Evaluation of an on-farm preconditioning program and novel spore-based mucosal vaccine as tools to mitigate bovine respiratory disease

by

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Abstract

Bovine respiratory disease (BRD) is the most costly disease in the North American feedlot industry. Antimicrobials are mainly used to prevent BRD in feedlots. However, concerns over antimicrobial use and rising resistance in key BRD pathogens necessitate alternative strategies to reduce antimicrobial dependency and prevent BRD in feedlot cattle. The pathogenesis of BRD is associated with several stressors which impair calf immunity, such as abrupt weaning, transportation, auction barn sales, commingling, and feed adaptation. Preconditioning programs are designed to distribute stressful events over a period of time, so as to optimize animal health prior to feedlot placement. Despite this, there is limited information on how preconditioning affects immunity and respiratory microbiota. Study 1 (Chapter 2) investigated the impact of on-farm preconditioning (multiple vaccination, low-stress weaning, introduction to bunk feeding) on cattle stress, immunity, morbidity, and respiratory bacterial responses after feedlot placement. When transported to a feedlot, preconditioned calves exhibited reduced stress. However, BRD morbidity was not different between preconditioned and non-preconditioned calf groups up to 32 days after feedlot placement. This was attributed to low efficacy of the vaccine used to target *Histophilus somni*, which was the most abundant pathogenic genus in BRD cases from both cattle groups. Thus, this first study highlighted the need for improved mucosal vaccines to enhance protection against BRD pathogenesis. Therefore, a major component of this thesis aimed to develop a novel mucosal vaccine using spore adsorption technology. *Mannheimia haemolytica* was the targeted pathogen due to its significant role in BRD and well-characterized antigenic epitopes. *Bacillus subtilis* spores were used as adjuvant because they have been shown to adsorb antigens for presentation to immune cells, eliciting an antibody response. Study 2 (Chapter 3) developed a *M. haemolytica* chimeric protein (MhCP) containing a tandem repeat of *M. haemolytica* membrane protein PlpE, and the neutralizing epitope of leukotoxin (NLKT). The purified MhCP was

then adsorbed to *B. subtilis* spores, and the resultant spore-based vaccine (Spore-MhCP) was evaluated in mice. In addition to inducing a systemic immune response, intranasal immunization of Spore-MhCP elicited a strong secretory IgA-specific response against both PlpE and NLKT in bronchoalveolar lavage, saliva, and feces. Study 3 (Chapter 4) developed two additional spore-based *M. haemolytica* vaccines, Spore-MhCP1 and Spore-MhCP2, using leukotoxin as the core component, along with either PlpE or GS60 membrane protein. Intranasal immunization of mice with these spore-bound vaccines induced stronger mucosal and systemic antibody responses compared to free antigens, indicating that binding antigens to *Bacillus* spores led to an enhanced immune response, emphasizing the potential of spores as an effective adjuvant for respiratory vaccines. In study 4 (Chapter 5), Spore-MhCP was further characterized using sheep as a ruminant model. The vaccine was administered intranasally and intragastrically, while unbound MhCP was evaluated intranasally and intramuscularly. A control group was administered saline intranasally. Study 4 demonstrated that intranasal Spore-MhCP elicited the strongest secretory IgA response against PlpE and NLKT in nasal swabs, bronchoalveolar lavage, and feces compared to control sheep. Intranasal Spore-MhCP also showed a tendency towards enhanced immune responses compared to unbound MhCP. In addition, intragastric administration of Spore-MhCP elicited antigen-specific secretory IgA and serum IgG. Thus, it was shown that immunity in sheep was conferred by both intranasal and intragastric vaccine administration. Sequencing analysis of the 16S rRNA gene from nasopharyngeal samples revealed that 27 genera were altered in their abundances for the intranasal Spore-MhCP group compared to the control. Notably, *Mannheimia* abundance decreased consistently from days 14 onwards in the intranasal Spore-MhCP group, likely due to vaccine-induced antibodies. Since the proliferation of respiratory pathogens in the upper respiratory tract is a precursor to lung infection, this spore-based technology may provide protection against both pathogen proliferation and subsequent lung infection. In summary, the technology

developed in this thesis has the potential to expand protection against respiratory diseases through mucosal vaccination, and warrants further evaluation in challenge and field studies with cattle.

Preface

This thesis is an original work by Muhammed Salah Uddin. All the animal experiments involved in this thesis were carried out in strict accordance with the recommendations established in the Canadian Council on Animal Care Guidelines. The animal experiment for Chapter 2 was reviewed and approved by the Animal Care Committee of Lacombe Research and Development Centre (Animal Use Protocol number 202201). The mice experiments for Chapter 3 and 4 were reviewed and approved by Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol number 1712) before commencement. The sheep experiment for Chapter 5 was reviewed and approved by Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol number 1906). The research projects of this thesis was funded by Alberta Beef Producers, Results Driven Agriculture Research (Project #2023F281R), and Agriculture and Agri-Food Canada.

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Abbreviations

Symbols	Definition
Ab	Antibody
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
ASL	Air surface liquid
ASVs	amplicon sequence variants
BAL	Bronchoalveolar lavage
BCV	bovine coronavirus
BHI	Brain heart infusion
BHV-1	Bovine herpesvirus type 1
BIC	Bayesian information criterion
BRAV	bovine rhinitis A virus
BRBV	bovine rhinitis B virus
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
CDCS	colostrum-deprived calf serum
CT	Cholera toxin
DCA	Detrended correspondence analysis
DNA	Deoxyribonucleic acid

DNS	Deep nasopharyngeal swab
ELISA	Enzyme-linked immunosorbent assays
GLIMMIX	Generalized liner mixed model
GRAS	Generally regarded as safe
IDV	Influenza D virus
IG	Intragastric
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IM	Intramuscular
IN	Intranasal
IPTG	Isopropyl 1-thio-β-d-galactopyranoside
IQR	Interquartile range
LAB	Lactic acid-producing bacteria
LaRDC	Lacombe Research and Development Centre
LB agar	Luria–Bertani agar
LeRDC	Lethbridge Research and Development Centre
LKT	Leukotoxin
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
MALT	Mucosa-associated lymphoid tissue
MhCP	M. haemolytica chimeric protein
Mh-LKT	*M. haemolytica* leukotoxin

NAHMS	National animal health monitoring system
NALT	Nasal-associated lymphoid tissue
NETs	Neutrophil extracellular traps
NK cells	Natural killer cells
NLKT	Neutralizing epitope of *M. haemolytica* leukotoxin
NP	Nasopharyngeal
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate bufered saline
PCA	Principal coordinates analysis
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccines
PERMANOVA	Permutational multivariate analysis of variance
PI3	Parainfluenza virus type 3
PMSF	Phenyl methyl sulphonyl fuoride
PRRs	Pattern recognition receptors
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SBA	Serum bactericidal activity
sIgA	Secretory IgA
Spore-MhCP	Spore-bound MhCP
tet(H)	Tetracycline resistance gene
TLRs	Toll-like receptors

TTA	Trans tracheal aspiration
URT	Upper respiratory tract
USDA	United States Department of Agriculture

Chapter One

Literature review

1.1 Bovine respiratory disease

1.1.1 Background

The beef production cycle encompasses three primary stages, which include cow-calf, backgrounding, and feedlot finishing (Beauchemin et al., 2010). The cow-calf stage commences with cows breeding in summer and giving birth in spring. Calves are raised on hay and pasture with minimal interventions until five to eight months of age, at which point they are weaned and grouped with other calves. The backgrounding stage follows, where calves are fed a forage-based diet to gain weight, either on the same farm or elsewhere. Cattle then enter feedlots for finishing between nine and eleven months, receiving high-energy grains until they reach a market weight of 650 to 775 kilograms at approximately 18 to 24 months, after which they are sent to processing plants for slaughter. Bovine respiratory disease (BRD) is the leading cause of death and economic loss in nursing beef calves (Wang et al., 2018) and remains a significant issue post-weaning, being the primary cause of morbidity and mortality in North American feedlot cattle (Cernicchiaro et al., 2013; Smith, 2020). The development of BRD is interlinked with various management, host, and microbial factors (Taylor et al., 2010b). Bacteria, including *Mannheimia haemolytica*, *Histophilus somni*, *Mycoplasma bovis*, and *Pasteurella multocida* are frequently involved in BRD (Confer, 2009). These opportunistic pathogens inhabit the upper respiratory tract and can infect the lungs when cattle immunity is compromised by stress or primary viral infections. Cattle entering feedlots are often given metaphylactic antimicrobials as a routine measure to prevent infections. However, concerns about antimicrobial use in livestock (Cameron and McAllister, 2016), coupled with rising antimicrobial resistance in key BRD pathogens (Anholt et al.,

2017; Timsit et al., 2017a) highlight the need for alternative strategies to reduce antimicrobial dependency and as a method to prevent BRD in feedlot cattle.

1.1.2 Significance and economic impact of bovine respiratory disease

The prevalence of BRD has not waned in North American commercial feedlots, even with advances in veterinary medicine, animal husbandry and welfare, and increased use of metaphylactic antimicrobials upon feedlot arrival (Brooks et al., 2011; Cernicchiaro et al., 2013; Smith et al., 2020). Pre-weaning BRD affects up to 10% of the US beef calves (Hanzlicek et al., 2010), resulting in mortality rates of 0.6–1.4% (USDA 2010), with infected calves potentially weighing less at weaning than healthy ones (Smith et al., 2020; Snowder et al., 2005). Cattle are most commonly affected by BRD within the initial 45 days after being placed in a feedlot (Avra et al., 2017; Edwards, 2010). In the United States, 97% of feedlots reported cases of BRD in their cattle, affecting approximately 21.2% (2.29 million) of beef cattle placed in feedlots (USDA, 2013). Researchers assessed the economic impact of BRD treatment frequency on cattle through comparison to untreated counterparts and found that treated cattle experienced decreases in carcass value of $23.23, $30.15, and $54.01 for one, two, and three or more treatments, respectively (Schneider et al., 2009). Increased BRD treatments correlate with decreased net returns during cattle backgrounding and finishing phases (Brooks et al., 2011). The annual financial loss due to BRD, including mortality, reduced feed efficiency and performance, as well as treatment costs, is estimated to exceed $4 billion annually for the US beef industry (Cernicchiaro et al., 2013). This loss surpasses the economic impact of all other cattle diseases combined (Duff and Galyean, 2007), with BRD accounting for 70-80% of all morbidity and 40-50% of all mortality in US feedlots (Hilton, 2014). Given the similarities between the beef industries of Canada and the United

States, it is expected that similar economic losses on a proportional basis would affect the Canadian cattle industry.

1.2 Predisposing factors of bovine respiratory disease

1.2.1 Viral agents

The principal viral agents linked to BRD include bovine herpesvirus type 1 (BHV-1), bovine viral diarrhea virus (BVDV), parainfluenza-3 virus (PI3), and bovine respiratory syncytial virus (BRSV) (Grissett et al., 2015). In recent years, Influenza D virus (IDV), bovine coronavirus (BCV), bovine rhinitis A virus (BRAV), and bovine rhinitis B virus (BRBV) have been identified as additional pathogens that contribute to this disease complex (Fulton, 2020; Zhang et al., 2019). These viruses commonly infect the upper respiratory tract of cattle and can independently cause respiratory infections in cattle. However, in post-weaned beef calves, they also predispose the respiratory tract to bacterial infections by damaging the mucosa and increasing bacterial pathogen binding sites (Gershwin et al., 2015; Grissett et al., 2015). In addition to inflicting damage on the mucociliary escalator mechanism and lung parenchyma, viral infections have the potential to compromise the immune system (Caswell, 2014; Murray et al., 2016). Consequently, this compromised state facilitates the translocation of bacteria from the upper respiratory tract to the lung and the subsequent establishment of infection in the lower respiratory tract (Zhang et al., 2019). Fatal BRD often involves an initial viral infection followed by a secondary bacterial infection, and experimental evidence has demonstrated the synergistic role of viral and bacterial agents in causing BRD (Hodgson et al., 2005). Furthermore, calves that were challenged with BHV-1 followed by *M. haemolytica* exhibited greater abundance and more sustained colonization of *M. haemolytica* in their nasopharynx compared to those infected only with *M. haemolytica* (Jericho et al., 1986; Word et al., 2019).

1.2.2 Bacterial agents

Among the bacterial pathogens linked to BRD, *Mannheimia haemolytica* is the most commonly isolated agent, known for causing acute lung infections characterized by fibrinous pneumonia, significantly impacting the North American feedlot industry (Snyder et al., 2017). Taxonomically, *M. haemolytica* belongs to the genus *Mannheimia*, family *Pasteurellaceae*, and phylum Proteobacteria. *Mannheimia* are gram-negative, non-motile, facultative anaerobic coccobacilli or rods, and this genus currently includes five species: *M. haemolytica*, *M. glucosida*, *M. ruminalis*, *M. varigena*, and *M. granulomatis* (Angen et al., 1999). Within *Mannheimia*, *M. haemolytica* is the most prevalent species in the respiratory tract, with a recent metagenomic analysis of bronchoalveolar lavage samples from BRD cases showing minimal sequences from other *Mannheimia* species (<0.05%) (Klima et al., 2019). *M. haemolytica* includes a dozen serotypes (1, 2, 5-9, 12-14 and 16-17), with serotypes 1, 2, and 6 most frequently found in respiratory tract of beef cattle at feedlot. The pathogenicity of *M. haemolytica* depends on its capsular serotype and the host species. Serotypes 1 and 6 are pathogenic in cattle, whereas serotype 2, while less pathogenic in cattle, is more virulent in sheep (Klima et al., 2014a; Klima et al., 2016; Rice et al., 2007). *M. haemolytica* serotype 1 is more commonly isolated from cattle with BRD compared to serotype 6. Klima et al. (2014a) reported that 70.7% of *M. haemolytica* isolates from the nasopharynx of feedlot cattle with BRD were serotype 1, while serotype 6 accounted for 19.5% (Klima et al., 2014a). Although serotype 2 is less pathogenic in cattle, it is frequently found in the nasopharynx of healthy feedlot cattle, with a prevalence of up to 75.5% (Klima et al., 2014a).

