The Effect of Aerosolized Bacterial Lysate on the Development of Pneumonia in Cattle

by

Laura L Bassel

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ABSTRACT

THE EFFECT OF AEROSOLIZED BACTERIAL LYSATE ON THE DEVELOPMENT OF PNEUMONIA IN CATTLE

Dr. Laura Bassel
University of Guelph, 2020

Advisor:
Dr. Jeff Caswell

Bovine respiratory disease (BRD) is a significant problem affecting the health, welfare and productivity of beef cattle. Disease most commonly occurs shortly after arrival to the feedlot following a number of stressors including weaning, transportation, co-mingling, processing, and ration changes. This leads to stress and viral infections which contribute to alterations in immune responses and a high incidence of bacterial pneumonia. Control of BRD often relies on mass administration of antimicrobial medication to high-risk groups. There is increasing concern about antimicrobial use in food production and a need for alternative control strategies. The innate immune system responds rapidly to stimuli, and induced responses can protect against a number of different pathogens. Stimulation of the innate immune system was investigated as a potential target for intervention to prevent BRD. For proof of concept, respiratory innate responses were stimulated via delivery of aerosolized bacterial lysate. Pulmonary cellular and humoral responses and systemic responses were characterized. Lysate administration triggered cytokine production, neutrophil infiltration, and alterations in bronchoalveolar lavage fluid, and caused a mild and transitory systemic inflammatory reaction with no significant lung lesions. When delivered to clean-catch colostrum-

deprived Holstein calves prior to aerosol *Mannheimia haemolytica* challenge, aerosolized bacterial lysate provided mild protection as evidenced by reduced neutrophilia early after infection and a tendency for reduced lung lesions at necropsy. However, lysate administration prior to challenge did not reduce nasal or lung *M. haemolytica* numbers. Unexpectedly, in high-risk feedlot steers, administration of bacterial lysate following arrival increased BRD. Lysate-treated cattle had increased serum haptoglobin and body temperature and reduced weight gain over the first 28 days. Lysate administration increased nasal *Mycoplasma bovis* and increased mortality due to *M. bovis*. The combined findings of these studies provide insight into the mechanisms behind increased susceptibility to respiratory disease in newly arrived feedlot cattle and suggest that stress and virus-induced pro-inflammatory responses may contribute to pathogenesis. Furthermore, this study suggests that pre-existing inflammation contributes to the development of BRD and that mortalities due to *M. bovis* occurring in the second month are influenced by factors initiated in the immediate post-arrival period.

DEDICATION

This thesis is dedicated to my family whose unwavering support made completion of this degree possible.

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TABLE OF CONTENTS

Abstract ii
Dedication iv
Acknowledgements v
Table of Contents vi
List of Tables xi
List of Figures xiii
List of Abbreviations xvi
List of Appendices xviii
1 The use of aerosolized innate immune agonists to aid in prevention of bovine respiratory disease 1
1.1 Introduction 1
1.2 The role of epithelial innate immunity in antibacterial defenses in cattle 2
1.2.1 Introduction 2
1.2.2 Anatomic defenses & mucociliary clearance 3
1.2.3 Humoral defenses in the ASL 8
1.2.4 DUOX Peroxidase System 11
1.2.5 Mucins 11
1.2.6 Lactoferrin and lysozyme 12
1.2.7 Surfactant proteins 13
1.2.8 Host defense peptides 16
1.2.9 Complement 21
1.3 Stimulation of the innate immune response for the prevention of pulmonary disease 22
1.3.1 Innate immune stimulation in ruminants 23
1.3.2 Currently available respiratory immunostimulants in other species 31
1.3.3 Laboratory animal models 34
1.4 Conclusions and thesis proposal 43
1.4.1 Objectives 44
1.4.2 Hypotheses 45
1.5 Tables 46
1.6 Reference List 49
2 Pulmonary and systemic responses to aerosolized lysate of *Staphylococcus aureus* and *Escherichia coli* in calves 66

2.1 Abstract 66
2.2 Introduction 67
2.3 Materials and Methods 70
2.3.1 Preparation of bacterial lysate 70
2.3.2 Cell culture experiments 71
2.3.3 Aerosolization of bacterial lysate to calves 72
2.3.4 Clinical and hematologic responses 73
2.3.5 Bronchoalveolar lavage fluid cellular analysis 73
2.3.6 Mass spectrometry analysis 74
2.3.7 Cytokine analysis 75
2.3.8 Post-mortem examination 77
2.3.9 Statistical analysis 77
2.4 Results 78
2.4.1 Induction of TAP and LAP expression in tracheal epithelial cells 78
2.4.2 Clinical and clinical pathologic findings 79
2.4.3 Changes in bronchoalveolar lavage fluid 80
2.4.4 Pathologic findings 82
2.5 Discussion 83
2.6 Conclusions 89
2.7 Reference List 90
2.8 Figures 95
2.9 Tables 106
2.10 Supplemental Material 110
3 Development of an aerosolized *Mannheimia haemolytica* pneumonia model in calves 128
3.1 Abstract 128
3.2 Introduction 129
3.3 Materials and Methods 131
3.3.1 Animals 131
3.3.2 Preparation and characterization of *Mannheimia haemolytica* 133
3.3.3 Delivery of aerosols 135
3.3.4 Clinical data and sampling 136
3.3.5 Euthanasia and post-mortem evaluation and sampling 137
3.3.6 Statistical Analysis 138
3.4 Results 139

3.4.1 Characterization of *Mannheimia haemolytica* challenge strains 139
3.4.2 Anatomic distribution of aerosols 139
3.4.3 Pilot Experiment 1: Aerosolized *Mannheimia haemolytica* serotype 1 challenge in conventional calves 140
3.4.4 Aerosolized *Mannheimia haemolytica* serotype 2 challenge in clean-catch colostrum-deprived calves 141
3.4.5 Aerosolized *Mannheimia haemolytica* serotype 1 challenge in clean-catch colostrum-deprived calves 142
3.5 Discussion 145
3.6 Conclusion 152
3.6 Reference List 153
3.7 Tables 157
3.8 Figures 160
3.9 Supplemental Material 175
4 The effect of aerosolized bacterial lysate on experimentally induced *Mannheimia haemolytica* pneumonia in calves 195
4.1 Abstract 195
4.2 Introduction 196
4.3 Materials and Methods 199
4.3.1 Animals 199
4.3.2 Preparation of Mannheimia haemolytica for challenge 200
4.3.3 Delivery of aerosols 200
4.3.4 Clinical monitoring and antemortem sampling 201
4.3.5 Bronchoalveolar lavage fluid sampling and analysis 202
4.3.6 Study endpoints and assessment of gross and histologic lesions 202
4.3.7 Quantitative bacterial culture 204
4.3.8 Statistical analysis 204
4.4 Results 205
4.4.1 Baseline parameters 205
4.4.2 Response to aerosolized bacterial lysate 206
4.4.3 Clinical parameters and mortality 207
4.4.4 Blood leukocytes and acute phase proteins 209
4.4.5 Bacterial culture 210
4.4.6 Postmortem lung lesions 210
4.5 Discussion 211

4.6 Conclusions 219
4.7 Reference List 220
4.8 Tables 222
4.9 Figures 225
4.10 Supplemental Material 235
5 Stimulation of respiratory innate immune responses worsens naturally occurring respiratory disease in high-risk feedlot calves 243
5.1 Abstract 243
5.2 Introduction 244
5.3 Materials and Methods 246
5.3.1 Study animals 246
5.3.2 Creation and delivery of the bacterial lysate 247
5.3.3 Evaluation and sample collection 248
5.3.4 Targeted thoracic ultrasound 248
5.3.5 Clinical Illness & Mortality 249
5.3.6 Nasal Swab Processing and PCR 250
5.3.7 Statistical analysis 252
5.4 Results 253
5.4.1 Body weight 254
5.4.2 Clinical parameters 254
5.4.3 Bacteria in nasal swabs 255
5.4.4 Ultrasound lesions 256
5.4.5 Clinical disease 256
5.4.6 Mortality 257
5.5 Discussion 258
5.6 Conclusions 269
5.7 Reference List 270
5.8 Tables 277
5.9 Figures 283
5.10 Supplementary Material 289
6 Discussion and Conclusions 300
6.1 Summary of Chapter 2 301
6.2 Summary of Chapter 3 302
6.3 Summary of Chapter 4 305
6.4 Summary of Chapter 5 306

6.5 Disparity between the experimental model and natural disease 307
6.6 Inflammation..... 309
6.7 Mycoplasma bovis..... 314
6.8 Mannheimia haemolytica 315
6.9 Need for alternate strategies to prevent BRD 318
6.10 Conclusion 319
6.11 Reference List..... 321
7 Appendices 326

LIST OF TABLES

Table 1.1: Summary of licensed immunostimulants in Canada and the United States of America as of April 2020 indicated to prevent respiratory disease in animals. 46

Table 1.2: A summary of studies using innate immune stimulation to prevent respiratory disease in laboratory animals. 47

Table 2.1: Summary of calves used for each study, samples collected, and tests performed. 106

Table 2.2: Objective clinical scoring system for calves. 107

Table 2.3: Elevated proteins in bronchoalveolar fluid following aerosolization of bacterial lysate. 108

Table 2.4: Proteins that were reduced in bronchoalveolar fluid following aerosolization of bacterial lysate. 109

Table 3.1: Clinical signs following *Mannheimia* haemolytica challenge. 157

Table 3.2: Gross lesions and isolation of *Mannheimia haemolytica* from the lung 48 to 72 hours after aerosol challenge of 8 colostrum-deprived calves. 158

Table 3.3: Lung histopathology following *Mannheimia haemolytica* challenge. 159

Table 4.1: Clinical signs in control and lysate-treated calves at different times relative to challenge. 222

Table 4.2: Results of multivariate mixed model analysis of acute phase proteins and hematologic values in clean-catch colostrum-deprived calves following aerosol challenge with *Mannheimia haemolytica.* 223

Table 4.3: Histologic findings in control and lysate-treated calves following *Mannheimia haemolytica* challenge. 224

Table 5.1: Baseline parameters in lysate- and saline-treated calves, measured on arrival, prior to treatment. 277

Table 5.2: The effect of on-arrival treatment with aerosolized bacterial lysate on body temperature, serum haptoglobin, and plasma fibrinogen over time after arrival 278

Table 5.3: The effects of aerosol treatment with bacterial lysate one day after arrival on the number of nasal *Mycoplasma bovis* determined by quantitative PCR. 279

Table 5.4: The effects of aerosol treatment with bacterial lysate one day after arrival on nasal colonization with *Mannheimia haemolytica* serotype 1 determined by multiplex PCR 280

Table 5.5: The effects of aerosol treatment with bacterial lysate one day after arrival to a feedlot on the presence of pulmonary consolidation greater than 3 cm detected by targeted trans-thoracic ultrasound. 281

Table 5.6: The effects of aerosol treatment with bacterial lysate on the risk of clinical disease, mortality due to *Mycoplasma* bovis, and overall mortality in 59 feedlot calves. 282

LIST OF FIGURES

Figure 2.1: Effect of bacterial lysate on relative tracheal antimicrobial peptide and lingual antimicrobial peptide gene expression in tracheal epithelial cells……..95

Figure 2.2: Clinical scores in conventional calves after aerosol administration of bacterial lysate. 96

Figure 2.3: Clinical parameters in conventional calves after aerosol administration of bacterial lysate. 97

Figure 2.4: Hematologic values in calves before and after aerosol administration of bacterial lysate. 98

Figure 2.5: Cytology of bronchoalveolar lavage fluid following aerosol administration of bacterial lysate and saline. 99

Figure 2.6: Bronchoalveolar lavage fluid cell counts prior to and post aerosolization of bacterial lysate. 100

Figure 2.7: Bronchoalveolar lavage fluid cytology 24-hours following aerosolized bacterial lysate. 101

Figure 2.8: Cytokine responses to aerosolized bacterial lysate..... 102

Figure 2.9: Relationship between challenge dose of *Staphylococcus aureus* and *Escherichia coli* lysate and lung: heart weight ratios. 103

Figure 2.10: Post-lysate histology of bronchioles. 104

Figure 2.11: Post-lysate lung histology..... 105

Figure 3.1: Anatomic distribution of aerosolized particles. 160

Figure 3.2: Histological distribution of aerosolized dye..... 161

Figure 3.3: Gross lung lesions following aerosolized *Mannheimia haemolytica* challenge to a calf with positive leukotoxin antibody..... 162

Figure 3.4: Histologic lung lesions following aerosolized *Mannheimia haemolytica* challenge to a calf with positive leukotoxin antibody. 163

Figure 3.5: Clinical parameters over time after challenge with aerosolized Mannheimia haemolytica serotype 1. 164

Figure 3.6: Individual animal clinical parameters over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1 in 8 colostrum-deprived Holstein bull calves. 165

Figure 3.7: Acute phase proteins in blood following *Mannheimia haemolytica* challenge. 166

Figure 3.8: Individual animal acute phase proteins in blood following *Mannheimia haemolytica* challenge. 167

Figure 3.9: Blood leukocyte counts over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1. 168

Figure 3.10: Individual animal blood leukocyte counts over time after challenge of 8 colostrum-deprived Holstein bull calves with aerosolized *Mannheimia haemolytica* serotype 1. 169

Figure 3.11: Ultrasonographic findings in the lungs of calves. 170

Figure 3.12: Gross lung lesions in colostrum-deprived calves challenged with *Mannheimia haemolytica.* 171

Figure 3.13: Digital evaluation of the extent of lung lesions. 172

Figure 3.14: Relationship among 3 quantitative measures of gross lung lesions including subjective visual estimation of the percentage of lung affected, software-based image analysis, and lung: heart weight ratios. 173

Figure 3.15: Histologic lesions in lung from a colostrum-deprived calf challenged with *Mannheimia haemolytica* 174

Figure 4.1: Experimental timeline with sampling points. 225

Figure 4.2: Bronchoalveolar fluid cytology in control and lysate-treated calves. 226

Figure 4.3: Cytokine concentrations in bronchoalveolar lavage fluid sampled at 24 hours following aerosolization of bacterial lysate or saline 227

Figure 4.4: Clinical scores over time relative to challenge with *Mannheimia haemolytica.* 228

Figure 4.5: Clinical parameters over time relative to challenge with *Mannheimia haemolytica* in control and lysate-treated calves. 229

Figure 4.6: Blood leukocytes over time relative to *Mannheimia haemolytica challenge* in calves that were treated with bacterial lysate or saline. 230

Figure 4.7: Acute phase protein concentrations over time relative to *Mannheimia haemolytica* challenge. 231

Figure 4.8: Nasal carriage of *Mannheimia haemolytica* following challenge in control and lysate-treated colostrum-deprived calves. 232

Figure 4.9: Quantitative culture of *Mannheimia haemolytica* from the lung of calves following challenge. 233

Figure 4.10: Severity of lung lesions at postmortem examination in control and lysate-treated calves following challenge with *Mannheimia haemolytica.* 234

Figure 5.1: Weight over time after arrival in control and lysate-treated steers..... 283

Figure 5.2: Rectal temperature, serum haptoglobin and plasma fibrinogen over time after arrival in control and lysate-treated steers. 284

Figure 5.3: Nasal *Mannheimia haemolytica* in control and lysate-treated steers..... 285

Figure 5.4: Nasal *Mycoplasma bovis* in control and lysate-treated steers over time after arrival. 286

Figure 5.5: Ultrasound lesions over time after arrival in control and lysate-treated steers. 287

Figure 5.6: Clinical illness in control and lysate-treated steers. 288

LIST OF ABBREVIATIONS

AHL	Animal Health Laboratory
AMP	antimicrobial peptides
ANOVA	analysis of variance
ASL	airway surface liquid
BALF	bronchoalveolar lavage fluid
BNBD	bovine neutrophil beta defensin
BHV	bovine herpes virus
BPIV	bovine parainfluenza virus
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
bTEC	bovine tracheal epithelial cell
BVDV	bovine viral diarrhea virus
CFU	colony forming units
CI	confidence interval
DNA	deoxyribonucleic acid
DUOX	dual oxidase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase granulocyte
HBD	human beta defensin
HDP	host defense peptide
HMGB	high mobility group box
IFN	interferon
IL	interleukin
Ig	immunoglobulin
iNOS	inducible nitric oxide synthase
IPAF	interleukin-1β -converting enzyme protease-activating factor
iPPVO	inactivated parapoxvirus ovis
IRF	interferon response factor
LAP	lingual antimicrobial peptide
LPS	lipopolysaccharide
Maldi-Tof	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinases
MAVS	mitochondrial antiviral-signalling protein
MDA	melanoma differentiation associated gene
mRNA	messenger ribonucleic acid
MUC	mucin
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NLR	nucleotide oligomerization domain-like receptor
NOD	nucleotide oligomerization domain
NTHi	non-typeable *Haemophilus influenzae*

ODN	oligodeoxynucleotide
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RIG	retinoic-acid-inducible gene-like helicases
ROS	reactive oxygen species
RT-PCR	reverse transcription quantitative PCR
SEM	standard error of the mean
SP	surfactant protein
STING	stimulator of interferon genes
TAP	tracheal antimicrobial peptide
Th	T helper type
TLR	toll-like receptor
TMT	tandem mass tag
TNF-α	tumor necrosis factor-alpha

LIST OF APPENDICES

Appendix 1: Calf Feeding Record and Health Check…….326
Appendix 2**:** Necropsy protocol for sampling and scoring *Mannheimia haemolytica*-challenged calves……..…….....327
Appendix 3: Supplemental Figure A3.: Postmortem scoring sheet for estimating the extent of lung lesions. …….328
Appendix 4: Ultrasound scoring sheets……329

1 The use of aerosolized innate immune agonists to aid in prevention of bovine respiratory disease

1.1 Introduction

Compromised innate immune function is an important predisposing factor for the development of pneumonia, and is considered to be a major contributor to bovine respiratory disease (BRD)(Caswell, 2013). The innate immune system is a multifaceted first line of protection of the airways and lungs against a range of potential pathogens and consists of both constitutive and inducible components. The surface of the alveoli and airways represents the largest area to which the body is constantly exposed to the outside world, including potentially pathogenic bacteria, viruses, fungal spores, particulate matter and other irritants. Vulnerability of the lung is compounded by the need to maintain a thin barrier for effective respiratory exchange. This function can be severely compromised by inflammatory infiltrates and edema, as well as through bystander damage to the respiratory epithelium resulting from inflammation occurring during the elimination of pathogens. The respiratory epithelium is a critical component of the defenses of the respiratory tract, providing both constitutive protective, sentinel function, and inducible responses when needed. The purpose of the first section of this literature review is to provide an overview of the contribution of respiratory epithelial cells to innate immunity in the respiratory system, with more detailed consideration of their role in inducible innate immunity to extracellular bacteria in cattle. The following section focuses on studies investigating the stimulation of innate immunity for prevention of respiratory disease in cattle and other species. Finally, the research proposal addresses the rationale for this project where stimulation of innate immune defenses is explored as a potential strategy to prevent bovine respiratory disease.

1.2 The role of epithelial innate immunity in antibacterial defenses in cattle

1.2.1 Introduction

Cattle are particularly vulnerable to lung disease due to a number of factors. These include a low alveolar surface area-to-body weight ratio, a high resting oxygen requirement when compared with other similarly sized animals, a lack of collateral ventilation, and the presence of a right tracheal bronchus (Veit and Farrell, 1978; Kainer and Will, 1981). Despite this vulnerability, the numerous structural and functional protective mechanisms are, in most cases, effective in excluding or neutralizing pathogens and preventing disease. However, if these barriers are breached, induced innate responses can rapidly be initiated and upregulated and, over time, an adaptive immune response may result. When inflammatory responses are triggered, it is important to limit the negative effects and bystander damage to host tissue. Thus, timely resolution of inflammatory processes is another vital function of the innate immune system.

A better understanding of these innate immune defenses, including pathways involved in stimulation and upregulation of antimicrobial defenses, may lead to strategies for disease prevention. This section will focus on the inducible components of the innate immune system, which could serve as potential therapeutic targets. As well, excessive inflammation can occur due to dysregulated responses, and may be important in the development of BRD lesions. While some aspects of bovine innate immunity are well-studied, not all aspects have been investigated in this species. Where available, cattle-specific information will be discussed, but in some cases the information will be extrapolated from research conducted in other species. Although it is

well-recognized that stress can affect the effectiveness and intensity of the innate responses, with both increased and decreased immune responsiveness seen depending on the duration of the stressor and timing relative to pathogen exposure, as well as the outcome being investigated, this topic has been recently reviewed elsewhere and will not be thoroughly explored here (Caswell, 2013; Dhabhar, 2014).

1.2.2 Anatomic defenses & mucociliary clearance

Airways are lined by pseudostratified ciliated epithelial cells, club cells, goblet cells and neuroendocrine cells. In addition, submucosal glands are seromucinous glands responsible for the majority of airway secretions. Alveolar epithelial cells consist of membranous type I pneumocytes, which facilitate gas exchange between alveolar spaces and capillaries; plump type II epithelial cells, which produce surfactant and act as regenerative stem cells; and the poorly described brush cells (Grubor et al., 2006). Far from being solely a passive physical barrier to infection with tight and adherens junctions, respiratory epithelial cells play an active, responsive, and coordinating role in host defenses (Whitsett and Alenghat, 2014). Epithelial cells contribute to pulmonary protection through the secretion of mucus and the beating of cilia, resulting in mucociliary clearance whereby inhaled pathogens and particulates impact the wall of airways, are entrapped in mucus and are then moved away from the lungs to the oral cavity. Epithelial cells can detect pathogens, activate and recruit other cells via cytokines including chemokines, express adhesion molecules, and produce many constitutive and inducible antimicrobial products such as β-defensin, nitric oxide (NO), and lactoferrin (Parker and Prince, 2011). They also play a role in the adaptive immune system, activating local dendritic cells through the release of cytokines (Parker and Prince, 2011).

The respiratory tract begins at the nares, extends through the nasal cavity, sinuses and pharynx, is gated at the larynx, continues down the trachea to the bronchi and bronchioles, and terminates in the alveoli where gas exchange occurs. Numerous defense mechanisms are present along the length of the respiratory tract. Hairs around the nostrils block large particulate matter, and turbulent airflow in nasal cavities and sinuses causes larger inhaled particles to impact the walls of the airways, where they are entrapped in the airway surface liquid (ASL) produced by submucosal glands and goblet cells. The ciliated epithelial cells lining the airways work in a coordinated fashion to move ASL and the entrapped particles up the trachea, away from the lungs or caudally in the nasal passages, toward the nasopharynx where the ASL-particulate mixture can be swallowed.

The ASL has a periciliary liquid or “sol” layer and a luminal gel/mucus layer (Ackermann et al., 2010). The periciliary layer is more fluid and maintains a pH that is slightly more acidic than blood, whereas the mucus layer is more viscous and contains a larger proportion of mucin glycoproteins and proteoglycans. The recent “Gel on Brush” model of ASL suggests that the periciliary layer contains tethered membrane-spanning mucins and mucopolysaccharides that prevent inhaled particles and other mucins from penetrating (Button et al., 2012). The ASL consists of 97% water, with the remaining solids consisting of mucins, proteins, lipids, salts and cellular debris (Munkholm and Mortensen, 2014). Most inhaled bacteria and other pathogens are quickly cleared via entrapment in the ASL and expulsion by the mucociliary apparatus. When larger or more abundant particulates are present in the airways, coughing and sneezing can more forcibly expel inhaled irritants from the nasal cavity and airways.

Effective production and clearance of mucus is of major importance to respiratory health, as is evidenced by the severity of disease seen in human patients when this process is compromised, such as with cystic fibrosis (characterized by thick mucus) and primary cilia dyskinesia (ineffective ciliary beating) (Randell and Boucher, 2006). Structurally, cilia consist of microtubules arranged in a 9 plus 2 arrangement, with outer and inner dynein arms responsible for movement (Salathe, 2007). Ciliary beat frequency can be increased in response to environmental conditions through cyclic AMP-dependent phosphorylation and potentially cyclic GMP-dependent kinases, calcium and intracellular pH (Salathe, 2007). In healthy animals, mucociliary movement of particles has been reported to range from 4.2 mm/minute to 6.2 mm/minute to 15 mm/minute varying with mucus quality and location (Reinhold et al., 1993; Mars et al., 1994). Under normal circumstances, 90% of aerosolized *Mannheimia haemolytica* are removed from the lungs by four hours (Lillie and Thomson, 1972). Ciliary beat frequency can be increased in response to cytokines, including tumour necrosis factor (TNF)-α and interleukin (IL)-1β (Jain et al., 1995).

In cattle, reduced function of the mucociliary apparatus occurs secondary to viral infection, air quality, cold air, and potentially dehydration. It is well accepted that viral infections often precede development of bacterial pneumonia, and one way in which viral infections predispose to bacterial infection is by altering mucociliary clearance. Bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus (BPIV) 3, bovine herpes virus (BHV)1 and bovine coronavirus have been shown to infect respiratory epithelial cells. Ultrastructural studies of BRSV demonstrated a loss of cilia in epithelial cells following viral infection (Castleman et al., 1985; Gershwin et al., 2008). Mucociliary

clearance is altered by both loss of cells due to virus-induced necrosis and loss of function due to cilia loss , and this has been shown *in vivo* where infection with BPIV-3 decreased inhaled pulmonary cobalt oxide particle clearance, and BRSV infection reduced pulmonary clearance of (99m)Tc-labeled ovalbumin (Slauson, 1989).

Poor air quality on trucks during transportation, in sales barns, and on the feedlot might increase the risk of pneumonia. In young calves housed indoors, the number of airborne bacterial colony forming units was shown to be strongly predictive for increased risk of pneumonia; however, the mechanism by which this occurs was not determined (Lago et al., 2006). Hog barn dust extract has been shown to increase baseline ciliary beat frequency in cultured bovine tracheal epithelial cells, but pre-treatment with hog barn dust decreases responsiveness to beta agonists (Wyatt et al., 2008). Air pollution is a known risk factor for many human respiratory diseases, but the mechanisms of effect vary with the pollutant. A recent panel discussion at the Agricultural Safety and Health Council of America/National Institute for Occupational Safety and Health conference indicated that there is a high prevalence of respiratory symptoms in hog barn workers; the prevalence of respiratory disease in feedlot workers, however, is unknown (MPH et al., 2010). One study investigating air quality on feedlots and other agricultural locations demonstrated a high amount of airborne lipopolysaccharide (LPS) on feedlots, and correlated the level of its reactive constituent 3-hydroxy fatty acid (3-OHFA) with increased respiratory inflammation, as measured by analysis of nasal lavage fluid (Burch et al., 2009). These results suggest that reduced air quality during transportation, at the sales barn, or at the feedlot, might also negatively affect the respiratory tract of cattle; however, direct evidence for this is

lacking. More research is required to understand the relationship between air quality and BRD.

Cold-exposed calves have been shown to have both altered pulmonary deposition of particles and decreased nasal mucus velocity, as well as decreased tracheal mucus velocity *ex vivo* (Diesel et al., 1991). Experiments on murine tracheal epithelial cells showed decreased ciliary beat frequency with cooler air (Christopher et al., 2014). There is no epidemiological evidence correlating BRD with low temperatures, and contradictory evidence on the effect of large temperature variations, with different studies reporting a positive association, negative association, or lack of association between meteorological data and BRD (Taylor et al., 2010).

Cattle cohorts with significant weight loss during shipping (i.e. “shrink”) are at increased risk for BRD following arrival to feedlots (Cernicchiaro et al., 2012), and it has been hypothesized that this is due to dehydration-induced increased mucus viscosity. Thickened mucus is of major importance in the pathogenesis of cystic fibrosis and chronic obstructive pulmonary disease in humans due, in part, to impaired clearance and obstruction of small airways. The pathogenesis of these lesions, however, involves locally disordered ion and water transport and mucus production rather than systemic dehydration, and therefore it is difficult to extrapolate from these conditions to BRD (Seys et al., 2015). Systemic dehydration has been demonstrated to decrease mucus velocity in dogs (Chopra et al., 1977), but this has not been demonstrated in cattle, and it is likely that “shrink” is a proxy for other confounding management and calf factors that increase the risk of BRD.

1.2.3 Humoral defenses in the ASL

In addition to entrapping particles, the ASL contains many humoral factors, such as surfactant proteins (SP), lactoferrin, lysozyme, collectins, defensins, cathelicidins, complement, and reactive oxygen species produced by the dual oxidase (DUOX) lactoperoxidase system, produced by epithelial cells, as well as components of the adaptive immune system (e.g. immunoglobulins (Ig)A and IgG). These various components have numerous, redundant and pleiotropic effects, including opsonization, killing or limiting growth of pathogens, antioxidant effects, protection from proteolytic injury, and regulation of inflammation (Grubor et al., 2006). Most of these substances are produced by the respiratory epithelial cells, but they can also originate from recruited or resident leukocytes and serum. Importantly, the production of many of these defenses can be increased when needed.

To coordinate the transcriptional and secretory activity necessary to mount an inducible innate response, epithelial cells are able to detect microbes, damage to host tissue, and autocrine or paracrine signals (e.g. cytokines, interferon, and antimicrobial peptides). Compared to the adaptive immune response, which is highly antigen-specific, many of the effector mechanisms of the innate immune system have broad activity against entire groups of pathogens, including bacteria, fungi and viruses. However, the manner in which individual and combinations of receptors are stimulated allows some degree of specificity of response.

Epithelial cells of the conducting airways and alveoli possess a number of PRRs that are capable of sensing pathogen-associated molecular patterns (PAMPs), molecular motifs unique to groups of microbes, and damage-associated molecular patterns (DAMPs), substances released following cellular stress, damage and

inflammation. As well, epithelial cells possess receptors capable of binding and responding to various signalling cytokines. Some of the more well-characterized PRRs include Toll-like receptors (TLR)s, retinoic-acid-inducible gene-like helicases (RIGs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs).

The TLRs are transmembrane glycoproteins that possess a conserved intracellular cytoplasmic Toll/interleukin-1 receptor domain, a membrane spanning α-helix, and a sensing area consisting of leucine-rich repeats, which is responsible for the variable pathogen specificity. Details of TLR structure and signaling pathways have been reviewed elsewhere (Kawai and Akira, 2006). In general, stimulation of TLRs by their cognate ligands results in the expression of pro-inflammatory and antimicrobial mediators, with the combination of receptors activated allowing for variations in specificity and intensity of response. There have been 10 TLRs identified in cattle (Jungi et al., 2010), all of which have been shown to be expressed in lung and tracheal cells (Berghuis et al., 2014). Signaling via PRRs initiates intracellular cascades leading to transcription of a number of substances important in respiratory defense, including β-defensins, cytokines, mucins, and lactoferrin.

The cytoplasmic RIG-I-like receptors, including RIG-I and melanoma differentiation associated gene (MDA) 5, detect viral RNA and signal through mitochondrial antiviral-signaling protein (MAVS) to trigger an antiviral response, including the production of type 1 interferons which result in the transcription of interferon response genes that have widespread antiviral effects (Loo and Gale, 2011). NOD- like receptors are cytosolic PRRs found in leukocytes and epithelial cells that consist of a C-terminal domain consisting of leucine-rich repeats capable of sensing

bacterial PAMPs, a central NOD, and an N-terminal signaling domain. NOD-like receptors include NOD1 and NOD2, which recognize different forms of bacterial peptidoglycan and use mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) to produce proinflammatory mediators (Evans et al., 2010b). Other NLRs include the NALPs and IPAF, which recognize host danger signals as well as microbial products, and activate inflammasomes, which activate caspases to convert precursors of proinflammatory cytokines to their active forms, including pro-IL-1β and pro-IL-18 to active IL-1β and IL-18, which can then be secreted (Martinon et al., 2007; Ting et al., 2008). Activation of the NLR inflammasome may also lead to a form of regulated cell death known as pyroptosis, which results in rapid cell membrane rupture and release of intracellular pro-inflammatory products (Bergsbaken et al., 2009). Necroptosis, another form of programmed pro-inflammatory cell death, is caspase-independent and can be triggered by activation of various PRRs (Stephenson et al., 2016). This form of cell death is beneficial in viral infections where it can restrict viral replication in the presence of viral caspase inhibitors that prevent apoptosis (Stephenson et al., 2016). The host also benefits by these forms of cell death as the release of inflammatory mediators will lead to the recruitment and activation of additional effector cells, as well as the blockade of further virus production.

In addition to directly sensing microbial PAMPs, respiratory epithelial cells also indirectly sense pathogens via various signal pathways. Damage to neighbouring cells results in the release of DAMPs, such as adenosine triphosphate and high mobility group box 1 (HMGB1) proteins, which can stimulate TLRs and other PRRs. Epithelial

cells possess C3a and C5a receptors and can respond when complement pathways are activated (Evans et al., 2010b). Cytokines (e.g. IL-17 and IL-22), produced by leukocytes (e.g. lymphocytes, natural killer cells, macrophages), as well as other cells including respiratory epithelia, can enhance epithelial resistance by promoting transcription of various antimicrobial molecules.

1.2.4 DUOX Peroxidase System

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), generated by the dual oxidases DUOX1 and DUOX2 and the NADPH oxidase system, are other important defense mechanisms of the ciliated respiratory epithelium. When the DUOX system is activated, H_2O_2 is released and converted by lactoperoxidase to bactericidal OSCN-, leading to killing of bacteria (Ryu et al., 2010). The Duox1 and Duox2 enzymes have been shown to be upregulated by T helper type (Th) 2 cytokines (IL-4 and IL-13), interferon (IFN)-γ, polyinosine-polycytidylic acid (poly(I:C)) and rhinovirus infection (Harper et al., 2005). Nitric oxide production by respiratory epithelium can also be increased following various stimuli. *Staphylococcus aureus* increased NO production in nasal epithelium (Carey et al., 2015). As well, LPS was shown to increase inducible nitric oxide synthase iNOS and NO in a calcium-dependent manner in alveolar epithelium (Boncoeur et al., 2013). Thus, the generation of the antimicrobial ROS can be upregulated via multiple pathways.

1.2.5 Mucins

Mucins are large glycoproteins that provide the structure to, and prevent dehydration of, airway mucus. They can be tethered to epithelial cells or secreted and can function as anchors to other host defense proteins or directly bind and prevent aggregation of pathogens. Within the respiratory tract, mucins are primarily produced by

goblet cells, club cells, mucosal glands and alveolar cells (Whitsett and Alenghat, 2014). Mucin is stored in a dehydrated form in granules that can be released in response to various stimuli (Thornton et al., 2008). Mucus secretion is responsive to airway particulate matter and pathogens via TLR signalling, and increased production helps to remove pathogens and debris during infection (Whitsett and Alenghat, 2014). Studies in knockout mice have demonstrated the importance of mucin in pulmonary health, and that mucin (MUC)5B is more critical to pulmonary health than MUC5AC (Whitsett and Alenghat, 2014). Mucin expression is upregulated in response to a variety of bacteria and viruses, and pro-inflammatory cytokines including TNF-α and IL-8 (Parker and Prince, 2011). In some situations, excess mucus production may also contribute to reduced respiratory function and is an important component of some chronic lung diseases in humans including asthma, chronic obstructive pulmonary disease and bronchitis.

1.2.6 Lactoferrin and lysozyme

Of the antimicrobial factors present in the ASL, lactoferrin and lysozyme are most abundant (Ganz, 2003). These are constitutively produced, but some components can be upregulated by airway epithelial cells and macrophages upon detection of a perceived threat. Lactoferrin is a glycoprotein that binds and sequesters iron from bacteria, modulates the immune response, has direct anti-bacterial, anti-viral, anti-parasitic, and anti-fungal properties, and can modulate the adaptive immune response (Actor et al., 2009). In mice, bovine lactoferrin has been shown to reduce neutrophil numbers and inflammatory cytokines following challenge with *Pseudomonas aeruginosa* (Valenti et al., 2017) and reduce hyperoxic lung injury (Chen et al., 2014). The factors which regulate lactoferrin production in airways are poorly understood, but upregulation

does occur after LPS stimulation. Murine mammary epithelial cells had induced lactoferrin production in supernatant after LPS stimulation, as did cultured bovine tracheal epithelial cells (Al-Haddawi et al., 2007; LI et al., 2009). Infection with type 2 bovine viral diarrhea virus (BVDV) inhibited the LPS-induced secretion of lactoferrin, and this might represent one of the ways in which viral infection can predispose to bacterial disease (Al-Haddawi et al., 2007).

Lysozyme is a constitutively secreted cationic enzyme produced by the serous cells of the submucosal glands that degrades peptidoglycan in gram-positive bacterial cell walls (Moon et al., 2010). In conjunction with membrane disruption by lactoferrin, lysozyme is also capable of killing gram-negative bacteria (Ellison and Giehl, 1991). Production of lysozyme is upregulated during inflammation, but the mechanism by which this occurs is poorly understood. A recent study indicated that phorbol 12-myristate 13-acetate (PMA) upregulated lysozyme expression via activation of protein kinase C and stimulating extracellular signal-regulated kinase-2 (ERK2) MAPK signaling (Moon et al., 2010); however, the factors influencing *in vivo* upregulation are not known. Both lysozyme and lactoferrin have reduced antibacterial activity in high salt concentrations, and are especially sensitive to divalent cations (Ellison and Giehl, 1991).

1.2.7 Surfactant proteins

Collectins are a group of proteins, with a common structure consisting of a carbohydrate recognition domain (C-type lectin domain) attached to a collagen-like region via a helical neck region, that bind to oligosaccharides on microorganisms (Grubor et al., 2006). Collectins function as soluble PRRs, and binding of microorganisms can result in aggregation, opsonization, complement activation,

enhanced phagocytosis, and inhibition of bacterial growth. As well, collectins modulate the inflammatory and adaptive immune responses. Cattle have several collectins, but within the local environment of the lung, surfactant proteins (SPs) are most important (Grubor et al., 2006). Surfactant is a fluid, primarily produced by type II pneumocytes, made up of lipid (90%) and protein (10%) that, in addition to its role in reducing surface tension in the alveoli, plays an important role in pulmonary innate defenses (McCormack and Whitsett, 2002).

Whereas SP-B and SP-C are hydrophobic, SP-A and SP-D are hydrophilic calcium-dependent PRRs that bind oligosaccharides and lipid on the surface of microorganisms (Grubor et al., 2006). Surfactant protein-A and SP-D have immunomodulatory and antimicrobial properties: they can form aggregates which inhibit cellular invasion, opsonize pathogens (via the macrophage receptors C1q and SP-R210), prevent nutrient absorption, and increase bacterial wall permeability (McCormack, 2006). The importance of SP-A and SP-D in pulmonary immunity has been demonstrated by knockout mouse studies that showed reduced clearance of a number of bacterial, mycoplasmal, viral, and fungal pathogens, in addition to increased inflammation (Ariki et al., 2012). Surfactant protein-A and SP-D can have both pro-inflammatory effects (by activating the NF-κB pathway via calreticulin/CD91) and anti-inflammatory effects (via interference with TLR2 and TLR4 signalling and, in the absence of microbes, binding of their carbohydrate recognition domain to the inhibitory receptor SIRPα) (Ariki et al., 2012).

The level of expression of SP-A and SP-D appears to be variable; however, the mechanisms of induction are poorly understood and may be pathogen-, age-,

environment- and time-dependent. In mice, aerosol exposure to a lysate of non-typable *Haemophilus influenzae* (NTHI) increased SP-D in ASL (Clement et al., 2008). Bovine parainfluenza virus 3 infection in lambs increased expression of SP-A and SP-D; however, *M. haemolytica* infection had no effect, with a trend toward decreased expression of SP-D mRNA with more chronic infection (Grubor et al., 2004; Meyerholz et al., 2004). In contrast, another study found elevated SP-D levels in lung lobes of young calves infected with either *M. haemolytica* or bovine adenovirus-3 when compared to uninfected lung lobes from the same animal or uninfected lung lobes from control calves (Miyamoto et al., 2010). Experiments on sheep demonstrated that respiratory syncytial virus infection increased SP-A and SP-D mRNA in infected cells when compared to uninfected cells from the same animal, and that the uninfected cells from infected sheep had decreased SP-D expression compared to cells from uninfected sheep (Kawashima et al., 2006). The authors suggest that cells infected with RSV may downregulate SP-D production in neighbouring cells (Kawashima et al., 2006). Interestingly, although normal bronchiolar epithelium contained SP-D identified with immunohistochemistry, hyperplastic reparative epithelium had reduced or absent labelling for SP-D (Grubor et al., 2004). Reduced surfactant production secondary to viral infection or immature differentiation during regeneration may represent another mechanism by which viral infection predisposes to bacterial disease. Environmental factors may also act via altered surfactant production to increase the risk of disease. When applied to cultured human alveolar epithelial cells, organic dust from a poultry farm reduced expression of both SP-A and SP-B, but did not affect SP-D (Natarajan et al., 2019).

Surfactant protein-B and SP-C have been generally thought to function primarily with dipalmitoylphosphatidylcholine to reduce the surface tension of alveolar fluid and facilitate lung expansion. Transgenic mice experiments, however, suggest SP-B and SP-C may also have immunomodulatory effects: SP-B deficient mice had an increased inflammatory response in alveolar macrophages and type II epithelial cells, and SP-C deficient mice were more susceptible to bacterial and viral infections, as well as increased inflammation (Augusto et al., 2003; Ikegami et al., 2005; Chroneos et al., 2009). In SP-C deficient mice, LPS treatment resulted in increased and prolonged inflammation compared to wildtype mice (Glasser et al., 2013). More work is needed to further define the role of SP-B and SP-C in bovine immune function.

1.2.8 Host defense peptides

Host defense peptides (HDPs) include the defensins and cathelicidins and consist of a number of different short (less than 60 amino acid) peptides with the shared feature of segregation of hydrophobic and hydrophilic amino acids, resulting in an amphipathic shape (Diamond et al., 2009). Host defense peptides can directly inhibit pathogens, including Gram-positive and Gram-negative bacteria, fungi, mycoplasma, protozoa and enveloped viruses (Kościuczuk et al., 2012). Most HDPs have a positive charge, which creates an electrostatic interaction with negatively charged molecules on the surface of bacteria, whereas host cells are protected by their high amounts of membrane cholesterol (Diamond et al., 2009). Host defense peptide interactions with bacteria disrupt and permeabilize the cell membrane, but they can also lead to bacterial agglutination. Other important effects of HDPs include inactivation of bacterial or host proteases, binding bacterial products including endotoxin, triggering chemotaxis of

macrophages, T-cells, and neutrophils, and stimulating both pro- and anti-inflammatory pathways in other cells (Hancock et al., 2016).

β-defensins are small cationic, cysteine-rich peptides with three disulfide bonds, that are expressed in the epithelia of the respiratory tract, gastrointestinal tract, skin, and mammary gland, as well as in bovine leukocytes, with higher expression in tissues constantly exposed to higher levels of microbes (Mackenzie-Dyck et al., 2014). The other classes of defensins are α-defensins and the circular θ-defensins, which cattle due not possess (Fjell et al., 2008). Cattle have a diverse repertoire of β-defensins with 57 genes identified (Cormican et al., 2008). The first identified β-defensin was tracheal antimicrobial peptide (TAP), identified in ciliated respiratory epithelia of cattle, followed by lingual antimicrobial peptide (LAP), first identified in the tongue and airways (Diamond et al., 1993; Russell et al., 1996). Tracheal antimicrobial peptide is expressed along the entire airway, by columnar ciliated epithelial cells (Diamond et al., 1993). In the lung, defensins are released from epithelial cells, have broad spectrum antiviral, antifungal and antibacterial activity, and can help induce an immune or inflammatory response (Tecle et al., 2010).

Expression of β-defensins can be both constitutive and inducible. TAP expression varies significantly between individuals and is highly correlated with IL-8 expression, with a trend to upregulation of TAP and LAP in lung tissue from calves infected with *M. haemolytica* (Caverly et al., 2003). It is possible to induce TAP expression in cultured tracheal epithelial cells with LPS, and LAP expression with TNF-α and LPS (Russell et al., 1996; Diamond et al., 2000). Other studies have demonstrated that Pam3CSK4 (TLR1/2 agonist), IL-17A, LPS (TLR4), IFN-α and flagellin, but not

class B CpG ODN 2007 (TLR9 agonist) or lipoteichoic acid (TLR2), increases TAP expression, acting via NF-κB pathways (Berghuis et al., 2014; Taha-Abdelaziz et al., 2016). In this study there was high variability between animals with respect to TAP expression (Berghuis et al., 2014). Genetic influences of innate ability to express antimicrobial peptides may influence disease resistance. Of relevance to BRD, both dexamethasone and viral infection with type 2 bovine viral diarrhea virus (BVDV) have been shown to ameliorate LPS-induced upregulation of TAP expression (Mitchell et al., 2006; Al-Haddawi et al., 2007).

Experiments have demonstrated *in vitro* bactericidal activity of synthetic TAP against some of the common bacteria implicated in BRD, namely *M. haemolytica*, *Histophilus somni*, and *Pasteurella multocida* (but not *Mycoplasma bovis*) (Taha-Abdelaziz et al., 2013; Vulikh et al., 2019). The minimum inhibitory concentration of TAP against the BRD pathogens was 1.56-6.25 μg/ml but bactericidal effects were inhibited by physiologic concentrations of sodium chloride and serum (Taha-Abdelaziz et al., 2013). Experiments conducted in calves showed that the administration of TAP following *M. haemolytica* challenge did not protect against disease or significantly affect the number of lung or nasal bacteria (Vulikh et al., 2019). In addition to inactivation by salts, challenges of therapeutic administration of exogenous HDP are reported to include a short half-life and protease sensitivity which may affect efficacy in vivo (Adlakha et al., 2019).

Additive or synergistic effects between different antimicrobial factors, as is seen with lysozyme, lysozyme-secretory leukocyte protease inhibitor (SLPI) and lactoferrin, (Singh PK, 2000; Grubor et al., 2006; André et al., 2015) may contribute to the

effectiveness of pathogen killing *in vivo*. Additive and synergistic effects have also been demonstrated between different combinations of human β-defensins, cathelicidin and lysozyme (Chen et al., 2005). Thus, examining the antimicrobial efficacy of a single antimicrobial factor might underestimate the efficacy of an induced innate response, as multiple antimicrobial compounds would be upregulated and additive, or synergistic effects would likely occur.

Infection with type 2 BVDV (Al-Haddawi et al., 2007) and corticosteroids (Mitchell et al., 2006) have been shown to reduce inducible expression of TAP by bovine cultured tracheal epithelial cells, and calves treated with dexamethasone had lower LPS-induced expression of TAP and LAP in bronchi (Mitchell et al., 2006). This might be another mechanism by which these risk factors lead to bacterial pneumonia. In human pathogens, various bacteria possess a number of mechanisms of resistance to antimicrobial peptides, including transcriptional interference (Cole and Nizet, 2016). It is unknown if any of the other viral components of BRD interfere with TAP function.

Alveolar macrophages constitutively express bovine neutrophil β-defensin (BNBD)-4, BNBD-5, TAP, bovine epithelial β-defensin, and enteric β-defensin, but stimulation with LPS did not increase transcription of these defensin genes (Ryan et al., 1998). However, increased human β-defensin (HBD)-3 expression was observed in human alveolar macrophages infected with *Legionella pneumophila* (Scharf et al., 2010). As well, both LPS and IFN-γ increased HBD-2 expression, whereas the expression of HBD-1 could not be further increased (Duits et al., 2002). Cultured bovine endothelial cells have demonstrated increased expression of the β-defensins LAP, bovine β-defensin 1 (DEFB1), and bovine neutrophil β-defensin 4 (BNBD4) in response

to LPS and *Staphylococcus aureus* (Alva-Murillo et al., 2012), but the relevance to pulmonary immunity is unknown. Other bovine tissues expressing various combinations of β-defensins include mammary gland, endometrium, and gastrointestinal cells (Davies et al., 2008; Tetens et al., 2010; Malmuthuge et al., 2012).

Cathelicidins are a family of host defense peptides that are stored in neutrophil and macrophage granules as prepropeptides that need to be cleaved by elastase to their active form (Tecle et al., 2010). They have a common cathelin domain at their N-terminus and a diverse antimicrobial domain located at the C-terminus (Kościuczuk et al., 2012). In addition to a wide spectrum of antimicrobial activity, various cathelicidins have been reported to be chemotactic for neutrophils, monocytes, mast cells, and T cells. Cattle possess at least 5 different cathelicidins with demonstrated antimicrobial activity (Kościuczuk et al., 2012). Bovine cathelicidins are structurally and functionally diverse. The bovine myeloid antimicrobial peptides (BMAP)-27 and BMAP-2, which have an α-helical structure, permeabilize a wide range of bacterial and fungal agents (Zanetti, 2004). The bovine peptides Bac5 and Bac7 are rich in proline and primarily target Gram-negative bacteria (Zanetti, 2004). Activated neutrophils have high expression of the cathelicidin-encoding gene Bac5 in pneumonic lungs, and *in vitro* studies showed increased neutrophil expression of Bac5 in response to LPS and *E. coli* (Tomasinsig et al., 2002). Only BMAP28 expression has been demonstrated in nasal mucosa and lung tissue (Burucúa et al., 2019). Increased levels of cathelicidin-1 and cathelicidin-4 were found in bronchoalveolar lavage fluid from cattle challenged with *M. haemolytica* versus unchallenged cattle (Boehmer et al., 2011). Increased production of cathelicidin in activated neutrophils may aid in resolution of bacterial infection, but the

capacity for induced production of the spectrum of bovine cathelicidins by bovine airway epithelial cells, as has been demonstrated in other species, has not been explored (Kovach et al., 2012).

1.2.9 Complement

The complement system consists of a number of proteins which, when activated, trigger a chain reaction of events, ultimately leading to a number of effector functions including formation of the membrane attack complex, opsonization and stimulation of inflammation via the release of C5a and C3a, the “anaphylatoxins” which lead to smooth muscle contraction, mast cell histamine release, increased vascular permeability, and leukocyte chemotaxis. Respiratory epithelial cells possess receptors for C5a and C3a and binding can trigger mucous metaplasia in the face of chronic inflammation (Evans et al., 2010b). Although most complement proteins are produced by the liver and circulate in serum, some proteins (including C2, C3, C4 and C5) are produced by type II alveolar and bronchiolar epithelial cells (Pandya and Wilkes, 2014). Thus, complement components can be present in the lungs as a result of local production, in addition to leakage of serum induced by inflammatory conditions. Although its purpose is protective, excessive complement activation can contribute to tissue damage during inflammation (Hiemstra, 2015).

Several other substances are reported to have antimicrobial activity in the respiratory tract. Anionic peptides are small molecules requiring zinc as a cofactor to exert antimicrobial activity against Gram-positive and Gram-negative organisms (Fales-Williams et al., 2002). In cattle, they are constitutively present throughout the lungs, and are not upregulated by infection with *M. haemolytica* (Fales-Williams et al., 2002). Lactoperoxidase generates hypothiocyanite ($OSCN^-$) and is important in bacterial

clearance (Gerson et al., 2000). Antiproteases such as secretory leukoprotease inhibitor (SLPI) and secretory type II phospholipase A2 (PLA2) are induced in response to cytokines and are important in protecting host tissue against damage from proteolytic enzymes in addition to their own bactericidal activity (Grubor et al., 2006). There are many more peptides and proteins, about which less is known, that will not be described in this review.

In summary, an enormous number of defense systems work together to prevent bacterial infection of the deeper respiratory tract, including numerous anatomic, cellular, and humoral factors. These defense mechanisms are highly interrelated and are often both redundant and pleiotropic, with many substances having multiple effects and mechanisms of action. In addition, many of the components of the cellular and humoral defenses share the ability to be upregulated when a threat is detected, most commonly through the stimulation of PRRs. Harnessing the potential for rapid upregulation of defenses that are protective against a broad spectrum of agents may represent a novel target for control of disease.

1.3 Stimulation of the innate immune response for the prevention of pulmonary disease

Stimulation of the innate immune system can result in a rapid, general, non-antigen-specific innate immune cellular and humoral response. Activation of the innate immune system has long been recognized as necessary for the generation of an effective adaptive immune response, and vaccine adjuvants have been developed to this end. The recognition of PAMPs activates dendritic cells, which can then migrate to the local lymph nodes to initiate an adaptive immune response through the presentation of antigen and costimulatory molecules to T cells, resulting in activation (Iwasaki and

Medzhitov, 2015). Less investigation has been conducted on the stimulation of an innate response as its own strategy for disease prevention. At the time of writing this review there are only a few licensed products that specifically target innate responses on their own. Recently, mouse models of human pneumonia have been used to investigate the utility of stimulating innate responses for prevention or treatment, with several of these demonstrating promising results. Currently licensed veterinary immunostimulants indicated to prevent respiratory disease are summarized in Table 1.1 and are briefly reviewed in this section.

1.3.1 Innate immune stimulation in ruminants

There are few *in vivo* studies investigating the effectiveness of innate immune stimulation in the prevention of respiratory disease in cattle, and only one currently licensed product. The recently released immunostimulant (Zelnate, Bayer HealthCare LLC, Shawnee Mission, KS) is described as a noncoding DNA plasmid containing multiple cytosine-guanine rich (CpG) sequences that is encased in cationic liposomes (Ilg, 2017). The product label indicated that it is “for use as an aid in the treatment of Bovine Respiratory Disease due to *Mannheimia haemolytica* in cattle 4 months of age or older, when administered at the time of, or within 24 hours after, a perceived stressful event.” In experimental studies, the administration of this DNA immunostimulant at the time of, or one day after challenge with *M. haemolytica* reduced lung lesions at necropsy compared to control calves, and treatment one day after challenge reduced mortality prior to scheduled euthanasia (Nickell, J.S. et al., 2016). Although a similar percentage of calves had *M. haemolytica* isolated from the lungs at necropsy, quantitative evaluation was not conducted. In a field study of high-risk heifers, there was reduced mortality in calves treated with the immunostimulant after arrival, however, the

incidence of morbidity was similar (Rogers et al., 2016). There were no differences in weight gain.

The mechanism of action of this product is not well-defined in cattle. However, one study administered repeated doses of this DNA immunostimulant to calves following shipping and reported no differences in activated macrophages or gamma-delta T cells in blood but found that peripheral blood mononuclear cells isolated from treated calves had a higher ratio of IFN-γ to IL-4 following stimulation with concanavalin A and staphylococcal enterotoxin B (Woolums et al., 2019). This suggests their immune responses were skewed towards a Th1 response compared to control calves (Woolums et al., 2019). Although not statistically significant, peripheral blood mononuclear cells retrieved from some control animals had increased TNF-α following stimulation, whereas none of the cells from treated animals upregulated TNF-α (Woolums et al., 2019). Treatment did not affect serum haptoglobin, TNF-α, or IL-1β concentrations (Woolums et al., 2019). Another study investigated the mode of action in human monocytes and mouse melanoma cell lines and found that ZeInate did not activate TLR9 pathways as was expected, and instead activated the interferon response factor (IRF) 3 pathway via the stimulator of interferon genes (STING) pathway (Ilg, 2017). Rather than the pro-inflammatory stimulation of NF-κB pathways, the activation of IRF3 pathways leads to a type 1 interferon response which is associated with anti-inflammatory effects (González-Navajas et al., 2012; Ilg, 2017). Further research is necessary to understand how this product exerts its effect in cattle, and whether TLR9, STING or other pathways are activated.

There is some research in ruminants investigating the CpG oligodeoxynucleotides (ODN) which act as TLR9 agonists. CpG ODNs have been reported to have an immune stimulatory effect when administered subcutaneously in cattle, resulting in transient fever, haptoglobin elevations, and neutrophilia (Nichani et al., 2004, 2006). Potential mechanisms of action of CpG ODNs have been explored in several species. The effects of CpG ODN (either A-, or B- or C-class ODN) on cultured ovine lymphocytes and peripheral blood mononuclear cells were examined. All three classes of CpG ODN resulted in increased IFN-α, IFN-γ, and IL-12 in lymph nodes, with weaker and less consistent responses identified in peripheral blood mononuclear cells (Booth et al., 2007). An evaluation of their effects on cultured tracheal epithelial cells found no upregulation of TAP expression following administration of Type B CPG ODN 2007 (Berghuis et al., 2014). It is recognized that different CPGs have varying efficacy, so it is possible that different formulations would produce a more stimulatory effect, or that other unmeasured antimicrobial or immunostimulatory mechanisms could be upregulated. Alternately, *in vivo* there are other cell types present, such as lymphocytes, dendritic cells and macrophages that may respond to CpG ODNs, releasing cytokines and stimulating additional immune responses.

There are currently no published studies investigating the effectiveness of CpG ODNs in the prevention of pneumonia in cattle, however there is some research investigating their ability to prevent viral disease in sheep. Newborn lambs showed reduced viral shedding of PI-3 and BHV-1 after subcutaneous CpG ODN treatment (Nichani et al., 2006, 2010). CpG ODNs were effective adjuvants for BHV-1, foot and mouth disease and resulted in enhanced Th1 responses and prolonged immunity, but

CpG ODNs did not improve response to malignant catarrhal fever vaccination (Ioannou et al., 2002; Parameswaran et al., 2014).

In addition, CpG ODNs have been shown to induce protective responses in mice (Mutwiri, 2012). One study demonstrated that repeated doses of CpG ODNs provided continued protection from *Listeria monocytogenes* and *Francisella tularensis* challenges over several months with no evidence of tolerance or adverse effects (Klinman et al., 1999). However, a review on the effectiveness of TLR9 agonists in domestic mammals suggests that they might not be as effective in regulating immune responses as has been reported in laboratory animals due to differing expression of TLR9 in target tissues (Mutwiri, 2012).

There are several studies investigating inactivated parapoxvirus ovis (iPPVO) as an immunomodulator. In one study, where iPPVO was injected in veal calves on arrival to the farm, the authors reported reduced clinical scores and antibiotic treatment versus control animals (Ziebell, 1997). However, the two treatment groups were housed in separate compartments, which limits the validity of the conclusion that it was iPPVO, and not environmental conditions that was responsible for reduced disease (Ziebell, 1997). Another group of studies investigated the ability of iPPVO to reduce clinical signs and viral shedding in response to BHV-1 exposure, with or without vaccination (Castrucci et al., 1995, 1996, 1998). These studies had very low experimental numbers (2-4 animals per group) and statistical analysis was not carried out (Castrucci et al., 1995, 1996, 1998). Vaccination at the time of iPPVO treatment confounded the results of one study, as treatment effects could have been due to stimulation of an adaptive immune response (Castrucci et al., 1996). Another study had 3 control animals and 6

treated animals (consisting of 3 different dosing regimes) (Castrucci et al., 1998). There was a more recent study that investigated the interaction between dexamethasone and iPPVO on phagocytosis and lymphocyte populations (Ryan et al., 2010). In this study, there was one group receiving the iPPVO prior to dexamethasone, and the other group received dexamethasone first (Ryan et al., 2010). It was reported that iPPVO initially increased phagocytosis, which was subsequently reduced by dexamethasone treatment (Winnicka et al., 2000). Results from this study would have been more easily interpreted had additional groups been examined, including a negative control, an iPPVO-only, and a dexamethasone-only treatment group.

Mechanistic studies for iPPVO in cattle are lacking, but research in other species is likely relevant. *In vitro* studies on porcine cells suggest iPPVO enhanced release of IL-2, interferon-α, and interferon-γ, but did not affect phagocytic activity, oxidative burst, or natural killer cell activity (Fachinger et al., 2000). In this study, the authors were interested in the direct effects of parapox virus on the innate response (versus indirect responses stimulated by cytokines produced by other cells) and thus minimized the incubation time. *In vivo* activation of the innate immune system may occur indirectly through activating cytokines. In studies of peripheral blood mononuclear cells, there was increased expression of IFN-αβ mRNA and, when combined with concavalin A, increased expression of IFN-γ and TNF-α following treatment with iPPVO (Castrucci et al., 2000). Canine blood monocytes and neutrophils treated with iPPVO showed enhanced phagocytosis and oxidative burst (Schütze et al., 2009). In mice, intraperitoneal iPPVO resulted in increased Type I IFN, IFN-γ, and IL-12, as well as increased phagocytic activity of peritoneal macrophages, and increased bactericidal

activity of sera against *Escherichia coli* (Anziliero et al., 2014). A recent study in rats demonstrated increased TNF-α and IL-6 in response to intraperitoneal injection of Zylexis (Zoetis Inc., Kalamazoo, MI), a commercially available iPPVO-based immunostimulant for horses (Avci et al., 2016). In this study, iPPVO did not affect concentrations of IL-12 or IL-10 (Avci et al., 2016). Thus, iPPVO does appear to alter immune responses but there is insufficient evidence to determine whether it can be effective in preventing respiratory disease in cattle. Additional summaries of studies reporting the effectiveness of iPPVO in horses are described below (section 1.3.2).

The administration of exogenous cytokines has also been investigated for their potential to stimulate immune responses and prevent BRD. One study investigated the use of recombinant bovine IL-1 as an immune stimulant but, despite finding increased numbers of neutrophils and monocytes as well as enhanced phagocytic ability in blood monocytes, reported no reduction in disease caused by BHV-1 and *M. haemolytica* (Klinman et al., 1999). Investigating the role of neutrophils in the pathogenesis of *M. haemolytica* pneumonia, calves pretreated with granulocyte colony stimulating factor (G-CSF) prior to inoculation were protected from neutropenia and found to have reduced lung lesions at post mortem, after controlling for significant effects of antibody (Youssef et al., 2004). In this study, at the higher inoculation dose, 2 control calves were euthanized due to profound depression with concurrent neutropenia 2 days after bacterial inoculation (Youssef et al., 2004). Paradoxically, neutrophils are often implicated as contributing to the pathogenesis of BRD caused by *M. haemolytica*. Several studies have investigated the effect of depletion of neutrophils on the development of pneumonia following *M. haemolytica* challenge and found protective

effects in neutrophil-depleted animals (Slocombe RF, 1985; Breider et al., 1988; Weiss et al., 1991). In experimentally-induced *Mycoplasma bovis* pneumonia, treatment with synthetic G-CSF pegbovigrastim, flunixin and florfenicol did not improve disease outcomes compared to florfenicol alone (Dudek et al., 2019). However, in this study a treatment group where pegbovigrastim was added without flunixin was not evaluated.

Type I interferons (IFNs) have been more extensively studied. Treatment of afebrile calves with detectable nasal interferon with oral human IFN-α prior to shipment resulted in improved weight gain in the first three weeks but did not reduce the incidence of clinical disease; treatment after arrival showed a trend towards a reduction in the number of sick animals ($p = 0.08$) (Cummins et al., 1999). Oral IFN-α also reduced clinical scores and improved weight gain in calves challenged with BHV-1 (Cummins et al., 1993). In other species, oral type I interferon protected mice from various viral infections, including lethal influenza infection (Beilharz et al., 2007). However, in another study, humans who received daily IFN administration did not experience a reduction in the incidence of viral respiratory disease; although the severity of clinical signs was reduced in selected subgroups under conditions of natural exposure (Bennett et al., 2013). There are many potential mechanisms of action that may have contributed to the observed protective effects as hundreds of genes respond to interferon (McNab et al., 2015). A recent study, using quantitative polymerase chain reaction (qPCR) microarray assays to assess the response in cattle to oral bovine type I interferon treatment, found differential expression in 41 of 92 tested immune and inflammatory genes (Mamber et al., 2016). At this point, it is unknown which specific mediators downstream of IFN are responsible for protection.

The administration of tracheal antimicrobial peptide after *M. haemolytica* challenge was not effective at reducing bacterial numbers and did not reduce disease (Vulikh et al., 2019). One study found a dose-related reduction in colonization by *M. haemolytica* following nasal challenge in calves that were exposed to plasmids containing genes for cercropin (Boudreaux et al., 2005). Cercropin is an antimicrobial peptide produced by insects with demonstrated *in vitro* inhibitory effects against *M. haemolytica* (Boudreaux et al., 2005). In this study, the control group received only the transfection reagent without a naked plasmid. In sheep, a pulmonary adenovirus vector construct encoding human beta defensin (HBD)-6 exacerbated inflammation, including recruitment of neutrophils after BPIV-3 infection (Ackermann and Brogden, 2000). Although adenovirus delivery to the lungs is associated with inflammation, in this study, the sheep that received gene therapy had worse inflammation and clinical signs than the adenovirus vector alone. It is unknown whether a different vector or different defensin would have the same negative effects, and whether this effect was pathogen-specific. Adenovirus delivery of defensins has been successfully used in a mouse model for otitis media, where adenoviral delivery of HBD-2 significantly improved clearance of *Haemophilus influenza* (Woo et al., 2015).

In summary, there are many studies investigating multiple strategies to augment innate immune defenses with varying results. When protective responses are reported, there is often a lack of understanding of the underlying mechanisms and pathways. However, despite these inconsistent results and lack of in-depth understanding, the protection afforded by innate immune stimulation in some studies suggests further exploration of this strategy is warranted.

1.3.2 Currently available respiratory immunostimulants in other species

There are a number of commercially available innate immune modulators available on the market to prevent respiratory disease in other species, including but not limited to horses and humans.

Equine respiratory disease complex shares some features with BRD, in that it occurs with stress, shipping, and weaning. However, contrary to BRD it is primarily a viral disease with only rare (and usually gram-positive) secondary bacterial infections. A recent systematic review concluded that evidence supports the use of two immune-modulators, Zylexis (Zoetis Inc., Kalamazoo, MI), which contains an iPPVO, and Eqstim (Neogen Corporation, Lexington, KY), which contains a non-viable *Proprionibacterium acnes*. Both are indicated as adjunct therapies to conventional management of equine respiratory disease complex (Paillot, 2013).

Some of the purported effects of Zylexis include decreased disease severity after equine herpes virus (EHV) -1 and EHV-4 experimental infection, and increased serum neutralizing antibody titres (Paillot, 2013). Administration of Zylexis reduced clinical signs and shedding of EHV-1 and *Streptococcus equi* ssp. *equi* versus controls in horses experimentally challenged with EHV-1 (Ons et al., 2014). Following treatment of foals with iPPVO, cells obtained from bronchoalveolar lavage and exposed to *Rhodococcus equi* showed improved neutrophil phagocytosis and oxidative burst, as well as TNF-α expression in monocyte-derived macrophages (Ryan et al., 2010). However, Zylexis administration did not decrease the incidence of *R. equi* in foals on an endemically infected farm (Sturgill et al., 2011).

Intravenous administration of *Propionibacterium acnes* to horses has been shown to augment leukocyte function. Peripheral blood showed increased CD4+ T

lymphocytes, increased non-opsonized phagocytosis in blood leukocytes, and increased expression of IFN-γ and NK-lysin by blood mononuclear cells (Flaminio et al., 1998; Davis EG, 2003). An *ex vivo* study demonstrated that *P. acnes*-treated horses had reduced proliferation of *R. equi* in monocyte-derived macrophages compared to control (Ryan et al., 2010).

Equimune®-I.V. (Bioniche, Animal Health USA, Athens, GA) is another stimulant containing *Mycobacterium* spp. cell wall fraction preparations, and is licensed to aid in the treatment of equine respiratory disease complex (*Plumb's Veterinary Drug Handbook, 8th Edition*, 2015); however, peer-reviewed studies are not available. All three of these products use PAMPs (targeting different PRRs) to stimulate an innate immune response against viral infections.

In humans, oral bacterial lysates, including Buccalin, OM-85 and LW-50020, have been used to prevent respiratory infection, with the notion that they promote protection by increasing innate defenses and by activating dendritic cells to promote adaptive immunity (Kearney et al., 2015). The bacterial lysate Buccalin®, a human product containing *Streptococcus pneumoniae, Streptococcus haemolyticus, Staphylococcus aureus and Haemophilus influenza*, has had one recent study to support its effectiveness. In a randomized, double blinded, placebo-controlled study, 188 patients received either Buccalin® or placebo, and it was shown to reduce the number of days with infectious episodes of respiratory disease (Carlone et al., 2014). In this and other studies, its specific mechanism of action was not described, and as with other available immunostimulants consisting of bacterial lysates, it is comprised of inactivated bacterial species that are themselves often associated with respiratory

disease. As such, its reported beneficial effects may have resulted from the development of a specific adaptive immune response targeting these bacteria. Several other systematic reviews investigating the effectiveness of oral bacterial lysates in humans have concluded that: 1) the evidence for protection is weak, but there is “a trend for fewer and shorter infections and a reduction of antibiotic use” (Steurer-Stey et al., 2007); 2) there were “significantly and consistently fewer cases of recurrent respiratory tract infections”, with stronger effects seen in patients at higher risk of recurrent respiratory tract infections (Schaad, 2010); 3) polyvalent bacterial lysate “is effective in both children and adults in preventing respiratory tract infections” (Cazzola et al., 2012); and 4) the studies were of poor quality, but showed that the use of immunostimulants “reduces incidence of acute respiratory tract infections in children”(Del-Rio-Navarro et al., 2006). The available research is intriguing, but there are few published high quality studies and there are very little available human data on mechanisms of action in response to this treatment. A common conclusion in the systematic reviews is that more high quality studies are needed. In addition, many characteristics of the studied populations might limit the applicability of results, such as studies on patients with chronic airway disease (e.g. chronic obstructive pulmonary disorder) or studies conducted in cities with high levels of air pollution (Rozy and Chorostowska-Wynimko, 2008).

Oral bacterial lysates are thought to stimulate an enhanced innate response in the respiratory tract by increasing expression of various adhesion molecules on monocytes, neutrophils, and endothelial cells, facilitating transmigration from the blood stream, stimulating phagocytic activity in alveolar macrophages and neutrophils through

enhanced respiratory bust, and increasing levels of pro-inflammatory cytokines including TNF-α, IL-8, IL-6 and monocyte chemotactic protein-1 (Rozy and Chorostowska-Wynimko, 2008). Additional effects of these bacterial lysates include stimulation of dendritic cells to induce an adaptive response, as well as increasing TGF-β and IL-10, potentially modulating excess inflammation and thus controlling clinical signs in those prone to airway hypersensitivity (Kearney et al., 2015). Because the lysate contains homologous strains to the organisms commonly causing secondary bacterial respiratory disease, adaptive immunity might have been promoted, and thus this treatment might have functioned more as an oral vaccine than solely as an innate immune stimulant. Alternately, the production of IL-10 and TGF-β might have downregulated excessive immune responses in hypersensitive individuals, promoting a tolerance effect rather than exerting its effect primarily as an innate immune stimulant. As most respiratory infections in humans are viral in origin, the ability to prevent bacterial pneumonia is unknown. Further discussion on the use of oral bacterial lysates in humans is beyond the scope of this review.

Alternately, although oral administration of lysates has been demonstrated to possess some beneficial effects, administration directly to the respiratory tract may result in even stronger protection. Although there is very little published information on innate immune stimulation for the prevention of bacterial respiratory disease in humans, mouse models have shown that this strategy might be effective and could provide insights as to how this effect can be achieved.

1.3.3 Laboratory animal models

In the face of growing concerns about antimicrobial resistance there has been increasing interest in the potential for stimulation of innate immune responses in the

lungs to prevent pneumonia. Such a strategy could be of use in situations of immunosuppression, such as during chemotherapy or organ transplantation, during assisted ventilation, or in the face of an outbreak of highly pathogenic viral disease (Evans et al., 2011). Several studies using mouse models have investigated the potential for induced innate defences to prevent experimentally induced pneumonia, many with promising results. A number of different agonists have been examined for the ability to upregulate innate immune responses. In addition, innate immune stimulation has been shown to result in protection from various classes of infectious organisms, including bacterial, viral, and fungal pathogens. A summary of a some of these studies is provided in Table 1.2. Many of the reported experiments used inhaled bacterial lysates or TLR ligands to stimulate responses prior to inoculation with pathogens. This discussion will focus on induced pulmonary innate immune responses and the prevention of bacterial pneumonia.

Several studies have used bacterial lysates to stimulate innate immune responses. These have the potential to simultaneously stimulate multiple different PRRs. Aerosolized lysate of non-typable *Haemophilus influenzae* (NTHi) was administered prior to challenge with *Streptococcus pneumoniae*, and provided complete protection when mice were challenged between 4-24 hours after stimulation; with additional, but reduced protection seen when the lysate was administered after challenge, or up to 72 hours prior to challenge (Clement et al., 2008).

Subsequent studies further investigated pulmonary administration of NTHi to immunosuppressed mice and reported decreased mortality following challenge with several different pathogens, including gram-positive bacteria (*S. aureus, Bacillus*

anthracis) and gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Yersinia pestis, Francisella tularensis*), as well as the fungal pathogen *Aspergillus fumigatus* (Evans et al., 2010a). Results from these experiments suggest that protection peaked between 18-24 hours, and gradually decreased over several days (Evans et al., 2010a). In this study, repeated weekly treatment remained protective against bacterial pathogens, with no loss of efficacy (Evans et al., 2010a).

Experimental leukocyte depletion in several studies highlighted the importance of respiratory epithelial cells in inducible innate resistance, as mice deficient in neutrophils, macrophages or mast cells were nonetheless protected from challenge (Clement et al., 2008; Yu et al., 2010; Cleaver et al., 2013). As well, there was an immediate reduction in bacterial numbers in lung homogenate in treated mice indicating protective factors were present in the lungs at the time of challenge (Clement et al., 2008). Most of the protective antimicrobial molecules in ASL are produced by the respiratory epithelium, and various studies have documented increases in different types of innate defense molecules in response to stimulation, including increased lactoferrin, lysozyme, haptoglobin, calregulin, and cathelicidin-related antimicrobial peptide (Evans et al., 2010b; Yu et al., 2010). Analysis of gene expression of respiratory epithelial cells showed 3011 differentially expressed genes including cytokines, chemokines, PRRs, intracellular signalling proteins, antimicrobial polypeptides, and oxygen radicals (Evans et al., 2010a). Lungs were lavaged and the pulmonary circulatory system was perfused prior to analysis thus the effect of iNTHI on gene expression in leukocytes was not determined (Evans et al., 2010a). With the vast number of genes demonstrating changes in expression, it is difficult to determine which are responsible for protection

and it is likely that the induction of a combination of antimicrobial factors led to the robust protective responses. Cultured epithelial cells treated with NTHi lysate and then inoculated with *S. pneumoniae*, *B. anthracis*, or *P. aeruginosa* had reduced numbers of viable bacteria when compared to untreated cells, but a similar protective effect was not seen with cultured dendritic cells or macrophages (Evans et al., 2010a). There are, however, some organisms that may require stimulation of pulmonary bone marrow-derived cells for protection. An example is the intracellular pathogen *Legionella pneumonophila*, from which mice were only protected following administration of CPG ODN when bone marrow-derived cells expressed TLR9 (Bhan et al., 2008). Thus, there were multiple mechanisms by which NTHi lysate conferred resistance in epithelial cells, and the stimulation of epithelial resistance mechanisms was sufficient to prevent disease.

Several studies have demonstrated that iNTHI lysate and TLR ligands cause upregulation of various pro-inflammatory cytokines by epithelial cells, including TNF-α, IL-6, IL-1β, and IFN-γ (Tuvim et al., 2009; Alfaro et al., 2014). However, other studies demonstrated a lack of type I, II or III IFN in cultured cells and lung homogenate, and a lack of effect on type I IFN in bronchoalveolar lavage fluid following immunostimulation (Tuvim et al., 2012; Mathieu et al., 2013). Increased levels of two pro-inflammatory cytokines, TNF-α and IL-6, were found in bronchoalveolar lavage fluid from treated mice, but the use of anti-TNF antibody-treated and IL-$6^{-/-}$ mice indicated these were not required for induced protection (Evans et al., 2010a). Various cell types, including neutrophils, are recruited to the lungs and are identified in bronchoalveolar lavage fluid following immunostimulation (Deng et al., 2004; Alfaro et al., 2014).

