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Effect of aerosolized bacterial lysate on development of naturally occurring respiratory disease in beef calves

Laura L. Bassel[1] | Emily I. Kaufman[1] | Sarah-Nicole A. Alsop[1] | Jordan Buchan[1] | Joanne Hewson[2] | Erin E. McCandless[3] | Raksha Tiwari[3] | Shayan Sharif[1] | Ksenia Vulikh[1] | Jeff L. Caswell[1]

[1]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

[2]Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

[3]Global Therapeutics Research, Veterinary Medicine Research and Development, Zoetis Inc., Kalamazoo, Michigan

Correspondence
Jeff L. Caswell, Department of Pathobiology, University of Guelph, Guelph, ON, Canada N1G 2W1.
Email: jcaswell@uoguelph.ca

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Abstract

Background: Bovine respiratory disease (BRD) is a major problem affecting beef cattle after arrival to feedlots. Alternatives to antibiotics are needed for prevention.

Hypothesis: Stimulation of pulmonary innate immune responses at the time of arrival to a feedlot reduces the occurrence and severity of BRD.

Animals: Sixty beef steers at high risk of BRD.

Methods: Randomized, double-blinded, placebo-controlled study. Calves received saline or a lysate of *Staphylococcus aureus* and *Escherichia coli* by aerosol, at 16 hours after feedlot arrival. Calves were monitored for 28 days for disease outcomes and levels of *Mycoplasma bovis* and *Mannheimia haemolytica* in nasal swabs.

Results: Death from *M bovis* pneumonia was significantly greater in lysate-treated animals (6/29, 24%) compared to controls (1/29, 3%; odds ratio = 10.2; 95% confidence interval [CI] = 1.1-96.0; P = .04). By 28 days after arrival, 29/29 lysate-treated calves had ultrasonographic pulmonary consolidation compared to 24/29 control calves (P = .05). Lysate-treated calves had lower weight gain compared to control calves (−8.8 kg, 95% CI = −17.1 to −0.5; P = .04), and higher body temperatures on days 4, 7, and 21 (0.19°C; 95% CI = 0.01-0.37; P = .04). Nasal *M bovis* numbers increased over time and were higher in lysate-treated calves (0.76 log CFU, 95% CI = 0.3-1.2; P = .001).

Conclusions and Clinical Importance: Aerosol administration of a bacterial lysate exacerbated BRD in healthy high-risk beef calves, suggesting that respiratory tract inflammation adversely affects how calves respond to subsequent natural infection with *M bovis* and other respiratory pathogens.

KEYWORDS
cattle, immunostimulation, inflammation, innate immunity, *Mycoplasma bovis*, pneumonia, trained immunity, ultrasound

Abbreviations: ANOVA, analysis of variance; BRD, bovine respiratory disease; CFU, colony forming units; CI, confidence interval; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

1 | INTRODUCTION

Bovine respiratory disease (BRD) is the most common and costly disease of beef cattle in North America. Metaphylaxis is a common control strategy, where calves are treated with antibiotics if their penmates develop a critical threshold of disease or if calves are otherwise considered at high risk of BRD. Control of BRD is reportedly the principal reason for antimicrobial use in feedlots.[1] There is increasing public scrutiny of antimicrobial use in agriculture due to concerns over development of antimicrobial resistance. With inconsistent effectiveness of vaccination and concerns about mass treatment with antimicrobials, alternative means of BRD control are needed.

Several studies reported that various components of the innate immune system can be less effective or reduced in stressed or virus-infected cattle.[2] However, some in vitro and in vivo innate immune responses, such as antimicrobial peptides expressed by tracheal epithelial cells, can be induced by stimulation with bacterial lysates or Toll-like receptor agonists.[3-6] Similarly, aerosol delivery of bacterial lysate causes a mild and transient innate immune and inflammatory response characterized by elevation in rectal temperature and recruitment of neutrophils to the lungs.[6] Finally, in mice, aerosolization of bacterial lysate before challenge with respiratory pathogens is highly effective in preventing pneumonia caused by a number of viral, bacterial, and fungal agents.[7] Thus, stimulation of innate immune responses might represent a viable strategy to rapidly increase the resistance of cattle to a broad range of respiratory pathogens.

We hypothesized that stimulating innate immune responses in beef cattle at the time of arrival to a feedlot would reduce the incidence and severity of respiratory disease. Specifically, we investigated the effects of aerosolized bacterial lysate versus saline on the development of clinical disease, rectal temperature, serum haptoglobin, plasma fibrinogen, nasal carriage of *Mannheimia haemolytica* and *Mycoplasma bovis*, pulmonary consolidation identified by transthoracic ultrasound, and weight gain in the first 28 days; and on mortality rate in the first 60 days after arrival.

2 | MATERIALS AND METHODS

2.1 | Study animals

Use of animals was approved by the Animal Care Committee of the University of Guelph (#3286) according to Canadian Council on Animal Care guidelines. This was a randomized, double-blinded, placebo-controlled study completed at the Ontario Beef Research Centre in southwestern Ontario, Canada. Sixty unvaccinated beef steers of mixed breeds (mainly Angus, Limousin, Charolais, and Simmental) were obtained at auction between November 8 and December 10. The 60 calves comprised 4 cohorts of 14 to 16 animals, each cohort arriving 1 to 2 weeks apart, and each cohort housed in 1 pen. The calves were considered to be at high risk of BRD because they were of low body weight and no more than 4 steers per cohort were obtained from a single source farm. The steers did not receive metaphylactic antibiotic treatment on arrival. The calves were housed outside in roofed pens, bedded on wood shavings, and fed a ration containing 50% haylage and 50% corn silage.

