaga-Robles et al., 2010; Hanzlicek et al., 2010) and indicates that the MH-challenge model successfully induced an acute-immune response. Total leukocyte count response to the MH challenge in the present study is consistent with previous reports (Gånheim et al., 2003; Corrigan et al., 2007; Burciaga-Robles et al., 2010; Hanzlicek et al., 2010) that reported elevated leukocyte concentrations 24 to 48 h post-MH challenge.

Haptoglobin is an acute phase protein that performs multiple roles during the upregulation of the innate immune response. Through the binding of free hemoglobin in the blood, haptoglobin stabilizes iron-reducing oxidative stress (Ceciliani et al., 2012) and imposes a bacteriostatic effect by making iron unavailable for proliferating bacteria (Idoate et al., 2015). The haptoglobin-hemoglobin complex also exerts anti-inflammatory effects, when this complex binds with macrophages they in turn release anti-inflammatory cytokines such as interleukin-10 (Ceciliani et al., 2012). Haptoglobin has been shown to be an effective indicator for multiple disease processes in beef cattle including BRD

(Godson et al., 1996; Eckersall and Bell, 2010). In the current study, steers challenged with MH exhibited a marked increase in haptoglobin concentration 24 h after MH challenge which persisted for 5 d. In contrast, the PBS-treated steers exhibited a minimal haptoglobin response during the study. Similarly, Burciaga-Robles et al. (2010) reported increased concentrations of haptoglobin for 4 d following MH challenge. Similarly, Corrigan et al. (2007) reported an 8-fold increase in haptoglobin concentration 2 d post-MH challenge. These results further indicate that the MH-challenge model induced an immune response in this study.

Supplementation with LY did not affect the leukogram or haptoglobin concentrations. However, steers supplemented with LY exhibited increased serum cortisol concentrations. The impact of LY supplementation on the hypothalamic-pituitary-adrenal axis is unclear. In agreement with the current study, Buntyn et al. (2014) reported increased serum concentration of cortisol in steers supplemented with live *S. cerevisiae* prior to an endotoxin challenge. However, post-endotoxin challenge, the cortisol concentrations of steers supplemented with *S. cerevisiae* were less than the control animals. They also reported steers supplemented with *S. cerevisiae* had a reduction in proinflammatory cytokines (tumor necrosis factor- α , interleukin-6, and interferon gamma) during the prechallenge period when cortisol concentrations were elevated. Buntyn et al. (2014) concluded that yeast supplementation may reduce negative effects associated with innate inflammatory responses via cortisol-induced proinflammatory cytokine messaging mechanisms. Increased levels of cortisol concentration due to supplementation with *S. cerevisiae* have also been reported in other species. Iwashita et al. (2015) reported increases of cortisol concentrations in juvenile tilapia following 4 wk of supplementation with *Bacillus subtilis*, *Aspergillus oryzae*, and *S. cerevisiae*. The elevated cortisol concentrations were only observed on the initial measurement and were not different from the controls during the 2-wk period following the disease challenge. However, supplementation with DFM improved feed conversion ratio by 24% ($P < 0.05$) and increased the survivability rate to an intraperitoneal challenge with *Aeromonas hydrophila* (21.3 vs. 5.3%, $P < 0.05$) and *Streptococcus iniae* (34.7 vs. 4.3%, $P < 0.05$). It is not possible to determine if the positive effects observed by Iwashita et al. (2015) were due to the fact that 3 DFM products were fed in combination or if the effect was due to *S. cerevisiae*. Contrary to these findings, Zaworski

et al. (2014) reported decreased concentrations of cortisol in postpartum dairy heifers supplemented with *S. cerevisiae* (fermentation product) compared with the controls. Burdick Sanchez et al. (2013) reported no differences in cortisol concentrations in heifers supplemented with *S. cerevisiae* (cell wall) prior to an intravenous challenge with endotoxin. However, post-challenge heifers supplemented with *S. cerevisiae* exhibited reduced concentrations of cortisol as well as interleukin-6. Similarly, Collier et al. (2011) reported supplementation with *S. cerevisiae* *boulardii* reduced cortisol, interferon gamma, and interleukin-6 post-intravenous endotoxin challenge in newly weaned piglets. Reduction in interleukin-6 concentrations due to yeast supplementation appears to be fairly repeatable; however, the impact that yeast supplementation exerts upon the relationship between cortisol and proinflammatory cytokines is yet to be fully determined. Serum cortisol concentrations in the current study were not affected by the MH-challenge model as seen in the aforementioned studies. Even though proinflammatory cytokines were not measured in this study, there appears to be no impact on other measures of inflammation (body temperature, haptoglobin, and neutrophils) affected by cortisol concentrations or supplementation with LY.