Dysregulated lung inflammation is a significant contributor to influenza virus-related morbidity, and as such, concern exists that stimulating an innate immune response and inflammation could exacerbate this effect. However, the association between inflammation and morbidity may be due to ineffective, rather than increased, inflammation. Despite increased inflammation triggered by the aerosol administration of NTHi three days prior to, or one day after, influenza A infection in mice, there was a significant protective effect against mortality (Tuvim et al., 2009). Initial inflammatory cytokines (IL-6 and TNF-α) were higher in treated mice, but three days after influenza infection, the treated mice had lower levels of inflammatory cytokines, suggesting a more rapid resolution of influenza-induced inflammation in immune stimulated mice (Tuvim et al., 2009). Treated mice had lower IFN-γ and inflammatory cell infiltrate three days after influenza infection (Tuvim et al., 2009). Another important finding of this study was that repeated dosing did not result in tachyphylaxis, and mice treated three times still experienced a protective effect from NTHi despite having a reduced neutrophil response in lung bronchoalveolar lavage fluid (Tuvim et al., 2009). This is consistent with the paradigm of gene-specific regulation of TLR-induced responses, where inflammatory genes develop tolerance to repeated agonists, and antimicrobial genes maintain (and even show enhanced) sensitivity to increased expression (Foster et al., 2007; Foster and Medzhitov, 2009).

The use of more purified agonists, as well as gene knock-out mice, allow for a better understanding of which individual components of the bacteria are responsible for the protective immune response, as well as the pathways which are stimulated. The administration of flagellin, a TLR5 ligand, resulted in protection against *P. aeruginosa*

challenge, and this protection was partially dependent on cathelicidin-related antimicrobial peptide (Yu et al., 2010). Pulmonary administration of LPS, a TLR4 ligand, was also protective against *P. aeruginosa* (Jean et al., 1998). The administration of synthetic macrophage-activating lipopeptide-2 (MALP-2), a TLR2 ligand, prior to infection with *S. pneumoniae* increased survival and improved bacterial clearance (Reppe et al., 2009). In another experiment MALP-2 was also protective against *S. pneumoniae* in mice infected with influenza, with no evidence of exacerbation of clinical signs (Reppe et al., 2015).

Some investigators have fine-tuned administration of TLR ligands by various methods. Conjugation of a TLR7 ligand with phospholipid prior to pulmonary delivery provided variable protection from lethal infection with *Bacillus anthracis*, Venezuelan equine encephalitis virus, and H1N1 influenza virus, but did not result in systemic effects such as changes in blood cytokines or anorexia (Wu et al., 2013). Another study investigated nanoparticles composed of the coat protein of the plant virus, papaya mosaic virus, and a single-stranded RNA virus affording protection from influenza A and *S. pneumoniae* (Mathieu et al., 2013). Interestingly, in this study, mice were fully protected at five days following administration, had partial protection at seven days, and experienced no decrease in protection following ten treatments at weekly intervals (Mathieu et al., 2013). The signalling pathways used by the nanoparticle-delivered RNA are not known, and the results indicate that increases in inflammatory cytokines occur only after a second dose administered one week later, suggesting a potential memory component (Mathieu et al., 2013). Liposomal delivery of CPG ODNs and Poly I:C (a TLR3 agonist) has also been shown to induce protection against several different

influenza viruses and western equine encephalitis virus challenge for up to three weeks (Wong et al., 2005, 2009).

The use of multiple, rather than individual, TLR ligands has been shown to be beneficial with some combinations of agonists showing synergistic effects. Combinations of agonists with synergistic protective effects include Pam2CSK4, a TLR2/6 ligand, and class C CPG ODN, a TLR9 ligand which has shown protective effects against influenza*, S. pneumoniae* and *P. aeruginosa* (Duggan et al., 2011; Tuvim et al., 2012; Cleaver et al., 2013). Increased effects from various signalling pathways were also demonstrated when multiple versus a single bacterial species were used to stimulate dendritic cell maturation (Morandi et al., 2011). However, the administration of multiple TLR ligands to cultured bovine tracheal epithelial cells did not result in synergistic effects on TAP expression (Berghuis et al., 2014).

The use of knock-out mice has helped to determine which intracellular signalling pathways create protection from challenge in response to immunostimulation. The signalling adapter protein MyD88, but not TRIF, was necessary for NTHi to induce a protective effect from *P. aeruginosa* (Lee et al., 2008). Lung epithelial expression of MyD88 and IRAK4 were necessary for protection from bacterial challenge in mice stimulated with a combination of TLR2/6 and TLR9 ligands (Lee et al., 2008). The large number of genes that are affected following pulmonary administration of immunostimulants makes it difficult to discern which are important, but further investigation using inhibitory antibodies or knockout mice and correlating results with protein levels should be informative. Investigation of specific pathways can also be achieved by administering individual cytokines to determine their individual effects on

the innate immune responses. As an example, intranasal IL-17F, given daily for 7 days prior to *S. pneumoniae* challenge, increased several inflammatory cytokines and expression of β-defensin-2 while reducing the *S. pneumoniae* colonization in the lung; however, in this study mice were euthanized three days following infection and no differences in survival were reported (Chen et al., 2015).

Upon aerosolization of innate immune ligands, induced innate resistance appears to be locally compartmentalized; aerosolized immunostimulation afforded no protection against pathogens administered by intraperitoneal or intravenous routes (Clement et al., 2008). While pro-inflammatory cytokines (including TNF-α) were increased in the lungs following aerosol exposure to TLR2/6 and TLR9 agonists, no increases were seen in other examined organs (Walker et al., 2014).

Most of the studies investigated pulmonary administration of innate immune ligands, but other routes of administration have also been examined. Intraperitoneal administration of CPG ODNs failed to improve survival after challenge with *K. pneumoniae* or *Legionella pneumonophila* (Deng et al., 2004; Bhan et al., 2008). Conversely, subcutaneous and intranasal administration of c-di-GMp were equally protective against *K. pneumoniae* (Karaolis et al., 2007).

From these studies, there appears to be significant potential for innate immune stimulation to prevent respiratory disease. Some innate immune stimulants have shown protective effects that last as long as three days, and occur even when administered after exposure, depending on treatment. Response continues with repeated treatments, albeit with less induced inflammation. Stimulation is protective against a number of different bacterial pathogens and can be evoked via multiple PRRs. Responses include

elevated inflammatory cytokines and antimicrobial proteins and peptides, with questionable contributions by recruited leukocytes, but the specific induced factors necessary to evoke the protective response have not yet been defined. At this point, a key question that remains is whether these results, obtained from young, inbred laboratory animals, with minimal genetic variability, reared under tightly controlled and pristine environmental conditions, challenged using non-host-adapted pathogens, and provided minimal exposure to other pathogens, can be extrapolated to domestic animals and humans. Can this experimental efficacy translate into real-world effectiveness?

Although most research on respiratory innate immune stimulation has been conducted on species other than cattle, some conclusions can be drawn nonetheless: innate immune stimulation has the potential to reduce disease caused by a number of pathogens, including Gram-positive and Gram-negative bacteria and viruses; it can be induced using a variety of substances and routes of administration; and it appears to be well-tolerated. The early immune modulators iPPVO and *P. acnes* were developed many years ago, and although they have demonstrated the potential to provide protection from disease, increased understanding of innate immune system pathways and PRRs may be able to guide and refine further development of novel immune therapeutics with improved efficacy.

Extrapolation from the work done in other species suggests that: 1) innate immune stimulation is well tolerated; 2) innate stimulation can be protective against various microbial pathogens; 3) bacterial and viral PAMPs can be cross-protective; 4) stimulated responses occur within several hours, and in some cases can be protective if

given shortly after instead of only prior to infection; 5) effective stimulation can result from direct stimulation of the lower respiratory tract, stimulation of the upper airways or systemic stimulation by parenteral administration; and 6) innate immune stimulation can be protective in complex biological situations, e.g. during the development of equine respiratory disease. Much more research needs to be carried out in cattle to establish the potential for innate immune stimulation to prevent respiratory disease.

1.4 Conclusions and thesis proposal

BRD is the most economically important disease affecting the beef industry due to the costs of treatment, direct losses to mortality, reduced growth performance and carcass quality, and compromised animal welfare (Duff and Galyean, 2007). Despite its profound impact, control of this disease has remained elusive, due to the combination of well-entrenched stress-inducing management practices (e.g. shipping), the mixing of animals from various sources (creating novel exposure to respiratory pathogens), the sheer number of different bacterial and viral species involved in pathogenesis, and practical limitations for effective vaccination. Furthermore, the most important bacterial agents—*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*—are opportunistic pathogens, and as such take advantage of virus- and stress-induced altered host immune defenses and damaged respiratory mucosal epithelium in order to initiate clinical disease (Mosier, 2014).

Given the significant negative economic impact of BRD and the strong desire to reduce the use of antimicrobials in food production, alternative strategies for prevention would be enormously beneficial to the beef industry. Because BRD typically occurs within a very specific and relatively short period in time, and may involve one or more of

several different pathogens, targeting the innate immune system is a promising strategy for disease prevention. Innate immune responses develop rapidly and are broadly protective against a wide number of bacterial, viral and fungal pathogens. Research has demonstrated enhancement of *in vitro* expression of the β-defensin tracheal antimicrobial peptide by a number of innate immune agonists (Berghuis et al., 2014). Furthermore, *in vivo* research in non-bovine species has shown that stimulation of the innate immune system can be successful in preventing both viral and bacterial respiratory disease in response to experimental challenge and natural disease. The goal of this research project is to investigate the potential for innate immune stimulation to prevent BRD. Specifically, these investigations will focus on the potential for aerosolization of bacterial lysate to stimulate innate immune responses and subsequently, their impact on the severity of disease in calves challenged with *M. haemolytica* and in feedlot calves at high risk of developing disease.

1.4.1 Objectives

1) Characterize the nature and magnitude of innate responses stimulated by aerosolization of selected agonists (Chapter 2).
2) Identify any adverse effects of immunostimulation (Chapter 2).
3) Determine optimal dose and timing to trigger innate immune responses and minimize adverse outcomes (Chapter 2).
4) Develop an experimental challenge model using aerosol delivery of *M. haemolytica* to more closely mimic natural exposure (Chapter 3).
5) Identify quantitative or qualitative outcomes that can be used to evaluate whether immunostimulation is protective against *M. haemolytica* challenge (Chapter 3).

6) Determine whether innate immune stimulation is protective against experimental challenge as measured by clinical scoring, thoracic ultrasonography, hematology, gross and histological evaluation of respiratory tissues, and quantification of bacteria in nasal cavity and lungs (Chapter 4).
7) Determine whether innate immune stimulation is protective against natural disease in a feedlot setting as measured by incidence of clinical disease, mortality, hematologic changes, nasal bacteria, and average daily gain (Chapter 5).

1.4.2 Hypotheses

1) Aerosolization of innate immune agonists (heat-killed *Staphyloccus aureus* and *Escherichia coli*) induces an innate immune response in the respiratory tract of calves.
2) Stimulation of an innate immune response protects against experimental *M. haemolytica* infection in calves.
3) Stimulation of an innate immune response protects against naturally-occurring bovine respiratory disease in high-risk cattle.

1.5 Tables

Table 1.1: Summary of licensed immunostimulants in Canada and the United States of America as of April 2020 indicated to prevent respiratory disease in animals.

Product (Manufacturer) active ingredient	Species	Label indications
Zelnate (Bayer Animal Health, Mississauga, Ontario) DNA liposome	Cattle	Treatment at the time of, or within 24 hours of, stressful events to prevent bovine respiratory disease
Eqstim (Neogen, Lansing, Michigan) *Propionibacterium acnes*	Horses	As an adjunct to conventional therapy in the treatment of Equine Respiratory Disease Complex
Equimune-I.V. (NovaVive USA, Athens, Georgia) Mycobacterial cell wall	Horses	Use as an immunotherapeutic agent for the treatment of Equine Respiratory Disease Complex

Table 1.2: A summary of studies using innate immune stimulation to prevent respiratory disease in laboratory animals.

Reference	Immunostimulant/ method of administration	Pathogen(s)	Summary of key findings
Evans et al. 2010	NTHi lysate / aerosol	Gram-negative and positive bacteria, *Aspergillus fumigatus*	Increased host survival, decreased lung pathogens, increased haptoglobin, lactoferrin, lysozyme, and calgranulin, but not defensins and no tachyphylaxis.
Clement et al. 2008	NTHi lyasate / aerosol	*Streptococcus pneumoniae*	Complete protection from lethal dose when given 4-24 hours prior to challenge. Some protection was seen when stimulated 72 hours prior to, or 2 hours after, challenge. Neutrophils, mast cells and alveolar macrophages were not required for protection.
Tuvim et al. 2009	NTHi / aerosol	Influenza A virus	Treated mice had increased survival when NTHi was administered 3 days prior to, or 1 day post, influenza administration. No tachyphylaxis was seen, but decreased lung neutrophils were seen with subsequent treatments.
Jean et al. 1998	LPS/ intratracheal	*Pseudomonas aeruginosa*	Treated rats had no mortality and decreased protein leakage into the air spaces, as well as enhanced bacterial clearance versus controls.
Reppe et al., 2009, 2015	MALP-2 / intratracheal	*S. pneumoniae*	MALP-2 protective against S. pneumoniae in the face of influenza virus infection.
Bhan et al. 2008	CPG ODN / intratracheal	*Legionella pneumonophila*	Needed expression of TLR-9 in bone marrow-derived cells for protection.
Wong et al., 2005, 2009	Lysosome-encapsulated poly I:C or CpG ODNs / IN	Influenza A and western equine encephalitis viruses	Protection lasted up to 3 weeks for poly I:C and 2 weeks for CPG ODN.
Deng et al., 2004	CpG ODN / intratracheal	*Klebsiella pneumoniae*	Treatment 48 hours prior to challenge; increased survival and decreased bacteria in lungs and blood.
Karaolis et al., 2007	c-di-GMp / IN or SC	*Klebsiella pneumoniae*	Both treatment routes were equally effective in improving survival and decreasing CFU in lungs. Treatment not effective when given concurrent with, or after challenge. There was increased iNOS expression in lung macrophages from c-di-GMP-pretreated mice.
Duggan et al., 2011	Combinations of Pam3CSK4, Pam2CSK4, poly (I:C), MPLA, synthetic flagellin, imiquimod, and ODN 2395 / aerosol	*P. aeruginosa*	MyD88, but not TRIF signalling required for protection. Combination of TLR/2/6 agonist and TLR 9 agonist provided strong protection. Not all individual or combinations of agonists were protective.

Reference	Immunostimulant/ method of administration	Pathogen(s)	Summary of key findings
Tuvim et al., 2012	TLR2/6 and TLR 9 agonists / IN	Influenza A (H1N1)	Protection when given up to 3 days prior or 1 day following challenge. Protection was not dependent on induction of interferon – unknown mechanism. Induction of IL-6, TNF, IL-1α, IL-1β, and multiple chemokines
Cleaver et al., 2013	TLR2/6 and TLR 9 agonists / IN	*P. aeruginosa, S. pneumoniae*	Protection is inducible in mice depleted of multiple leukocytes indicating primacy of epithelial responses. MyD88 and IRAK4 are required for protection.
Wu et al., 2013	phospholipid conjugated TLR7 ligand / IN or IT	*Bacillus anthracis*, Venezuelan equine encephalitis virus, and H1N1 influenza virus	Increased innate cytokines and chemokines in bronchoalveolar lavage fluid.
Chen et al., 2015	IL-17F / IN	*S. pneumoniae*	Increased neutrophils, macrophages MIP-1α, MIP-2β, interferon-γ and HBD-2 in bronchoalveolar lavage fluid and reduced bacterial colonization. No data on survival or clearance of infection past 3 days.
Yu et al., 2010	Flagellin (TLR-5 agonist) / IN	*P. aeruginosa*	Strong protection, increased bacterial clearance and reduced lung damage. Neutrophils not required for protection. Upregulation of cathelicidin-related antimicrobial peptide was important for protection.

LPS, lipopolysaccharide; CFU, colony forming units; IL, interleukin; TLR, Toll-like receptor; IN, intranasal; SC, subcutaneous; BALF, bronchoalveolar lavage fluid; TNF, tumour necrosis factor; MALP-2, macrophage-activating lipopeptide-2; iNOS; inducible nitric oxide synthase; MPLA, monophosphoryl lipid A; IRAK, interleukin-1 receptor-associated kinase; MIP, macrophage inflammatory protein

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2 Pulmonary and systemic responses to aerosolized lysate of *Staphylococcus aureus* and *Escherichia coli* in calves

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2.1 Abstract

Constitutive and inducible defenses protect the respiratory tract from bacterial infection. The objective of this study was to characterize the response to an aerosolized lysate of killed bacteria, as a basis for studying the regulation and in vivo effects of these inducible innate immune responses.

Bacterial lysate consisting of heat-killed and sonicated *Staphylococcus aureus* and *Escherichia coli* was aerosolized to 6 calves and systemic and pulmonary responses were measured in the first 24 hours relative to baseline. Mild clinical signs with increased heart rates and rectal temperatures developed following administration of the lysate, with resolution by 24 hours. Serum haptoglobin ($p = 0.04$) and plasma fibrinogen ($p = 0.06$) concentrations were elevated at 24 hours relative to baseline. Bronchoalveolar lavage fluid (BALF) had increased cellularity and increased proportion of neutrophils, as well as higher concentrations of interleukin (IL)-8, IL-10 and total protein at 24 hours relative to baseline. Mass spectrometry identified 965 unique proteins in BALF: 19 proteins were increased, and 26 proteins were decreased relative to baseline. The upregulated proteins included those involved in complement activation and platelet activation. At post-mortem examination, calves receiving higher doses of lysate had areas of lobular consolidation and interlobular edema. Histologically, neutrophils were present within bronchioles and to a lesser extent within alveoli. Calves

receiving higher doses of lysate had patchy areas of neutrophils, hemorrhage and hyaline membranes within alveoli.

These findings indicate that aerosolization of bacterial lysate stimulates an innate immune response in lungs and airways, with alveolar damage observed at higher doses. Such a stimulus could be of value for investigating the effects of inducible innate immune responses on occurrence of disease, or for evaluating how stress, drugs or genetics affect these dynamic responses of the respiratory tract.

2.2 Introduction

The respiratory tract has not only static defences but also dynamic cellular and humoral responses to inhaled substances. For the most part, these responses protect the lungs from infection and other threats. Pattern recognition receptors on epithelial cells and leukocytes (including alveolar macrophages), as well as humoral factors such as complement proteins, act as sentinels to detect various pathogen-associated molecular patterns (PAMPs) that are unique to microbes. Once these receptors bind their ligands, signaling cascades are activated that induce the production of various mediators of inflammation and host defense, leading to rapid recruitment of neutrophils and monocytes as well as increased production of host defense proteins such as defensins. This may increase disease resistance but excessive inflammation can damage tissue and impair pulmonary function (Pechous, 2017). Thus, inflammatory responses should be inducible under circumstances where they are needed, but tightly controlled and quickly downregulated when the threat has passed.

In mice, administering PAMPs by aerosol to the respiratory tract has been shown to stimulate innate immune responses that protect against various pathogen challenges.

For example, aerosolization of a lysate of nontypeable *Haemophilus influenzae* (NTHi) bacteria induced pulmonary inflammation characterized by increased concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)-α as well as increased neutrophils in BALF (Tuvim et al., 2009). In pulmonary tissue collected 2 hours following administration of NTHi, numerous genes related to host defense were upregulated including cytokines, pattern recognition receptors, antimicrobial peptides and oxygen radicals (Evans et al., 2010a). Similarly, administration of aerosolized Toll-like receptor (TLR) ligands to mice increased IL-6, TNF, and CXCL2 concentrations in BALF with no observed signs of illness or behavioural changes (Alfaro et al., 2014). In these mice, neutrophils in BALF increased by 4 hours, peaked at 48 hours and returned to baseline by 7 days (Alfaro et al., 2014). Aerosolization of NTHi prior to challenge with influenza A virus, *Streptococcus pneumoniae*, *Bacillus anthracis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Yersinia pestis, Francisella tularensis,* or *Aspergillus fumigatus* protected against mortality (Clement et al., 2008; Tuvim et al., 2009; Evans et al., 2010a). Thus, upregulation of respiratory innate immune responses prior to pathogen challenge can be protective against bacterial pathogens in mice.

Induction of innate immune defenses has also been demonstrated in cattle in response to various PAMPs and inflammatory cytokines. In primary cultures of bovine tracheal epithelial cells, administration of lipopolysaccharide (LPS, a TLR4 agonist), Pam3CSK4 (a TLR1/2 agonist), flagellin (a TLR5 agonist), IL-1β, TNF-α or IL-17 resulted in the induction of innate defenses such as tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP), and lactoferrin (Russell et al., 1996; Diamond et al., 2000; Mitchell et al., 2006; Al-Haddawi et al., 2007; Berghuis et al., 2014; Taha-

Abdelaziz et al., 2016). Similarly, adenosine-5′-triphosphate, which has been shown to have antimicrobial effects (Tatano et al., 2015), was released from cultured pulmonary epithelial cells in response to LPS, heat-killed bacteria and IL-1β (Craddick et al., 2012). Lipopolysaccharide induced inflammatory cytokine expression (IL-1α, IL-1β, TNF-α, and IL-8) and the release of histamine and leukotriene B4 in cultured pulmonary epithelial cells (Saban et al., 1997; McClenahan et al., 2008). Similarly, isolated bovine alveolar macrophages increased expression of TNF-α, IL-1β and IL-8 in response to LPS (Lafleur et al., 1998; Malazdrewich et al., 2004a), whereas beta-defensin expression was unchanged (Ryan et al., 1998). Following instillation of LPS into the lungs of cattle, neutrophils and macrophages were identified histologically within alveoli (Brogden et al., 1995), and cell counts and the proportion of neutrophils in BALF increased, although TAP expression in bronchial mucosal biopsies did not change (Mitchell et al., 2006).

Thus, our hypothesis was that delivery of an aerosolized bacterial lysate to cattle would trigger innate immune responses without significant adverse effects. This was initially investigated using cell culture experiments to determine whether bacterial lysate upregulated TAP and LAP expression in cultured bovine tracheal epithelial cells. In vivo studies evaluated the clinical, hematologic and bronchoalveolar lavage fluid changes and postmortem findings in calves following aerosol delivery of a lysate of killed *Escherichia coli* and *Staphylococcus aureus*. The purpose was to establish a dose of lysate that induced an innate immune response without causing adverse effects, for use in further experiments. Thus, relatively low numbers of calves were used in the study, and comparisons were made between pre-treatment and post-treatment data in the same calf. We identified an inflammatory stimulus that could be tested as a novel

method to prevent respiratory disease at times when immune defenses are compromised, such as in stressed beef calves at the time of arrival to feedlots. Furthermore, the use of a standardized stimulus could be useful to measure how stress or pharmacologic interventions affect the inducible responses of the respiratory tract, and to investigate the contribution of genetics or prior life events to these dynamic respiratory innate immune responses.

2.3 Materials and Methods

2.3.1 Preparation of bacterial lysate

An isolate of *Staphylococcus aureus* from a case of bovine mastitis and an isolate of *Escherichia coli* from a case of bovine diarrhea were kindly provided by the Animal Health Laboratory (AHL), University of Guelph. The *E. coli* isolate was considered enterotoxigenic based on identification of F41, F5/K99 and STa genes but was not enteropathogenic or verotoxigenic based on absence of eaeA, hlyA, or Shiga toxins 1 or 2 genes (AHL). A stock suspension of each isolate was streaked onto a Columbia agar plates with 5% sheep blood (Oxoid Canada, Nepean, Ontario, Canada) and cultured at 37°C for 24 hours. A single colony of *S. aureus* and of *E. coli* were each inoculated into separate flasks containing 250 mL tryptose soy broth and incubated overnight at 37°C with shaking (stationary phase). The concentration of viable bacteria (colony forming units (CFU)/mL) was determined by overnight incubation at 37° C of serial dilutions of the suspension plated onto Columbia agar with 5% sheep blood and counting the number of bacterial colonies. The preparation of bacteria was killed by incubation in a 65°C water bath for 90 minutes and placed on ice. Bacterial killing was

confirmed by lack of growth on blood agar. The suspension was centrifuged at 4°C at 10 000 x *g* for 15 min and washed twice with cold PBS. The bacterial pellets were re-suspended in PBS to create a final stock solution containing $1x10^{11}$ CFU-equivalents. The heat-killed bacteria were sonicated using a dismembranator (Model 120, Fisher Scientific) at 90% amplitude alternating 20 second pulses with 30 seconds rest for a total of 5 minutes. Aliquots of bacterial lysates were stored at -20°C. Concentrations of the bacterial lysate are reported as CFU-equivalents.

2.3.2 Cell culture experiments

Primary cultures of bovine tracheal epithelial cells were established as previously described (Taha-Abdelaziz et al., 2016; Bourque et al., 2018). Once the cells reached 80% confluency, triplicate wells were treated with medium only (negative control) or a combination of 10^7 CFU-equivalents each of *S. aureus and E*. *coli* lysate. Cells treated with 0.1 µg/mL LPS (Sigma Aldrich, MO, USA, L9143) were used as a calibrator. Following treatment, the cells were incubated for 16 hours before being harvested for RNA extraction.

RNA extraction and cDNA synthesis were carried out as previously described (Bourque et al., 2018). Real-time reverse transcription quantitative PCR was used to evaluate the effect of bacterial lysate on TAP and LAP expression relative to expression of the reference gene GAPDH as previously described (Berghuis et al., 2014; Taha-Abdelaziz et al., 2016; Bourque et al., 2018). Stability of expression of GAPDH across treatments was assessed using ANOVA. Template-negative wells were included in each run, and data of technical triplicate samples were averaged. The specificity of the

reaction was confirmed by examining melting curves and identification of a single peak at approximately 82.8ºC (TAP), 86.9 ºC (LAP) and 85.4ºC (GAPDH).

2.3.3 Aerosolization of bacterial lysate to calves

Use of animals in these studies was approved by the Animal Care Committee of the University of Guelph (AUP #3286) according to Canadian Council on Animal Care guidelines. Calves were obtained from the Elora Dairy Research Centre (Ontario Ministry of Agriculture, Food and Rural Affairs). Eight 3-month-old Holstein bull calves were housed in pairs within climate-controlled, biosecurity level 2 pens in an isolation facility. Shavings were used as bedding, and hay, water and calf starter pellets (Sharpe Farms Supplies Limited, Guelph, Ontario) were offered *ad libidum*.

An aerosol of *S. aureus* and *E. coli* lysate was administered to 6 calves. Calves were housed separately in pairs, and were randomly assigned to receive either 10^8 (calf 1), 10^9 (calf 2), 10^{10} (calf 3), 10^{11} (calf 4) or 10^{12} (calves 5 and 6) CFU-equivalents suspended in a total volume of 10 mL PBS (Table 2.1). The bacterial lysates and PBS were delivered by aerosol using a compressor (Precision Medical, Northampton, PA) that produced approximately 25 psi of air pressure and was attached to a Whisper Jet nebulizer (Marquest, Englewood, Colo) connected to a small Equine Aeromask (Trudell Medical International, London, ON) placed over the calf's muzzle. A prior study showed that this system delivered aerosol to the upper respiratory tract as well as the bronchioles and alveoli (Bassel et al, 2019). Personnel wore powered air-purifying respirators (PAPRs) and N95 respirators were placed over the one-way exhalation valves on the calf masks to reduce room air contamination. Two control calves (calves

C1 and C2) received only PBS, to ensure that the aerosolization procedure did not affect the measured parameters.

2.3.4 Clinical and hematologic responses

Calves were assessed prior to and at 1, 2, 4, 6, 12, 18 and 24 hours following administration of aerosolized bacterial lysate or PBS. At these times, rectal temperature, respiratory rate and effort, heart rate, and mental status were assessed. Additional clinical signs including lack of appetite, coughing or nasal discharge were noted when present. Objective clinical scores were assigned, as described in Table 2.2. Blood was collected into sodium citrate anticoagulant for measuring plasma fibrinogen and without anticoagulant for evaluation of serum haptoglobin (AHL, University of Guelph) prior to and 24 hours following aerosolization of bacterial lysate or PBS.

2.3.5 Bronchoalveolar lavage fluid cellular analysis

Bronchoalveolar lavage fluid was collected from all calves prior to and 24 hours following administration of the bacterial lysate. Calves were sedated with xylazine and bronchoalveolar lavage fluid was collected from the right caudodorsal lung for the baseline sample collection and the left caudodorsal lung for the post-aerosol sample collection, by infusing 120 mL sterile saline (120 mL, 0.9%) through an endoscope and retrieving the lavage fluid by manual suction through a 60 mL syringe. The sample was placed on ice until further processing (within 2 hours). Samples were filtered through gauze prior to determination of total nucleated cell counts using electrical impedance (Z2 Coulter counter, Beckman Coulter, Mississauga, ON) and cytocentrifuge preparations of slides using 200 µL of fluid. Differential cell counts were performed on 200 cells on the Wright-stained slide preparations. Olympus CellSens software was

used to trace the outline of individual cells and measure the area of 15 macrophages in a single central 400x field.

2.3.6 Mass spectrometry analysis

For mass spectrometry, the remaining bronchoalveolar lavage fluid was centrifuged (500 × *g*, 5 min, 4°C), then desalted and concentrated by centrifugal filtration (Amicon® Ultra-15, Millipore, Bedford, MA, USA). Protein concentrations were measured using a Bradford assay (Bradford, 1976). Tandem mass tag (TMT) mass spectrometry (SPARC BioCentre, The Hospital for Sick Children, Toronto, Canada) was conducted on four pre- and post-aerosolization samples (calves 1-4). The normalized protein concentrations were reported. Samples were reduced, alkylated, digested, and TMT labelled according to manufacturer's directions (Thermo Fisher TMT 10 Plex, Product 90110). Labelled peptides from all samples were combined and lyophilized. Peptides were cleaned up using a C18 ZipTip (Millpore) and then lyophilized.

Samples were analyzed on a Thermo Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrometer (ThermoFisher, San Jose, CA) outfitted with a nanospray source and EASY-nLC 1200 nano-LC system (ThermoFisher, San Jose, CA) and equipped with ETD mode as described in Supplemental Material 2.1. Data analysis was performed using Proteome Discoverer 2.2 against a Uniprot Bovine Database (6002 sequences). TMT modifications of lysine and the peptide N-termini as well as carbamidomethyl of cysteine were considered fixed modifications while oxidation of methionine and protein N-terminal acetylation were considered variable. Parent mass tolerance was set to 10 ppm, fragment mass tolerance was set to 0.6 Da. Reporter ion quantification for the 8 TMT channels was done on the MS3, and lot-specific correction

factors were used. Proteins that were significantly increased or decreased following lysate treatment were analysed in PANTHER v14.1 (www.pantherdb.org/) to determine function. The pathways overrepresented among the upregulated and down regulated proteins were determined (Reactome version 68, www.Reactome.org; Fabregat et al., 2018) using a Fisher's exact test to produce a probability score that was corrected for false discovery rate using the Benjamani-Hochberg method.

2.3.7 Cytokine analysis

The following cytokines were measured in bronchoalveolar lavage fluid: IFN-α, IL-8, IL-2, IL-6, tumour necrosis factor alpha (TNF-α), IFN-γ, IL-10, and IL-17 using a multiplexed electrochemiluminescent ligand-binding assay with the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer's instructions. The assay was developed using two separate Meso Scale Diagnostics (MSD) U-Plex panels, following the MSD U-Plex protocol guide. Cytokines were quantified using the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer's instructions using a chemiluminescent readout. Panel 1 included biotinylated capture antibodies: IL-1β (Biorad, Hercules, CA), IL-2 (R&D Systems Minneapolis, MN), IL-6 (R&D Systems), IFN-α (Kingfisher Biotech, Minneapolis, MN), and TNF-α (R&D Systems). Panel 2 included: IL-8 (Mabtech, Cincinnati, OH), IL-10 (Biorad), IL-17A (Mabtech), and IFN-γ (Mabtech). The IL-1β antibody was biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) at a challenge ratio of 1:20 according to manufacturer instructions.

All calibrators used in the assay were purchased from Kingfisher Biotech. The panel 1 calibrators were diluted to a concentration of 40,000 pg/mL in 1X Dulbecco's PBS without calcium or magnesium, followed by 4-fold serial dilutions into 1X DPBS.

The panel 2 calibrators were diluted to a concentration of 10,000 pg/mL in 1X DPBS followed by 4-fold serial dilutions.

The detection antibodies were sulfo-tagged following the MSD quick guide conjugation protocol using a challenge ratio of 1:20. Panel 1 detection antibodies were used at the following concentrations: IL-1β at 1 µg/mL (Biorad), IL-2 at 0.5 µg/mL (R&D Systems), IL-6 at 2 µg/mL (R&D Systems), IFN-α at 1 µg/mL (Kingfisher Biotech) and TNF-α at 0.5 µg/mL (R&D systems). Panel 2 detection antibodies were used at the following concentrations: IL-8 at 0.5 µg/mL (Mabtech), IL-10 at 1 µg/mL (Biorad), IL-17A at 0.2 µg/mL (Kingfisher Biotech) and IFN-γ at 1 µg/mL (Mabtech).

Cytokines were quantified using the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer's instructions using a chemiluminescent readout. In brief, 0.5 mL of all biotinylated antibodies were diluted to 10 µg/mL with Diluent 100 and paired to their respective linkers by adding 750 mL of supplied Linker solution and incubated for 30 minutes followed by addition of Stop solution for another 30 minutes. The linked antibodies for panel 1 were then diluted 10-fold into Stop solution. The panel 2 linked antibodies were diluted 20-fold (IL-10 and IL-17A) or 40-fold (IL-8 and IFN-γ) into Stop solution. Plates were coated by adding 50 µL of antibody solutions per well and were incubated covered with shaking for 1 hour at room temperature. Plates were washed 3 times with PBS/Tween20 prior to samples or standards being added at 30 mL per well and incubated as above. Plates were washed 3 times before detection antibodies were added along with 2% rabbit serum to block and incubated as above. Plates were washed 3 times before addition of 150 µL 2X Read buffer and read immediately on the SI6000 instrument (MSD, Rockville, MD). The results were reported

as pg/mL. For soluble protein levels, a BCA protein kit (Thermofisher, Rockford, IL) was used. The cytokine concentrations were normalized to the total protein levels for each sample.

2.3.8 Post-mortem examination

All but one of the lysate-treated calves were euthanized at 24 hours following administration of aerosols, by intravenous injection of pentobarbital. Gross postmortem examination was performed within 2 hours of death including visual inspection and palpation of respiratory tissues. The lungs (trachea removed) and heart were weighed and the lung: heart weight ratio was calculated for each calf. Samples of nasal mucosa, trachea and cranioventral, caudodorsal and caudoventral regions of lungs were placed in 10% formalin, and histologic sections were routinely prepared and stained with hematoxylin and eosin.

2.3.9 Statistical analysis

Data were compared between samples obtained before and after aerosolization of bacterial lysate within the same animal. Descriptive statistics, t-tests and ANOVA were performed (Graphpad Prism v8.0, San Diego, CA, USA) and 2-sided tests were considered significant when $p < 0.05$. Outcome variables were evaluated for normality using a D'Agostino-Pearson omnibus K2 test, Shapiro-Wilk test and Kolmogorov-Smirnov test and transformed as indicated. Grubb's test, with alpha set at 1%, was used to detect outliers, which were subsequently removed from further analysis.

ANOVA compared the effects of bacterial lysate on normalized ratios of TAP: GAPDH and LAP: GAPDH expression in cultured tracheal epithelial cells. Repeated measures of clinical parameters including rectal temperatures, respiratory rate, and

heart rate were evaluated using repeated measures one-way ANOVA with a Geisser-Greenhouse correction to adjust for unequal variability of differences. Multiple comparisons of post-aerosolization values against baseline (time 0) values were evaluated using a Dunnett's test. Residuals were evaluated to determine whether ANOVA assumptions were met. For clinical scores, a repeated measure non-parametric Friedman test was conducted, with post hoc Dunn's tests to compare post-aerosolization clinical scores with baseline.

Paired t-tests were used to compare baseline and post-stimulation concentrations of serum haptoglobin, plasma fibrinogen, hematology values and bronchoalveolar lavage fluid parameters (cell counts and cytokines). For the cytokine assays, analytes that were below the limit of detection were assigned a value that was equal to the lower limit of detection divided by the square root of 2 (Hornung and Reed, 1990). Outcome variables that were not normally distributed and were not normalized following transformation were assessed non-parametrically using a Wilcoxon matched-pairs sign rank test. Two-sided tests with $\alpha < 0.05$ were considered significant.

2.4 Results

2.4.1 Induction of TAP and LAP expression in tracheal epithelial cells

Treatment of primary cultures of bovine tracheal epithelial cells with a lysate of heat-killed *E. coli* and *S. aureus* significantly increased relative TAP gene expression compared to that of cells treated with medium alone ($p = 0.002$; Figure 2.1A). Similarly, LAP expression was upregulated ($p = 0.018$) in tracheal epithelial cells following administration of bacterial lysate. Treatment with bacterial lysate did not affect

expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on analysis of the crossing points ($p > 0.05$; Figure 2.1B).

2.4.2 Clinical and clinical pathologic findings

Six 3-month-old Holstein bull calves received aerosolized lysate of heat-killed *E. coli* and *S. aureus* (calves 1-6). All calves had clinical scores of zero prior to the administration of aerosol. Between 1 and 12 hours following lysate administration, all 6 calves that received bacterial lysate developed subtle clinical signs consisting of mildly increased respiratory effort and mild depression that resolved by 18 hours (Figure 2.2; Supplemental Figure S2. 1). All 6 calves coughed at one or more time points following administration of the lysate. Two calves (5 and 6) were reluctant to rise at 2 and 4 hours respectively, following administration of the lysate. One calf (1) had a reduced appetite at 2 hours following lysate administration. Two control calves (calves C1 and C2) received only aerosolized phosphate-buffered saline (PBS) to ensure that the aerosolization procedure did not affect the measured parameters; these 2 calves did not cough or show any clinical signs following aerosolization of PBS.

Rectal temperature was increased relative to baseline in calves that received aerosolized bacterial lysate ($p = 0.02$), with significant elevations at 4 and 8 hours (Figure 3A; Supplemental Table S2. 1). Heart rate increased following administration of bacterial lysate (one-way analysis of variance [ANOVA]*;* $p < 0.05$); however, upon adjustment for multiple comparisons (Dunnett's post hoc test), there were no significant differences in heart rate between baseline and any single post-aerosolization time point (Figure 2.3B). Respiratory rate was not affected by the administration of aerosolized

lysate (p = 0.18; Figure 3C). In calves that received PBS, rectal temperature, heart rate, and respiratory rate did not change.

In calves treated with bacterial lysate, serum haptoglobin concentration significantly increased between baseline (mean: 0.17 g/L; 95% CI: 0.15, 0.18) and 24 hours following aerosolization of lysate (mean: 0.27 g/L; 95% CI: 0.19, 0.70; paired t-test; p = 0.044) (Figure 2.4A). Plasma fibrinogen concentration increased numerically between baseline (mean: 3.28 g/L; 95% CI: 2.60, 3.97) and 24 hours following aerosolization of bacterial lysate (mean: 3.64 g/L; 95% CI = 3.29, 3.99) but the difference was not significant (p = 0.058; paired t-test of log-transformed values) (Figure 2.4B). Administration of aerosolized bacterial lysate did not change hematologic values from baseline including total blood leukocytes (p = 0.92), neutrophils (p = 0.88), monocytes (p = 0.08) and lymphocytes (p = 0.19) (Figure 2.4D-F).

2.4.3 Changes in bronchoalveolar lavage fluid

The cellular composition of bronchoalveolar lavage fluid was significantly different following administration of bacterial lysate relative to baseline (Figure 2.5; Figure 2.6; Supplemental Table S2. 2; Supplemental Figure S2. 2; Supplemental Figure S2. 3). Administration of bacterial lysate significantly increased the number of nucleated cells (p = 0.036*)* and the proportion of neutrophils (p < 0.0001), while the proportion of macrophages was reduced (p < 0.0001) and the proportion of lymphocytes was unchanged (p = 0.95). Microscopically, cytocentrifuge preparations of the fluid from calves administered bacterial lysate contained large numbers of non-degenerate neutrophils as well as large macrophages with foamy cytoplasm containing occasional phagocytosed neutrophils (Figure 2.7). Macrophages were significantly larger in post-

lysate samples compared to baseline, based on morphometric analysis of the area of these cells in the stained cytocentrifuge preparation ($p < 0.0001$; Supplemental Figure S2. 4).

Protein concentrations in bronchoalveolar lavage fluid (as determined by BCA protein assay) were higher at 24 hours following delivery of aerosolized bacterial lysate (mean: 122 µg/mL; 95% CI: 58, 186) compared to baseline (mean: 55 µg/mL; 95% CI: 33, 77) ($p = 0.016$, Supplemental Table S2. 2). In this analysis, one pair was excluded because the post-aerosol protein concentration was an outlier (870.8 µg/mL) as determined by Grubb's test.

Cytokine concentrations in bronchoalveolar lavage fluid were measured before and 24 hours after aerosolization of bacterial lysate (Figure 2.8:). Following aerosolization, there was increased concentration of IL-8 ($p = 0.005$) and IL-10 ($p = 0.048$) compared to baseline. Interleukin 17 concentration increased numerically following aerosolization of bacterial lysate, but this change was not statistically significant ($p = 0.14$). The following cytokines were generally below the lower limit of the assay and were not statistically analyzed: interferon (IFN)-α (<9.8 pg/mL), IL-1β (<9.8 pg/mL), IL-2 (<39.1 pg/mL), IL-6 (<156.3 pg/mL), TNF-α (<39.1 pg/mL), and IFN-γ (<2.4 pg/mL).

Bronchoalveolar lavage fluid samples collected before and 24 hours after administration of aerosolized bacterial lysate were analyzed by tandem mass tag (TMT)-labelled quantitative mass spectrometry. There were 965 different proteins identified in the bronchoalveolar lavage fluid (Supplemental Table S2.3). Individual paired t-tests identified 19 unique proteins that had increased abundance (Table 2.3)

and 26 proteins that had reduced abundance (Table 2.4) following aerosolization of bacterial lysate. The 19 proteins with increased abundance in post-lysate bronchoalveolar lavage fluid included the following protein classes (as determined by PANTHER analysis): transfer/carrier proteins (PC00219) (n=3), signaling molecules (PC00207; n=2), receptors (PC00197; n=2), defense/immunity proteins (PC00090; n=2), hydrolases (PC00121; n=4), and 1 of each of enzyme modulator (PC00095), oxidoreductase (PC00176), and extracellular matrix protein (PC00102) (Supplemental Table S2.). The 26 proteins that were decreased in post-lysate bronchoalveolar lavage fluid included the following protein classes: membrane traffic proteins (PC00150) (n=3), hydrolase (PC00121) (n=3), enzyme modulator (PC00095) (n=3), nucleic acid binding (PC00171) (n=6), and cytoskeletal proteins (PC00085) (n=2) (Supplemental Table S2.5). The remaining protein classes had only a single protein in each category. Reactome analysis recognized 13 of the 19 significantly upregulated proteins and identified overrepresentation of pathways related to complement cascade, platelet activation and degranulation, neutrophil degranulation, innate immune system, and noncanonical NF-κB signaling (Supplemental Table S2.).

2.4.4 Pathologic findings

At postmortem examination, the calculated lungs-to-heart weight ratio was positively associated with the dose of aerosolized bacterial lysate (R^2=0.96, p = 0.003; Figure 2.9). Grossly, lesions were predominately in the cranioventral areas of the lungs and included red mottling of the lung tissue, interlobular edema, and scattered discrete angular red-purple foci of lobular consolidation (Supplemental Figure S2. 5). One calf (10^9 colony forming unit [CFU]-equivalents) had an area of consolidation of a cranial

lung lobe that was determined by histopathology to have been present for greater than 3 days prior to euthanasia (that is, preceding aerosolization of bacterial lysate).

Histologically, bronchioles and, to a lesser extent, alveoli contained neutrophils in airspaces and occasionally in the bronchiolar lamina propria and alveolar septa (Figure 2.10; Supplemental Figure S2. 7). In addition, in the calves that received 1010 to 1012 CFU-equivalents, there were several small foci of acute alveolar damage evidenced by hyaline membranes and intra-alveolar neutrophils, macrophages and brightly eosinophilic (protein-rich) edema fluid (Figure 2.11).

2.5 Discussion

The pulmonary innate immune system is critical for detecting and responding to inhaled pathogens, yet there has been relatively little investigation into ways to stimulate or increase the effectiveness of these responses. In situations where a pathogen reaches the lung, the course of disease depends on many factors including host immunity and pathogen virulence. There is considerable variation among individuals in both the magnitude of inducible innate immune defenses (Caverly et al., 2003; Bourque et al., 2018) and the severity of disease following bacterial pathogen challenge (Jericho and Kozub, 2004). Thus, the standardized stimulus characterized in these studies could provide a platform to investigate the genetic or acquired basis for this variability.

Evaluating the response to bacterial challenge is complicated by survival or host-mediated killing of the bacteria, and by the effects of bacterial virulence factors that may modulate the host innate immune response. By using killed bacterial lysate, we were able to characterize pulmonary and systemic responses to an inflammatory stimulus delivered to the lung, independent of pathogen survival. Gaining an understanding of

these responses to bacterial lysate can allow us to compare innate immune responsiveness between individuals, examine the effects of various external influences, and investigate the impacts of immunomodulatory drugs on these responses.

Initial in vitro experiments confirmed that bacterial lysate upregulated the host defense peptides LAP and TAP in cultured tracheal epithelial cells. This is consistent with previous studies that demonstrated upregulation of TAP and LAP in response to the purified TLR agonists LPS and Pam3CSK4 (Mitchell et al., 2006; Berghuis et al., 2014; Taha-Abdelaziz et al., 2016). Similarly, administration of non-typeable *Haemophilus influenzae* lysate to cultured murine epithelial cells resulted in upregulation of 18 of the 30 antimicrobial peptides examined and enhanced bacterial killing (Evans et al., 2010a). As the focus of this investigation was on the *in vivo* effects of aerosolization of bacterial lysate, we did not investigate additional genes in cultured epithelial cells or the *in vitro* responses of other cell types.

We used a bacterial lysate rather than purified agonists to stimulate innate immune responses. It has been shown that combinations of PAMPs can induce synergistic or complementary innate immune responses (Trinchieri and Sher, 2007; Mäkelä et al., 2009). In the bovine lung, it is likely that the simultaneous stimulation of multiple pattern recognition receptors by lysed bacteria, similar to that in natural pathogen exposure, could similarly induce a more robust response than individual purified agonists, although this was not specifically investigated in these experiments.

The aerosol treatments were well-tolerated by the calves. Although mild clinical signs including elevated respiratory effort, cough, and nasal discharge developed shortly after aerosolization of bacterial lysate, these resolved by 24 hours. Similarly, in

mice that received a sub-lethal dose of *Pseudomonas aeruginosa*, lung function and rectal temperature were most significantly decreased and behavioural signs of illness were highest between 6 and 12 hours post-inoculation (Wölbeling et al., 2011).

Neutrophils were markedly increased in bronchoalveolar lavage fluid following aerosolization of bacterial lysate. Similarly, experiments in mice demonstrated that neutrophils appeared in lung lavage fluid at 4 hours after treatment with the TLR agonists oligodeoxynucleotide and Pam2CSK4 (Alfaro et al., 2014). In that study, the number of neutrophils in bronchoalveolar lavage fluid peaked at 48 hours and returned to baseline by 7 days. In the current study, there was no apparent relationship between the dose of lysate and the magnitude of the increase in neutrophils in bronchoalveolar lavage fluid. This suggests that each of the tested lysate doses were above the threshold for a maximal response. In support of this, the neutrophil response to oligodeoxynucleotide and Pam2CSK4 is reported to follow a sigmoidal dose-response curve, with a plateau at higher doses (Alfaro et al., 2014).

In cattle, aerosolization of *Mannheimia* (*Pasteurella) haemolytica* significantly increased the neutrophil: macrophage ratio in bronchoalveolar lavage fluid, beginning at 30 minutes and increasing up to 4 hours after challenge (Walker et al., 1985; Lopez et al., 1986). Conversely, although inhalation of endotoxin from *Salmonella typhimurium* induced increases in the percentage of neutrophils within the bronchoalveolar lavage fluid, inhalation of *Staphylococcus epidermidis* did not alter the cell populations (Walker et al., 1985). This is consistent with a pilot experiment we conducted (data not shown) where aerosolization of heat-killed *S. aureus* did not induce clinical effects or significantly increased neutrophils in bronchoalveolar lavage fluid. Thus, the inclusion of

E. coli lysate augmented the pulmonary inflammatory response compared to *S. aureus* alone. Similarly, killed Gram-negative bacteria were found to contribute to robust induction of inflammatory responses (Walker et al., 1985). In the present study, at 24 hours after administering bacterial lysate, macrophages in bronchoalveolar lavage fluid demonstrated evidence of activation with a larger cell size than at baseline, vacuolated cytoplasm, and frequent binucleation. The phagocytosis of apoptotic neutrophils (efferocytosis) observed in these calves is thought to be important in the resolution of inflammation (Robb et al., 2016).

The most consistent histologic observations were neutrophils within bronchiolar walls and lumens, whereas neutrophils were more variably observed within alveoli. The absence of neutrophils in baseline bronchoalveolar lavage fluid and their presence after stimulation confirmed that neutrophil infiltration occurred after and in response to the bacterial lysate. Neutrophils, lymphocytes and plasma cells were also present within bronchial and tracheal submucosa but these can be present in conventionally-reared calves (Senthilkumaran et al., 2015).

At higher doses, the aerosolized lysate induced histologically evident lung injury, including intra-alveolar neutrophils, hyaline membranes and hypercellularity of alveolar septa. In addition, the gross lung: heart weight ratios increased with increasing dose, presumably due to edema as a result of increased vascular permeability. However, the presence of edema did not affect respiratory rates at the time of euthanasia. In mice, lung weights were also increased following aerosolization of oligodeoxynucleotide and Pam2CSK4 (Alfaro et al., 2014). Aerosolization of LPS has been shown to induce lung injury in various species and has been used to model septic lung injury, similar to what

was seen in the calves receiving a high dose of lysate (Liu et al., 2013; Roos et al., 2014; Lin et al., 2019).

Consistent with other studies that delivered aerosolized lysates or innate immune agonists, aerosolization of bacterial lysate in the present study significantly increased bronchoalveolar lavage fluid protein concentration and inflammatory cytokines (Roos et al., 2014). Interleukin-8 and IL-10 concentrations were elevated in bronchoalveolar lavage fluid after stimulation. Interleukin 8 has been shown to recruit neutrophils to the bovine lung (Mitchell et al., 2003). Interleukin-10 functions to downregulate inflammatory responses and inhibits release of pro-inflammatory cytokines from macrophages and other leukocytes; its induced expression in response to bacterial infection or inflammatory cytokines is thought to represent a feedback loop to protect against excessive inflammation and tissue damage (Ouyang et al., 2011). It is likely that the pro-inflammatory IL-8 was induced following administration of bacterial lysate to initiate antimicrobial defenses, but in the absence of a persistent and severe stimulus IL-10 was also increased as a negative feedback mechanism to limit potential damage caused by prolonged or severe inflammatory responses. Additional cytokines were below the limit of quantification (TNF-α, IFN-α, IL-1β, IL-2, IL-6). This may be due to the timing of the sample collection, as IL-1β and TNF-α were shown to peak and return to baseline within 8 hours of exposure to M. haemolytica (Malazdrewich et al., 2001). In mice, the cytokines IL-6, CCCL2 and TNF peaked within 8 hours and returned almost to normal following aerosol administration of CpG oligodeoxynucleotide and Pam2CSK4 (Alfaro et al., 2014). Similarly, following non-lethal administration of Pseudomonas aeruginosa, TNF-α levels peaked at 4 hours and returned to baseline by 24 hours

(Wölbeling et al., 2011). It is therefore possible that additional changes in cytokine concentrations occurred at an earlier time point.

Mass spectrometry identified numerous proteins within bovine bronchoalveolar lavage fluid, and there were significant increases in the levels of several proteins related to innate immunity and the acute phase response, including complement components, metal-scavenging proteins, protease inhibitors, and chemokines. As we did not limit which proteins were included in the statistical analysis, large numbers of proteins (965) were evaluated, many of which were not expected to change following administration of the lysate. We therefore did not adjust for multiple comparisons in the statistical analysis. Had we made this adjustment, none of the differences between baseline and post-lysate bronchoalveolar lavage proteins would have been significant.

Many of the upregulated proteins identified by our analysis were part of pathways of the innate immune system including complement pathways and neutrophil degranulation. Complement-related pathways can be activated by the alternative and lectin pathways, both of which were likely stimulated by the bacterial lysate. Previous experiments have demonstrated that the NF-kB pathway is activated in tracheal epithelial cells following the administration of various agonists (Taha-Abdelaziz et al., 2016) as well as in a multitude of inflammatory pathways relevant to leukocytes (D'Ignazio et al., 2016) Activation of neutrophil-related pathways corresponds to the increased neutrophils in bronchoalveolar lavage fluid and histological sections. The overrepresentation of pathways related to platelet activation and degranulation are consistent with their increasingly recognized role in pulmonary innate immunity (Gomez-Casado et al., 2019). In mice, the presence of membrane TLR receptors capable of

recognizing and responding to bacterial components supports the potential of bovine platelets to respond directly to bacterial lysate. However, the role of platelets in bovine pulmonary immune responses has not been well examined.

Thus, these results support activation of a number of innate immune pathways following aerosolization of bacterial lysate. It should be noted that this analysis does not determine the cellular source of the detected proteins, nor whether the increased levels result from greater synthesis of the protein, secretion from infiltrating cells such as leukocytes, exudation of proteins from plasma, or reduced degradation or clearance.

2.6 Conclusions

In vitro studies demonstrated that the bacterial lysate induced gene expression of host defense peptides. In vivo aerosol administration of bacterial lysate stimulated a transient systemic inflammatory response with short-term elevations in rectal temperature and heart rate, mild increases in blood biomarkers of inflammation, but no differences in blood leukocytes. Between baseline and 24 hours after administering lysate, the bronchoalveolar lavage fluid had a significant increase in neutrophil numbers, enlargement of macrophages with foamy cytoplasm, and increased concentrations of total protein, IL-8 and IL-10. Mass spectrometric analysis of bronchoalveolar lavage fluid proteins identified 965 proteins, with altered levels of many of them after administration of the lysate. Understanding and characterizing the pulmonary inflammatory responses could provide a platform to investigate innate immune responses, measure the effect of host or environmental influences on these responses, and evaluate immunomodulatory therapeutics. Further studies will investigate the effects of this immune stimulant on development of respiratory disease.

2.7 Reference List

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2.8 Figures

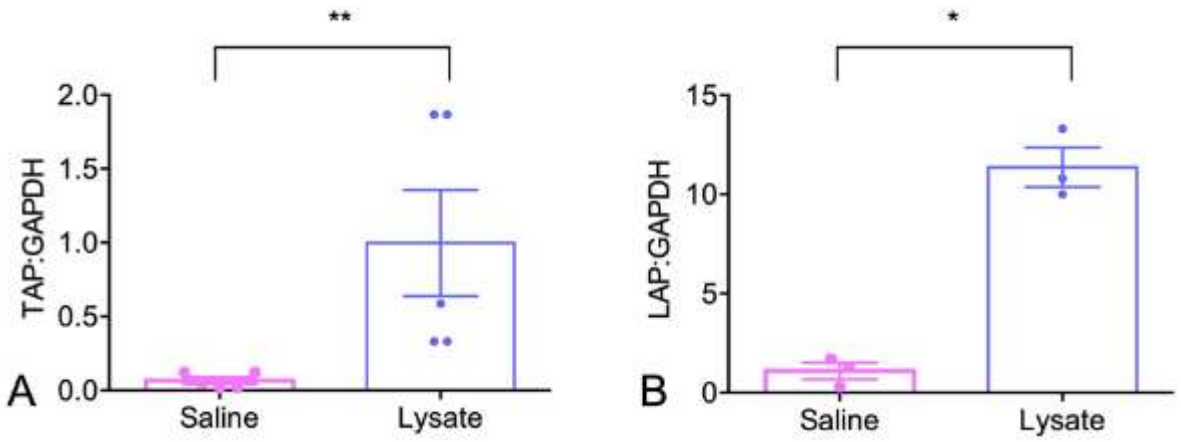


Figure 2.1: Effect of bacterial lysate on relative tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) gene expression in tracheal epithelial cells. Primary cultures of bovine tracheal epithelial cells from 5 calves (TAP) and 3 calves (LAP) were exposed for 16 hours to medium treated with saline (negative control) or lysate of *Staphylococcus aureus* and *Escherichia coli*. Following exposure, TAP, LAP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression were measured using RT-qPCR. Data are presented as the mean normalized TAP: GAPDH or LAP:GAPDH ratios plus or minus the standard error of the mean. Treatment with bacterial lysate resulted in an average of 15-fold greater TAP expression (P=0.002; Fig. 2.1A) and 13-fold greater LAP expression (P=0.018; Fig. 2.1B) compared to saline-treated cells.

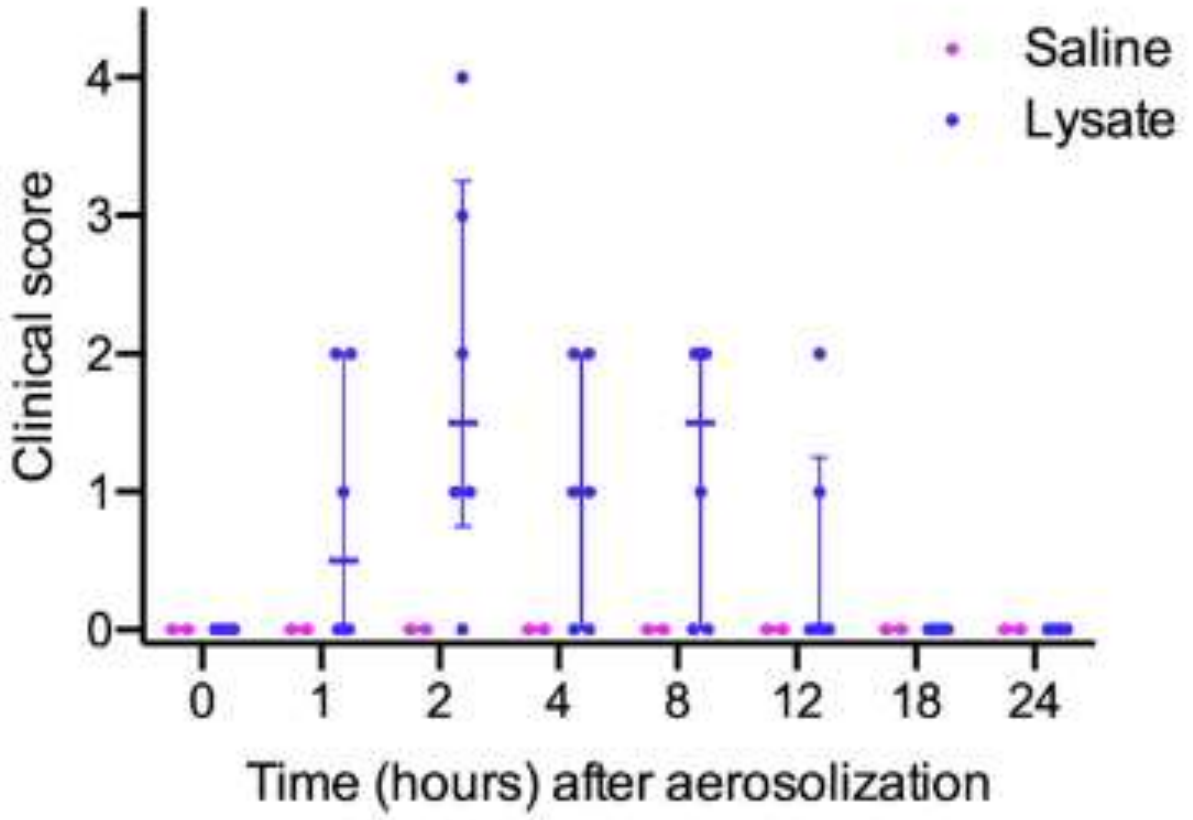


Figure 2.2: Clinical scores in conventional calves after aerosol administration of bacterial lysate. Six 3-month-old Holstein calves received aerosolized *Staphylococcus aureus* and *Escherichia coli* lysate at doses ranging from 10^8-10^{12} CFU-equivalents and 2 calves received aerosolized saline (control). Clinical scores are the sum of individual scores for demeanor (0–4), strength (0–4), appetite (0–2), and respiratory effort (0–3) for a maximum possible score of 13 at each time point.The graph shows individual data points, median and interquartile ranges.

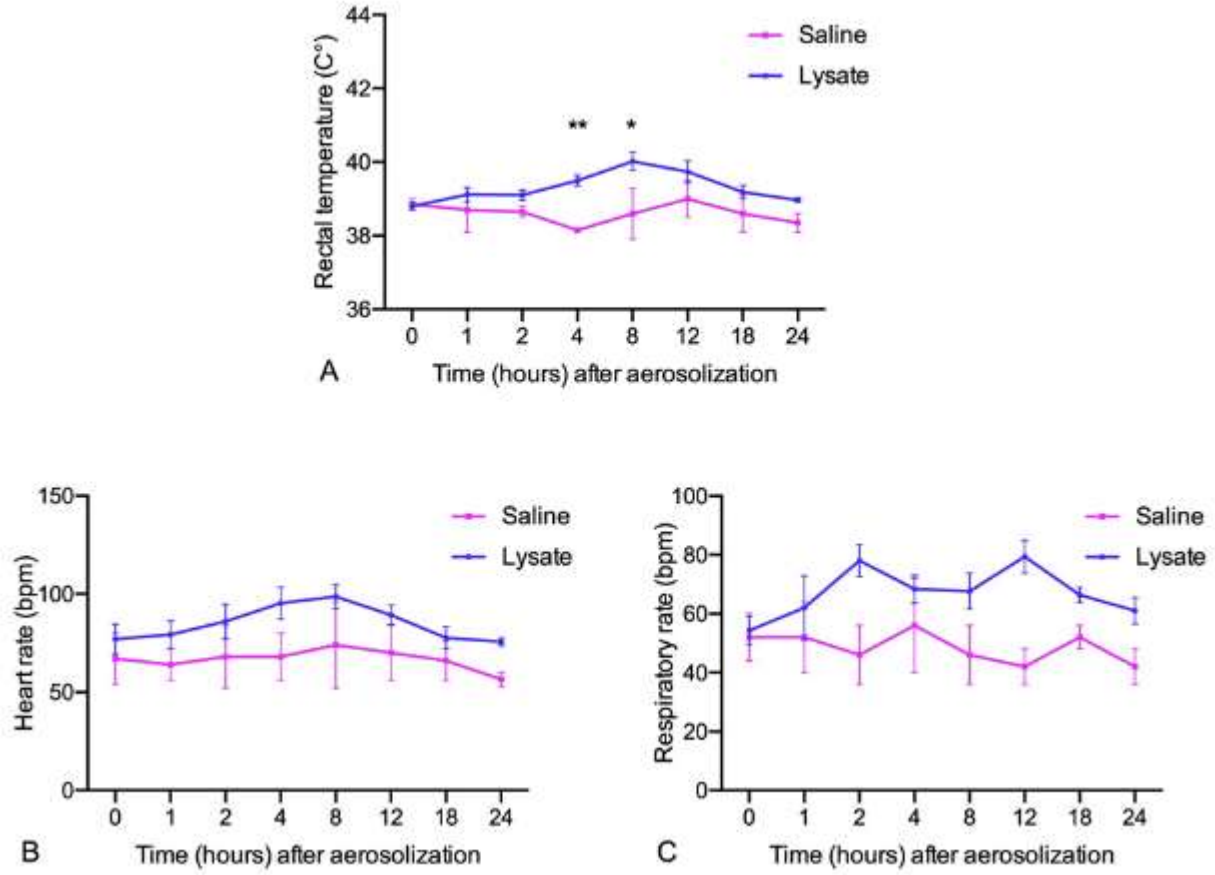


Figure 2.3: Clinical parameters in conventional calves after aerosol administration of bacterial lysate. Six 3-month-old Holstein calves received aerosolized*Staphylococcus aureus* and *Escherichia coli* lysate at doses ranging from 10^8-10^{12} CFU equivalents (n=6) or saline (n=2). The data are mean ± standard error of the mean. A) rectal temperature, B) heart rate, and C) respiratory rate over time. Individual time points were compared to baseline (time=0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * p <0.05, ** p <0.01.

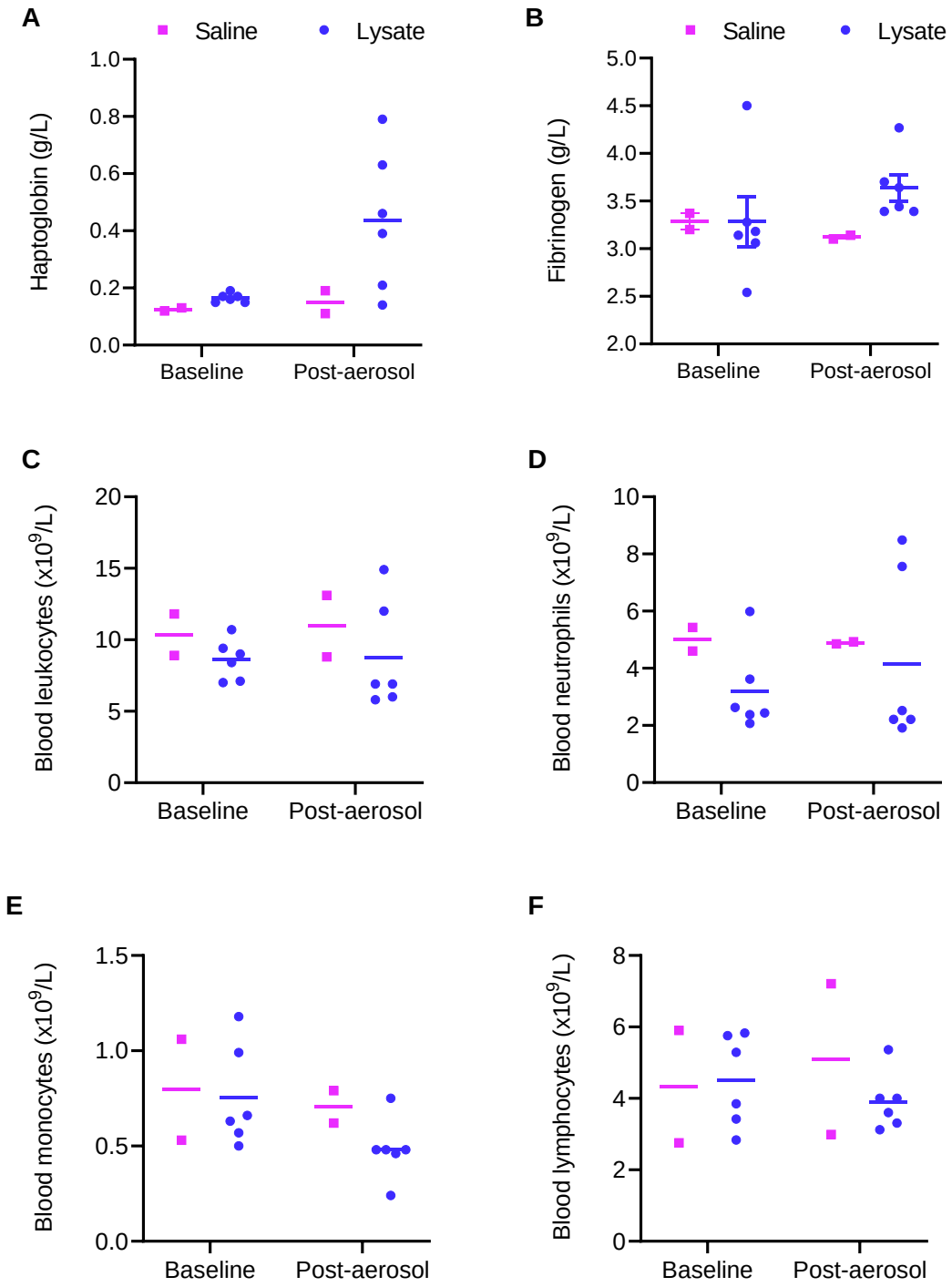


Figure 2.4: Hematologic values in calves before and after aerosol administration of bacterial lysate. Blood samples were collected at baseline and 24 hours after aerosolization of *Staphylococcus aureus* and *Escherichia coli* lysate (n=6) or saline (n=2). Horizontal bars indicate the mean. A) serum haptoglobin B) plasma fibrinogen C) total blood leukocytes, D) blood neutrophils, E) blood monocytes, and F) blood lymphocytes. * p <0.05.

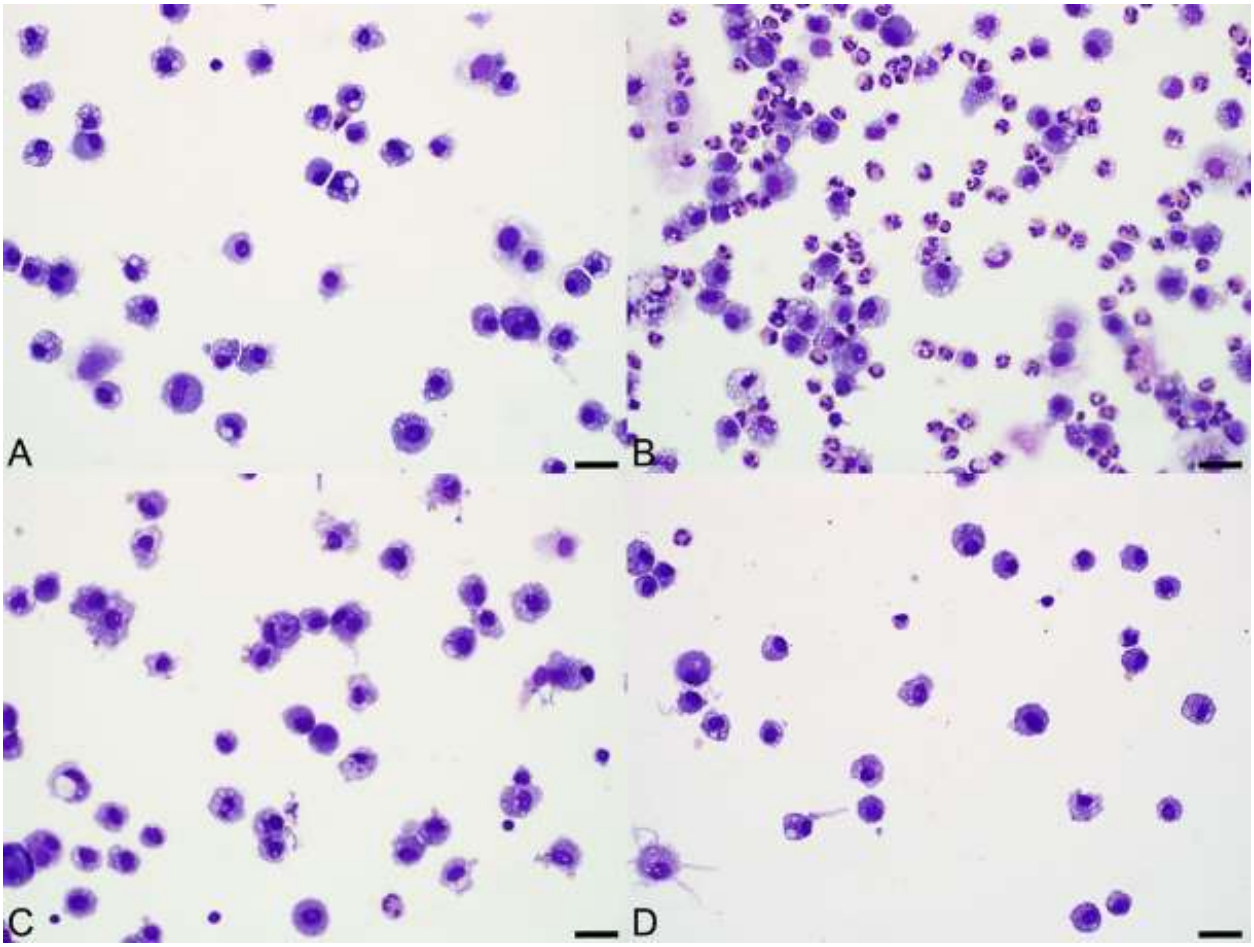


Figure 2.5: Cytology of bronchoalveolar lavage fluid following aerosol administration of bacterial lysate and saline. Bronchoalveolar lavage fluid was sampled from 2 calves, sampled before (A and C) and 24 hours after aerosolization of either *Staphylococcus aureus* and *Escherichia coli* lysate (B) or phosphate-buffered saline (D). The sample obtained after administration of the lysate (B) contains many neutrophils whereas the other samples (A, C, D) contain mainly macrophages and lymphocytes. Cytocentrifuge preparation, Wright's stain. Bar=25 µm.

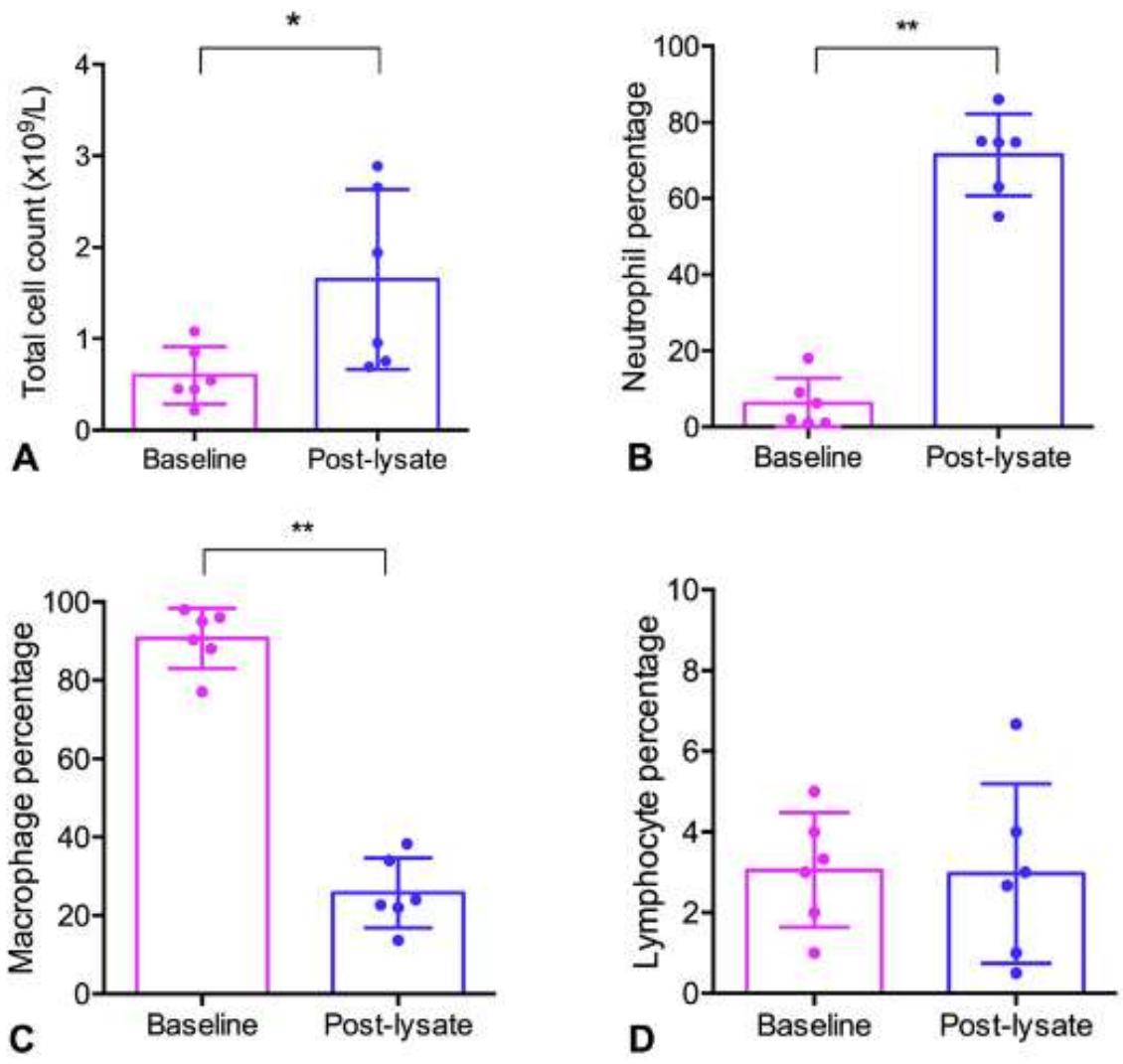


Figure 2.6: Bronchoalveolar lavage fluid cell counts prior to and post aerosolization of bacterial lysate. Bronchoalveolar lavage fluid cells (mean ± SEM) before and 24 hours after aerosol administration of bacterial lysate to 6 Holstein bull calves. A) Total nucleated cell count. B) Percentage of neutrophils. C) Percentage of macrophages. D) Percentage of lymphocytes (y-axis scale is different than B and C).