Calves were transported approximately 100 km from the auction barn and arrived at the research facility in the evening (day 0). The next morning, within each cohort, calves were stratified in pairs by weight at arrival. Block randomization to treatment within each pair was assigned by a computer-generated random number; within each weight-ranked pair, 1 calf received the aerosolized bacterial lysate and the other calf received aerosolized phosphate-buffered saline (PBS). Bacterial lysate was prepared and administered as previously described.[6] Briefly, *S. aureus* and *E. coli* were heat-killed and non-viability was confirmed by culture on blood agar, then washed twice with PBS, lysed by sonication, and stored at −20°C. This treatment was defined as day 1 of the study. Bacterial lysate suspension or PBS was delivered through a Whisper Jet nebulizer (Marquest) connected to an Equine Aeromask (Trudell Medical International). The bacterial lysate or PBS was administered on day 1 of the study using a nebulizer and facemask placed over placed over the calves' muzzles as previously described (Supplementary File S1).[6,8] Researchers collecting data and performing statistical analysis were blinded to treatment groups, as were the facility personnel who made therapeutic decisions.

2.2 | Evaluation and sample collection

The steers were examined on the morning after arrival to the research station (day 1, baseline), twice weekly for 2 weeks, then once weekly for 2 weeks for a total of 28 days. Body weights, rectal temperatures, and thoracic ultrasound findings (scored as 0 or 1 based on detecting lesions ≥3 cm; see Supplementary File S1) were recorded. Serum haptoglobin and plasma fibrinogen were measured within 3 hours of collection (Animal Health Laboratory, University of Guelph). Bilateral nasal swabs were collected (7 cm depth) and tested by quantitative polymerase chain reaction (PCR) for *M bovis* and by multiplex PCR for *M haemolytica* serotypes 1, 2, and 6 (see Supplementary File S1).[9] In addition to the times listed above, body weight and thoracic ultrasound findings were also recorded at 122 days (cohort 1), 115 days (cohort 2), 130 days (cohort 3), and 111 days (cohort 4).

2.3 | Clinical illness and death losses

All animals were observed twice daily by experienced farm operators blinded to aerosol treatment status, clinicopathologic data, and ultrasound findings. Calves were considered clinically ill if they had depression, coughing, reluctance to move, or poor appetite; and confirmed with an increased rectal temperature (≥40°C). Affected calves were treated according to standard protocols determined by the herd veterinarians (Supplementary File S1).

Decisions regarding euthanasia were made by the herd veterinarians, who were blinded to aerosol treatment and study data. Calves

that died or were euthanized during the study period were subjected to postmortem examination. Lungs were examined grossly and histologically, cultured for isolation of aerobic bacteria and mycoplasmas, and tested by PCR for bovine respiratory syncytial virus, bovine herpes virus-1, bovine parainfluenza-3 virus, and bovine viral diarrhea virus (Supplementary File S1).

2.4 | Statistical analysis

Simple descriptive statistics and comparisons between treatment groups, including means and 95% confidence interval (CI), 1-way analysis of variance (ANOVA) with post hoc Tukey test, paired *t* test, and Fisher's exact test, were calculated using Prism version 8.2.1 for Windows (GraphPad Software). Results with a *P* value less than .05 were considered statistically significant. Survival analysis was performed to determine time to first treatment in lysate-treated and control steers, and survival curves were compared using the log-rank (Mantel-Cox) test. Outcomes measured repeatedly over the course of the study including rectal temperature, serum haptoglobin, plasma fibrinogen, nasal bacteria numbers, lung consolidation detected by ultrasound, and body weight were evaluated using multivariate mixed-model linear or logistical regression (StataCorp 2017, Stata Statistical Software: Release 15). Cohort and calf were included as random effects. The additional investigated covariates included baseline measurements for each outcome, antimicrobial treatment in the past 4 days, time relative to arrival, weight on arrival, and interaction terms. Backward stepwise removal of nonsignificant interactions and individual factors was performed until only significant variables ($P < .05$) and random effects ($P < .2$) remained in the models. Data were transformed if indicated by evaluation of residual variance for normality and homogeneity. Initial analyses assessed if there were significant differences between the 2 doses of bacterial lysate. As no differences were demonstrated between the 2 doses, animals receiving bacterial lysate were grouped for the subsequent and reported analysis.

3 | RESULTS

All 60 calves received a randomly assigned aerosol treatment (PBS or lysate of killed bacteria) between 14 and 18 hours after arrival (day 1). No adverse effects were seen at the time of administration. At the time of arrival, 1 steer in the lysate-treated group had increased plasma fibrinogen and serum haptoglobin concentrations and large areas of lung consolidation detected by ultrasound, and thus was excluded from further analysis. The other 59 calves were included in the analysis.

The mean body weight on arrival was 218 kg (95% CI = 213-224). There were no statistically significant differences in baseline (on-arrival) measurements between the lysate-treated group and the control group including body weight (paired *t* test, $P = .85$), rectal temperature ($P = .4$), fibrinogen ($P = .33$), or haptoglobin ($P = .25$; Tables 1 and S1). At the time of arrival, the percentage of steers with lung lesions identified by targeted thoracic ultrasound was similar between groups, with 2/29 calves in the lysate-treated group and 5/30 calves in the control group having lung consolidation measuring greater than 3 cm (Fisher's exact test; $P = .42$).

At the time of arrival to the feedlot, the mean body weights differed between the 4 cohorts (ANOVA ($F_{3,56} = 1.361$, $P = .007$). In the first cohort, the mean weight on arrival was 218 kg (95% CI = 205-230), the second was 207 kg (95% CI = 200-213), the third was 232 kg (95% CI = 220-245), and the fourth was 216 kg (95% CI = 203-229). The mean body weight was significantly lower in the second than the third cohort (post hoc Tukey-Kramer test: $P = .007$). Other baseline measurements did not differ significantly between cohorts.