M. haemolytica encodes various virulence factors, including capsular polysaccharides, adhesion molecules, and fimbriae, which facilitate attachment to and colonization of the respiratory tract.

Additionally, the bacterium utilizes sialoglycoprotease, transferrin-binding proteins, neuraminidase, leukotoxins, lipopolysaccharide (LPS), and lipoproteins to evade host mucosal and immune defenses (Confer and Ayalew, 2018). The leukotoxins produced by *M. haemolytica* interact with host defenses in the lung, leading to the necrosis of apoptotic cells. This necrosis releases antimicrobial agents, reactive oxygen species, and lysozyme enzymes into the surrounding tissue, ultimately causing the characteristic lung tissue damage seen in *M. haemolytica* - associated pneumonia (Rice et al., 2007; Singh et al., 2011). *M. haemolytica* is frequently used in experimental models to induce bronchopneumonia in cattle due to its role as a primary bacterial pathogen in BRD (Hanzlicek et al., 2010; Theurer et al., 2013). The virulence factors of *M. haemolytica* differ among serotypes, and these differences have been reported to contribute to the genetic differences across serotypes (Klima et al., 2016). Additionally, this bacterium employs a quorum-sensing system believed to regulate the expression of virulence factors (Czuprynski et al., 2004).

H. somni, formerly known as *Haemophilus somnus*, is a Gram-negative cocco-bacillus that is non-motile, non-capsulated, and fastidious (Confer, 2009). This opportunistic bacterial pathogen is associated with a range of systemic infections including bronchopneumonia, thrombotic meningoencephalitis, myocarditis, septicemia, pleuritis, and reproductive tract disorders, posing a significant challenge in North America (Headley et al., 2013). The prevalence of *H. somni* in clinically healthy cattle upon feedlot entry ranges from 15% to over 50% (Angen et al., 2009). For feeder cattle exhibiting clinical signs of BRD, the isolation rate is typically higher than in clinically healthy counterparts (Corbeil, 2007). Studies indicate an inverse relationship between the isolation rate of *H. somni* and the geometric mean of antibody titers against it in newly received cattle at feedlot (Corbeil, 2007). The prevalence of *H. somni* is higher in cattle during the later stages of feedlot placement (Timsit

et al., 2017b). *H. somni* strains vary in their ability to induce pneumonia in calves and can persist as commensals in both the genital and respiratory tracts (Murray et al., 2016). *H. somni* can trigger the expression of immunoglobulin binding proteins and cause endothelial cell apoptosis, and has the ability to evade host immune cells through phase and antigen variation, as well as the endotoxin activity of LPS (Griffin et al., 2010). These virulence factors enable *H. somni* to cause lung infections in cattle.

P. multocida is also an opportunistic bacterial pathogen involved in bronchopneumonia in cattle with clinical signs similar to *M. haemolytica*-associated pneumonia (Taylor et al., 2010a). *P. multocida* is frequently isolated from nasal secretions and deep pharyngeal samples in young calves and feeder cattle (Dabo et al., 2007). Isolation rates range from 20% to 60% in clinically healthy cattle, and can double in calves with clinical respiratory disease compared to healthy calves (Dabo et al., 2007; Timsit et al., 2017b). *P. multocida* induces pneumonia through its virulence factors including LPS, cytotoxic proteins, and iron acquisition proteins (Griffin et al., 2010).

While *Mycoplasma* is commonly found in both the nasopharynx and lower airways of cattle (Caswell et al., 2010), the pathogenesis of *M. bovis* is less understood compared to the other three BRD bacterial pathogens described above. *M. bovis* is fastidious and requires special growth media and conditions, which complicates the study of its pathogenic mechanisms contributing to chronic pneumonia (Panciera and Confer, 2010a). *M. bovis* has a trilayered membrane, and its presence is more often detected in the deeper respiratory system than in nasal swabs (Caswell et al., 2010).

1.2.3 Host, management and environmental factors

The development of BRD involves multiple factors. In addition to viral and bacterial pathogens, host, management, and environmental components are considered major predisposing factors (Mosier,

2015). Host factors such as age, body weight, immune status, and genetics can influence the susceptibility of cattle to BRD (Taylor et al., 2010a). Calves that are younger and lighter when they enter the feedlot are more likely to develop BRD during the feeding phase compared to older and heavier calves (Sanderson et al., 2008). In North America, the segmented nature of beef cattle production subjects calves to various management practices, including castration, dehorning, abrupt weaning, auction sales, transportation, commingling, diet changes, as well as exposure to adverse environmental conditions (Tucker et al., 2015). These practices can stress calves, impacting their immune system, health, and performance. The combination of stress and exposure to bacterial and viral pathogens due to commingling, often compromises host defenses against respiratory infection, predisposing cattle to primary viral infections followed by the secondary bacterial infections associated with BRD (Taylor et al., 2010a). Shipping is a leading environmental risk factor for BRD in cattle, affecting their resilience to respiratory infections upon feedlot arrival (Baruch, 2020; Taylor et al., 2010a). Auction market-sourced calves are at greater risk for BRD compared to farm-purchased calves due to increased exposure to pathogens, multiple episodes of transit, and stress from mixing with calves from different sources (Step et al., 2008). Weather conditions, particularly sudden and extreme temperature changes in the fall, have been closely linked to the occurrence of BRD, as this period typically shows the highest incidence of the disease (Taylor et al., 2010a). Overall, the multi-factorial nature and complexity of BRD makes it challenging to identify the role of individual factors.

1.3 Bovine respiratory defense mechanisms against pathogens

1.3.1 Physical and biochemical barriers

The respiratory tract's first line of defense includes the physical barriers in the nasal cavity. Hairs in the external nares block large particles, while nasal conchae create air vortices to trap

pathogens (Ackermann et al., 2010; Srikumaran et al., 2007). Mucosal clearance, involving the airway surface liquid layer, ciliated epithelial cells, and goblet cells, restricts pathogen adherence (Lopez and Martinson, 2017). The mucus gel layer captures inhaled particles, and coordinated cilia movement expels them to the throat for swallowing into the digestive tract (Ackermann et al., 2010; Bansil and Turner, 2018). The airway surface liquid contains antimicrobial molecules like mucin, lactoferrin, and lysozymes. Mucin helps remove bacterial pathogens by binding and shedding them, while lactoferrin and lysozymes directly kill bacteria (van Putten and Strijbis, 2017). The respiratory tract also harbors antimicrobial peptides, such as defensins and cathelicidins, which target pathogens and modulate host defenses (Bartlett et al., 2008). Among antimicrobial proteins, surfactant proteins A and D, located on lung epithelial surfaces, enhance pathogen killing by phagocytic immune cells via coating the pathogens (Pastva et al., 2007). The innate immune system employs a variety of soluble, surface-bound, and intracellular receptors to detect invading pathogens. These receptors, known as pattern recognition receptors (PRRs), identify conserved molecular patterns called pathogen-associated molecular patterns (PAMPs), such as peptidoglycan and lipoteichoic acids from gram-positive bacteria, and lipopolysaccharide from gram-negative bacteria (Li et al., 2012). The cells of the bovine respiratory tract express a wide range of surface-bound and intracellular PRRs, including Toll-like receptors (TLRs), NOD-like receptors, and RNA helicases (McGill and Sacco, 2020). All pathogens involved in the bovine respiratory disease (BRD) complex produce PAMPs that activate the innate immune system (Ackermann et al., 2010). Infection leads to the up-regulation of many PRRs, enabling the animal to mount a robust immune response. Immunoglobulins A (IgA) and G (IgG) in nasal secretions provide primary defense, with secretory IgA entrapping pathogens in mucus and blocking their access to epithelial receptors, and IgG protecting against respiratory invasion (Brandtzaeg, 2007; Ellis et al.,

2018). Combined, physical and biochemical barriers are key defense mechanisms for rapidly and efficiently countering pathogen attachment and colonization.

1.3.2 Cellular barriers

An array of immune cells within the respiratory tract, including dendritic cells, T and B cells, vascular endothelium, alveolar and intravascular macrophages, NK cells, eosinophils, neutrophils, NK T cells, and mast cells, coordinate immune responses against pathogens (Ackermann et al., 2010). In cattle, macrophages and neutrophils play a critical role in eliminating pathogens from the lower respiratory tract through phagocytosis. Initially, alveolar macrophages engage in phagocytosis and release pro-inflammatory cytokines like IL-8 (Gordon and Read, 2002; Srikumaran et al., 2007). Neutrophils are among the first responders at infection sites, attracted by proinflammatory cytokines and chemotactic factors like IL-8. These highly phagocytic cells are crucial for defending the host against extracellular bacterial threats (McGill and Sacco, 2020; Srikumaran et al., 2007). Neutrophil extracellular traps (NETs) contribute significantly to BRD pathogenesis, where neutrophils release nuclear DNA and associated proteins into the extracellular environment through NETosis. *In vitro* studies have shown that *M. haemolytica* (Aulik et al., 2010), *M. bovis* (Gondaira et al., 2017), and *H. somni* (Hellenbrand et al., 2013) induce NET formation, with evidence of NETs observed in the lungs of calves infected with *M. haemolytica* (Aulik et al., 2010) and *H. somni* (Hellenbrand et al., 2013). Antigen-presenting cells (APCs) such as monocytes, macrophages, and dendritic cells play a crucial role in connecting the innate and adaptive immune responses (McGill and Sacco, 2020). Dendritic cells are particularly vital for initiating effective T-cell and B-cell responses, while monocytes and macrophages also serve as APCs and actively engage in phagocytosis of dead cells, clearance of pathogens, and production

of inflammatory cytokines. The adaptive immune response is crucial for controlling and clearing respiratory pathogens. Following infection, cattle initiate antibody (Ab) and antigen-specific T-cell responses. However, pathogens often evade these immune responses through various immune evasion strategies (Srikumaran et al., 2007). Gamma delta T cells, abundant in mucosal sites such as the respiratory tract, play an early and important role in both innate and adaptive immunity, serving as frontline defenders against invading pathogens (McGill and Sacco, 2020).

1.4 BRD management strategies

1.4.1 Preconditioning

Preconditioning, proposed as a strategy to manage BRD (Lalman and Ward, 2005), involves implementing management practices to reduce stressors and better prepare beef calves for subsequent production stages such as stocker and feedlot placement. These practices include vaccinating against viral and bacterial pathogens, optimizing the timing of dehorning and castration, employing effective weaning strategies, and acclimating calves to feeding from a bunk and drinking from a water source at least 45 days prior to transport to the feedlot (González et al., 2010; Taylor et al., 2010a). Preconditioning programs aim to enhance calf resistance to respiratory infections at weaning, minimize stress related to maternal separation, and bolster defenses against respiratory viral and bacterial infections during the transition from cow-calf operations to commercial feedlots. These measures collectively reduce the incidence of BRD in cattle during feedlot placement. The incorporation of preconditioning programs has been found to yield a net profit increase of $14 per head compared to non-preconditioned cattle (Dhuyvetter et al., 2005). Additionally, preconditioning has exhibited a 42.6% decrease in morbidity and a 10.3% reduction in mortality rates when compared to auction market-derived calves in Kentucky (Roeber et al.,

2001). Despite these beneficial outcomes, the widespread adoption of preconditioning remains limited due to the associated costs and limited incentive for cow-calf producers.

1.4.2 Antimicrobial administration

Prevention and control of BRD in North American feedlot cattle primarily focus on combating respiratory bacterial pathogens with antimicrobials. High-risk cattle receive metaphylactic antimicrobial treatment upon feedlot arrival, which reduces BRD-related morbidity and mortality by eliminating existing bacterial infections and preventing pathogen colonization and proliferation in immunosuppressed and vulnerable animals (Ives and Richeson, 2015). The widespread use of antimicrobials in Canadian and American feedlots, although effective in reducing the incidence of bovine respiratory disease (BRD), is facing increased scrutiny due to the development of resistant BRD pathogens (Snyder et al., 2017; Timsit et al., 2017b). A study conducted in Alberta found that over 60% of *M. haemolytica* and *P. multocida* isolates from the lower respiratory tract of feedlot cattle exhibited multidrug resistance to the commonly used metaphylaxis antimicrobials tulathromycin and oxytetracycline (Timsit et al., 2017b). Similarly, a study in the United States reported a significant increase in tulathromycin-resistant *M. haemolytica* in newly-received feedlot cattle, with resistance prevalence increasing from 4% to 99% within two weeks after tulathromycin metaphylaxis (Snyder et al., 2017). Recent studies have highlighted the impact of metaphylaxis at feedlot entry on antimicrobial resistance in the nasopharyngeal (NP) microbiome of cattle. Holman et al. (2018) found increased levels of the tetracycline resistance gene *tet*(H) in nasal swabs of feedlot cattle treated with oxytetracycline (Holman et al., 2018). A follow-up study (Holman et al., 2019) showed that oxytetracycline injection led to elevated abundances of resistance genes *erm*(X), *sul*2, *tet*(H), *tet*(M), and *tet*(W) in NP samples after 12

days. Cattle administered tulathromycin also exhibited higher levels of *erm*(X), *sul*2, and *tet*(M) genes compared to controls, indicating a clear link between metaphylactic antimicrobial use and antimicrobial gene in respiratory bacteria. Metaphylactic use of antimicrobials at feedlot entry has also been shown to disrupt respiratory microbiota homeostasis, leading to changes in community structure and composition (Holman et al., 2018). Studies have identified multidrug-resistant BRD pathogens in feedlot calves, including resistance genes within mobile genetic elements conveying resistance to all available BRD antimicrobials, highlighting a significant challenge in effectively managing and treating BRD in feedlots due to antimicrobial resistance (Bhatt et al., 2018; Klima et al., 2019; Klima et al., 2014b).