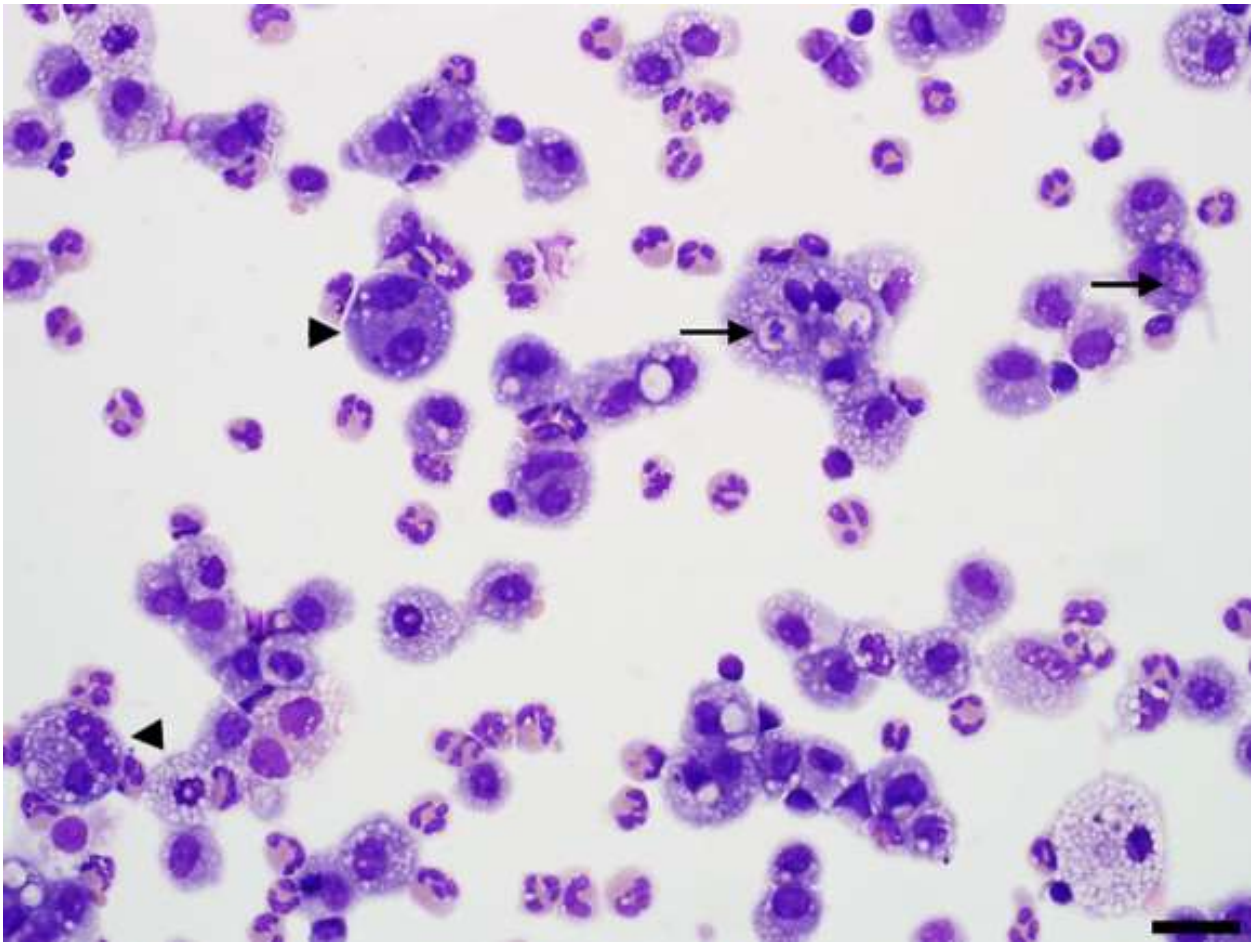


Figure 2.7: Bronchoalveolar lavage fluid cytology 24-hours following aerosolized bacterial lysate. Cytocentrifuge preparation of bronchoalveolar lavage fluid from a calf that received 10^{11} CFU-equivalents of *Escherichia coli* and *Staphylococcus aureus* lysate. There are large numbers of macrophages and neutrophils. Macrophages are large, with foamy cytoplasm and frequent binucleation (arrowheads). Occasional phagocytosed neutrophils are present within the cytoplasm of macrophages (arrows). Wright stain. Bar = 25 µm.

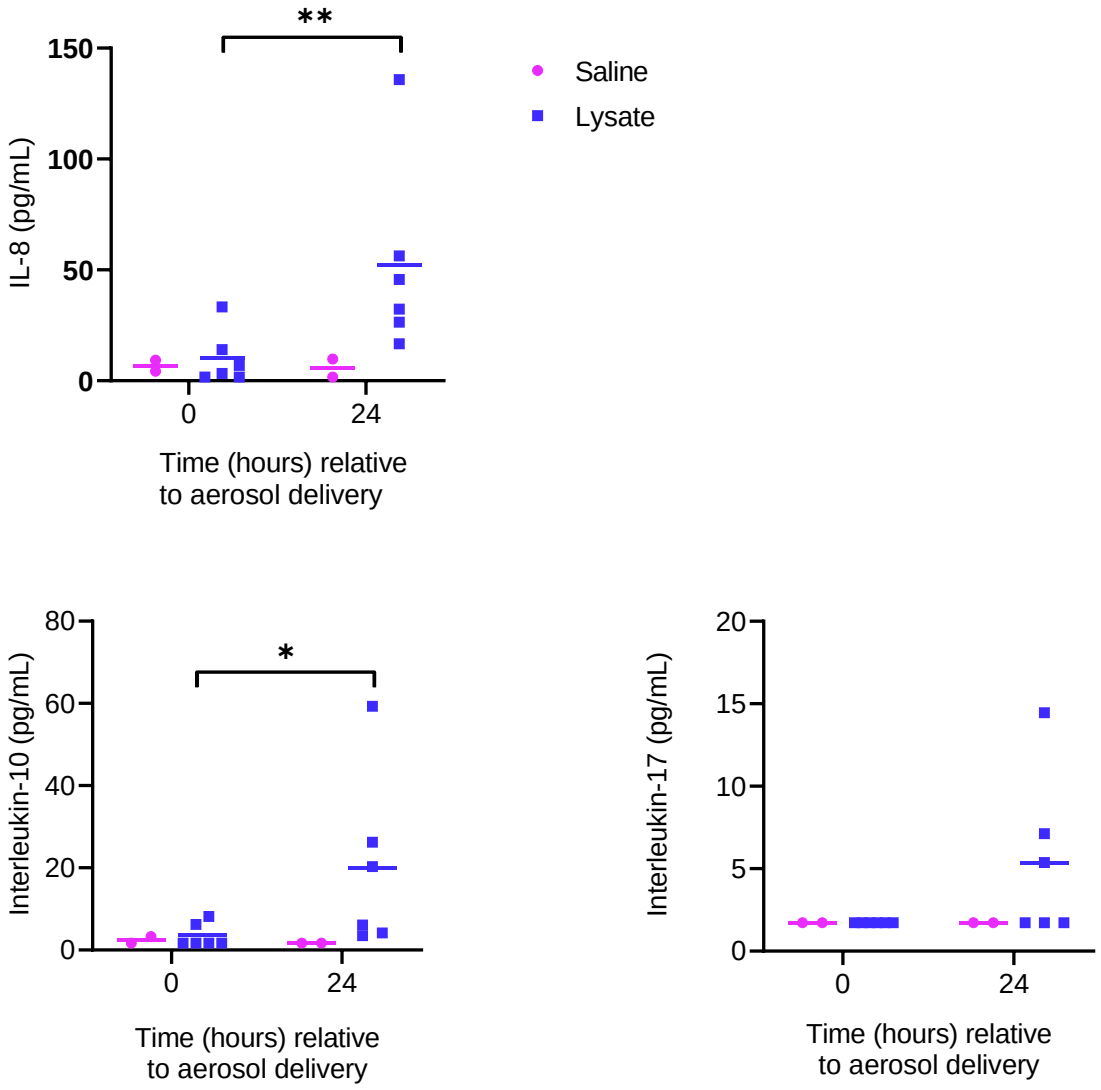


Figure 2.8: Cytokine responses to aerosolized bacterial lysate. Cytokine concentrations in bronchoalveolar lavage fluid prior to and 24 hours following aerosolization of *Staphylococcus aureus* and *Escherichia coli* lysate (n=6) or saline (n=2). Horizontal bars indicate the mean. A) Interleukin-8. B) Interleukin-10. C) Interleukin-17. * $p < 0.05$, ** $p < 0.01$; (paired t-test).

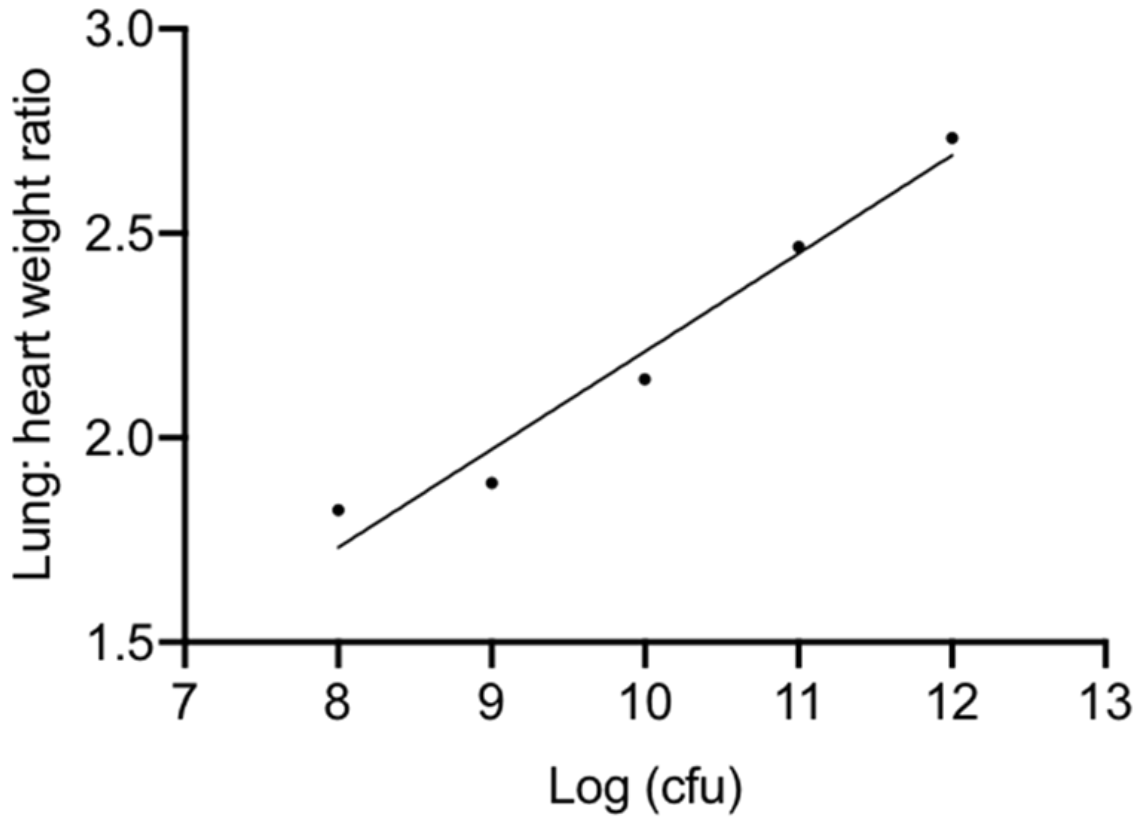


Figure 2.9: Relationship between challenge dose of *Staphylococcus aureus* and *Escherichia coli* lysate and lung: heart weight ratios. The data show the ratio of lung and heart weights measured at the time of postmortem examination, 24 hours after 4 calves were administered 10^8, 10^9, 10^{10} or 10^{11} CFU-equivalents of *Staphylococcus aureus* and *Escherichia coli* lysate by aerosol. The ratio of lung to heart weight was correlated with the dose of bacterial lysate ($R^2 = 0.96$, $p = 0.003$).

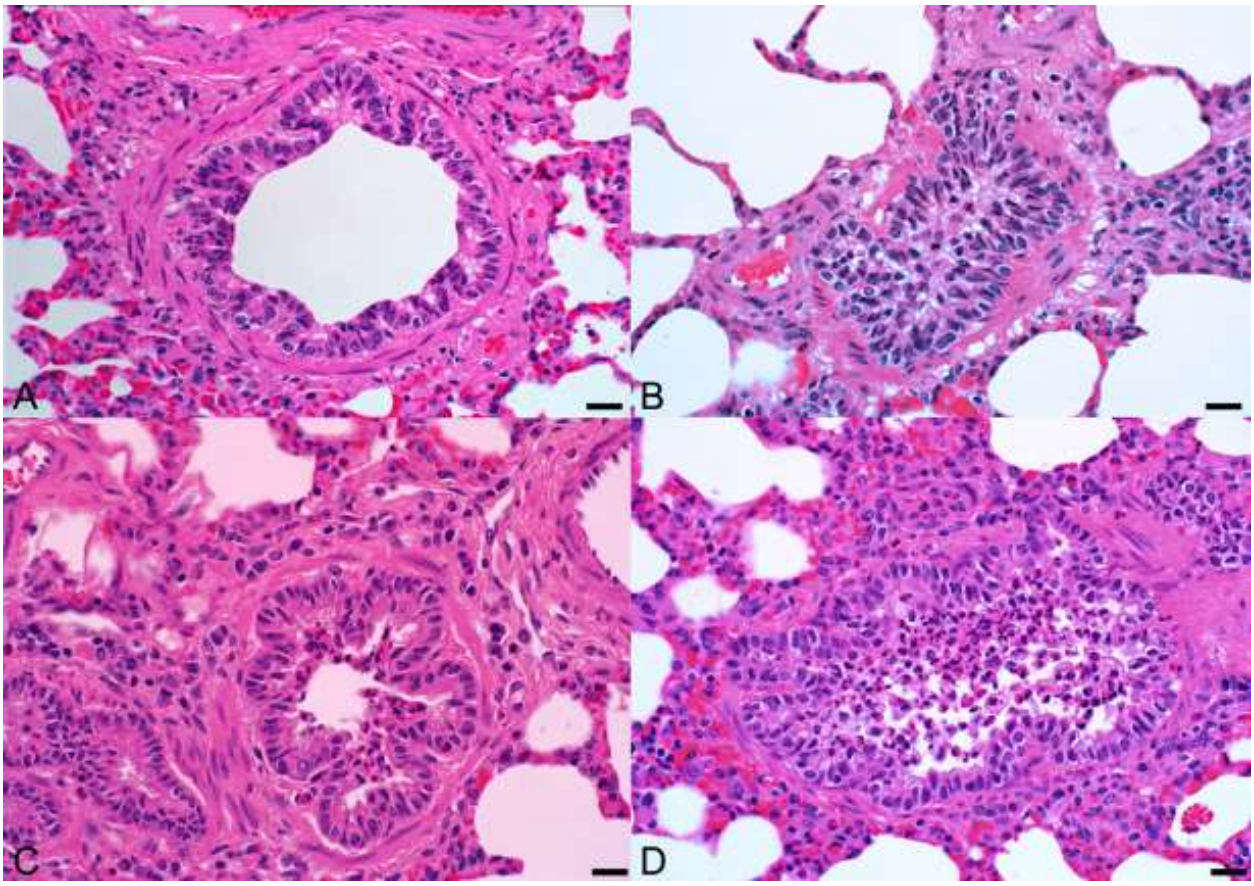


Figure 2.10: Post-lysate histology of bronchioles. Histologic sections of bronchioles from calves that received aerosolized bacterial lysate 24 hours prior to euthanasia. At lower doses of bacterial lysate (A, calf 1, 10^8 CFU-equivalents; and B, calf 2, 10^9 CFU equivalents), the bronchioles have no or few neutrophils within their lumens. At higher doses of bacterial lysate (C, calf 3, 10^{10} CFU-equivalents; and D, calf 4, 10^{11} CFU-equivalents) there are increased numbers of neutrophils within bronchioles. Hematoxylin and eosin. Bar=40 μm.

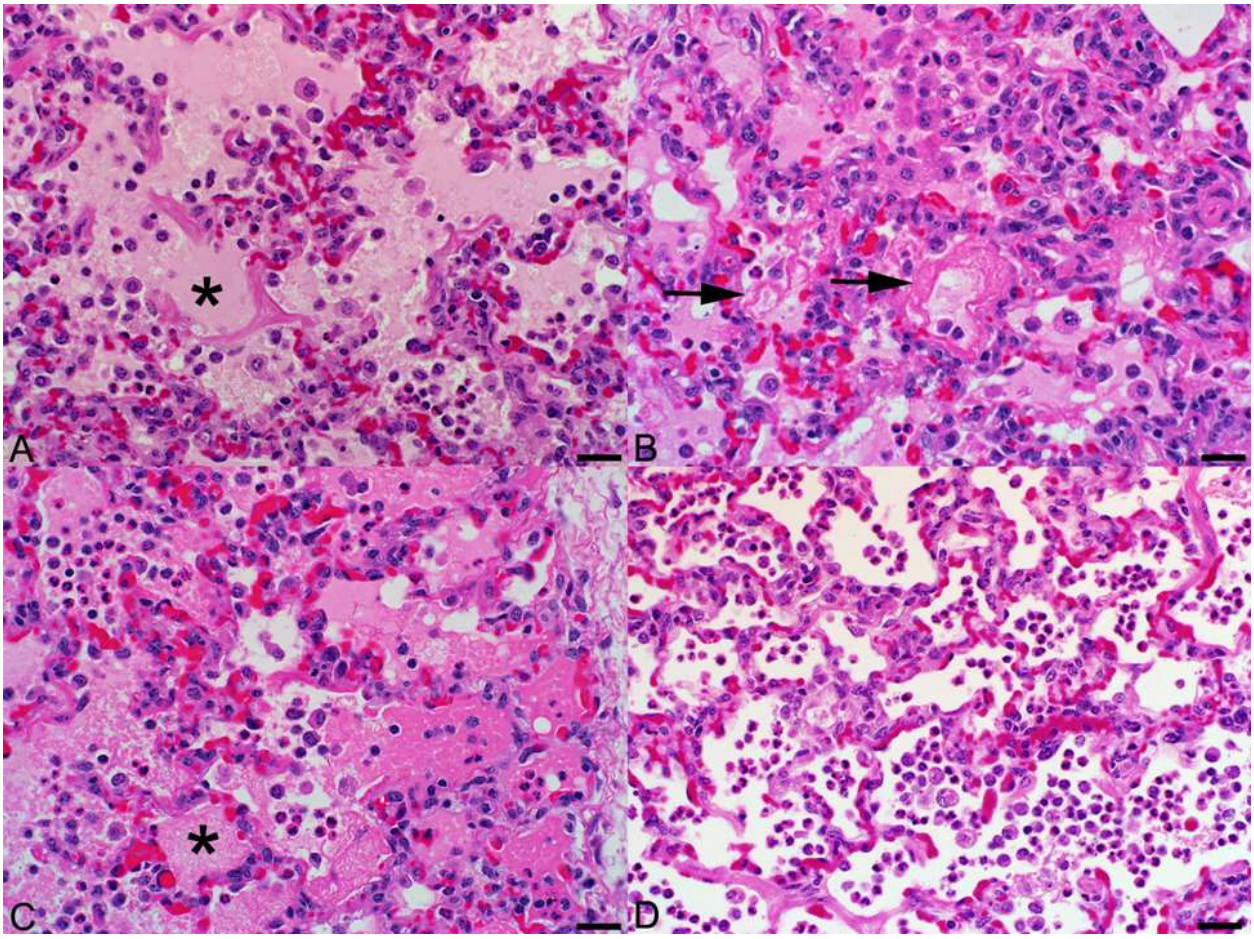


Figure 2.11: Post-lysate lung histology. Histologic sections of lungs from calves receiving 10^{12} (A, B), 10^{11} (C) and 10^{10} (D) cfu-equivalents of *Escherichia coli* and *Staphylococcus aureus* lysate. There is histological evidence of acute diffuse alveolar damage consisting of hyaline membranes (arrows), large amounts of eosinophilic fluid in alveoli (asterisks), neutrophils and macrophages in alveoli, and thickened hypercellular alveolar septa. Hematoxylin and eosin. Bar = 40 μm.

2.9 Tables

Table 2.1: Summary of calves used for each study, samples collected, and tests performed.

Trial	Calf ID	Aerosol treatment and dose (CFU-equivalents)	Clinical findings	Post-mortem examination	Blood-work[a]	BALF cytology	BALF cytokines[b]	BALF mass spectrometry	Notes
1	A1	Lysate ($1x10^{12}$)	X	X	X	X	X		Developed mild depression, dyspnea, pulmonary edema
1	B1	Control	X	X	X	X	X		Pre-existent lung lesions, coughed prior to study
2	A2	Lysate ($1x10^{12}$)	X		X	X	X		
2	B2	Control	X		X	X	X		Pre-existing pneumonia, high baseline neutrophils in BALF
3	A3	Lysate ($1x10^{8}$)	X	X	X	X	X	X	High neutrophils in BALF prior to lysate administration
3	A4	Lysate ($1x10^{9}$)	X	X	X	X	X	X	
3	A5	Lysate ($1x10^{10}$)	X	X	X	X	X	X	
3	A6	Lysate ($1x10^{11}$)	X	X	X	X	X	X	

CFU—colony-forming units; ID—study identification; Lysate—aerosolized lysate of killed *S. aureus* and *E. coli*; Control— aerosolized phosphate-buffered saline as negative control.
[a]Blood work includes complete blood count, fibrinogen, and haptoglobin; [b]Cytokines evaluated were interleukin-8, -10 and -17.

Table 2.2: Objective clinical scoring system for calves. Adapted from Potter et al., 1999.

Feature	Score	Description
Demeanor	0	Not depressed.
	1	Rarely stands alone, ears droop slightly, moves away when people approach.
	2	Walks slowly, lethargic, sometimes stands with head low, easy to corner but difficult to catch.
	3	Uninterested in environment, little response when people enter pen, easy to catch, lies in sternal recumbency frequently.
	4	Lies down most of the time, stands only occasionally, doesn't respond when people enter pen.
Appetite	0	Animal is seen eating with normal vigour, finishes milk.
(only assessed at feedings)	1	Starts, but doesn't finish milk. Nibbles food but little is consumed.
	2	Doesn't eat milk, hay or grain.
Strength	0	Normal and difficult to catch.
	1	Walks slowly, mildly unsteady gait, easier to catch than normal.
	2	Staggers or knuckles occasionally, recumbent less than 50% of the time, becomes recumbent from standing when people are in the pen, obviously unsteady gait.
	3	Recumbent most of the time but will rise when stimulated.
	4	Recumbent and will not rise when stimulated.
Effort of breathing	0	Normal breathing pattern. Mild but detectable increase in respiratory effort when stressed.
	1	Obvious increase in respiratory effort when stressed, subtle increase when not stressed.
	2	Obvious increase in respiratory effort when not stressed (observed from a distance).
	3	Open mouth breathing marked increase in respiratory effort.

Table 2.3: Elevated proteins in bronchoalveolar fluid following aerosolization of bacterial lysate. Bronchoalveolar lavage fluid proteins that were significantly elevated (n=19) following aerosolization of bacterial lysate relative to baseline, as determined by a paired t-test. Tandem mass-tag mass spectrometry was performed on bronchoalveolar lavage fluid from 4 calves obtained at baseline and 24 hours after aerosol administration of bacterial lysate, with data analysis and protein normalization in Proteome Discoverer 2.2 against Uniprot Bovine Database.

Accession	**Description**	**Ratio of post-lysate to baseline**	***P*-value**
G3MYZ3	Afamin	2.06	0.04
Q7SIH1	Alpha-2-macroglobulin	3.05	0.04
P01888	Beta-2-microglobulin	2.25	0.00
Q0V8R6	Beta-hexosaminidase subunit alpha	1.67	0.01
F1N076	Ceruloplasmin	2.92	0.04
P81187	Complement factor B	2.22	0.00
Q28085	Complement factor H	3.39	0.04
A5PJT7	ECM1 protein	2.78	0.02
Q29437	Primary amine oxidase, liver isozyme	3.23	0.01
P82943	Regakine-1	2.50	0.04
Q29443	Serotransferrin	1.92	0.01
G3N1U4	Serpin A3-3	2.46	0.05
Q2KIS7	Tetranectin	3.02	0.04
F1MMR5	Tetratricopeptide repeat domain 38	2.30	0.03
Q3ZBS7	Vitronectin	2.24	0.02
E1BH06	Uncharacterized protein*	2.46	0.05
F1MCF8	Uncharacterized protein	1.40	0.02
F1MJK3	Uncharacterized protein	4.34	0.04
F1MVK1	Uncharacterized protein	4.19	0.05

*Four uncharacterized proteins were increased following treatment

Table 2.4: Proteins that were reduced in bronchoalveolar fluid following aerosolization of bacterial lysate. Bronchoalveolar lavage fluid proteins (n=26) that were significantly reduced following aerosolization of bacterial lysate relative to baseline, as determined by a paired t-test.

Accession	Description	Ratio of post-lysate to baseline	*P*-value
A6QLI0	Mammalian ependymin-related protein 1	0.30	0.03
P31404	V-type proton ATPase catalytic subunit A	0.33	0.02
Q5E9J1	Heterogeneous nuclear ribonucleoprotein F	0.35	0.04
Q3T0D0	Heterogeneous nuclear ribonucleoprotein K	0.35	0.03
Q5E9E2	Myosin regulatory light polypeptide 9	0.37	0.03
G3MYX8	Tyrosine-protein kinase receptor	0.41	<0.001
P04272	Annexin A2	0.41	0.01
Q3T0F7	Myotrophin	0.42	0.04
Q3T0M0	Vacuolar protein sorting-associated protein	0.44	0.03
Q3SZA6	Syndecan binding protein (Syntenin)	0.52	0.02
Q2HJH1	Aspartyl aminopeptidase	0.54	0.05
F1MC48	IQ motif containing GTPase activating protein 1	0.56	<0.001
Q3T0S6	60S ribosomal protein L8	0.56	0.02
A0A140T843	Beta-2-glycoprotein 1	0.57	0.05
Q2YDE4	Proteasome subunit alpha type-6	0.57	0.05
F1MG05	Elongation factor 1-gamma	0.58	0.02
F1MSE7	Sushi domain containing 2	0.60	0.05
P11116	Galectin-1	0.62	0.04
Q32PH8	Elongation factor 1-alpha 2	0.65	0.05
F1MM32	Sulfhydryl oxidase	0.68	0.01
G3MXB5	Uncharacterized protein	0.69	0.00
A6QLG5	40S ribosomal protein S9	0.69	0.01
Q0IIM3	Heat shock protein 105 kDa	0.71	0.05
Q3ZC44	Heterogeneous nuclear ribonucleoprotein A/B	0.72	0.03
Q1RMR9	Protein kinase C and casein kinase substrate in neurons 2	0.72	0.03
A5PKK0	FAM151B protein	0.81	0.05

2.10 Supplemental Material

Supplemental Material 2.1: Methods for mass spectrometry analysis of bronchoalveolar lavage fluid.

Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm x 50 cm PepMax RSLC EASY-Spray column filled with 2 µM C18 beads (ThermoFisher San, Jose CA) at a pressure of 900 Bar and a temperature of 60C. Peptides were eluted over 180 minutes at a rate of 250 nL/min using a gradient set up as follows, where Buffer A is 0.1% Formic acid and Buffer B is 80% Acetonitrile, 0.1% Formic Acid, all v/v in HPLC grade water.

Time (total minutes)	Duration (minutes)	% Buffer B
0	0	0
168	168	42
170	2	100
180	10	100

Peptides were introduced by nano-electrospray into the Fusion- Lumos mass spectrometer (Thermo-Fisher). Data were acquired using the MultiNotch MS3 acquisition with synchronous precursor selection (SPS) with a cycle time of 5 seconds. MS1 acquisition was performed with a scan range of 550m/z - 1800 m/z with resolution set to 120 000, maximum injection time of 50ms and AGC target set to 4e5. Isolation for MS2 scans was performed in the quadrupole, with an isolation window of 0.7. MS2 scans were done in the linear ion trap with a maximum injection time of 50ms and a normalized collision energy of 35%. For MS3 scans, HCD was used, with a collision energy of 30% and scans were measured in the orbitrap with a resolution of 50000, a scan range of 100m/z-500m/z, an AGC Target of 3e4, and a maximum injection time of 50ms. The dynamic exclusion was applied using a maximum exclusion list of 500 with one an exclusion duration of 20 s.

Supplemental Material 2.2: Quantification of bronchoalveolar lavage fluid cytokines using Meso Scale Discovery electrochemiluminescent U-plex.

A multiplexed electrochemiluminscent ligand binding assay was developed using two separate Meso Scale Diagnostics (MSD) U-Plex panels, following the MSD U-Plex protocol guide. Cytokines were quantified using the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer's instructions using a chemiluminescent readout. Panel 1 included biotinylated capture antibodies: IL-1β (Biorad, Hercules, CA), IL-2 (R&D Systems Minneapolis, MN), IL-6 (R&D Systems), IFN-α (Kingfisher Biotech, Minneapolis, MN), and TNF-α (R&D Systems). Panel 2 included: IL-8 (Mabtech, Cincinnati, OH), IL-10 (Biorad), IL-17A (Mabtech), and IFN-γ (Mabtech). The IL-1β antibody was biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) at a challenge ratio of 1:20 according to manufacturer instructions.

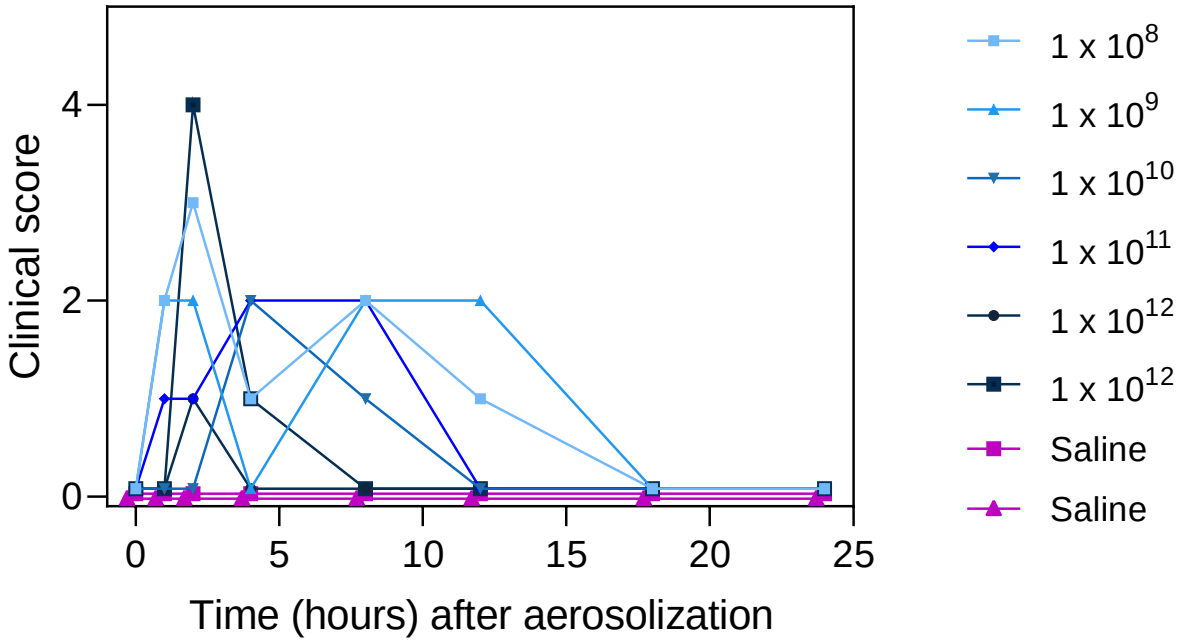
All calibrators used in the assay were purchased from Kingfisher Biotech. The panel 1 calibrators were diluted to a concentration of 40,000 pg/mL in 1X Dulbecco's phosphate Buffered Saline (DPBS) without calcium or magnesium, followed by 4-fold serial dilutions into 1X DPBS. The panel 2 calibrators were diluted to a concentration of 10,000 pg/mL in 1X DPBS followed by 4-fold serial dilutions.

The detection antibodies were sulfo-tagged following the MSD quick guide conjugation protocol using a challenge ratio of 1:20. Panel 1 detection antibodies were used at the following concentrations: IL-1β at 1 µg/mL(Biorad), IL-2 at 0.5 µg/mL (R&D Systems), IL-6 at 2 µg/mL (R&D Systems), IFN-α at 1 µg/mL (Kingfisher Biotech) and TNF-α at 0.5 µg/mL (R&D systems). Panel 2 detection antibodies were used at the following concentrations: IL-8 at 0.5 µg/mL (Mabtech), IL-10 at 1 µg/mL (Biorad), IL-17A at 0.2 µg/mL (Kingfisher Biotech) and IFN-γ at 1 µg/mL (Mabtech).

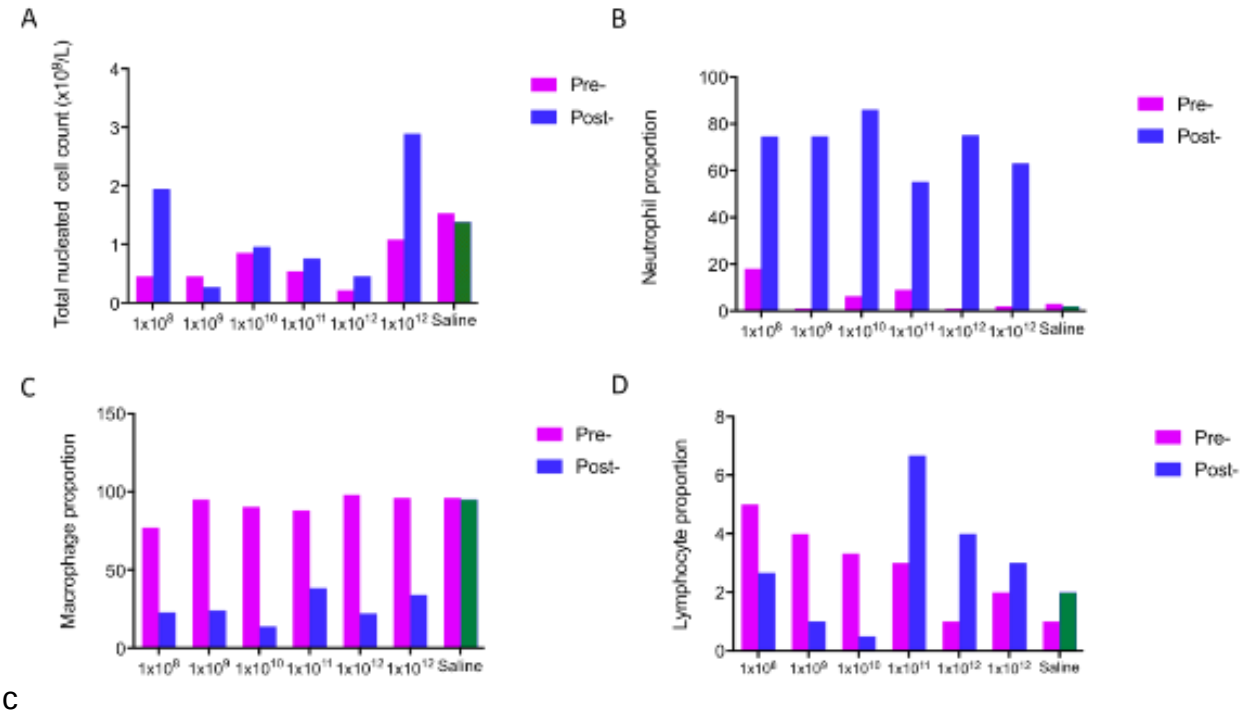
Cytokines were quantified using the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer's instructions using a chemiluminescent readout. In brief, 0.5 mL of all biotinylated antibodies were diluted to 10 µg/mL with Diluent 100, and paired to their respective linkers by adding 750 mL of supplied Linker solution and incubated for 30 minutes followed by addition of Stop solution for another 30 minutes. The linked antibodies for panel 1 were then diluted 10-fold into Stop solution. The panel 2 linked antibodies were diluted 20-fold (IL-10 and IL-17A) or 40-fold (IL-8 and IFN-γ) into Stop solution. Plates were coated by adding 50 µL of antibody solutions per well and were incubated covered with shaking for 1 hour at room temperature. Plates were washed 3 times with PBS/Tween20 prior to samples or standards being added at 30 µL per well and incubated as above. Plates were washed 3 times before detection antibodies were added along with 2% rabbit serum to block and incubated as above. Plates were washed 3 times before addition of 150 µL 2X Read buffer and read immediately on the SI6000 instrument (MSD, Rockville, MD). The results were reported as pg/mL. For soluble protein levels, a bicinchoninic acid (BCA) protein kit (Thermofisher, Rockford, IL) was used. The cytokine concentrations were normalized to the total protein levels for each sample.

Supplemental Table S2. 1: Clinical parameters over time in six, 3-month-old Holstein calves following aerosolization of bacterial lysate. Data were compared to pre-challenge (time=0) using repeated measures one-way analysis of variance (ANOVA) with post hoc Dunnett's multiple comparison test. **p* < 0.05, ***p* < 0.01. The mean, standard error of the mean and lower and upper confidence intervals (CI) are presented.

Outcome variable	ANOVA results	P-value	Time (hours)	Mean	SEM	Lower 95% CI	Upper 95% CI
Rectal temperature	F (2.65, 13.27) = 4.95	0.019*	0	38.8	0.24	38.5	39.1
			1	39.1	0.48	38.6	39.6
			2	39.1	0.33	38.8	39.5
			4	39.5**	0.36	39.1	39.9
			8	40.0*	0.62	39.4	40.7
			12	39.7	0.72	39.0	40.5
			18	39.2	0.44	38.7	39.7
			24	39.0	0.15	38.8	39.1
Heart rate	F (3.52, 17.60) = 3.05)	<0.050*	0	77.0	7.6	57.4	96.6
			1	79.3	7.0	61.4	97.2
			2	86.0	8.8	63.4	108.6
			4	95.3	8.1	74.5	116.1
			8	98.7	6.0	83.3	114.1
			12	89.3	4.9	76.7	102.0
			18	77.7	5.7	63.1	92.2
			24	75.7	2.0	70.6	80.7
Respiratory rate	F (2.25, 11.24) = 1.89	0.195	0	54.3	4.7	42.1	66.5
			1	62.0	10.9	34.1	89.9
			2	78.0	5.4	64.0	92.0
			4	68.3	4.7	56.3	80.4
			8	67.7	6.1	51.9	83.4
			12	79.3	5.6	64.9	93.7
			18	66.3	2.6	59.8	72.9
			24	61.0	4.5	49.5	72.6



Supplemental Figure S2. 1: Individual clinical scores over time in Holstein bull calves receiving saline or different doses of bacterial lysate. Six 3-month-old Holstein calves received aerosolized *Staphylococcus aureus* and *Escherichia coli* lysate at doses ranging from 10^8-10^{12} CFU-equivalents and 2 calves received aerosolized saline (control). Clinical scores are the sum of individual scores for demeanor (0–4), strength (0–4), appetite (0–2), and respiratory effort (0–3), for a maximum possible score of 13 at each time point.



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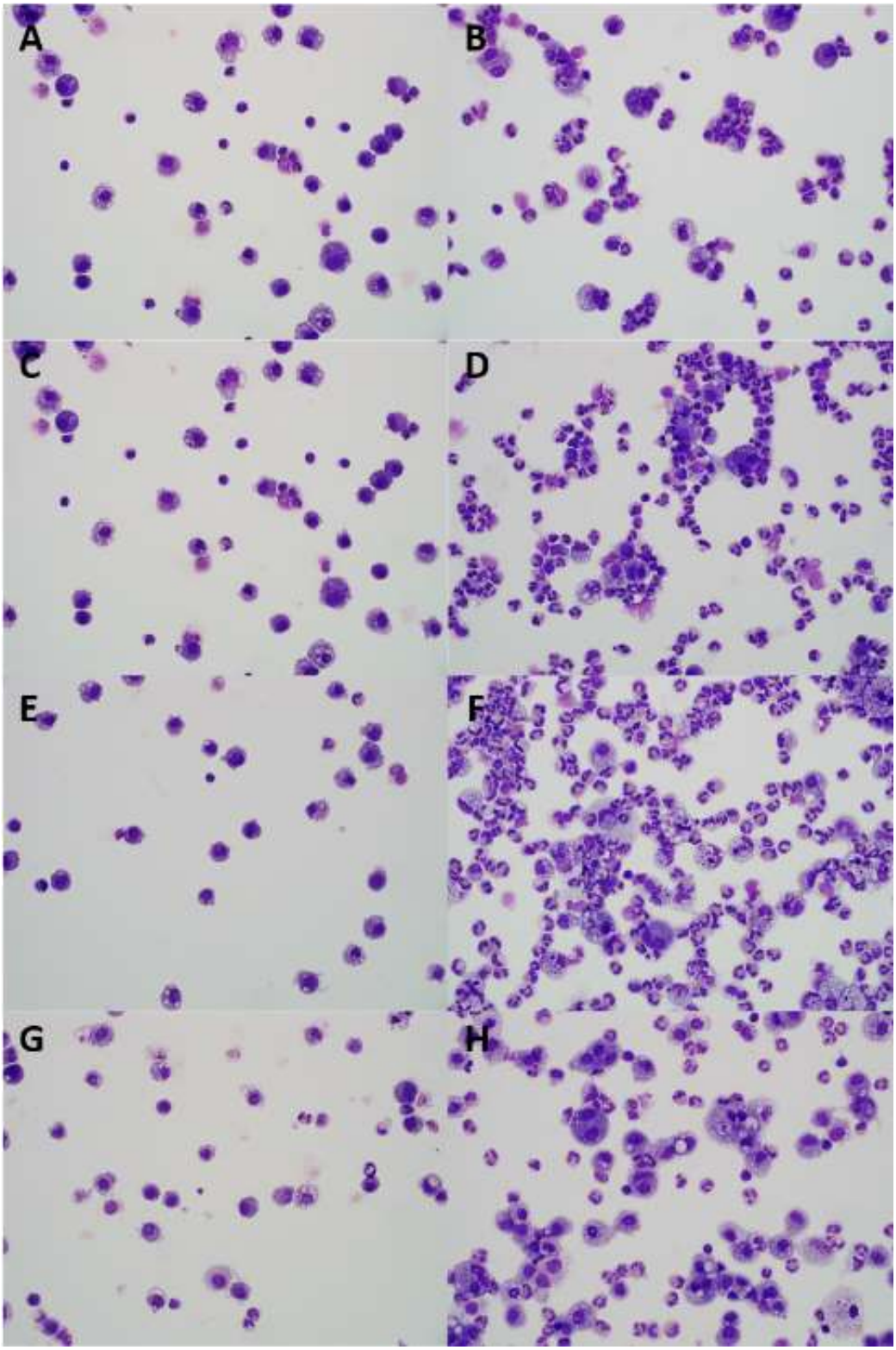
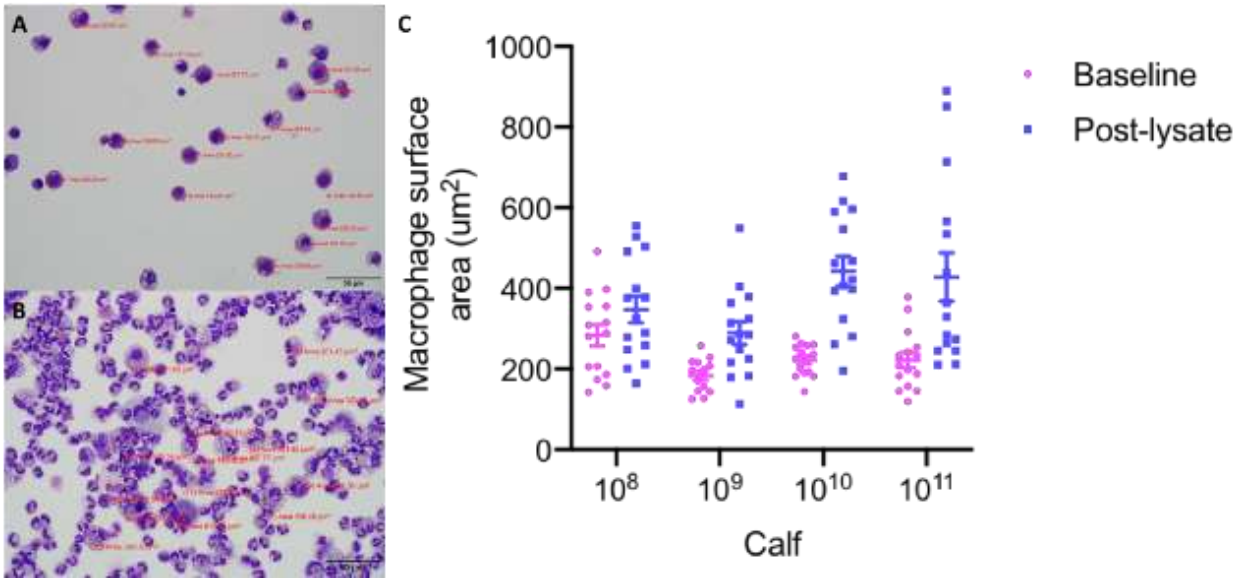
Supplemental Figure S2. 2: Bronchoalveolar lavage fluid cells pre- and post-administration of differing doses of bacterial lysate or saline in Holstein bull calves. A) Total nucleated cell count. B) Percentage of neutrophils. C) Percentage of macrophages. D) Percentage of lymphocytes.



Supplemental Figure S2. 3: Bronchoalveolar lavage fluid cytology pre and post-aerosolization of bacterial lysate. The left column shows baseline cytology and the right column was collected 24 hours after aerosolization of lysate at 10^8 (B), 10^9 (D), 10^{10} (F), and 10^{11} (H) colony forming unit equivalents. Wright stain.



Supplemental Figure S2. 4: Surface area of macrophages pre- and post-aerosolization of bacterial lysate. Baseline cytocentrifuge preparation (A) and 24 hours post aerosol delivery (B) in a calf that received 10^{10} colony forming unit (CFU)equivalents of bacterial lysate. Wright stain. C) Macrophage surface area with bars showing mean and standard error of the standard error of 15 cells prior to and 24-hours post aerosolization of bacterial lysate at dosages ranging from 10^8 to 10^{11} CFU-equivalents. Cell size increased following lysate treatment ($p < 0.0001$, 2-way ANOVA).

Supplemental Table S2. 2: Bronchoalveolar lavage fluid protein concentrations (g/L) in calves prior to and 24 hours post aerosolization of bacterial lysate or saline.

ID	Treatment	Baseline	After aerosolization
B1	Control	73.08	65.66
B2	Control	81.57	42.67
A1	Lysate ($1x10^{12}$)	77.65	195.37
A2	Lysate ($1x10^{12}$)	42.23	119.5
A3	Lysate ($1x10^{8}$)	69.89	144.58
A4	Lysate ($1x10^{9}$)	69.81	870.81
A5	Lysate ($1x10^{10}$)	40.00	91.46
A6	Lysate ($1x10^{11}$)	45.11	60.56

Lysate—aerosolized lysate of killed *S. aureus* and *E. coli*; Control— aerosolized phosphate-buffered saline as negative control.

Supplemental Table S2.3: Mass spectrometry data for proteins in bronchoalveolar lavage fluid in 4 calves (A4, A3, A5, A6) before and 24 hours after aerosolization with heat-killed lysate of *Escherichia coli* and *Staphylococcus aureus* https://atrium.lib.uoguelph.ca/xmlui/handle/10214/17840

Supplemental Table S2. 4: PANTHER classification of 19 upregulated proteins in bronchoalveolar lavage fluid following aerosolization of bacterial lysate in 4 calves.

Protein ID	Gene Name	Panther Protein Class
P82943	Regakine-1	Chemokine (PC00074)
E1BH06	Uncharacterized protein	complement component (PC00078); cytokine (PC00083); serine protease inhibitor (PC00204)
P01888	Beta-2-microglobulin	major histocompatibility complex antigen (PC00149)
Q29437	Primary amine oxidase, liver isozyme	
Q3ZBS7	Vitronectin	
G3MYZ3	Afamin	transfer/carrier protein (PC00219)
G3N1U4	Serpin A3-3	serine protease inhibitor (PC00204)
F1MMR5	Tetratricopeptide repeat domain 38	
F1MJK3	Uncharacterized protein	complement component (PC00078); cytokine (PC00083); serine protease inhibitor (PC00204)
Q0V8R6	Beta-hexosaminidase subunit alpha	Glycosidase (PC00110)
F1N076	Ceruloplasmin	Oxidase (PC00175)
Q2KIS7	Tetranectin	extracellular matrix structural protein (PC00103)
A5PJT7	ECM1 protein	
Q28085	Complement factor H	
Q7SIH1	Alpha-2-macroglobulin	complement component (PC00078); cytokine (PC00083); serine protease inhibitor (PC00204)
Q29443	Serotransferrin;TF;ortholog	Receptor (PC00197); serine protease (PC00203); transfer/carrier protein (PC00219)
F1MCF8	Uncharacterized protein	Immunoglobulin (PC00123)
P81187	Complement factor B	
F1MVK1	Uncharacterized protein	complement component (PC00078); cytokine (PC00083); serine protease inhibitor (PC00204)

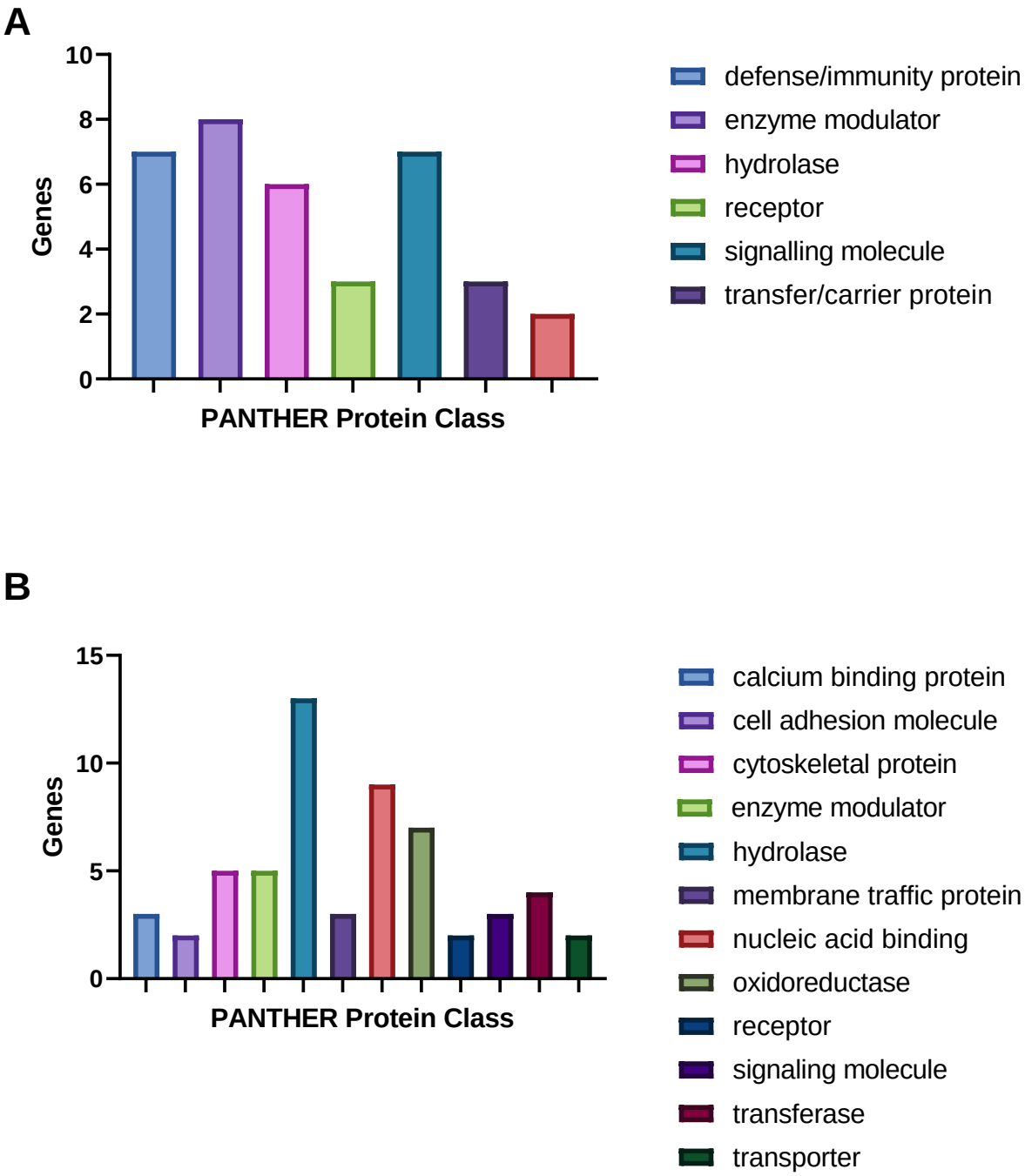
Supplemental Table S2. 5: Panther classification of 26 proteins in bronchoalveolar lavage fluid that were reduced 24 hours after aerosolization of bacterial lysate in 4 calves.

Accession	Gene Name	Panther Protein Class
A6QLG5	40S ribosomal protein S9	ribosomal protein (PC00202)
Q3T0S6	60S ribosomal protein L8	ribosomal protein (PC00202)
P04272	Annexin A2	
A0A140T843	Beta-2-glycoprotein 1	
F1MG05	Elongation factor 1-gamma	
P11116	Galectin-1	cell adhesion molecule (PC00069); signaling molecule (PC00207)
Q0IIM3	Heat shock protein 105 kDa	
Q3ZC44	Heterogeneous nuclear ribonucleoprotein A/B	
Q5E9J1	Heterogeneous nuclear ribonucleoprotein F	ribosomal protein (PC00202)
Q3T0D0	Heterogeneous nuclear ribonucleoprotein K	enzyme modulator (PC00095); mRNA splicing factor (PC00148); ribonucleoprotein (PC00201); serine protease (PC00203)
G3MXB5	IG alpha chain C region	immunoglobulin receptor superfamily
F1MC48	IQ motif containing GTPase activating protein 1	G-protein modulator (PC00022)
A6QLI0	Mammalian ependymin-related protein 1	

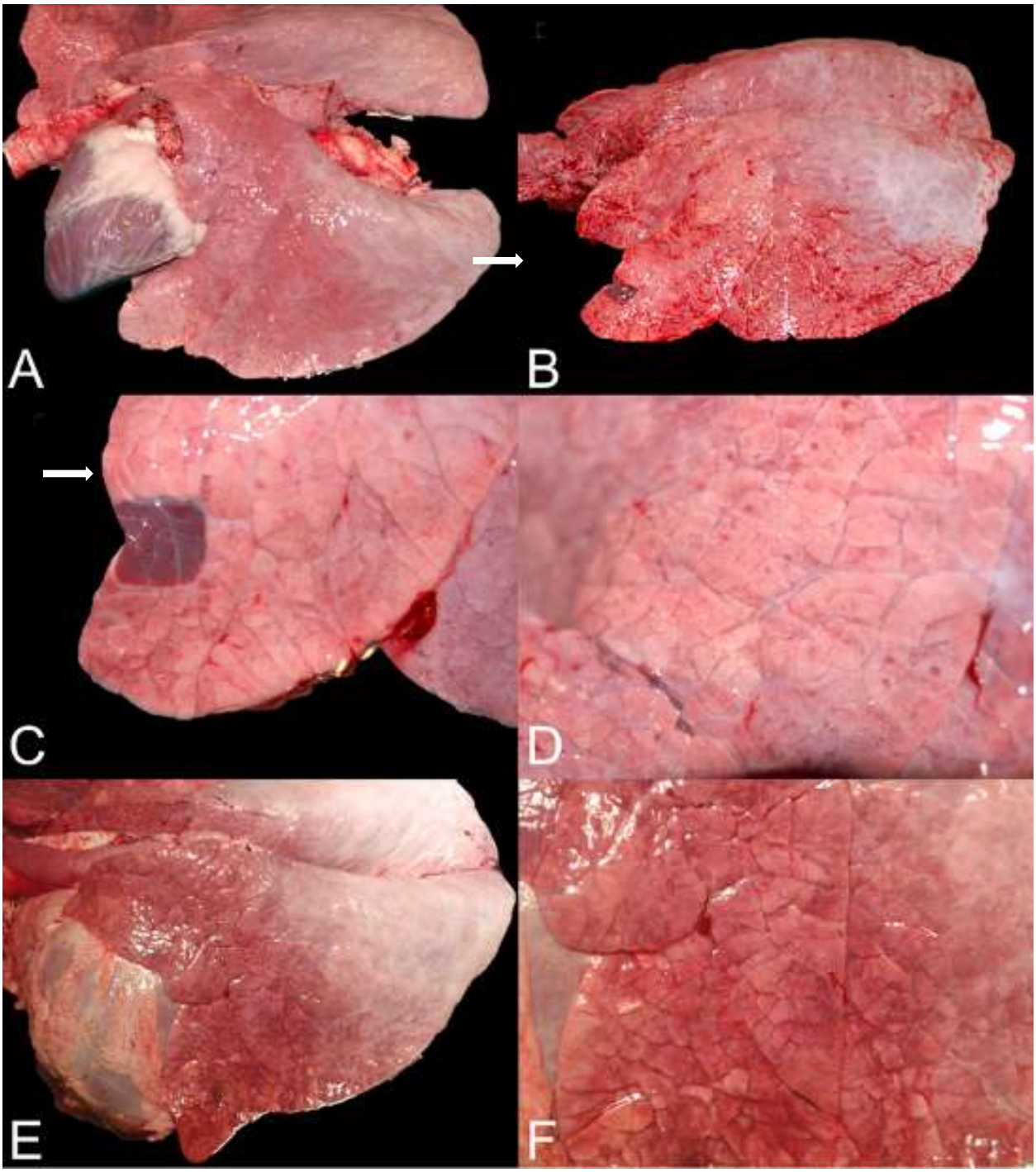
Accession	Gene Name	Panther Protein Class
Q5E9E2	Myosin regulatory light polypeptide 9	actin family cytoskeletal protein (PC00041); calmodulin (PC00061)
Q3T0F7	Myotrophin	
Q2YDE4	Proteasome subunit alpha type-6	
A5PKK0	Protein Fam151B	
Q1RMR9	Protein kinase C and casein kinase substrate in neurons 2	actin family cytoskeletal protein (PC00041); membrane trafficking regulatory protein (PC00151)
G3MYX8	Proto-oncogene tyrosine-protein kinase ROS	
F1MM32	Sulfhydryl oxidase	Oxidase (PC00175)
F1MSE7	Sushi domain containing 2	
Q3SZA6	Syndecan binding protein	membrane trafficking regulatory protein (PC00151)
G3MYX8	Tyrosine-protein kinase receptor	
G3MXB5	Uncharacterized protein	immunoglobulin receptor superfamily (PC00124)
Q3T0M0	Vacuolar protein sorting-associated protein 29	vesicle coat protein (PC00235)
P31404	V-type proton ATPase catalytic subunit A	ATP synthase (PC00002); DNA binding protein (PC00009); anion channel (PC00049); ligand-gated ion channel (PC00141); protease (PC00190)

Supplemental Table S2. 6: Reactome pathway analysis for differently expressed proteins in bronchoalveolar lavage fluid in 4 calves. For each Reactome pathway, the listed proteins were increased ($p < 0.05$, or $p > 0.05$ but <0.10) following aerosolization of bacterial lysate compared to baseline levels.

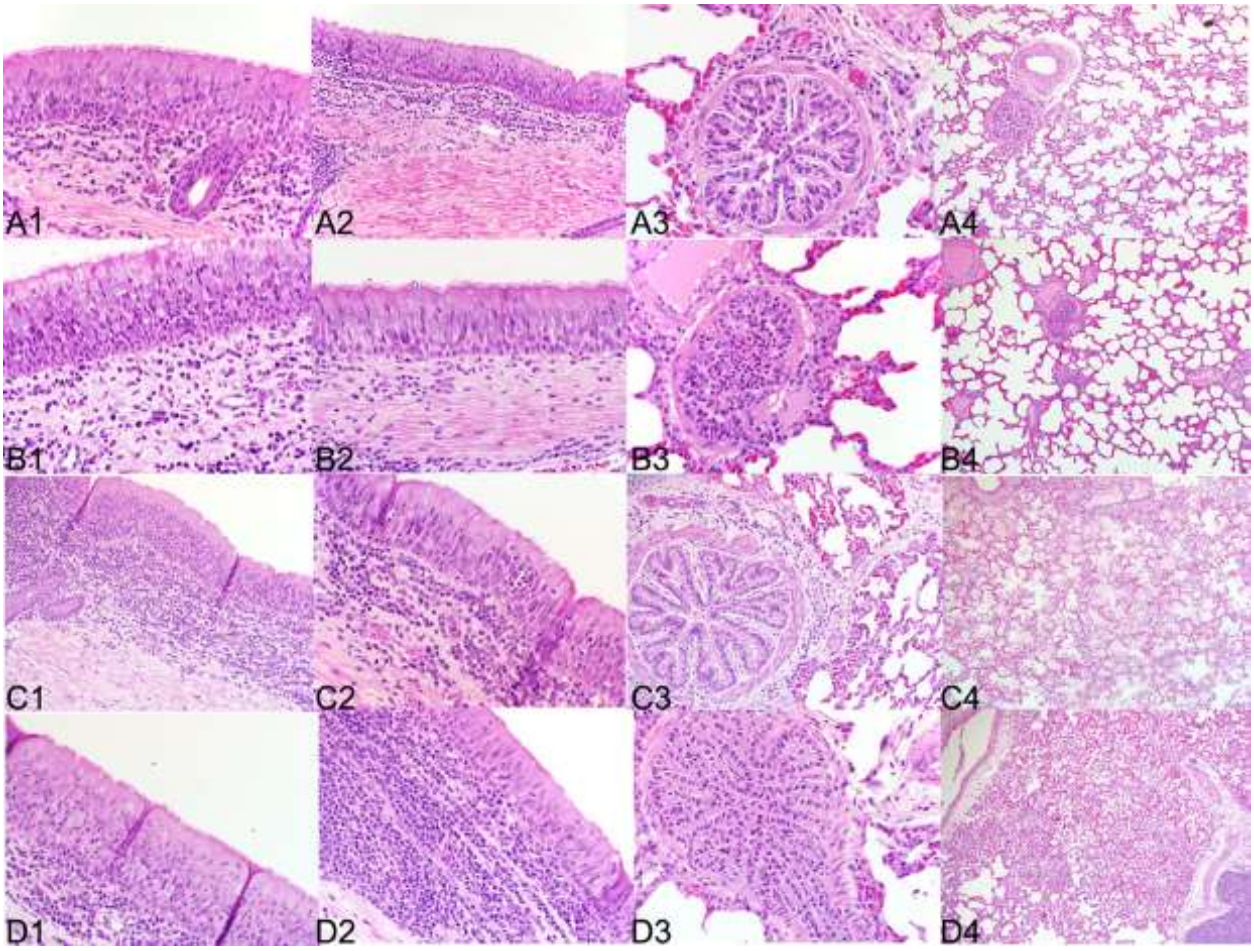
Reactome pathway	**P<0.05**	**0.05<P<0.10**
Complement (activation of C3 and C5 / regulation of Complement cascade)	Uncharacterized protein, C4a gene (E1BH06) Vitronectin (Q3ZBS7) Uncharacterized protein (F1MVK1)	Complement component C9 (Q3MHN2) Complement factor I (F1N4M7)
Platelet responses (platelet degranulation/response to eleavated platelet cytosolic Ca2+/platelet signaling and activation)	ECM1 protein (A5PJT7) Tetranectin (Q2KIS7) Alpha-2-macroglobulin (Q7SIH1)	Inter-alpha-trypsin inhibitor heavy chain H4 (F1MMD7)
Post-translational protein phosphorylation	Uncharacterized protein (F1MVK1) Ceruloplasmin (F1N076)	Kininogen-1 (A0A140T8C8)
Regulation of Insulin-like Growth Factor transport and uptake by Insulin-like Growth Factor Binding Proteins	Uncharacterized protein (F1MVK1) Ceruloplasmin (F1N076)	Kininogen-1 (A0A140T8C8)



Supplemental Figure S2. 5: PANTHER protein class for bronchoalveolar lavage proteins. Mass spectrometry measured proteins that were increased (A) or decreased (B) in bronchoalveolar lavage 24 hours after aerosolization of bacterial lysate. Included are proteins that were different from baseline, $p \leq 0.10$, paired t-test. Protein classes that contained only one protein were excluded.



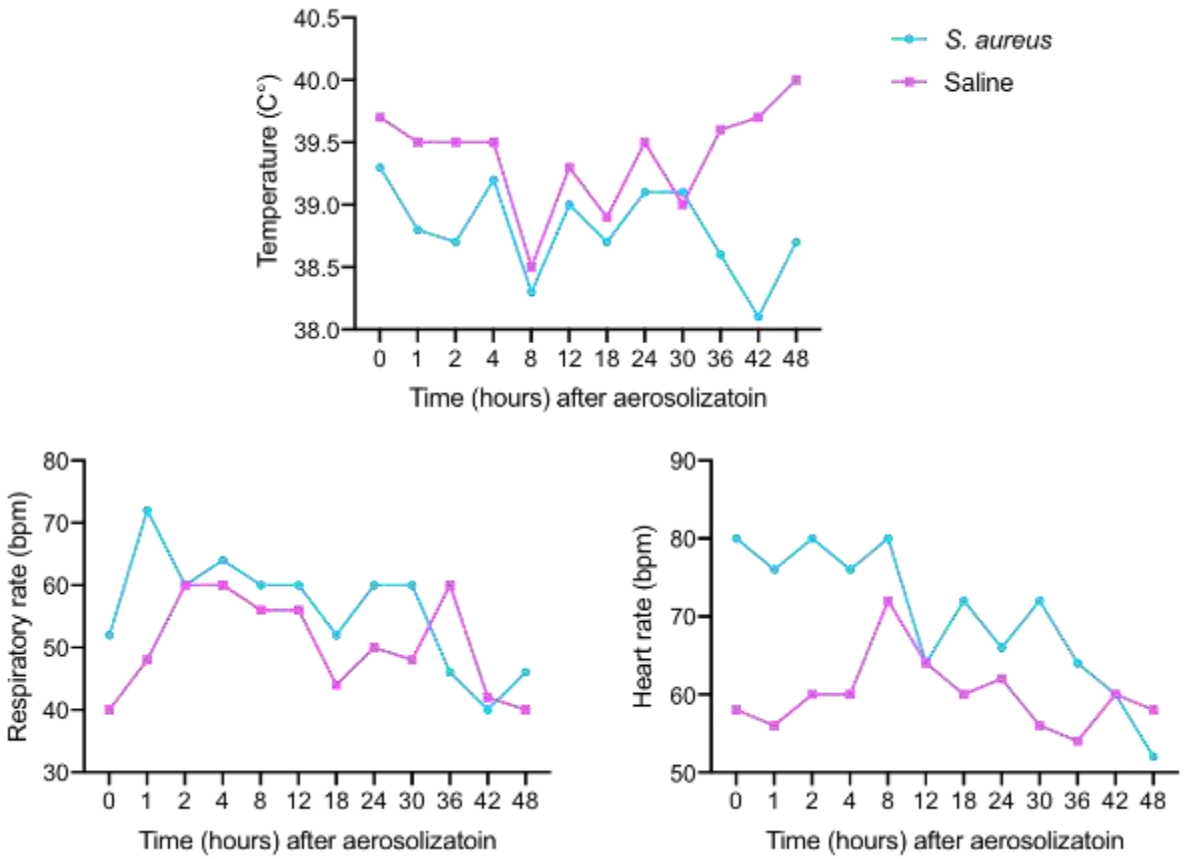
Supplemental Figure S2. 6:: Gross lesions following aerosolization of bacterial lysate. Gross images of lungs at 24 hours after aerosol administration of *Staphylococcus aureus* and *Escherichia coli* lysate. Lungs from calves 24 hours after aerosol administration of *Staphylococcus aureus* and *Escherichia coli* lysate. A) Calf A3 received 10^8 CFU-equivalents and had no gross lesions in the lungs. B-D**)** Calf A6**;** 10^{11} CFU-equivalents**;** multifocal areas of consolidation (arrow), interlobular edema and small foci of hemorrhage on the pleural surface. E-F) Calf B1; 10^{12} CFU-equivalents*;* mild edema with red mottling and rare small foci of hemorrhage.



Supplemental Figure S2. 7: Respiratory tract histology following aerosolization of bacterial lysate. Histologic sections of respiratory tissues from 4 calves treated with aerosolized *Staphylococcus aureus* and *Escherichia coli* lysate at 10^8 (A1-4), 10^9 (B1-4), 10^{10} (C1-4), and 10^{11} (D1-4) colony forming unit equivalents. All sections of upper (thoracic) trachea (A1-D1) and lower (cervical) trachea (A2-D2) have small numbers of neutrophils within the lamina epithelium and submucosa, and variable numbers of lymphocytes and plasma cells. Bronchioles (A3-D3) have neutrophils within the submucosa, epithelium and lumens. Alveoli (A4-D4) contain increasing numbers of neutrophils with higher doses of lysate.

Supplemental Material 2.3: Summary of pilot study: Response to aerosolized heat-killed S. aureus

The experiment was similar to what was described previously. In this experiment, two calves (calves S1 and C1) were randomly assigned to receive either 6 mL of aerosolized PBS containing 6 X 10^{11} colony forming units of heat-killed intact S. aureus or 6 mL of PBS. Bronchoalveolar lavage fluid was collected 48 hours following S. aureus or PBS aerosolization. Calves were euthanized at 48 hours. Both calves remained bright and alert following aerosol treatment, with good appetites up to the point of euthanasia at 48 hours. The calf treated with saline (C1) coughed prior to and following aerosolization with saline, and post mortem examination identified chronic pneumonia with abscessation indicating this lesion was present prior to the start of the experiment. The only clinical sign that was observed in the S. aureus-treated calf was a bilateral serous nasal discharge which was first identified at 4 hours post-aerosol and was intermittently present until 18 hours. Rectal temperatures, respiratory rates and heart rates over time after aerosolization for both calves are shown below. There were no notable findings. Serum haptoglobin and plasma fibrinogen did not change. Cytological analysis of bronchoalveolar lavage fluid from the calf treated with aerosolized S. aureus, collected at 48 hours following aerosolization yielded 85% macrophages, 9% neutrophils and 6% lymphocytes. Gross examination of lung tissue from the *S. aureus*-treated calf identified no abnormalities. Histological examination identified rare numbers of neutrophils in bronchioles and alveoli.



Supplemental Figure S2. 8: Clinical parameters (rectal temperature, respiratory rate and heart rate) from 2 calves in pilot experiment 1 treated with aerosolized *Staphylococcus aureus* and saline.

3 Development of an aerosolized *Mannheimia haemolytica* pneumonia model in calves

Selected data and text from this chapter are included in the manuscript:

Bassel LL, Kaufman EI, Alsop SNA, Stinson KJ, Hewson J, Sharif S, et al. Development of an aerosolized Mannheimia haemolytica experimental pneumonia model in clean-catch colostrum-deprived calves. Veterinary Microbiology [Internet]. 2019; 234:34–43. http://www.sciencedirect.com/science/article/pii/S0378113519302524

3.1 Abstract

Mannheimia haemolytica is an important cause of bovine respiratory disease (BRD). It is usually a multifactorial disease with host factors and viral infections influencing pathogenesis. Previous studies that have attempted to experimentally produce pneumonia using aerosolized *M. haemolytica* alone have produced inconsistent results, yet an aerosol model would be useful to study the details of early infection and to investigate the role of innate defenses in pathogenesis. The objective of these studies was to develop and characterize an aerosolized *M. haemolytica* disease model. In early pilot studies, conventionally raised calves with higher levels of blood antibodies against *M. haemolytica* leukotoxin developed acute respiratory distress and diffuse alveolar damage but did not develop bronchopneumonia following challenge with *M. haemolytica* serotype 1. Clean-catch colostrum-deprived calves that had low levels of blood antibodies against *M. haemolytica* leukotoxin and were challenged with *M. haemolytica* serotype 2 did not develop clinical illness or postmortem lesions of pneumonia, and even failed to produce consistent nasal colonization. However, clean-catch colostrum-deprived calves challenged with 1 x 10^{10} colony forming units of *M. haemolytica* serotype 1 consistently developed pneumonia, with elevations in rectal temperature, serum haptoglobin, plasma fibrinogen, blood neutrophils, and reductions in blood lymphocytes. *Mannheimia haemolytica* was consistently isolated from the nasal

cavities and lungs of challenged calves. Gross and histologic lesions included neutrophilic bronchopneumonia and fibrinous pleuritis, with oat cells (necrotic neutrophils with streaming nuclei), and areas of coagulative necrosis, which are similar to lesions found in naturally occurring BRD. Thus, challenge with *M. haemolytica* serotype 1 of and clean-catch colostrum-deprived calves with low or absent antibody titres allowed development of an effective experimental challenge model that induced typical clinical disease and lesions.

3.2 Introduction

Bovine respiratory disease (BRD) is the most costly disease affecting the North American beef industry causing significant economic losses due to morbidity, mortality and decreased production and meat quality (Griffin, 1997). A number of bacterial and viral pathogens contribute to its development, but of these *Mannheimia haemolytica* is the most important bacterial agent in acute pneumonia of feedlot calves (Griffin et al., 2010). This bacterium can be frequently isolated from the nasopharynx of healthy cattle, although it has been reported to be more prevalent in those with respiratory disease (Allen 1991; Timsit 2017). It is therefore considered an opportunistic pathogen, associated with disease under circumstances when host defenses have been altered such as following stressors or viral infection (Caswell 2014).

Experimental models of *M. haemolytica* pneumonia include: i) prior viral infection such as bovine herpesvirus 1 (BHV-1) followed by aerosolized *M. haemolytica* challenge (Jericho et al, 1978, Stockdale et al. 1979), and ii) inoculation of *M. haemolytica* directly into the trachea or bronchi (Hodgins and Shewen, 2000; J. Hewson et al., 2011; Amrine et al., 2014; Gershwin et al., 2015). However, these

experimental models are not well-suited to investigating the role of pulmonary innate defenses. Intra-tracheal or intra-bronchial fluid inoculation is expected to bypass and dilute many of the innate immune defenses that are important in preventing the initial establishment of infection, and it is not the natural mode by which bacteria enter the lungs. Infection with viruses such as BHV-1 alters innate immune responses, either by damaging or altering the function of epithelial cells or leukocytes, or by induction of inflammatory responses (Levings and Roth, 2013). Although pre-infection with virus might model the events in some cases of naturally occurring BRD, viral infection is not a necessary requirement for the development of BRD. For example, animals treated for BRD did not differ from untreated animals with respect to detection of viruses in nasal swabs or seroconversion to viral pathogens (Martin and Bohac, 1986; Martin et al., 1999; Mitra et al., 2016). Furthermore, although BHV-1 is used in an *M. haemolytica* model, it might not be a common predisposing infection in natural disease. In Ontario feedlots during the first 28 days after arrival, seroconversion to BHV-1 was infrequent (less than 5%) and was not associated with BRD, suggesting that BHV-1 is not a significant predisposing risk factor for BRD in this high-risk period (Martin and Bohac, 1986; Martin et al., 1989, 1999).