3.1 | Deaths

One animal was euthanized due to severe pneumonia within the first 28 days (specifically, on day 22), and 7 additional steers died or were euthanized due to severe disease before the final evaluation at 3 months. In 7/8 cases, the cause of death was *M bovis* pneumonia based on the predominance of caseonecrotic lesions at postmortem and isolation of *M bovis*. Six of 29 lysate-treated steers (24%) died of *M bovis* pneumonia compared to 1/29 (3%) control steers. The other death was due to chronic pneumonia not caused by *M bovis*; in this calf, *M bovis* was not isolated in culture or identified by PCR and the lung did not have caseonecrotic lesions. Thus, lysate-treated steers had 10.2 times (95% CI = 1.1-96.0) greater odds of dying from *M bovis* pneumonia within the first 3 months after arrival than did control steers ($P = .04$; multivariate logistic regression, controlling for effect of on-arrival body weight and pen).

TABLE 1 Baseline measurements in lysate-treated (n = 29) and saline-treated (n = 30) calves, measured on arrival before treatment

Variable (value on arrival)	Control steers mean (95% CI)	Lysate-treated mean (95% CI)	Paired *t* test (control vs lysate)
Body weight (kg)	218.5 (209.9-227.0)	218.2 (210.2-226.3)	$t(28) = 0.193$ $P = .85$
Rectal temperature (°C)	39.7 (39.3-39.8)	39.6 (39.3-39.8)	$t(28) = 0.851$ $P = .4$
Serum haptoglobin (g/L)	0.18 (0.13-0.22)	0.24 (0.15-0.32)	$t(28) = 1.178$ $P = .25$
Plasma fibrinogen (g/L)	3.21 (2.94-3.48)	3.35 (3.04-3.65)	$t(28) = 0.992$ $P = .33$

Note: Paired *t* tests compared means between treatment groups (calves that later received saline or bacterial lysate).

TABLE 2 The effects of aerosol treatment with bacterial lysate on the risk of clinical disease, deaths due to *Mycoplasma* bovis, and overall deaths in 59 feedlot calves

	Clinical disease			Death due to *M bovis*			Deaths, all causes		
	Odds ratio	*P* value	95% CI	Odds ratio	*P* value	95% CI	Odds ratio	*P* value	95% CI
Aerosol treatment									
Lysate	2.55	.11	0.80-8.11	10.20	.04	1.085-96.01	4.79	.1	0.73-31.40
Saline	REF			REF			REF		
Weight (kg)	0.97	.02	0.94-0.996	0.948	.05	0.899-1.000	0.92	.01	0.87-0.98

Note: Summaries of odds ratios, *P* values, and 95% confidence intervals were determined by multivariate logistic regression, controlling for the effect of on-arrival body weight and pen.
Abbreviation: REF, reference category.

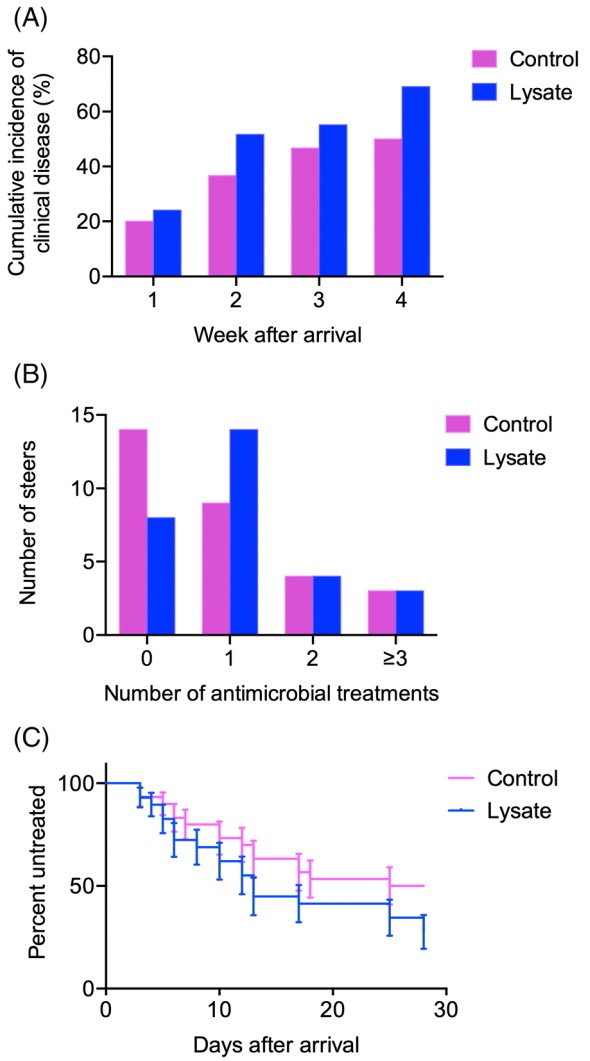

FIGURE 1 Clinical illness requiring antimicrobial treatment in steers that received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n = 29)1 day after arrival to the feedlot. A, Cumulative incidence of clinical disease. B, The number of antimicrobial treatments administered. C, A survival curve showing the time to first treatment in control and lysate-treated steers. None of the measures shown were significantly different between aerosolized treatment groups