1.4.3 Vaccination

Vaccination is widely acknowledged as a cost-effective strategy for managing infectious diseases in livestock, though this is dependent on the efficacy of the vaccine (Meeusen et al., 2007). In North America, vaccines targeting both viral and bacterial agents associated with BRD in feedlot cattle are widely used, and typically are combination formulations for multiple pathogens. The viral targets include bovine rhinotracheitis, bovine viral diarrhea (types 1 and 2), bovine parainfluenza 3, and bovine respiratory syncytial viruses. Current commercial vaccines against *M. haemolytica*, *P. multocida*, *H. somni*, and *M. bovis* are typically based on bacterins or killed whole bacteria. However, these traditional bacterial vaccines demonstrate limited efficacy, offering only partial protection against *M. haemolytica* and *P. multocida*, and no benefit against *H. somni* (Larson and Step, 2012). This suboptimal efficacy may partially arise from the timing of vaccine administration. Although larger feedlots often implement vaccination protocols upon calf entry, complete immune protection may not be immediately achieved due to the stress calves experience

during placement. The efficacy of vaccines can be influenced by factors such as the timing of vaccination (e.g., before or after feedlot arrival), the combination of antigens (e.g., mixing bacterial and viral antigens), stress levels, and maternal antibody presence (Chamorro and Palomares, 2020b; Richeson and Falkner, 2020b). Administering BRD vaccines during periods of physiological stress can cause transient immunosuppression, potentially making the vaccines more harmful than beneficial, so vaccine timing and safety must be carefully considered in beef production systems (Richeson and Falkner, 2020b). Additionally, the evolving pathogenic strains in feedlot environments may diminish the effectiveness of current vaccines, indicating a need for new vaccine approaches to enhance BRD protection. The reduced effectiveness of current vaccines may stem from vaccines providing limited protection across bacterial strains, with systemic immunity targeted by intramuscular injection also playing a role. Mucosal immunization, in contrast, can induce both mucosal and systemic immunity, offering protection at the site of infection (Shewen et al., 2009).

1.5 Mucosal vaccines

1.5.1 Advantages of mucosal vaccines

The mucosal surface, a vast and exposed area lining the respiratory, digestive, and genitourinary tracts, is the primary entry point for most infectious diseases across species. As a result, there has been significant interest in studying immune responses at these sites and in developing vaccines that specifically target these entry points (Hodgins et al., 2015). However, the majority of the vaccines for respiratory infections in ruminants are currently administered parenterally, inducing systemic immunity rather than mucosal immunity (Shewen et al., 2009). Vaccines administered by injection often have limited ability to trigger mucosal immunity,

resulting in reduced effectiveness against mucosal surface infections (Lamm, 1997; Neutra and Kozlowski, 2006). Protection resulting from injected vaccines is generally achieved through the transfer of immune mediators to mucosal surfaces or by preventing infection after the mucosal barrier has been compromised (Shewen et al., 2009). Mucosal immunization through oral, nasal, rectal, or vaginal routes is the most effective method for eliciting protective mucosal immune responses. Mucosal immunization presents distinct advantages compared to parenteral delivery, as it triggers both mucosal and systemic immunity, thereby providing protection at the primary sites of pathogen entry and subsequent infection (Holmgren and Czerkinsky, 2005). The activation of adaptive immunity at mucosal surfaces, encompassing secretory antibody responses and tissue-resident T cells, has the potential to prevent the establishment of infections rather than simply limiting the infection and preventing the disease progression (Lavelle and Ward, 2022). Parenteral vaccination necessitates trained personnel and the use of needles, which may potentially result in injection site lesions, impacting beef cattle carcass and animal welfare quality. In contrast, mucosal immunization offers a relatively painless and cost-effective method for mass vaccine delivery through feed, water, or aerosol, reducing labor costs for producers and stress on animals.

1.5.2 Routes of administration for mucosal vaccines

Mucosal immunization can be achieved through traditional oral and nasal routes, as well as rectal, vaginal, sublingual, and transcutaneous routes. While oral and nasal routes are commonly utilized, other routes of administration have been primarily explored for experimental purposes. Oral immunization with immunogenic antigens, either alone or with adjuvants, can elicit a significant antibody response in the small intestine, with the strongest response observed in the proximal segment, ascending colon, stomach, and mammary and salivary glands (Czerkinsky and

Holmgren, 2010). Nasal or tonsillar immunization in humans stimulates the production of IgA antibodies in the upper airway mucosa, regional nasal or salivary secretions, and also in distant sites such as the genital mucosa (Johansson et al., 2001). A study comparing nasal and oral vaccination in humans found that nasal administration induced higher levels of antigen-specific IgA in nasal secretions and sustained antibody responses in serum and mucosal sites longer than oral vaccination (Rudin et al., 1998). Studies indicate that the intestinal immune response following oral immunization is rapid but short-lived, although it establishes long-lasting immunological memory (Czerkinsky and Holmgren, 2010). The sublingual route for vaccine administration is gaining attention in humans and small animals for its potential to induce widespread mucosal and systemic immune responses. Advances in mucoadhesive formulations enhancing antigen permeability and prolonging epithelial contact may aid in development of sublingual vaccines (Czerkinsky and Holmgren, 2010).

1.5.3 Adjuvants and delivery strategies

Mucosal vaccine adjuvants enhance immune responses by stabilizing antigens, optimizing their delivery, activating innate immune receptors, and recruiting immune cells (Song et al., 2024). They also help in achieving robust immune responses with reduced antigen doses or fewer vaccine administrations. Studied mucosal vaccine adjuvants include cholera toxin (CT), *Escherichia coli* heat-labile toxin (LT), CpG-containing DNA, lectins, polyelectrolytes, ISCOM, actins, avridine, low-oil emulsion (MF59), lipid A, lysophosphatidyl glycerol, cytokines (IL-5), and chemokines (Verma et al., 2023). Mucosal vaccine adjuvants based on natural products, such as biopolymers, nanoparticles, virus-like particles, and extracellular vesicles, are being researched due to their potential benefits over traditional adjuvants. These adjuvants can enhance both specific and

nonspecific immunity while reducing toxicity and side effects, because of their biocompatibility and biodegradability properties (Gao and Guo, 2023). Mucosal vaccine adjuvants must overcome challenges related to safety, toxicity, stability, and regulatory approval before they can be widely used in licensed vaccines (Song et al., 2024). A variety of techniques have been utilized for the administration of mucosal vaccines, encompassing oral delivery through pills, liquids, dissolvable substrates, or patches, as well as intranasal or respiratory delivery through nebulizers, nasal sprays, and inhalers (Song et al., 2024). Various strategies for mucosal immunization have been explored, including the use of recombinant proteins, live vectors, subunit vaccines, DNA vaccines, transgenic edible plants, non-living microparticle carriers (such as copolymer microspheres and liposomes), particle-based vaccines (such as VLP), and immune-stimulating-complexes (Ogra et al., 2001). The primary objective of these methods is to reduce virulence, increase antigen load, and improve delivery into the mucosa.

1.5.4 Hurdles in mucosal vaccine development

Delivery of antigens to mucosal surfaces presents several challenges, including overcoming innate clearance mechanisms for intranasal vaccines, protecting oral vaccines from digestive degradation, promoting antigen adherence to mucosal epithelium while evading clearance by the mucociliary blanket in the gut, and addressing the potential for tolerance induction rather than active immunity (Shewen et al., 2009). Developing orally administered vaccines for ruminats with non-replicating antigens presents significant challenges, including susceptibility to digestion, poor mucosal adherence, and inadequate activation of necessary danger signals for initiating robust cellular immune responses (Shewen et al., 2009). One potential approach to enhancing digestive mucosal immunity involves using potent adjuvants and encapsulating antigens

in polymer microparticles or microspheres that resist digestion in the rumen, aiming to stimulate mucosal immune responses in Peyer's patches through oral delivery.

Live attenuated mucosal vaccines, common in practice, pose a risk of reverting to the pathogenic wild type (Bandyopadhyay et al., 2015). Intranasal vaccines, especially those that are live or viral-vectored, require rigorous safety testing due to the potential proximity of nasal passages to the central nervous system via the olfactory bulbs (Song et al., 2024). Inactivated or subunit mucosal vaccines, while potentially safer than live vaccines, often necessitate the use of adjuvants, which can introduce additional safety concerns. Thus, development of mucosal vaccines can be challenging in terms of maintaining antigen survival and host health following vaccine use.

1.5.5 *Bacillus subtilis* spores as an adjuvant

Bacillus subtilis, a gram-positive nonpathogenic and endospore-forming bacterium, is versatile in biotechnological applications. It is utilized in probiotic formulations, as an expression system for heterologous proteins, and as a vehicle for mucosal antigen delivery (Cutting, 2011). Engineered *B. subtilis* can express antigens post-spore germination or display them on the spore surface via fusion with spore coat proteins (De Souza et al., 2014). The endospores of *B. subtilis* exhibit exceptional resistance to various stressors like high temperatures, desiccation, nutrient deprivation, and chemical solvents, enabling their long-term storage and transport (Nicholson et al., 2000). Previous studies showed that *B. subtilis* spores have a strong adjuvant effect, enhancing antibody responses to co-administered antigens or those adsorbed onto the spore surface (De Souza et al., 2014; Huang et al., 2010b). Additionally, non-transgenic *B. subtilis* spores, when adsorbed with antigens in their native state for vaccination via oral or intranasal administration, have shown

to stimulate mucosal immunity and provide protection against bacterial and viral infections in mice (Huang et al., 2010b; Song et al., 2012b). The use of *B. subtilis* spores for vaccinating cattle against BRD pathogens offers potential commercial benefits due to its established production, low-cost inputs, and generally regarded as safe (GRAS) status. Its recognized role as a probiotic further supports industry adoption as a low-cost and safe vaccine component. To date, *B. subtilis* spores have not been tested as vaccine delivery systems for cattle pathogens.

1.6 Animal models for mucosal vaccine development

Animal models have a crucial role in vaccine development, to evaluate the quality and quantity of immune response, determine optimal formulation and best route of delivery, and assess protection from infection (Mukherjee et al., 2022). Studies involving animals should be performed according to Canadian Council on Animal Care guidelines, with ethical considerations prioritizing humane treatment, minimal pain, and suffering. In Canada, livestock administered novel vaccines cannot enter the food chain system. As a result, the cost of conducting vaccine studies using beef cattle is significantly high, and other animal models are routinely used to minimize these costs.

1.6.1 Mice

Mice have been widely utilized as small animal models in biomedical research and serve as the cornerstone of immunological and infection research (Ehret et al., 2017; Lee et al., 2012). Typically belonging to the species *Mus musculus*, laboratory mice are the most frequently employed animal research model for the initial screening of vaccine candidates and for testing the efficacy of new vaccines (Gong et al., 2020). Mice are considered to be a good animal model for evaluating the immunogenicity of vaccine candidates (Gong et al., 2020; Griffin, 2002). This is

due to their low cost, ease of handling, convenient group maintenance within cage systems, well-characterized immune system, and the availability of a wide range of commercial reagents for studying immunity at the cellular or molecular level.,. BALB/c and C57BL/6 mice strains are commonly used to assess vaccine-induced immune responses. Other strains, including knock-in or knock-out derivatives and humanized mice, are used to evaluate certain qualities of the immune response including shifts in Th1, Th2, or Th17 responses and the induction of mucosal versus systemic immunity (Guzman and Montoya, 2018b).

The innate immune system is largely conserved among animals, but there are significant variations in pattern-recognition-receptor structures and responses between species (Grabiec et al., 2004; Jungi et al., 2011). Differences between mice and larger animals, including humans, include variations in toll-like receptors, antibody subsets, cytokine expression, Th17 inducers, and defensins (Gibbons and Spencer, 2011; Mestas and Hughes, 2004). Leukocyte balance varies significantly; for example, human blood is neutrophil-rich, while mouse and ruminant blood is lymphocyte-dominant (Jones and Allison, 2007). Additionally, fragment crystallizable region (Fc) receptor expression and immunoglobulin isotypes differ between species, with mice and cattle having different IgG subtypes. Recent studies suggest that immunoglobulin subclasses in one species may not functionally correspond to those in another (Guzman and Montoya, 2018b; Morgan et al., 2018; Qin et al., 2008), and the Th1/Th2 polarization of T cells observed in response to a particular antigen is different between laboratory mice and outbred mammals including farm animals and humans (Dicks et al., 2015; Estes and Brown, 2002). This indicates the need for caution when extrapolating immunological findings from mice to other mammals. Intranasal vaccination in mice often leads to digestion of the inoculum, complicating the distinction between

intranasal and oral vaccination (Kiros et al., 2012). Larger animals allow for controlled vaccine delivery to the nasal passages and easier access to mucosal surfaces. Additionally, larger animals like pigs, sheep, and cattle have well-defined tonsils, whereas mice lack tonsils and have undefined mucosa-associated lymphoid tissue (MALT) networks (Haley, 2017; Mestas and Hughes, 2004). While keeping these caveats in mind, mice remain a preferred choice as an *in vivo* model for studying bovine and human vaccine development, providing valuable insights into immune system function in both health and disease contexts, at a low cost for replicated experiments.