Existing models of *M. haemolytica* pneumonia either bypass upper respiratory tract defenses or use pre-infection with respiratory viruses. Therefore, they do not allow investigation of early innate immune responses of the respiratory tract to a pure *M. haemolytica* infection and they are problematic when the research objective is to understand the early stages of bacterial colonization of the lungs, or the effect of induced innate immune responses to prevent disease caused by *M. haemolytica*. Thus,

our goal was to develop an experimental disease model in calves using aerosolized *M. haemolytica* without prior viral infection. Such a model would be relevant to natural disease because *M. haemolytica* has been demonstrated in exhaled air from infected calves (Jericho 1986), and thus aerosolization of droplets containing bacteria may be a biologically plausible route of infection.

Published experiments using aerosolized *M. haemolytica* alone, summarized in (Supplemental Table S3. 1: Published studies of aerosol *Mannheimia haemolytica* challenge experiments in calves.), have not consistently produced respiratory disease (Jericho and Langford, 1978; Yates et al., 1983; Filion et al., 1984; Bielefeldt Ohmann and Babiuk, 1985; Jericho, 1989). Only one of these challenge studies created post-mortem lesions of pneumonia, and only in 2 of 3 challenged calves (Jericho, 1989). Here we present and characterize a novel model for *M. haemolytica* pneumonia in clean-catch colostrum-deprived calves using aerosol bacterial challenge. The clinical and pathological findings were similar to those observed during natural disease. In addition, this chapter summarizes early pilot studies that were unsuccessful at producing respiratory disease. In these pilot experiments *M. haemolytica* serotype 2 was delivered to clean-catch colostrum-deprived calves and *M. haemolytica* serotype 1 was administered to conventionally raised calves with positive antibody titres.

3.3 Materials and Methods

3.3.1 Animals

The use of animals in this study was approved by the Animal Care Committee of the University of Guelph (#3286) in accordance with the Canadian Council on Animal Care.

Sequential studies were conducted to develop a disease model using aerosolized *M. haemolytica* in calves. The first pilot experiment (i.e. *Mannheimia haemolytica* serotype 1 challenge in conventional calves) used a single pair of 3-month old conventionally raised male Holstein calves, obtained from the Elora Dairy Research Centre, University of Guelph. These calves were transported to the Isolation Unit of the Central Animal Facility, University of Guelph and were housed together in a pen, allowing 10 days of acclimatization prior to pathogen challenge.

Subsequent studies used clean-catch colostrum-deprived calves that were obtained and housed as previously described (Hodgins and Shewen, 2000). Briefly, male Holstein calves were obtained at birth from the Elora Dairy Research Centre, University of Guelph, and were delivered by members of the research team onto single-use plastic sheets and immediately removed from the calving pen. The muzzle and umbilicus were disinfected with 7% tincture of iodine. Calves received 2 mg/kg of ceftiofur (Excenel® RTU, Zoetis, Kirkland, Quebec) subcutaneously and, once able to lie in sternal recumbency, were transported to the University of Guelph Central Animal Facility Isolation Unit in a clean plastic crate. Only healthy vigorous calves free of congenital anomalies were accepted.

Calves were housed indoors, in separate rooms in groups of 2-3 similarly aged animals until the time of challenge. Lab-grade shavings were used as bedding and all personnel entering the room followed a biosecurity protocol. On arrival, calves received 1 mL/45 kg vitamin E and selenium (Dystosel, Zoetis, Kirkland, Quebec). In the first 48 hours of life, calves were fed 2 L of commercially available evaporated milk (Carnation Evaporated Milk, Markham, Ontario) mixed 50:50 with water, three times daily.

Colostrum and milk replacer were not offered until 48 hours after birth to ensure that calves did not absorb antibody to *M. haemolytica*. From 48 hours, calves were bottle-fed milk replacer (Grober High Conversion 20/15, Grober Nutrition, Cambridge, Ontario) three times daily until they were able to transition to a nipple bucket feeder. At one week of age, unmedicated calf starter pellets (Sharpe Farm Supplies, Guelph, Ontario) and hay were offered ad libitum. The calves received 1 mg/kg ceftiofur hydrochloride (Excenel RTU, Zoetis, Kirkland, Quebec) subcutaneously twice daily for 7 days. From birth to the start of the experiments, all calves were assessed three times daily at feeding, including measurements of rectal temperature, assessment of stool consistency and evaluation of demeanor and appetite (Appendix 1). During the first 2 weeks after birth, joints and umbilici were palpated once daily.

3.3.2 Preparation and characterization of Mannheimia haemolytica

Mannheimia haemolytica isolates (B158 and B159) were kindly provided by the Animal Health Laboratory (AHL), Guelph, Ontario and stored at -80°C. For each isolate, a one-time multiplex polymerase chain reaction (PCR) was performed to determine serotype (Klima et al., 2017). Briefly, DNA was isolated by scraping a single colony of *M. haemolytica* into 40 µL TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.00) in individual PCR tubes and incubating at 98°C for 5 min. Samples were centrifuged at 13000 x *g* for 5 min and the supernatant was retained as template. A Qiagen multiplex PCR kit (Qiagen Inc., Toronto, ON), was used to prepare 50 µL of reaction mixture as directed by the manufacturer with 2 µL of template used for each reaction. A no-template negative control was included. The following cycle conditions were used: denaturation for 15 minutes at 95°C, 35 cycles of 94°C for 30 seconds, 55°C for 45

seconds and elongation at 72°C for 1 minute, and a final 10 min elongation at 72°C. The PCR products were analyzed by gel electrophoresis using 5 µL of SYBR Safe gel dye (Thermo Fisher Scientific, Burlington, Ontario, Canada) in a 1% agarose gel in Tris-borate-ethylenediamine tetra-acetic acid buffer with a 100 base pair DNA ladder (DNA Ladderer RTU, GeneDireX). Bands were excised from the gel and submitted to Laboratory Services, University of Guelph for sequencing (Supplemental Table S3. 2).

Frozen bacterial stock was inoculated onto a Columbia agar with 5% sheep blood plate (Oxoid Canada, Nepean, Ontario, Canada) and incubated overnight at 37°C. A single colony was inoculated in 10 mL brain-heart infusion broth (BHIB; Bacto Brain Heart Infusion, Sparks, MD) and incubated overnight on a rotary shaker at 37°C. On the morning of challenge, 500 µL of overnight culture was placed in 150 mL of BHIB and incubated until log-phase growth, with an OD_{600} of 0.5 (approximately 1 x 10^{10} CFU/mL) was reached (Supplemental Figure S3. 2).

Optical density $(OD)_{600}$ was measured (Genesys 10S VIS spectrophotometer, Thermo Scientific, Waltham, MA, USA) to estimate the number of colony forming units (CFU)/mL using a previously obtained growth curve and to confirm the logarithmic phase of growth had been reached (Supplemental Figure S3. 2).

A calculated volume of log-phase *M. haemolytica* at OD600 0.5, based on inoculation dose, was centrifuged for 20 minutes at 1500 x g at 4°C for 20 min, and the pellet was re-suspended in 10 mL of cold phosphate-buffered saline (PBS) and kept on ice until the time of challenge (within 1 hour).

Prior to challenge, the purity of the *M. haemolytica* inoculum was confirmed by microscopic examination of a Gram-stained smear (Supplemental Figure S3. 3). For

each experiment, the concentration and purity of the inoculum were determined by plating serial dilutions on blood agar and incubating the dilutions overnight at 37°C. The final intended challenge doses for the pilot studies were as follows: conventionally-raised calves received 2 X 10^{10} and 1 X 10^{11} CFU, clean-catch colostrum-deprived calves challenged with *M. haemolytica* serotype 2 received 1 x 10^{7} to 1 x10^{9} CFU, and clean-catch colostrum-deprived calves challenged with *M. haemolytica* serotype 1 received 2 X 10^{9} CFU to 1 X 10^{10} CFU. For each challenge, representative colonies were analyzed by matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) to confirm the identity and genotype of the *M. haemolytica* (AHL, University of Guelph)(Loy and Clawson, 2017). The data presented are from 6 separate challenge studies; calves C2 and C6 were challenged in the same study, as were C5 and C6 (Table 2.1).

3.3.3 Delivery of aerosols

A compressor (Precision Medical, Northampton, PA) delivered approximately 25 psi of air pressure through a Whisper Jet nebulizer (Marquest, Englewood, Colo), to a large canine anesthesia mask with an added one-way exhalation valve that was placed over the calves' muzzles to deliver aerosolized *M. haemolytica*. The anatomic distribution of aerosols was determined in one calf by nebulization of a mixture of 5 mL of green tissue dye (Davidson Marking System, Bradley Products Inc., Minneapolis, MN.) and Higgins Black Magic waterproof ink (Chartpak Inc., Leeds, MA) diluted in 15 mL PBS immediately prior to euthanasia. The anatomic distribution of the nebulized dye was evaluated by gross and histologic evaluation of the respiratory tract from the nares to the lungs.

3.3.4 Clinical data and sampling

Immediately prior to challenge, baseline clinical parameters including temperature, heart rate, respiration rate, demeanor and appetite were recorded. Calves were visually assessed multiple times daily, as indicated by the severity of disease following pathogen challenge. Full evaluation and scoring were performed in a blinded manner by the same investigator twice daily. In addition to the parameters described above, the presence or absence of coughing and nasal discharge was also recorded. Calves were scored according to a system designed to identify respiratory disease on-farm in pre-weaned calves (McGuirk and Peek, 2014).

Once-daily blood samples were collected from the jugular vein for measurement of complete blood counts and serum haptoglobin and plasma fibrinogen concentrations (AHL, University of Guelph). Antibodies to *M. haemolytica* leukotoxin were measured (Prairie Diagnostic Laboratory, Saskatoon, Saskatchewan) in pre-challenge serum as previously described (Harland et al., 1992). ELISA values were expressed as the reciprocal of the final dilution, giving a positive value compared to background. Right and left nasal swabs at a depth of 7 cm were collected at baseline and once daily thereafter, stored in transport medium, and aerobic bacterial cultures were routinely prepared (AHL, University of Guelph). In pilot studies, serum was collected from 2 calves at 72 hours, 24 hours following the introduction of milk replacer, to confirm gut closure and a lack of passive transfer. These samples were submitted to the AHL for serum electrophoresis to evaluate globulin concentrations (Supplemental Figure S3. 4).

Baseline and subsequent daily systematic thoracic ultrasound of clean-catch colostrum-deprived calves was performed using a portable linear rectal ultrasound unit (Ibex Pro, E. I. Medical, Loveland, CO), with a frequency of 6.2 MHz, depth set to 6 cm,

and an 18 dB gain (near 31 dB, far 35 dB) as previously described (Ollivett et al., 2015). Isopropyl alcohol (70%) was applied to the unshaved skin, and the lungs were visualized by moving the transducer dorsal to ventral, beginning at the 10th intercostal space and proceeding cranially, to examine each intercostal space. Any identified lesions including lung consolidation (hypoechoic regions within the parenchyma, with a loss of the normally observed parallel horizontal lines (known as A-lines, or reverberation artifacts) and pleural fluid were recorded, along with location (including intrathoracic space and vertical position (dorsal, mid, and ventral) and approximate size (Supplemental Figure S3. 5 and Supplemental Figure S3. 6).

3.3.5 Euthanasia and post-mortem evaluation and sampling

Scheduled euthanasia occurred 3 days after *M. haemolytica* inoculation via intravenous injection of 102 mg/kg pentobarbital sodium solution (Euthansol®, Merck, Kirkland Quebec). Calves were euthanized prior to the planned study endpoint if they were in respiratory distress or were unwilling to rise or eat.

Gross postmortem examination was performed within one hour of euthanasia, and lesions were evaluated in a blinded manner. Several measures of the severity of pneumonia were determined. First, the ratio of lung-to-heart weight was determined by separating the heart from the lungs, removing the blood and major vessels from the heart, removing the aorta, esophagus, vena cava and trachea cranial to the bifurcation from the lungs, and weighing the heart and lungs separately. Second, the lungs were examined visually and by palpation, and areas were considered to be pneumonic if they were discoloured and firmer than normal and if the red-purple discoloration was sharply demarcated from normal lung tissue and typically in an angular or lobular pattern. The portion of each lung lobe that was diseased was recorded on a schematic map of the

lungs (Appendix 3) and the proportion of diseased lobe was multiplied by the relative size of the lobe to calculate the total proportion of lung tissue that was diseased (Jericho and Kozub, 2004). Third, digital photographs of the lateral surface of the left and right lungs were obtained after they were removed from the thoracic cavity. Identifiers were removed, and the images were assigned random numbers for blinded analysis. Image analysis was used (with knowledge of the notes taken at postmortem examination) to calculate the extent of lesions in the left and right lungs of each calf (ImageJ software, Schneider et al., 2012). Each focus of consolidated lung was traced, and their areas were added together to estimate the total affected surface area, which was then divided by the total surface area of the lungs in the image.

Fresh and formalin-fixed samples of the left and right cranioventral, caudodorsal and caudoventral lung lobes and fresh samples of accessory lung lobes were collected in a standardized manner, focusing on lesional lung tissue if it was present. The right kidney was also sampled. All fresh samples were kept on ice and then frozen until aerobic bacterial culture (AHL, University of Guelph). The formalin-fixed tissues were routinely processed, and histologic sections were stained with hematoxylin and eosin. Histologic sections were scored for the presence or absence of bronchopneumonia (neutrophils in alveoli and bronchioles), oat cells (necrotic neutrophils with streaming chromatin), coagulation necrosis, pleuritis (fibrin and neutrophils on the pleural surface), alveolar damage (hemorrhage or fibrin in alveoli) and thrombi (fibrin within venules).

3.3.6 Statistical Analysis

Two-way repeated measures analysis of variance (ANOVA) was conducted using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA) and Dunnett's test was used to compare pre-challenge to post-

challenge parameters measured every 12 hours (temperature, heart rate, respiratory rate, plasma fibrinogen, serum haptoglobin, clinical scores). Linear regression was used to calculate an R-squared value correlating various measures of postmortem lung lesion severity including visual analysis of the proportion of affected lung, image analysis of proportion of affected lung, and lungs to heart weight ratio.

3.4 Results

3.4.1 Characterization of Mannheimia haemolytica challenge strains

The *M. haemolytica* strains used in these studies were identified by multiplex PCR as serotype 1 (B158) and serotype 2 (B159) (Supplemental Table S3. 2). The genotype of the isolates determined by MALDI-TOF MS were genotype 2 (B158) and genotype 1 (B159). The amplification product of B158 was sequenced and results were consistent with that expected for *M. haemolytica*.

3.4.2 Anatomic distribution of aerosols

The experimental apparatus was used to deliver aerosolized dye to one calf. The dye was detected throughout the respiratory tract including on the epithelium of the nares, nasal conchae, nasopharynx, larynx, trachea, bronchi, and bronchioles. Grossly, the majority was concentrated in the nasal passages, with some accumulation at the larynx, minimal deposition in the majority of the trachea, and increased deposition at airway branching points and in the smaller airways (Figure 3.1).

Histologic examination demonstrated similar patterns in the larger airways but also highlighted widespread deposition of dye in the bronchioles with rare deposition in alveoli (Figure 3.2). The amount of dye deposited in the bronchioles was similar across the examined cranioventral, caudodorsal and caudoventral lung sections. Throughout

each experiment, all calves tolerated restraint in the head gate and delivery of aerosols through the mask with no signs of discomfort or distress.

3.4.3 Pilot Experiment 1: Aerosolized Mannheimia haemolytica serotype 1 challenge in conventional calves

Two conventionally raised calves with similar positive anti-leukotoxin antibody titres of 19800 (Calf A1) and 13700 (Calf A2) were challenged by aerosol with 2×10^{10} CFU (Calf A1, lower dose) and 1×10^{11} CFU (Calf A2, higher dose) of *M. haemolytica* serotype 1. Within 1 hour of administration of the challenge dose, both calves displayed similar clinical signs, with increased rectal temperature, depression, increased respiratory rate and effort. One hour following challenge, both calves had increased respiratory effort. By 2 hours, both were moderately depressed, continued to have increased respiratory rate and effort, and were reluctant to rise when researchers entered the pen. At 6 hours, the calf that received the higher dose of *M. haemolytica* became severely dyspneic, refused to rise when researchers entered the pen, and was euthanized. The second calf's demeanor and respiratory effort improved between 6 and 12 hours after challenge and remained normal from this point until euthanasia 3 days post challenge. Clinical findings are summarized in Supplemental Figure S3. 7.

On gross postmortem examination of the calf that received the higher dose of *M. haemolytica* (euthanized 6 hours after challenge), the lungs were diffusely wet and heavy, with marked interlobular and subpleural edema and emphysema (Figure 3.3). Histologically, the lungs had diffuse alveolar damage with hyaline membranes lining alveoli, a large amount of proteinaceous fluid and variable fibrin strands, neutrophils and hemorrhage filling alveoli, and occasional intravascular thrombi Figure 3.4). In contrast, the calf that received the lower dose (euthanized at 3 days after challenge) had grossly

normal lungs (Supplemental Figure S3.9). Histologically, the lungs had occasional areas of atelectasis with small numbers of neutrophils and rare bronchioles filled with neutrophils (Supplemental Figure S3.9). *Mannheimia haemolytica* was isolated from the cranioventral and dorsocaudal areas of the lungs of both calves.

3.4.4 Aerosolized Mannheimia haemolytica serotype 2 challenge in clean-catch colostrum-deprived calves

A second pilot study was conducted using 2 clean-catch colostrum-deprived calves with anti-leukotoxin antibody titres of 3500 and 600 that received respectively aerosolized *M. haemolytica* serotype 2 at doses of 1×10^8 and 1×10^9 CFU. Neither of these calves exhibited clinical respiratory illness. In the calf that received 1×10^8 CFU *M. haemolytica*, there was a mild increase in the serum haptoglobin concentration over baseline on days 2 and 3 following challenge but these were still within the reference range (Supplemental Figure S3. 10A). In the calf that received 109 CFU of *M. haemolytica*, the plasma fibrinogen concentration was slightly increased over baseline on day 3 (Supplemental Figure S3. 10B).

At post mortem examination, the calf that received 1×10^8 *M. haemolytica* serotype 2 had rare areas of red mottling of the surface of the lungs (Supplemental Figure S3. 11A). The calf receiving 1×10^9 *M. haemolytica* had an area of dark red consolidation on the ventral aspect of the right middle lung lobe (Supplemental Figure S3. 11B). Bacterial culture of this tissue revealed high number of *Enterococcus* species and *Streptococcus equinus*, but *M. haemolytica* was not isolated from any of the samples. Histologically, lungs from the calf that received 1×10^8 *M. haemolytica* had occasional areas of atelectasis and neutrophils within bronchioles and alveoli, but most

of the examined sections were normal. The calf that received 1 x 10^9 CFU had a single small focus of neutrophilic bronchopneumonia (Supplemental Figure S3. 12).

3.4.5 Aerosolized Mannheimia haemolytica serotype 1 challenge in clean-catch colostrum-deprived calves

Eight clean-catch colostrum-deprived calves (with anti-leukotoxin antibodies ranging from 100-2225) were challenged by aerosol with *M. haemolytica* serotype 1. The target dose of 1 x 10^{10} CFU, based on OD600; was subsequently confirmed using colony counts to range from 2 x10^9 to 8.9 x 10^{10} CFU) (Supplemental Table S3. 4 for CFU counts for challenge doses). All calves developed elevated rectal temperature by 12 hours after inoculation, and this persisted until euthanasia 3-4 days later (P<0.001) (

Figure **3.5** &

Figure **3.6**). The mean respiratory rate was not affected by *M. haemolytica* challenge (p=0.354) (

Figure **3.5** &

Figure **3.6**). Heart rate increased after challenge, but this was only significant at 36 hours following inoculation (P<0.05). Clinical signs are summarized in Table 3.1. The most frequently identified clinical signs were (in order of decreasing frequency) cough, increased respiratory effort, depression and reduced appetite. Infrequently identified parameters (observed in 1 or 2 calves) included nasal discharge, ear flicking or droop, and ocular discharge. Three of the 8 calves developed severe clinical signs and were euthanized two days after challenge, prior to the study endpoint.

Blood indicators of inflammation increased on the first day following challenge with *M. haemolytica* serotype 1. Serum haptoglobin and plasma fibrinogen concentrations increased at 24 hours following challenge and remained elevated until

euthanasia (*P*<0. 01) (Figure 3.7 & Figure 3.8). Total blood leukocyte counts increased at 24 hours following challenge (p <0.001) (Figure 3.9A & Figure 3.10A), primarily due to an increase in blood neutrophils (p <0.001) (Figure 3.9C). The number of blood monocytes did not increase significantly during the course of the study (p =0.176). Blood lymphocytes decreased following challenge and remained lower than baseline until euthanasia (p <0.001) (Figure 3.9D). The ratio of neutrophils: lymphocytes in blood increased following challenge and this persisted to the end of the study (data not shown).

Bilateral nasal swabs were collected daily from each calf and aerobic bacterial culture was performed. *Mannheimia haemolytica* was not isolated prior to challenge. However, all post-challenge swabs contained large numbers of *M. haemolytica* (reported as 3+ to 4+).

Thoracic ultrasound evaluation of calves prior to challenge demonstrated occasional comet tails but there was no evidence of lung consolidation (Figure 3.11). Evidence of consolidation—hypoechoic regions lacking the white pleural band and reverberation lines—appeared one day following challenge in 7/8 calves and at 2 days following challenge in 1/8 calves. Once identified, lesions persisted, and the amount of lung affected increased over time until the calves were euthanized. One calf had pleural fluid detected by ultrasound examination on day 2 after challenge and was euthanized due to the severity of the clinical signs.

At postmortem evaluation, all calves had some degree of consolidation of the cranioventral lung. The proportion of affected lung ranged from 1% to 55% (mean 20%; 95% confidence interval 3-36%). The consolidation primarily affected the cranial and

right middle lung lobes (Table 3.2). Most frequently, the lesions appeared as angular (lobular) areas of red-purple discoloration that were neither raised nor collapsed. When these coalesced, they formed larger lesions that were palpably firmer than normal. Less frequently, lesions affected most or all of a lobe. On cut section, the affected lung was homogeneously red-purple or contained irregular foci delineated by a white line (coagulation necrosis). Three calves had prominent pleuritis, with sheets of fibrin covering a large portion of the lung surface, while 3/8 calves had focal areas of pleuritis measuring less than 2 cm, which appeared as pleural opacity with a ground-glass appearance and a rough texture (Figure 3.12). All measures of the severity of post mortem lung disease were significantly correlated with one another ($P < 0.05$) including visual estimates of the proportion of diseased lung (including and excluding the accessory lobe), software-based image analysis, and lung: heart weight ratios (Figures 3.13 and 3.14A-F and Supplemental Table 3.6).

Histological examination of lung tissue from all animals with consolidation confirmed the presence of acute bronchopneumonia (Table 3.3). These areas often contained irregularly shaped foci of coagulation necrosis and large numbers of degenerate leukocytes with streaming nuclei (oat cells) were frequently present within alveoli and bronchioles, particularly at the periphery of the foci of necrosis (Figure 3.15). In other areas, the alveoli and bronchioles contained non-necrotic neutrophils and mononuclear cells, and some also had fibrin and hemorrhage with occasional intravascular thrombi or pleuritis. Cranioventral lung sections tended to have more severe histologic lesions than dorsocaudal lung sections.

Large number of *M. haemolytica* were isolated from one or more samples from lungs of all calves (8/8), and pleural fluid when present (2/2) (Table 3.3). In 1 out of 8 calves, *M. haemolytica* was isolated from kidney, indicating bacteremia. *Mannheimia haemolytica* was isolated from 15/16 cranial lung lobes and 12/16 caudal lung lobes. Seven of the lung lobes had no observed gross lesions, and of these, *M. haemolytica* was isolated from 2 samples. Conversely, of the 25 lung samples with observable gross lesions, we failed to culture *M. haemolytica* from 3 samples.

3.5 Discussion

This study describes the development of a novel experimental model in which clean-catch colostrum-deprived calves were challenged by aerosol with a virulent strain of *M. haemolytica* without prior viral challenge. Inhalation of *M. haemolytica* from aerosolized particles from the nasopharynx represents the most plausible natural route of infection and prior research has demonstrated the presence of *M. haemolytica* in exhaled and tracheal air from infected cattle (Jericho et al., 1986).This aerosol challenge consistently resulted in *M. haemolytica* nasal and lung infection, with clinical and pathologic changes that were comparable to the natural disease (Rehmtulla and Thomson, 1981). We expect this model will be useful to evaluate how modulating innate immune responses affects the development of *M. haemolytica* pneumonia.

The use of clean-catch colostrum-deprived calves resulted in uniformly low anti-leukotoxin antibody titres. These values were considerably lower than those measured in young (3 week to 7 month) calves and in on-arrival feedlot calves prior to vaccination (Harland et al., 1992; Van Donkersgoed et al., 1993, 1994). Disinfection of the nares and the prevention of contact with adult cattle prior to removal from the farm of origin

prevented colonization of the upper respiratory tract by *M. haemolytica*, confirmed by bacterial culture of paired nasal swabs.

The dose of *M. haemolytica* in this study was higher than reported in studies that inoculated a bacterial suspension directly into the trachea or bronchus (Hewson et al., 2011; Gershwin et al., 2015). However, it is likely that only a small percentage of the nebulized *M. haemolytica* reached the lungs. One study estimated only a maximum of 0.42% of *M. haemolytica*-containing aerosols reached the trachea (Jericho et al 1986). In several previous studies, lower challenge doses of aerosolized *M. haemolytica* failed to produce disease (Jericho and Langford, 1978; Yates et al., 1983; Bielefeldt Ohmann and Babiuk, 1985). However, in one report, the challenge dose was identical but failed to produce disease (Filion et al. 1984). In another study, calves received 1×10^8 CFU of *M. haemolytica* by intrabronchial aerosolization; 3 days later, *M. haemolytica* was isolated from bronchoalveolar lavage fluid from only 1 of 8 calves (McBride et al., 1999). There are several potential differences from the current study, including the experimental apparatus used to produce and deliver the aerosols (which might affect the number of bacteria reaching the lungs), the use of calves with low antibody titres to *M. haemolytica*, and the use of fresh log-phase *M. haemolytica* serotype 1 bacteria (culture methods were not reported in the referenced study) (Filion et al. 1984). In addition to using calves with low antibody titres to *M. haemolytica*, the use of bacteria at the log-phase of growth may be critical because the important virulence factor leukotoxin is produced during this phase (Baluyut et al., 1981; Gibbs et al., 1984; Shewen and Wilkie, 1985). It is noteworthy that in the one study where aerosolization of *M. haemolytica* produced pneumonia, the challenged calves did not have detectable *M.*

haemolytica antibody in serum and that fresh log-phase bacteria were used for challenge, providing further evidence for the importance of these methods (Jericho, 1989).

One drawback to this model is the variability in the severity of the lung lesions with visual estimates of the proportion of affected lung ranging from 1.7 to 54.7%. However, this variability has been reported in other models as well. In the study reporting pneumonia induced by aerosolized *M. haemolytica*, one of the three challenged calves did not develop any evidence of pneumonia, and in the other two calves the lesions affected only 5% of the left anterior cranial lobe in one calf and 40% of the right anterior cranial and 25% of the posterior cranial in the other calf (Jericho 1989). Similar variability was observed between calves in our study, which might have resulted from differences among individuals in innate defences, the nature of the inflammatory response, or breathing patterns at the time of bacterial challenge. Amrine et al. (2014), using an intratracheal challenge with a suspension of *M. haemolytica*, also reported a variable proportion of lung with pneumonia following challenge (range of 13-40% in non-antibiotic-treated calves).

A retrospective analysis of the combined BHV-1 and *M. haemolytica* challenge studies conducted at one institute over 15 years also identified a high variance of pneumonia within and between experiments (Jericho and Kozub, 2004). Of note, the authors reported that there was not a linear relationship between the challenge dose of *M. haemolytica* and the severity of clinical signs (Jericho and Kozub, 2004). Similarly, in our experiments, although attempts were made to standardize the challenge dose of *M. haemolytica* using OD_{600} measurements, subsequent counts of colony forming units

indicated that the actual challenge dose differed between experiments, but dose was not correlated with the severity of disease. One of the most severely affected calves (1621) received the lowest challenge dose. An experiment where lambs were challenged with *M. haemolytica* (*Pasteurella haemolytica* serotype A2, the pathogenic serotype in sheep) reported no difference in the proportion of lambs developing gross lesions of pneumonia 5 days after challenge with a 1000-fold difference in challenge dose (Gilmour et al., 1984). The main problem with the variability of the severity of disease is that high variance increases the sample size needed to demonstrate a statistically significant difference between groups.

Another drawback to this model is the significant time investment required to procure and care for calves up to the point of challenge, due to the need to be present at parturition and their susceptibility to infection in the first few weeks of life. In addition to being labour-intensive, it was not possible with our production system to assemble sufficient numbers of calves to conduct the entire challenge study at one time. Although these calves had uniform signalments, environments, diets, external stressors, and antibody levels, the variability of the manifested disease suggests that individual calf factors may greatly influence the severity of the disease that developed.

Aerosolization of dye immediately prior to euthanasia demonstrated widespread distribution throughout the lungs. However, at the time of post-mortem examination, the pneumonic lesions were primarily in the cranioventral area of the lung. In the combined BHV-1/*M. haemolytica* model, a similar pattern of pneumonia developed, with ventral distribution of bacterial pneumonia, despite generalized petechial and ecchymotic viral lesions seen throughout the lungs (Jericho and Kozub, 2004).

Mannheimia haemolytica serotype 1 is considered to be more pathogenic than serotype 2. Similarly, recent work has identified two genotypes, predicted by MALDI-TOF, with genotype 2 more commonly associated with pneumonia than genotype 1 (Clawson et al., 2016; Loy and Clawson, 2017). We used multiplex PCR to identify serotype and MALDI-TOF to identify genotype. One of our *M. haemolytica* isolates was serotype 1 and genotype 2, and the other isolate was serotype 2 and genotype 1. Similarly, we found that in *M. haemolytica* isolates from nasal swabs sequentially collected from 60 auction-sourced calves arriving to a research station feedlot, those that were identified as *M. haemolytica* genotype 2 by MALDI-TOF were identified as serotype 1 by multiplex PCR (data not shown).

In the first experiment, using conventionally raised leukotoxin antibody-positive calves that received 1 x 10^{10} or 5 x 10^{10} CFU of *M. haemolytica* serotype 1, both calves developed severe dyspnea in the first few hours following challenge that required euthanasia of one of the calves. This severe acute reaction was not seen in the clean-catch colostrum-deprived calves that received a similar dose. The pathogenesis of this reaction is not certain, but similar patterns have been reported. In an investigation of *M. haemolytica* vaccination, cattle with greater titres had more severe lung lesions following challenge (Friend et al., 1977). In a study investigating the efficacy of vaccination with capsular polysaccharide, 36% of calves developed an anaphylactoid reaction at the time of challenge (Rice et al., 1993). In another study, although antibody levels weren't reported, increased bronchoalveolar lavage fluid neutrophils were evident only after repeated exposures to intrabronchial aerosols of *M. haemolytica* (McBride et al., 1999). It is possible that in these calves with higher antibody titres and an intense

and generalized exposure to *M. haemolytica*, there was excessive promotion of inflammatory pathways such as complement activation. Clinically this resulted in severe dyspnea resembling acute respiratory distress syndrome requiring euthanasia of one of the calves. This pronounced inflammatory response to *M. haemolytica* was associated with histologic lesions of diffuse alveolar damage, which differ from the lesions in naturally occurring *M. haemolytica* bronchopneumonia. Antibody can be protective against natural or other experimental *M. haemolytica* infection, such as in studies using intrabronchial inoculation of a bacterial suspension (Hodgins and Shewen, 2000; Shewen et al., 2003), and thus we speculate that this adverse reaction may occur only when the entire lung is exposed to a large dose of aerosolized *M. haemolytica*. In the calf that received the lower dose, the dyspnea resolved within 12 hours after challenge, and by the time of postmortem evaluation there were no lesions of bronchopneumonia although *M. haemolytica* was isolated from the lung.

Multiple clinical and pathologic outcomes were evaluated in the aerosol-challenged calves. The most consistent clinical signs were cough, increased respiratory effort, depression, and reduced appetite. Nasal discharge, ear flicking or droop, and ocular discharge were only infrequently identified, and thus are not considered useful measures of disease in this experimental model. All challenged calves in our studies developed an elevation in rectal temperature early after challenge that persisted until euthanasia. Although Jericho (1989) reported temperature elevations in the challenged calves following aerosolized *M. haemolytica,* this was only seen on day 1 (1 calf) or day 2 (2 calves) following challenge up to the point of euthanasia at day 4. This may be due to a lower challenge dose or differences in the age of the calves, immune status or

other factors. Complete blood counts were informative as blood neutrophil counts increased whereas blood lymphocyte counts decreased following challenge. Similarly, serum haptoglobin and plasma fibrinogen concentrations increased following challenge. Thoracic ultrasound was useful to rule out the presence of lesions prior to challenge and to follow development of pneumonia over time, but quantification of lesions was not attempted.

Several quantitative measures of postmortem disease severity were compared. There were significant correlations between visual estimates of the proportion of lung affected, image analysis (using software) to assess the proportion of lung affected, and the ratio of lung to heart weights. The accessory lobe was not included in the photographs used for image analysis. Although this lobe was frequently affected by pneumonia, it has a relatively minor contribution to the total lung volume, so there were minimal differences in the proportion of affected lung if the accessory lobe was included or excluded. Thus, digital analysis of images of the laterodorsal surfaces of the lung are justified as a measure of disease severity. Advantages to visually estimating the proportion of lung affected are that it is quick, and results are immediately available; however, the estimated proportion of diseased lung is not accurate (compared to image analysis), and variation is expected among individuals. Analysis of postmortem lung photographs using image analysis software does not consider the depth of the lesions, lesions not visible on the dorsolateral surfaces, and requires additional time. Furthermore, colour changes in the lung frequently develop after death, so notes of postmortem findings were useful to determine whether discolored areas in the digital images were considered to be antemortem lesions. However, image analysis is more

objective and more accurate than visual estimation. A study examining the correlations between image analysis, visual analysis and lung: body weight ratios in pigs challenged with *Actinobacillus pleuropneumoniae* found similar results (Sibila et al., 2014).The percent of organ to body weight is frequently used to assess the true proportional size of organs. However, we could not measure carcass weight in the isolation facility, and, in cattle, carcass weight is highly influenced by the degree of rumen fill. Thus, heart weight may be proxy for body weight for comparison of lung weights among calves.

3.6 Conclusion

Aerosolization of *M. haemolytica* serotype 1 to clean-catch colostrum-deprived calves caused clinical signs and gross and histological lesions similar to those seen in natural disease. There is some variability in disease outcomes which influences the number of animals required to make meaningful conclusions in experimental studies. Nonetheless, this model may be useful for studying early innate immune responses, virulence factors necessary for colonization and disease, and interventions to prevent *M. haemolytica* pneumonia. In contrast, conventional calves that had higher titres of antibodies against *M. haemolytica* developed acute respiratory distress with diffuse alveolar damage, but no evidence of progressive bronchopneumonia, showing the importance of using low antibody clean-catch colostrum-deprived calves. Finally, whereas the serotype 1 isolate induced severe disease, challenging clean-catch colostrum-deprived calves with a serotype 2 isolate resulted in no clinical signs and only minimal gross and histologic lesions of pneumonia.

3.6 Reference List

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3.7 Tables

Table 3.1: Clinical signs following *Mannheimia* haemolytica challenge. The prevalence of clinical signs prior to and following aerosol challenge with 1 x 10^{10} colony forming units *Mannheimia haemolytica* serotype 1 in eight colostrum-deprived Holstein bull calves.

	Pre-challenge	0-24 hours post challenge	25-48 hours post challenge
Cough	0/8 (0%)	6/8 (75%)	5/8 (63%)
Increased respiratory effort	0/8 (0%)	5/8 (63%)	4/8 (50%)
Depression	0/8 (0%)	3/8 (38%)	5/8 (63%)
Reduction in strength	0/8 (0%)	2/8 (25%)	4/8 (50%)
Loss of appetite	0/8 (0%)	1/8 (13%)	3/8 (38%)
Nasal discharge	0/8 (0%)	1/8 (13%)	2/8 (25%)
Ear flicking or droop	0/8 (0%)	2/8 (25%)	1/8 (13%)
Ocular discharge	0/8 (0%)	0/8 (0%)	1/8 (13%)

Table 3.2: Gross lesions and isolation of *Mannheimia haemolytica* from the lung 48 to 72 hours after aerosol challenge of 8 colostrum-deprived calves.

	Left lung lobes		Right lung lobes			
	Cranial	**Caudal**	**Cranial**	**Middle**	**Accessory**	**Caudal**
Number of animals with lesions	7/8	6/8	8/8	8/8	6/8	7/8

Calf ID	**Percentage of each lung lobe with gross lesions (+/-: Isolation of *M. haemolytica*)**					
1	68% (+)	10% (+)	77% (+)	80%	50%	80% (+)
2	8% (+)	5% (+)	10% (+)	2%	2% (-)	0% (+)
3	80% (+)	10% (+)	30% (+)	25%	95% (+)	1% (+)
4	50% (+)	15% (+)	53% (+)	50%	2% (+)	5% (+)
5	0% (-)	0% (-)	3% (+)	1%	0% (-)	0% (-)
6	3% (+)	3% (+)	70% (+)	100%	5% (+)	3% (+)
7	31% (+)	0% (+)	3% (+)	10%	0% (-)	1% (-)
8	30% (+)	1% (+)	3% (+)	15%	30% (-)	1% (+)

Table 3.3: Lung histopathology following *Mannheimia haemolytica* challenge. Histologic lesions in clean-catch colostrum deprived calves 2-3 days following challenge with 1 x 10^{10} colony forming units of aerosolized *M. haemolytica*. The data indicate whether histological bronchopneumonia, oat cells, coagulative necrosis, pleuritis, fibrin in alveoli, and positive culture of *M. haemolytica* were detected in one or more foci in the cranioventral (including right middle, and left and right cranial lobes) and caudodorsal areas of lung (dorsal aspect of the caudal lung lobes).

Calf ID	Day of euthanasia relative to challenge	Broncho-pneumonia		Oat cells		Coagulative necrosis		Pleuritis		Fibrin in alveoli		*M. haemolytica* cultured from lungs	
		CV[a]	CD[b]	CV	CD	CV	CD	CV	CD	CV	CD	CV	CD
C1	2	+	+	+	+	+	-	+	-	+	+	+	+
C2	3	+	+	-	-	-	-	-	-	-	+	+	+
C3	2	+	+	-	-	-	-	+	-	-	-	+	+
C4	2	+	+	+	-	+	-	+	-	+	+	+	+
C5	3	+	-	+	-	-	-	+	+	+	-	+	+
C6	3	+	+	+	-	+	-	+	-	+	-	+	+
C7	3	+	-	+	-	+	-	+	-	+	-	+	-
C8	3	+	-	+	-	+	-	+	-	+	-	+	+
Number of calves with lesion (n=8)		8	5	6	1	5	0	7	1	6	3	8	7

[a]CV – caudoventral
[b]CD - caudodorsal

3.8 Figures

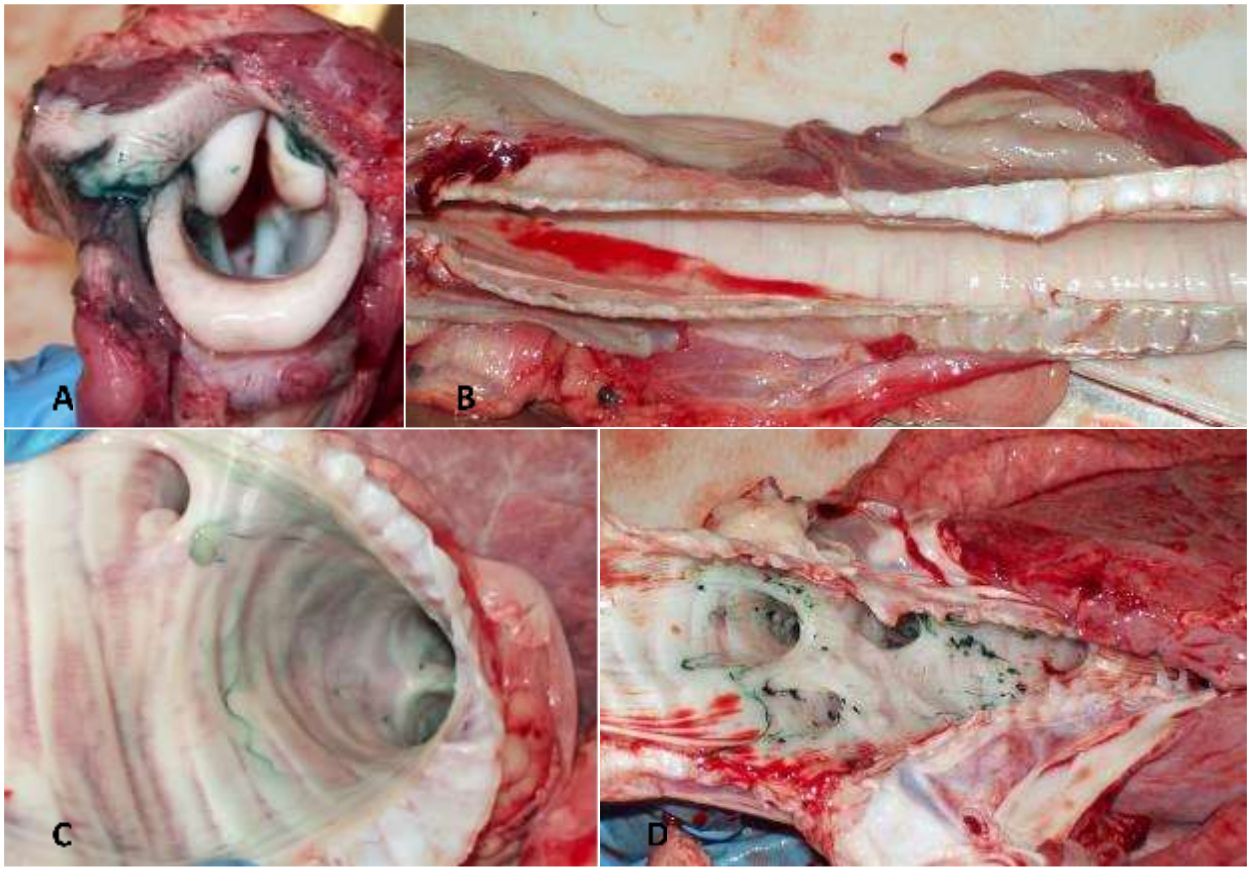


Figure 3.1: Anatomic distribution of aerosolized particles. Nebulization of green dye in Calf B4 resulted in significant deposition in the nasal passages (not shown) and larynx (A), minimal deposition in the trachea (B), increasing deposition in at airway bifurcations including carina (C), and in the tertiary and smaller bronchi (D).

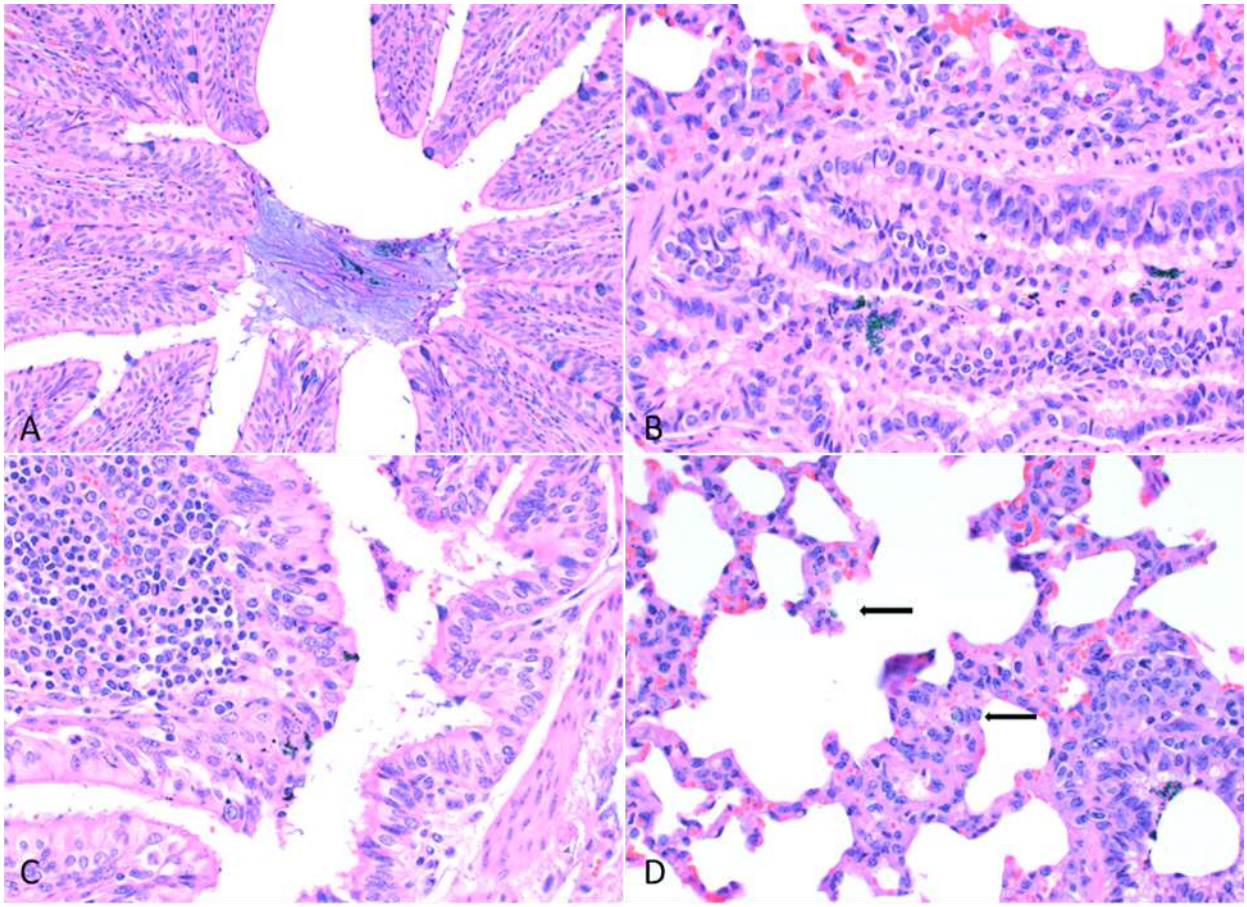


Figure 3.2: Histological distribution of aerosolized dye. In calf B4 following nebulization, the green dye is histologically evident in the bronchial mucus (A), on the apical surface of bronchiolar epithelium (B, C) and occasionally lining the alveoli (D, arrows). Hematoxylin and eosin.

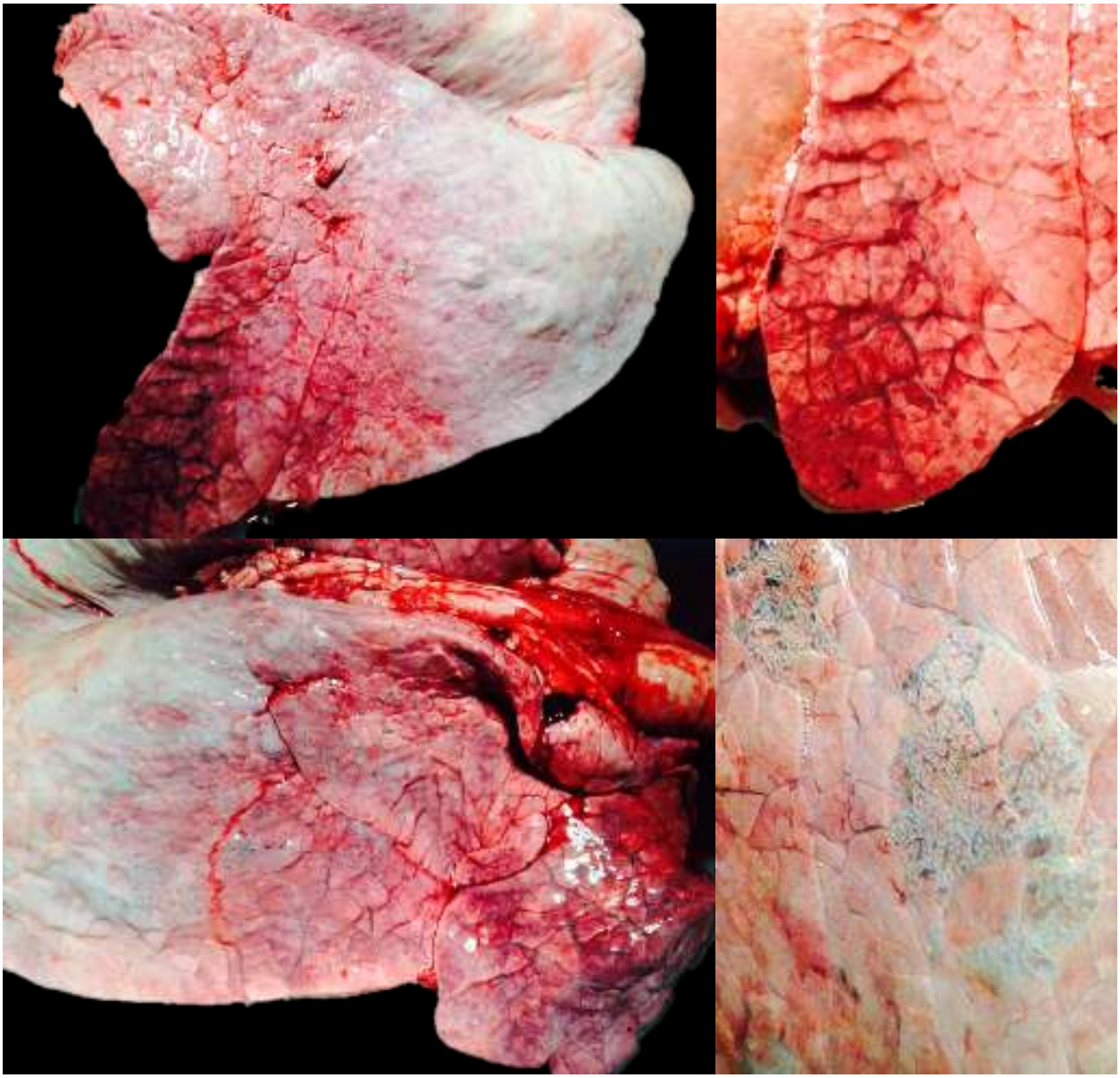


Figure 3.3: Gross lung lesions following aerosolized *Mannheimia haemolytica* challenge to a calf with positive leukotoxin antibody. Lungs of the conventionally raised calf (Calf A2; not colostrum-deprived), 6 hours after aerosol challenge with 5 x 10^{10} CFU of *M. haemolytica* type 1. Following euthanasia for severe respiratory distress, the lungs had marked interlobular and subpleural edema and emphysema, with red mottling of the cranioventral lung parenchyma.

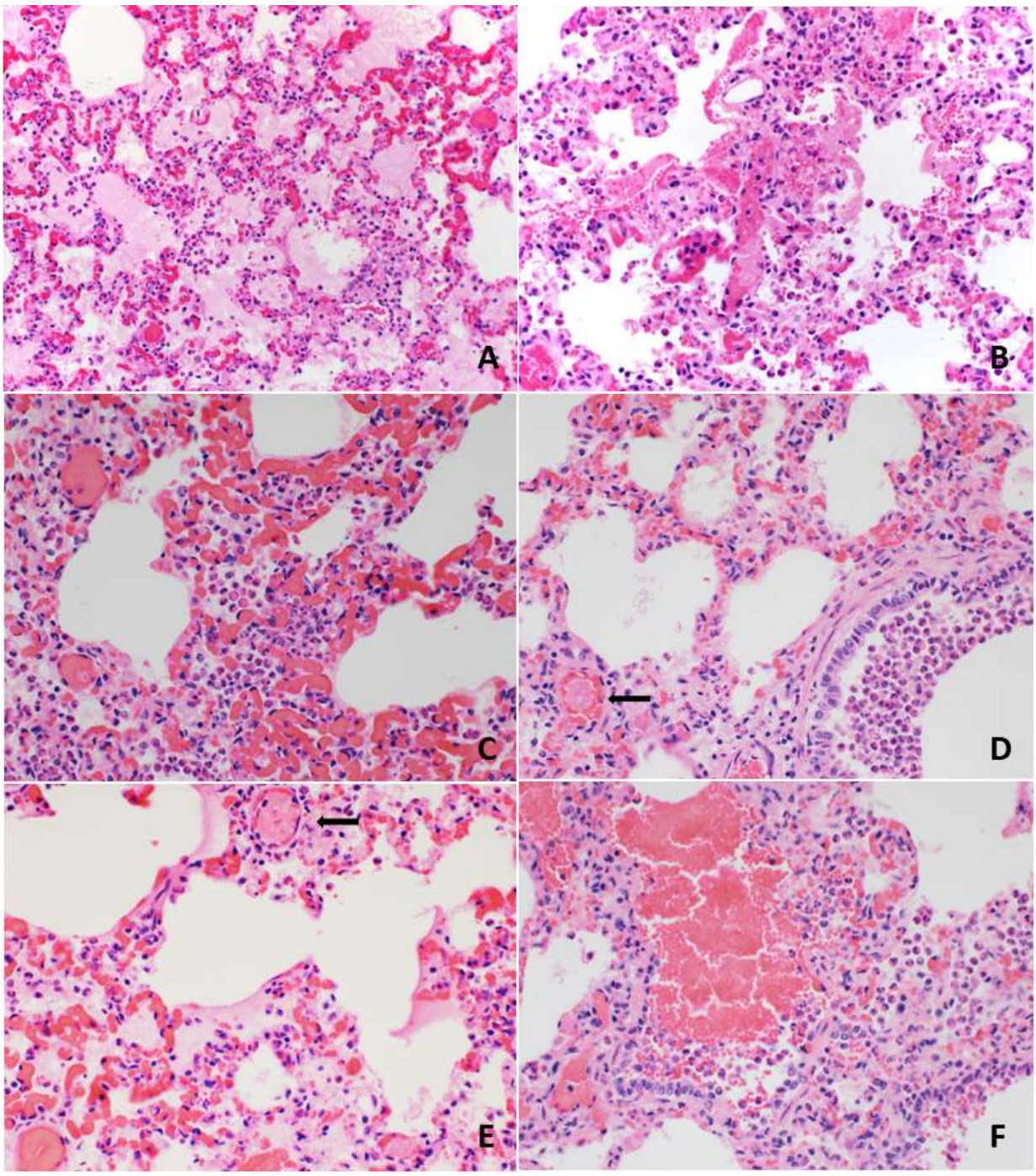


Figure 3.4: Histologic lung lesions following aerosolized *Mannheimia haemolytica* challenge to a calf with positive leukotoxin antibody. Lung tissue, conventionally raised calf (Calf A2), euthanized 6 h after challenge with 5×10^{10}CFU aerosolized *Mannheimia haemolytica* serotype 1. All examined sections had alveolar edema, fibrin and neutrophils. Lungs had eosinophilic (proteinaceous) alveolar fluid (A), alveolar fibrin and hyaline membranes (B), intact and necrotic neutrophils in alveoli (C), fibrin thrombi in blood vessels (arrows) (D, E), and alveolar and bronchiolar hemorrhage (F).

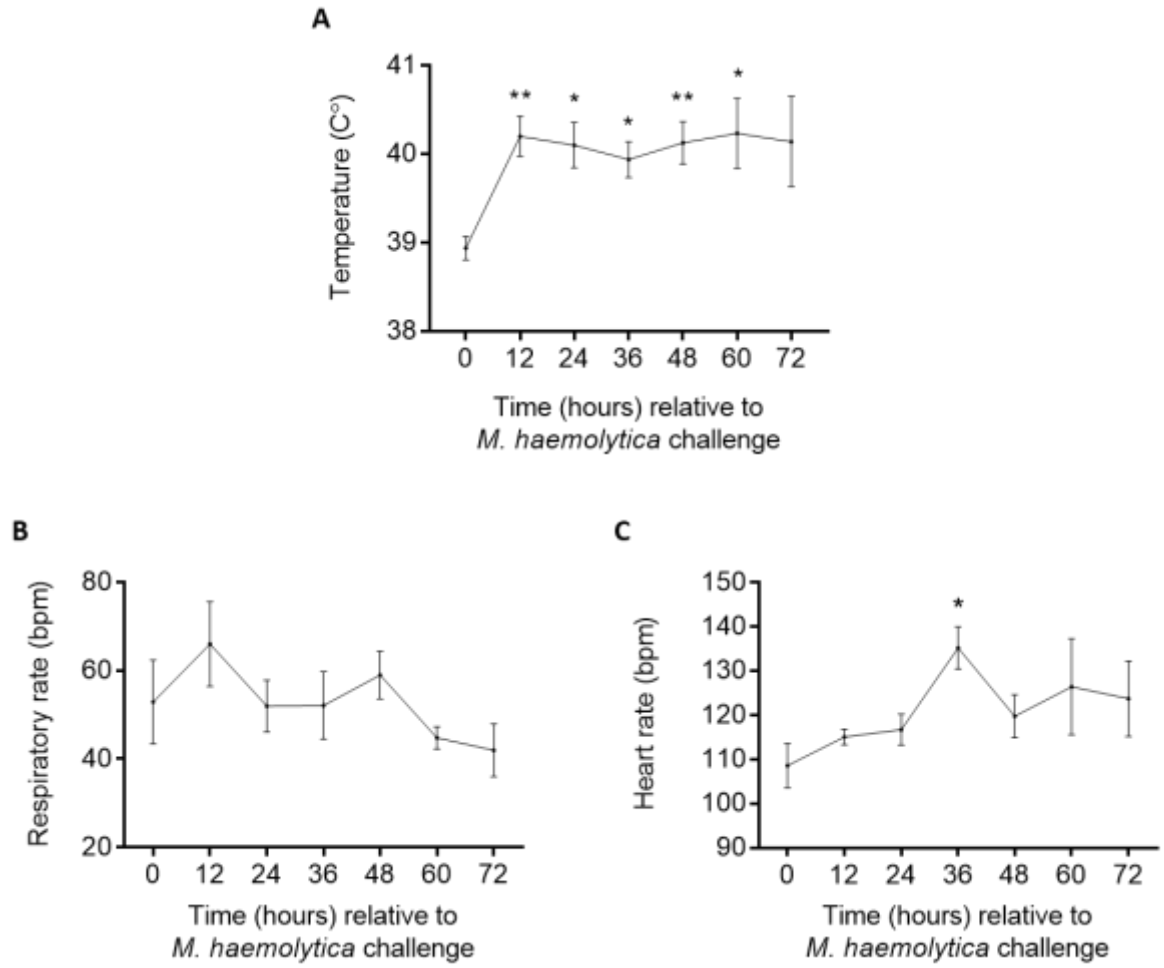


Figure 3.5: Clinical parameters over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1. The data are mean ± standard error of the mean from 8 colostrum-deprived Holstein bull calves. The data at 60 and 72 hours are from 6 and 5 surviving calves, respectively. A) Rectal temperature. B) Respiratory rate. C) Heart rate. Data were compared to pre-challenge (time=0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P<0.05$, ** $P<0.01$.

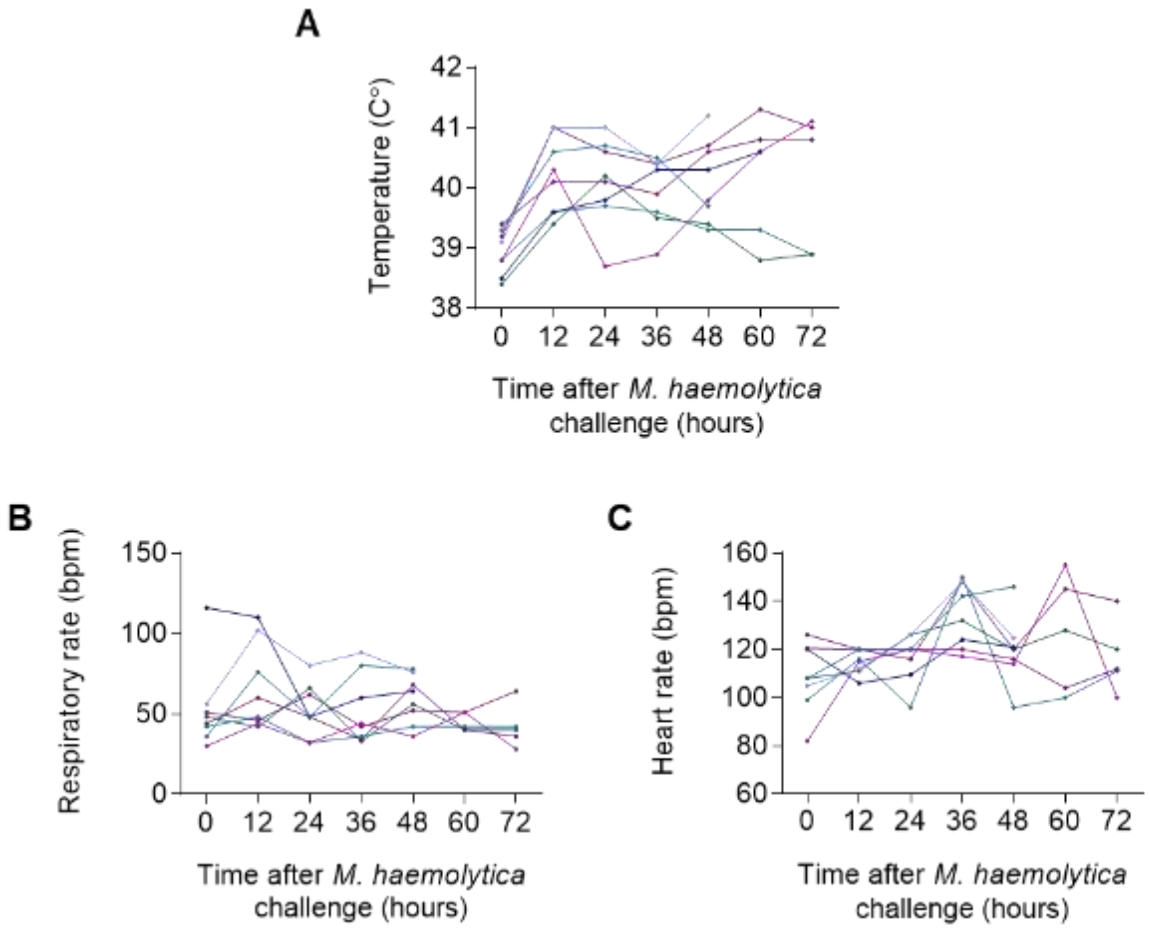


Figure 3.6: Individual animal clinical parameters over time after challenge with aerosolized Mannheimia haemolytica serotype 1 in 8 colostrum-deprived Holstein bull calves. A) Rectal temperature. B) Respiratory rate. C) Heart rate.

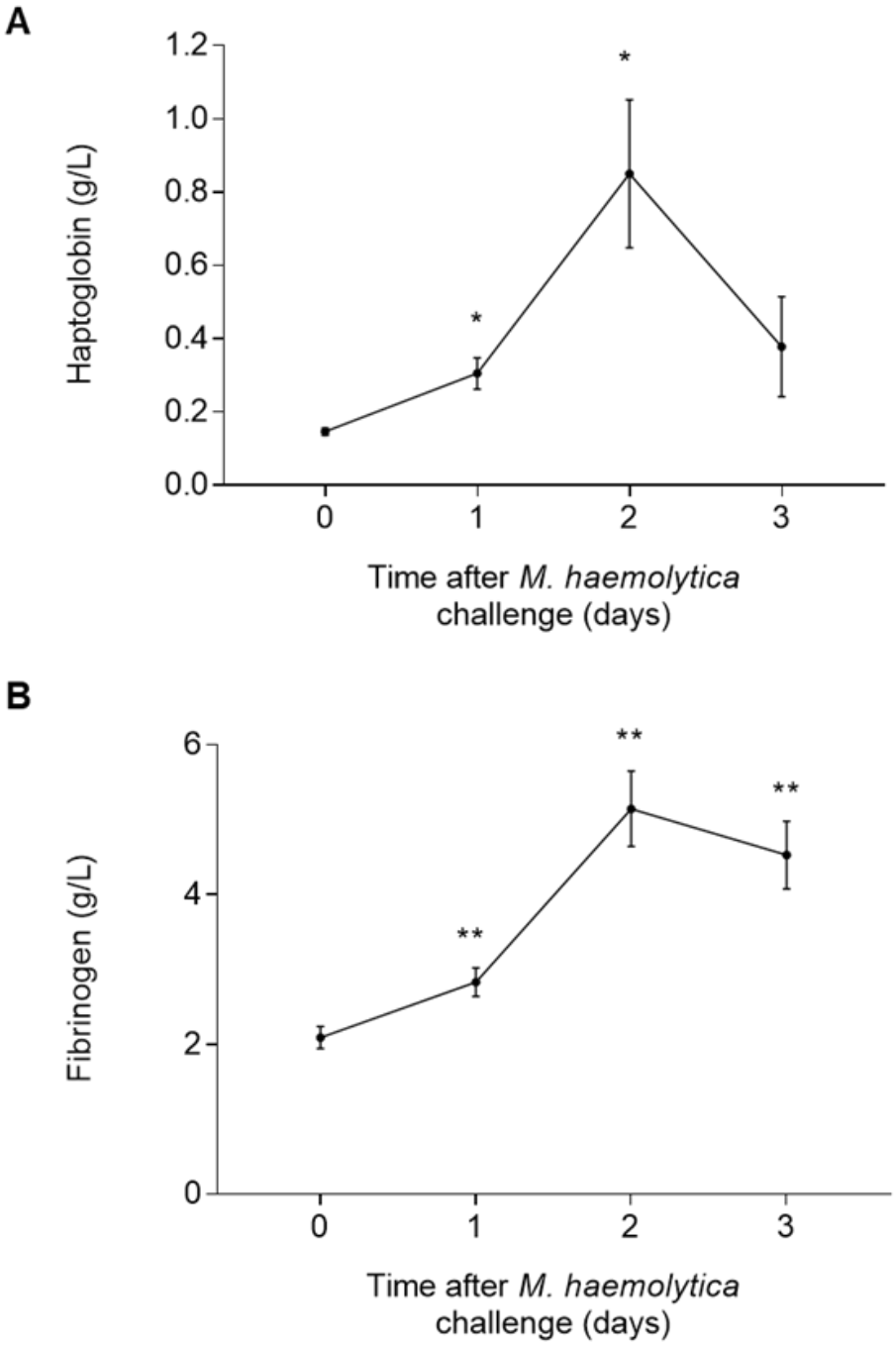


Figure 3.7: Acute phase proteins in blood following *Mannheimia haemolytica* challenge. A) Serum haptoglobin and B) plasma fibrinogen concentrations following challenge with aerosolized *M. haemolytica* serotype 1. The data are mean ± standard error of the mean from 8 colostrum-deprived Holstein bull calves. Data at day 3 are from 5 surviving calves. Data were compared to pre-challenge (time=0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P<0.05$. ** $P<0.01$.

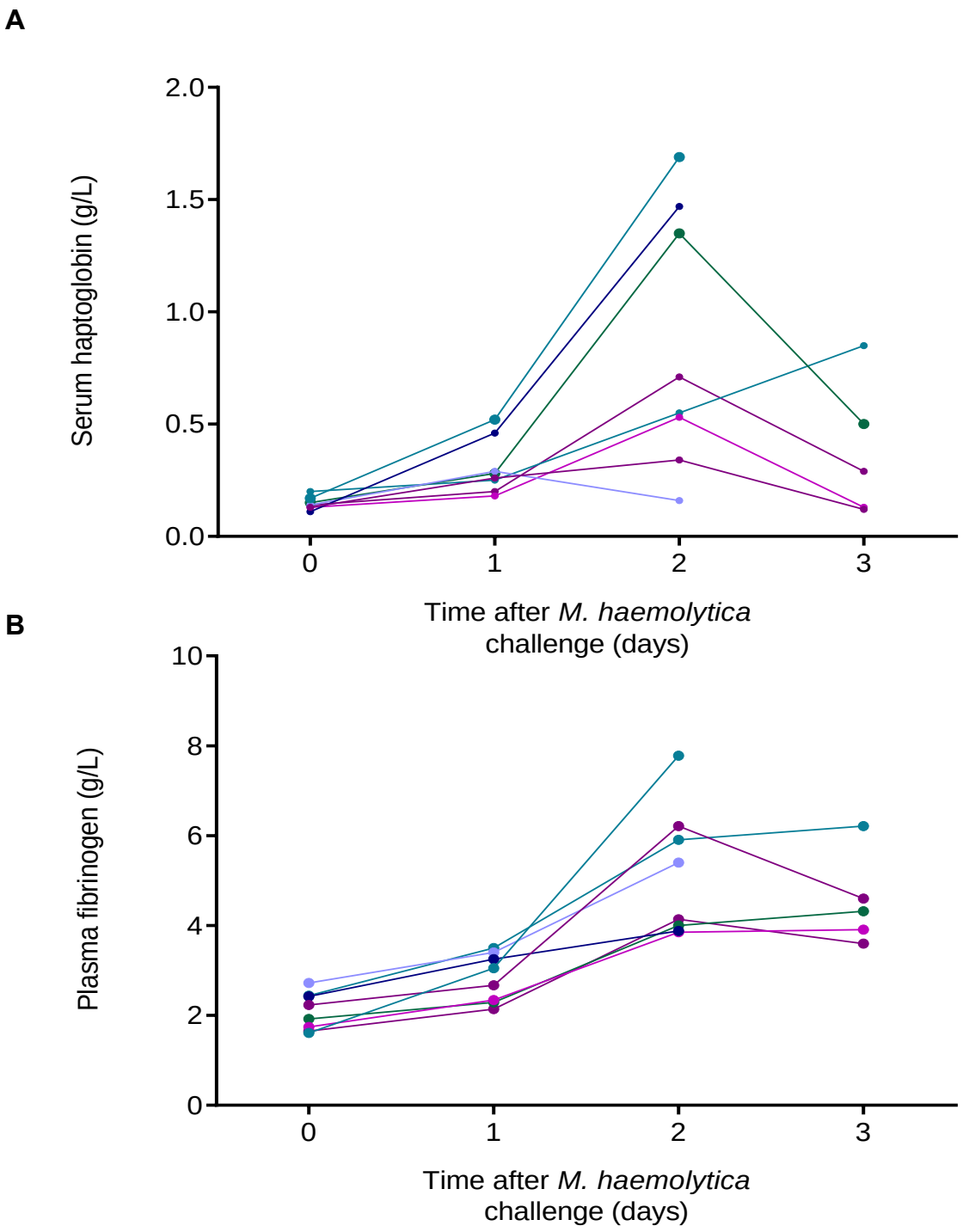


Figure 3.8: Individual animal acute phase proteins in blood following *Mannheimia haemolytica* challenge. A) Serum haptoglobin and B) plasma fibrinogen concentrations following challenge of 8 colostrum-deprived Holstein bull calves with aerosolized *M. haemolytica* serotype 1.

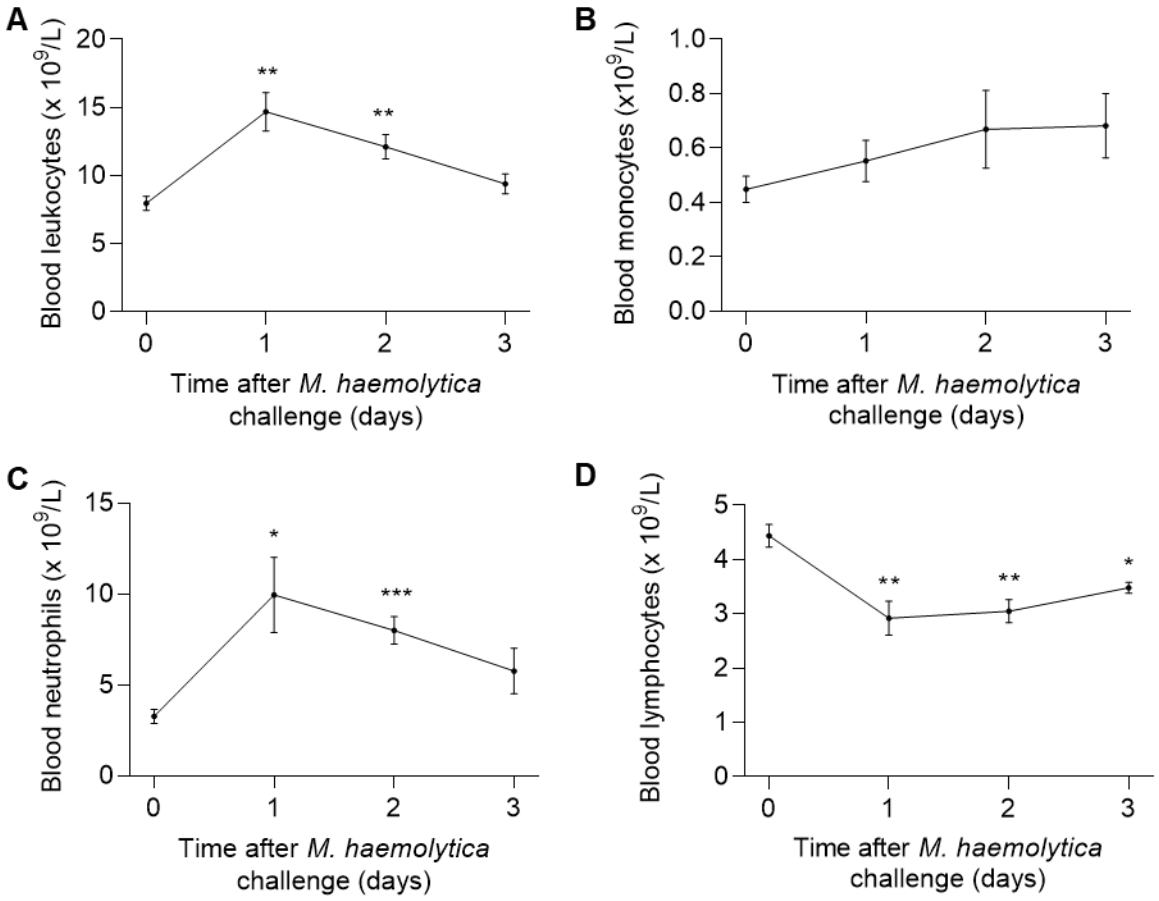


Figure 3.9: Blood leukocyte counts over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1. A) Total leukocytes. B) Monocytes. C) Neutrophils. D) Lymphocytes. The data are mean ± SEM from 8 colostrum-deprived Holstein bull calves. The data at day 3 are from 5 surviving calves. Data were compared to pre-challenge (time=0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P<0.05$. ** $P<0.01$.