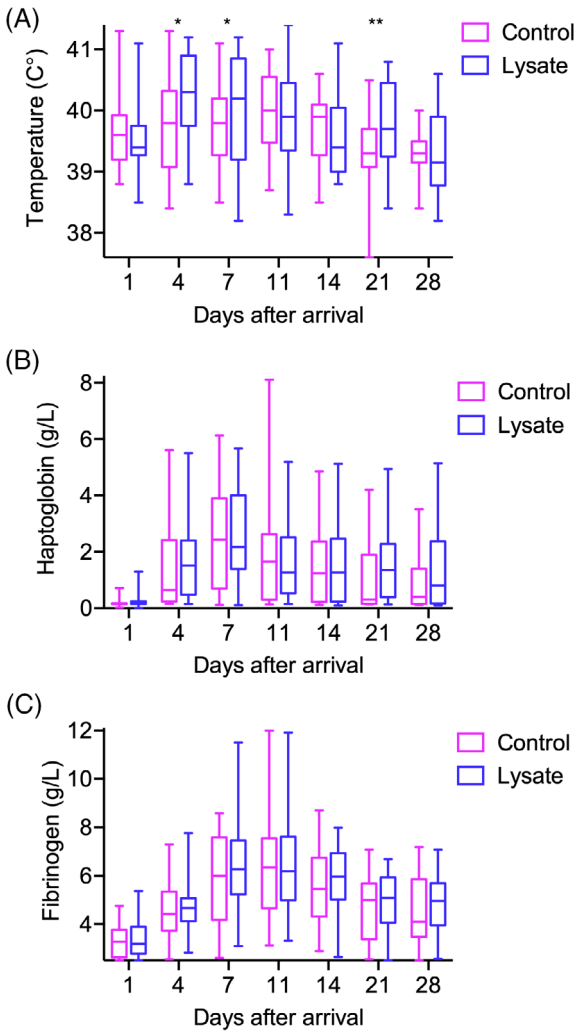

FIGURE 2 Rectal temperature, serum haptoglobin, and plasma fibrinogen concentrations over time. Steers received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n = 29) 1 day after arrival to the feedlot. Data show the median and quartiles (whiskers = range) for (A) rectal temperature, (B) serum haptoglobin, and (C) plasma fibrinogen over time after arrival. Significant differences between control and lysate-treated steers are shown (*$P < .05$, **$P < .01$)

TABLE 3 The effect of on-arrival treatment with aerosolized bacterial lysate on rectal temperature, serum haptoglobin, and plasma fibrinogen over time after arrival (days 4-28)

	Temperature[a]			Log (serum haptoglobin)[a]			Log (plasma fibrinogen)[a]		
Risk factor	Estimate of effect (β)	*P* value	95% CI	Estimate of effect (β)	*P* value	95% CI	Estimate of effect (β)	*P* value	95% CI
Aerosol									
Lysate	0.19	.04	0.01-0.37	0.14	.1	−0.03 to 0.31	0.03	.21	−0.02 to 0.07
Saline	REF			REF			REF		
Antimicrobial									
Within 4 days	−0.52	<.001	−0.72 to −0.32	—	—	—	—	—	—
No recent antimicrobial	REF								
Days after arrival	−0.03	<.001	−0.04 to −0.02	−0.015	<.001	−0.02 to −0.01	0.014	<.001	0.01-0.02
(Days after arrival)^2	—	—	—	—	—	—	−0.001	<.001	−0.001 to −0.003

Note: The results from mixed linear regression include model coefficients (β), *P* values, and 95% confidence intervals (CI) for body temperature (°C), serum haptoglobin (g/L), and plasma fibrinogen (g/L) for 59 steers randomly assigned to receive aerosolized bacterial lysate (n = 29) or saline (n = 30) on arrival to a feedlot with animal ID and cohort included as random effects. "—" indicates factors that were not significant in the final models.
Abbreviation: REF, reference category. (Days after arrival)^2: The square of the number of days was analyzed as an independent variable.
[a]Separate multivariable mixed linear regression analyses were performed for temperature, haptoglobin, and fibrinogen controlling for the random effects of herd and animal. Regression coefficients (β) indicate the direction and magnitude of the effect of listed factors relative to the reference category.

Postmortem gross, histologic findings, and microbiologic findings are summarized in Table S2. Of the 7 animals that were diagnosed with *M bovis* pneumonia, 3 had septic arthritis with *M bovis* cultured from 1 or more joints.

The effect of aerosolized bacterial lysate on overall mortality and mortality due to *M bovis* was evaluated with multivariate logistic regression models (Table 2). On-arrival administration of bacterial lysate significantly increased the risk of death due to *M bovis* (*P* = .04) but not overall mortality rate (*P* = .1). In these models, decreased body weight on arrival increased the overall risk of death and deaths due to *M bovis*.

3.2 | Body weight

Over the first 28 days, the mean change in body weight in lysate-treated steers was −4.3 kg (95% CI = −10.7 to 1.7) while that of control steers was +4.3 kg (95% CI = −1.5 to 10.2) and this difference was significant (*P* = .04; Figure S1). At the follow-up visit (mean of 120 days after arrival), the average daily gain was similar (*P* = .8) between the surviving lysate-treated steers (n = 23/29; mean = 0.588 kg/d; 95% CI = 0.446-0.729) vs the surviving control steers (n = 27/29; mean = 0.649 kg/d; 95% CI = 0.499-0.800).

3.3 | Clinical findings

Twice-daily pen-side evaluations of all steers were performed by farm operators blinded to lysate versus saline status, who administered treatment as per farm protocols and as directed by the herd veterinarian. Overall, 37/59 steers were identified as clinically ill. These included 16/30 (53%) control calves and 21/29 (70%) lysate-treated calves. Multivariate logistic regression (Table 2) evaluated the impact of lysate treatment on the risk of clinical disease, controlling for the effects of cohort and body weight on arrival. Administration of bacterial lysate did not significantly increase the risk of clinical respiratory disease (odds ratio = 2.55; 95% CI = 0.08-8.11; *P* = .11). Lower body weight on-arrival was associated with increased incidence of respiratory disease in the first month (*P* = .02). The effect of cohort was negligible. The highest incidence of newly identified respiratory disease occurred in the second week after arrival, when 24% of steers were treated with an antimicrobial for the first time. Time to first treatment was not different between lysate-treated and control animals (Mantel-Cox test, *P* = .1; Figure 1). The number of treatments administered to lysate-treated and control calves was also similar between treatment groups (Figure 1).