When animals become morbid, they enter into a state of hyper-metabolism, due to increased energy demands required to produce proinflammatory cytokines, acute phase proteins, lymphocytes, and mount a febrile response (Carroll and Forsberg, 2007). In human patients, Long (1977) observed alterations of resting metabolism measured by indirect respiratory calorimetry and reported a maximum increase in resting metabolism of 45% when the patient had peritonitis. To compensate for these increased energy demands, mammals display various behavioral responses such as increased sleep, and reductions in social activity, sexual behavior, and feed intake in an effort to conserve energy (Carroll and Forsberg, 2007). Lethargy and reduced feed intake have been well documented in studies monitoring the health of beef cattle (Hutcheson and Cole 1986; Galyean and Hubbert, 1995; Sowell et al., 1999; Quimby et al., 2001; Jackson et al., 2016). These metrics are often the basis for subjective disease detection paradigms. In the current study, steers challenged with MH had reduced DMI, and reductions in frequency and duration of BV events as well as reduction in eating rate. The reduction in frequency and duration of BV events only occurred the day following MH challenge, whereas DMI remained suppressed for 4 d following MH

challenge. Theurer et al. (2013) reported decreases in duration of time calves spent at a hay feeder that were similarly inoculated with MH. Interestingly, in the same study, the duration the MH calves spent at a grain feeder was less only 24 h after inoculation and returned to normal, as though the calves were self-selecting a higher energy diet to meet increased requirements. These alterations in behavior, which are synonymous with behavior changes observed in naturally occurring BRD cases, further illustrate the effectiveness of the MH-challenge model.

Although the intent of this study is not to evaluate the current methodologies for determining BRD in cattle, it is worth highlighting that in many of the challenge studies discussed, few reported gross clinical signs of disease typically observed in a commercial setting. However, these studies provide evidence of physiologic changes that are suggestive of a morbid/inflammatory state. There have been recent efforts to evaluate the effectiveness of using visual observation for disease detection. Comparing the presence of lung lesions at slaughter with previous animal treatment records, White and Renter (2009) reported that visual observation was 62% sensitive and 63% specific. Timsit et al. (2016) using the studies included in the White and Renter (2009) analysis and others concluded that the sensitivity of visual observation was only 27% sensitive; however, the specificity was 92%. The differences between the studies were affected by the different estimates used in the Bayesian estimation, with Timsit et al. (2016) results more closely resembling the averages of the studies. Regardless, these studies illustrate the challenge with relying on visual observation of clinical illness scores to detect BRD, and demonstrate that numerous BRD cases are not identified and treated. Numerous studies have examined behavioral changes prior to clinical signs of disease. Pillen et al. (2016) reported reductions in motion index and daily step counts 5 and 4 d prior to diagnosis, respectively. Jackson et al. (2016) used a 2-slope broken-line regression model to characterize deviations in DMI and feeding behavior patterns preceding the onset of observed clinical symptoms associated with BRD. The model-detected breakpoint for DMI occurred 6.8 d prior to observed clinical illness, whereas breakpoints for BV frequency and duration were 7.6 and 7.2 d prior to observed clinical illness. Quimby et al. (2001) monitored BV traits with a cumulative summation chart and was able to detect morbidity 4.1 d prior to observed clinical symptoms with an accuracy and sensitivity of 87% and 90%, respectively. White et al. (2015) developed a system that

monitors a suite of behavior traits that was capable of detecting morbid animals 0.75 d prior to visual observation. These results highlight that real-time measurements of behavioral traits may be useful for preclinical detection of BRD.

CONCLUSION

This study evaluated the effects of an experimental challenge with *M. haemolytica* on physiological, immunological, and behavioral responses in crossbred beef steers. The MH challenge effectively stimulated inflammation, and upregulated leukocytes and acute phase proteins synonymous with an acute immune response associated with naturally occurring BRD. Furthermore, this challenge model affected feeding behavior similarly to an acute BRD infection. Supplementation with LY minimally affected performance, physiological, and behavioral responses in this study. Future research endeavors should further examine the effects of LY supplementation on cortisol concentrations. Results from this study illustrate the potential value of using biosensors to monitor physiological and behavioral responses associated with subacute cases of BRD in beef cattle.

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