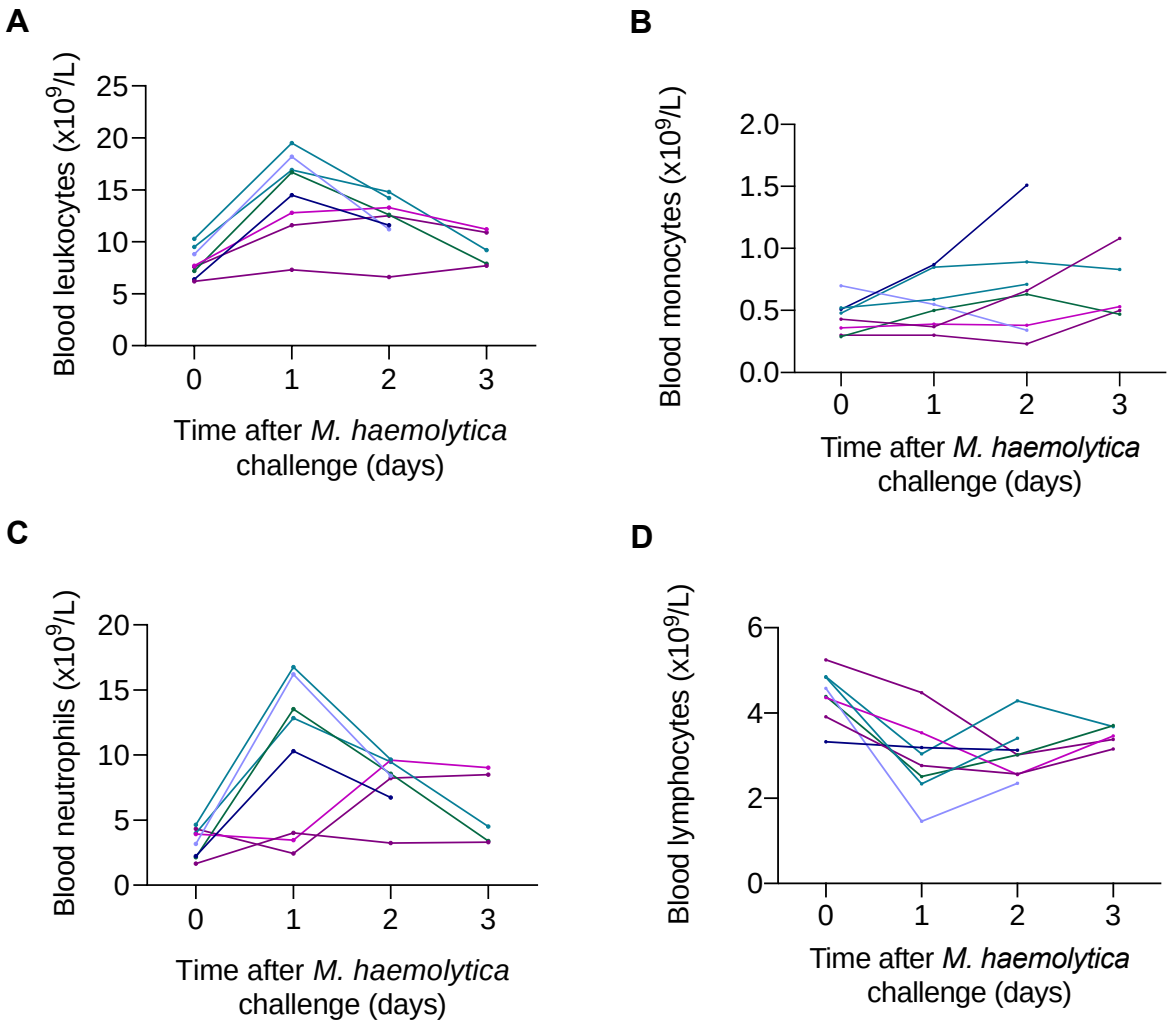


Figure 3.10: Individual animal blood leukocyte counts over time after challenge of 8 colostrum-deprived Holstein bull calves with aerosolized *Mannheimia haemolytica* serotype 1. A) Total leukocytes. B) Monocytes. C) Neutrophils. D) Lymphocytes.

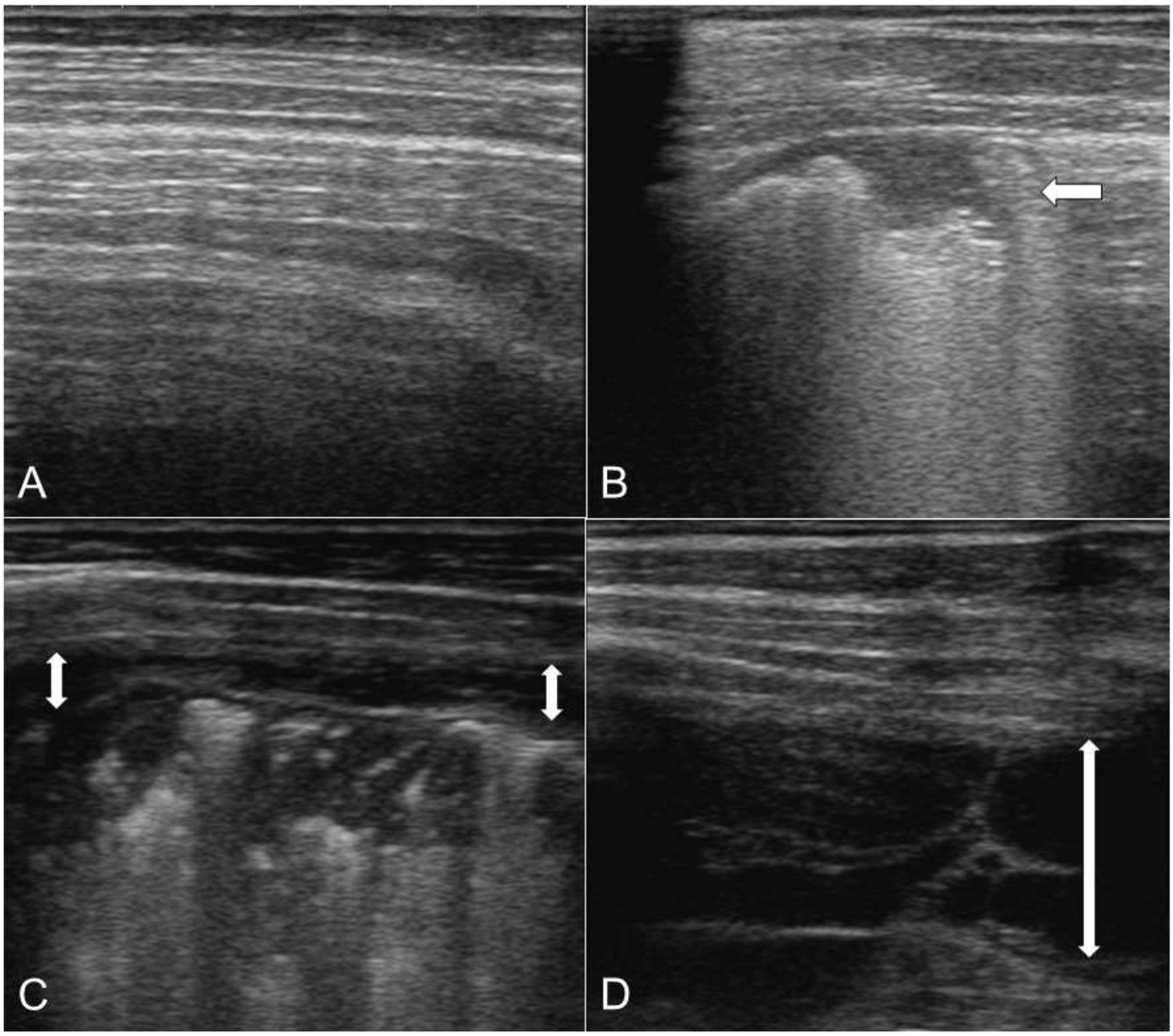


Figure 3.11: Ultrasonographic findings in the lungs of calves. A) Normal lung. The parallel horizontal white lines are A-lines or reverberation artifact and are characteristic of healthy lungs. B) Lung (Calf 8), 24 hours after *Mannheimia haemolytica* infection. The lung contains an area of consolidation (white arrow) that is hypoechoic and lacking reverberation artifact. C) Lung (Calf 6), 48 hours after *M. haemolytica* infection. The entire lung in this field is consolidated (hypoechoic and lacking reverberation artefact) and there is a small amount of pleural effusion identified as a black area (double arrows) between the body wall and the pleural surface of the lungs. D) Lung (Calf 6), 48 hours after *M. haemolytica* infection. Some areas had a large amount of pleural fluid (double arrow) and fibrin between the pleural surfaces.

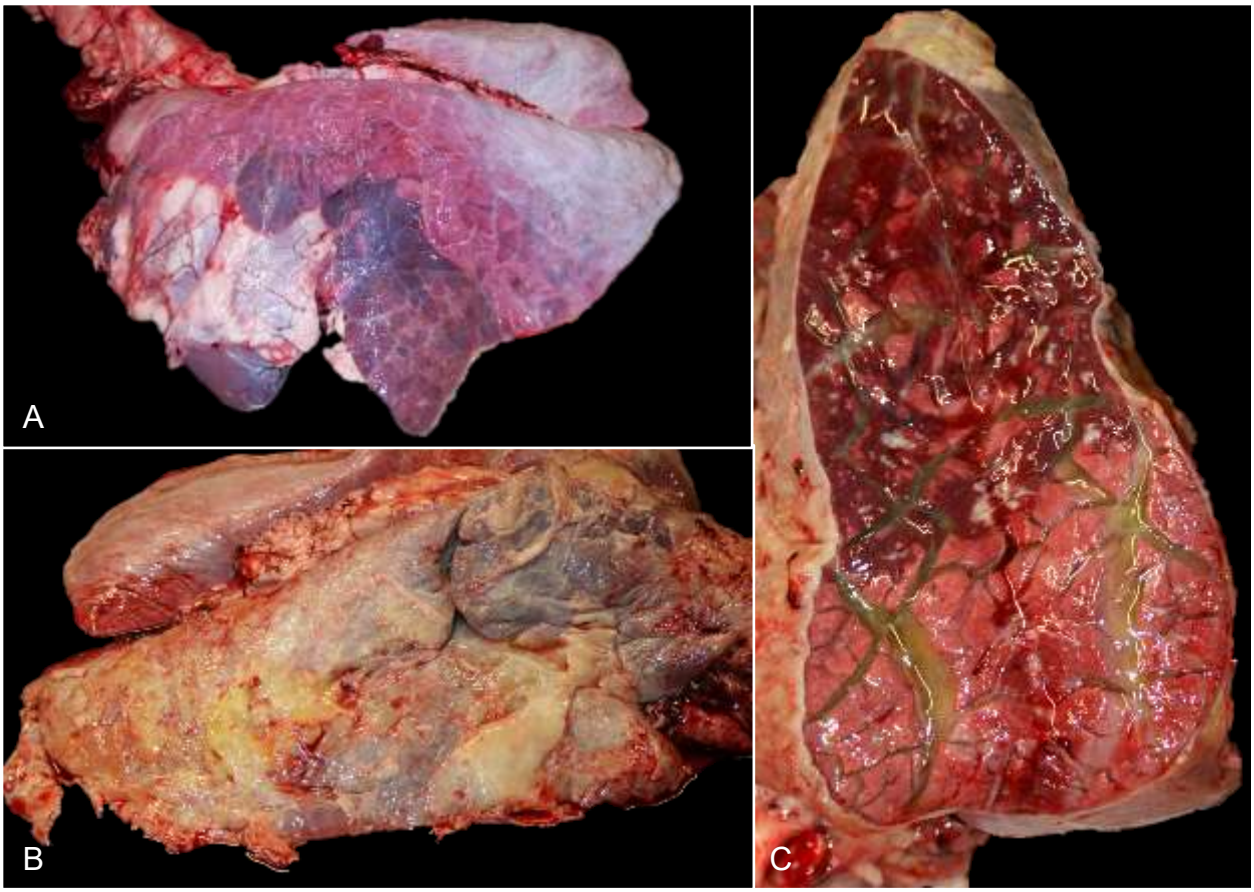


Figure 3.12: Gross lung lesions in colostrum-deprived calves challenged with *Mannheimia haemolytica.* Images were obtained following aerosol challenge of colostrum-deprived calves with 1 x 10^{10} CFU of *M. haemolytica* serotype 1. A) Calf 4, 2 days post-infection. There is lobular consolidation and purple-red-tan discoloration of the left cranioventral lung. B) Calf 6, euthanized 3 days post infection. Fibrin covers the entire pleural surface of the right lung, and there is consolidation of the cranial and middle lobes. C) Calf 6, euthanized 3 days post infection. On cut section, there is lobular consolidation, interlobular septa are markedly expanded by edema, and fibrinous pleuritis is present (at the top of the image).

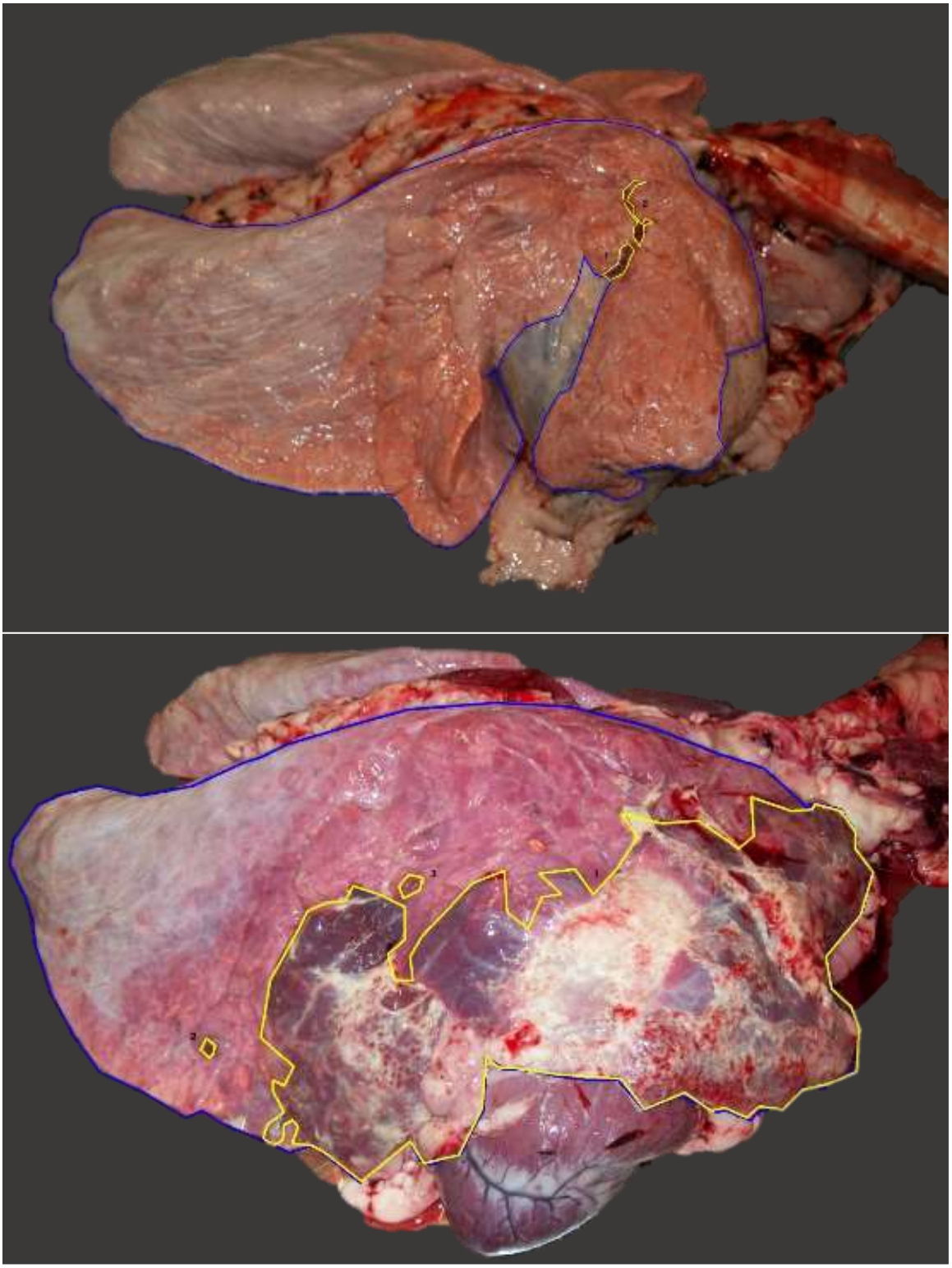


Figure 3.13: Digital evaluation of the extent of lung lesions. For image analysis to determine the proportion of lung affected, software was used to measure the diseased lung (yellow) and the total lung area (blue).

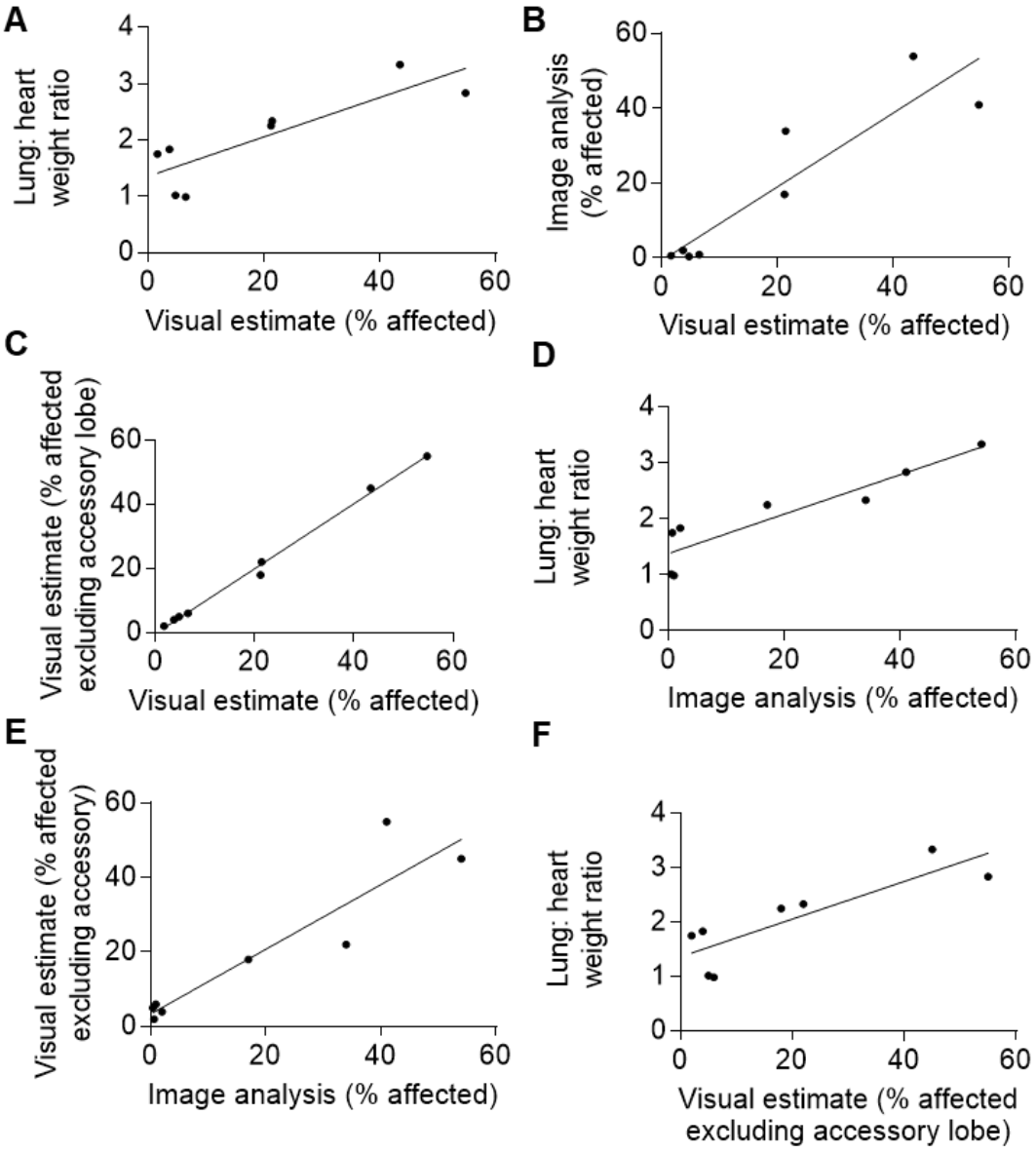


Figure 3.14: Relationship among 3 quantitative measures of gross lung lesions including subjective visual estimation of the percentage of lung affected, software-based image analysis, and lung: heart weight ratios. As the ventrally located accessory lobe was not included in the digital image analysis, additional comparisons of the visual estimates of the percentage of affected lung excluding the accessory lobe was calculated. A) Lung: heart weight ratio versus visual estimate ($R^2 = 0.72$, $p = 0.008$). B) Image analysis versus visual estimate ($R^2 = 0.838$, $p = 0.001$). C) Visual estimate (excluding the accessory lobe) versus visual estimate (including the accessory lobe) ($R^2 = 0.995$, $p < 0.001$). D) Lung: heart weight ratio versus image analysis ($R^2 = 0.846$, $p = 0.001$). E) Visual estimate (excluding accessory lobe) versus image analysis ($R^2 = 0.855$, p = 0.001). F) Lung: heart weight ratio versus visual estimate (excluding the accessory lobe) ($R^2 = 0.852$, $p = 0.007$).

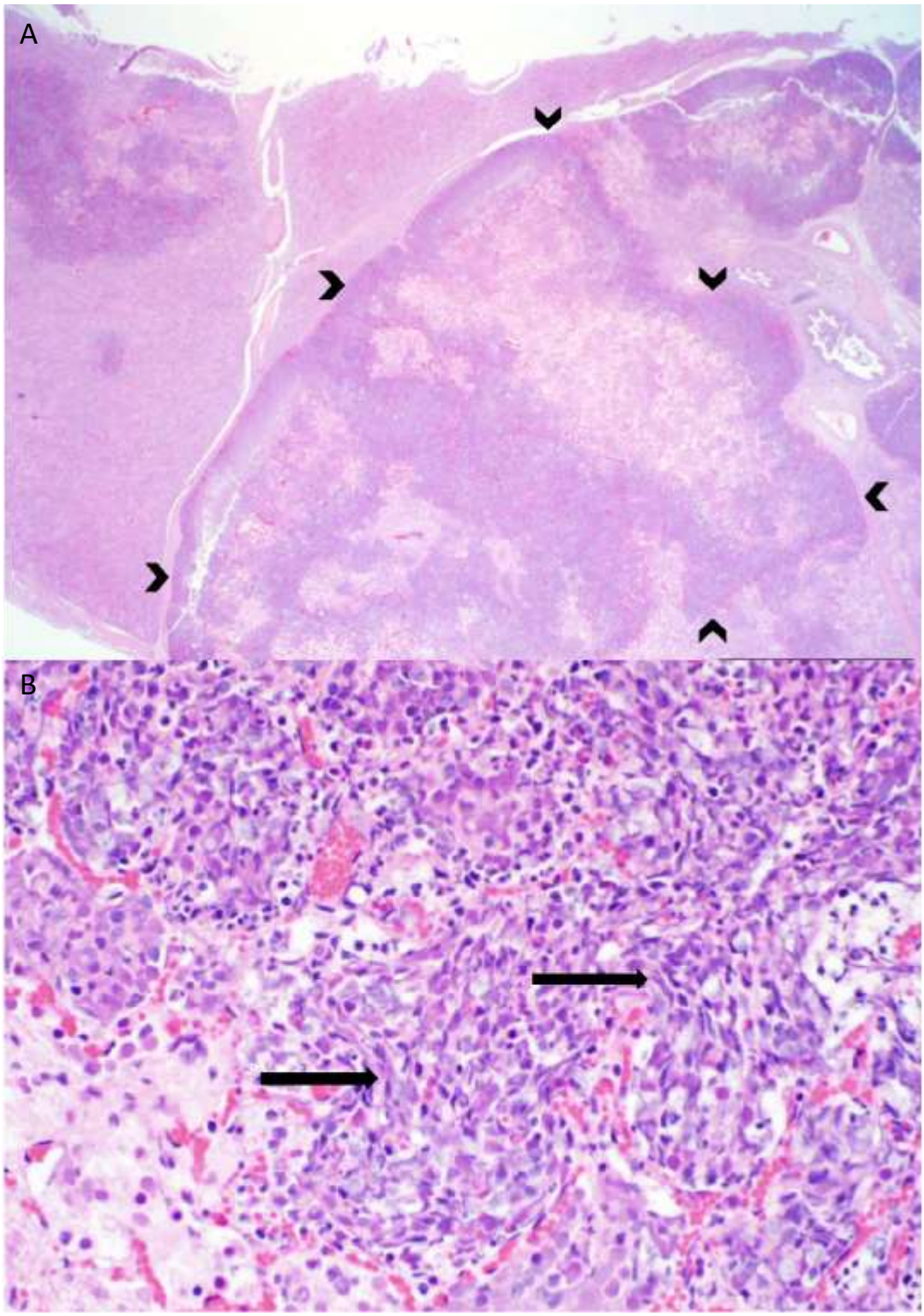


Figure 3.15: Histologic lesions in lung from a colostrum-deprived calf challenged with 1 x 10^{11} CFU of *Mannheimia haemolytica*. A) Calf 4: The lung contains irregularly shaped areas of coagulation necrosis (outlined with chevrons). B) Alveoli contain necrotic neutrophils with streaming nuclei (arrows). Hematoxylin and eosin.

3.9 Supplemental Material

Supplemental Table S3. 1: Published studies of aerosol *Mannheimia haemolytica* challenge experiments in calves.

Authors (year)	**Experimental animals**		***M. haemolytica***		**Results**
	Number	**Age (months)**	**Dose (CFU)**	**Serotype**	
Jericho and Langford (1978)	4	2-4	Not available[a]	Not reported	No gross lung lesions
Yates et al. (1983)	4	2-4	1.1×10^{6} [b]	1	No fever or pneumonia
Filion et al. (1984)	8	6-8	$1x10^{10}$ [c]	Not reported	No clinical signs
Bielefeldt-Ohmann and Babiuk (1985)	4	7-10	$1\text{-}2 \times 10^{9}$ [a]	1	No clinical signs
Jericho (1989)	3	8	3.4×10^{7} [a]	Not reported	2 of 3 developed pneumonia

[a] Fresh *M. haemolytica* culture
[b] Frozen and thawed *M. haemolytica* culture
[c] The *M. haemolytica* culture method was not described.

Supplemental Table S3. 2: Characterization of *Mannheimia haemolytica* strains used for experimental infection studies. DNA sequences of amplicons from multiplex PCR for *M. haemolytica* strains B158 and B159. A primer blast search (August 20, 2018) indicated that the amplicon for B158 was 100% homologous with published sequences for *M. haemolytica* serotype 1.

Strain	Source	Primers	Amplicon (size)	Serotype (PCR)	MALDI-TOF genotype
B158	Clinical isolate from fatal pneumonia in a dairy cow	F: CATTTCCTTAGGTTCAGC R: CAAGTCATCGTAATGCCT	TTCCTTTAGGTTCAGCTG CATGACCTGATTCATGAG AATACAAAATAACTTTTA CATCTTTGCTCTGTTGTT CTCTCCAATGGGTCTTTT CTATTTCCGATAGATCTA TCCCCTTTAACTCTAGGA ATGGGAGGAAATGCCAT TTATGATGAAATTCATCC TGAGTATTTTGAATATAC AAAATCTTTGATGTATTTT CCTTAAGTAATACTAAAG CATTAAATTTCTCTTGATT AGAAAGACTATCGTACTC TTCCGCATTAAAGTCAAA GCAAGCGTTTAAAAAGG CATTACGATGAC (306 base pairs)	1	2
B159	Isolate from brain of a dairy cow with no associated lesions	F: GGCATATCCTAAAGCCGT R: AGAATCCACTATTGGGCACC	Not sequenced (160 base pairs)	2	1

Supplemental Table S3. 3: A summary of dose, *Mannheimia haemolytica* serotype, clinical outcomes, and gross and histological lesions from pilot studies in conventionally raised Holstein bull calves.

Experiment	Calf ID Ear tag/name (leukotoxin antibody titre)	*M. haemolytica* inoculum: strain (dose)	*M. haemolytica* isolated		Clinical signs	Gross and histologic lesions
			Nasal	Lung		
Pilot 3	A1 1323 (19800)	Serotype 1 1 x 10^{10}	+	+	Severe clinical signs within 4 hours, resolved by 12 hours Euthanized day 3	Occasional atelectasis and rare neutrophils in alveoli
Pilot 3	A2 1324 (13700)	Serotype 1 5 x 10^{10}	+	+	Severe clinical signs, moribund at 6 hours, euthanized 6 hours post infection	Severe pulmonary edema, interlobular and subpleural emphysema. Severe alveolar damage with pulmonary edema, alveolar neutrophils, hemorrhage and hyaline membranes

Supplemental Table S3. 4: A summary of dose, *Mannheimia haemolytica* serotype, clinical outcomes, and gross and histological lesions from pilot studies in clean-catch colostrum-deprived Holstein bull calves.

Experiment	Calf ID Ear tag/name (leukotoxin antibody titre)	*M. haemolytica* inoculum: strain (dose)	*M. haemolytica* isolated		Clinical signs	Gross and histologic lesions
			Nasal	Lung		
Pilot 6	B1 1485/Littlefoot (3100)	Serotype 2 1×10^7	-	-	No	Single focus of bronchopneumonia.
Pilot 6	B2 No Tag/Wowow (3500)	Serotype 2 $1x\ 10^8$	-	-	No	Mild atelectasis and rare neutrophils in alveoli.
Pilot 6	B3 1493/Singer (600)	Serotype 2 $1x10^9$	Days 1, 3	-		
Pilot 7	B4 1567/Pepe	SH1217 ATCC Serotype 1 2×10^{10} day 0, 5.4×10^{10} day 1 Green dye 10 minutes before euthanasia	Not cultured	-	Brief period of dullness, increased temperature (39.9), cough.	Rare neutrophils in alveoli. Patchy distribution of dye on the surface of all respiratory epithelium.

Experiment	Calf ID Ear tag/name (leukotoxin antibody titre)	*M. haemolytica* inoculum: strain (dose)	*M. haemolytica* isolated		Clinical signs	Gross and histologic lesions
			Nasal	Lung		
1	C1 Dandy/1586	Serotype 1	+	+	Increased rectal temperature, coughing, depression, dyspnea. Euthanasia 2 days after challenge.	Severe gross and histologic bronchopneumonia with oat cells, coagulative necrosis, pleuritis and alveolar fibrin.
2	C2 Diego/1593	Serotype 1	+	+	Increased rectal temperature, coughing, depression, and respiratory effort..	Gross and histologic bronchopneumonia with alveolar fibrin.
2	C3 Dock/1597	Serotype 1	+	+	Increased rectal temperature, coughing, depression, dyspnea. Euthanasia 2 days after challenge.	Gross and histologic bronchopneumonia with severe pleuritis.
3	C4 1599 /Sebastian	Serotype 1	+	+	Increased rectal temperature, coughing, depression, dyspnea. Euthanasia 2 days after challenge.	Severe gross and histologic bronchopneumonia with oat cells, coagulative necrosis, pleuritis and alveolar fibrin.
4	C5 1615/Elvis	Serotype 1	+	+	Increased rectal temperature, mild depression and increased respiratory effort.	Gross and histologic bronchopneumonia with oat cells, pleuritis and fibrin in alveoli.
4	C6 1621/Cash (-)	Serotype 1 ($2x10^9$)	+	+	Increased rectal temperature, coughing, depression, increased respiratory effort.	Gross and histologic bronchopneumonia with oat cells, coagulative necrosis, pleuritis and fibrin in alveoli.
5	C7 1633/Dagmar (2225)	Serotype 1 $1x10^{11}$	+	+	Increased rectal temperature, coughing, and increased respiratory effort.	Gross and histologic bronchopneumonia with oat cells, coagulative necrosis, pleuritis and fibrin in alveoli.
5	C8 /Spotty (381)	Serotype 1 $1x10^{11}$	+	+	Increased rectal temperature and coughing.	Gross and histologic bronchopneumonia with oat cells, coagulative necrosis, pleuritis and fibrin in alveoli.

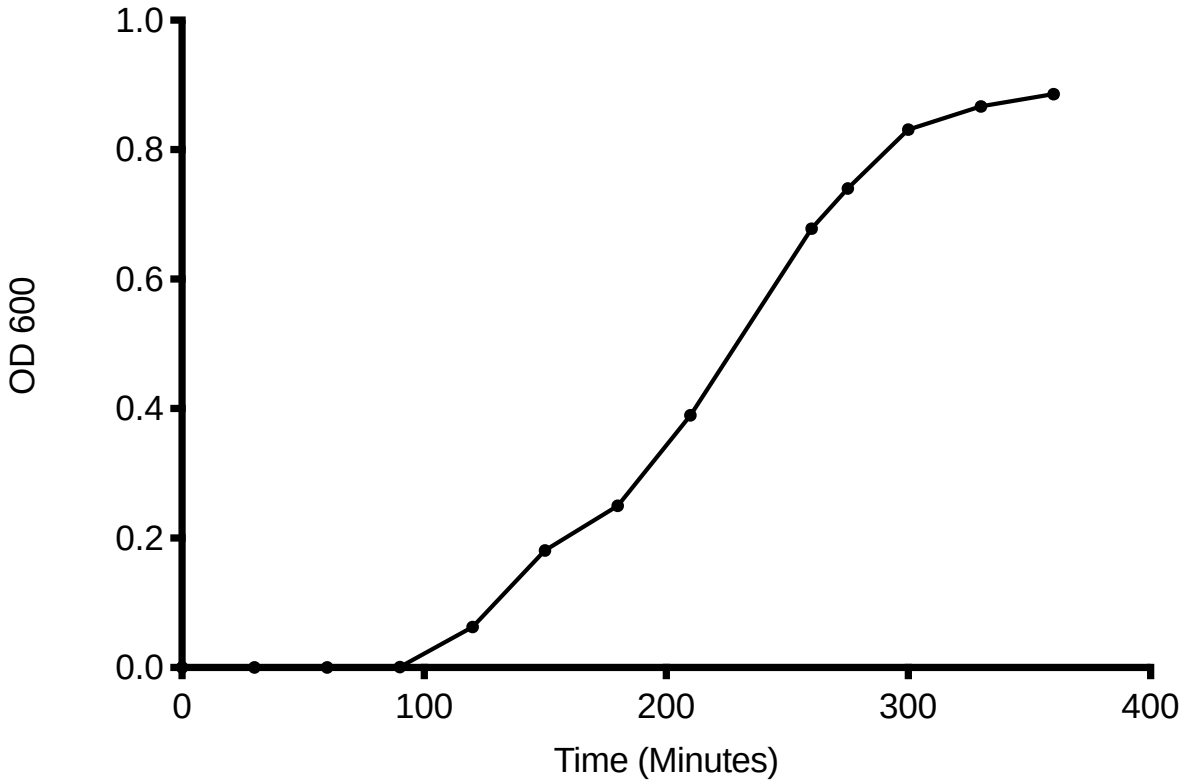
Supplemental Table S3. 5: Serum electrophoresis from two calves to confirm failure of passive transfer. One calf had blood collected at 48 hours of age, prior to feeding of milk replacer, 24 hours following the first feeding of milk replacer, and again at the time of challenge at 4 weeks of age. The other calf was sampled 24 hours following the first feeding of milk replacer and at the time of challenge, at 4 weeks of age.

Time of Sample	Calf ID	Total Protein (g/L) (RR[a] 66-86)	Albumin (g/L) (RR 30-42)	Globulin (g/L) (RR 30-53)	Albumin/ globulin ratio	Gamma (g/L) (RR 5-18)
Pre-milk replacer	Maple	41	25	16	1.56	2
24 hours post-milk replacer	Maple	44	27	17	1.65	2
4 weeks old	Maple	55	34	21	1.62	5
24 hours post-milk replacer	Windsor	45	29	16	1.73	2
4 weeks old	Windsor	51	34	17	2.00	2

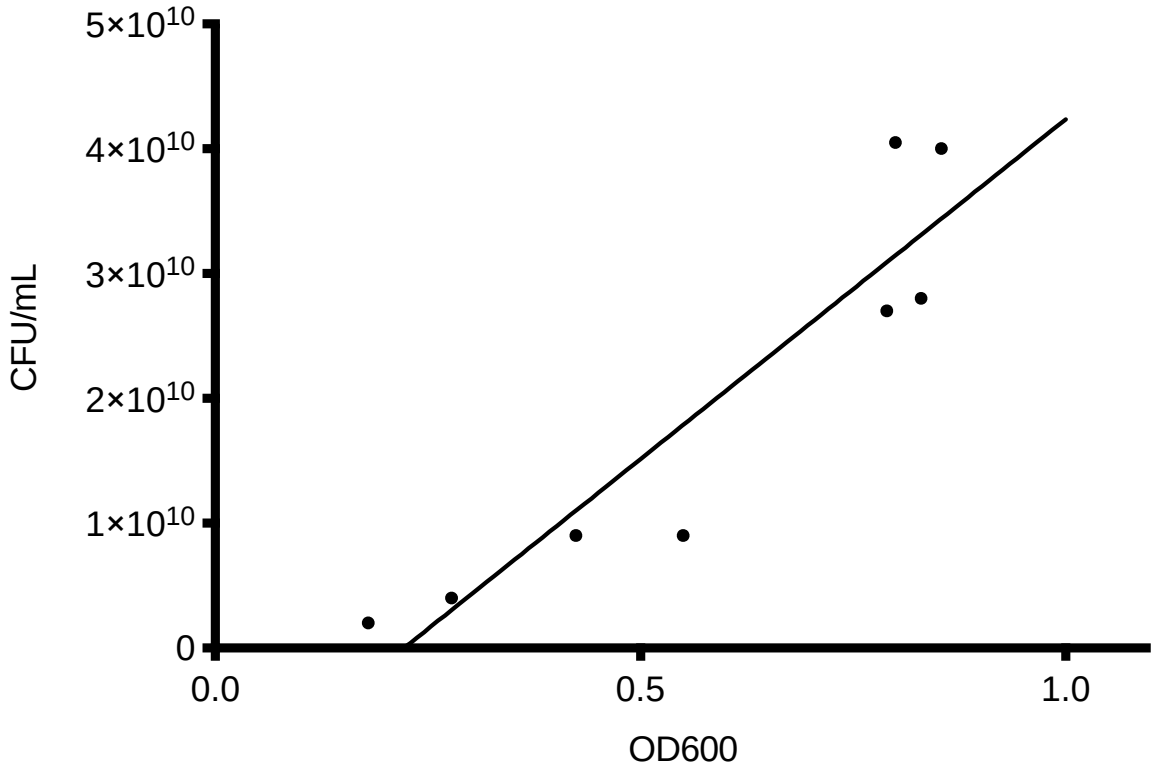
[a]RR – reference range

Supplemental Table S3. 6: Comparison of different methods of quantifying gross lung lesions, based on linear regression analysis. The 3 methods were subjective visual estimation of the percentage of lung affected (with or without the accessory lobe), software-based image analysis of the percentage of lung affected, and lung:heart weight ratios.

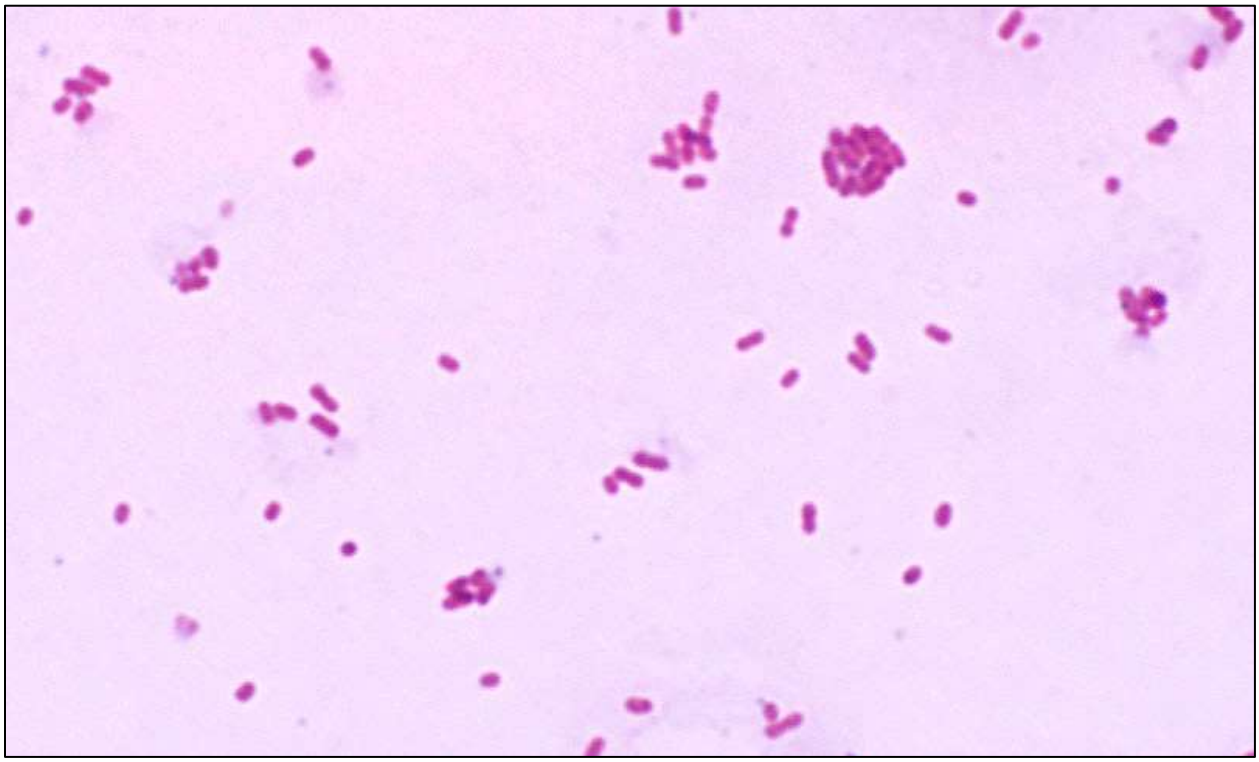
X	Y	R square	p value	Equation
Visual estimate	Image analysis	0.838	0.0014	Y = 0.1056*X - 0.5718
Visual estimate	Lung: heart weight ratio	0.720	0.0078	Y = 0.03496*X + 1.354
Visual estimate	Visual estimate (excluding accessory lobe)	0.995	<0.0001	Y = 1.012*X - 0.3034
Image analysis	Lung: heart weight ratio	0.846	0.0012	Y = 0.0351*X + 1.384
Image analysis	Visual estimate (excluding accessory lobe)	0.855	0.0010	Y = 0.8688*X + 3.345
Visual estimate (excluding accessory lobe)	Lung: heart weight ratio	0.726	0.0073	Y = 0.03461*X + 1.363



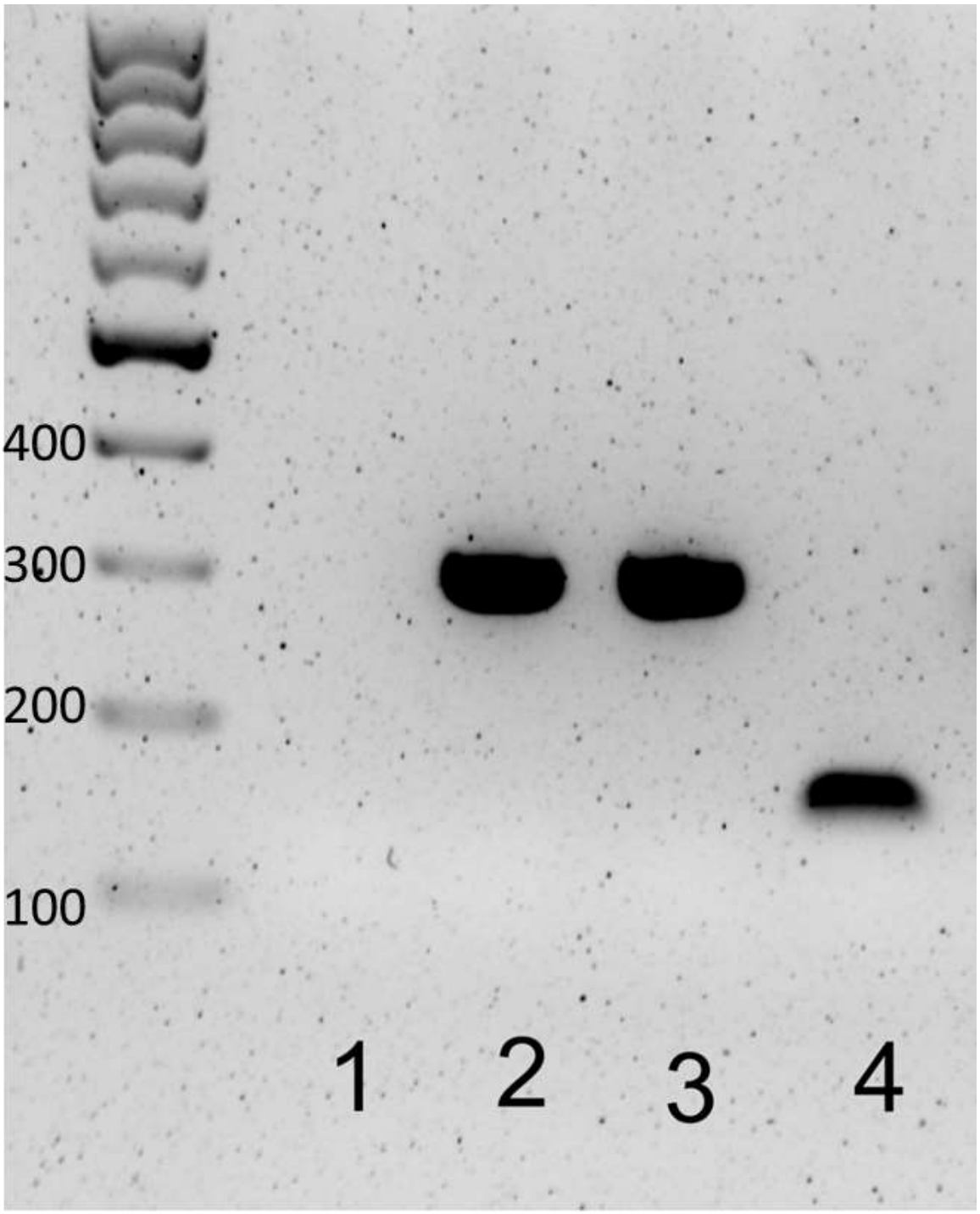
Supplemental Figure S3. 1: Growth curve using OD600 for *Mannheimia haemolytica* grown in brain heart infusion broth.



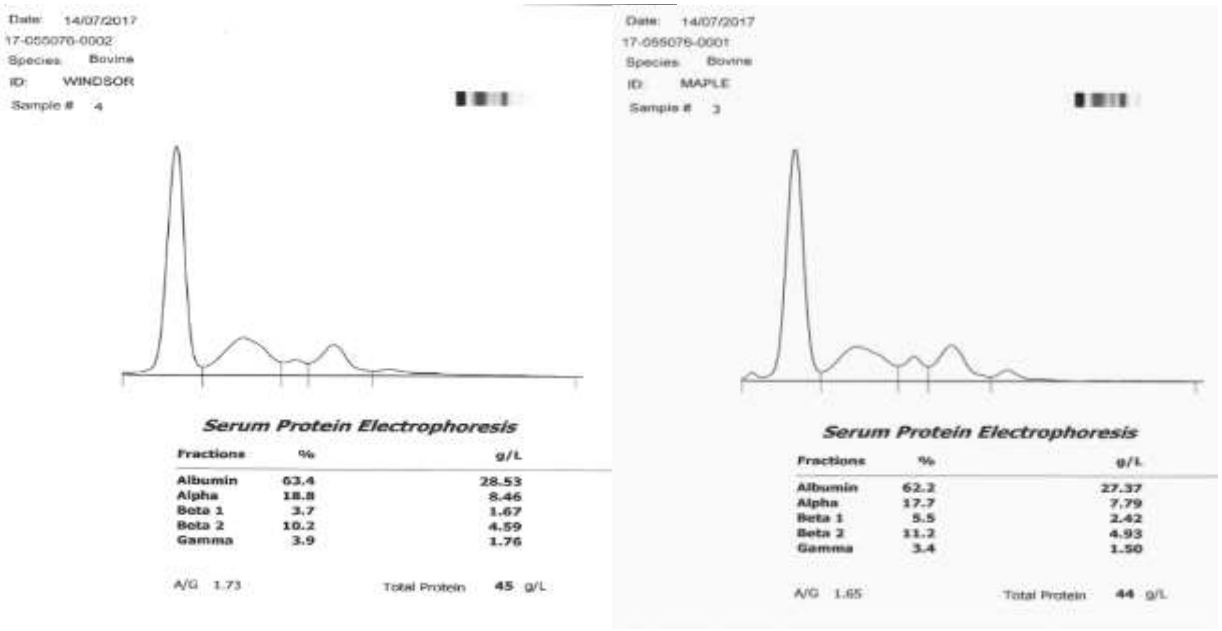
Supplemental Figure S3. 2: Comparison of optical density (OD600) and counts of colony-forming units (CFU/mL) for different concentrations of *Mannheimia haemolytica* in brain-heart infusion broth.



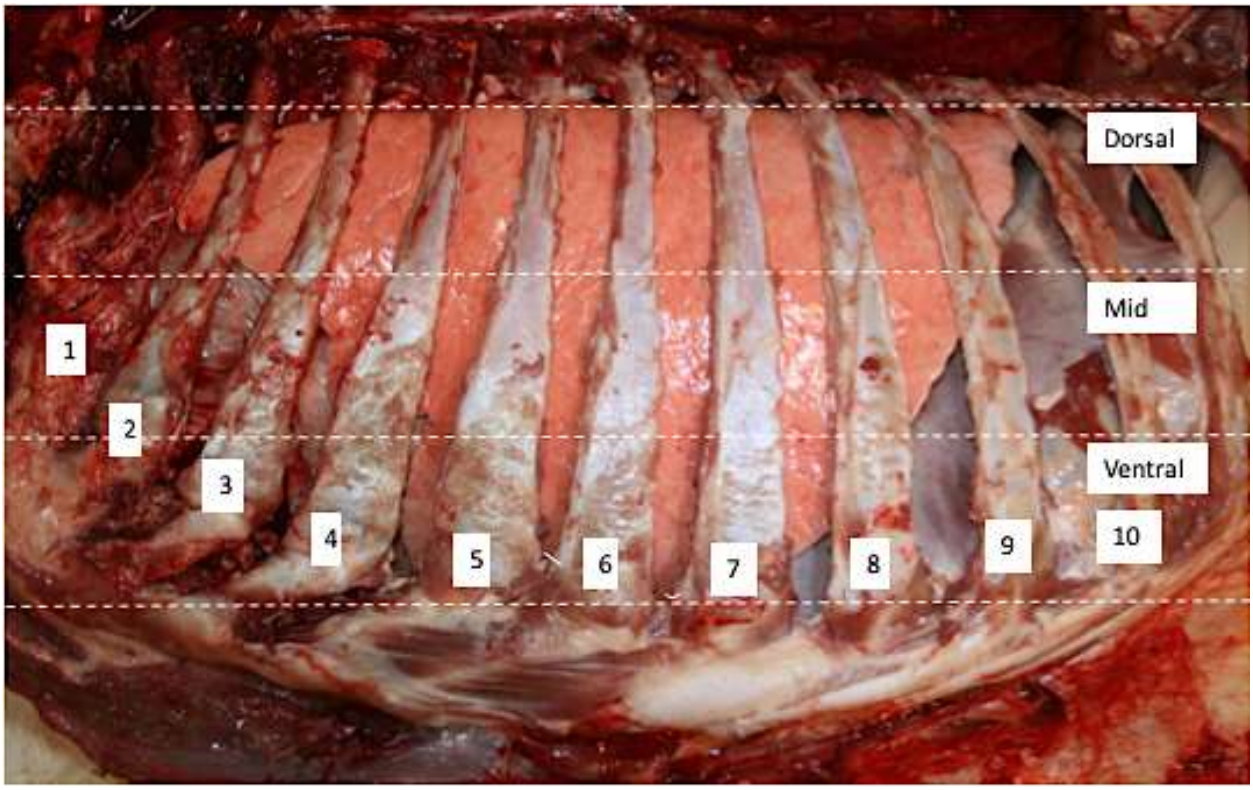
Supplemental Figure S3. 3: The inoculum used for experimental infections contained a pure population of short gram-negative rods, characteristic of *Mannheimia haemolytica.* Gram stain; 600x.



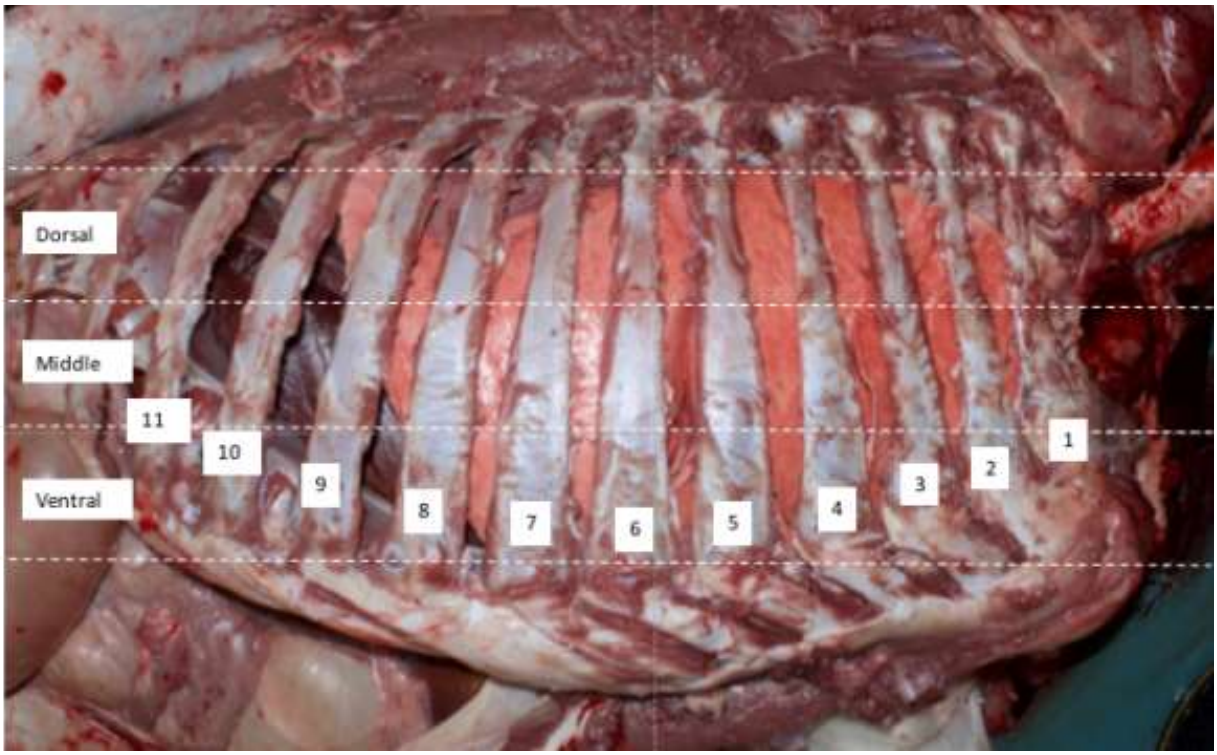
Supplemental Figure S3.3: Gel electrophoresis of amplification products following multiplex PCR for *Mannheimia haemolytica* serotypes 1, 2, and 6. Left: size marker (100 bp DNA Ladder RTU: GenedDireX); lane 1 is negative control (water), lanes 2 and 3 are DNA from strain B158 (306 bp, serotype 1 amplicon); lane 4 is DNA from strain B159 (160 bp, serotype 2 amplicon).



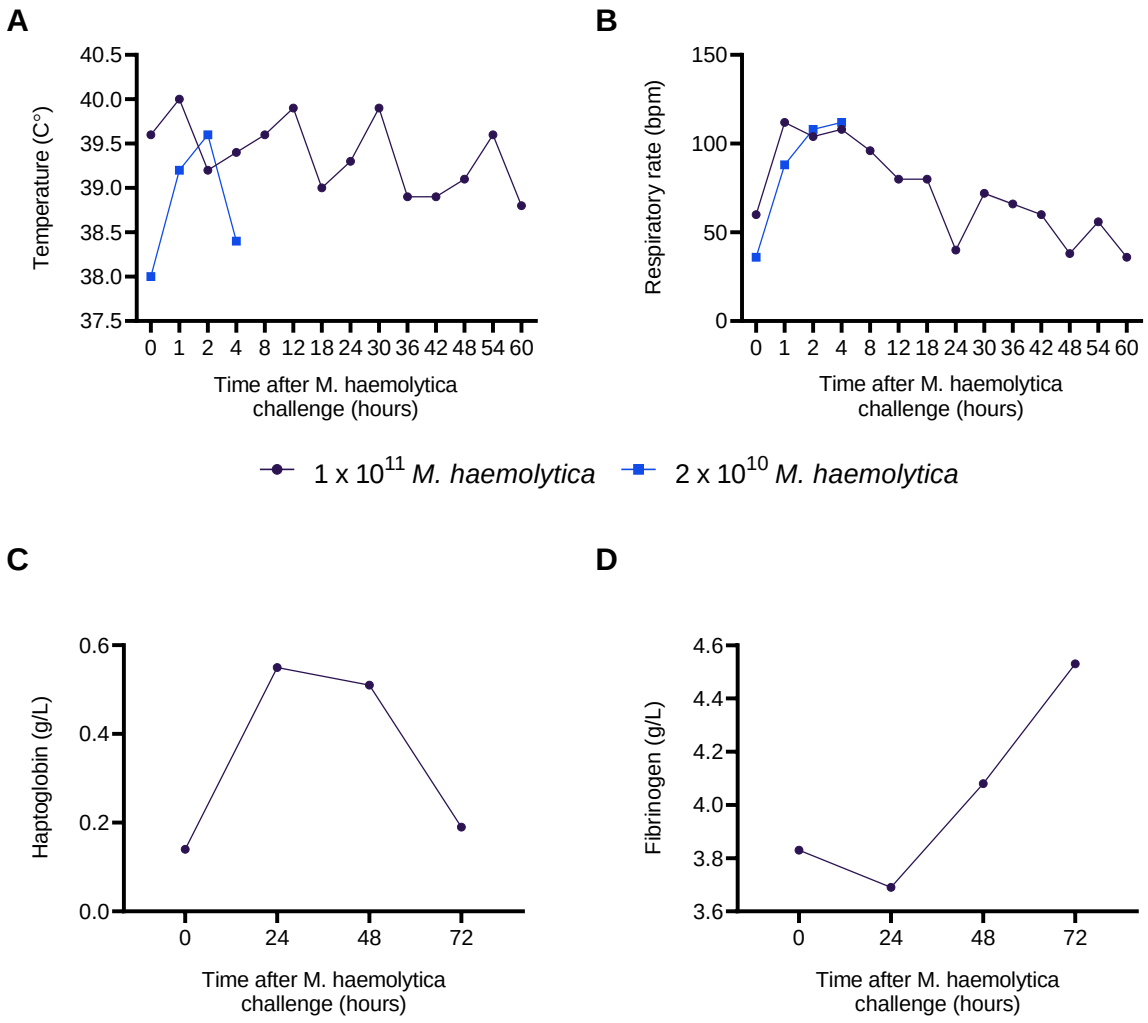
Supplemental Figure S3. 4: Serum electrophoretogram confirming failure of passive transfer in 2 calves at 72 hours of age. Failure of absorption of immunoglobulins from milk replacer was investigated in two calves using serum protein electrophoresis. At 72 hours of age, both calves evaluated had low total serum protein 44 and 45 g/L and serum gamma globulins of < 2 g/L. One calf was also tested prior to feeding milk replacer, and serum gamma globulins were identical pre- and 24 hours post-feeding of milk replacer.



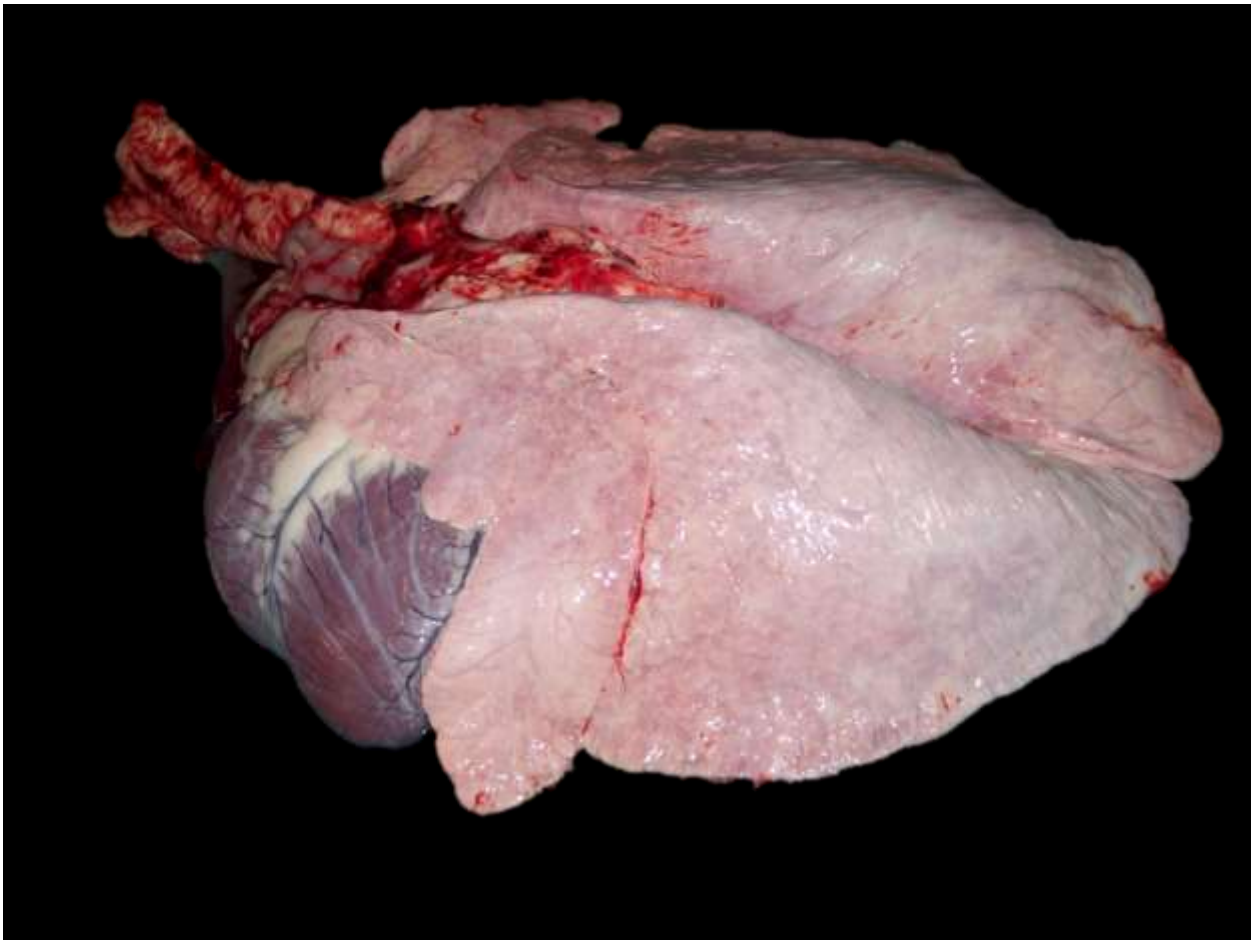
Supplemental Figure S3. 5: Left lung in situ with soft tissue of the body wall removed and ribs labelled to visualize the relationship between lung lobes and intercostal spaces. In this photograph, the head is to the left.



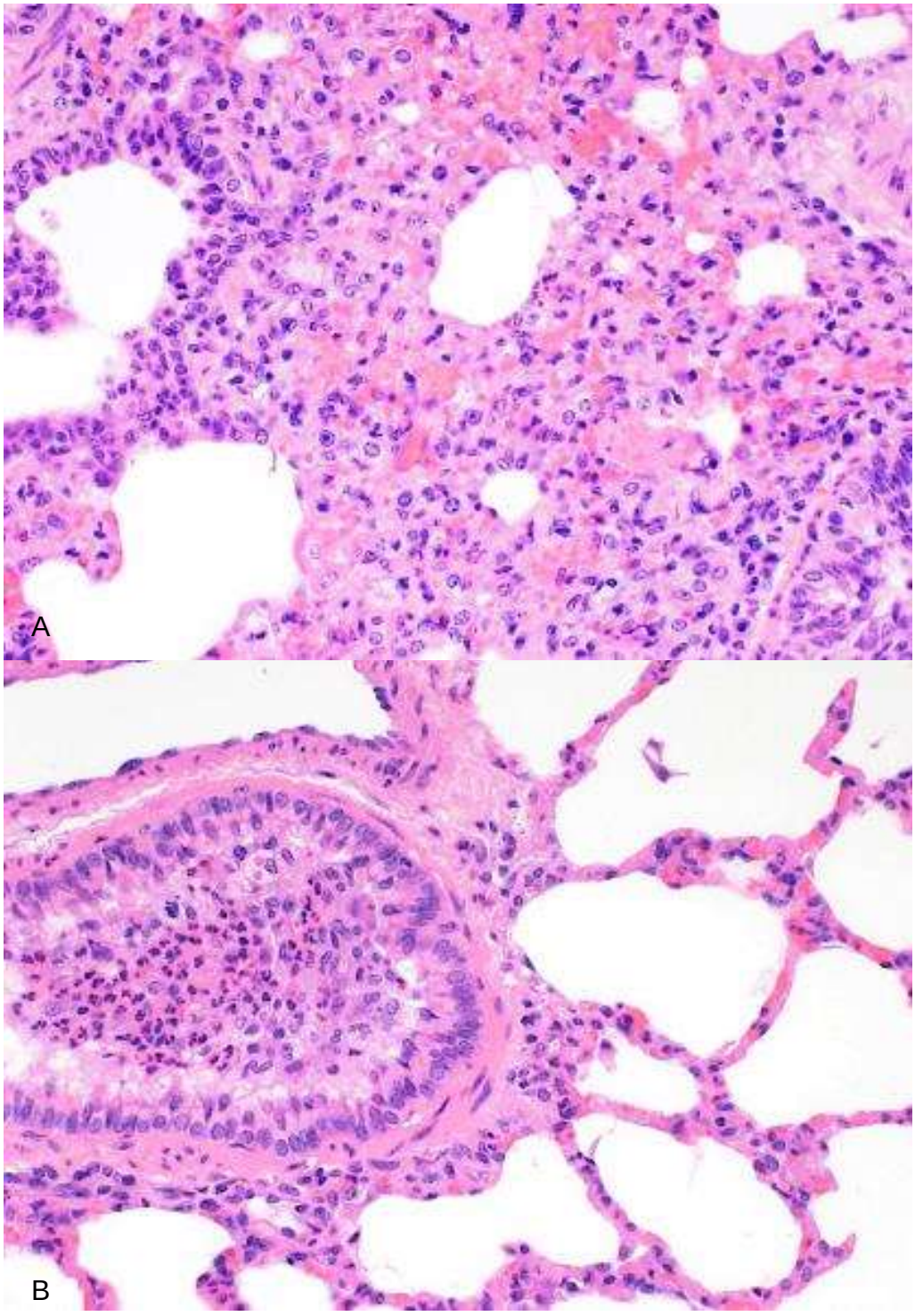
Supplemental Figure S3. 6: Right lungs in situ with soft tissue of the body wall removed and ribs labelled to visualize relationship between lung lobes and intercostal space. In this photograph, the head is to the right.



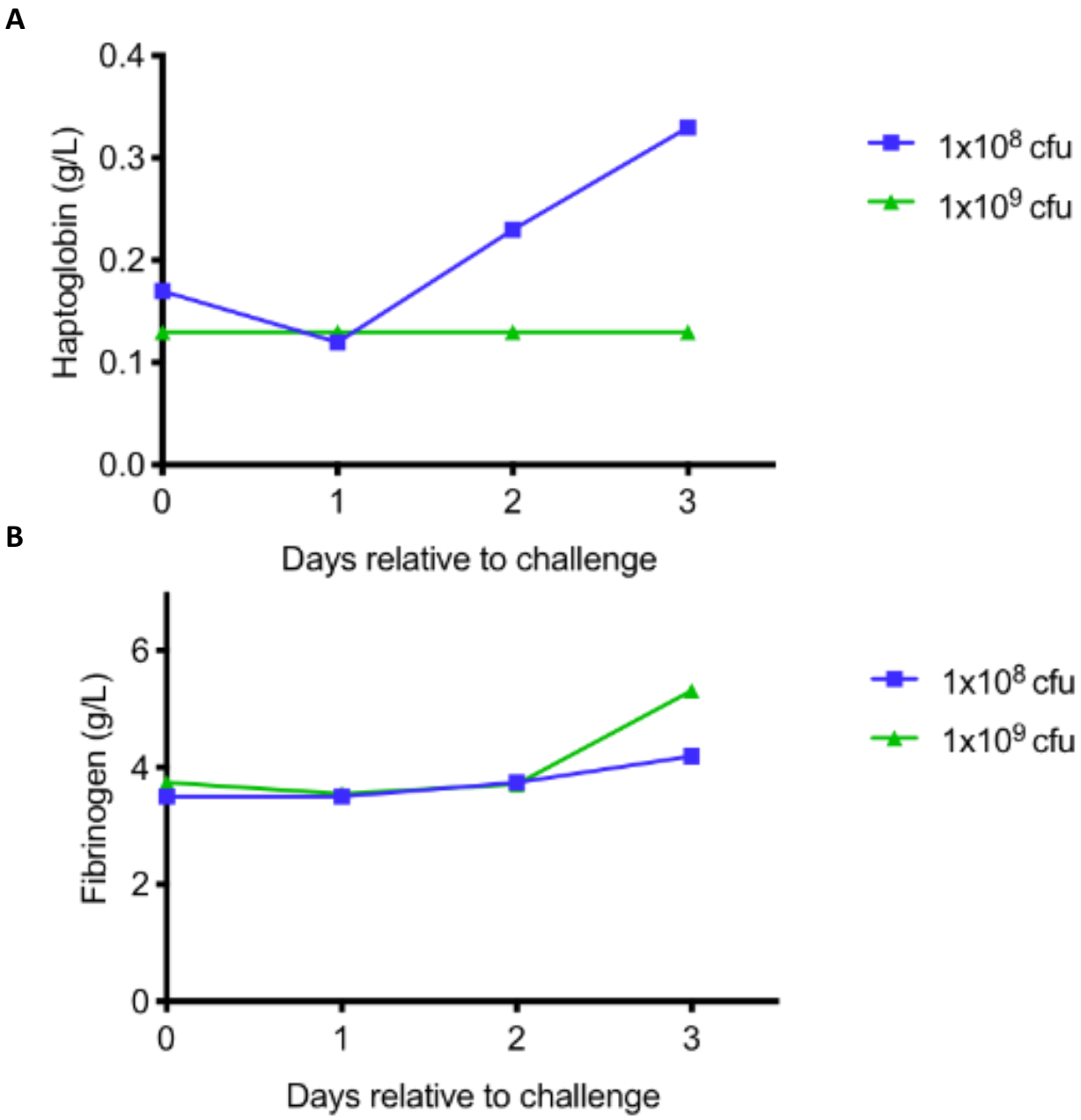
Supplemental Figure S3. 7: Two conventional (not colostrum-deprived) calves were challenged with aerosolized *Mannheimia haemolytica* serotype 1 at doses of $2x10^{10}$ colony forming units (CFU) (Calf A1) and $1x10^{11}$ CFU (Calf A2). The calf receiving $1x10^{11}$ CFU was euthanized 6 hours after challenge due to severe clinical signs. The graphs show the (A) rectal temperatures, (B) respiratory rates, (C) serum haptoglobin concentrations, and (D) plasma fibrinogen concentrations.



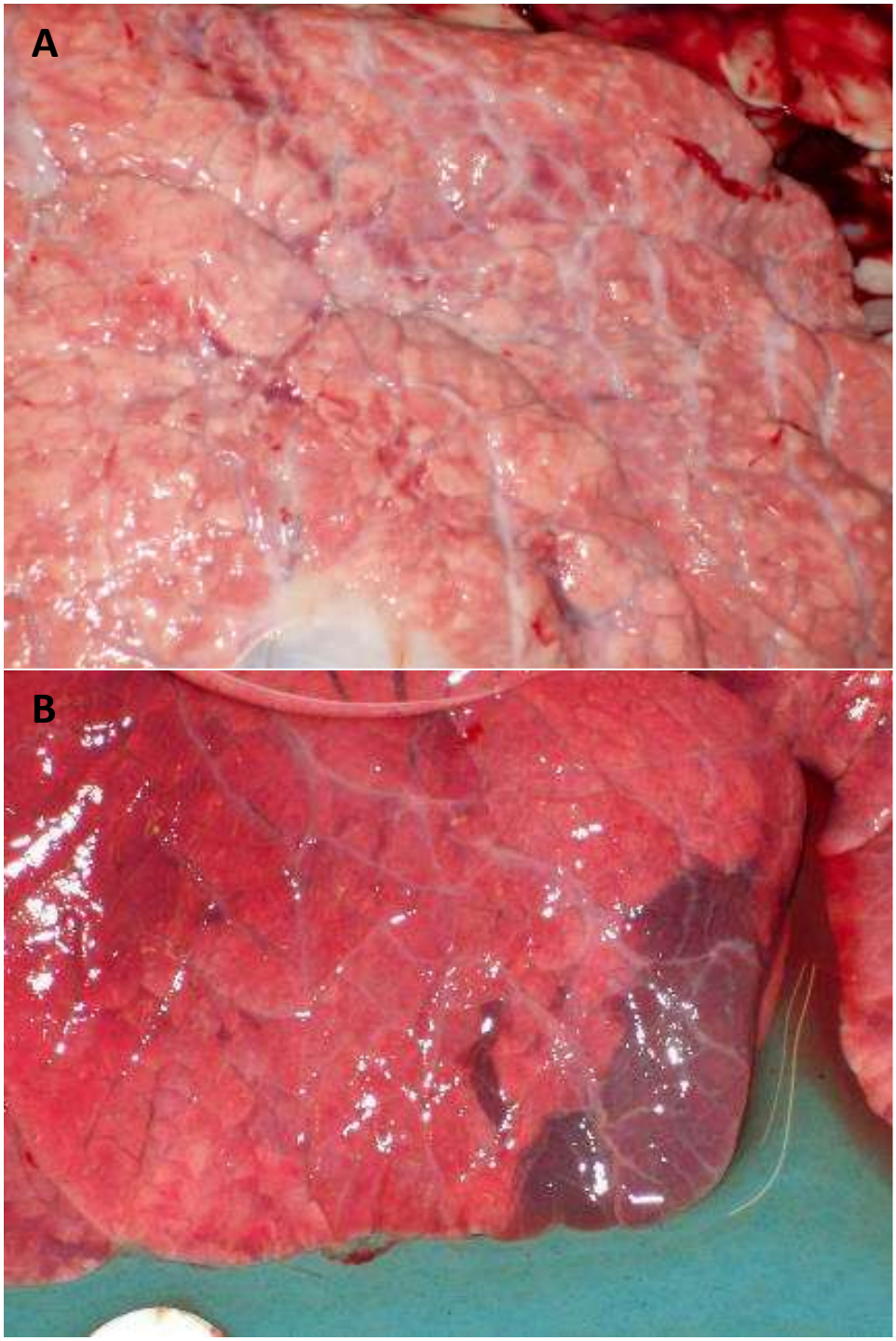
Supplemental Figure S3. 8: Lungs, conventionally raised calf (Calf A1), 3 days after challenge with 1 x 10^{10} CFU of *Mannheimia haemolytica* type 1. The lungs were grossly normal. The calf developed moderate respiratory distress after challenge, which resolved by 12 hours post-infection.