1.6.2 Sheep

Larger animals provide numerous advantages over mice while investigating immune response towards mucosal vaccine development (Guzman and Montoya, 2018a). Sheep are increasingly used for studying mucosal immunization due to their moderate cost, size, and ease of handling compared to other larger animals (Kiros et al., 2012; Meeusen, 2011). Their anatomical and physiological similarities to cattle make them valuable for investigating immune responses relevant to both species. Unlike mice, sheep allow for sequential sampling of peripheral blood and mucosal samples post-vaccination, enabling a detailed study of individual immune responses and subsequent protection against pathogens (Guzman and Montoya, 2018b). This approach, along with techniques like lymphatic cannulation, enhances the understanding of mucosal and systemic immune responses, making sheep an optimal model for respiratory disease research (Meeusen, 2011; Schwartz-Cornil et al., 2006).

In ruminants, the nasal mucosa is immunologically protected by nasal-associated lymphoid tissue (NALT) which belongs to MALT of the mucosal immune system (Kuper et al., 1992). In

larger animals, NALT has been identified as an alternative mucosal inductive tissue site for immunization (Davis, 2001; Fujimura, 2000). In sheep the epithelium overlying NALT can be distinguished from the neighboring respiratory epithelium by the presence of a specialized non-ciliated antigen presenting M cells which play a vital role in generating a mucosal immune response (Stanley et al., 2001). The presence of M cells and germinal centres have been revealed recently by ultrastructural and histological analysis of ovine NALT (Sedgmen et al., 2006; Stanley et al., 2001). These findings resemble those found in intestinal Peyer's patches, suggesting that NALT and gut-associated lymphoid tissue (GALT) may be closely related in their ability to induce mucosal immune responses in ruminants (Stanley et al., 2001). In Sheep, NALT along with the pharyngeal tonsil, are key components of defense in the upper respiratory tract, and due to their location and antigen-presenting capability, may play an important role in developing a mucosal vaccine (Stanley et al., 2001).

In developing a vaccine against the primary BRD causing bacteria, *M. haemolytica,* sheep can be considered an ideal animal model to predict vaccine responses in cattle because they are i) naturally colonized by *Mannheimia*, ii) ruminate similar to cattle, and iii) cost less than beef calves. Among the 12 different serotypes of *M. haemolytica*, serotypes A1, A2 and A6 are the most prevalent worldwide while serotype A2 are responsible for pneumonia in sheep (Lawrence et al., 2010). Repeated subcutaneous immunization of bighorn sheep with a multivalent *Mannheimia-Bibersteinia* vaccine protected them from a *M. haemolytica* challenge due to the presence of high antibody titers against leukotoxin (LKT) and a surface antigen of *M. haemolytica* (Subramaniam et al., 2011). Another study formulated a vaccine with recombinant small fragments of leukotoxin A (LKTA) and outer-membrane lipoprotein (PlpE) proteins, and demonstrated its ability to

generate high antibody titers in sheep (Guzman-Brambila et al., 2012). None of these studies have tried mucosal routes for the immunization where the protection could have been achieved both locally and systematically. It should be noted however, that because sheep are naturally colonized by *Mannheimia*, immune activity against this pathogen may be difficult to control in animal studies, should a response already have occurred due to colonization prior to experiment initiation.

1.6.3 Cattle

Cattle features an outbred, large animal species in which *M. haemolytica* is a natural inhabitant. Large size allows access to mucosal surfaces and various immune compartments. The mucosal immune system of cattle is fully functional at birth. The other advantages includes readily available reagents for the most common cytokines, isotypes and biomarkers. However, one should consider the cost of the animal and their care by trained staff, as well the availability of appropriate large animal facilities. *M. haemolytica* resides in the upper respiratory tract of cattle as an opportunistic pathogen and colonizes the nasopharynx in healthy cattle. However, in cattle with compromised immunity from stress and primary viral infections, *M. haemolytica* can proliferate in the nasopharynx and translocate to the lower respiratory tract, where it induces acute infections characteristic to fibrinous pleuropneumonia in the lung (Rice et al., 2007). Among the 12 different serotypes of *M. haemolytica,* serotypes 1 and 6 are most commonly isolated from sick cattle or from lesions of pneumonia (Klima et al., 2014a; Panciera and Confer, 2010b).

Several experimental approaches have been reported with regard to the development of improved *M. haemolytica* vaccines including outer membrane vesicles, recombinant proteins and chimeric proteins (Confer and Ayalew, 2018). A combination of subcutaneous vaccination with a

chimeric protein having membrane protein PlpE and LKT, two potent immunogens of *M. haemolytica*, plus formalin-killed bacteria in an oil-in-water adjuvant resulted in 75% lower lung lesion scores in vaccinated cattle compared with controls, whereas vaccination with the chimeric protein or bacterin alone was less effective (Confer et al., 2009a). Another study from this research group reported enhanced nasal antibodies to PlpE and LKTA antigens after intranasal vaccination of calves with *M. haemolytica* PlpE-LKTA chimeric protein where cholera toxin was used as adjuvant. However, this intranasal vaccination showed limited serum antibody responses (Confer et al., 2009b).

1.7 Respiratory tract microbiota of cattle

1.7.1 Structure and composition of respiratory tract microbiota

Cattle possess a complex respiratory system that can be broadly divided into the upper respiratory tract (URT) and the lower respiratory tract (LRT). The URT is comprised of nasal cavities, paranasal sinuses, nasal passages, nasopharynx, oropharynx, tonsils, and the upper portion of the larynx. The LRT consist of the lower portion of the larynx, trachea, bronchi, bronchioles, and alveoli (Beers and Berkow, 1999). Similar to other body sites, the cattle respiratory tract is colonized by a range of different bacterial microbiota immediately after birth (Lima et al., 2019). Recent studies have shown that the composition and diversity of microbiota have been associated with respiratory health in cattle (Lima et al., 2016; Timsit et al., 2016a).

The nasopharyngeal (NP) microbiota of feedlot cattle encompasses a diverse bacterial community and the nasopharynx is considered the primary niche for opportunistic BRD pathogen colonization and proliferation (Timsit et al., 2020). The NP has been shown to harbors

approximately 29 different phyla (Timsit et al., 2016a); among these *Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes* and *Tenericutes* are the predominant phyla and constitute over 90% of the total microbiome (Holman et al., 2017; Timsit et al., 2018; Zeineldin et al., 2017a). Approximately 300 genera colonize the nasopharynx and among them *Corynebacteria, Moraxella, Mycoplasma, Pasteurella, Mannheimia, Psychrobactor* and *Staphylococcus* are the most common (Holman et al., 2015; Timsit et al., 2018; Zeineldin et al., 2017a). Although the nasopharynx is colonized predominately by aerobic bacteria, some obligate anaerobic species associated with the rumen such as *Prevotella, Clostridium, Bacteriodes, Bifidobacterium* and *Ruminococcus,* have also been isolated from NP swabs.

Due to the invasiveness and difficulty of sample collection, the microbial community residing within the lower respiratory tract of feedlot cattle has been less characterized. Trans tracheal aspiration (TTA) and bronchoalveolar lavage (BAL) fluid samples have mainly been used to characterize the lower airway microbiota of feedlot cattle. The tracheal microbiota has been evaluated in healthy and BRD-diagnosed steers from commercial feedlots (Timsit et al., 2018) and weaned calves from smaller farms (Nicola et al., 2017a). In addition, tracheal samples from healthy calves sampled longitudinally before and after shipment to an auction market, and then upon feedlot placement have been collected (Stroebel et al., 2018). According to the 16S rRNA sequencing of TTA samples, a diverse microbial community is present in the trachea of cattle, with colonization by 9-21 different bacterial phyla and 91-182 different genera. *Tenericutes* (> 50% relative abundance), *Firmicutes, Proteobacteria* and *Actinobacteria* are the most predominant phyla in the trachea. The most relatively abundant bacterial genera include *Mycoplasma* (> 50%), *Moraxella, Pasteurella, Lactococcus, Histophilus* and *Bacteroides*. Similar to the tracheal

microbiota, bronchoalveolar microbial communities from healthy feedlot cattle contained mostly *Proteobacteria*, *Bacteroidetes, Actinobateria*, and *Tenericutes* (Zeineldin et al., 2017b).

The majority of the predominant phyla and genera found in nasopharyngeal (NP) samples are also present in tracheal (Nicola et al., 2017b; Timsit et al., 2018) and bronchoalveolar (Zeineldin et al. 2017b) samples from the same cattle. However, the bacterial communities in the lower respiratory tract differ from those in the nasopharynx, exhibiting lower bacterial richness and evenness (Nicola et al. 2017b; Timsit et al. 2018). Although the lung microbiota has a distinct microbial structure from NP microbiota, it is influenced by bacteria inhaled from the upper respiratory tract (Nicola et al. 2017b). Additionally, the respiratory tract's physiological conditions, including pH, relative humidity, temperature, and the partial pressures of oxygen and carbon dioxide impact microbiota composition (Man et al., 2017).

1.7.2 Factors affecting the respiratory microbiota

The composition of the airway microbiota evolves over time and in beef cattle the respiratory tract microbiota is influenced by multiple factors, including diet (Hall et al., 2017), host genetics, age (Woldehiwet et al., 1990), antimicrobial use (Timsit et al., 2017c), vaccination, season, management strategies, and the surrounding environment (McMullen et al., 2018; Timsit et al., 2016b). Weaning possesses a key impact on the respiratory microbial composition of calves, particularly when associated with other environmental stressors (Hall et al., 2017; Timsit et al., 2016b). It has been reported by several studies that the NP microbiota of beef calves changes after transportation to a feedlot. One study showed significant changes in calves' NP microbiota over time, with 92 operational taxonomic units (OTUs) varying from weaning to 40 days after feedlot

placement (Timsit et al., 2016b). Another study found increased diversity and richness in the NP microbiota within two days of feedlot placement (Holman et al., 2017). The composition of microbiota in the respiratory tract of cattle is influenced by both farm of origin and the practices employed at the feedlot (McMullen et al., 2018; Stroebel et al., 2018). Consequently, the composition of microbiota upon feedlot arrival is not uniform across all cattle, with differences attributed to their specific farm of origin and feedlot practices (Stroebel et al., 2018). The vulnerability of cattle to BRD in the initial weeks after weaning and arrival at a feedlot may be linked to the instability in their respiratory microbiota following feedlot placement. This instability may arise from stress-induced reduced immunity following weaning and transportation, as well as exposure to bacteria from the feedlot environment or new pen mates (Timsit et al. 2016). Additionally, changes in diet before or after being placed in the feedlot can also impact the respiratory microbiota (Hall et al., 2017).

Antimicrobials have been shown to impact resident, mucosal, microbial communities at various life stages (Jakobsson et al., 2010). Antimicrobial administration can affect the respiratory microbiota by inhibiting growth of certain bacteria, while promoting the growth of others that have intrinsic or acquired resistance to the administered antimicrobial. In children, antibiotic use has been linked to an altered microbial community structure in the upper respiratory tract for up to six months after administration (Santee et al., 2016), demonstrating that this practice has a prolonged impact. Recent studies have reported that alterations in the NP microbiota of commercial cattle were apparent 60 days after injection with either oxytetracycline or tulathromycin (Holman et al., 2018). A controlled study examining the impact of these two antimicrobials on the NP microbiota over 34 days found that the greatest disruption to the NP microbiota occurred two and five days

after administration (Holman et al., 2019). Although the indirect effects of antimicrobials on respiratory microbiota and disease progression in cattle are not well-studied, current research suggests that antimicrobial administration can have a significant impact on the NP microbiota in both the short and long term.

1.7.3 The effect of vaccination on the microbiota

In mammals, the immune system and microbiota co-evolve, and their balanced relationship is based on crosstalk between the two throughout their life. Host microbiota and immune responses from vaccination are interconnected and studies suggest that the diversity and composition of microbiota may influence vaccine efficacy (Valdez et al., 2014b). However, knowledge regarding the direct or indirect effects of vaccination on the microbiota is limited. Until recently, most research studies investigating the relationship between vaccination and microbial response have been conducted on children (Biesbroek et al., 2014; Boelsen et al., 2019; Feazel et al., 2015; Hilty et al., 2012; Mika et al., 2017), while there are a few studies in other animals such as macaques (Musich et al., 2020), pigs (van Sambeek et al., 2016) and cattle (Meira et al., 2020; Mir et al., 2019). Vaccination may alter the microbiota, thus it is important to understand their effects to minimize impacts on animal health (Mir et al., 2019). Ultimately, the best microbial outcome resulting from vaccination would be reduced colonization or shedding of pathogens targeted by the vaccine.

In children, studies have found that pneumococcal conjugate vaccines (PCV) may alter nasopharyngeal bacterial composition and diversity, although findings are not consistent (Biesbroek et al., 2014; Boelsen et al., 2019; Feazel et al., 2015). One study reported an association

between a 7-valent pneumococcal conjugate vaccine (PCV7) with shifts in microbial composition and increases in bacterial diversity in Dutch children at 12 months of age, but not at 24 months (Biesbroek et al., 2014). In contrast, a study conducted in Kenyan children (aged 12–59 months) reported that the 10-valent pneumococcal conjugate vaccine (PCV10) had no effect on the microbiome 180 days after vaccination (Feazel et al., 2015). The same vaccination may have a differential impact on microbiota depending on geographic region and socio-economic status (Adegbola et al., 2014). A higher prevalence of pneumococcal colonization is often found in children from low-income countries, and susceptible populations in high-income settings, proposing that the impact of vaccination on the microbiome could differ in these settings (Adegbola et al., 2014).There is also potential for changes in pathogen strain carriage, resulting from vaccines targeting one strain over others. Thus, vaccines that target broad pathogen subtypes are better able to reduce heterogenous pathogen populations.