Variables that were determined to significantly influence rectal temperature included administration of aerosolized bacterial lysate versus saline, day relative to arrival, and administration of antimicrobial treatment within 4 days preceding the sampling date (Figure 2, Table 3). Body weight on arrival, baseline temperature, and interaction terms were not significantly correlated with rectal temperature over time. The mean serum haptoglobin and plasma fibrinogen concentrations increased over baseline by day 4, with a peak at day 7 (haptoglobin) or day 11 (fibrinogen; Figure 2). Aerosol administration of bacterial lysate or saline did not significantly affect serum haptoglobin (*P* = .1) or plasma fibrinogen (*P* = .22) concentrations (Table 3).

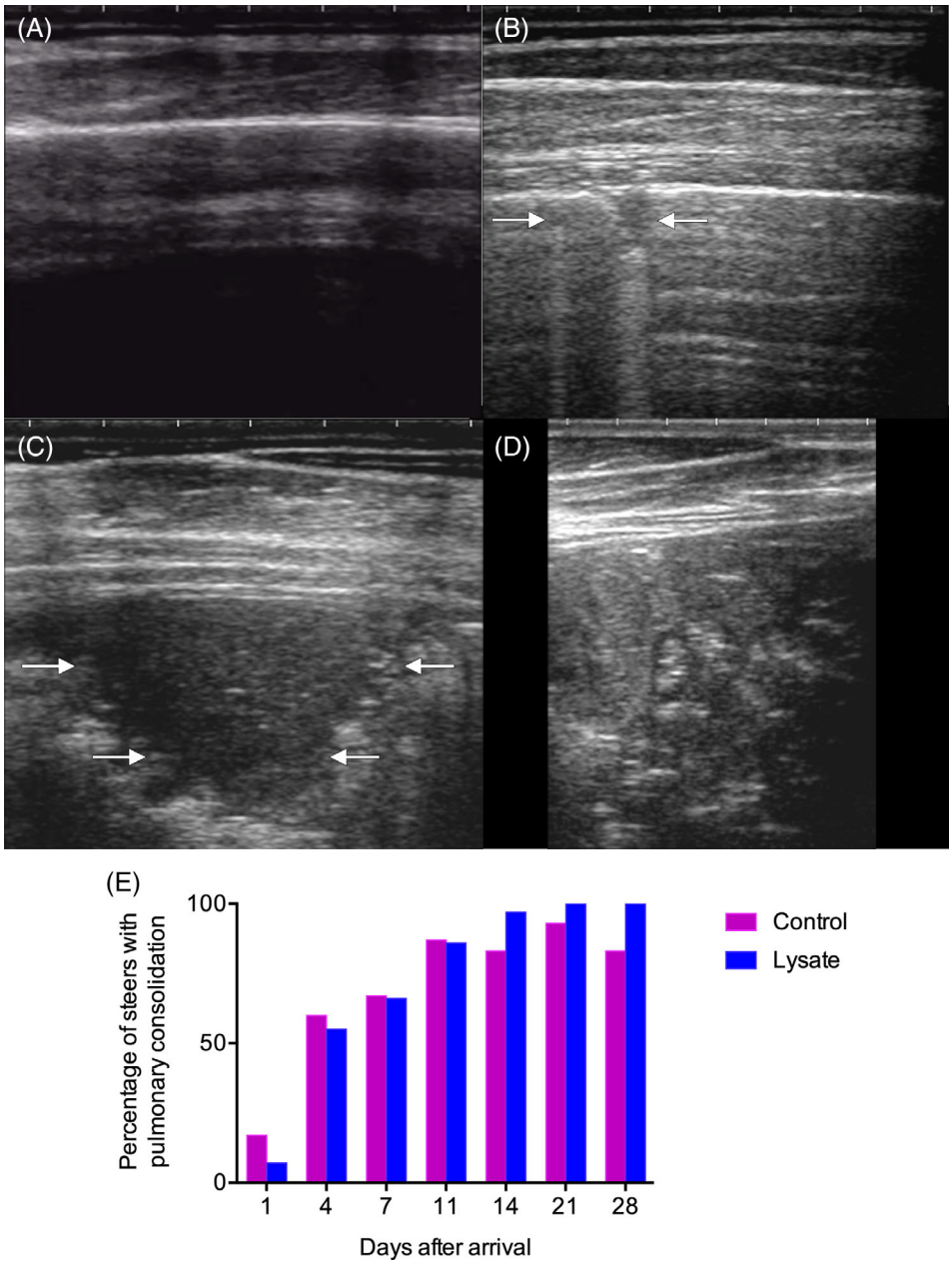

FIGURE 3 Ultrasound lesions over time after arrival to the feedlot. Steers received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n = 29) 1 day after arrival. Following arrival, targeted thoracic ultrasound was performed to evaluate the presence of pulmonary consolidation. A-D, Ultrasonographic images of the chest wall and lung from calves with (A) normal lung, (B) small foci of pulmonary consolidation (arrows), (C) large area of hypoechoic consolidation (arrows) with only a small amount of normal lung at the right edge of the field, and (D) entirely consolidated lung. E, Pulmonary consolidation over time in control and lysate-treated steers. Data represent the proportion of steers with pulmonary consolidation measuring greater than 3 cm detected by targeted thoracic ultrasound over time after arrival. There are no significant differences between treatment groups

In addition, baseline haptoglobin or fibrinogen concentrations, antimicrobial treatment, weight on arrival, or interaction terms were not correlated with either serum haptoglobin or plasma fibrinogen (as measured on days 4-28).