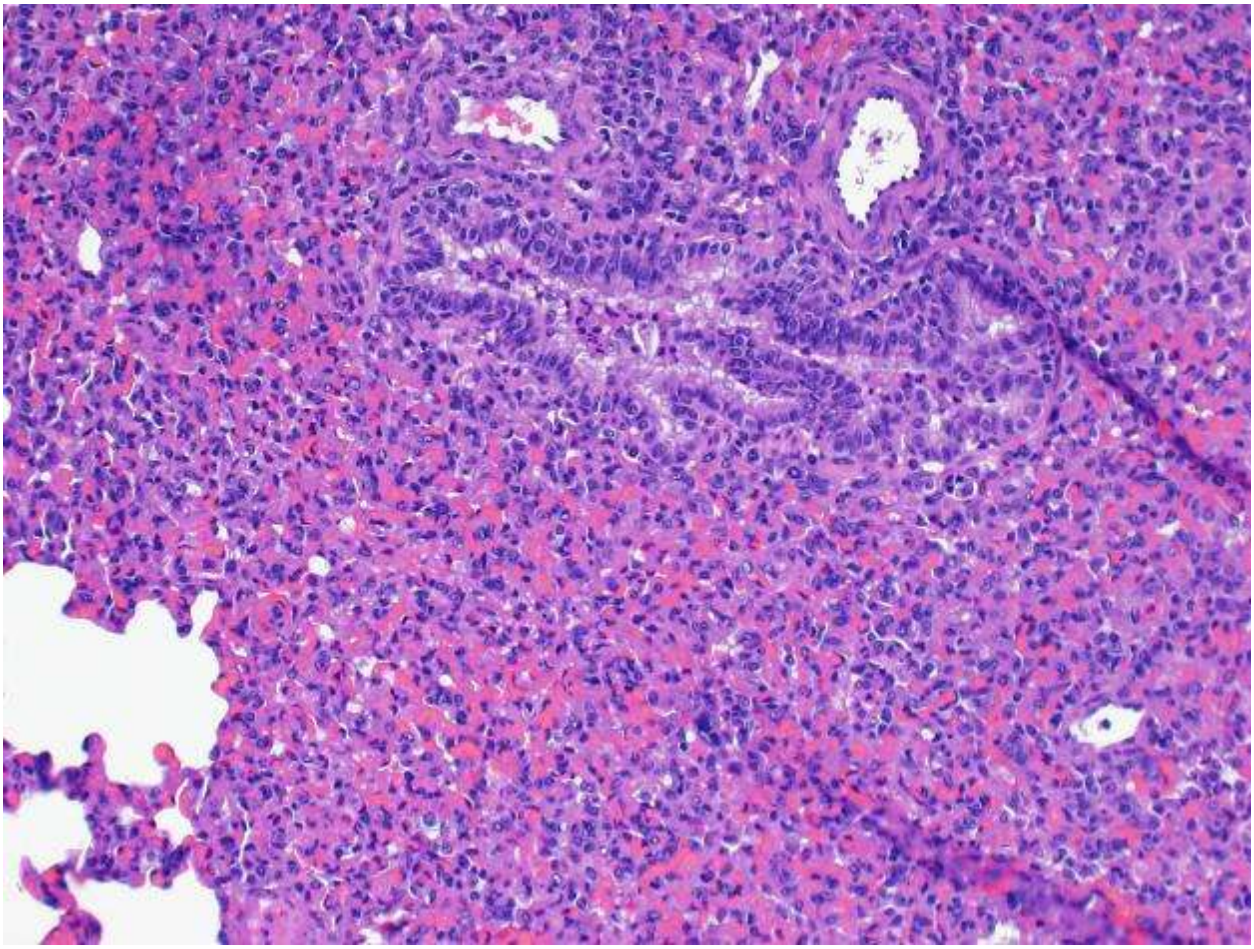
Supplemental Figure S3. 9: Lung, conventionally raised calf (Calf A1), euthanized 3 days after challenge with $1x10^{10}$ colony forming units of aerosolized *Mannheimia haemolytica* serotype 1. The majority of the lungs were histologically normal with rare areas of atelectasis, small amounts of alveolar hemorrhage and neutrophils (A), and occasional bronchioles filled with neutrophils (B). Hematoxylin and eosin.



Supplemental Figure S3. 10: Acute phase proteins following challenge with aerosolized *Mannheimia haemolytica* serotype 2 in clean-catch colostrum-deprived calves (n=2). A) Serum haptoglobin and B) plasma fibrinogen.



Supplemental Figure S3. 11: Gross lung lesions from colostrum-deprived calves challenged with aerosolized *Mannheimia haemolytica* serotype 2. A) Lung, (Calf B2) challenged with 1 x 108 colony forming units of *M. haemolytica* serotype 2 had patchy red mottling affecting less than 1% of the parenchyma. B) Lung, (Calf B3), challenged with 1 x 109 colony forming units of *M. haemolytica* serotype 2. There is a single focal area of consolidation in the cranioventral aspect of the right middle lung lobe. *Mannheimia haemolytica* was not isolated from the lungs.



Supplemental Figure S3. 12: Histologic sections of lung from a colostrum-deprived calf (Calf B3), following challenge with 1 x 10^9 CFU *Mannheimia haemolytica* type 2. There is atelectasis and rare areas with neutrophils and macrophages in alveoli and bronchioles. Oat cells were not present. Hematoxylin and eosin.

4 The effect of aerosolized bacterial lysate on experimentally induced *Mannheimia haemolytica* pneumonia in calves

4.1 Abstract

Bovine respiratory disease (BRD) affects beef cattle, predominantly within the first few weeks after arrival to the feedlot. Suppression of respiratory defenses due to stress and viral infection is considered to be a significant mechanism contributing to the high incidence of disease during this period. The innate immune system is rapidly responsive and broadly protective against a range of pathogens and may represent a potential target for intervention to prevent BRD. This study investigated whether stimulation of pulmonary innate immune responses protected calves against subsequent experimentally induced *Mannheimia haemolytica* pneumonia. Ten clean-catch colostrum-deprived Holstein calves were randomly assigned to receive either aerosolized bacterial lysate or saline 24 hours prior to challenge with *M. haemolytica*. The effects of this treatment on clinical, hematological, microbiological and pathological outcomes were assessed. Compared to controls, lysate-treated calves had lower serum haptoglobin and blood leukocyte and neutrophil concentrations following challenge. Body temperature, heart and respiratory rates, clinical scores and ultrasound lesions did not differ between treatment groups. There were no differences in the number of *M. haemolytica* in the nasal cavity on days 1-3 following challenge, or in lung tissue at postmortem examination. The proportion of diseased lung was similar in lysate-treated and saline-treated calves. Thus, treatment with bacterial lysate prior to *M. haemolytica* challenge appeared to ameliorate early measures of inflammation but did not provide sufficient protection to alter the course of disease.

4.2 Introduction

Bovine respiratory disease (BRD) continues to be the most economically important disease problem in beef cattle. Naïve calves entering the feedlot are at high risk of BRD due to a combination of stress and exposure to novel pathogens including various viruses and bacteria. Although preconditioning programs—including weaning and processing (to reduce stress at the time of arrival) and vaccination (to increase immunity) in advance of shipping—have been shown to reduce BRD, these strategies for disease prevention are not uniformly applied. Many cattle entering the feedlot are highly stressed from management practices such as abrupt weaning, processing, and disruption of social groups; and they are naïve to many of the important BRD pathogens. This results in animals with a high susceptibility to developing BRD and it is thus necessary to implement disease prevention strategies on arrival to mitigate the effects of disease during this high-risk period. Immunization on arrival can aid in the reduction of BRD, however, naïve cattle vaccinated on arrival to the feedlot may not have time to mount an immune response before they are exposed to pathogens during the early, highest risk period for developing disease. Metaphylactic administration of antimicrobials is a common disease prevention strategy employed for high-risk calves on arrival. However, with increasing desire to reduce the use of antimicrobials in food production, additional BRD control strategies are needed. In the absence of pre-conditioning, a useful approach to overcoming the factors which lead to this increased susceptibility to disease may be to target the innate immune system.

Suppression of immune defenses has been considered a key mechanism contributing to the high incidence of BRD in on-arrival beef cattle. Prior studies have shown alterations in various innate immune responses following stress and viral

infection that may represent a mechanism by which these risk factors contribute to the development of pneumonia (Caswell, 2013). For example, bovine viral diarrhea virus type (BVDV) 2 and dexamethasone treatment reduced the induction of tracheal antimicrobial peptide (TAP) in response to lipopolysaccharide (LPS) (Mitchell et al., 2006; M. Al-Haddawi et al., 2007). Infection with parainfluenza, BVDV and bovine respiratory syncytial virus reduce alveolar macrophage function (Liggitt et al., 1985; Liu et al., 1999), and corticosteroids reduce some neutrophil functions (Paape et al., 1981; Roth and Kaeberle, 1981; Bassel and Caswell, 2018). As viral infection and stress are known risk factors for BRD, suppression of innate immune responses may represent a mechanism by which these risk factors cause disease. Additional potential advantages of targeting the innate immune system are the rapidity of the response as well as its ability to provide broad-spectrum protection against various pathogens. The innate immune system can mount responses within hours and could be targeted at the time of arrival to the feedlot. Similarly, the broad protection from a range of pathogens could be beneficial in preventing infection and disease from the various bacteria and viruses that cause BRD complex in the early feeding period. Thus, stimulation of innate immune responses during the high-risk period is a potential strategy to reduce the development of BRD.

In mouse experiments, stimulation of pulmonary innate immune responses prior to pathogen challenge has been shown to prevent or reduce the severity of respiratory disease (S. E. Evans et al., 2011; Paillot, 2013). For example, in mice, aerosolization of non-typeable *Haemophilus influenzae* prior to pathogen challenge was shown to protect against a number of fatal bacterial, viral and fungal challenges, with maximum

protection observed within the first 24 hours (S. E. Evans et al., 2011). Previous experiments in calves (Chapter 2) demonstrated that aerosolization of *Staphylococcus aureus* and *Escherichia coli* lysate induced innate immune responses including mild systemic and pulmonary responses which were characterized by transiently increased rectal temperatures, mild increases in plasma acute phase proteins, recruitment of neutrophils into pulmonary bronchioles and alveoli, and changes in bronchoalveolar lavage fluid cytokines and other proteins related to platelet activation and complement pathways.

As immunosuppression has been considered to be the mechanism by which stress and viral infection lead to the high susceptibility to pneumonia that is recognized in feedlot calves, we hypothesized that stimulation of innate immune responses would thus reduce disease. The purpose of this study was to evaluate whether induction of innate immune defenses could protect against experimentally induced pneumonia caused by *Mannheimia haemolytica*. To evaluate this, 10 clean-catch colostrum-deprived calves were treated by aerosol with either a lysate of killed *E. coli* and *S. aureus* (to stimulate an innate immune response) or saline (control), 24 hours prior to aerosol *M. haemolytica* challenge. The main outcomes of interest were the postmortem evaluation of the proportion of diseased lung and the severity of clinical disease as assessed using clinical scores. Other outcomes were the number of *M. haemolytica* cultured from the lungs at postmortem, as well as antemortem rectal temperature, heart and respiratory rates, concentrations of leukocytes and acute phase proteins in blood, and nasal carriage of *M. haemolytica*.

4.3 Materials and Methods

4.3.1 Animals

Use of animals was approved by the Animal Care Committee of the University of Guelph (AUP #3286) following the guidelines of the Canadian Council on Animal Care. Between May and August 2017, 10 Holstein bull calves were obtained at birth as previously described (Bassel et al. 2019; Chapter 3). Briefly, the calves did not have any contact with the dam, did not receive colostrum, and were removed from the farm immediately following birth. The calves were raised at the Isolation Unit of the Central Animal Facility, University of Guelph in separate controlled-access rooms in isolated groups of 2-3. Calves were fed a 50:50 mixture of commercially available evaporated milk and water for the first 48 hours. From 48 hours, they were fed milk replacer (Grober High Conversion 20/15, Grober Nutrition, Cambridge, Ontario) three times daily. From one week of age, calves were offered hay and unmedicated calf starter pellets (Sharpe Farm Supplies, Guelph, Ontario).

At approximately 4 weeks of age, experiments were conducted in age-matched pairs, with one calf randomly assigned (by coin flip) to receive aerosolized bacterial lysate or phosphate-buffered saline (PBS, as a control). All calves were challenged with *M. haemolytica* one day after receiving the bacterial lysate or PBS (Figure 4.1; Supplemental Table S4.1).

The caregivers and the members of the research team that evaluated the clinical and postmortem findings were blinded to treatment status until final evaluation of samples was completed. A formal sample size calculation was not conducted as there were no data available to predict expected effects of the bacterial lysate on the development of pneumonia in calves. The experiments were terminated after 5

replicates as the magnitude of the observed differences were not sufficient to justify further animal use.

4.3.2 Preparation of Mannheimia haemolytica for challenge

These studies used *M. haemolytica* isolate B158 which was determined to be genotype 2, serotype 1 (Chapter 3). The bacteria were grown to log phase as described (Chapter 3), the concentration was estimated based on the OD_{600}, and the bacteria were washed once in PBS prior to challenge. The targeted challenge dose was 1×10^{10} CFU in a final volume of 10 mL, and the inoculum was kept on ice until the time of challenge. The purity of the *M. haemolytica* inoculum was confirmed by microscopic examination of a Gram-stained sample of broth. For each challenge, the actual challenge dose was later determined by colony counts on blood agar, and a representative colony was analyzed by matrix-assisted laser desorption/ionization—time of flight mass spectrometry (MALDI-TOF) mass spectroscopy (AHL, Guelph, Ontario) to confirm the identity of the *M. haemolytica*.

4.3.3 Delivery of aerosols

On day 0, calves received either 10^9 CFU-equivalents of bacterial lysate in 10 mL PBS (innate immune stimulant) or 10 mL of PBS alone (control) delivered by a compressor (Precision Medical, Northampton, PA) and Whisper Jet nebulizer (Marquest, Englewood, Colo) as previously described (Chapter 2). Between calves, the tubing, nebulizer and masks were rinsed and wiped with 70% ethanol followed by water. Twenty-four hours after administering the lysate, 10^{10} CFU of *M. haemolytica* was delivered to each calf using the same apparatus as described previously (Chapter 3).

4.3.4 Clinical monitoring and antemortem sampling

A baseline physical examination and systematic thoracic ultrasound examination (Ibex Pro, E. I. Medical, Loveland, CO, frequency of 6.2 MHz, depth set at 6 cm, with 18 dB gain—near 31 dB, far 35 dB) were performed to confirm the absence of lung disease, as previously described (Ollivett et al., 2015). Clinical data were collected and recorded by a blinded researcher at baseline and every 12 hours throughout the experiment. At each time point, rectal temperature, heart and respiratory rates, and clinical score were determined as described in Chapter 2. Clinical scores were assigned by totaling the scores for each of the following assessment parameters: strength, demeanor, appetite, and effort of breathing (Chapter 2, Table 2.3). The presence of spontaneous and induced coughing was also recorded. Additional evaluations were performed at 1, 2, 4, 12 and 18 hours after administration of the lysate or saline, and at 1, 2, 4, 12 and 18 hours after administration of *M. haemolytica*, with further evaluations performed as indicated by the severity of disease.

Blood was collected from the jugular vein once daily prior to administration of lysate or saline and continuing until euthanasia. Serum haptoglobin and plasma fibrinogen concentrations, as well as complete blood counts were determined (AHL). Daily systematic thoracic ultrasound examination was performed and the number of regions in each intercostal space (dorsal, middle and ventral) that had consolidated lung lesions were recorded (Appendix 4). The total number of regions with consolidation were added together to determine an ultrasound score for each day of the study (maximum score of 34 excludes dorsal and middle regions located subjacent to the scapula). Baseline leukotoxin antibody levels in serum were determined by Prairie Diagnostic Laboratory (Saskatoon, Saskatchewan). Bilateral deep nasal swabs were

collected at baseline and once daily until euthanasia. Swabs were stored in transport medium and semi-quantitative aerobic bacterial cultures (1+, 2+, 3+ 4+) were performed (AHL) to evaluate the presence of *M. haemolytica*.

4.3.5 Bronchoalveolar lavage fluid sampling and analysis

Bronchoalveolar lavage fluid (bronchoalveolar lavage fluid) was collected from 4/5 saline-treated calves and 4/5 lysate-treated calves one day after administration of saline or lysate as well as (i.e. immediately prior to administration of *M. haemolytica)*. Calves were sedated with xylazine prior to endoscopic collection of bronchoalveolar lavage fluid from the caudodorsal lung. The endoscope was used to infuse 120 mL of sterile 0.9% saline, and lavage fluid was retrieved by manual suction through a 60 mL syringe. The sample was placed on ice until further processing (within 2 hours). Samples were filtered through gauze. Total nucleated cell counts were determined by electrical impedance (Z2 Coulter counter, Beckman Coulter, Mississauga, ON) and differential cell counts were determined from Wright-stained cytocentrifuge preparations using 200 µL of fluid. Additional aliquots were stored at -80°C prior to analysis of cytokine concentrations by an external laboratory (Zoetis, Kalamazoo, Michigan) including interferon alpha (IFN-α), IL-8, IL-2, IL-6, tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-10, and IL-17 using a multiplexed electrochemiluminescent ligand-binding assay with the U-plex assay platform (MSD, Rockville, MD) as previously described (Chapter 2).

4.3.6 Study endpoints and assessment of gross and histologic lesions

On day 4, calves were euthanized by intravenous injection of 102 mg/kg pentobarbital sodium solution (Euthansol®, Merck, Kirkland, Quebec). Prior to this planned endpoint, calves were euthanized if they displayed significant clinical disease

evidenced by dyspnea (effort of breathing score = 3), reduced strength (score ≥ 3), depression (demeanor score ≥ 3), or anorexia at 2 consecutive feedings. No animals died prior to euthanasia. Four of 10 calves were euthanized due to severity of disease on day 2, prior to the planned endpoint. In addition, one calf was euthanized on day 2, at the same time as its matched pair, due to welfare concerns about potential distress from being housed alone. Subsequent experiments were arranged such that 3-4 calves were housed together at the time of challenge so that if one calf required euthanasia, its penmate could continue to the end of the study.

Postmortem examination was performed immediately following euthanasia. The lungs were removed from the thoracic cavity, and the heart and trachea were separated from the lungs. Photographs of the lateral surfaces of the left and right lungs were obtained at the time of postmortem examination and assigned a random number. Blinded evaluation and scoring of gross lung lesions were conducted by a single blinded member of the research team as previously described (Chapter 3). The percentage of lungs with pneumonia was estimated by visual inspection and palpation and confirmed by histologic examination of left and right cranioventral, caudoventral and caudodorsal lung lobes. The heart and lungs were weighed separately for determination of the lung: heart weight ratio. Lung sections from each region were preserved in 10% buffered formalin, processed routinely and stained with hematoxylin and eosin. A second blinded evaluator used ImageJ software (Schneider et al., 2012) to analyze the gross images and compute the proportion of surface area affected by consolidation in the each of the left and right lungs. Formalin-fixed tissues were routinely sectioned and stained with hematoxylin and eosin for histopathology. A blinded evaluator assigned scores (0 =

absent; 1 = present, but rare; or 2 = present, moderate to severe) for each of the following: bronchopneumonia, oat cells (leukocytes with streaming nuclear material), coagulative necrosis, pleuritis, and fibrin.

4.3.7 Quantitative bacterial culture

Standardized samples were collected from the left and right cranioventral, caudoventral and caudodorsal lungs as well as the accessory lobe, targeting lesions when present. Samples were immediately placed on ice and subsequently frozen at -80° C. For quantitative bacterial culture, the pleural surface of each lung sample was seared, and 100 mg of tissue was homogenized in 900 µL PBS. Serial dilutions were prepared, and 100 µL of each dilution were plated on blood agar and incubated at 37° C for 48 hours. Colonies were counted and the total number of CFU per gram of lung tissue was calculated. A swab of kidney from each calf was cultured on blood agar for 48 hours.

4.3.8 Statistical analysis

Paired evaluation of differences between baseline values and single point-in-time outcomes (including postmortem parameters) were examined using paired t tests and Chi-squared tests using statistical software (GraphPad, Prism 8). Simple linear regression was used to correlate various outcomes. Data were tested for normality of distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests and transformed if indicated. Associations were considered significant at *P*-values <0.05.

For evaluation of repeated measures, mixed effects multivariate models were built and analyzed using STATA 15.1 (StataCorp, College Station, Texas, USA). Models included a random intercept for individual animals to account for repeated

measurements taken from the same animal, and for pairs of animals to account for the effects of challenge group. Statistical analysis was performed on time points following *M. haemolytica* challenge up to 2 days after challenge. Statistical analysis of day 3 data was not possible due to non-random missing data resulting from euthanasia of severely affected animals. Mixed-model linear regression was used to evaluate the following outcomes: rectal temperature, heart rate, respiratory rate, clinical scores; concentrations of serum haptoglobin, plasma fibrinogen, blood leukocytes, neutrophils, monocytes and lymphocytes; ultrasound scores; and semiquantitative assessment of nasal *M. haemolytica*. The following data were log-transformed: haptoglobin, fibrinogen, blood leukocytes, and blood neutrophils. In addition to treatment, the day relative to challenge, baseline parameters, and interaction between treatment and day were included, with stepwise removal of non-significant variables ($p > 0.05$).

4.4 Results

4.4.1 Baseline parameters

Prior to administration of the bacterial lysate, there were no significant differences between treatment groups in any of the evaluated parameters including rectal temperature, serum haptoglobin, plasma fibrinogen, and complete blood counts. All calves had low ELISA readings that were considered negative for antibodies to *M. haemolytica* leukotoxin. There was no difference in anti-leukotoxin antibody ELISA readings between the saline- and lysate-treated calves ($p = 0.74$, two-tailed t-test). The mean log of the antibody titers was 2.59 (95% CI: 1.75, 3.43) in lysate-treated calves and 2.48 (95% CI:1.92, 3.05) in control calves. Prior to challenge, none of the submitted nasal swabs yielded *M. haemolytica* on aerobic bacterial cultures. There was no

evidence of consolidation on ultrasound examination of the lungs and no signs of clinical disease.

4.4.2 Response to aerosolized bacterial lysate

Following aerosol delivery, there were no observed clinical signs in the 5 calves that received bacterial lysate or the 5 control calves that received saline. Heart and respiratory rates were not affected by lysate treatment. One day after aerosolization, the lysate-treated calves had a slightly higher mean temperature of 39.5° C (95% CI: 39.1, 39.8) compared to control calves at 39.0° C (95% CI: 38.6, 39.5; p = 0.025). The serum haptoglobin concentrations and blood leukocyte counts were not significantly different at baseline compared to one day post-aerosolization of lysate or saline. One day after administration of aerosols, the lysate-treated animals had a higher concentration of plasma fibrinogen compared to the saline-treated animals (P=0.043).

In the bronchoalveolar lavage fluid, the mean percentage of neutrophils was higher in calves that received aerosolized bacterial lysate (20.0% (95%CI: 9.4, 30.6)) than in calves that received saline (7.3 % (95% CI: 0, 17.1)) (P=0.031; Figure 4.2). A corresponding decrease in the mean percentage of macrophages in lysate-treated calves (77.8% (95% CI: 66.0, 89.5)) compared to the saline-treated calves (91.0% (95% CI: 79.7, 100.0)) was also observed (p = 0.041; Figure 4.2). The percentage of bronchoalveolar lavage fluid lymphocytes was similar in both groups: 2.5% (95% CI: 0, 5.5) in lysate-treated calves, and 1.5% (95% CI: 0.6, 2.4) in saline-treated calves (p = 0.356).

Of the cytokines measured in bronchoalveolar lavage fluid, the mean IL-8 concentration in lysate-treated calves was 20.3 pg/L (95% CI 1.7, 14.0) and in control calves was 7.8 pg/L (95% CI: 0, 40.7); the differen0ce was not statistically significant (p

= 0.11). The mean concentration of IL-10 in lysate-treated calves was 3.5 pg/L (95% CI: 2.6. 4.3) and in control calves was 6.6 pg/L (95% CI: 1.0, 12.2); the difference was not statistically significant (p = 0.13; Figure 4.3). In lysate-treated calves, there was a strong correlation between the log-transformed concentration of IL-8 and the proportion of neutrophils (Supplemental Figure S4.1; R-squared = 0.92; p = 0.042). The additional cytokines that were evaluated in bronchoalveolar lavage fluid were generally below the limit of quantification and were not analyzed: IL-17 (<2.44 pg/mL), IFN-α (<9.8 pg/mL), IL-1β (<9.8 pg/mL), IL-2 (<39.1 pg/mL), IL-6 (<156.3 pg/mL), TNF-α (<39.1 pg/mL), and IFN-γ (<2.4 pg/mL).

4.4.3 Clinical parameters and mortality

All calves showed varying degrees and combinations of clinical signs at one or more time points between administration of the bacterial lysate and euthanasia. Clinical signs included coughing, depression, increased respiratory effort and rate, fever, inappetence and recumbency and were similar between groups. A summary of the prevalence of clinical signs in lysate-treated and control calves by day is shown in Table 4.1. Clinical scores were uniformly zero up to and at the time of challenge with aerosolized *M. haemolytica*. Following *M. haemolytica* challenge, clinical scores in both groups increased over time ($p < 0.003$) (

Figure **4.4**). Plots of individual-animal clinical scores are shown in Supplemental Figure S4.2. Treatment with bacterial lysate prior to challenge did not affect clinical scores relative to those control calves ($p = 0.272$).

In 3/5 control calves and 1/5 lysate-treated calves, the severity of clinical signs led to the decision to euthanize calves prior to the planned endpoint. These calves

showed marked depression, dyspnea, reluctance to rise, inappetence and fever. Additionally, one lysate-treated calf was euthanized at the same time as its ill lysate-treated penmate because of welfare concern for an individually housed calf. At this point, the lysate-treated calf lacked clinical signs and had a normal body temperature of 39.1^{o}C.

The mean rectal temperatures increased in both lysate-treated and control calves following challenge with *M. haemolytica* and remained elevated until euthanasia (Figure 4.5). All calves developed a fever greater than 40°C within 3-9 hours of administration of *M. haemolytica* (Supplemental Figure S4.3), and by 12 hours post-infection the mean rectal temperature was significantly higher than at all previous time points in both groups. Temperatures remained elevated until euthanasia ($p < 0.001$). There was no difference in rectal temperatures between control and lysate-treated calves ($p > 0.05$).

Respiratory and heart rates significantly increased following *M. haemolytica* challenge in both lysate-treated and control calves ($p < 0.05$) (Figure 4.5 & Supplemental Figure S4.3). Lysate treatment did not affect either respiratory or heart rate, as evaluated using multivariate mixed model linear regression with pair and group as random effects. However, respiratory and heart rates changed significantly over time relative to challenge ($p < 0.05$).

All calves had ultrasonographic evidence of pulmonary consolidation in one or more lung regions that was apparent by one day after aerosolization of *M. haemolytica* (Supplemental Figure S4.6). These areas of consolidation persisted until euthanasia, when they were confirmed to correlate with grossly evident pneumonia. Additional areas of consolidation appeared in some calves at later time points (i.e. 2-3 days after *M.*

haemolytica challenge), and some areas of consolidation expanded to encompass adjacent intercostal space regions. There was no difference in ultrasound scores between the 2 treatment groups.

4.4.4 Blood leukocytes and acute phase proteins

Complete blood counts had significant changes at 1 and 2 days following challenge with *M. haemolytica* (Figure 4.6). Total blood leukocyte concentrations increased over time following challenge ($p = 0.001$) due to an increase in blood neutrophils ($p < 0.001$). Calves that received bacterial lysate prior to *M. haemolytica* challenge had significantly lower blood neutrophil concentrations ($p < 0.001$) and total leukocyte concentrations ($p = 0.001$) at 1 and 2 days after challenge. Blood lymphocytes decreased at 1 day following challenge with *M. haemolytica* ($p < 0.01$) in both control and lysate-treated calves, before returning to baseline concentrations at 2 days after challenge. Although lysate-treated calves tended to have higher lymphocyte concentrations at on day 1 after challenge, this difference was not significant ($p = 0.093$).

The concentrations of the acute phase proteins serum haptoglobin and plasma fibrinogen increased over time after *M. haemolytica* challenge (Figure 4.7 & Supplemental Figure S4.4). Treatment with lysate did not affect serum haptoglobin concentrations prior to *M. haemolytica* challenge ($p = 0.28$). Calves that were treated with lysate had lower serum haptoglobin concentrations following *M. haemolytica* challenge compared to control animals ($p = 0.002$). Lysate-treated calves had higher plasma fibrinogen concentrations at baseline and at one day after *M. haemolytica* challenge compared to control calves ($p < 0.05$). The interaction between aerosol treatment and time was significant ($p < 0.05$).

4.4.5 Bacterial culture

No *M. haemolytica* was isolated from any of the nasal swabs prior to challenge. Following challenge, all calves had *M. haemolytica* isolated from 1 or both nasal swabs at multiple time points. Of the 50 nasal swabs that were collected post-challenge, 45/50 (94%) were positive for *M. haemolytica*. Of the post-challenge swabs, only 1/24 collected from a control calf and 4/26 collected from lysate-treated calves did not yield *M. haemolytica.* One lysate-treated calf was positive in both swabs 1 day post challenge, negative at 2 days post challenge, and positive in both swabs (4+) at 3 days post-challenge. There was no difference in nasal carriage of *M. haemolytica* between lysate-treated and control calves (Figure 4.8).

Postmortem bacterial culture yielded *M. haemolytica* in one or more lung lobes from all calves. Of the 68 lung lobes submitted for culture, *M. haemolytica* was isolated from 54 (79%). Quantitative culture yielded bacterial counts that ranged from none detected to 10^9 CFU/gram. The average number of bacteria from all lobes varied between animals but was not significantly affected by lysate treatment ($p = 0.72$; Figure 4.9). There was a strong correlation in the number of bacteria isolated from the different lung lobes tested from the same animal ($p < 0.001$), but the various anatomic locations (i.e. lung lobe) sampled did not significantly differ in the number of bacteria per gram of lung tissue ($p = 0.159$). None of the kidney cultures yielded *M. haemolytica.*

4.4.6 Postmortem lung lesions

All calves developed gross lesions of bronchopneumonia, characterized as a lobular pattern of well-demarcated, red-purple, firm lesions. Additionally, 4/5 control calves and 1/5 lysate-treated calves also had some degree of fibrinous pleuritis. Digital analysis of lung lesions indicated that between 1% and 43% of the lungs were

pneumonic Figure 4.10). The mean percentage of diseased lung in lysate-treated calves was 19% (95% CI: 0, 41.6) compared to 2.5% (95% CI: 0, 5.5) in saline-treated calves. Although there was tendency for a lower percentage of diseased lung in lysate-treated calves compared to control calves, this difference was not statistically significant (p = 0.055). There was no difference in the lung: heart weight ratio between control and lysate-treated calves (p = 0.51). The visual estimates and the digital image analysis estimates for the proportion of diseased lung were strongly correlated (R^2 = 0.82; p = 0.0003). There was a positive correlation between the extent of pneumonia and the average concentration of *M. haemolytica* in lung tissue (R^2 = 0.74, p = 0.001), and between the extent of pneumonia and the log-transformed lung: heart weight ratio (R^2 = 0.611, p = 0.008) (Supplemental Figure S4.7).

Histologic lesions are summarized in Table 4.3. All calves (10/10) had histologic lesions of bronchopneumonia, 5 of 10 had “oat cells”, and 4 of 10 had areas of coagulative necrosis. The lesions were variable among animals, corresponding with the severity of disease identified at postmortem examination. There were no differences in lesions in lysate-treated versus control animals.

4.5 Discussion

There is a strong need to reduce the reliance on antimicrobials in food production. Metaphylactic treatment of high-risk calves is frequently used to reduce the high incidence of bovine respiratory disease and is the most common reason for antimicrobial use in beef feedlot cattle (Brault et al., 2019). As certain respiratory innate immune defenses have been shown to be altered during stress or following viral infection, we hypothesized that stimulating these defenses prior to exposure to BRD

pathogens, such as *M. haemolytica* as used in this study, could be an alternative strategy to prevent disease. We hypothesized that the induced innate immune responses resulting from aerosolization of bacterial lysate would protect against pneumonia resulting from subsequent *M. haemolytica* challenge.

Previous work demonstrated that aerosolization of bacterial lysate increased the concentrations of inflammatory cytokines in bronchoalveolar lavage fluid and resulted in recruitment of neutrophils to the lungs. Neutrophils are highly effective phagocytic cells that are capable of killing bacteria. Similarly, exposure of tracheal epithelial cells to bacterial lysate caused induction of antimicrobial peptides (Chapter 2). Antimicrobial peptides have previously demonstrated bactericidal activity against some of the agents of BRD including *M. haemolytica* (Taha-Abdelaziz et al. 2013; Vulikh et al. 2019). Finally, induction of inflammatory responses has been shown to protect against pneumonia in experimental challenges in mice. Similar to this study, aerosolization of bacterial lysate in mice induced neutrophil recruitment to the lungs (Clement et al., 2008). In several mouse studies, aerosolization of bacterial lysate conferred significant protection against subsequent challenge with various pathogens (Evans et al., 2011; Paillot, 2013). Aerosol treatment of individual animals is not a practical method for stimulation of innate immunity in an agricultural production system, but it is effective for inducing responses that are targeted to the respiratory tract and could therefore be used for proof of concept.

Aerosol administration of the bacterial lysate caused an inflammatory response in calves in this study compared to control animals administered saline, based on comparison of clinical parameters, acute phase proteins, and cytology of

bronchoalveolar lavage fluid. Significant responses included increased proportions of neutrophils in bronchoalveolar lavage fluid, a mild increase in body temperature, and an increase in plasma fibrinogen concentrations relative to control calves. None of the calves showed clinical signs or reduced appetite as a result of this procedure.

An aerosol route for challenge with *M. haemolytica* was chosen to mimic the natural route of exposure, whereas some other *M. haemolytica* challenge models infuse fluid containing suspended bacteria. By using an aerosol challenge, we expected that the bacteria would be dispersed within the epithelial lining fluid and thus more directly exposed to leukocytes and antimicrobial proteins that had been induced by the bacterial lysate. Furthermore, when using an aerosol challenge model, we surmised that these innate immune cells and proteins would not be diluted by an instilled fluid as would occur with intrabronchial inoculation of a bacterial suspension.

This study used clean-catch colostrum-deprived calves raised in isolation to ensure that calves were not infected with *M. haemolytica*, that they did not have antibody against *M. haemolytica*, and that they had no underlying pneumonia. We expected that this would create a uniform study population and reduce the variation in responses to *M. haemolytica* challenge. Antibody against *M. haemolytica* was shown in our earlier experiments to interfere with development of disease, and conventionally raised calves from our source farm had frequent underlying pneumonia that complicated the interpretation of the data. Indeed, all animals in this study demonstrated qualitatively similar clinical signs and hematological responses after aerosol *M. haemolytica* challenge. However, despite attempts to create a uniform study population, there was variability in the severity of disease produced with this challenge model. This is

consistent with what has been reported in Chapter 3 using an aerosol challenge model and in studies using other challenge models.

We did not detect statistically significant differences between lysate-treated and control calves, with respect to the severity of disease that developed after M. haemolytica challenge. Only 1 lysate-treated calf compared to 3 control calves had to be euthanized prior to the planned endpoint, and the mean clinical score was lower in lysate-treated vs control calves at 1 and 2 days post-infection. However, these differences were not statistically significant. Lysate-treated calves did have significantly lower serum haptoglobin concentrations than control calves at 2 days post-infection, and significantly lower blood neutrophil levels on days 1 and 2 post-infection. However, differences in the severity of postmortem lung lesions was not statistically significant. Similarly, the administration of bacterial lysate did not reduce nasal carriage of M. haemolytica after challenge and did not reduce the number of M. haemolytica isolated from the lungs. Thus, the study demonstrates that administration of the bacterial lysate did not exacerbate the disease resulting from *M. haemolytica* challenge, which is addressed further in Chapter 6 (General Discussion) in relation to the findings in Chapter 5. Although increased numbers of experimental animals may have resulted in statistically significant differences in disease outcomes, the findings of this study suggest that administration of bacterial lysate 24 hours prior to challenge in this experimental model did not result in differences in disease outcome that were of sufficient magnitude to warrant further investigation.

All animals that were euthanized on day 2 due to severe clinical signs had postmortem lung lesions that affected more than 10% of the lungs, whereas animals

that survived to day 3 (the planned endpoint of the study) had lesions affecting less than 10% of the lungs. We expect that the calves euthanized at the early time points might have developed more extensive lesions if they had remained alive until the planned endpoint. This is supported by the observation that in all calves, the amount of consolidation detected by ultrasound examination remained stable or worsened over time. Thus, euthanasia on day 2 versus day 3 after *M. haemolytica* challenge might have under-estimated the degree of difference between the study groups. Due to the potential for progression of lesions, the decision was made to follow all calves to the planned end point (when ethically possible) to ensure that any protection persisted to 3 days post-infection.

The failure of all animals to reach the study endpoint also complicated analysis of the repeated measures data. On day 3, graphical representations of the clinical and hematological parameters appeared to show improvement. However, this was due to euthanasia of the more severely ill animals, and therefore only 5 of the original 10 animals (that is, the more mildly affected calves) were represented at this time point. As the other 5 animals were removed for non-random reasons, no statistical analysis could be performed for this time point.

Although nasal *M. haemolytica* were assessed every day following challenge, the bacterial load in the lungs was only measured after euthanasia. It remains possible that early after challenge, the induced innate defenses might have cleared a portion of the bacteria, and had we collected lung tissue earlier after challenge (e.g. less than 24 hours) we might have seen a reduction in the number of *M. haemolytica* in the lungs. The less severe neutrophilia and serum haptoglobin concentrations suggest that lysate-

treated calves had reduced induction of inflammatory responses, potentially due to fewer viable bacteria in the early stages after infection. However, if lysate administration did lead to a reduction in bacterial numbers soon after infection, it was not sufficient to clear enough of the bacteria to prevent pneumonia, nor to prevent the bacterial numbers from later increasing. Thus, even if there were partial reduction in *M. haemolytica* loads soon after challenge, this did not appear to confer a meaningful difference at the study's endpoint.

Calves with higher mean *M. haemolytica* bacterial counts in the lungs had a higher percentage of lungs affected by consolidation. However, the other calves with less extensive consolidation also had numerous *M. haemolytica* isolated from the lungs. The number of *M. haemolytica* bacteria per gram of tissue did not vary by lung lobe, while consolidation was primarily cranioventral. These findings are consistent with those reported in Chapter 3 and imply that the presence of bacteria is not the only determinant of why bronchopneumonia tends to affect the cranioventral part of the lung. This observation conflicts with the commonly held belief that gravitational settling of bacteria within the cranioventral lung lobes is the reason that bronchopneumonia selectively affects this area of the lung.

Prior to conducting this experiment there was some concern that recruitment of neutrophils would worsen disease due to the well-known virulence factor leukotoxin that is produced by *M. haemolytica* (Singh et al., 2010). At high concentrations, this exotoxin binds to the β2-integrin CD11a/CD18 (LFA-1) and subsequently creates pores in the cell membrane that lyse neutrophils and macrophages, releasing tissue-damaging cellular contents and resulting in formation of neutrophil extracellular traps (Aulik et al.,

2010). An in vitro study demonstrated that prior exposure to *M. haemolytica* lipopolysaccharide enhanced LFA-1 expression and worsened leukotoxin-induced cytotoxicity (Leite et al., 2003). As well, depletion of neutrophils prior to experimental challenge ameliorated disease (Slocombe et al., 1985; Breider et al., 1988). However, in the current study, aerosolization of bacterial lysate led to recruitment of neutrophils to the lung, but this was not associated with a worse disease outcome compared to the saline-treated control group. This might be related to the timing of exposure to *M. haemolytica* and potentially to the activation state of the neutrophils. In this study, the neutrophils were recruited to the lungs prior to *M. haemolytica* challenge, whereas in naturally occurring pneumonia it is presumed that the neutrophils are recruited after the bacteria have become established in the lungs and started producing leukotoxin.

There are few experimental challenge studies investigating the effect of immune stimulation or modulation on development of *M. haemolytica* pneumonia. In one study, calves were treated with bovine granulocyte colony-stimulating factor prior to challenge with *M. haemolytica* (Youssef et al., 2004). The treated calves had neutrophilia prior to challenge and were afforded some protection against disease (Youssef et al., 2004), although the low number of calves per group limited the reliability of the conclusions. In another study, calves that were treated with dexamethasone around the time of *M. haemolytica* challenge had reduced disease scores and postmortem lesions of pneumonia (Malazdrewich et al., 2004b). Thus, the impact of neutrophils on the progression of respiratory disease might depend on the timing of their recruitment relative to bacterial challenge, and their effects are further confounded by the

neutrophil's multiple roles as a protector against bacterial survival, as a contributor to lung dysfunction, and a basis for the pathologic diagnosis of bronchopneumonia.

Although administration of bacterial lysate induced innate immune responses characterized by expression of antimicrobial peptides in tracheal epithelial cells and neutrophil recruitment to the lungs (Chapter 2), this did not protect from disease following aerosol challenge with *M. haemolytica*. It is possible that bacterial lysate could provide greater protection if the magnitude of the bacterial challenge had been lower. However, previously published experiments using a similar experimental model that administered lower numbers of *M. haemolytica* resulted in inconsistent isolation of M. haemolytica from the lungs at postmortem examination, and occasionally minimal lesions of pneumonia (Vulikh et al. 2019). We also considered whether experimental challenge at a different timepoint following lysate administration could result in better protection. Specifically, if the immune response induced by the lysate was transient and was downregulated by 24 hours, then it is possible that challenge at an earlier timepoint after administration of the lysate would be more protective. Finally, it is well recognized that there are significant animal-level risk factors such as stress and viral infection that alter innate immune responses and play a significant role in *M. haemolytica* pathogenesis (Caswell, 2013). We did not reproduce these underlying risk factors in this experimental infection model, but these factors (stress and virus-infection) could potentially influence the disease outcomes following *M. haemolytica* challenge. Thus, it is possible that calves at high risk of respiratory disease might show greater benefit from innate immune stimulation due to their altered immune status.

4.6 Conclusions

Aerosolization of bacterial lysate prior to *M. haemolytica* challenge induced an immuno-inflammatory response but did not provide a clinically meaningful or statistically significant level of protection from pneumonia under the conditions used in this study. Lysate-treated calves had lower blood neutrophil numbers and serum haptoglobin concentration and increased plasma fibrinogen concentration following *M. haemolytica* challenge. Lysate treatment did not reduce nasal or lung *M. haemolytica* loads. Pulmonary neutrophil recruitment prior to challenge did not worsen disease, despite the potential for leukotoxin production by *M. haemolytica* to exacerbate the disease. Further work is necessary to determine whether the induction of innate immune responses could be protective in high-risk populations of calves.

4.7 Reference List

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4.8 Tables

Table 4.1: Clinical signs in control and lysate-treated calves at different times relative to challenge. Calves were aerosolized with either bacterial lysate (n=5) or saline (n=5) at day -1, and then challenged with aerosolized *M. haemolytica* at day 0.

	Day -1 to day 0		**Day 0 to day 1**		**Day 1 to day 2**	
	Control	**Lysate**	**Control**	**Lysate**	**Control**	**Lysate**
Coughing	0	0	4	4	2	3
Depression	0	0	3	5	4	3
Loss of appetite	0	0	1	2	3	1
Increased respiratory effort	0	0	4	5	4	4
Decreased strength	0	0	2	2	4	2

Table 4.2: Results of multivariate mixed model analysis of acute phase proteins and hematologic values in clean-catch colostrum-deprived calves following aerosol challenge with *Mannheimia haemolytica.* Final model coefficients (β), 95% confidence intervals (CI), and *P*-values are shown for statistically significant (*P*<0.05) variables of interest.

Outcome			β coefficient of effect	95% CI		*P*-value
Serum haptoglobin (log-transformed)	Treatment	Control[a]	ref			
		Lysate	-0.705	-1.147	-0.264	0.002
	Time after challenge	Day 1[b]	ref			
		Day 2	0.468	0.093	0.844	0.015
	Baseline	Day 0	-0.460	-0.806	-0.113	0.009
Plasma fibrinogen (log-transformed)	Treatment	Control	ref			
		Lysate	0.397	0.090	0.704	0.011
	Time after challenge	Day 1	ref			
		Day 2	0.268	0.185	0.350	<0.001
	Interaction	Treatment* time	-0.139	-0.257	-0.020	0.022
Blood leukocyte count (log-transformed)	Treatment	Control	ref			
		Lysate	-0.360	-0.572	-0.147	0.001
	Time after challenge	Day 1	ref			
		Day 2	0.161	0.053	0.269	0.004
	Baseline	Day 0	1.025	0.521	1.529	0
Blood neutrophil count (log-transformed)	Treatment	Control	ref			
		Lysate	-0.690	-1.011	-0.369	<0.001
	Time after challenge	Day 1	ref			
		Day 2	-0.299	-0.465	-0.132	<0.001
	Baseline	Day 0	0.251	0.117	0.384	<0.001
Blood lymphocyte count	Treatment	Control				
		Lysate	0.538	-0.090	1.166	0.093
	Time after challenge	Day 1	0.000			
		Day 2	-0.852	-1.480	-0.224	0.008

[a]Saline-treated (control) calves were the referent category in each analysis
[b]Day 1 following *M. haemolytica* challenge was the referent category

Table 4.3: Histologic findings in control and lysate-treated calves following *Mannheimia haemolytica* challenge. Calves (n=10) were assigned a score by a blinded evaluator for the severity of histologic lesions. For each category, severity scores were assigned as follows: 0 = absent; 1 = present, but rare; or 2 = present, moderate to severe.

Treatment	Pair	Broncho-pneumonia	Oat cells	Coagulative necrosis	Pleuritis	Fibrin
Control	1	2	2	2	2	2
	2	1	0	0	1	0
	3	1	0	0	2	0
	4	2	2	2	2	2
	5	2	2	2	2	2
Lysate	1	2	0	0	0	2
	2	1	0	0	0	0
	3	2	0	0	0	2
	4	2	2	2	2	2
	5	2	2	0	0	2

4.9 Figures

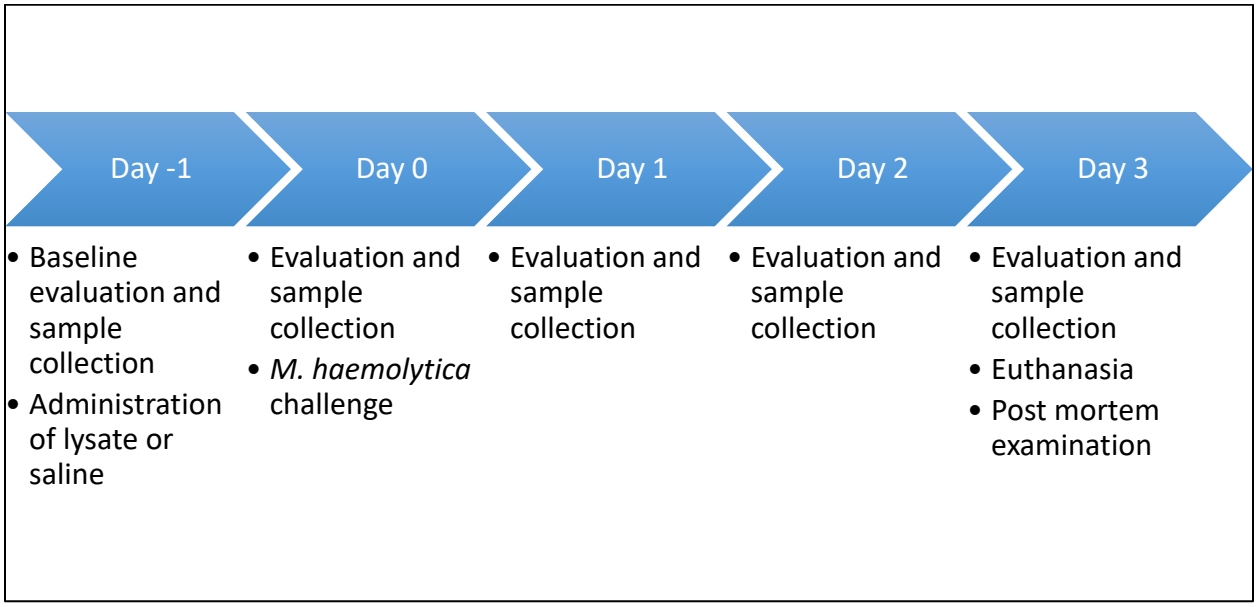


Figure 4.1: Experimental timeline with sampling points. Additional clinical evaluations were conducted every 6 hours.

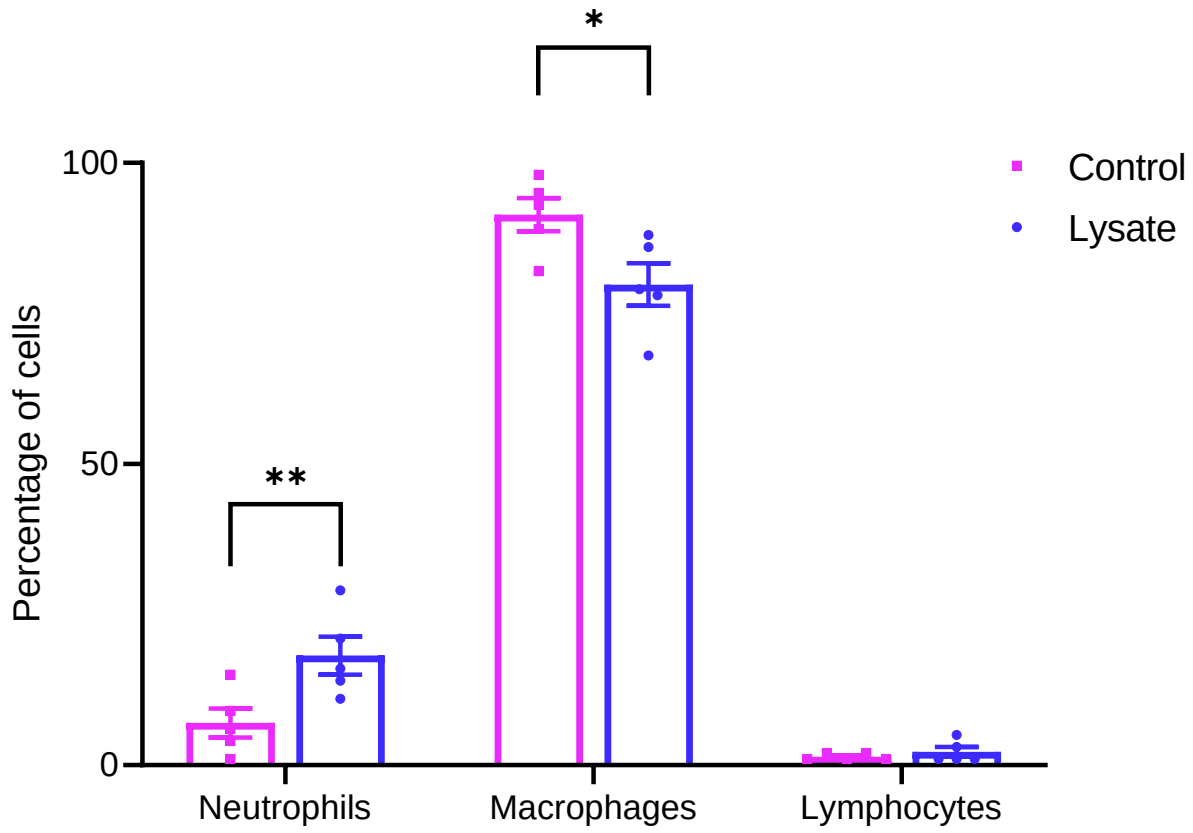


Figure 4.2: Bronchoalveolar fluid cytology in control and lysate-treated calves. The percentage of neutrophils, macrophages and lymphocytes in bronchoalveolar lavage fluid 24 hours after calves received aerosolized bacterial lysate or saline (control). *p < 0.05 , **p < 0.01; (n = 4 per group).

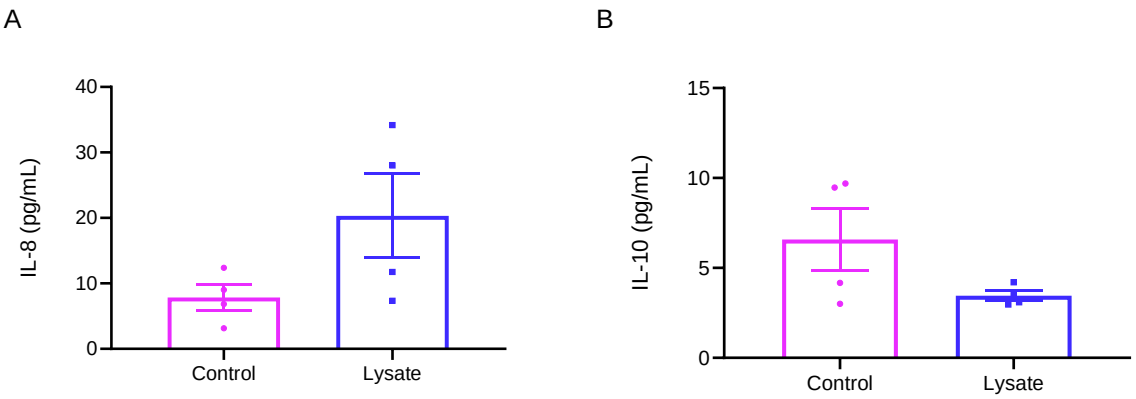


Figure 4.3: Cytokine concentrations in bronchoalveolar lavage fluid sampled at 24 hours following aerosolization of bacterial lysate or saline (controls) (n=4).

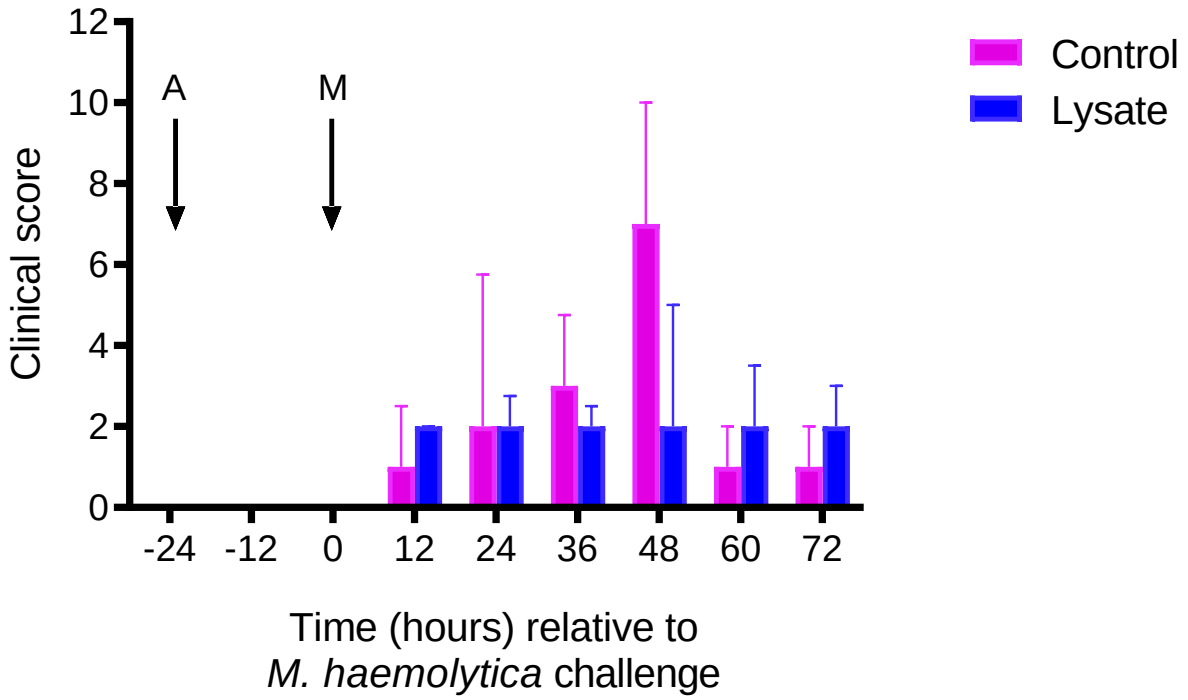


Figure 4.4: Clinical scores over time relative to challenge with *Mannheimia haemolytica.* Five pairs of calves received aerosolized bacterial lysate or saline at time -24h (arrow A). All calves received aerosolized *M. haemolytica* at time 0h (arrow M). Calves were examined every 12 hours. Clinical scores were assigned based on the sum of individual assigned scores for demeanor (0-4), strength (0-4), appetite (0-3), respiratory effort (0-3) for a maximum score of 14. Based on evaluation of time points 0-48 hours, clinical scores increased following *M. haemolytica* challenge ($p < 0.001$, ANOVA, n = 10) but were not different in calves administered bacterial lysate or saline. Data shown represent the mean and interquartile range for surviving calves. There were 5 surviving calves at 60 and 72 hours post challenge and these timepoints were excluded from the analysis.

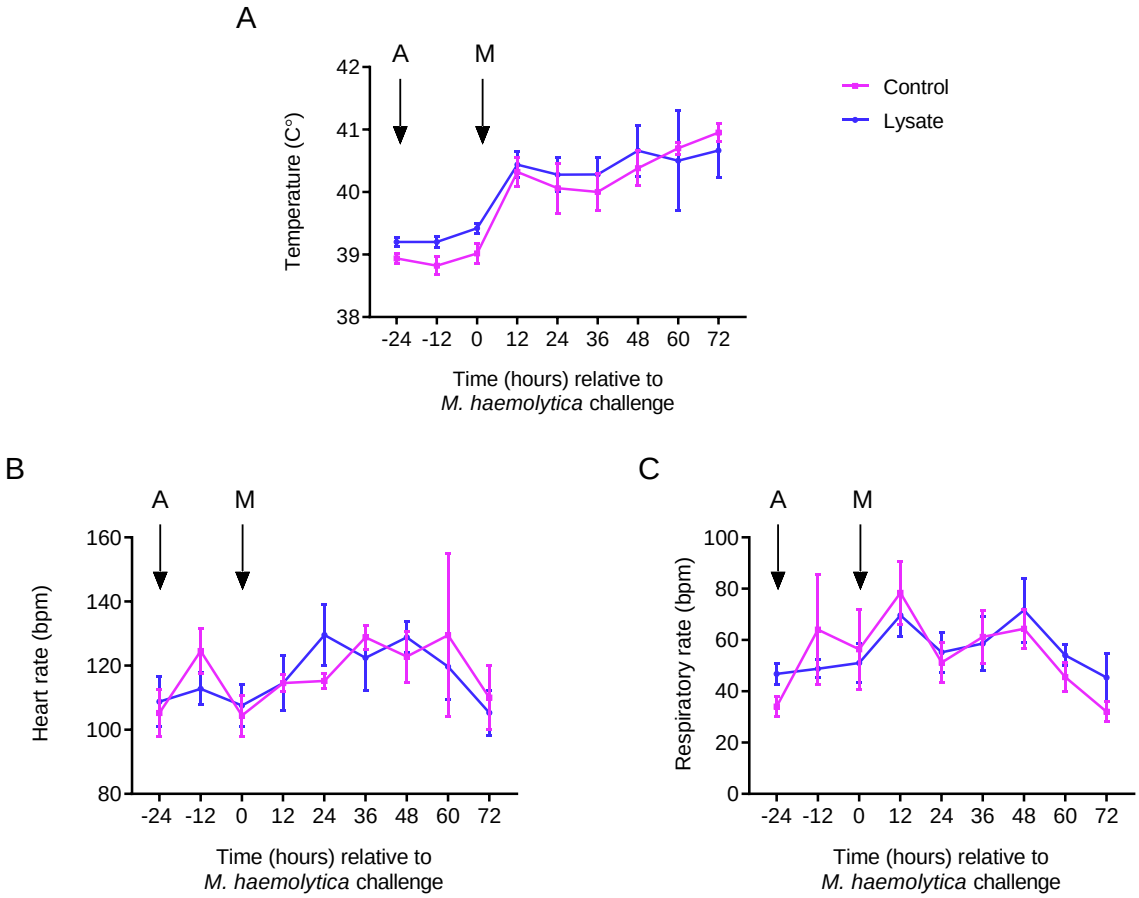


Figure 4.5: Clinical parameters over time relative to challenge with *Mannheimia haemolytica* in control and lysate-treated calves. Five pairs of calves received aerosolized bacterial lysate or saline at time -24h (arrow A). All calves received aerosolized M. haemolytica at time 0h (arrow M). Calves were examined every 12 hours to evaluate: A) body temperature, B) heart rate and C) respiratory rate. Based on evaluation of timepoints 0-48 hours, body temperature significantly increased following *M. haemolytica* challenge with elevations apparent by 12 hours ($p < 0.001$). There were no differences between calves that received bacterial lysate and control calves. Data points represent mean ± SEM. There were 5 surviving calves at 60 and 72 hours post challenge, and these timepoints were excluded from the statistical analysis.

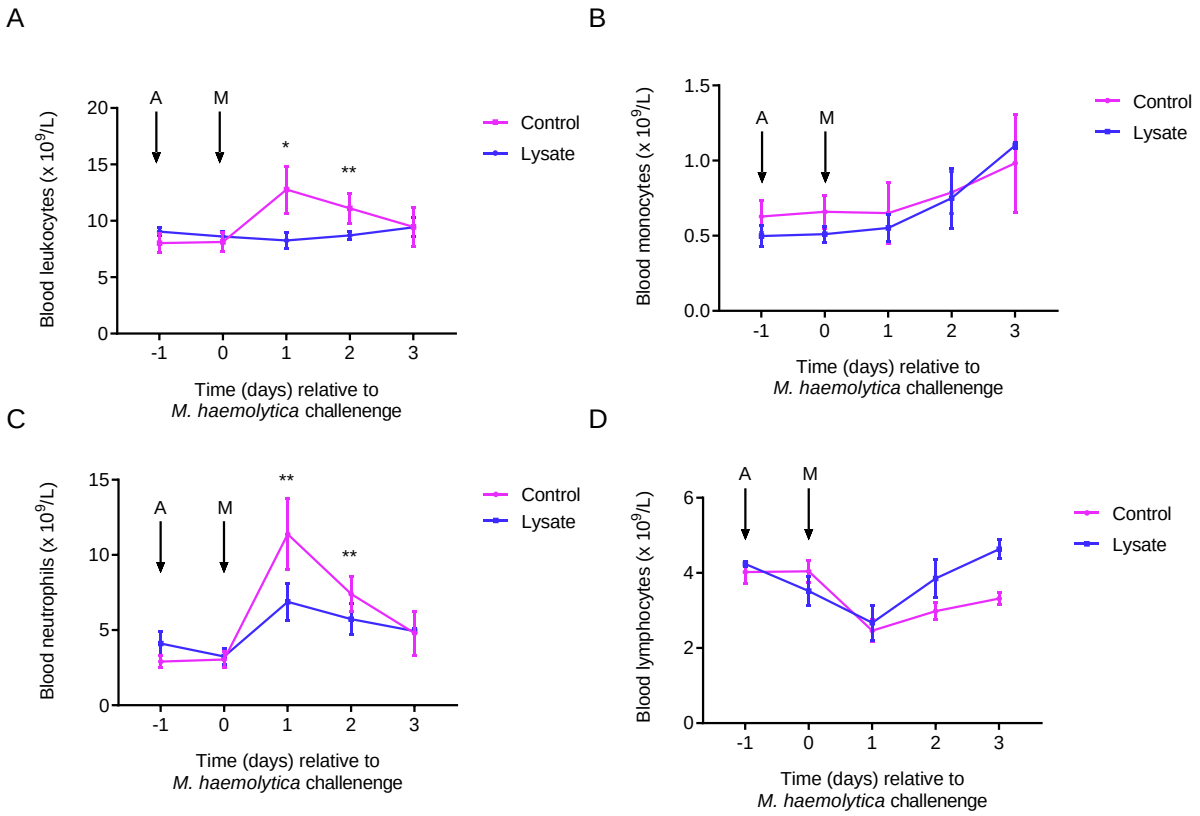


Figure 4.6: Blood leukocytes over time relative to *Mannheimia haemolytica* challenge in calves that were treated with bacterial lysate or saline. Five pairs of calves received aerosolized bacterial lysate or saline at day -1 (arrow A). All calves received aerosolized *M. haemolytica* at time 0 (arrow M). Calves had daily blood collection for evaluation of: A) total leukocytes, B) monocytes, C) neutrophils and D) lymphocytes. Data points represent the mean ± standard error. Significant differences between lysate and control calves are shown (*$p < 0.05$, **$p < 0.01$). There were 5 surviving calves at 3 days post challenge and this timepoint was excluded from the statistical analysis.

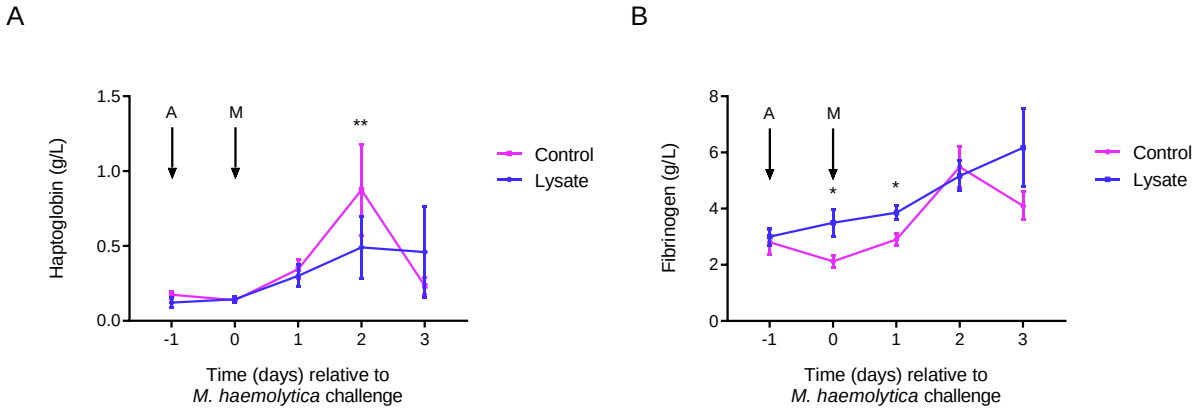


Figure 4.7: Acute phase protein concentrations over time relative to *Mannheimia haemolytica* challenge. Five pairs of calves received aerosolized bacterial lysate or saline at day -1 (arrow A). All calves received aerosolized *M. haemolytica* at time 0 (arrow M). Calves were examined every 24 hours to evaluate: A) haptoglobin and B) fibrinogen. Data points represent the mean ± standard error of the mean. Significant differences between lysate and control calves are shown (**p* < 0.05, ***p* < 0.01). There were 5 surviving calves at 3 days post challenge and this timepoint was excluded from the statistical analysis.

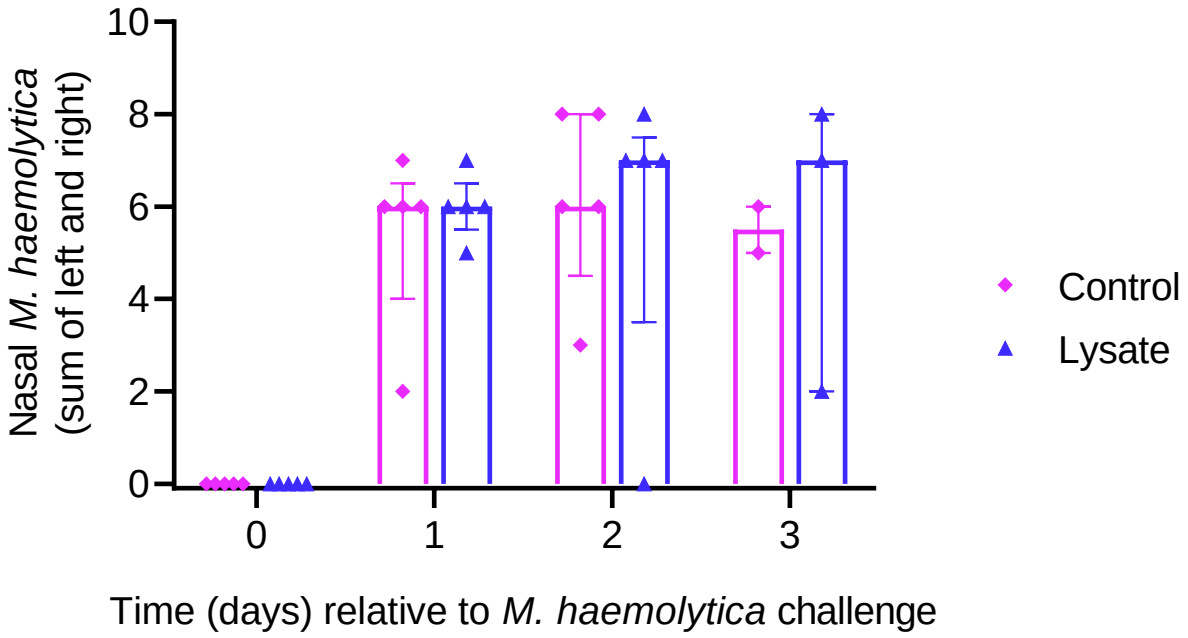


Figure 4.8: Nasal carriage of *Mannheimia haemolytica* following challenge in control and lysate-treated colostrum-deprived calves. Five pairs of calves received aerosolized bacterial lysate or saline at day -1. All calves received aerosolized *M. haemolytica* at day 0. The number of *M. haemolytica* colonies isolated by aerobic culture of left and right nasal swabs were semi-quantified as 0, 1+, 2+, 3+, 4+, and the individual data points show the sum of these scores (maximum score = 8). The bars and error bars show the median ± interquartile range for control and lysate-treated calves.

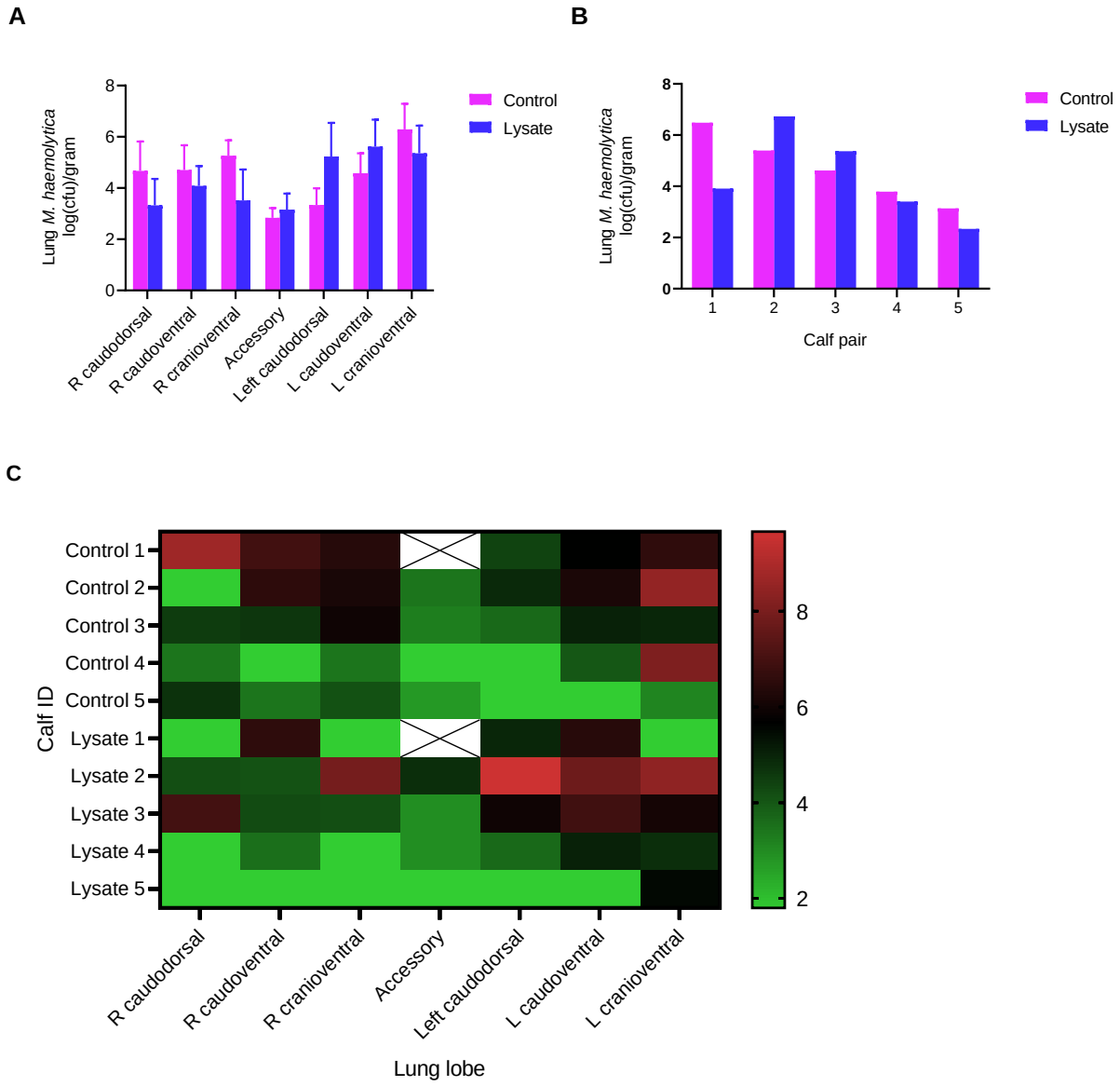


Figure 4.9: Quantitative culture of *Mannheimia haemolytica* from the lung of calves following challenge (n = 5 per group). Five pairs of calves received aerosolized bacterial lysate or saline 1 day prior to aerosolized *M. haemolytica* challenge. Quantitative culture was performed on individual lung lobes. A) *M. haemolytica* concentrations (CFU/g) by lung lobe in control and lysate-treated calves. Data shown represent mean ± standard error of the mean. B) Pairwise comparison of mean *M. haemolytica* concentrations for all lung lobes in control and lysate-treated calves. C) Heat map representation of mean *M. haemolytica* concentrations by calf and lung lobe. Lobes not sampled are represented by an X in Figure 4.9.C.