One recent study in cattle reported an association between a recombinant protein subunit vaccine against puerperal metritis and the vaginal microbiome (Meira et al., 2020). This study showed that the vaccine effectively modulated the vaginal microbiome by decreasing the total bacterial load and improving the immune response postpartum by suppressing one of the primary targeted bacteria, *Fusobacterium necrophorum*. Mir et al. reported shifts in gastrointestinal microbiota following *E. coli* O157:H7 vaccination and colonization in cattle (Mir et al., 2019). This study reported that the change in bacterial community structure was significantly associated with vaccination but independent of the adjuvants used in vaccine formulation. Substantial differences in the GIT bacterial community structure and taxonomic profiles had been observed between vaccinated and non-vaccinated calves. Additionally, this study demonstrated that

subsequent experimental exposure of vaccinated calves to *E. coli* O157:H7 resulted in additional changes in the GIT microbiota community structure but this was not the case for unvaccinated but *E. coli* O157:H7-exposed animals.

1.7.4 Role of the microbiome in vaccine response

The structural composition and richness of the microbiota play a potent role in the modulation of host immunity and may impact vaccine immune response. Recent studies have shown that the microbiome plays a crucial role in the development and regulation of the immune system and therefore its composition might influence the immune response to vaccination in individuals (Jamieson, 2015; Zimmermann and Curtis, 2018). During the first months of infancy, the microbiome and human immune response develop in tandem while children receive the majority of vaccinations. Thus, the composition of the early life microbiota potentially has a vital role in the response to vaccination (Ciabattini et al., 2019). The mechanisms that can affect the immune response to vaccination are multifaceted and include factors related to the vaccine, the host immune system, and the microbiota (Ciabattini et al., 2019).

Studies have demonstrated that the microbiota constitutes a constant source of natural adjuvants and through this role they are capable of activating a multitude of pathways that control innate and adaptive immunity (Pabst and Hornef, 2014). A study using germ-free (GF) or antibiotic-treated mice demonstrated this potential adjuvanticity of host microbiota by reporting a limited immune response to an influenza vaccine, compared to control mice harboring a diverse microbiota (Oh et al., 2014). In that study, there was a strong correlation between the expression of TLR5 and the magnitude of the antibody response. It was demonstrated that commensal bacteria

were the source of TLR5 ligands responsible for the enhancement of immune response to the vaccine. Vaccine antibody responses were restored by oral reconstitution with a flagellated strain of *E. coli,* demonstrating the effect of the microbiome on vaccination (Oh et al., 2014). Other components of the microbiota, such as the peptidoglycan component muramyl dipeptide (MDP), are agonists of nucleotide-binding oligomerization domain containing 2 (Nod2) (Ciabattini et al., 2019). The Nod2 sensor has also been shown to enhance the adjuvanticity of cholera toxin in the nasal cavity of mice (Kim et al., 2016).

The efficacy of any vaccine relies on the ability of the antigen to elicit a protective immune response and on the competency of the host immune system to respond appropriately to an antigenic stimulation. Recent studies demonstrate that the microbiota could potentially enhance vaccine responses by acting as an immunologic modulator as well as natural vaccine adjuvant (Ciabattini et al., 2019). Huda and colleagues reported that adaptive immune responses were positively correlated to gastrointestinal tract *Actinobacteria* when children were vaccinated against hepatitis B virus (Huda et al., 2014). The composition of gastrointestinal microbiota is also related to antibody response after immunization (Harris et al., 2018). When the host microbiota experience dysbiosis due to antimicrobial use, it can lead to impaired antibody responses after vaccination (Lynn et al., 2018). This impairment however can reinstated when the microbiota recovers from antimicrobial dysbiosis (Lynn et al. 2018). Consequently, the microbiota is important for vaccine efficacy, both in terms of pathogen reduction and host response to the vaccine.

1.8 Knowledge gaps

Despite advancements of management practices and continuous efforts to control BRD, this disease remains a significant economic and welfare challenge in feedlot cattle. The emergence of multidrug-resistant bacteria associated with BRD, along with growing public pressure to reduce antimicrobial use in food animals, highlights the pressing need to develop alternative strategies to metaphylactic antimicrobials. Preconditioning programs involve implementing various management practices, including vaccination, and are aimed at minimizing stress and better preparing beef calves for subsequent production stages that are predisposed to BRD. Research has demonstrated that this program can reduce morbidity and mortality rates in comparison to non-preconditioned auction market-derived calves (Roeber et al., 2001). However, there have also been studies showing limited effects of preconditioning. This apparent discrepancy may be due to multiple factors, including preconditioning products tested, age and breed of cattle, and geographical location. It is also possible that the formulation of vaccines differed between studies or had limited efficacy. Despite vaccination being incorporated into most Canadian feedlots, BRD infections remain unchanged. While *M. haemolytica* and *P. multocida* vaccines were shown to have a limited positive impact on BRD, vaccination against *H. somni* had no effect (Larson and Step, 2012). The reasons for vaccine failure may be due to timing of administration but perhaps also due to a lack of a immune response in cattle. The feedlot environment can select for certain pathogenic strains, possibly as a result of antimicrobial use (Guo et al., 2020), and older vaccines may lack specificity for newly evolved pathogenic strains. Novel vaccines using broad spectrum antigen targets are likely to be more effective than single bacterin-derived vaccines. However, there is a lack of data specifically investigating precondition programs, that include vaccination regimes, on host health and microbiota. The significance of respiratory microbiota for host health is now widely acknowledged, yet there remains a paucity of research investigating the impact of

preconditioning on respiratory microbiota, particularly when specific stressors such as transportation and commingling with auction market calves at the feedlot are involved. There is also has been little effort to develop new mucosal vaccines that can be delivered to the upper respiratory or gastrointestinal tracts.

1.9 Hypotheses

1. Preconditioning will decrease stress responses, increase antigen specific immunity, and reduce the abundance of genera linked to BRD in calves transitioned from farm to feedlot.

2. *B. subtilis* spores will be an effective mucosal adjuvant in augmenting immune responses against *M. haemolytica*, paving the way for potential use in developing novel immunization strategies against BRD pathogens.

1.10 Objectives

1. To assess the impact of preconditioning on stress, immunity, and respiratory bacteria responses in cattle originating from a single farm (Chapter 2).

2. To develop spore-based candidate vaccines targeting *M. haemolytica*, and assess the immunogenicity of these vaccines after delivery through mucosal (intranasal and intragastric) routes using mice models (Chapter 3 and Chapter 4).

3. To evaluate the immunogenicity of a novel spore-based *M. haemolytica* vaccine in sheep after delivery through oral or intranasal routes, and to investigate the effect of the spore-based vaccine on sheep respiratory microbiota (Chapter 5).

Chapter 2

The effect of preconditioning on stress, immune, and respiratory microbiota responses in beef cattle

2.1 Introduction

Bovine respiratory disease (BRD), also known as shipping fever, continues to be the leading cause of morbidity and mortality after cattle are placed in feedlots (Booker et al., 2008), resulting in substantial economic losses estimated at over $3 billion annually for the North American feedlot cattle industry (Andrés-Lasheras et al., 2021). The etiology of BRD is complex and multifactorial in nature, but often associated with bacterial species such as *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida* (Confer, 2009). These opportunistic pathogens typically reside in the upper respiratory tract and can proliferate and infect the lungs when cattle immunity is compromised due to stress or primary viral infections (Hodgson et al., 2005). High-risk cattle entering feedlots, such as those with unknown immunization history, recently weaned, commingled at the auction market, and transported long distances, are particularly susceptible to BRD and frequently receive metaphylactic antibiotics to minimize respiratory infections upon arrival. The rise in antimicrobial-resistant BRD pathogens in cattle highlights the need for alternative disease management strategies, including modifications to beef production management practices (Sultana et al., 2023).

The lifecycle of beef cattle typically involves several stages, including cow-calf, post-weaned backgrounding, stocker, livestock auction market, and feedlot finishing phases

(Drouillard, 2018). Throughout these stages, calves undergo various management practices, such as castration, dehorning, abrupt weaning, sale through an auction market, transportation, commingling, changes in diet, as well as exposure to adverse environmental conditions (Tucker et al., 2015). These practices can stress the calves, impacting their immune system, health, performance, and microbiome (Chen et al., 2015), outcomes that have been linked to the pathogenesis of BRD (Taylor et al., 2010a). For instance, research has shown that cattle from multiple sources purchased through auctions (morbidity 22%) were more likely to be treated for BRD in the feedlot compared to ranch-sourced steers (morbidity 11%) (Step et al., 2008). Similarly, pens with fewer sources of cattle had lower BRD incidence compared to pens with a greater variety of cattle sources (Wiegand et al., 2020). Commingling cattle from multiple sources can increase the risk of exposure to infectious agents, weaken immune responses due to stress, and negatively impact calf health, performance, and value compared to single-source calves (Step et al., 2008). An increase in cortisol levels can result from these stressors, which can weaken host defenses and lead to dysbiosis of microbiota in the upper respiratory tract (Rice et al., 2007). It has also been shown that the abundance of *M. haemolytica* in the upper respiratory tract was increased in recently weaned calves that were transported over 1,600 km (Frank and Smith, 1983), and that *M. haemolytica* prevalence at feedlot entrance was a risk factor for BRD (Noyes et al., 2015). This suggests that transportation can disrupt the composition of the bovine respiratory microbiota and stimulate the proliferation of pathogenic bacteria.

Preconditioning, first suggested several decades ago to manage BRD (Lalman and Ward, 2005), involves implementing management practices to minimize various stressors and better prepare beef calves for subsequent production stages like stocker and feedlot. These practices

include vaccinating against viral and bacterial pathogens, optimizing the timing of dehorning and castration, implementing the best weaning strategies, and acclimating calves to eating from a bunk and drinking from a water source at least 45 days before being transported to the feedlot (González et al., 2010; Taylor et al., 2010a). It has been shown that low-stress fence-line weaning resulted in higher diversity in post-weaning rumen samples compared to traditional abrupt weaning (Diaz et al., 2023), highlighting that preconditioning may impact the microbiota of ruminants. Vaccination, a key element of preconditioning programs, is recognized as effective in reducing morbidity and mortality due to BRD (O'Connor et al., 2019) by targeting BRD pathogens. Ideally, cattle should be vaccinated against causative agents several weeks before they are naturally exposed to the disease challenge (Chamorro and Palomares, 2020b; Richeson and Falkner, 2020b). The implementation of a 45-day post-weaning preconditioning program has been shown to increase net profit by $14 per head compared to those that were not preconditioned (Dhuyvetter et al., 2005). Additionally, preconditioning has demonstrated a 42.6% reduction in morbidity and a 10.3% reduction in mortality rates compared to auction market-derived calves in Kentucky (Roeber et al., 2001). Despite these positive impacts, the widespread adoption of preconditioning remains limited, and there is a lack of studies exploring how specific stressors affect host health and respiratory microbiota. Therefore, the objective of this study was to assess the impact of preconditioning on stress, immunity, and respiratory bacteria responses in cattle originating from a single farm.

2.2 Materials and Methods

The animal experiment was reviewed and approved by the Animal Care Committee of Lacombe Research and Development Centre (LaRDC; Animal Use Protocol number 202201). All

animals involved in the study were handled and cared in strict accordance with the guidelines established by the Canadian Council of Animal Care (CCAC, 2009).

2.2.1 Animals and study design

One hundred beef calves with a known history of antibiotic use and vaccination were enrolled from the closed herd at the LaRDC, Alberta. Additionally, 100 beef calves with unknown background were sourced from a local auction market at Lethbridge, Alberta, for a total of 200 calves used in this study. At the start of the study, the 100 calves at LaRDC were randomly assigned to a preconditioning program (n=50; vaccinated three times prior to feedlot arrival, low stress two-stage weaning using anti-sucking nose tags, introduction to feed bunk) and non-preconditioned program (n=50; vaccination only at 2 months of age, abrupt weaning at time of shipment to feedlot) group. The groups were kept separated by fencing at LaRDC. The preconditioned group was vaccinated at first processing (approximately 2 months of age), weaning (approximately 6 months of age; at time of nose-flap application), and three weeks after weaning. The non-preconditioned group was only vaccinated at 2 months of age. Commercial subcutaneous vaccines were used, including: Pyramid FP 5 + Presponse SQ vaccinates against IBR, BVD types 1 and 2, PI3, and BRSV, and *M. haemolytica*) and Vision 8 Somnus vaccinates against *Clostridium chauvoei* (Blackleg), septicum (Malignant edema), haemolyticum (Bacillary hemoglobinuria), novyi (Black disease), sordellii, perfringens Types C & D (Enterotoxemia) and *H. somni*.

Five weeks after weaning of the preconditioned calves, both groups were transported separately for 12 h by commercial liner from LaRDC to the Lethbridge Research and Development Centre (LeRDC) feedlot, with the non-preconditioned group being abruptly weaned prior to

transportation. An additional 100 weaned beef calves with unknown background were sourced from a local auction market and transported for 12 h prior to arrival to the LeRDC feedlot. Upon arrival at the LeRDC feedlot, preconditioned and non-preconditioned calves were kept separately but placed in pens with an equal number of auction market calves (Figure 2.1). In each pen, 5 auction market calves were commingled with five preconditioned or non-preconditioned calves, where a total of 20 pens were used for 200 calves. At the LeRDC, all calves were processed according to the feedlot's health protocol, including ear tagging and vaccination with the same vaccine used at LaRDC. Calves were not administered antimicrobials at feedlot arrival. Sick calves were diagnosed (fever greater than 40^{o}C and respiratory symptoms including nasal or ocular discharge, coughing) and treated for BRD (florfenicol injection), according to LeRDC feedlot protocols. Treated animals remained in the study for sampling.