3.4 | Ultrasound lesions

On day 1, 7/59 (12%) animals had ultrasonographic evidence of pulmonary consolidation, including 2/29 (7%) lysate-treated animals and 5/30 (17%) control animals (Figure 3). Multivariate logistic regression demonstrated significant effects of days relative to arrival ($P < .001$), days squared ($P < .001$), and the presence of lung consolidation on arrival ($P = .02$) on the risk of ultrasound consolidation (Table 4). As all lysate-treated animals had pulmonary consolidation at days 21 and 28, logistic regression could not be performed to evaluate the effect of treatment; however, evaluation at 28 days after arrival demonstrated a trend toward increased prevalence of consolidation in lysate-treated steers (Fisher's exact test; $P = .05$). The proportion of animals with lung consolidation increased over time ($P < .001$), with a peak overall prevalence at day 21 when pulmonary consolidation was identified in 29/29 (100%) lysate-treated calves and 27/29 (93%) control calves. At the end of 28 days, pulmonary consolidation was identified in 29/29 (100%) lysate-treated calves and 24/29 (83%) control calves. Pulmonary consolidation was identified in all animals at 1 or more time points, with the exception of 1 lysate-treated calf.

At the 4-month examination, 11/23 (48%) surviving lysate-treated steers and 13/28 (46%) surviving control steers had

TABLE 4 The effects of aerosol treatment with bacterial lysate 1 day after arrival to a feedlot on the presence of pulmonary consolidation greater than 3 cm detected by targeted transthoracic ultrasound between days 4 and 28

	Odds ratio	*P* value	95% CI
Aerosol treatment			
Lysate	Not testable[a]	—	—
Saline			
Days relative to arrival[a]	1.69	<.001	1.35-2.12
(Days relative to arrival)^2	0.99	.001	0.98-0.995
Lung consolidation at baseline	17.28	.02	1.60-186.46

Note: Odds ratios, *P* values, and 95% confidence intervals were determined by multivariate logistic regression controlling for lysate treatment, time relative to arrival, weight on arrival, and the presence of pulmonary consolidation before aerosol treatment (baseline). Random effects of cohort and individual animal were also included in the final model. (Days relative to arrival)^2: The square of the number of days was analyzed as an independent variable.
[a]Due to 100% correlation between lysate treatment and ultrasound lesions at 21 and 28 days, logistic regression was not performed.

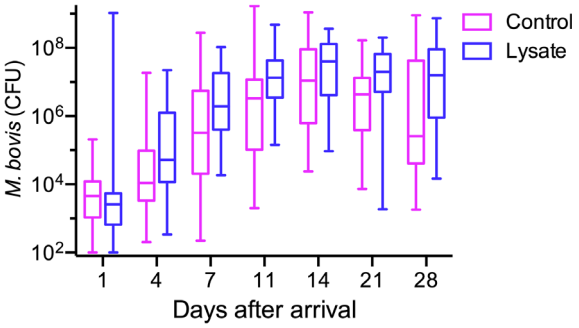

FIGURE 4 Nasal *Mycoplasma bovis* bacterial loads in control and lysate-treated steers over time after arrival. Steers received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n = 29) 1 day after arrival. Quantitative PCR was used to evaluate the numbers of *M bovis* in nasal swabs in control (n = 30) and lysate-treated (n = 29) steers. Data show the number of *M bovis* as median and quartiles (whiskers = range). The number of nasal *M bovis* bacteria was greater in lysate-treated than control calves (*P* = .001)

pulmonary consolidation greater than 3 cm. Severe consolidation of the entirety of 1 or more lung lobes was identified in 5/23 (22%) lysate-treated steers compared to 4/28 (14%) control steers. Differences between treatment groups were not statistically significant.

3.5 | Bacteria in nasal swabs

The number of nasal *M bovis* bacteria increased over time based on analysis by quantitative PCR. This increase was first identified at day 4 and persisted to the final sample point at day 28 (*P* < .001; Figure 4). This increase in the number of nasal *M bovis* bacteria was greater in lysate-treated than control calves (*P* = .001, Table 5). Body weight or antibiotic treatment in the prior 4 days was not associated with the number of nasal *M bovis* bacteria. On day 1 (the morning after arrival to the feedlot), 80% of steers had detectable *M bovis* in nasal swabs and this percentage increased to 100% by day 14.

The prevalence of nasal colonization by *M haemolytica* serotype 1 tended to be higher in calves that received bacterial lysate compared to controls (multivariate logistic regression, *P* = .08; odds ratio = 2.14 [95% CI = 0.90-7.07]; Table 6).

4 | DISCUSSION

Administration of aerosolized bacterial lysate worsened rather than improved many evaluated outcomes in these 4 cohorts of feedlot steers. The steers treated with aerosolized bacterial lysate had reduced weight gain over the first 28 days, increased numbers of *M bovis* in their nasal cavities, increased rectal temperatures, increased pulmonary consolidation, and an increased mortality rate due *M bovis* pneumonia. These results show that stimulation of inflammatory responses in the respiratory tract of these high-risk calves did not confer protection from BRD and instead exacerbated the development of *M bovis* pneumonia. These results do not support the notion that immunosuppression is the mechanism by which BRD risk factors cause disease in high-risk beef calves.

We chose to administer a lysate of *E coli* and *S aureus* rather than a lysate of BRD pathogens to avoid induction of specific acquired immune responses (ie, vaccination). We delivered the bacterial lysate on day 1 because this is typically when a preventative would be given to high-risk calves. Furthermore, this immediately precedes the time point when reduced gene expression of tracheal and lingual antimicrobial peptides were demonstrated in bronchial biopsies (ie, day 2 after dexamethasone treatment),[3] supporting this as a time when immune stimulation could be valuable. The dose of lysate was based on pilot studies performed in bull calves,[6] to ensure safety and effectiveness in stimulating innate immune responses.