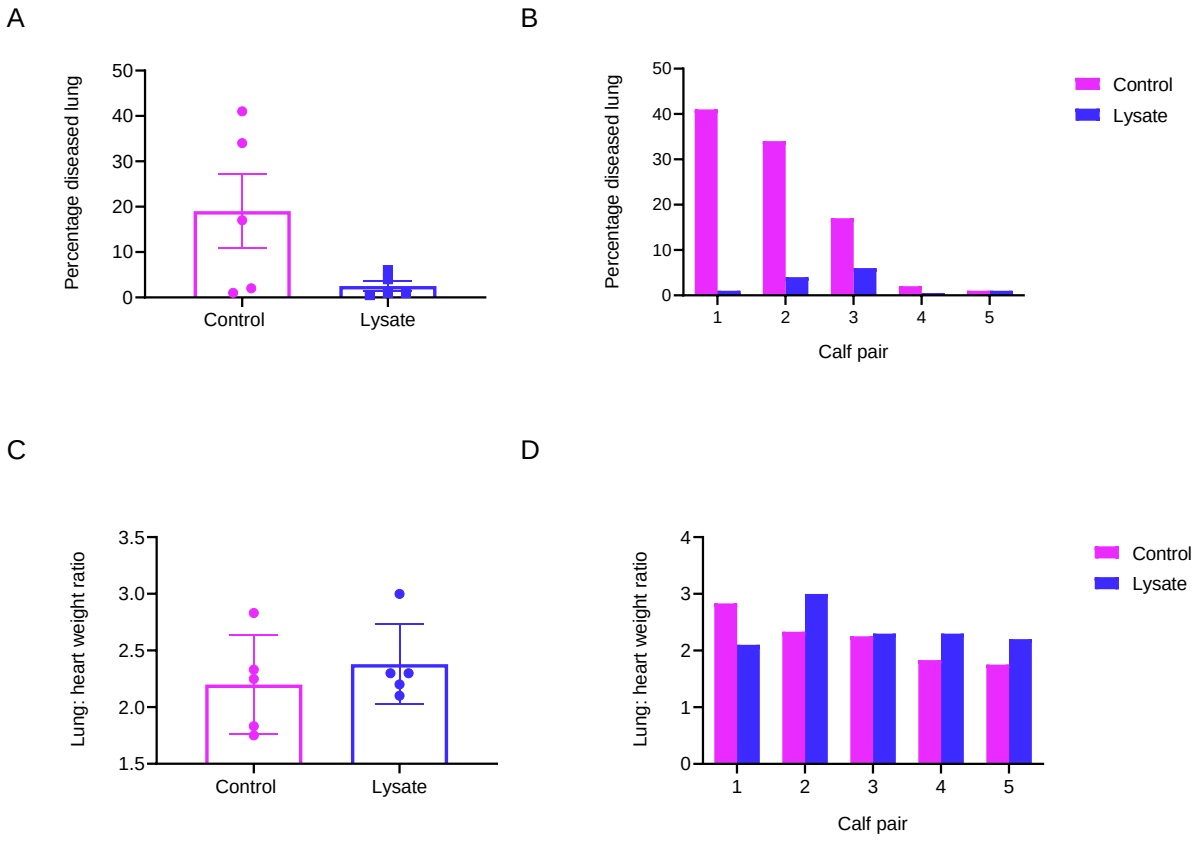


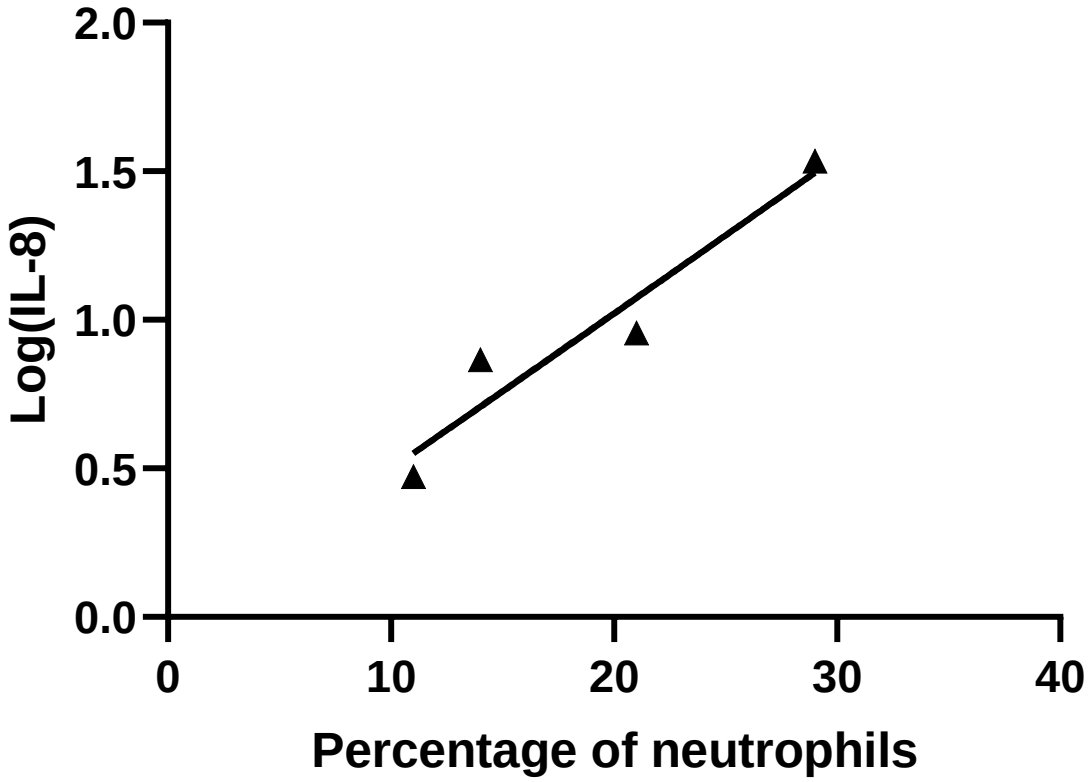
Figure 4.10: Severity of lung lesions at postmortem examination in control and lysate-treated calves following challenge with *Mannheimia haemolytica.* A, B) The percentage of diseased lung identified at postmortem examination based on image analysis. C, D) The lung: heart weight ratio. A and C show individual-animal data in lysate-treated and control animals; the bars show mean ± SEM, n=5 per group. B and D show individual-animal data with matching of calf by pair.

4.10 Supplemental Material

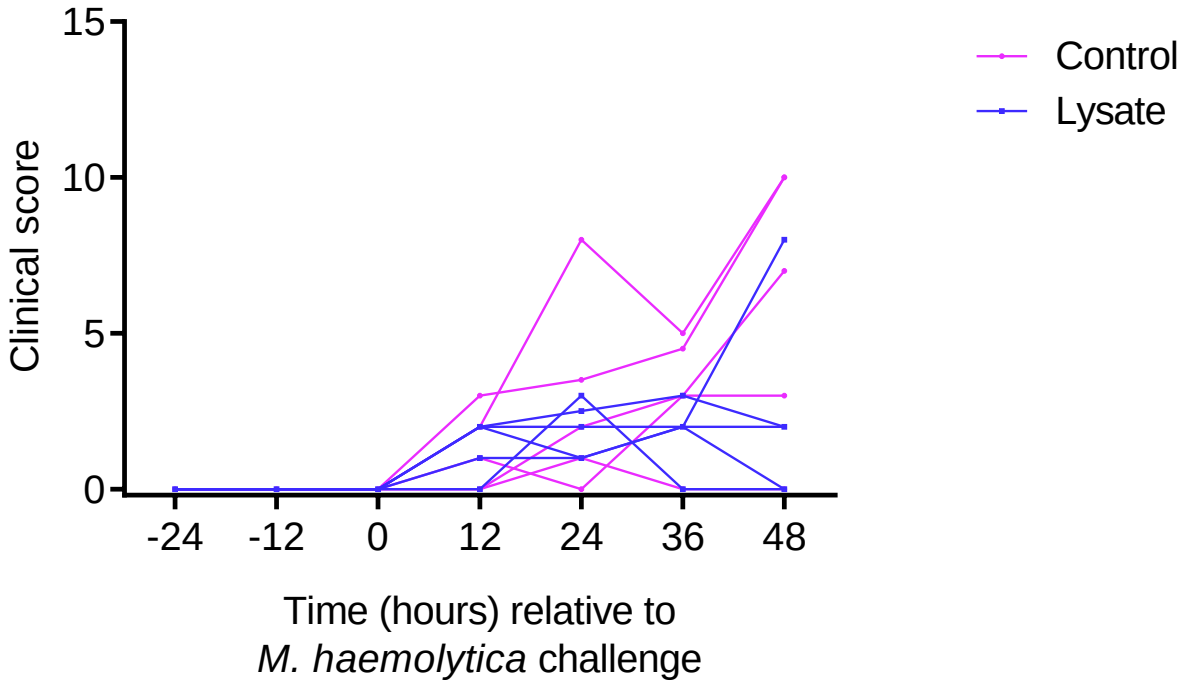
Supplemental Table S4.1: A record of ear tags, names, date of birth, calving ease and treatment.

Calf ID Ear tag/name	Date of birth	Calving Notes	Treatment	Leukotoxin titre[a]	Challenge dose
C1 Dandy/1586	27-Apr-17	Easy pull	Control	700	$8.6x10^{10}$
L1 Smurf/1587	10-May-17	Unassisted	Lysate	1300	$8.6x10^{10}$
L2 Diego/1593	30-May-17	Hard pull	Lysate	300	$5.4x10^{10}$
C2 Pedro/1595	1-Jun-17	Dystocia with C-section	Control	900	$5.4x10^{10}$
C3 Dock/1597	7-Jun-17	Dystocia with C-section	Control	100	$5.4x10^{10}$
L3 Benny/1598	9-Jun-17	Easy pull	Lysate	100	$5.4x10^{10}$
L4 Sebastian/1599	13-Jun-17	Easy pull	Control	400	$7.4x10^{9}$
C4 Finn	21-Jun-17	Dystocia with C-section	Lysate	200	$7.4x10^{9}$
C5 Elvis/1615	22-Jul-17	Easy pull	Control	N/A[b]	$2.2x10^{9}$
L5 Jerry Lee/1624	21-Jul-17	Easy pull	Lysate	N/A[b]	$2.2x10^{9}$

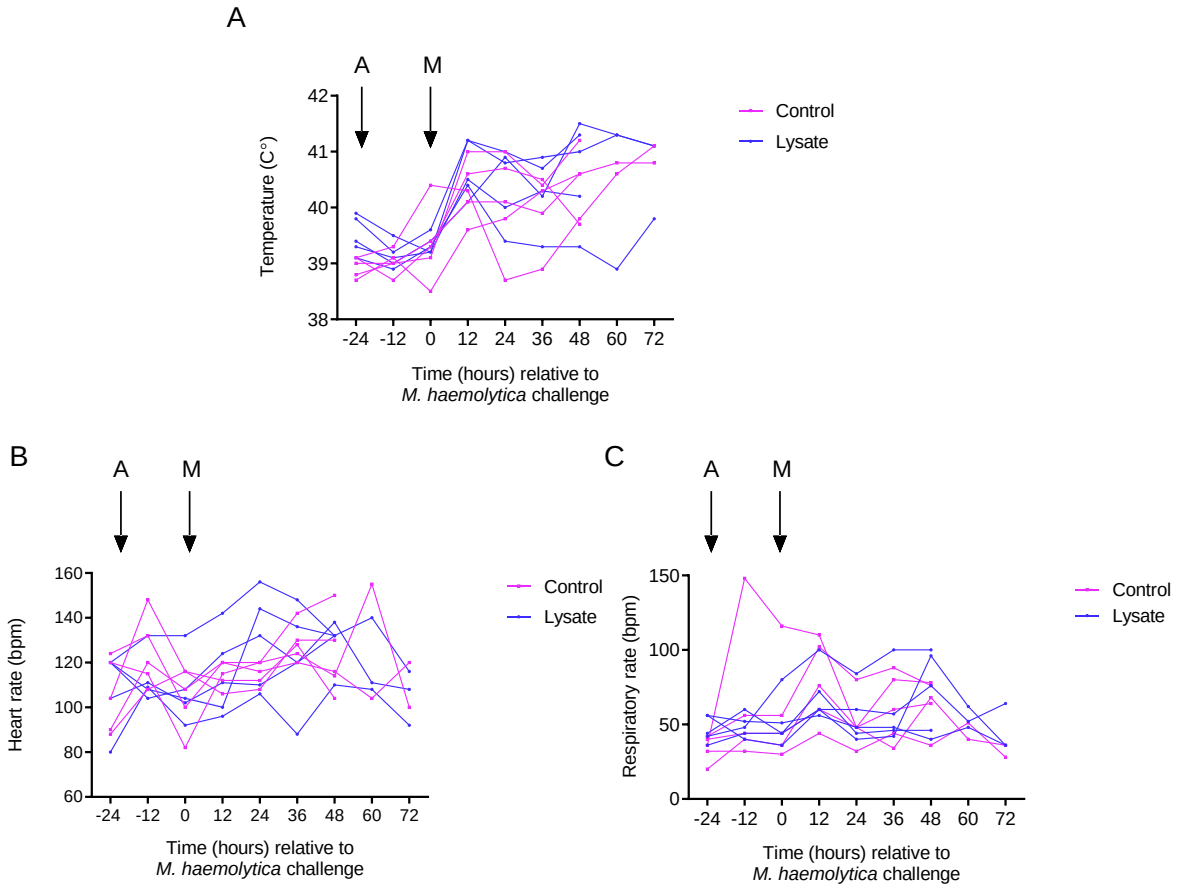
[a] Antibody levels to *Mannheimia haemolytica* leukotoxin determined by ELISA at Prairie Diagnostic Services, Saskatoon, Saskatchewan. Reported reference values range from <100 to >100000 and specific positive cut-off values have not been reported.
[b] Samples lost by lab. Leukotoxin antibody levels are not available.



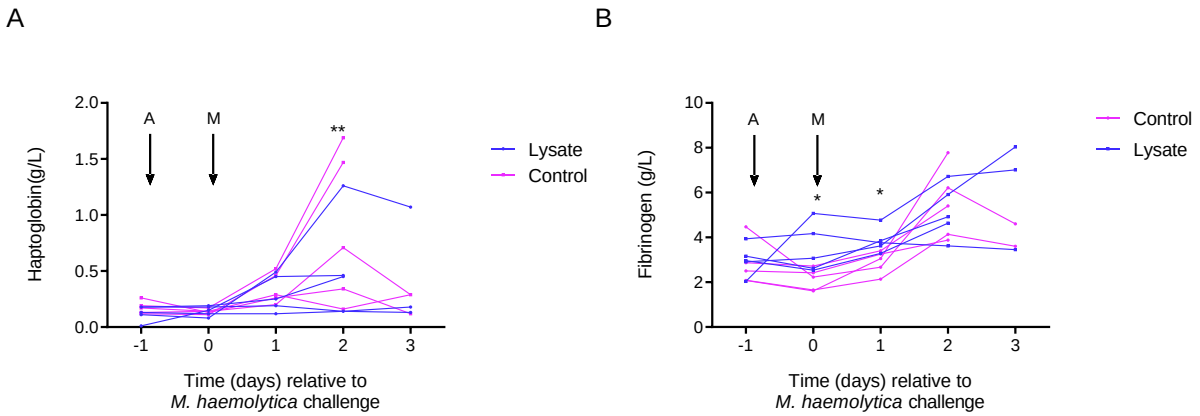
Supplemental Figure S4.1: The proportion of neutrophils is correlated with the concentration of IL-8 in in bronchoalveolar lavage fluid of lysate treated calves (n = 4) (R2 =0.92' *p* = 0.042).



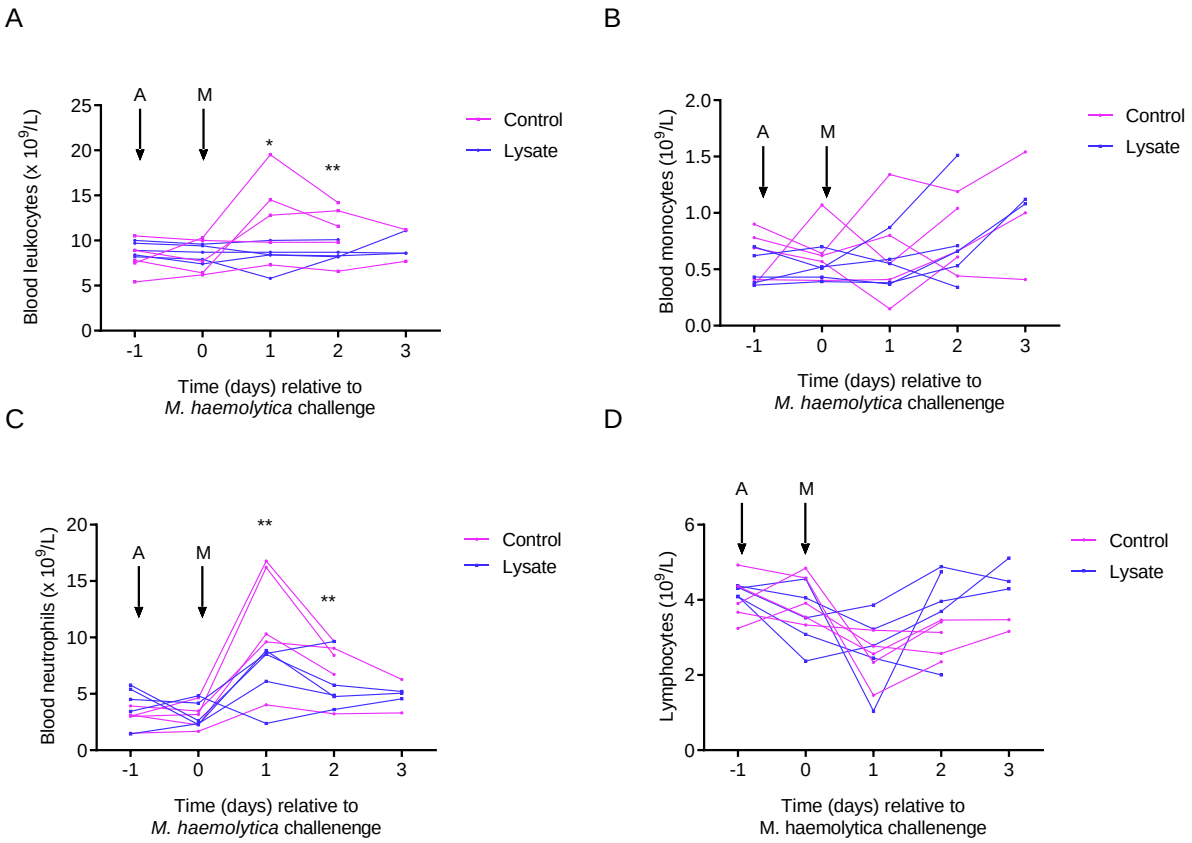
Supplemental Figure S4.2: Clinical scores over time relative to challenge with *Mannheimia haemolytica.* Five pairs of calves received aerosolized bacterial lysate or saline at time -24h (arrow A) (n=5 per group). All calves received aerosolized *M. haemolytica* at time 0h (arrow M). Calves were examined every 12 hours. Clinical scores were assigned based on the sum of individual assigned scores for demeanor (0-4), strength (0-4), appetite (0-3), respiratory effort (0-3) for a maximum score of 14.



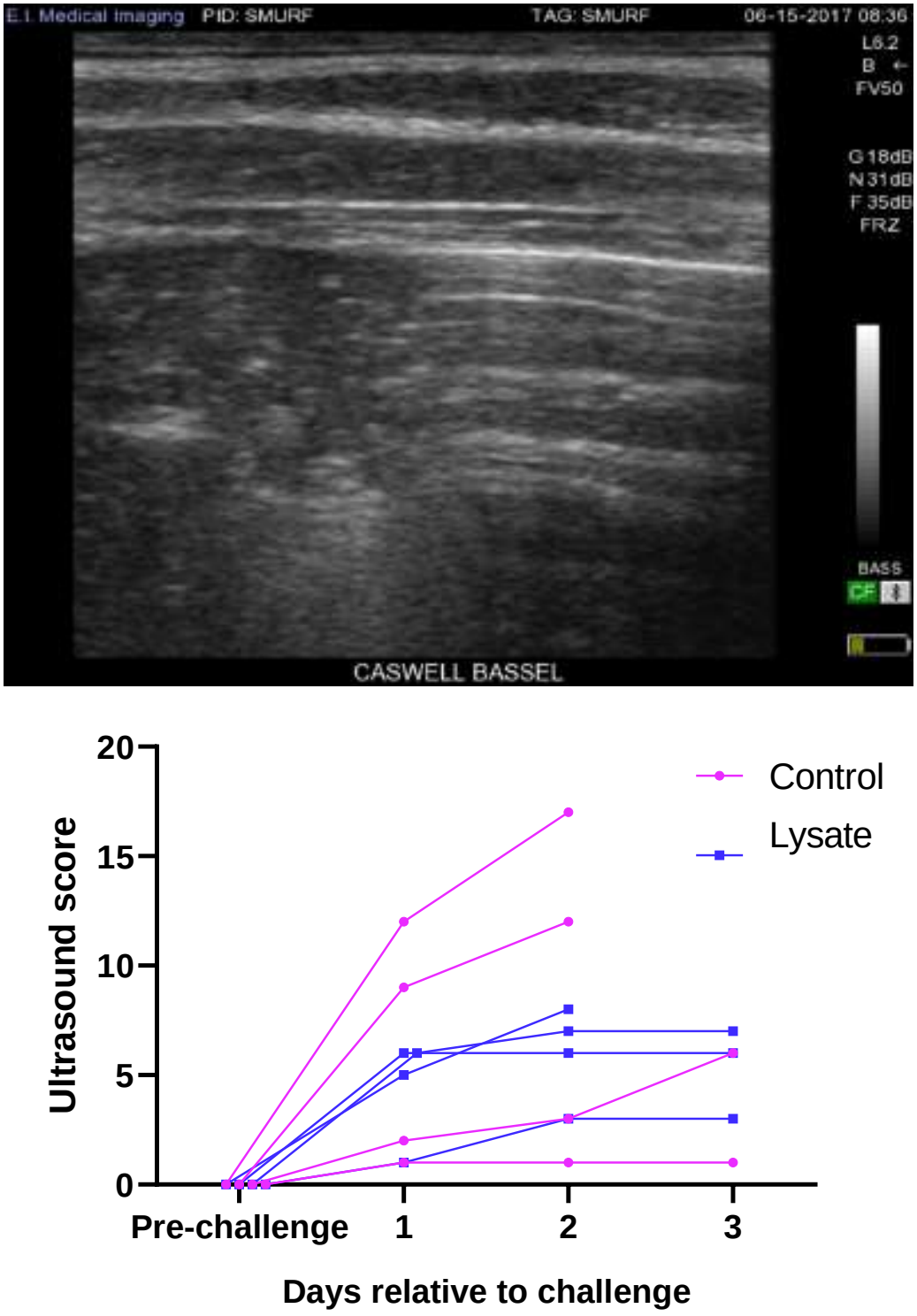
Supplemental Figure S4.3: Individual animal clinical parameters over time relative to challenge with *Mannheimia haemolytica* in control and lysate-treated calves. Five pairs of calves received aerosolized bacterial lysate or saline at time -24h (arrow A) (n=5 per group). All calves received aerosolized M. haemolytica at time 0h (arrow M). Calves were examined every 12 hours to evaluate: A) body temperature, B) heart rate and C) respiratory rate.



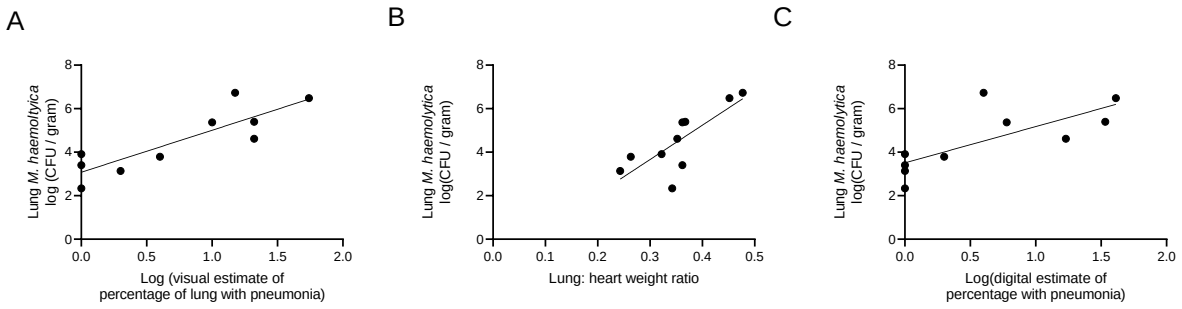
Supplemental Figure S4.4: Acute phase protein concentrations over time relative to *Mannheimia haemolytica* challenge. Five pairs of calves received aerosolized bacterial lysate or saline at day -1 (arrow A). All calves received aerosolized *M. haemolytica* at time 0 (arrow M). Calves had daily blood collection for evaluation of: A) haptoglobin and B) fibrinogen. *$p < 0.05$, **$p < 0.01$; (n = 5 per group).



Supplemental Figure S4.5: Individual-animal blood leukocytes over time relative to *Mannheimia haemolytica* challenge in calves that were treated with bacterial lysate or saline. Five pairs of calves received aerosolized bacterial lysate or saline at day -1 (arrow A) (n = 5 per group). All calves received aerosolized *M. haemolytica* at time 0 (arrow M). Calves had daily blood collection for evaluation of: A) total leukocytes, B) monocytes, C) neutrophils and D) lymphocytes.



Supplemental Figure S4.6: Thoracic ultrasound following challenge with *Mannheimia haemolytica* in colostrum-deprived calves. A) Ultrasound evidence of consolidation (left of image) at 2 days after challenge with *M. haemolytica*. B) Individual-animal ultrasound scores over time (maximum score 34) (n = 8).



Supplemental Figure S4.7: Relationship among 3 quantitative measures of gross lung lesions and the number of *Mannheimia haemolytica* isolated from lungs. A) Log-transformed mean colony forming units (CFU) of *M. haemolytica* in lungs versus visual estimation of the percentage of lungs with pneumonia ($R^2 = 0.741$, $p = 0.001$). B) Log-transformed mean CFU of *M. haemolytica* in lungs versus the lung to heart weight ratio ($R^2 = 0.8610$, $p = 0.001$). C) Log-transformed mean CFU of *M. haemolytica* in lungs versus digital estimate of percentage with pneumonia ($R^2 = 0.561$, $p < 0.013$).

5 Stimulation of respiratory innate immune responses worsens naturally occurring respiratory disease in high-risk feedlot calves

5.1 Abstract

Bovine respiratory disease (BRD) is a major problem affecting beef cattle after arrival to feedlots. Alternatives to antibiotics are needed for prevention of BRD, such as by enhancing innate immune responses that are broadly protective against BRD pathogens. This study investigated whether stimulation of pulmonary innate immune responses on arrival to a feedlot would reduce the impact of BRD. Sixty high-risk feedlot calves were randomly assigned to receive saline or a lysate of *Staphylococcus* aureus and *Escherichia coli*, by aerosol at 16 hours after arrival. The calves were monitored for 28 days for body weight, body temperature, serum haptoglobin, plasma fibrinogen, targeted thoracic ultrasound for lung consolidation, PCR on nasal swabs (*Mycoplasma* bovis and *Mannheimia haemolytica*), morbidity and mortality. Unexpectedly, aerosol administration of bacterial lysate resulted in lower weight gain. Lysate-treated calves had higher body temperatures on days 4 and 21 compared to control calves. At the end of the study, all 29 lysate-treated calves had ultrasonographic pulmonary consolidation compared to 24/29 (83%) control calves. The number of *Mycoplasma bovis* in the nasal cavity increased significantly over time and was higher in lysate-treated calves. There was a high mortality rate caused by chronic *M. bovis* pneumonia that was significantly greater in lysate-treated animals (6/29, 24%) compared to controls (1/29, 3%). Thus, aerosol administration of a bacterial lysate exacerbated BRD in healthy beef calves at high risk of disease, suggesting that respiratory tract inflammation might adversely

affect how calves respond to subsequent natural infection with *M. bovis* and other respiratory pathogens.

5.2 Introduction

Bovine respiratory disease (BRD) is the most common and costly disease of beef cattle in North America (Griffin, 1997). The current production system, where calves are finished in feedlots that are remote from their farm of birth, involves a combination of stressful events and pathogen exposure at the time of entry to the feedlot. At this time, calves can be stressed by weaning, transportation, ration changes, castration, dehorning, and vaccination. Furthermore, co-mingling with calves from other sources disrupts social groups and exposes calves to novel viruses and bacteria. These events lead to a predictable period of high risk for respiratory disease after arrival to the feedlot or stocker operation (Sweiger and Nichols, 2010).

Although weaning, vaccination and dietary transitions in advance of transportation and co-mingling have long been recognized to offer protection from BRD and improved production (Macartney et al., 2003; Hay et al., 2016) these practices have not been universally adopted. Vaccination on-arrival is widely practiced, but reduced responses due to stress may reduce vaccination efficacy (Lynch et al., 2010; O'Loughlin et al., 2011) and there may be inadequate time for immunity to develop before animals are exposed to pathogens. As such, 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 considered to be at high risk of BRD. Prevention of BRD is reported to be the biggest reason for antimicrobial use in feedlots representing 90% of

individually dosed antimicrobials and 40% of in-feed antimicrobial use (Brault et al., 2019).

There is increasing public scrutiny of antimicrobial use in agriculture due to concerns over the risk of development of antimicrobial resistance. The reported level of antimicrobial resistance in BRD pathogens varies, but multidrug-resistant bacteria have been identified in BRD mortalities at levels up to 45% (Klima et al., 2014). Furthermore, the prevalence of nasopharyngeal or enteric bacteria that were resistant to one or more antimicrobials increased in the weeks following arrival (Snyder et al., 2017; Woolums et al., 2018) and multi-drug resistance increased following antimicrobial treatment (Zaheer et al., 2013; Catry et al., 2016). Animals whose penmates received antimicrobial treatment had a higher prevalence of recovery of multi-resistant *M. haemolytica* (Noyes et al., 2015). With inconsistent effectiveness of vaccination and concerns about preventative use of antimicrobials, alternative means of BRD control are needed.

Several studies have reported that various components of the innate immune system may be less effective or reduced in stressed or virus-infected cattle (Caswell, 2013). However, in vitro and in vivo studies have demonstrated that it is possible to induce innate immune system responses, such as the expression of antimicrobial peptides by tracheal epithelial cells, following stimulation with Toll-like receptor agonists (Mitchell et al., 2006; Berghuis et al., 2014; Taha-Abdelaziz et al., 2016). Similarly, aerosol delivery of bacterial lysate to calves has been shown to cause a mild and transient immuno-inflammatory response characterized by elevation in temperature and recruitment of neutrophils to the lungs, and induction of antimicrobial peptide gene expression following in vitro treatment of tracheal epithelial cells (Chapter 3). Finally,

experiments in mice have demonstrated that aerosolization of bacterial lysate prior to challenge with respiratory pathogens is highly effective in preventing pneumonia caused by a number of viral, bacterial and fungal agents (Evans et al., 2010a). 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 clinically identified disease, rectal temperature, serum haptoglobin, plasma fibrinogen, nasal carriage of *M. haemolytica* and *Mycoplasma bovis*, pulmonary consolidation identified by transthoracic ultrasound, and weight gain in the first 28 days. We also evaluated the effect on mortality in the first 60 days after arrival.

5.3 Materials and Methods

5.3.1 Study animals

This study was approved by the Animal Care Committee of the University of Guelph (AUP #3286). This was a randomized, double-blinded, placebo-controlled study completed at the Elora Beef Research Centre (EBRC) in southwestern Ontario, Canada. Sixty unvaccinated beef steers of mixed breeds were obtained from Ontario Stockyards Inc, Cookstown, ON, approximately 100 km from the ERBC between November 8, 2016 and December 10. The sixty calves comprised 4 cohorts of 14-16 animals, each cohort arriving 1-2 weeks apart. The calves were considered to be at high risk of developing respiratory disease 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 and bedded on wood shavings for the duration of the study. Calves were fed a ration that contained 50% haylage and 50% corn silage. Calves arrived at the feedlot in the evening. 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, one calf was randomly assigned to receive the aerosolized bacterial lysate and the other calf received aerosolized phosphate-buffered saline.

5.3.2 Creation and delivery of the bacterial lysate

Bacterial lysate was prepared and administered as previously described (Chapter 2). Briefly, isolates of *S. aureus* and *E. coli* (kindly provided by the Animal Health Laboratory, Guelph, Ontario) were inoculated into 250 mL tryptose soy broth and incubated at 37°C with shaking for 18 hours. Bacteria were killed by immersion in a 65°C hot water bath for 60 minutes; non-viability was confirmed by culture on blood agar. The heat-killed bacteria were centrifuged at 7,700 x *g* for 10 min at 4ºC (H4000 rotor, Sorvall RC-3B) to pellet the bacteria and were washed twice with PBS. The bacterial pellets were suspended in PBS and sonicated in an ice water bath using a probe sonicator (Fisherbrand™ Model 120 Sonic Dismembrator) with 10 cycles of 10 seconds (*E. coli*) or 10 cycles of 30 seconds (*S. aureus*) alternating with 20 seconds cooling at an amplitude of 80%. Bacterial lysate was stored in aliquots at -20ºC until use. From this stock suspension, 10^9 or 10^{10} cfu-equivalents each of *S. aureus* and *E. coli* were mixed with PBS to a final volume of 2 mL. The 15 calves in the first two cohorts received 10^9 cfu-equivalents each of *S. aureus* and *E. coli*; the 15 calves in the last two cohorts received 10^{10} cfu-equivalents of each bacterial lysate (n=15). This

treatment was defined as day 1 of the study. Animals were restrained in a hydraulic headgate for administration of the aerosol treatments and collection of clinical parameters and samples. Using a compressor (Precision Medical, Northampton, Pa) delivering approximately 25 psi of air pressure, bacterial lysate suspension or PBS was delivered through a Whisper Jet nebulizer (Marquest, Englewood, Colorado) connected to an Equine Aeromask (Trudell Medical International, London, ON) placed over the calves' muzzles.

5.3.3 Evaluation and sample collection

The steers were examined on the morning after arrival to the research station (baseline), twice weekly for 2 weeks, and then once weekly for 2 weeks for a total of 28 days. At these times, they were visually inspected and weighed, and rectal temperatures were recorded. Bilateral nasal swabs were collected at a depth of 7 cm and the swab tips were stored in 1.5 mL tubes at -80° C until DNA extraction was performed. Blood was collected from the jugular vein and serum haptoglobin and plasma fibrinogen were measured within 3 hours of collection (Animal Health Laboratory, University of Guelph). In addition to the times listed above, body weight and thoracic ultrasound findings were also recorded at 122 days (group 1), 115 days (group 2), 130 days (group 3) and 111 days (group 4).

5.3.4 Targeted thoracic ultrasound

Targeted thoracic ultrasound was performed at the times listed above using a portable linear rectal ultrasound (Ibex Pro, E.I. Medical Imaging) by a blinded evaluator (LLB). Approximately 500 mL of 70% isopropyl alcohol was used as a transducing agent. Scanning was performed starting at the epaxial muscles of the 7th intercostal space (ICS) and moving ventrally within the ICS to the border of the lung (Ollivett et al.,

2015). If no lesions were identified, the probe was advanced cranially, and this was repeated until the entire cranial left and right lungs were examined. To examine the most cranial aspects of the lungs, the transducer was placed between the forelimb and the ventral thoracic body wall. Lung tissue was considered normal when the pleural line was visualized with accompanying reverberation artifact (A-lines). Small vertical hyperechoic lines emanating from the pleural surface were recorded as comet tails (B-lines). Consolidated lung appeared as hypoechoic tissue, lacking the normal white pleural surface line and A-lines. Left and right lungs were given a score of 0 (no lesions, comet tails only, or consolidated lesions less than 3 cm) or 1 (lesions greater than or equal to 3 cm, as measured using the 1 cm grid of the ultrasound unit). An overall ultrasound score was assigned as 0-1 based on the most severely affected side. At the final ultrasound conducted at 4 months, an additional ultrasound measure of severe consolidation was recorded if there was evidence of consolidation of an entire lung lobe.

5.3.5 Clinical Illness & Mortality

All animals were observed twice daily by experienced farm operators blinded to treatment status. Calves were identified as clinically ill based on depression, coughing, reluctance to move, or poor appetite; and confirmed with an elevated rectal temperature (≥40^{o}C); affected calves were treated according to standard protocols determined by the herd veterinarians. Treatments included label doses of florfenicol (Nuflor®, Merck Animal Health, Kirkland, Quebec), tulathromycin (Draxxin®, Zoetis, Kirkland, Quebec), enrofloxacin (Baytril 100®, Bayer Animal Health, Mississauga, Ontario), and/or tilmicosin (Micotil®, Elanco Canda Limited, Guelph, Ontario). In addition to antimicrobial injections, 11/59 steers received one or more doses of meloxicam by subcutaneous injection (Metacam 20 mg/mL, Boehringer Ingelhein, Burlington, Ontario, Canada).

Calves that died or were euthanized during the study period were subjected to full postmortem examinations by pathologists blinded to treatment status at the Ontario Veterinary College, Guelph, Ontario. Lungs were examined grossly and histologically, cultured for isolation of aerobic bacteria and mycoplasmas, and tested by PCR for bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), bovine parainfluenza-3 virus and bovine viral diarrhea virus (BVDV).

5.3.6 Nasal Swab Processing and PCR

DNA was extracted from the left nasal swabs. Briefly, 500 μL of PBS was added to a 2 mL tube containing the swab, vortexed and left at room temperature for 30 minutes. The swabs were vortexed again and 400 μL of fluid was removed for DNA extraction using a commercial kit following manufacturer's instructions (QIAamp DNA Mini Kit®, QIAGEN Inc, Mississauga, ON). The final DNA concentration and quality of the eluted sample were determined using a Nanodrop spectrophotometer. Extracted DNA was stored at -20˚ C until PCR analysis.

Quantitative PCR for *M. bovis* was performed using published primers and probe for the *uvrC* gene (Mbov_uvrC_L, 50 -GAATTTACGCAAGAGAATG CTTCA-30 ; Mbov_uvrC_R, 50 -GCAATGCCTCTTTATTTGTTTTACAG-30 ; Mbov_uvrC_P, 50 -TGACGGCGCTATTAA-30 (FAM-MGBNFQ))(Rossetti et al., 2010). The PCR reactions were performed in triplicate for each DNA sample using LightCycler 480 technology (Roche Diagnostics). The reaction conditions consisted of an initial step at 50º C for 2 min and at 95º C for 10 min, then PCR amplification was carried out for 40 cycles with denaturation at 95º C for 15 s and extension at 60º C for 1 min. Ten-fold serial dilutions of a known concentration of pure cultured *M. bovis* was used to spike *M. bovis*-negative

nasal swabs to measure the detection limit of the assay (which was found to be 10^3 cfu-equivalents per swab), to determine the reaction efficiency, and to serve as an internal positive control and standard for each PCR plate. The calibration curve was used to correlate cycle threshold to CFU. Each plate contained template-negative control wells. LightCycler 480 software was used for quantification against a within-run standard. Nasal swabs with crossing threshold values greater than the determined lower limit of detection were assigned a total bacteria number that was equal to the lower limit of detection divided by $\sqrt{2}$ (i.e.707).

Using the same nasal swab DNA preparation, multiplex PCR was used to determine the presence or absence of *M. haemolytica* serotypes 1, 2 and 6 (Klima et al., 2017). Briefly 2 μL of DNA template was added to the primers and Qiagen Multiplex PCR Master Mix (QIAGEN Inc, Mississauga, ON) and a thermocycler was used for amplification with an initial step at 94º C for 7 min, followed by 35 cycles at 94º C for 30 s , 55º C for 55 s, and 72º C for 1 min, followed by a final step of 72º C for 7 minutes. Following amplification, 20 μL of each sample was analyzed by gel electrophoresis using a product was resolved by electrophoresis in a1% agarose gel with SYBR Safe gel dye (Thermo Fisher Scientific, Burlington, Ontario, Canada) in Tris–borate–ethylenediamine tetra-acetic acid buffer with a 50 bp DNA marker ladder. Gels were visualized after staining with ethidium bromide. Amplicon bands representing four strains of each of serotypes 1, 2 and 6 were excised from the agarose gel and sequenced to confirm amplification of the desired gene targets in the assay.

5.3.7 Statistical analysis

Simple descriptive statistics and comparisons between treatment groups, including means and 95% confidence interval (CI), one-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, San Diego, California USA, www.graphpad.com). Results with a *p*-value less than 0.05 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 temperature, serum haptoglobin, plasma fibrinogen, nasal bacteria numbers, and body weight were evaluated using multivariate mixed-model linear or logistical regression (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.). Group and calf were included as random effects, as were the additional covariates of the baseline parameters for each outcome, antimicrobial treatment in the past 4 days, time relative to arrival, weight on arrival and interaction terms. Stepwise removal of non-significant interactions and individual factors was performed until only significant variables ($p < 0.05$) and random effects ($p < 0.20$) remained in the models. Data was transformed if indicated by evaluation of residual variance for normality and homogeneity. Initial analyses assessed if there were significant differences between the two doses of bacterial lysate. As no differences were demonstrated between the 2 doses, animals receiving bacterial lysate were grouped together for the subsequent and reported analysis.

5.4 Results

All 60 calves received a randomly assigned aerosol treatment (bacterial lysate or PBS) between 14-18 hours after arrival (day 1). No adverse effects were seen at the time of administration. One steer in the lysate-treated group (10029) had elevated baseline fibrinogen and haptoglobin as well as large areas of lung consolidation on arrival and thus was excluded from further analysis. The other 59 calves were included in the subsequent analysis.

The mean body weight on arrival was 218 kg (95% CI: 213, 224). There were no statistically significant differences in baseline (on-arrival) parameters between the lysate-treated group and the control group including body weight (paired *t*-test; p = 0.85), temperature (paired *t*-test; p = 0.40), fibrinogen (paired *t*-test p = 0.33), or haptoglobin (paired *t*-test p = 0.25) (Supplemental Table S5.1). 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 with lung consolidation measuring greater than 3 cm (Fisher's exact test; p = 0.42).

At the time of arrival to the feedlot, the mean body weights differed between the 4 cohorts (ANOVA (F(3,56) = 1.361, p =0.007). In the first cohort the mean weight on arrival was 218 kg (95% CI: 205-230), the second cohort was 207 kg (95% CI: 200-213), the third cohort was 232 (95% CI: 220-245), and the fourth was 216 (95% CI: 203-229). The mean body weight in the second cohort was significantly lower than the mean body weight in the third cohort (post hoc Tukey-Kramer test: p = 0.007). Other baseline parameters did not differ between cohorts. One animal was euthanized due to severe

pneumonia and septic arthritis within the first 28 days, and 7 additional steers died due to severe disease prior to the final evaluation at 3 months.

5.4.1 Body weight

Body weight and total weight gain relative to arrival weight over time in surviving calves are shown in Figure 5.1. Over the first 28 days, the mean change in body weight in lysate-treated steers was -4.3 kg (95%CI: -10.7, 1.7) while that of control steers was +4.3 kg (95% CI: -1.5, 10.2) and this difference was significant (p = 0.04). Body weight on arrival to the feedlot did not significantly affect the weight gain at 28 days. At the follow-up visit (mean of 120 days after arrival), the average daily gain was similar (p = 0.80) between the surviving lysate-treated steers (n = 23/29) with a mean of 0.588 kg/day (95% CI: 0.446, 0.729) versus the surviving control steers (n = 27/29) with a mean of 0.649 kg/day (95%CI: 0.499, 0.800).

5.4.2 Clinical parameters

Rectal temperature over time is shown in Figure 5.2. Variables that were determined to significantly influence rectal temperature included treatment with aerosolized lysate versus saline, day relative to arrival, and administration of antimicrobial treatment within 4 days preceding the sampling date (Table 5.2). 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 by day 4, with a peak at day 7 (haptoglobin) or day 11 (fibrinogen) (Figure 5.2). Aerosol treatment with lysate or saline did not significantly affect serum haptoglobin (p = 0.10) or plasma fibrinogen (p = 0.22) concentrations (Table 5.2). 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).

5.4.3 Bacteria in nasal swabs

The number of nasal *M. bovis* increased over time based on analysis by quantitative PCR. This increase was first identified at day 4 (i.e., 3 days after administration of lysate or saline) and persisted to the final sample point at day 28 ($p < 0.001$). This increase was greater in lysate-treated than control calves ($p = 0.001$, Table 5.3). Body weight or antibiotic treatment in the past 4 days were not associated with the number of nasal *M. bovis*. 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.

Multiplex PCR for *M. haemolytica* serotypes 1, 2 and 6 was performed on 412 samples of DNA isolated from nasal swabs. Of these, 174 (42.2%) samples were positive for *M. haemolytica* serotype 1, 114 (27.7%) were positive for serotype 2, and 10 (2.4%) were positive for serotype 6. The prevalence of serotype 1 increased significantly from day 1 when 4/59 (7%) were positive, to a peak at day 4 when 43/59 (73%) were positive, and then declined ($p < 0.001$)(Figure 5.3). There was no change in the prevalence of serotype 2 between day 1 and 4, with 24/59 (83%) of calves positive on both days, however, there was a gradual decrease in prevalence over the course of the study ($p < 0.001$) (Figure 5.3).

The prevalence of *M. haemolytica* serotypes 1 and 2 were not affected by lysate administration, although there was a trend toward increased prevalence of nasal colonization by *M. haemolytica* serotype 1 in calves that received bacterial lysate (multivariate logistic regression, $p = 0.08$) (Table 5.3). Body weight on arrival or

antimicrobial treatment were not associated with nasal prevalence of *M. haemolytica.* There were too few isolates of *M. haemolytica* serotype 6 to analyze. Of the swabs that were positive for serotype 6, most (9/10) were from 5 animals in group 4.

5.4.4 Ultrasound lesions

On day 1, 7/59 (12%) cattle had ultrasonographic evidence of pulmonary consolidation, with 2/29 (7%) lysate-treated cattle and 5/30 (17%) control animals affected (Figure 5.5). The proportion of animals with lung consolidation increased over time ($p < 0.001$) with a peak prevalence at day 21 where 29/29 (100%) lysate-treated calves had pulmonary consolidation and 27/29 (93%) control calves had consolidation. ($p < 0.05$; Table 5.5). At the end of 28 days, 29/29 (100%) lysate treated calves had pulmonary consolidation compared to 24/29 (83%) control calves. All but one (control) steer had pulmonary consolidation at one or more time points. As all lysate-treated animals had pulmonary consolidation at days 21 and 28, logistic regression could not be performed; however, at 28 days after arrival there was a trend towards increased consolidation in lysate treated steers (Fisher's exact test; $p = 0.052$). Time after arrival and the presence of lung consolidation at arrival (baseline lesions) were significantly associated with lesions on pulmonary ultrasound (Table 5.5). At the 4 month visit, 11/23 (48%) of surviving lysate-treated steers and 13/28 (46%) of surviving control steers had pulmonary consolidation greater than 3 cm. Severe consolidation of the entirety of one or more lung lobes was identified in 5/23 (22%) lysate-treated steers compared to 4/28 (14%) control steers. These differences were not statistically significant.

5.4.5 Clinical disease

Twice-daily pen-side evaluations of all steers were performed by farm operators blinded to lysate versus saline status, who proceeded to administer therapy as per farm

protocols and as directed by the herd veterinarian. Overall, 37/59 steers were identified as clinically ill by farm operators. These included 16/30 (53%) control calves and 21/29 (70%) lysate-treated calves. Multivariate logistic regression, evaluating the impact of lysate treatment on the risk of clinical disease, controlling for the effects of time after arrival, cohort and body weight on arrival, is shown in Table 5.4. Treatment with lysate did not significantly increase the risk of clinical respiratory disease (odds ratio = 2.55 (95% CI: 0.08-8,11; p = 0.113). Lower body weight on arrival was associated with increased prevalence of respiratory disease ($p = 0.025$). The effect of group was negligible. The incidence of respiratory disease was highest in week 2, when 24% of steers received treatment. The cumulative incidence of clinical disease and time to first treatment are shown in Figure 5.6. Time to first treatment was not different between lysate-treated and control animals (Mantel-Cox test, p = 0.10). The number of treatments administered to lysate-treated and control calves was similar between treatment groups (Figure 5.6).

5.4.6 Mortality

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 prior to 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 and isolation of M. bovis. Six of 29 lysate treated steers (24%) died of *M. bovis* pneumonia compared to 1/29 control steers (the other mortality was due to chronic pneumonia not caused by *M. bovis*; mycoplasma culture and PCR were performed but *M. bovis* was not isolated and caseonecrotic lesions were absent.). Thus, lysate-treated steers had 7.2 times (95% CI: 1.3, 43.9) greater risk of dying from *M. bovis* pneumonia within the first three

months after arrival than did control steers (Fisher's exact test: $p = 0.03$). Post-mortem gross, histologic and microbiologic findings are summarized in Supplemental Table S5.4. Of the 7 animals that were diagnosed with *M. bovis* pneumonia, 3 had septic arthritis caused by *M. bovis* in one or more joints. The effect of bacterial lysate treatment on overall mortality and mortality due to *M. bovis* was evaluated with multivariate logistic regression models (Table 5.6). On-arrival treatment with bacterial lysate significantly increased the risk of mortality due to *M. bovis* ($p = 0.042$) but not overall mortality ($p = 0.102$). In these models, decreased body weight on arrival increased the risk of overall mortality and mortality due to *M. bovis.*

5.5 Discussion

Unexpectedly, administration of aerosolized bacterial lysate worsened rather than improved many of the evaluated outcomes in these four groups of feedlot steers. The steers treated with aerosolized bacterial lysate had reduced weight gain over the first 28 days, increased body temperatures, increased numbers of M. bovis in their nasal cavities, increased pulmonary consolidation, and increased mortality due M. bovis pneumonia. Furthermore, although not statistically significant, lysate-treated steers had a tendency to increased serum haptoglobin concentrations ($p = 0.10$) and a higher prevalence of nasal M. haemolytica serotype 1 ($p = 0.08$). These results demonstrate that stimulation of inflammatory responses in the respiratory tract of high-risk calves did not confer protection from BRD and instead exacerbated the development of M. bovis pneumonia.

We chose to administer a lysate of *E. coli* and *S. aureus* rather than a lysate which consisted of one or more BRD pathogens to avoid induction of specific acquired

immune responses (i.e. vaccination). Although a single-time high-dose aerosol treatment was given, the effects of repeated administration or administration at a different time point or dose remain unknown. We delivered the lysate on day 1 because this is typically when a preventative would be given to high-risk calves. Furthermore, this time point immediately precedes that when reduced TAP and LAP expression were demonstrated in bronchial biopsies (Mitchell et al., 2006), supporting this as a time when immune stimulation could be valuable. The dose of lysate was based on pilot studies performed in bull calves (Chapter 2), to ensure safety and effectiveness in stimulating innate immune responses.

Previous *in vitro* experiments demonstrated that lysate administration induced expression of tracheal antimicrobial peptide and lingual antimicrobial peptide in cultured tracheal epithelial cells. As well, pilot studies in calves showed that aerosolized lysate resulted in neutrophil recruitment to the lungs and a mild and transient acute phase response, however, significant lung damage did not develop (Chapter 2). Similarly, a small initial pilot study to determine safety and assess responses in beef cattle (data not shown) performed at the ERBC in non-stressed resident steers did not demonstrate adverse effects. As the lysate does not directly cause pneumonia, we speculate that the induced inflammatory responses were only detrimental in cattle at high risk of developing disease. These results do not support the theory that immunosuppression is the reason on-arrival cattle develop BRD.

Purposeful selection of beef calves at high risk of developing respiratory disease for inclusion in this study was done to ensure adequate disease incidence to allow us to detect an effect of the intervention. The clinical disease that occurred in these study

animals was typical of BRD in North American feedlot cattle with respect to timing after arrival, clinical signs and the bacteria identified in the nasal cavity and lung. The prevalence was within the expected range for high-risk calves without metaphylaxis (Baptiste and Kyvsgaard, 2017). Similarly, it is typical to find the predominance *of M. bovis* mortalities occurring in the second month after arrival (Gagea et al., 2006a) even if the onset of disease occurs soon after arrival (Castillo-Alcala et al., 2012). 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 this study underscores the need for alternate prevention strategies in the absence of metaphylactic/prophylactic antimicrobial treatment for high-risk calves.

In this study, although the characteristic caseonecrotic bronchopneumonia (+/- arthritis) 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 multoc*ida. Rather than directly increasing the risk of severe *M. bovis* pneumonia, it is possible that lysate administration led to increased pneumonia from other causes, which in turn accelerated and worsened the development of *M. bovis* pneumonia. 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 additional objective production outcome that is of considerable interest to producers but takes time to manifest and can be considered a non-specific outcome, susceptible to influences other than BRD. Thoracic ultrasound is an immediate chute-side test that is highly specific for identifying lung consolidation and in this study cranioventral consolidation allowed us to confirm the presence of pneumonia. However, ultrasound could be considered more subjective, with the potential for operator-associated variability in performance and interpretation. In addition, although the presence of left and right sided consolidation was recorded, more detailed evaluation such as measurements of lesion size were not analyzed.

Clinical illness and need for antimicrobial therapy was another outcome that was evaluated but is less objective and is problematic as a sole outcome due to the low sensitivity in detecting clinically ill animals (Timsit et al., 2016). The low sensitivity of using clinical illness to detect BRD was similarly demonstrated in this work where the treatment rate (37/59) was lower than the prevalence of pulmonary consolidation, which was detected in 58/59 animals at one or more time points. In other words, only 37/58 (64%) animals with ultrasonographic pulmonary consolidation were identified as clinically ill and received antimicrobial treatment.

Finally, although not intended as a major outcome, the high rate of mortality, an objective and severe negative outcome, allowed definitive evaluation of lung lesions causing death, including correlation with ultrasound findings and evaluation of the pathogens present within the lung tissue. Together, evaluation of these various

parameters provided a detailed evaluation of the effect of aerosolized lysate on feedlot calves. The concordant negative results following evaluation of multiple parameters strengthen the conclusion that lysate administration worsened disease in feedlot calves.

Average daily gain was significantly lower in lysate treated steers in the first 28 days 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. It was also notable that 4 months after arrival, after having lost 6 of the most poor-doing animals to *M. bovis* pneumonia, the surviving lysate-treated steers had an ADG that was not statistically different (but was numerically lower) than control calves. Similarly, although pulmonary consolidation (as detected by ultrasound) did not statistically differ between treatment groups, the higher proportion of steers with consolidation 4 months after arrival, in spite of the high rate of mortality, further supports the long-term negative effects of bacterial lysate. Although other measures of inflammation, including body temperature and acute phase proteins, did not consistently differ between treatment groups, the overall trend for each of these was toward a negative effect of bacterial lysate.

It is well-accepted that stress and viral infections predispose to bacterial pneumonia in cattle and other species, and the mechanism behind this is commonly attributed to immunosuppression. However, the findings of this study suggest a role for inflammation in increasing susceptibility to BRD. Previous studies (chapter 2) confirmed that administration of the lysate caused mild inflammation in the lungs, and the results of this study demonstrated increased BRD mortality and poor performance following lysate treatment. A contributing role for inflammation in the pathogenesis of *M. haemolytica* has been reported in prior experimental studies. Both depletion of

neutrophils (Slocombe et al., 1985; Breider et al., 1988) and administration of the anti-inflammatory corticosteroid dexamethasone (Malazdrewich et al., 2004b) prior to challenge with *M. haemolytica* have been shown to reduce subsequent clinical disease and lesions.

There is evidence that reduced inflammation may lessen the development of natural BRD. One study demonstrated that the administration of meloxicam to calves at the time of castration (a significant stressor and inducer of inflammatory responses), reduced the subsequent incidence of BRD morbidity, although this could also be attributed to analgesic effects (Coetzee et al., 2012). Although not directly evaluating the effect of anti-inflammatory agents on BRD, another study demonstrated that following transportation, meloxicam treatment reduced acute phase proteins in blood and improved average daily gain at 21 days (Guarnieri Filho et al., 2014). In this study, of the animals that died, 4 of 8 received meloxicam treatment at some point. Controlling inflammation prior to the development of disease may be beneficial in some circumstances.

Endogenous capacity to control inflammation may also be important in the development of BRD. In one study, healthy calves that later developed BRD had lower levels of annexin A1 (a modulator of inflammation) in bronchoalveolar lavage fluid on arrival to the feedlot (Senthilkumaran et al., 2013). It is possible that calves with lower annexin levels on arrival had increased tendency to pulmonary inflammation, and this resulted in increased risk of BRD. A recent study evaluated differentially expressed genes in on-arrival blood transcriptomes from cattle that subsequently developed BRD versus calves that remained healthy. Calves that remained healthy had increased

expression of anti-inflammatory pro-resolving mediators (Scott et al., 2020). Thus, evidence for a negative role of inflammation in BRD pathogenesis is provided by the results of the current study that shows induced inflammation can worsen BRD, and by prior studies that show protective effects of anti-inflammatory treatments, leukocyte depletion or increased endogenous anti-inflammatory substances.

As an important risk factor for BRD, stress has been shown to increase inflammatory responses. Although stress is commonly considered immunosuppressive, it is not uniformly so. The net effect of stress depends on duration, with acute stress (eustress) enhancing immune responses and long-term stress leading to immunosuppression (Dhabhar, 2014). From an evolutionary perspective, acute stress is often associated with the potential for harm or injury (e.g. an attack by a predator) and thus enhancing the effectiveness of innate immune responses would be beneficial to protect from infections, whereas immunosuppression would seem to be maladaptive (Dhabhar, 2014). In mice, social disruption (a recognized stressor for mice) was shown to induce pulmonary inflammation including neutrophil and monocyte recruitment, inflammatory cytokines levels, and adhesion molecule expression (Curry et al., 2010). Furthermore, in mice, social disruption increased the mortality and inflammatory responses following a lipopolysaccharide challenge (Quan et al., 2001).

Corresponding findings have been reported in cattle. Increases in baseline or induced inflammatory cytokines (including interleukin (IL)-1β, IL-8, IFN-γ, and tumor necrosis factor-α), blood ceruloplasmin, serum amyloid A, haptoglobin and neutrophils have been reported in stressed cattle (Yagi et al., 2004; Lomborg et al., 2008; Cooke and Bohnert, 2011; O'Loughlin et al., 2011; Cooke, 2017; Masmeijer et al., 2019).

Injections of dexamethasone and corticotrophin releasing hormone have been shown to trigger (inflammatory) acute phase responses in cattle, and increased the magnitude of the response to inflammatory stimuli including increased neutrophils and haptoglobin in blood and bronchoalveolar fluid (Mitchell et al., 2008; Cooke and Bohnert, 2011). Stress and glucocorticoids have been shown to enhance tissue migration and reduce apoptosis in neutrophils, although other functions, such as oxidative burst, phagocytosis and production of the anti-inflammatory protein annexin A1 are reduced (Bassel and Caswell, 2018). Thus, some studies have shown that short term stress or single injections of corticosteroids can promote pro-inflammatory responses.

Similarly, viruses can also stimulate inflammation. Bovine herpes virus-1 causes secretion of pro-inflammatory cytokines and neutrophil adhesion and activation (Leite et al., 2004; Rivera-Rivas et al., 2009). Likewise bovine respiratory syncytial virus (BRSV) has been shown to induce inflammatory cytokines and recruitment and activation of neutrophils (Soethout et al., 2004; Engen et al., 2014; Guzman and Taylor, 2015; Hägglund et al., 2017). In humans, mortality due to pneumonia is frequently attributed to combined infections with influenza and other viruses and bacteria such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae*; however, it remains controversial whether exaggerated or attenuated innate immune responses are responsible for lethality (Seki et al., 2004; Lee et al., 2010; Short et al., 2013). There has been little work investigating the influence of virus-induced inflammation on the development of bacterial pneumonia in cattle; however, one study reported an exacerbated IL-17 response in cells co-infected with BRSV and *M. haemolytica* (McGill et al., 2016). However, it should also be noted that several innate immune responses

are decreased following viral infection. For example, bovine viral diarrhea virus type 2 leads to neutropenia (Keller et al., 2006) and reduced expression of tracheal antimicrobial peptide and lactoferrin (M. Al-Haddawi et al., 2007). As well direct virus-induced damage to airway epithelial cells inhibits mucociliary clearance. Both stress and viral infection are common in cattle arriving at the feedlot and both risk factors have been shown to trigger inflammatory responses.

Although there are several studies that have experimentally investigated the role of inflammation in the development of *M. haemolytica* pneumonia, there have not been similar experiments to look at the role of inflammation on the subsequent development of *M. bovis* pneumonia. In this study, administration of bacterial lysate resulted in increased number of *M. bovis* in the nasal cavity, more severe disease caused by *M. bovis*, and increased risk of mortality due to *M. bovis* pneumonia. It is notable that the latter effect occurred more than 30 days after administration of the lysate but reflected the increased severity of disease that was seen during the earlier period. Although the innate immune responses directly stimulated by the lysate would no longer be in effect, 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 is potentially relevant to the pathogenesis of *M. bovis*, which has been shown to be associated with lesions of coagulative necrosis attributed to *M. haemolytica* (Gagea et al., 2006b). As *M. bovis* can be found in healthy animals and normal lungs (Gagea et al., 2006b; Castillo-Alcala et al., 2012), it is likely that additional factors are necessary for development of the caseonecrotic bronchopneumonia that is responsible for mortality. The later onset of *M.*

bovis pneumonia compared to other BRD mortalities as well as the frequent isolation of additional pathogens together with *M. bovis* suggests that caseonecrotic bronchopneumonia may develop in pre-existing lung lesions (Caswell et al., 2010). Once established, *M. bovis* pneumonia is often poorly responsive to treatment (Lysnyansky and Ayling, 2016), and thus further understanding of factors that contribute to establishment of infection and disease progression are needed. The results of this study and others suggest a role for inflammation in development of *M. bovis* pneumonia.

Bacterial lysate administration increased the number of *M. bovis* and tended to increase the prevalence of *M. haemolytica* serotype 1 in the nasal cavity. In this study, the number of animals with *M. bovis* increased significantly increased over time, consistent with other studies (McMullen et al., 2019). A significant increase in the prevalence of *M. haemolytica* in the nasal cavity soon after arrival has been reported in other studies (Frank et al., 1994; Woolums et al., 2018), whereas in the current study the overall prevalence of nasal *M. haemolytica* infection did not change over time. However, we further evaluated nasal *M. haemolytica* serotypes based on multiplex PCR analysis, as pathogenicity varies significantly 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. This suggests that in addition to differences in virulence, the 2 strains are differentially influenced by factors at play in on-arrival feedlot cattle. In the current study, the administration of antimicrobials did not reduce nasal colonization by *M. haemolytica*. Although nasal *M. haemolytica* can be identified in healthy animals, it is lung *M. haemolytica* that is

relevant to pneumonia. The reported predictive value of nasal swabs on the presence of pathogens in bronchoalveolar lavage fluid varies between studies with reported kappa values for *M. haemolytica* ranging from 0.47 to 0.86 (Allen et al., 1991; Capik et al., 2017; Doyle et al., 2017).

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. Aerosol 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 mortality occurring later in the feeding period can be influenced by microbiologic and pathologic changes that occur soon after arrival.

Limitations of this study were the lack of data on pathogens other than *M. haemolytica* and *M. bovis* in the nasal cavity, as well as the lack of data on pathogens in the lungs at the time of and soon after arrival. Although several other important BRD pathogens were identified on the postmortem examinations of animals that died during the study, 7/8 mortalities were attributed to *M. bovis*. Furthermore, as the ultimate goal of lysate administration was to reduce the incidence and severity of disease in the lungs rather prevent infection by individual pathogens, identification of additional bacteria or viruses was not included in the study design. Expanding the study to include additional

animals may have led to increased statistical power, however, once the negative outcomes were identified, evaluating further cohorts was not considered. To further understand how bacterial lysate administration led to increased disease, it would be necessary to sample lung tissues 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*.

5.6 Conclusions

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. Further investigation how innate immune responses are altered in high-risk cattle and what predisposing factors (e.g. stress or viral infection) are responsible for these effects may lead to further understanding and the potential for interventions to reduce the impact of BRD.

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5.8 Tables

Table 5.1: Baseline parameters in lysate- and saline-treated calves, measured on arrival, prior to treatment. Paired *t*-tests compared means between treatment groups.

Parameter	Control steers mean (95% CI)	Lysate-treated Mean (95% CI)	Paired *t*-test for statistical difference
Body weight (kg)	218.5 (209.9, 227.0)	218.2 (210.2, 226.3)	$t(28) = 0.193$ $p = 0.85$
Rectal temperature (Celsius)	39.7 (39.3,39.8)	39.6 (39.3,39.8)	$t(28) = 0.851$ $p = 0.40$
Serum haptoglobin (g/L)	0.18 (0.13, 0.22)	0.24 (0.15, 0.32)	$t(28) = 1.178$ $p = 0.25$
Plasma fibrinogen (g/L)	3.21 (2.94, 3.48)	3.35 (3.04, 3.65)	$t(28) = 0.992$ $p = 0.33$

Table 5.2: The effect of on-arrival treatment with aerosolized bacterial lysate on body temperature, serum haptoglobin, and plasma fibrinogen over time after arrival (days 4-28). 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 or saline on arrival to a feedlot with animal ID and group included as random effects.

	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	0.041	0.01, 0.37	0.14	0.102	-0.03, 0.31	0.03	0.215	-0.02, 0.07
Saline	REF[b]			REF			REF		
Antimicrobial									
Within 4 days	-0.52	<0.001	-0.72, -0.32	-[c]	-	-	-	-	-
No recent antimicrobial	REF								
Days after arrival	-0.03	<0.001	-0.04, -0.02	-0.015	<0.001	-0.02, -0.01	0.014	<0.001	0.01, 0.02
Days^2	-	-	-	-	-	-	-0.001	<0.001	-0.001, -0.003

[a] Separate multivariate 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.

[b] REF= Reference

[c] – indicates factors that were not significant in the final models

Table 5.3: The effects of aerosol treatment with bacterial lysate one day after arrival on the number of nasal *Mycoplasma bovis* determined by quantitative PCR. Coefficients (β), 95% confidence intervals (CI) and *P*-values were determined by multivariate linear regression.

	Estimate of effect (β)	*p*-value	95% CI
Aerosol treatment			
Lysate	0.758	0.001	0.307, 1.208
Saline	REF[a]		
Days relative to arrival[b]	0.397	<0.001	0.339, 0.455
(Days relative to arrival)^2	-0.010	<0.001	-0.012, -0.009

[a] REF= Reference category
[b] Nasal swabs were collected from days 4 to 28 after arrival

Table 5.4: The effects of aerosol treatment with bacterial lysate one day after arrival on nasal colonization with *Mannheimia haemolytica* serotype 1 determined by multiplex PCR. Summaries of 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.

	Serotype 1			Serotype 2		
	Odds Ratio	*p*-value	95% CI	Odds Ratio	*p*-value	95% CI
Aerosol treatment						
Lysate	2.14	0.084	0.90, 7.07	0.66	0.33	0.29, 1.51
Saline	REF[a]			REF[a]		
Days relative to arrival[b]	0.90	<0.001	0.87, 0.93	0.87	<0.001	0.83, 0.92

[a] REF= Reference category
[b] Nasal swabs were collected from days 4 to 28 after arrival

Table 5.5: The effects of aerosol treatment with bacterial lysate one day after arrival to a feedlot on the presence of pulmonary consolidation greater than 3 cm detected by targeted trans-thoracic ultrasound between days 4 and 28. Summaries of 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 prior to aerosol treatment (baseline) as well as random effects of cohort and individual animal were included in the final model.

	Odds Ratio	*p*- value	95% CI
Aerosol treatment			
Lysate Saline	Not testable[c]	-	-
Days relative to arrival[b]	1.69	<0.001	1.35, 2.12
(Days relative to arrival)^2	0.99	0.001	0.98, 0.995
Lung consolidation at baseline	17.28	0.019	1.60, 186.46

[a] REF= Reference category
[b] Nasal swabs were collected from days 4 to 28 after arrival
[c] Due to 100% correlation between lysate treatment and ultrasound lesions at 21 and 28 days, logistic regression was not performed.

Table 5.6: The effects of aerosol treatment with bacterial lysate on the risk of clinical disease, mortality due to *Mycoplasma* bovis, and overall mortality in 59 feedlot calves. 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.

	Clinical disease			Mortality due to *Mycoplasma bovis*			Mortality, 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	0.113	0.80, 8.11	10.20	0.042	1.085, 96.01	4.79	0.102	0.73, 31.40
Saline	REF[1]			REF					
Weight (kg)	0.97	0.025	0.94, 0.996	0.948	0.050	0.899, 1.000	0.92	0.014	0.87, 0.98

[1] REF= Reference category

5.9 Figures

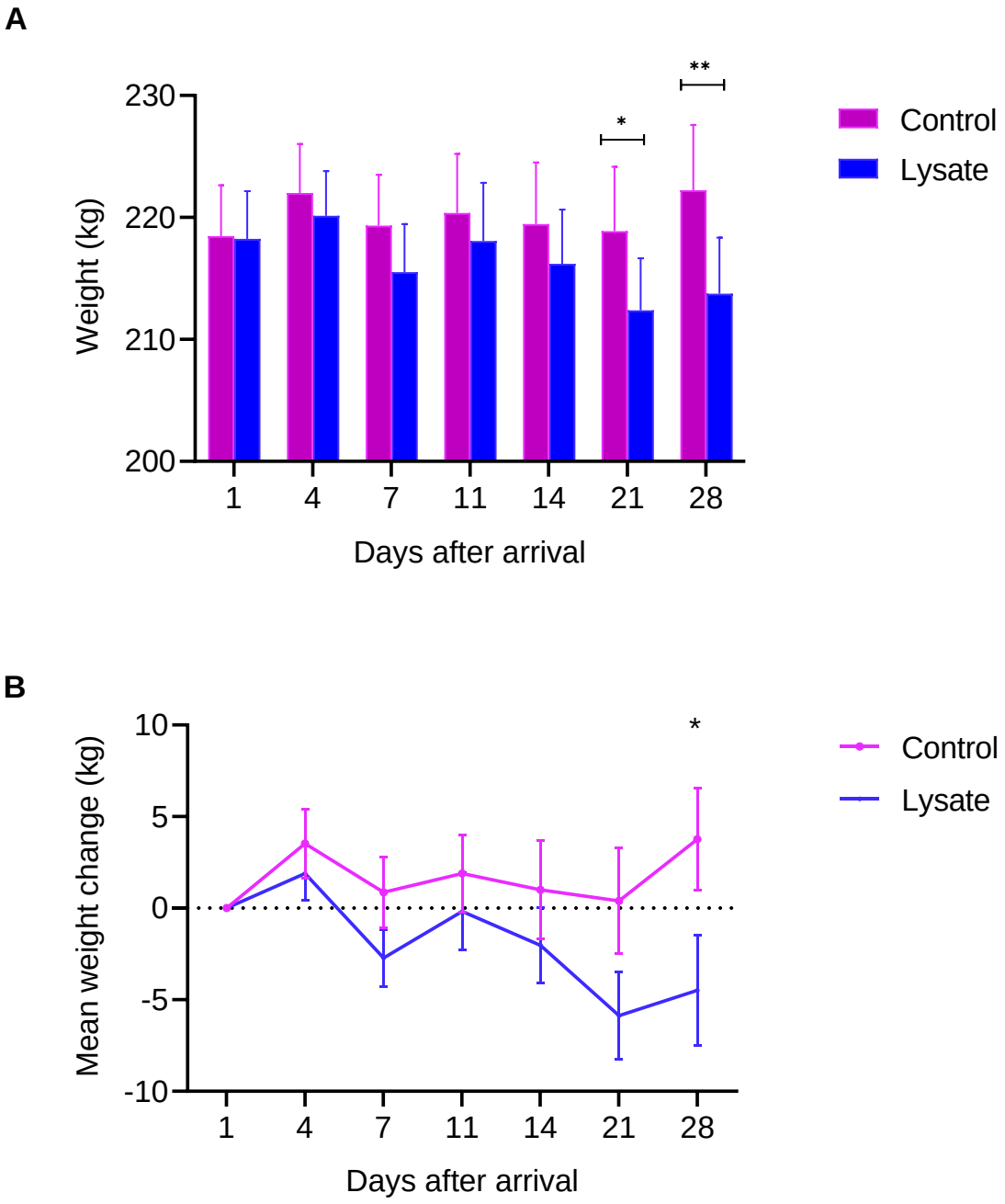


Figure 5.1: Weight over time after arrival in control and lysate-treated steers. Weight over time in steers that received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival to a feedlot. Data are means +/- standard errors of the mean of A) body weight (kg) and B) total weight gain (kg) relative to weight at arrival to the feedlot. Significant differences between control and lysate-treated steers are shown (*$p < 0.05$, **$p < 0.01$).

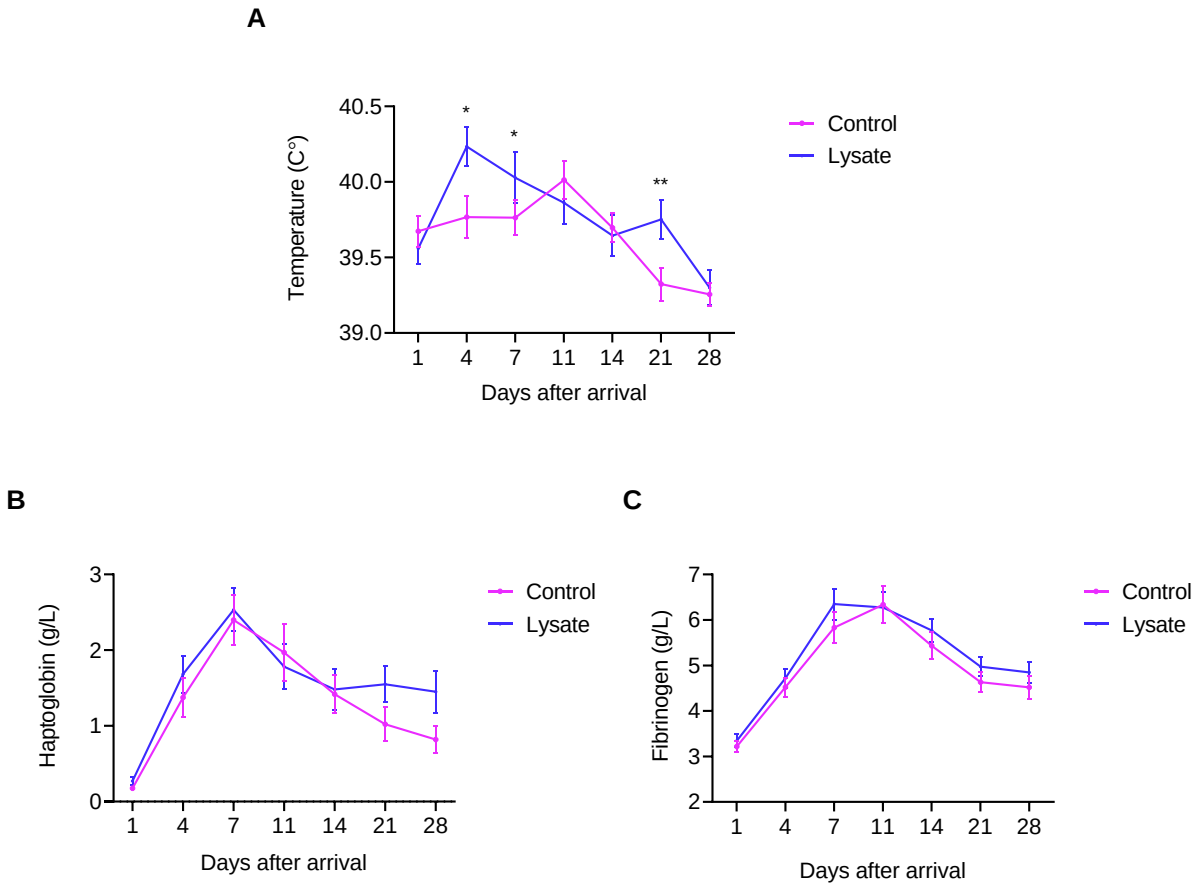


Figure 5.2: Rectal temperature, serum haptoglobin and plasma fibrinogen over time after arrival in control and lysate-treated steers. Steers received aerosolized saline (n = 30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival. Data are means +/- standard errors of the mean of A) rectal temperature, B) serum haptoglobin (g/L) and D) plasma fibrinogen (g/L) over time over days after arrival. Significant differences between control and lysate-treated steers are shown (**p* < 0.05, ***p* < 0.01).