2.2.2 Sample collection

Nasopharyngeal (NP) swabs were collected from the preconditioned and non-preconditioned calves six weeks before transport (Baseline), immediately before transport (Pre-transport), on arrival at the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement. Nasopharyngeal swabs were also collected from the auction market calves on arrival at the feedlot (D0), and day 6 (D6) after feedlot placement. In addition, a subset of NP swabs was collected from sick animals prior to treatment (auction market, n=23; preconditioned, n=13; non-preconditioned, n=13). The NP swabs were collected from the right nasal cavity while calves were restrained in a chute. Prior to sampling, the nostril was wiped clean with 70% ethanol. Extended guarded swabs (27cm) with a rayon bud were used for sampling. Swab tips were then cut and placed into sterile 1.5-mL tubes and kept on ice. Samples were

transported to the lab and stored at -80°C within one hour of collection. Blood samples were collected from the preconditioned and non-preconditioned calves via jugular venipuncture at Pre-transport, and D0, D2, and D6 to measure cortisol levels, and antibody titres against *M. haemolytica* and *H. somni*.

2.2.3 Antibody responses to *M. haemolytica* and *H. somni,* and serum cortisol quantification

Enzyme-linked immunosorbent assays (ELISA) were performed to quantify serum IgG and nasal IgA antibody against *M. haemolytica* and *H. somni* as described previously (Malmuthuge et al., 2021). Briefly, purified recombinant *M. haemolytica* leukotoxin (Mh-LKT) and soluble bacterial lysates prepared from *H. somni* were used as a ligand to capture antibodies. The purified recombinant Mh-LKT or bacterial lysate protein was suspended in coating buffer to coat ELISA plates at a concentration of 50 ng per well. The plates were then incubated overnight at 4°C before being used the next morning to measure serum IgG and Nasal IgA levels. Peroxidase-conjugated AffiniPure Goat Anti-Bovine IgG (H+L) from Jackson Immuno Research Inc. (Cat # 101-035-003) and Sheep anti Bovine IgA:HRP from Bio Rad (Cat # AAI49P) were used as secondary antibody for IgG and IgA evaluation, respectively. Color development was carried out using TMB (3,3′,5,5′-tetramethylbenzidine; ThermoFisher, Canada) and the reactions were stopped by adding ELISA stop solution (0.2 M H2SO4). Optical densities were measured at a wavelength of 450 nm using a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA) and analyzed with Gen5 software.

Blood cortisol levels were measured using an immunoassay kit (DetectX Kit, Arbor Assays, Ann Arbor, MI, USA) and following the manufacturer's instructions. Briefly, the serum

samples were initially treated with the provided dissociation reagent and subsequently diluted in assay buffer. Following this, the diluted samples and the standards were pipetted into respective wells in duplicate. DetectX cortisol conjugate and antibody were then added to the wells. The plate was covered with a plate sealer and incubated in a shaker incubator (700–900 rpm) at room temperature for 1 h. Subsequently, the TMB substrate was added, which reacted with the bound cortisol-peroxidase conjugate. After a brief incubation without shaking, the reaction was stopped, and the optical densities were measured at a wavelength of 450 nm using a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA). Finally, the concentration of cortisol in the sample was analyzed using Gen5 software.

The ELISA data were analyzed using SAS PROC GLIMMIX (SAS version 9.4, SAS Inst. Inc., Cary, NC). For each generalized linear model, a log-normal distribution was selected based on the respective values of the Bayesian information criterion (BIC). A lower value of the BIC associated with the model indicated superior model fit. Statistical significance was indicated by p-values less than 0.05. Microsoft Excel 2010 and GraphPad Prism version 10.1.0 were utilized for visual representation of all ELISA analyses.

2.2.4 DNA extraction, amplicon sequencing and analysis

Nucleic acids were extracted from NP swabs using the method previously described (Holman et al., 2017). DNA was amplified with primers 515-F (5′-GTGYCAGCMGCCGCGGTAA-′3) and 806-R (5′-GGACTACNVGGGTWTCTAAT-′3) targeting the V4 region of the 16S rRNA gene, as described earlier (Holman et al., 2019). The

amplicon was sequenced on a MiSeq instrument (PE250) using the MiSeq Reagent Kit v2, generating a read coverage of 25,000 at Genome Quebec in Montreal, Quebec, Canada.

The quality of the raw paired reads was assessed using FastQC v0.11.9 and MultiQC v1.12 (Ewels et al., 2016). Sequence trimming to remove primer sequences and low-quality regions in the reads was performed with Trimmomatic v0.39 (Bolger et al., 2014) with the following settings: HEADCROP:10, SLIDINGWINDOW:5:20, LEADING:20, and TRAILING:20. Subsequent data analysis was carried out with R version 4.2.2 (Team, 2013). The trimmed paired reads underwent additional filtering with the DADA2 v1.22.0 'filterAndTrim' function using default settings, and were then merged. Bimeric sequences were removed from the merged dataset using the DADA2 'removeBimeraDenovo' function. Taxonomic classification of the 16S sequences was accomplished using the DADA2 'IdTaxa' function and the SILVA 138 database (Quast et al., 2013), generating the amplicon sequence variants (ASVs) table. Analysis was performed with Phyloseq v1.42.0 (McMurdie and Holmes, 2013), and visualization was done with ggplot2 v3.4.1 (Wickham, 2011). The Shannon diversity index and richness were computed using the 'vegan' v2.6-4 package (Oksanen et al., 2019). The ASV table was filtered to retain ASVs present with a count of at least 2 in a minimum of 1% of the samples. One-way analysis of variance (ANOVA) was utilized to assess α-diversity with respect to treatment and time. β-dispersion and permutational multivariate analysis of variance (PERMANOVA) were performed with 9999 permutations using the 'vegan' package to examine the effects of treatment and time on the microbial community structure. The β-diversity analysis was performed on the filtered ASV counts after size factor normalization using 'GMPR' within DESeq2 v1.40.2 (Love et al., 2014). Sample-to-sample distances were determined using the Bray-Curtis metric through Phyloseq ordination

and presented as a detrended correspondence analysis (DCA). Analyses of the significant ($P < 0.05$) differences in the abundances of taxa were identified using DESeq2 by fitting a negative binomial model to two equations. The equation 'time X vs time Y' within a treatment group (~Time), was applied to analyses with alterations to the time variables as required, and, the equation 'sampling time X vs sampling time Y' with respect to 'treatment group X vs treatment group Y' comparisons (~ Treatment + Time + Time:Treatment) was applied for a single analysis. Significant ASVs ($P < 0.05$) within the significant genera were visualized with heatmaps.

2.3 Results

2.3.1 16S rRNA gene sequencing overview

Pre-processing steps produced 16,826 ASVs in 929 samples from 6,623,808 merged paired reads. At this stage the median number of sequences per sample was 7,008 ± 2,378.1, with a minimum of 15 and a maximum of 24,046. After filtering for ASVs that occur in at least 1% of the samples, 277 ASVs comprised of 5,801,557 reads remained. The final median number of sequences was 6,361 ± 2,685.8 with a minimum of 1,526 and a maximum of 23,696 sequences per sample.

2.3.2 Diversity and community structure of the NP microbiota

For both Richness (Figure 2.2A) and Shannon diversity index (Figure 2.2B), ANOVA indicated that treatment, time, and the interaction between treatment and time affected diversity metrics ($P < 0.001$). The non-preconditioned group had greater microbial diversity at the Pre-transport time point compared to their baseline level. For both preconditioned and non-conditioned groups, there was a decline in diversity metrics from day 0 to day 6 of their feedlot placement, and

remained lower than baseline timepoint for the remaining feedlot timepoints. On days 0 and 6, Shannon diversity index and richness were not different between auction market and preconditioned or non-preconditioned animals. PERMANOVA was used to evaluate the structure of the NP communities and indicated that the centroids and dispersions of the samples grouped by sampling time ($P < 0.001$, $R2 = 0.104$), treatment group ($P < 0.001$, $R2 = 0.016$), and the interaction of time and treatment ($P < 0.001$, $R2 = 0.013$). This was confirmed using DCA plots (Figure 2.3), which showed variance in microbial structure. While non-preconditioned and preconditioned samples had highly similar dispersions at Baseline time, a divergence in microbial structure over time and between groups was observed.

2.3.3 Composition of the microbiota

A total 10 bacterial phyla were identified across all groups and sampling times. These consisted of Proteobacteria (57.1%), Mycoplasmatota (24.9%), Actinobacteriota (9.9%), Firmicutes (3.9%), Bacteroidota (1.6%) Fusobacteriota (0.9%) Deinococcota (0.5%), Chloroflexi (0.1%), Euryarchaeota (0.1%), and Acidobacteriota (0.1%) (data not shown). The 9 most relatively abundant genera from all groups and sampling times of healthy animals (Figure 2.4) were *Mycoplasma* (24.6%), *Moraxella* (16.5%), *Pasteurella* (8.9%), *Histophilus* (8.9%), *Mannheimia* (4.1%), *Lactobacillus* (1.3%), *Streptococcus* (1.1%), *Caviibacter* (0.9%), and *Acinetobacter* (0.5%) with those genera representing 66.8% of all sequences. While *Mycoplasma* levels in both preconditioned and non-preconditioned calves increased over time, *Histophilus* abundance increased at the final two sampling times in both treatments. *Moraxella* and *Pasteurella* had abundances that rose and decreased between Pre-transport to D6 and D14 respectively. The

abundances of the 9 most relatively abundant genera in auction market calves (Appendix A) were generally similar to preconditioned and non-preconditioned calves.

2.3.4 Changes in the microbiota across sampling time and between treatments

β-binomial regression was performed separately on non-preconditioned and preconditioned samples to identify genera that exhibited significant ($P < 0.05$) changes in abundance levels at each time compared with the Baseline time (Figure 2.5A). There were 30 genera that were significantly different at least at one timepoint, with both treatment groups exhibiting changes in 29 of these genera. In the non-preconditioned group, 9 genera had decreases in relative abundance during the majority of sample times, while 5 genera decreased at most time points in preconditioned animals. Shared between the two treatments were overall decreases in *Biberstenia*, *Lactobacillus*, and *Streptococcus* relative to the Baseline sampling time. Relative increases were observed in 19 genera for the non-preconditioned treatment and 18 genera in the preconditioned treatment, with the remaining genera fluctuating in around and across timepoints. Both groups experienced an increase in *Histophilus* levels from D6 to D32, and an overall increase in *Myocplasma*, *Moraxella*, and *Pasteurella* at most timepoints. The preconditioned NP microbiota had consistently lower levels of *Mannheimia* at each timepoint, compared to the Baseline timepoint.

β-binomial regression analysis was performed to compare changes in samples from non-preconditioned and preconditioned groups relative to the pre-transportation timepoint, evaluating the impact of feedlot placement (Figure 2.5B). In total, 28 genera were identified that showed significant difference between the two conditions in at least one timepoint. Of these 28 genera,

relative abundances of 19 genera decreased in non-preconditioned animals for the majority of timepoints when compared to the preconditioned samples. Relative abundance of eight genera were increased at most timepoints in the non-preconditioned animals, including *Bibersteinia*, *Pasteurella*, *Moraxella*, and *Staphylococcus*. *Histophilus* had higher relative abundance in the non-preconditioned calves, but only at a single timepoint. Both *Mycoplasma* and *Mannheimia* had a mixture of increases and decreases of their relative abundance across timepoints.

2.3.5 The NP microbiota of sick animals

After feedlot placement, 22 calves from the preconditioned group and 25 calves from the non-preconditioned group were treated for BRD (Table 2.1). There were no differences in the morbidity rate and weight gain between the preconditioned and non-preconditioned calves ($P > 0.05$; Table 2.1). Animals that became sick had a differing set of the 9 most relatively abundant genera on the day of treatment (Figure 2.6). These were *Histophilus* (26.42%), *Mycoplasma* (24.94%), *Moraxella* (7.21%), *Pasteurella* (6.00%), *Mannheimia* (2.70%), *Bibersteinia* (1.79%), *Acinetobacter* (0.82%), *Deinococcus* (0.80%), and *Streptococcus* (0.78%), for a total of 71.46% of sequence reads. On the day of treatment, sick calves within the auction market group had lower levels of *Histophilus* ($P < 0.05$) and higher levels of *Mycoplasma* when contrasted against preconditioned and non-preconditioned calves. β-Binomial regression was conducted on samples from sick animals across all treatments that identified a total 17 genera that showed ($P < 0.05$) changes in abundance levels between the day of treatment and their arrival at the feedlot (D0) sampling (Figure 2.7). Compared to D0, the relative abundances of *Histophilus* and *Mycoplasma* increased on the day of treatment across sick animals from all treatment groups, where those of *Moraxella* and *Streptococcus* decreased in all treatments. While *Mannheimia* and *Staphylococcus*

remained unchanged for the sick animals in the non-preconditioned group, a significant decrease was observed for the sick animals in the preconditioned group. When sick animals were spread over the time and treatment including their day of sick/treatment, *Histophilus* showed an interesting trend, being higher in abundance on days 0 and 6 compared to other two treatment groups (Appendix B). Preconditioned and non-preconditioned groups showed a higher abundance of *Histophilus* from D6 onwards. Further investigation revealed that *Histophilus* identified from auction market calves on days 0 and 6 corresponded to four ASVs (ASV4, ASV8, ASV26, and ASV35; data not shown). Interestingly, a higher relative abundance of these same four ASVs was found in both preconditioned and non-preconditioned calves during the later period of their feedlot placement (day 6 onwards).

2.3.6 Serum cortisol levels in preconditioned and non-preconditioned calves:

Cortisol concentrations were greater in preconditioned calves at the pre-transport timepoint compared to non-preconditioned calves ($P<0.05$; Figure 2.8A). However, in the preconditioned calves the cortisol concentrations did not change after transportation. In contrast, non-preconditioned calves showed higher concentrations of blood cortisol levels at all post-transport time points ($P<0.05$), and remained elevated until D6 ($P<0.05$) compared to the pre-transport sampling time (Figure 2.8B).