Previous in vitro experiments demonstrated that bacterial lysate induced gene expression of tracheal and lingual antimicrobial peptides in cultured tracheal epithelial cells.[6] As well, pilot studies showed that aerosolized bacterial lysate stimulated an inflammatory response characterized by neutrophil recruitment to the lungs, increased interleukin-8 and -10 concentrations in lung fluids, and a mild and transient acute phase response. However, lung damage did not

TABLE 5 The effects of aerosol treatment with bacterial lysate 1 day after arrival to the feedlot on the number of nasal *Mycoplasma bovis* bacteria as determined by quantitative PCR

	Estimate of effect (β)	*P* value	95% CI
Aerosol treatment			
Lysate	0.758	.001	0.307-1.208
Saline	REF		
Days relative to arrival[a]	0.397	<.001	0.339-0.455
(Days relative to arrival)^2	−0.010	<.001	−0.012 to −0.009

Note: Coefficients (β), 95% confidence intervals (CI), and *P* values were determined by multivariate linear regression.
Abbreviation: REF, reference category. (Days relative to arrival)^2: The square of the number of days was analyzed as an independent variable.
[a]Nasal swabs were collected from days 4 to 28 after arrival.

TABLE 6 The effects of aerosol treatment with bacterial lysate 1 day after arrival to the feedlot on nasal colonization with *Mannheimia haemolytica* serotypes 1 and 2 as determined by multiplex PCR

	Serotype 1			Serotype 2		
	Odds ratio	*P* value	95% CI	Odds ratio	*P* value	95% CI
Aerosol treatment						
Lysate	2.14	.08	0.90-7.07	0.66	.33	0.29-1.51
Saline	REF			REF		
Days relative to arrival[a]	0.90	<.001	0.87-0.93	0.87	<.001	0.83-0.92

Note: Odds ratios, *P* values, and 95% confidence intervals were determined by multivariate logistic regression controlling for random effects of cohort and repeated measures on individual animals.
Abbreviation: REF, reference category.
[a]Data are based on nasal swabs collected from days 4 to 28 after arrival.

develop.[6] Similarly, a small pilot study to determine safety and assess responses in nonstressed beef cattle (data not shown) did not demonstrate adverse effects. As the bacterial lysate does not directly cause clinically significant pneumonia,[6] we speculate that the induced inflammatory responses were only detrimental in cattle at high risk of developing disease.

Beef calves at high risk of BRD were used in our study to ensure an adequate disease incidence to detect an effect of the intervention. The clinical disease in these study animals was typical of BRD in feedlot cattle with respect to timing after arrival, clinical signs, and the bacteria identified in the nasal cavity and lung. The incidence was within the expected range for high-risk calves without metaphylaxis.[10] Similarly, it is typical to find the predominance of *M bovis* deaths occurring in the second month after arrival even if the onset of disease occurs soon after arrival.[11,12] Although lysate-treated steers had reduced weight gain and a numerically higher incidence of clinical disease in the first 28 days, poor performance and a high incidence of clinical disease was also seen in the control steers. The high incidence and severity of BRD in our study underscores the need for prevention strategies in the absence of metaphylactic antimicrobial treatment for high-risk calves.

In our study, although the characteristic caseonecrotic bronchopneumonia caused by *M bovis* was the predominant cause of death, it is presumed that other viral and bacterial pathogens in addition to *M bovis* were involved in the early stages of disease. Even at the time of postmortem examination, several other pathogens were identified including bovine respiratory syncytial virus, *Trueperella pyogenes*, *Histophilus somni*, and *Pasteurella multocida*. However, sampling of lungs in the early days after arrival was not conducted.

Multiple outcomes were used to evaluate the impact of bacterial lysate on the development of respiratory disease. Rectal temperature and blood acute phase proteins are objective outcomes that correlate with systemic inflammatory responses, but do not differentiate pneumonia from diseases of other organs. Weight gain is an objective outcome of considerable relevance to producers but takes time to manifest and is dependent on influences other than BRD. Thoracic ultrasound is a chute-side test that is highly specific for identifying lung consolidation and allowed us to monitor the presence of cranioventral consolidation over time. Illness requiring antimicrobial treatment is clinically relevant but is less objective and has low sensitivity in detecting diseased animals.[13] The low sensitivity of using clinical illness to detect BRD was similarly demonstrated in our study where the treatment rate (37/59) was lower than the prevalence of ultrasonographic pulmonary consolidation (detected in 58/59 animals at 1 or more time points). Thus, only 37/58 (64%) animals with ultrasonographic pulmonary consolidation were identified as clinically ill and received antimicrobial treatment. The high mortality rate, although unexpected, was an objective and clinically relevant outcome, which furthermore allowed us to identify *M bovis* as the major cause of death in our study. Average daily gain was significantly lower in lysate-treated steers in the first 28 days after arrival compared to control steers. At this time, the steers had lost an average of 4.3 kg of body weight compared to their weight on arrival. Finally, a high proportion of steers with lung consolidation at 4 months after arrival

further supports the long-term negative effect of the bacterial lysate. The concordant results among multiple variables strengthen the conclusion that administration of bacterial lysate worsened disease in feedlot calves.

It is well accepted that stress and viral infections predispose to bacterial pneumonia in cattle and other species, and the mechanism is commonly assumed to be immunosuppression. In contrast, the findings of our study suggest a role for inflammation induced by the bacterial lysate[6] in increasing susceptibility to BRD. A contributing role for inflammation in the pathogenesis of *M haemolytica* pneumonia has been reported in prior experimental and natural-disease studies. Depletion of neutrophils[14,15] and administration of dexamethasone before experimental challenge with *M haemolytica* were each shown to reduce subsequent clinical disease and lesions.[16] In a study of natural disease, administration of meloxicam to calves at the time of castration reduced the subsequent incidence of BRD, although this could also be attributed to analgesic effects.[17] In another study, meloxicam treatment after transportation reduced acute phase proteins in blood and improved average daily gain at 21 days.[18] Thus, controlling inflammation might be beneficial in cattle during the period of increased risk of BRD, before the onset of disease.