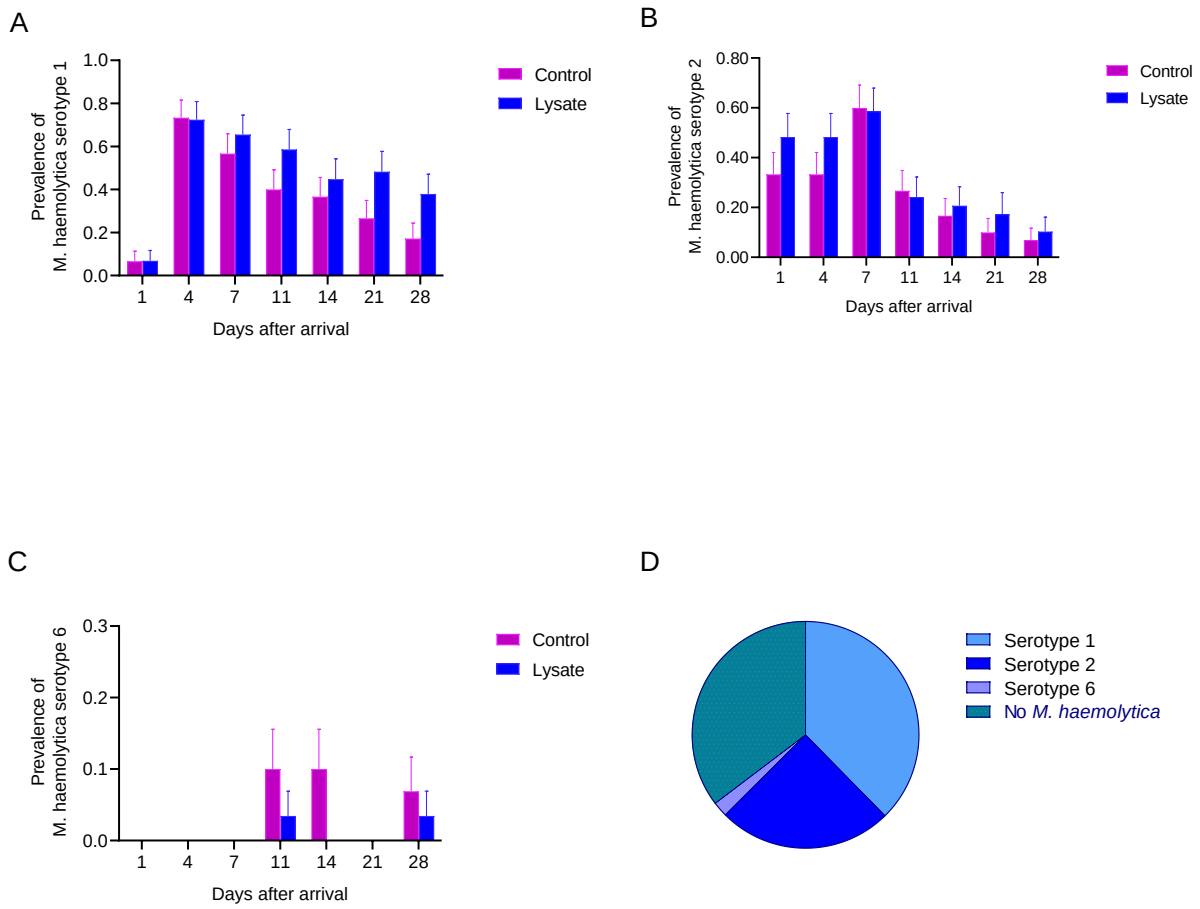


Figure 5.3: Nasal *Mannheimia haemolytica* in control and lysate-treated steers. Steers received aerosolized saline (n =30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival. *Mannheimia haemolytica* serotypes were identified by multiplex PCR. A) The prevalence of nasal *M. haemolytica* serotype 1 in control and lysate-treated steers over time after arrival. B) The prevalence of nasal *M. haemolytica* serotype 2 in control and lysate-treated steers over time after arrival. C) The prevalence of nasal *M. haemolytica* serotype 6 in control and lysate-treated steers over time after arrival. D) The relative number of nasal swabs that yielded no bacteria or *M. haemolytica* serotypes 1, 2 or 6 (n= 412 swabs evaluated).

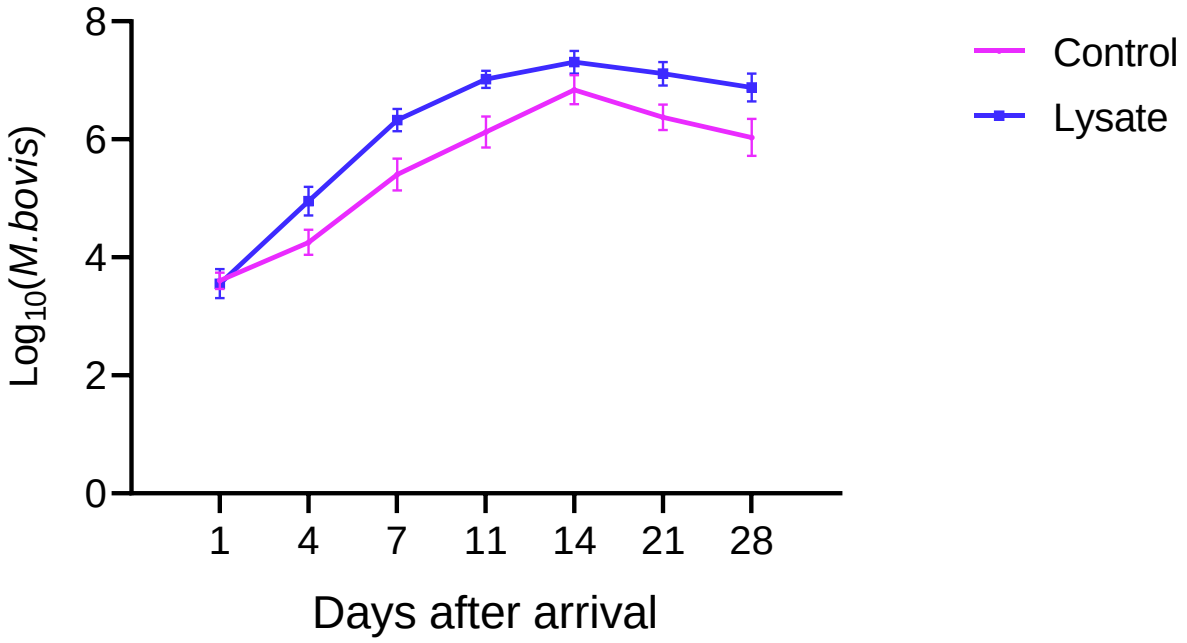


Figure 5.4: Nasal *Mycoplasma bovis* in control and lysate-treated steers over time after arrival. Steers received aerosolized saline (n =30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival. Quantitative PCR was used to evaluate the numbers of *M. bovis* in nasal swabs in control (n = 30) and lysate-treated (n = 29) steers. Data shown are the mean log-transformed number of *M. bovis* +/- standard errors of the mean.

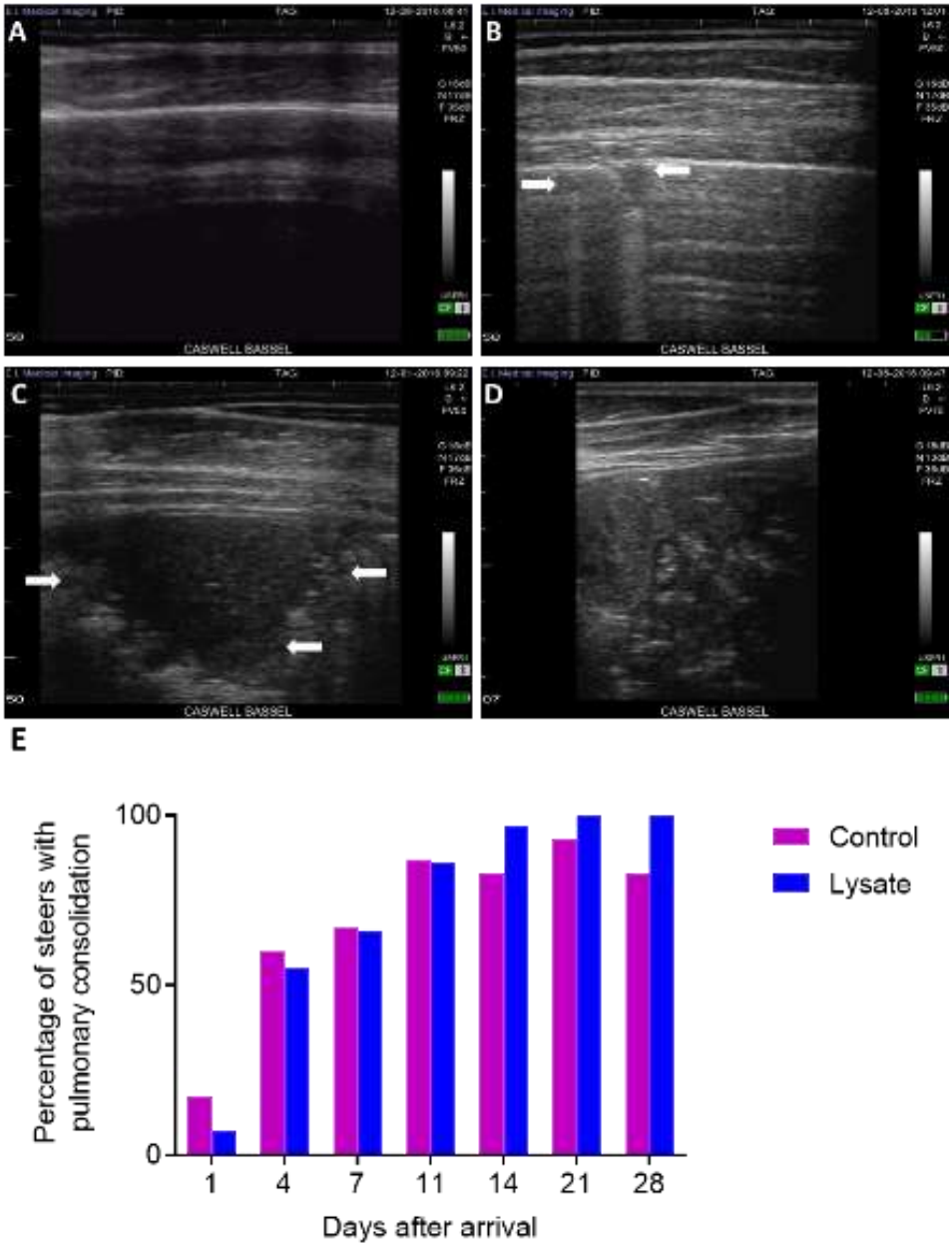


Figure 5.5: Ultrasound lesions over time after arrival in control and lysate-treated steers. Steers received aerosolized saline (n =30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival. Following arrival, targeted thoracic ultrasound was performed to evaluate the presence of pulmonary consolidation. A-D) Still images of thoracic ultrasound from A) normal lung, B) lung with small foci of consolidation (white arrows), C) large area of hypoechoic consolidation (white arrows) with only a small amount of normal lung at the right edge of the field, D) entirely consolidated lung. E) Pulmonary consolidation over time in control and lysate-treated steers. Data shown represent the proportion of steers with pulmonary consolidation measuring greater than 3 cm detected by targeted thoracic ultrasound over time after arrival.

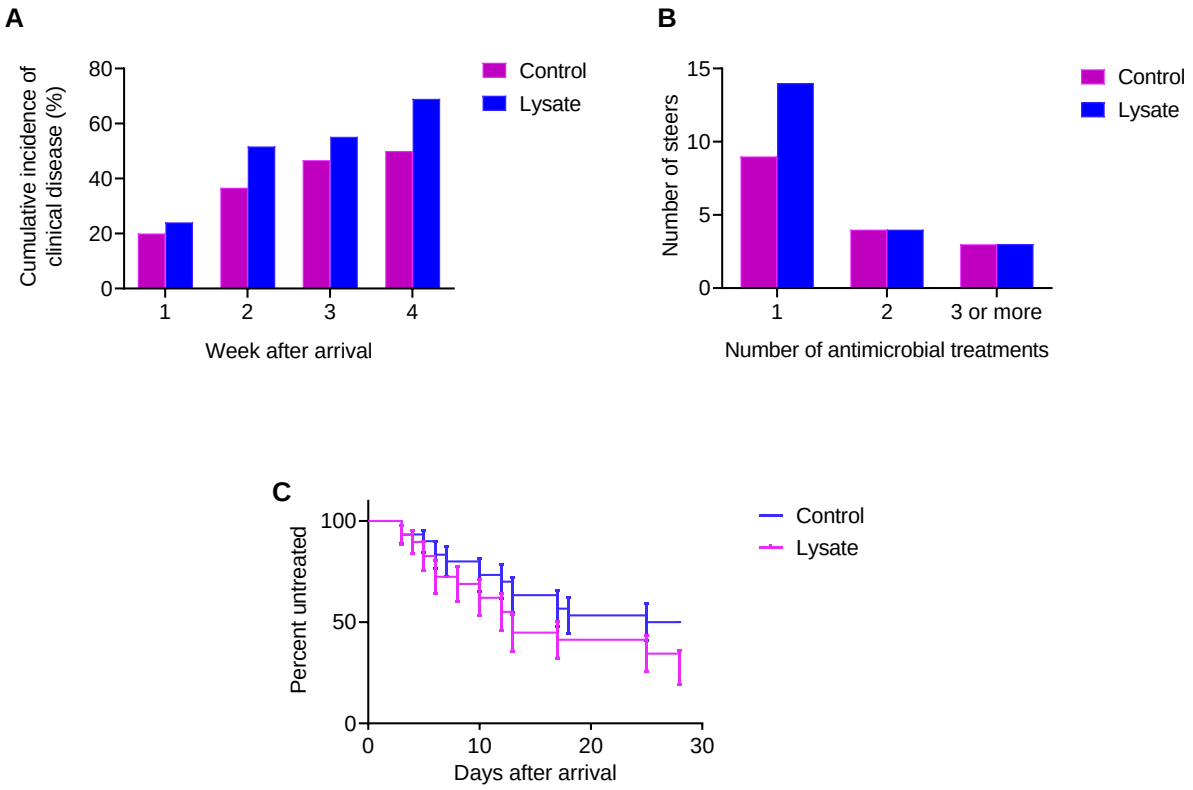


Figure 5.6: Clinical illness in control and lysate-treated steers. Steers received aerosolized saline (n =30) or *Staphylococcus aureus* and *Escherichia coli* lysate (n=29) one day after arrival. A) Cumulative incidence by week in control and lysate-treated steers. B) The number of antimicrobial treatments administered to control and lysate-treated steers. C) A survival curve showing the time to first treatment in control and lysate-treated steers. None of the measures shown had statistically significant differences between aerosolized treatment groups.

5.10 Supplementary Material

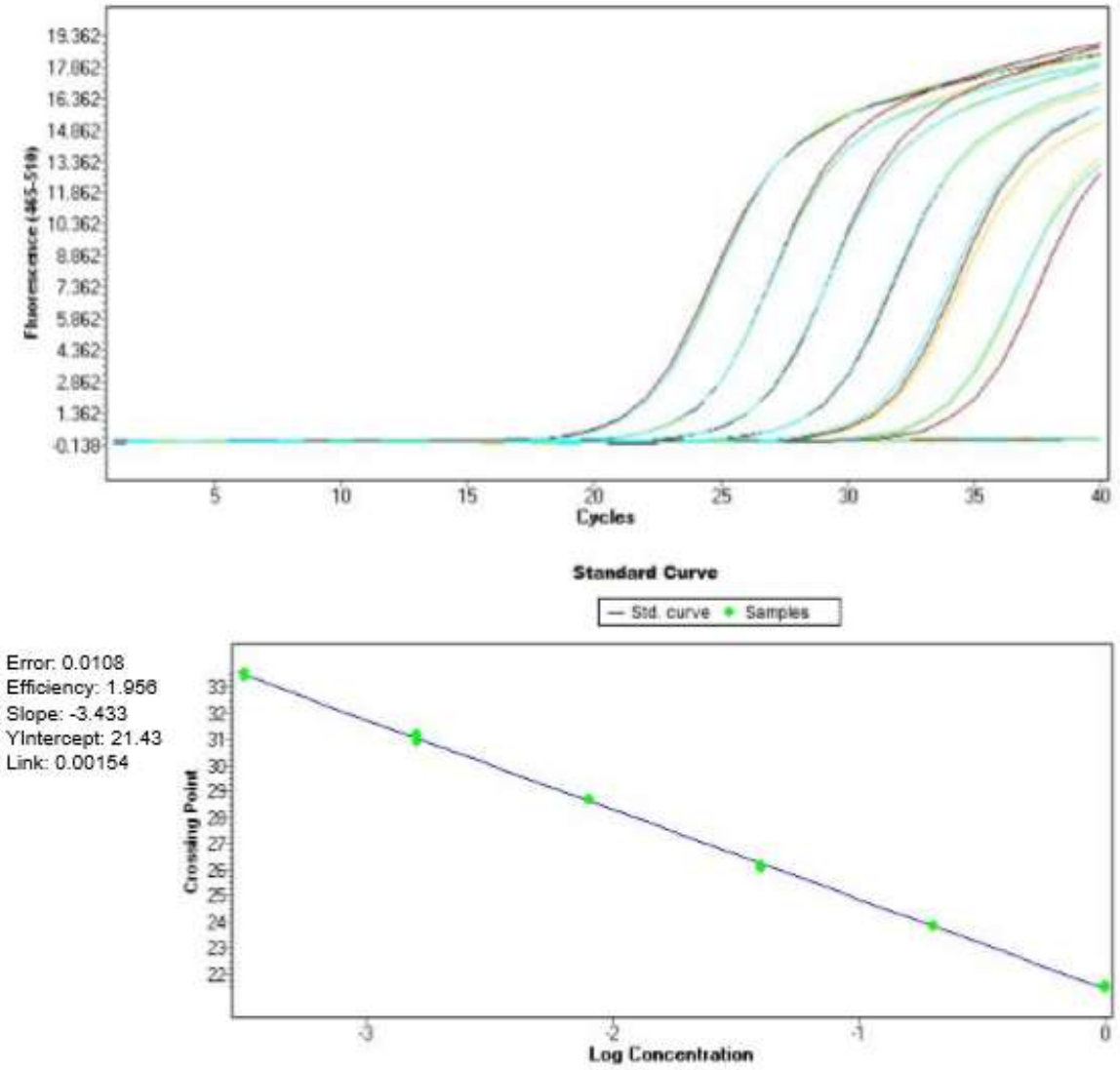
Supplemental Table S5.1: Baseline data for calves arriving at Elora Beef Research Centre including randomly allocated treatment group

Group	Calf ID	Breed	Weight (kg)	Treatment	Temperature	Left US Score	Right US Score	Fibrin-ogen (g/L)	Hapto-globin (g/L)
1	9977	AN	216	Lysate	39.4	0	0	3.02	0.24
1	9979	CH	220	Lysate	39.3	0	3	3.02	0.18
1	9983	CH-AN	224	Lysate	39.9	0	0	5.37	1.28
1	9984	LM	258	Lysate	39.7	2	0	2.53	0.13
1	9986	CH-AN	207	Lysate	40.4	1	1	3.92	0.48
1	9987	LM	206	Lysate	41.1	0	0	1.92	0.14
1	9988	AN	189	Lysate	39.9	0	0	4.12	0.61
2	9992	LM	205	Lysate	39.3	1	0	2.4	0.01
2	9994	CH-LM	211	Lysate	39.9	0	0	2.78	0.18
2	9995	LM	215	Lysate	39.2	0	0	2.95	0.18
2	9997	LM	211	Lysate	39.4	2	1	3.02	0.19
2	10000	LM	180	Lysate	39.4	0	1	2.72	0.14
2	10001	CH-SM	198	Lysate	39.2	0	0	2.44	0.15
2	10002	CH-LM	224	Lysate	39.6	0	1	2.78	0.16
2	10005	AN	207	Lysate	39.7	0	0	2.71	0.16
3	10008	SM-AN	213	Lysate	39.3	0	0	4.75	0.15
3	10011	SM-AN	228	Lysate	39	0	0	3.19	0.15
3	10012	SM-AN	207	Lysate	39.1	2	1	3.63	0.15
3	10013	SM-AN	218	Lysate	39.4	0	4	2.84	0.15
3	10016	SM-AN	236	Lysate	39.1	3	0	4.8	0.24
3	10017	SM-AN	246	Lysate	39.3	1	1	3.21	0.31
3	10018	AN	262	Lysate	40.9	1	1	2.93	0.26
3	10021	SM-AN	245	Lysate	38.5	3	1	4.29	0.19
4	10022	AN	255	Lysate	39.6	1	0.5	3.21	0.14
4	10025	LM	207	Lysate	38.9	3	3	3.21	0.13
4	10026	CH	230	Lysate	39.3	3	0	4.05	0.35
4	10028	SM	228	Lysate	39.4	0	0	3.86	0.14
~~4~~	~~10029~~	~~AN~~	~~222~~	~~Lysate~~	~~39.4~~	~~5~~	~~3~~	~~4.45~~	~~1.3~~
4	10033	CH	204	Lysate	39.4	0	3	3.71	0.11
4	10035	CH	179	Lysate	40.8	4	4	3.66	0.14
1	9976	CH-AN	214	Control	39.1	0	0	3.32	0.14
1	9978	LM	265	Control	39.1	1	0		0.25
1	9980	CH	186	Control	40.1	0	0	3.23	0.25
1	9981	AN	205	Control	41.3	1	1	3.17	0.35
1	9982	AN	219	Control	39.9	0	0	3.98	0.13
1	9985	LM	214	Control	40.2	0	0	2.54	0.13
1	9989	LM	224	Control	39.9	1	3	3.32	0.16
2	9990	LM	204	Control	40	0	0	2.65	0.17
2	9991	BWF	224	Control	40.9	0	2	2.88	0.15
2	9993	LM	206	Control	39.4	1	1	2.1	0.18
2	9996	LM	216	Control	39.4	0	0	2.57	0.19
2	9998	LM	215	Control	39.8	0	0	2.21	0.16

2	9999	LM	209	Control	38.9	0	1	2.49	0.02
2	10003	CH-LM	197	Control	40.4	2	0	3.27	0.17
2	10004	LM	186	Control	39.2	0	1	3.36	0.19
3	10006	SM-AN	215	Control	39.1	0	4	4.12	0.13
3	10007	SM-AN	197	Control	39.6	0	4	2.77	0.17
3	10009	AN	248	Control	39.9	3	0	3.89	0.13
3	10010	SM-AN	232	Control	39.2	0	3	2.84	0.12
3	10014	SM	225	Control	40	0	1	3.27	0.16
3	10015	SM-AN	217	Control	39.1	0	1	4.66	0.71
3	10019	SM-AN	238	Control	39.4	0	4	4.75	0.17
3	10020	AN	292	Control	39.4	0	0	2.29	0.16
4	10023	LM	224	Control	39.9	1	1	3.77	0.17
4	10024	CH	190	Control	39.9	0	3	3.77	0.12
4	10027	CH	229	Control	39.3	1	1	2.65	0.11
4	10030	AN	244	Control	39.9	0	3	2.27	0.12
4	10031	CH	217	Control	38.8	0	1	3.41	0.1
4	10032	AN	210	Control	39.6	3	0	3.66	0.15
4	10034	CH	192	Control	39.5	5	4	3.95	0.1

Supplemental Table S5.2: Mean weight on arrival for calves in cohorts 1 - 4.

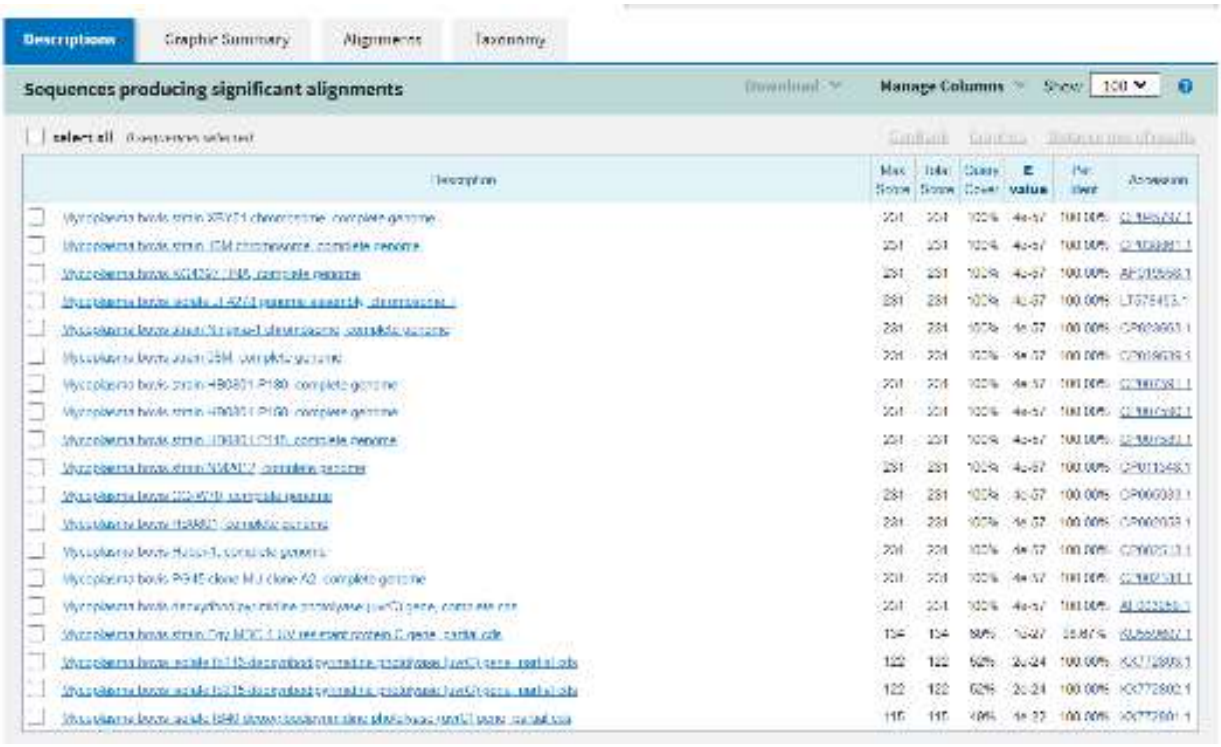
Group	**1**	**2**	**3**	**4**
Mean weight (kg) (95% confidence interval)	217.6 (205.0-230.3)	206.8 (200.3-213.2)	232.4 (220-244.9)	216.5 (204.3-228.7)



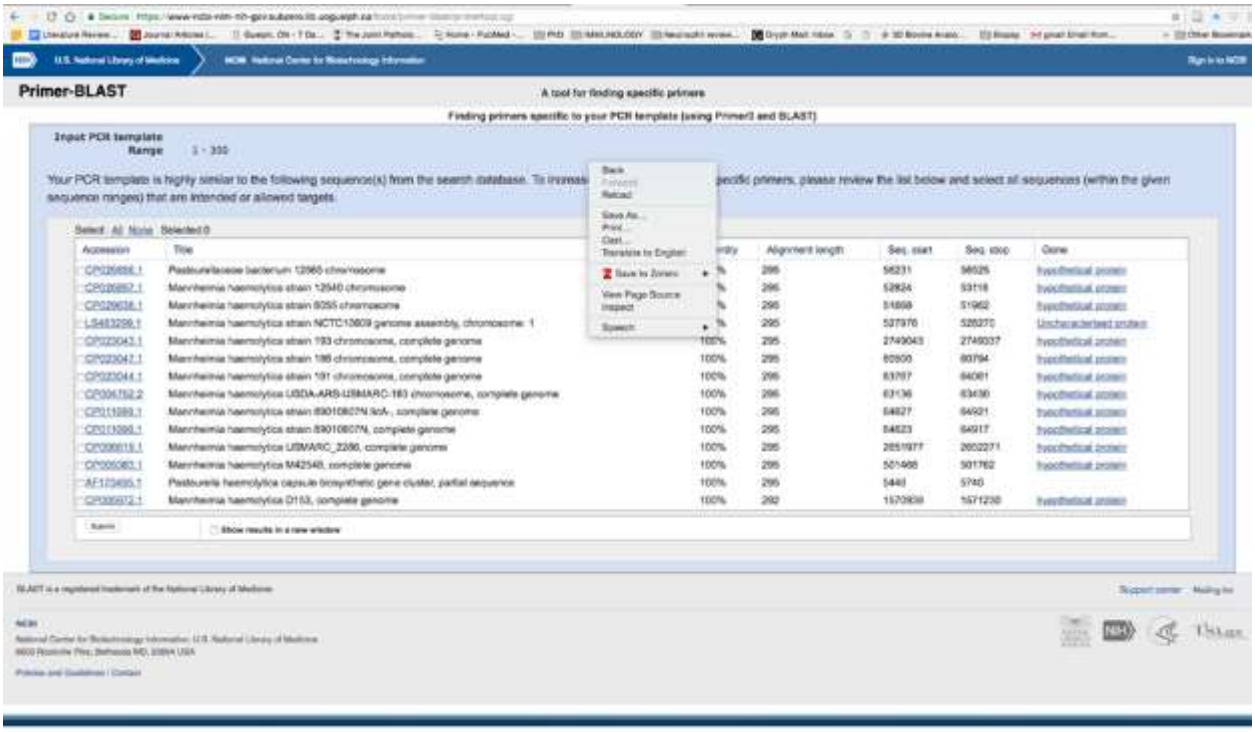
Supplemental Figure S5.1: Standard curve and efficiency for RT-qPCR analysis of *Mycoplasma bovis*.

Supplemental Table S5.3: Standard Result of qPCR analysis of nasal swabs spiked with known numbers of *Mycoplasma* bovis.

Number of *Mycoplasma bovis*	Crossing point
10^1	-
10^2	-
10^3	34.34
10^4	30.06
10^5	25.94
10^6	22.50
10^7	18.88



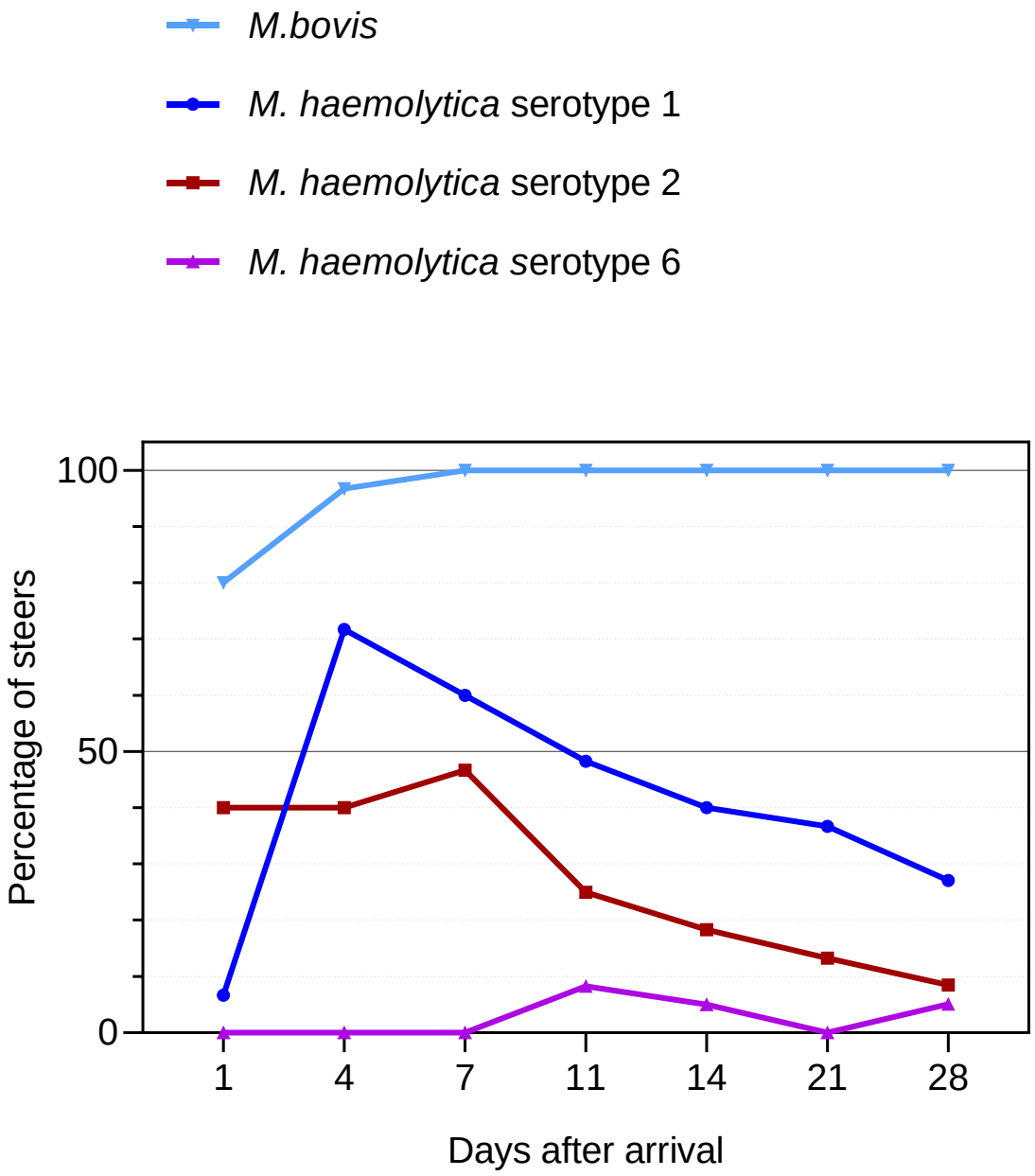
Supplemental Figure S5.2: Supplemental Figure S5.3: Results from Primer-BLAST query of sequenced amplification product from *Mycoplasma bovis* PCR reaction (Ye et al., 2012).



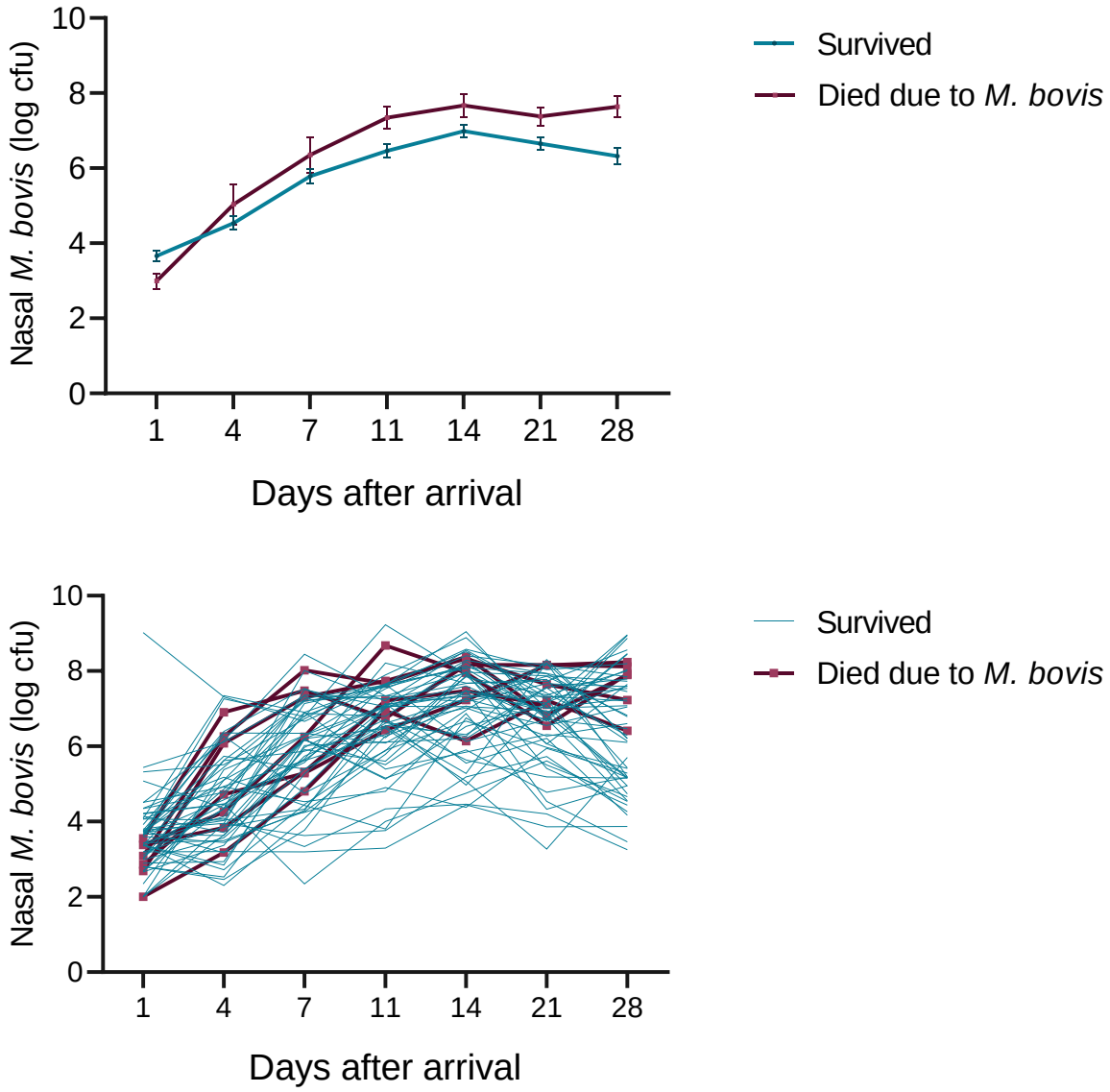
Supplemental Figure S5.4: Results from Primer-BLAST query of sequenced amplification product from *Mannheimia haemolytica* serotype 1 PCR reaction (Ye et al., 2012).

Supplemental Table S5.4: Post mortem findings on 8 calves that died or were euthanized within 3 months of arrival**.**

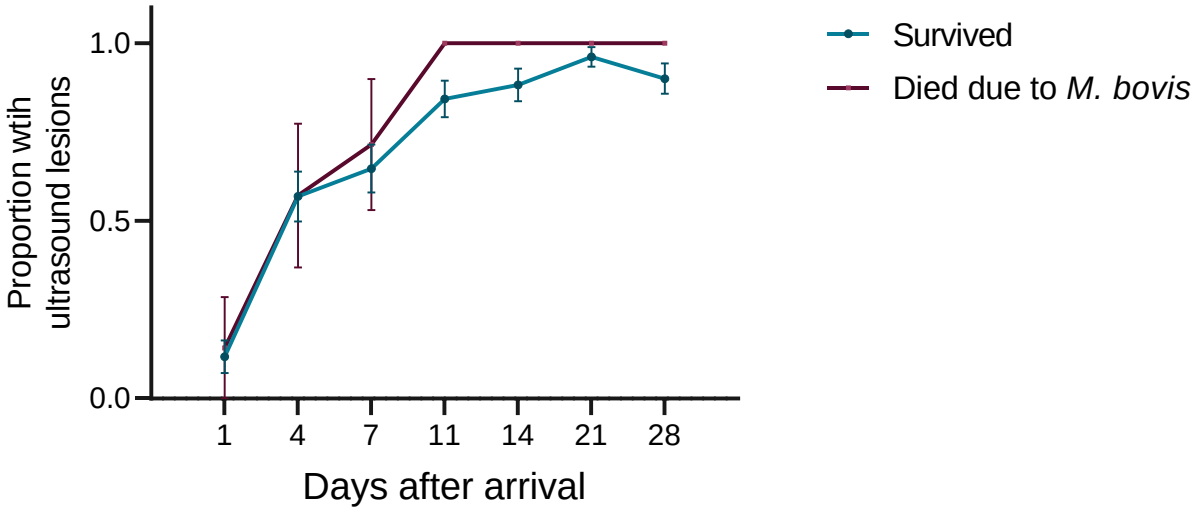
CALF ID	HISTORY	NECROPSY FINDINGS	PATHOGENS IN LUNG TISSUE
9990 (CONTROL)	Treated for pneumonia and septic arthritis (2 treatments). Failure to respond. Euthanized	Caseonecrotic and fibrinosuppurative bronchointerstitial pneumonia and bronchiolitis, Fibrinous and caseonecrotic synovitis and myositis	BRSV (+). *Mycoplasma bovis* (4+) *Mycoplasma arginini (4+) Ureaplasma sp.(4+)*
10001 (LYSATE)	Failure to respond to treatment for pneumonia and arthritis (3 treatments). Found dead.	Fibrinosuppurative and necrotizing bronchopneumonia	*Pasteurella multocida* (4+), *M. bovis* (4+), *Ureaplasma* (4+)
9986 (LYSATE)	Found in lateral recumbancy. previously treated for respiratory disease (1 treatment). Euthanized.	Severe fibrinosuppurative bronchopneumonia	*Trueperella pyogenes*, *M. bovis* (4+), *M. arginini* (3+), *Ureaplasma* spp. (2+)
9992 (LYSATE)	History of fever, depression. (1 treatment). Found dead.	Severe fibrinous suppurative necrotizing bronchopneumonia Trachea: Multifocal ulceration with serocellular crusts	BRSV, *P. multocida, M. bovis* (4+) *M. arginini* (2+) Ureaplasma spp. (4+)
10005 (LYSATE)	Chronic pneumonia with multiple treatments given (3 treatments). Found dead.	Bronchopneumonia, fibrinosuppurative, chronic, diffuse, with multifocal pulmonary abscesses	*Histophilus somni* (4+), *M. bovis* (4+), *M. arginini* (3+) *Ureaplasma* sp.(3+)
10025 (LYSATE)	Treated for respiratory disease with no response (5 treatments). Euthanized	Severe necrosuppurative bronchopneumonia with bronchiectasis and pleural adhesions	*Pasteurella caballi, T. pyogenes, M. bovis* (4+), *M. arginini* (4+), *Ureaplasma* (4+)
10034 (CONTROL)	Stunted growth, poor doing. Resp issues -- tachypnea. 1 treatment, no response.	Lungs: Chronic suppurative bronchopneumonia with abscessation	*Escherichia coli, Streptococcus equinus, Mycoplasma alkalescens* (4+), *Ureaplasma* spp. 4+
10035 (LYSATE)	Dull, fever (3 treatments). Euthanized.	Chronic multifocal severe fibrinosuppurative bronchopneumonia.	*H. somni* 4+, *M. bovis* 4+, *M. arginini 3+, Ureaplasma* spp.4+



Supplemental Figure S5.5: Prevalence of steers with nasal bacteria over time after arrival to the feedlot. Bacteria evaluated were *Mycoplasma bovis* and *Mannheimia haemolytica* serotypes 1, 2, and 6.



Supplemental Figure S5.6: Nasal *M. bovis* in animals that died from *Mycoplasma bovis* and animals that survived. Mean nasal *M. bovis* numbers in animals that died from *M. bovis* pneumonia (n = 7) versus animals that survived to the end of the study (n = 51). Data shown are the mean log-transformed number of *M. bovis* +/- standard errors of the mean B) Individual animal nasal *M. bovis* in surviving animals versus animals that died due to *M. bovis*.



Supplemental Figure S5.7: The prevalence of ultrasound lesions over time in animals that died from *Mycoplasma bovis* and those that survived to the end of the study. Proportion with ultrasound lesions > 3cm in animals that died from *M. bovis* pneumonia (n = 7) versus animals that survived to the end of the study (n = 51). Data shown are the +/- standard errors of the mean

6 Discussion and Conclusions

The work done for this thesis created and characterized an aerosolized bacterial lysate designed to stimulate respiratory innate immune responses, developed a novel aerosolized challenge model for *Mannheimia haemolytica* pneumonia using colostrum-deprived calves, and determined the effectiveness of aerosolization of bacterial lysate in preventing experimentally-induced pneumonia in calves as well as naturally occurring respiratory disease is high-risk beef steers. The rationale for this project was that various innate immune responses have been known to be altered secondary to stresses and viral infection and it was thought that the resulting immunosuppression leads to increased susceptibility to BRD. As the innate system is rapidly responsive and broadly protective against pathogens, it represents a potential target for interventions during the transient high-risk period when cattle are maximally at risk from BRD pathogens. The main hypothesis was that stimulation of innate immune responses during these times of high risk could overcome this immunosuppression and thus reduce disease.

Although we were able to demonstrate induction of innate immune responses following aerosolization of bacterial lysate, this conferred only mild protection against experimentally induced *M. haemolytica* pneumonia in clean-catch colostrum-deprived Holstein calves, and it worsened natural respiratory disease in high-risk feedlot steers. Thus, broad stimulation of innate immune responses was not an effective strategy to reduce BRD. Although this was not the anticipated outcome for this project, the combined results of these experiments provided insight into host and agent interactions in the pathogenesis of BRD and direct us to areas for further investigation. Evaluation of the combined data gathered from each experiment, including pilot studies, has led to

additional insights regarding the pathophysiology behind BRD. Results from this work suggest that the traditional paradigm of stress and viral infection causing immunosuppression and infection by the normally commensal *M. haemolytica* may be oversimplified. The pro-inflammatory effects resulting from stress and viral infections may also contribute to the pathogenesis of BRD. Improved understanding of BRD pathogenesis may lead to better interventions to reduce disease.

6.1 Summary of Chapter 2

As expected, aerosolization of a lysate of heat-killed *Escherichia coli* and *Staphylococcus aureus* stimulated both localized and systemic responses. The local innate immune responses included production of cytokines (IL-8, IL-10 and IL-17) in the lungs and recruitment of neutrophils to alveoli and bronchioles. The systemic inflammatory responses were mild, characterized by transitory elevations in body temperature and small increases in serum haptoglobin (conventionally raised calves) and fibrinogen (colostrum-deprived calves) concentrations. Although neutrophils were detected in the lungs using histopathology and cytology of bronchoalveolar lavage fluid, there was no change in blood neutrophils. Mass spectrometry analysis of bronchoalveolar proteins suggested that platelet activation and complement cascade pathways were induced. Thus, both cellular and humoral components of the innate immune system were stimulated by aerosolization of bacterial lysate.

There were no clinically significant adverse effects identified in these studies, although small foci of alveolar damage were identified in several animals that received higher doses. Although we tested a range of doses from 10^8-10^{12} colony forming unit equivalents, our objective was not to determine the lowest effective dose, nor to

determine the upper threshold for safety. The goal of the study reported in Chapter 2 was to ensure that calves received a dose of lysate that resulted in induction of responses, without significant adverse effects. However, it is notable that with the exception of histological lesions, at these doses, we did not observe obvious dose-related trends for differences in responses. However, this conclusion is limited by the small number of animals evaluated. The lack of observed dose-related responses may also be due to the inherent variability in responsiveness due to genetics, previous life events, environment and other factors which precluded identification of dose-related trends in the small number of animals used for these studies. It is also possible that there was a threshold effect, whereby doses above a certain level triggered similar responses. The lower limit of this response was not determined. In addition, it is possible that administration of higher doses of bacterial lysate than were tested could trigger excessive inflammation and significant adverse effects. The objective of this experiment was to determine whether we could induce measurable innate immune responses and determine a dose without significant adverse effects. Future work could address the range of doses and responses. Similarly, experiments to compare the responses to a standardized dose of bacterial lysate between cattle of different ages, stress levels, viral status and genetic background may provide further insight into the influence of these factors on innate immune responses.

6.2 Summary of Chapter 3

The work summarized in Chapter 3 represents multiple pilot experiments conducted to develop an aerosol *M. haemolytica* challenge model. The initial experiment challenged conventionally raised Holstein calves with positive antibody titers

against *M. haemolytica* leukotoxin. Within the first few hours after challenge with *M. haemolytica* serotype 1, both calves had pronounced dyspnea that was severe enough to lead to euthanasia in one calf at 6 hours after challenge. In this calf, the gross lesions consisted of hemorrhage, edema and subpleural and interlobular emphysema. Histologically, there was marked hemorrhage, fibrin and neutrophils within alveoli and bronchioles. The second calf (that received a lower challenge dose of *M. haemolytica*), showed severe dyspnea but subsequently recovered and showed no further clinical signs from 12-48 hours when it was euthanized. At necropsy this calf had no gross lesions, and only patchy neutrophilic infiltration of alveoli and bronchioles, with mild atelectasis. Bacterial culture of lung tissues did not yield *M. haemolytica*.

We speculate that *M. haemolytica* challenge throughout the lungs in these calves with positive antibody titres triggered an acute antibody-mediated inflammatory response (such as type III hypersensitivity). The kinetics of the clinical signs were very similar to those reported in calves that were parenterally sensitized to human serum albumin and then challenged by aerosol with the same antigen (Taylor et al. 1987), in terms of calf attitude and behaviour, body temperature elevations and resolution of signs. It has been shown that calves vaccinated with bacterins developed more severe clinical disease and more extensive lung lesions following challenge with *M. haemolytica* (Friend et al. 1977; Wilkie et al. 1980). Immunohistochemical evaluation of lungs from natural cases of *M. haemolytica* identified immune complexes along alveolar walls (McBride et al. 1999). This reaction could have also been IgE-mediated or a direct response to LPS. We concluded that aerosol challenge was unlikely to produce progressive pneumonia in antibody-positive calves because if we used a higher dose of

M. haemolytica, the hypersensitivity reaction would likely have necessitated euthanasia within the first 12 hours.

By using clean-catch colostrum-deprived calves we were able to ensure that calves used in challenge experiments were not colonized by *M. haemolytica* and did not have positive antibody titres. Following challenge with *M. haemolytica* serotype 1, these calves developed pneumonia that was pathologically consistent with natural *M. haemolytica* infection and yielded large numbers of lung *M. haemolytica* from postmortem cultures. Furthermore, they did not display the severe early dyspnea that was observed in the conventionally raised antibody-positive calves.

In early pilot experiments (prior to using multiplex PCR to determine the serotypes of *M. haemolytica* strains), three calves were challenged with *M. haemolytica* serotype 2 using doses between 10^7 to 10^9 colony forming units. The highest dose of *M. haemolytica* serotype 2 used was within the same log concentration that produced severe disease in another calf challenged by *M. haemolytica* serotype 1. Interestingly, none of these serotype 2-challenged calves developed persistent nasal colonization, clinical signs, bloodwork changes, significant bronchopneumonia, or positive lung culture at necropsy. Although only one strain of *M. haemolytica* serotype 1 and one strain of *M. haemolytica* serotype 2 were tested, these results support the long-recognized increased virulence attributed to *M. haemolytica* serotype 1 vs serotype 2. *Mannheimia haemolytica* serotype 6 is also considered to be a virulent serotype, but was not tested here.

6.3 Summary of Chapter 4

In the experiment where aerosolized lysate or saline was administered to colostrum-deprived calves prior to *M. haemolytica* challenge, a mild reduction in inflammatory responses was seen in lysate-treated calves in the first 2 days after infection. This was characterized by a less severe increases in serum haptoglobin and blood neutrophilia after challenge. However, this apparent initial protective effect appeared to diminish over time. Following aerosol challenge, the delivered *M. haemolytica* would likely have triggered similar innate immune responses as the bacterial lysate. Although lysate-treated calves tended to have slightly less severe clinical signs and reduced lesion severity at necropsy compared to control animals, these differences were not statistically significant, and the magnitudes of the differences were small. Furthermore, the number of bacteria in the lungs was not reduced in lysate-treated animals. It is possible, if sampling of the lungs had been conducted earlier after infection, that there might have been a reduction in bacterial numbers at that time as a result of the induced cellular and/or humoral responses. However, if this did occur, the number of bacteria must have subsequently rebounded until there was no difference compared to control calves at the time of necropsy. In addition, this potential reduction in bacterial numbers was not sufficient to prevent the subsequent development of pneumonia. Thus, in this experimental model, although the inflammatory responses were altered, the induction of innate immune responses was not sufficient to prevent pneumonia and bacterial numbers in the lungs were not reduced at necropsy.

The colostrum-deprived calves, although immunologically naïve, were not considered to be immunosuppressed. It is likely that their age, genetics, environment, stress levels and background all might contribute to differences in responses when

compared to feedlot calves. Although direct experiments to compare responses between the colostrum-deprived calves and conventional dairy calves were not conducted, the conventional calves did appear to mount more robust responses (specifically, more neutrophils and higher cytokine concentrations in bronchoalveolar lavage fluid following aerosolization) compared to the colostrum-deprived calves. Additional experiments were thus necessary to determine whether the clinically relevant population (high-risk calves recently arrived to a feedlot) might be better protected from natural disease following aerosol delivery of the lysate. Lysate-induced innate immune responses might be more successful at preventing disease in high-risk cattle following natural exposure than they were at preventing disease from a large dose of aerosolized bacteria delivered directly to the respiratory tract.

6.4 Summary of Chapter 5

Instead of conferring protection from pneumonia in high-risk feedlot calves, aerosolization of lysate worsened many of the evaluated outcomes. Lysate-treated calves had lower weight gains, higher body temperatures, increased pulmonary consolidation, and increased nasal carriage and higher mortality due to *Mycoplasma bovis* pneumonia compared to control calves. The lysate-induced innate immune responses were not expected to persist beyond a few days, and direct adverse clinical effects were not identified in prior experiments. It is most likely that lysate administration and the resultant inflammatory responses increased susceptibility to (or worsened disease caused by) *M. bovis* and potentially other BRD pathogens. These adverse outcomes became more apparent towards the end of the study. The reduced weight gain in lysate-treated calves was evident at 28 days after arrival and the increased

mortality did not manifest until the 2nd month after arrival. Thus, in addition to the conclusion that lysate did not confer protection against BRD, the results of this study suggest that in these calves, the induced pulmonary inflammation in the first few days after arrival affected the health and performance in these calves for weeks thereafter and contributed to the development of *M. bovis* pneumonia.

6.5 Disparity between the experimental model and natural disease

One of the more interesting and puzzling findings from this research was the disparity between the experimental disease study, where the bacterial lysate provided mild protection; and the natural disease study, where it worsened many of the outcomes of interest. These studies cannot be directly compared due to the multitude of differences between the animals involved (age, breed, immune status, stress levels, etc), their environments (climate-controlled isolation pens versus feedlot in winter) and their pathogen exposures (single-agent *M. haemolytica* versus the myriad of viruses and bacteria including *Mycoplasma bovis*). However, we can conclude that our "natural" experimental disease model did not predict disease outcomes in response to lysate treatment in feedlot calves.

Bovine respiratory disease in feedlot calves has been widely cited as a classic example of a multifactorial disease, and thus experimentally recreating the circumstances that lead to BRD is complicated. We were able to produce morphologically similar disease in our experimental model, however, stripping away many of the host and environmental factors did not give the same outcome as observed with natural disease. The importance of host factors is highlighted by the need to use colostrum-deprived calves (rather than conventionally raised Holstein calves) in order to

consistently produce *M. haemolytica* pneumonia, even with large numbers of bacteria delivered by aerosol. Similarly, the fragile health of colostrum-deprived calves precluded exposure to the harsh, cold and crowded environmental conditions during transport and auction that are frequently experienced by feedlot calves in the fall and winter.

Finally, our challenge experiments involved only *M. haemolytica* whereas the calves at the Elora Beef Research Station were stressed and were likely infected with other viruses and bacteria during the course of this research. Notably, in the Elora Beef study, *M. bovis* was responsible for the severe disease and mortality identified in the second month but this was absent in the experimental challenge model. Similarly, in contrast to the high-risk calves in the Elora Beef study, the colostrum-deprived calves were neither stressed nor exposed to viruses which are considered key risk factors for the development of BRD. The experimental model was able to produce disease that was morphologically similar to natural BRD, however it is probable that by omitting key factors important in pathogenesis the resultant disease differed enough from natural BRD that we achieved opposing effects of innate immune stimulation on disease development. Vaccine research has been fraught with similar issues, where BRD vaccines appear to perform well under experimental conditions, but were less successful in the field (Confer and Ayalew 2018). Despite this model's failure to predict the effectiveness of lysate administration on preventing natural BRD in feedlot calves, there is potential utility of the aerosolized *M. haemolytica* challenge model. Understanding early events in lung colonization, virulence factor production, and

interventions not related to innate immune responses may be well served by aerosolized *M. haemolytica* challenge.

6.6 Inflammation

Increased inflammation may contribute to the development of BRD. The outcomes of the natural disease study at Elora Beef suggest that in naturally occurring BRD, the pro-inflammatory effects of stress and viruses may be important in the pathogenesis of BRD, perhaps in addition to immunosuppressive effects. If suppression of innate immune responses were the only mechanism by which these factors contribute to BRD pathogenesis in high-risk cattle, then it follows that stimulation of innate immune responses should have improved outcomes or at worst, had no effect. It is more likely that immune responses in high-risk cattle are neither uniformly suppressed nor enhanced, but instead the risk factors experienced by these calves may alter the nature of the inflammatory response to stimuli.

Although several studies show reduced immune responses in stressed and virus-infected calves there is also a significant body of work that has shown increased inflammatory responses in such animals (Caswell, 2013). Inflammatory cytokines, acute phase proteins and blood neutrophils are all reported to increase in stressed cattle (Yagi et al. 2004; Lomborg et al. 2008; Cooke and Bohnert 2011; O'Loughlin et al. 2011; Cooke 2017; Masmeijer et al. 2019). Following transportation, increases in blood concentrations of inflammatory cytokines (tumour necrosis factor alpha), neutrophils, platelets, and monocytes have been reported (Engen et al. 2014). Transportation has also been shown to alter neutrophil gene expression, with reduced expression of the apoptosis-related gene Fas, and increased expression of L-selectin and genes

encoding bactericidal factors (Buckham Sporer et al. 2007). Similarly, dexamethasone was shown to increase neutrophil recruitment and haptoglobin levels in the lungs (Mitchell et al. 2008) and corticotrophin releasing hormone increased blood acute phase proteins and IL-6 (Cooke and Bohnert 2011). Various bovine respiratory viruses are considered to increase susceptibility to BRD via suppression of pulmonary defenses including damaging epithelial cells, and reducing leukocyte numbers and function (Caswell 2013). However, lung inflammation (with neutrophils) is also recognized in uncomplicated infections by many of the common BRD-associated viruses including bovine parainfluenza virus 3, bovine respiratory syncytial virus, and bovine herpesvirus 1 (Maxie 2016). In addition, pro-inflammatory IL-17 production by γδ T cells was synergistically increased by exposure to BRSV and *M. haemolytica* (McGill et al. 2016). Thus, in addition to immunosuppression, there are significant pro-inflammatory effects resulting from stress and respiratory virus infection. Dissecting these 2 potential mechanisms for stress- and virus-induced susceptibility to BRD is complicated in natural disease because both result from co-mingling calves from different sources.

In prior studies of experimental *M. haemolytica* challenge, data on the role of inflammation are variable. A harmful role of inflammation in experimentally induced *M. haemolytica* pneumonia is suggested by studies showing protective effects from depletion of neutrophils prior to challenge (Slocombe et al. 1985; Breider et al. 1988). Similarly, the administration of dexamethasone also reduced disease induced by experimental *M. haemolytica* infection (Malazdrewich et al. 2004). However, in another study, administration of granulocyte colony-stimulating factor prior to *M. haemolytica* challenge increased blood neutrophils and resulted in partial protection from disease

(Youssef et al. 2004). Similarly, in our experimental challenge study, bacterial lysate administration also had a mild protective effect from *M. haemolytica* challenge. Thus, the reported role of inflammation in experimental *M. haemolytica* challenge varies between studies.

In naturally occurring BRD, investigation into the role of inflammation on disease development is sparse, but consistently report that strategies to ameliorate inflammation provide some protection from disease. Administration of anti-inflammatory drugs has been shown to reduce transportation-associated inflammatory responses and may impact natural respiratory disease. Meloxicam administration prior to transportation reduced the stress-associated neutrophilia and monocytosis (Engen et al. 2014). Following transportation, meloxicam treatment reduced acute phase protein levels in serum and improved weight gain over the first few weeks after arrival (Guarnieri Filho et al. 2014). In another feedlot study, meloxicam administration at castration reduced the number of clinical cases of BRD (Coetzee et al. 2012). Flunixin meglumine treatment prior to long distance transport and at the time of unloading similarly reduced cortisol and acute phase proteins, but did not improve weight gain compared to untreated control animals (Cooke et al. 2013). In addition, there is evidence indicating cattle that are better able to downregulate inflammation may be afforded protection from clinical BRD. Cattle that developed BRD early after arrival to the feedlot had lower levels of annexin A1 (a glucocorticoid-regulated protein that downregulates inflammation) in bronchoalveolar fluid at the time of arrival (Senthilkumaran et al. 2013; Sugimoto et al. 2016). Transcriptome analysis of blood in on-arrival calves showed that calves that

went on to develop BRD had lower expression of anti-inflammatory mediators and pro-resolving mediators (Scott et al. 2020).

In addition to the above analysis of inflammation in clinically healthy animals soon after arrival to the feedlot, severe inflammation is a hallmark of BRD mortalities and dampening of the inflammatory responses with corticosteroids or non-steroidal anti-inflammatory drugs is a common ancillary treatment in clinical cases of BRD. Adjunct anti-inflammatory treatment improves clinical signs, reduces body temperature, and improves appetite in animals being treated for clinical BRD (Bednarek et al. 2003; Friton et al. 2005; Hewson et al. 2011). In addition to short term improvements, performance improvements have been reported. In one study, animals that received meloxicam in addition to oxytetracycline at the time of BRD diagnosis had higher body weights up to and including the day of slaughter and less severe lung lesions (Friton et al. 2005). The results of these studies suggest that reduction of inflammation provides protection from BRD. Our research demonstrated the inverse correlate: increased inflammation worsened BRD.

In comparing the natural disease (feedlot) study with the experimental challenge study, one potential explanation for the disparate results is that in cattle that may already have stress-induced inflammation of the respiratory tract, additional inflammatory stimuli may enhance their development of clinical BRD. We also considered that stress or virus infection in these feedlot calves might have predisposed to lysate-induced lung injury, which did not occur in the clean-catch, colostrum-deprived calf studies. However, evidence contradicting this theory includes the timing of clinical illness. In the feedlot calves, severe clinical disease and death occurred later (several

weeks) than would be expected from a direct lung injury (hours to days). Additionally, the ultrasound lesions showed localized consolidation rather than diffuse lung injury, and the pathological findings at the time of death were of bronchopneumonia and not diffuse alveolar damage.

Future investigation: Immunomodulation as a strategy to prevent bovine respiratory disease.

In these studies, enhancing innate immune responses in feedlot calves worsened bovine respiratory disease. Although feedlot calves are frequently considered “immunosuppressed” due to the stresses of weaning, transportation and processing, the reality is more complicated. Studies have shown that some responses are diminished in stressed calves, but others including some neutrophil responses are enhanced. It is plausible that in feedlot calves, exacerbated inflammatory responses contribute to pathogenesis of *Mannheimia haemolytica* pneumonia. *Mannheimia haemolytica* leukotoxin targets neutrophils, causing lysis and the release of toxic granule contents which may contribute to increased tissue damage. Increased numbers of pulmonary neutrophils and increased expression of the leukotoxin target receptor (CD18) caused by stress in these calves may contribute to the development of disease (Czuprynski et al. 1991). As such, strategies to dampen the transportation- and stress-associated increases in neutrophil infiltration and/or CD18 expression may reduce the incidence of disease.

6.7 Mycoplasma bovis

The severity of the *M. bovis* pneumonia and the apparent increase in nasal *M. bovis* bacteria in lysate-treated calves suggests an important role for inflammation in the pathogenesis of this condition. As the majority of *M. bovis* mortalities occur in the second month after arrival to the feedlot (Gagea et al. 2006a), it is possible that earlier (inflammatory) lesions caused by infections with other BRD pathogens, including *M. haemolytica*, might provide a favourable lung environment in which *M. bovis* can proliferate and persist. Histological examination of naturally occurring mortalities has demonstrated *M.* bovis-induced caseonecrotic lesions within lesions of coagulative necrosis attributed to *M. haemolytica* (Gagea et al. 2006b). In the present study, it appears that lysate-treated calves, with induced pulmonary inflammation, had more severe *M. bovis* pneumonia. As *M. bovis* is difficult to treat once it become established, understanding the early steps of pathogenesis may present important strategies to controlling this disease (Maunsell and Chase 2019). The critical importance of the first few days after arrival is suggested by the adverse outcomes caused by lysate administration during this time.

Future investigation: The role of pre-existing inflammation in the pathogenesis of *Mycoplasma bovis* pneumonia

Severe *M. bovis* pneumonia developed in the lysate-treated calves in the natural disease study. This suggests that lung inflammation is important in pathogenesis of this disease. Since cattle with naturally occurring *M. bovis* pneumonia usually have co-infection with other pathogens (i.e. *M. haemolytica*, other BRD bacteria, and sometimes viruses) and, in this study, seemed to have

initially developed pneumonia at similar times after arrival as calves that died from other forms of pneumonia (e.g. *Mannheimia haemolytica* broncho-pneumonia), this finding may be relevant to pathogenesis. *Mycoplasma bovis* may benefit from the presence of pre-existing inflammation to cause more severe lesions, or be more difficult to eliminate when pneumonia or necrosis from other causes is present. Thus, controlling this early pneumonia (or inflammation) may prevent the development of *M. bovis* pneumonia before it becomes severe and resistant to therapy. Although in most cases *M. bovis* pneumonia occurs in the second month, controlling inflammation soon after arrival may aid in prevention.

6.8 Mannheimia haemolytica

Due to the lack of a widely available method to classify (serotype) *M. haemolytica* strains, the early unsuccessful challenge experiments (Chapter 3) inadvertently used a strain of serotype 2, generally considered less pathogenic. Aerosol challenge of colostrum-deprived calves with *M. haemolytica* serotype 2 at doses up to 1×10^9 CFU failed to produce clinical disease or lung lesions, and *M. haemolytica* could not be isolated from the lungs at necropsy. In comparison, a calf in one pilot study was challenged with 2×10^9 cfu of *M. haemolytica* serotype 1 developed severe disease. This calf had severe clinical signs necessitating euthanasia, with 43% lung consolidation at post-mortem examination, and large numbers of *M. haemolytica* serotype 1 were cultured from lungs and the kidney. Although one strain of each serotype was tested, these results support what has been published in the literature and

highlight the need for precise characterization of *M. haemolytica* strains. Many studies report that *M. haemolytica* serotype 1 is more frequent in the nasal cavity and lungs of sick cattle, whereas serotype 2 is more common in healthy cattle (Purdy et al. 1997; Al-Ghamdi et al. 2000; Rice et al. 2008; Griffin et al. 2010; Klima et al. 2014) (Klima et al. 2014).

There are several more recent studies reporting differences in genetics, virulence and behaviour between the different serotypes. Recently, comparison of whole genome sequences of several *M. haemolytica* isolates of each serotype found that serotype 2 isolates had a smaller genome with fewer prophages and higher sequence diversity in several virulence factors compared to serotypes 1 and 6 (Klima et al. 2016). Invasion and intracellular replication within bronchial epithelial cells has been demonstrated in serotype 1 but not 2 (Cozens et al. 2019), albeit based on a single isolate of each. Finally, during stress and viral infections, *M. haemolytica* serotype 1 proliferate in the nasal cavity (Frank and Smith 1983; Frank et al. 1986, 1987; Jones 1987; Cozens et al. 2019). Our feedlot study corroborated this, with a marked increase in the prevalence of nasal serotype 1 demonstrated between days 1 and 4 and no change in serotype 2. Thus, consideration should be given to differences between *M. haemolytica* serotypes, and factors (including microbiome, and stress responses) which affect the nasal population shifts from serotype 2 to serotype 1, to further understand pathogenesis. Recent development of MALDI-TOF genotype identification (Loy and Clawson 2017) and multiplex PCR for serotype (Klima et al. 2017) will help aid future researchers in easily characterizing bacteria in their studies.

Future investigation: Factors influencing shifts in nasal bacteria, with emphasis on proliferation of *Mannheimia haemolytica* serotype 1

Although the dramatic increase in nasal *M. haemolytica* serotype 1 numbers following transportation is well recognized, how it occurs is relatively unexplored. The influence of microbial endocrinology may be worth investigation. Microbial endocrinology is defined as “the study of the ability of microorganisms to both produce and recognize neurochemicals that originate either within the microorganisms themselves or within the host they inhabit” (Lyte 2014). The impact of stress on immune responses in feedlot cattle has been the focus of investigation for many years; however, little research has investigated the direct effects of cortisol, epinephrine and norepinephrine on BRD pathogens. It is possible that *M. haemolytica* serotype 1 can also detect and respond to alterations in cortisol and neuropeptides leading to altered growth rates or virulence factor expression.

It has been demonstrated that other pathogens *including Yersinia enterocolitica*, *E. coli* and various *Salmonella* and *Shigella* species have increased multiplication and expression of virulence factors in response to catecholamines (Lyte 2014). Of relevance to respiratory disease in veterinary species, both *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica* responded to norepinephrine with altered transcription or growth (Anderson and Armstrong 2006; Oneal et al. 2008). Thus far, little work has been conducted on direct effects of stress hormones on *M. haemolytica*, although, one study did

demonstrate that norepinephrine and epinephrine led to dispersion of biofilms (Pillai et al. 2018). Investigating the direct effects of hormones on *M. haemolytica* growth, invasiveness and virulence factor production may aid in understanding the shifts in nasal populations and increased pneumonia seen in stressed cattle.

6.9 Need for alternate strategies to prevent BRD

Although this study was not designed to test the effectiveness of metaphylactic therapy, it is strongly suspected, based on other studies (Baptiste and Kyvsgaard 2017), that our disease burden would have been lower had we administered antimicrobial treatment on arrival. However, as our study objective was to investigate alternative strategies to antimicrobial metaphylaxis, antimicrobial use was restricted to treatment of animals with clinically detected disease. By failing to use metaphylaxis in these high-risk cattle, we had a large burden of respiratory disease in our study population. However, increasing concerns about antimicrobial resistance and growing public scrutiny regarding the reliance on antimicrobials in food production have made prudent use and the need for alternate strategies for disease prevention a priority for Canadian agriculture (Prescott et al. 2012). In a recent study of Western Canadian feedlots, 39% of cattle were considered high risk for BRD, and 95% of these received metaphylaxis (Brault et al. 2019). In that study, metaphylaxis was the principal reason for administration of individually dosed antimicrobial treatments and the most frequently administered drugs used are considered category III (of medium importance to human medicine as categorized by Health Canada Veterinary Drugs Directorate) (Brault et al. 2019). Ideally, lowering the proportion of high-risk animals through pre-conditioning programs could decrease the incidence of BRD and the number of animals requiring

antimicrobial treatments, but these practices have not been universally adopted. This results in animals that, without metaphylactic treatment, will have a high risk of disease upon entry to the feedlot as demonstrated by the severe respiratory disease that occurred in this study. This strongly emphasizes our current reliance on antimicrobials in intensive beef production and the need for alternative strategies for the future. It is neither economical nor humane to withhold antimicrobial therapy when doing so results in such severe disease, but a system reliant on mass antimicrobial treatments is likewise not sustainable.

6.10 Conclusion

This research demonstrated that administration of bacterial lysate conferred mild protection from experimental *M. haemolytica* challenge, but the induced lung inflammation was detrimental to feedlot calves. Although it is commonly said that immunosuppression leads to colonization of the lungs by opportunistic *M. haemolytica*, the results of this and other research suggest that to better understand the pathogenesis of BRD it is necessary to more finely parse the details of what is known. Rather than focusing on the suppressed immune responses, researchers may also consider the pro-inflammatory aspects of stress and viral infection in BRD pathogenesis. Even development of *M. bovis* pneumonia, which typically occurs during the second month after arrival, may be affected by inflammation in the respiratory tract occurring soon after arrival. This and other research also demonstrate the importance of characterizing *M. haemolytica* strains. Failure to differentiate the pathogenic *M. haemolytica* serotypes 1 and 6 from the less pathogenic serotype 2 may cloud our understanding of the nasal and lung epidemiology of these bacteria, whereas more

precise classification of *M. haemolytica* strains will enhance our understanding of BRD pathogenesis.

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7 Appendices

Appendix 1: Calf Feeding Record and Health Check

Calf ID: Date of arrival:
AUP#:3286 PI: Jeff Caswell

Date/ Time	Litres of milk consumed	Stool score	Rectal temperature	Other notes

- Stool scores: A: solid, B: pudding-like, C: watery pudding and stays on top of shavings, D: watery and goes through shavings

Notes to animal care attendants:

- Visual appearance is assessed at each feeding.
- Milk consumption is assessed at each feeding (the amount fed by bottle, and the approximate amount removed from the bulk feeder since the prior feeding.
- Rectal temperature and palpation of joints and umbilicus are assessed daily
- Clinical signs (contact Laura or Jeff if these signs occur):
  - Septicemia: fever, depression/ sulkiness/ reduced mentation, recumbency / won't rise when stimulated, swollen or hot joints, lameness, or neurologic signs such as seizures or twitching.
  - Enteritis/diarrhea: worsening of stool scores such as liquid feces, fecal staining of the perineal area, or dehydration (skin tent, sunken eyes); and by a worsening in the stool score (see chart;).
  - Navel infection: fever, presence of a warm, swollen, or tender umbilicus.
  - Decreased appetite

Appendix 2: Necropsy protocol for sampling and scoring *Mannheimia haemolytica*-challenged calves.

Necropsy scoring sheet

Parameters

- Estimated percentage affected
    - Left cranial, cranial part (5%)
    - Left cranial, caudal part (6%)
    - Left caudal (32%)
    - Right cranial, cranial part (6%)
    - Right cranial, caudal part (5%)
    - Right middle (7%)
    - Right caudal (35%)
    - Accessory (4%)
- Subjective ranking of lesion severity (1=worst, 12=most normal)
- Lung weight: heart weight

Samples

PHOTOGRAPHS: Lung, left and right and ventral, with identification
BACTERIOLOGY: Targeting lesions, sample all lung lobes (2 cm^3)
HISTO: Targeting the margin of lesions, left and right cranioventral, caudodorsal and caudoventral lung lobes

Equipment:

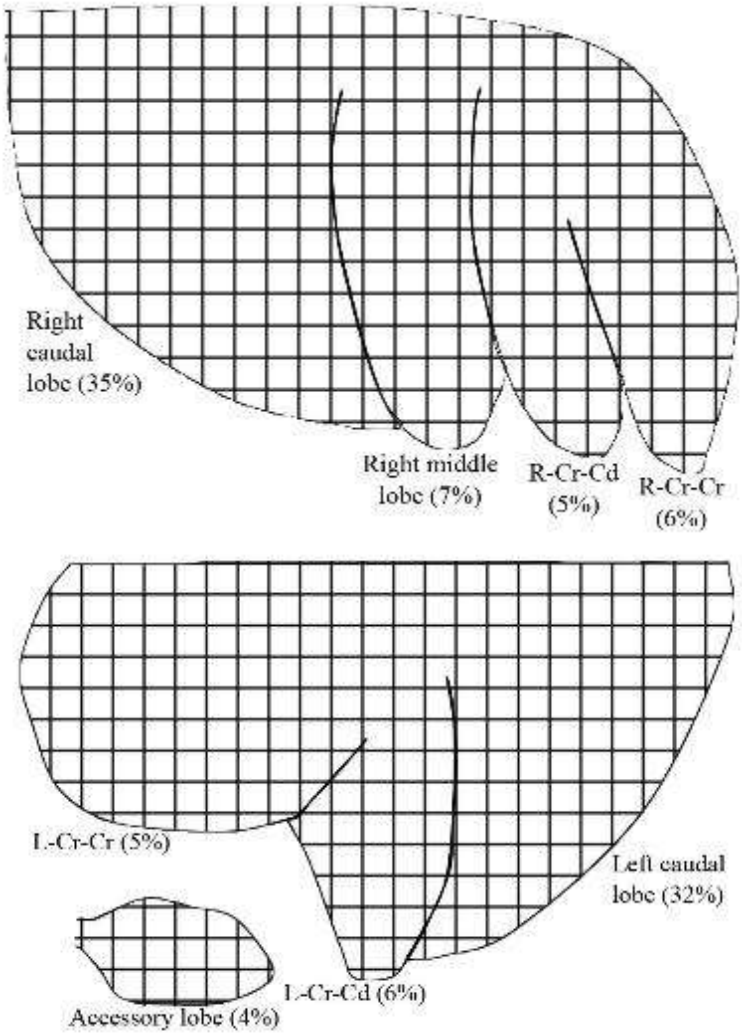
- Euthanyl, syringes, needles, drug logbook
- Knives, scalpel blades, scalpel handle, forceps, scissors, hacksaw
- Cutting board
- Camera and batteries
- Ice
- Whirl paks
- Formalin jars (3 per calf)
- Scoring sheets and pens

Appendix 3: Postmortem scoring sheet for estimating the extent of lung lesions.

Necropsy scoring sheet:

Calf ID: Time/order of necropsy: Subjective ranking of severity:
Lung weight:
Heart weight:

Notes:



Appendix 4: Ultrasound scoring sheets.

Calf:

			Right										Left									
Date	Day	Location	ICS 10	ICS 9	ICS 8	ICS 7	ICS 6	ICS 5	ICS 4	ICS 3	ICS 2	ICS 1	ICS 1	ICS 2	ICS 3	ICS 4	ICS 5	ICS 6	ICS 7	ICS 8	ICS 9	ICS 10
	0	D																				
	0	M																				
	0	V																				
	1	D																				
	1	M																				
	1	V																				
	2	D																				
	2	M																				
	2	V																				
	3	D																				
	3	M																				
	3	V																				
	4	D																				
	4	M																				
	4	V																				

Post-Mortem Notes