2.3.7 Antibody titres to *M. haemolytica* and *H. somni*

At the pre-transport time point, serum IgG concentrations against *M. haemolytica* were higher in preconditioned compared to non-preconditioned calves ($P<0.05$; Figure 2.9A). Although serum IgG remained unchanged at later time points for preconditioned calves ($P>0.05$), it increased

on days 2 and 6 in the non-preconditioned calves ($P<0.05$) when compared to their pre-transport levels. Unlike *M. haemolytica*, Serum IgG concentrations against *H. somni* did not different at the pre-transport time point between groups ($P>0.05$; Figure 2.9B), or later time points after feedlot placement. Rather, serum IgG concentrations against *H. somni* showed a consistent trend both for preconditioned and non-preconditioned calves (Figure 2.9B), with both groups having increased antibody concentrations on days 2 and 6, compared to their pre-transport time point ($P<0.05$).

Secretory IgA concentration against *M. haemolytica* from nasal samples were greater in preconditioned calves, compared to non-preconditioned calves, at the pre-transport time point ($P<0.05$; Figure 2.9C). However, the IgA concentrations decreased by day 32 for preconditioned group ($P<0.05$) compared to their pre-feedlot time point. On days 14 and 32, nasal IgA concentrations were similar between the two groups. Nasal IgA concentrations against *H. somni* had a similar trend to IgA responses against *M. haemolytica*, where preconditioned calves showed higher nasal IgA compared to non-preconditioned calves at the pre-transport time point ($P<0.05$; Figure 2.9D). However, the nasal IgA concentration decreased over times for both groups ($P<0.05$; Figure 2.9D), and were not different between the treatments on days 14 and 32.

2.4 Discussion

This study evaluated the effect of preconditioning on the health and NP microbiota of calves transported long-distance prior to feedlot arrival. Several advantages were realised in this study, including having a high number of replicates at each sampling time for microbial analysis, using calves from the same closed herd for preconditioning designation, and mixing the groups of preconditioned/non-preconditioned calves with auction market-derived calves. In addition, calves

did not receive metaphylaxis at feedlot entry which can confound results by increasing NP microbial diversity (Holman et al., 2019) and selecting for specific bacterial pathogens (Guo et al., 2020). A limitation of the study was placement of calves in pens housing 10 animals which is not undertaken in commercial settings. However, despite this limitation, calves experienced BRD, highlighting that the research model worked in stressing the calves and inducing respiratory illness similar to larger commercial feedlots.

Diversity of the NP microbiota decreased in both preconditioned and non-preconditioned calves after feedlot placement. While this is in contrast to a previous study showing diversity to increase after feedlot arrival (Holman et al., 2017), it likely reflects how the feedlot and ambient conditions can impact the diversity of respiratory microbiota, as a source of bacteria that can be aspirated. It is interesting that microbial diversity of the preconditioned group was lower than the non-preconditioned group at the Pre-transport timepoint, possibly due to the acclimatization period as part of the preconditioning program. Diets fed to cattle can affect the respiratory microbiota (Hall et al., 2017), potentially through causing changes in the rumen bacteria which then colonize the respiratory tract via oropharyngeal transfer. Indeed, genera associated with the gastrointestinal tract were identified in NP samples of the cattle. The preconditioned calves were bunk-fed as part of the preconditioning program, thus the diversity of their nasopharynx may have started to decrease prior to feedlot arrival, as a result of feed transition.

The structure of the NP microbiota of preconditioned and non-preconditioned calves changed greatly after feedlot placement, similar to observations reported previously (Holman et al., 2017; Holman et al., 2019). It is interesting to note that dispersion of the community structures

increased after feedlot placement, indicating that relatedness of bacterial communities became more variable between individual animals. Thus, host response to transportation and feedlot placement likely affects the composition of the NP microbiota. In support of this, while several trends in general abundances of the top 9 genera were observed, individual animals were occasionally dominated by specific genera at various timepoints, falling outside of these trends. For example, while there was an overall increase in the abundance of *Mycoplasma* across time, in some calves, this genus accounted for >95% of sequencing reads.

Previous studies have shown that *Mycoplasma*, *Moraxella*, *Pasteurella*, *Mannheimia*, and *Histophilus* can account for the main genera in the NP of healthy feedlot calves (Holman et al., 2017; Holman et al., 2018; Stroebel et al., 2018; Zeineldin et al., 2017b). Our findings are consistent with these studies, as these genera also represented the majority of sequence reads in NP samples. Animals that were treated with antimicrobials for BRD throughout the feeding period remained in the study. Both tetracycline and macrolide antimicrobials have been shown to alter the NP bacteria of calves (Holman et al., 2019), likely through secretion into the mucosa of the upper respiratory tract. Thus, it is likely that antimicrobial (florfenicol) administration in our study affected the NP bacteria of treated calves. However, the results therefore reflect the NP microbiota of calves under normal practices employed in feedlots.

It is noted that *Histophilus* increased significantly in relative abundance at days 14 and 32. This is supported by a previous study that showed tracheal samples of cattle were 32.5 times more likely to be culture-positive for *H. somni* in cattle sampled at 40 days, compared to those sampled less than 20 days after feedlot arrival (Timsit et al., 2017b). In contrast to the increase in

Histophilus, we observed a strong decline in *Lactobacillus*. *Lactobacillus* comprised a relatively large component of the NP bacteria in both preconditioned and non-preconditioned calves at the Baseline timepoint (approximately 6 months of age). Both calf groups had similar management practices to this point. Families of lactic-acid producing bacteria have been shown to be negatively correlated with the BRD-associated *Pasteurellaceae* family when cattle were transported to a feedlot (Amat et al., 2019). Specifically, *Lactobacillus*-derived probiotics have been shown to reduce colonization against *M. haemolytica* when administered intranasally (Amat et al., 2020). It is therefore intriguing to speculate that methods to maintain or enhance the NP *Lactobacillus* populations prior to feedlot placement may also enhance respiratory health.

We used β-binomial regression to analyse the NP microbiota of preconditioned and non-preconditioned calves across time, relative to the Baseline timepoint. At this timepoint, both groups had comparable vaccination programs (i.e. two months of age). Interestingly, in the preconditioned calves, *Mannheimia* was consistently reduced, compared to the Baseline, at every timepoint. This resembles an earlier report in which grazing beef calves that underwent preconditioning exhibited a reduction of *Mannheimia* when compared to non-preconditioned calves (Brooks et al., 2021). In contrast, for the non-preconditioned group, *Mannheimia* abundance was increased relative to the baseline up to D14, at which point this genus was reduced in abundance compared to the Baseline. While subcutaneous vaccines elicit a primary serum IgG antibody response, mucosal spill can occur in the form of secretory IgA antibodies. It was notable that both systemic IgG and secretory IgA against *M. haemolytica* were significantly greater in preconditioned calves at the pre-transport timepoint, likely reflecting the three vaccinations against *M. haemolytica* in that the preconditioned calves received prior to transport. However, by D6 in

the feedlot, non-preconditioned calves had similar antibody concentrations, which corresponded to 6 days after a second vaccination against *M. haemolytica* in these calves. While not definitive, we speculate that a booster vaccination against *M. haemolytica* reduced colonization of *Mannheimia* in the upper respiratory tract. This would account for the reduction in *Mannheimia* observed at the various timepoints in both preconditioned and non-preconditioned calves. Although a decrease in *Mannheimia* relative abundance was observed at later time points in the feedlot, the *Mannheimia*-specific secretory IgA concentrations significantly declined over time in the feedlot, which may suggest that the subcutaneous vaccine used for *Mannheimia* did not sustain consistent protection.

A similar trend to *Mannheimia* was observed for *Histophilus*, up to D2. *Histophilus* was reduced in preconditioned calves, which were vaccinated against *H. somni* three times prior to transport, while it remained unchanged in non-preconditioned calves to D2. Unlike *Mannheimia* however, *Histophilus* strongly increased in relative abundance on days 6, 14, and 32 of feedlot placement, in both preconditioned and non-preconditioned calves, despite vaccination at feedlot entry. It is possible that the increase in *Histophilus* relative abundance on days 6, 14, and 32, was either due to colonization by a *Histophilus* strain that the vaccine did not protect against, or the subcutaneous vaccine failed to maintain an effective immunity in the upper respiratory tract. Clonal transfer of *P. multocida* between feedlot cattle has been reported previously, highlighting that BRD pathogen transfer can occur in pens (Guo et al., 2020), thus it is possible that an *H. somni* strain spread among calves. The presence of *Histophilus* in auction market calves on days 0 and 6 was attributed to four distinct ASVs, and these ASVs later exhibited higher relative abundance in both preconditioned and non-preconditioned calves from day 6 onward. This indicates that

Histophilus bacteria found in the preconditioned and non-preconditioned calves likely originated from the auction market calves. Lack of vaccine protection was also supported by the microbiota of BRD cases. In sick animals, *Histophilus* was the predominant pathogenic genus detected in NP samples. In contrast, while present in all animal groups, *Mannheimia* comprised a lower percentage of the relative abundance of sequence reads. Although our analysis was limited to NP samples, we have shown before that the nasopharynx shows similar increases in pathogenic genera in BRD cases, compared to lung samples (Timsit et al., 2018). Thus, it may be possible that the *M. haemolytica* vaccine protected against strains of *Mannheimia* colonizing the calves, while the *H. somni* vaccine had limited efficacy against strains of *Histophilus*. This would suggest that development of novel *H. somni* vaccines is needed to mitigate BRD caused by this pathogen. A previous meta-analysis supports this finding, reporting limited efficacy of *H. somni* vaccination in feedlot cattle (Larson and Step, 2012).

Other BRD-associated genera, including *Bibersteinia*, *Moraxella*, and *Pasteurella* had greater increases in relative abundance in non-preconditioned calves. None of the calves were vaccinated against pathogens within these genera. This implies that preconditioning may have protected against increases in these genera after feedlot arrival, and may be related to stress as indicated by cortisol concentrations in the calves. Cortisol levels increased after transport for non-preconditioned calves, which is consistent with other studies (Cooke et al., 2013a; Grandin, 1994). However, the concentrations remained elevated up to 6 days after feedlot placement in non-preconditioned calves and was higher compared to preconditioned calves. This difference might be a combined effect of weaning and transport for non-preconditioned calves, as stress indicators like cortisol are directly related with psychological stress (Mellor et al., 2002). Studies have shown

that increased cortisol levels are linked to dysbiosis in oral (Duran-Pinedo et al., 2018), nasal (Zhao et al., 2020), and gut microbiota (Mudd et al., 2017). Recently a study evaluating long-distance transportation of weaned calves, found that several respiratory bacterial genera including *Mannheimia*, *Moraxella*, and *Streptococcus* were positively correlated with blood cortisol concentrations (Uddin et al., 2023b). Thus, it is possible that sustained stress in the non-preconditioned calves in this study may have compromised the host defences against these genera, resulting in greater growth compared to preconditioned calves.

2.5 Conclusion:

The study aimed to evaluate how preconditioning affects host immune, stress, and respiratory microbiota, in the presence of stressors including weaning, transportation and commingling. Overall, preconditioning resulted in a decreased stress response and reduced abundances of several genera linked to BRD in calves at the feedlot. Although the animals from both treatment groups showed similar morbidity in this study, the preconditioning group showed a better response against stress, and immunity compared to the non-preconditioned animals. However, host immunity upon arrival at the feedlot had varying effectiveness against different pathogen targets. Therefore, there is potential to further extend protection against BRD by developing novel mucosal vaccines with improved efficacy.

2.6 Tables and figures

Table 2.1: Comparison of BRD morbidity and weight gain (Mean ± Std. dev.) between preconditioned (N=50) and non-preconditioned (N=50) calves in the feedlot.

Details	Preconditioned	Non-preconditioned
Sick	22 (44%)	25 (50%)
First pull	22 (44%)	25 (50%)
Second pull	2 (4%)	2 (4%)
Entry weight (lbs)	651.72±61.62	684.76±63.06
Exit weight (lbs)	736.58±66.14	766.08±69.92
Weight gain at feedlot (lbs)	84.86±4.04	81.32±3.78

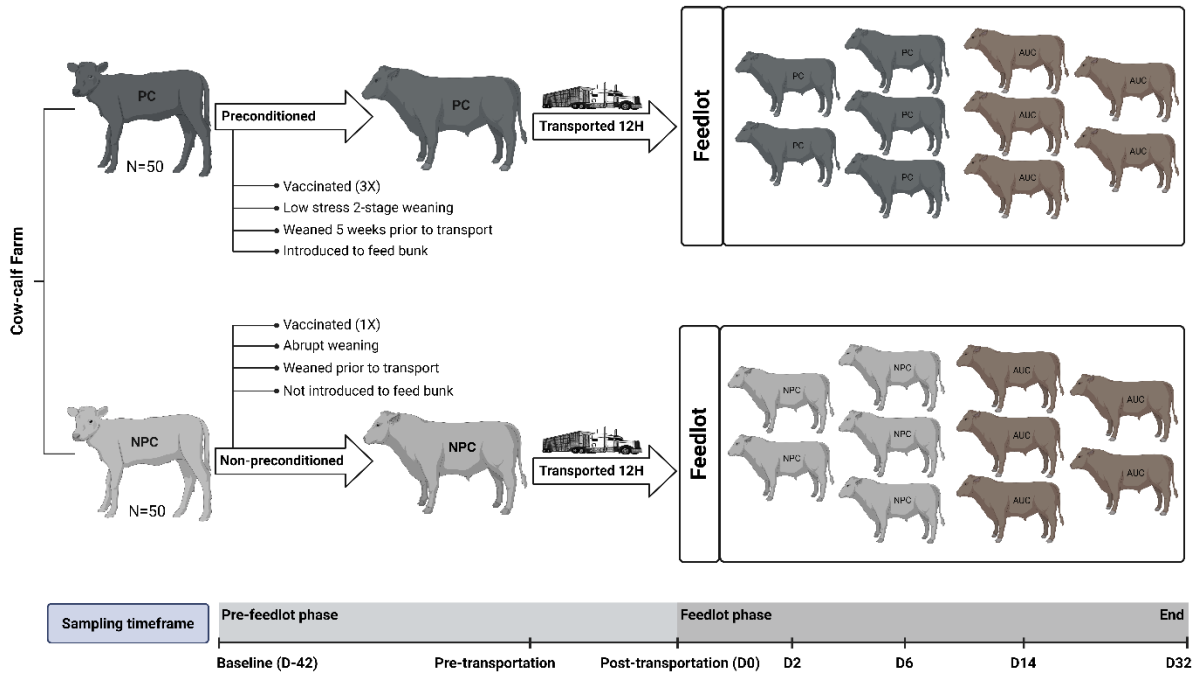


Figure 2.1: Experimental design. Preconditioned (n=50) and Non-preconditioned (n=50) calves were raised separately according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot, Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected six weeks before transportation (Baseline), right before the transportation (Pre-transportation), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement. The Auction Market calves were sampled on arrival to the feedlot (D0), and day 6 (D6) of feedlot placement.