Endogenous capacity to control inflammation might also be important in the development of BRD. In 1 study of bronchoalveolar lavage fluid proteins sampled at the time of arrival to the feedlot, calves that did not develop BRD had higher levels of the anti-inflammatory protein annexin A1 compared to healthy calves that later developed BRD.[19] It was suggested that calves with lower annexin levels on arrival had an increased tendency to pulmonary inflammation that might have resulted in increased risk of BRD. In a study of transcriptomes in blood at the time of arrival, calves that remained healthy had increased gene expression of anti-inflammatory pro-resolving mediators compared to calves that subsequently developed BRD.[20] Thus, evidence for a negative role of inflammation in BRD pathogenesis is provided by the results of the current study that shows inflammation induced by the bacterial lysate can worsen BRD, and by prior studies that show protective effects of anti-inflammatory treatments, leukocyte depletion, or increased endogenous anti-inflammatory substances. Whether this represents an inability to regulate harmful inflammatory and tissue-damaging responses to infection requires further study.[21]

It is biologically plausible that BRD risk factors—such as stress and viral infection—could lead to disease by inducing inflammation or by dysregulating inflammatory responses. For example, although chronic stress is immunosuppressive, acute stress can enhance immune responses.[22] In mice, stress (social disruption) can induce pulmonary inflammation including neutrophil and monocyte recruitment, inflammatory cytokines, and leukocyte adhesion molecules,[23] and increase inflammatory responses and death following lipopolysaccharide challenge.[24] Stressed cattle can have increased levels of inflammatory cytokines, acute phase proteins, and neutrophils.[25-30] In cattle, dexamethasone and corticotrophin-releasing hormone trigger acute phase responses that amplify the response to inflammatory stimuli[28,31] and reduce neutrophil clearance.[32] Similarly, viruses stimulate inflammation.[33-35]

Bovine herpesvirus-1 causes secretion of pro-inflammatory cytokines and neutrophil adhesion and activation;[36,37] and bovine respiratory syncytial virus induces inflammatory cytokines and neutrophil responses,[38-41] and exacerbates interleukin-17 responses in cells co-infected with bovine respiratory syncytial virus and *M haemolytica*.[42] These pro-inflammatory effects of BRD risk factors—stress and viral infection—support the idea that induced inflammation in the respiratory tract increases susceptibility to disease when calves are later exposed to pathogens.

In contrast to the above research on *M haemolytica*, the role of inflammation on the early development of *M bovis* pneumonia has not been investigated. In our study, administration of bacterial lysate resulted in increased number of *M bovis* in the nasal cavity and more severe disease with higher risk of death due to *M bovis* pneumonia. The latter outcome occurred more than 30 days after administration of the bacterial lysate but reflected the greater severity of disease that was seen during the earlier period. Although the innate immune responses directly stimulated by the lysate would presumably have subsided by this time, the initial effects of the lysate occurring in the few days after arrival appeared to have launched these calves on a trajectory to severe respiratory disease.

The longer-term effects of inflammation early after arrival are potentially relevant to the pathogenesis of *M bovis* pneumonia. As *M bovis* can be found in healthy animals and normal lungs,[12,43] it is likely that additional factors are necessary for development of the caseonecrotic bronchopneumonia that is responsible for death. The later onset of *M bovis* pneumonia compared to other BRD mortalities as well as the frequent isolation of not only *M bovis* but also additional pathogens suggests that caseonecrotic bronchopneumonia arises from earlier lung lesions.[44]

Once established, *M bovis* caseonecrotic pneumonia is often poorly responsive to treatment,[45] and thus further understanding of factors that contribute to establishment of infection and disease progression are needed. The results of our study and others suggest a role for inflammation in the early development of *M bovis* pneumonia.

Bacterial lysate administration increased the number of *M bovis*, consistent with other studies. In our study, we evaluated nasal *M haemolytica* serotypes based on multiplex PCR analysis, as pathogenicity varies between serotypes. Whereas the pathogenic *M haemolytica* serotype 1 increased significantly between day 1 and day 4, the prevalence of *M haemolytica* serotype 2 remained stable (data not shown).

The importance of the first few days after arrival with respect to subsequent development of BRD is highlighted by the marked early shifts in bacterial populations in the nasal cavity, the rapid increase in markers of systemic inflammation (fever, acute phase proteins), and the early development of lung consolidation that often persisted to the end of the study. Aerosolized bacterial lysate given on the day following arrival influenced nasal *M bovis* numbers out to 28 days and mortality rates in the second month. Of the 7 animals that died due to *M bovis*, 6 deaths occurred in the second month after arrival, although high levels of *M bovis* were detected in the nasal cavity and extensive pulmonary consolidation was evident on thoracic ultrasound in the

second week. This suggests that even deaths occurring later in the feeding period can be influenced by microbiologic and pathologic changes that occur soon after arrival to the feedlot.

As the ultimate goal of bacterial lysate administration was to reduce the incidence and severity of lung disease rather than prevent infection by individual pathogens, additional bacteria or viruses were not evaluated in our study. Expanding the study to include additional animals might have identified additional differences between treatment groups that were statistically significant. To further understand how bacterial lysate administration led to increased disease, it would be necessary to sample the lung early after arrival to investigate how bacterial populations are affected as well as the interaction between lung lesions, inflammation, and the various pathogens including *M haemolytica* and *M bovis*.

5 | CONCLUSIONS AND CLINICAL IMPORTANCE

Stimulation of respiratory innate immune responses worsened respiratory disease outcomes in high-risk feedlot calves. These results provide new insights into the role of inflammation in BRD pathogenesis and dispute the paradigm that immunosuppression is responsible for the development of BRD in high-risk feedlot calves. These findings are important for developing interventions to reduce the impact of BRD.

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CONFLICT OF INTEREST DECLARATION

E. E. M. and R. T. are employees of Zoetis. All the other authors have no conflicts of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Animal Care Committee of the University of Guelph (AUP #3286) according to Canadian Council on Animal Care guidelines. Calves were provided by the funding agency (Ontario Ministry of Agriculture, Food and Rural Affairs; UofG2013-1488) for the purpose of this research.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Jeff L. Caswell https://orcid.org/0000-0002-1991-3219

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