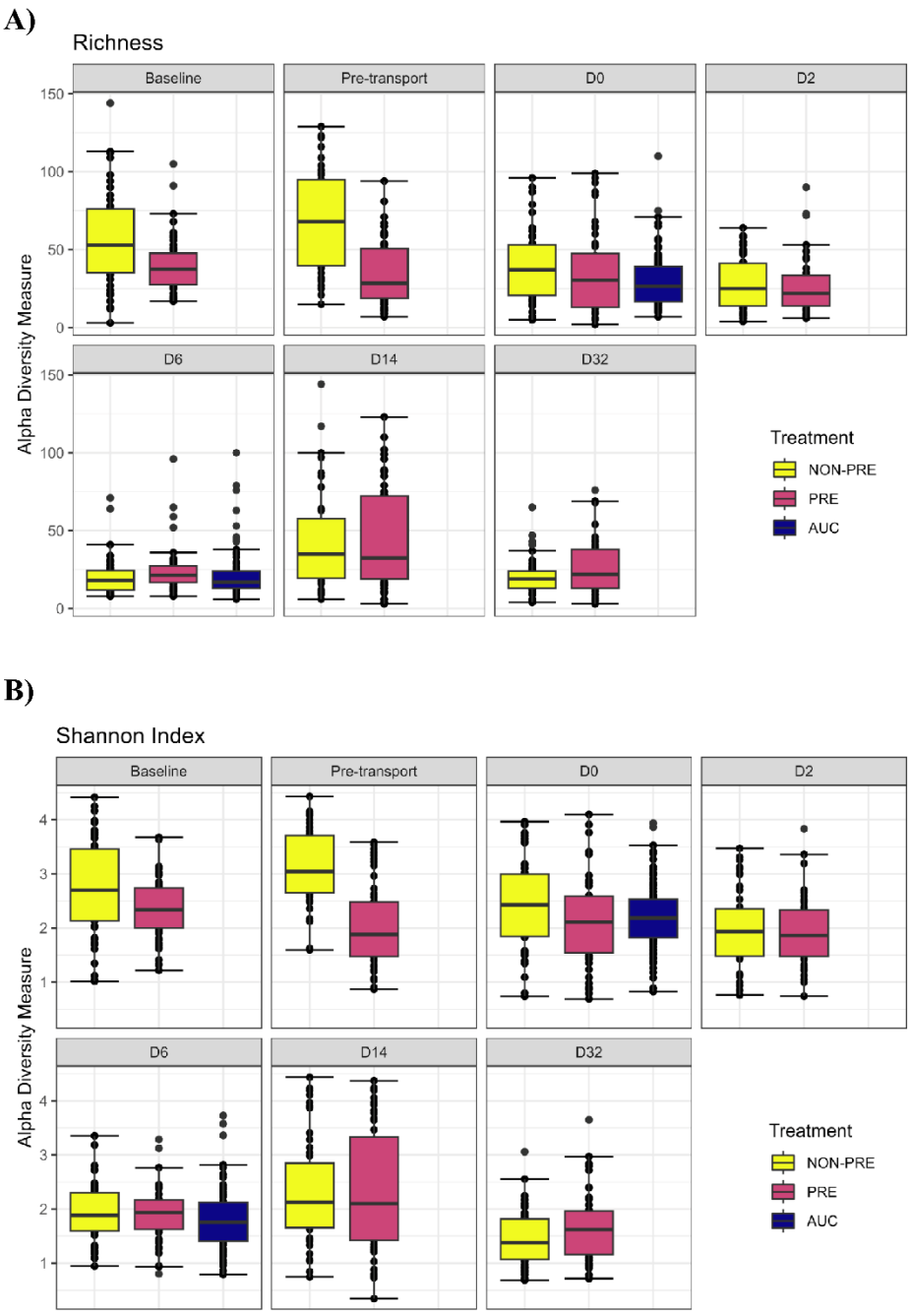


Figure 2.2: Alpha diversity of nasopharyngeal samples. Upper plot shows the bacterial richness or counts of taxa observed (A), and lower plot shows the Shannon diversity index (B). Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected six weeks before transportation (Baseline), right before the transportation (Pre-transport), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement. The Auction Market calves were sampled on arrival to the feedlot (D0), and day 6 (D6) of feedlot placement. Error bars indicate ± standard error of the mean. The box in the plots indicates the interquartile range (IQR) (middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.

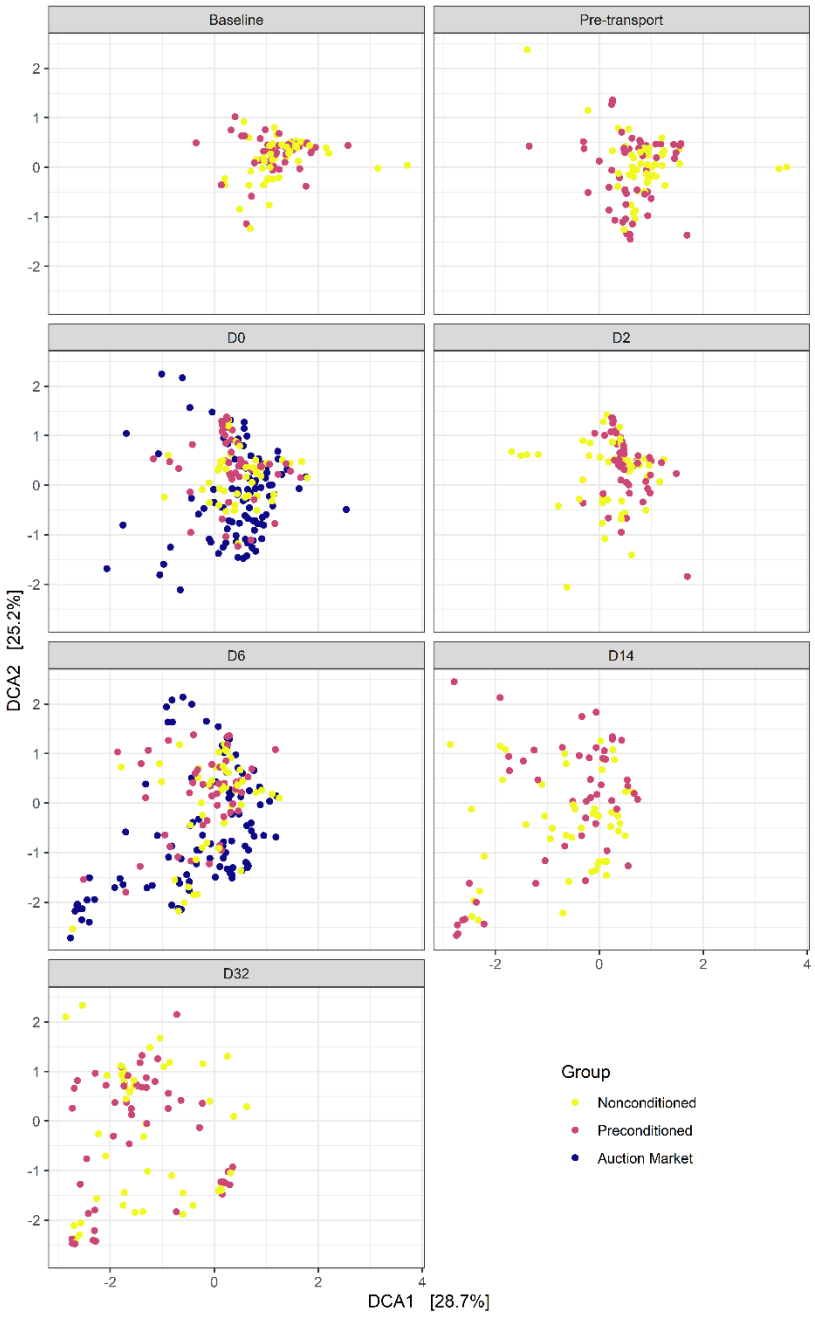


Figure 2.3: Detrended correspondence analysis (DCA) of the Bray-Curtis metric showing clustering of each treatment over time for the nasopharyngeal samples. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected six weeks before transportation (Baseline), right before the transportation (Pre-transport), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement. The Auction Market calves were sampled on arrival to the feedlot (D0), and day 6 (D6) of feedlot placement.

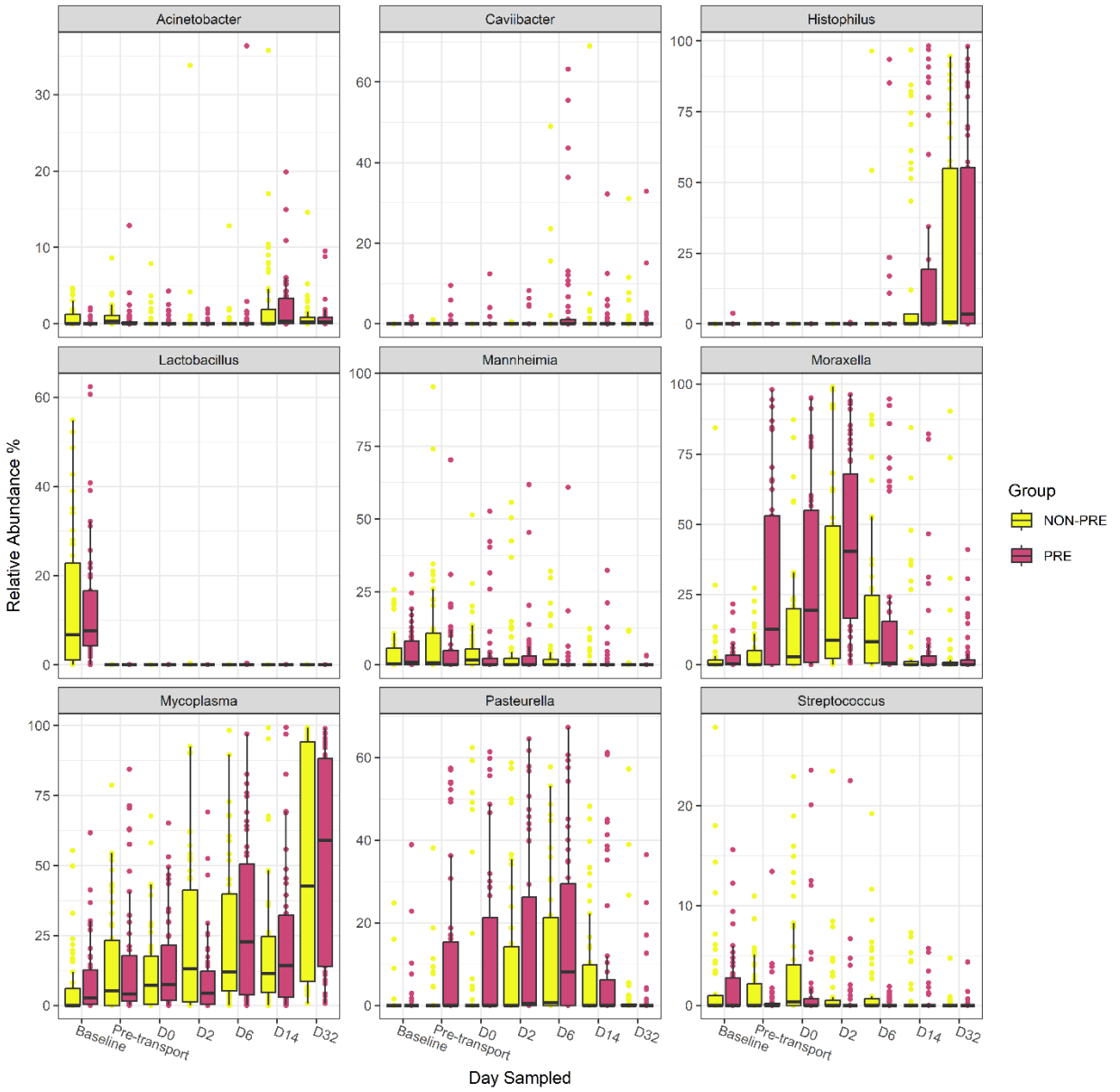


Figure 2.4: Relative abundance of the nine most abundant genera identified in nasopharyngeal swabs from preconditioned and non-preconditioned calves across time. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected six weeks before transportation (Baseline), right before the transportation (Pre-transport), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement. The Auction Market calves were sampled on arrival to the feedlot (D0), and day 6 (D6) of feedlot placement. Error bars indicate ± standard error of the mean. The box in the plots indicates the interquartile range (IQR) (middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.

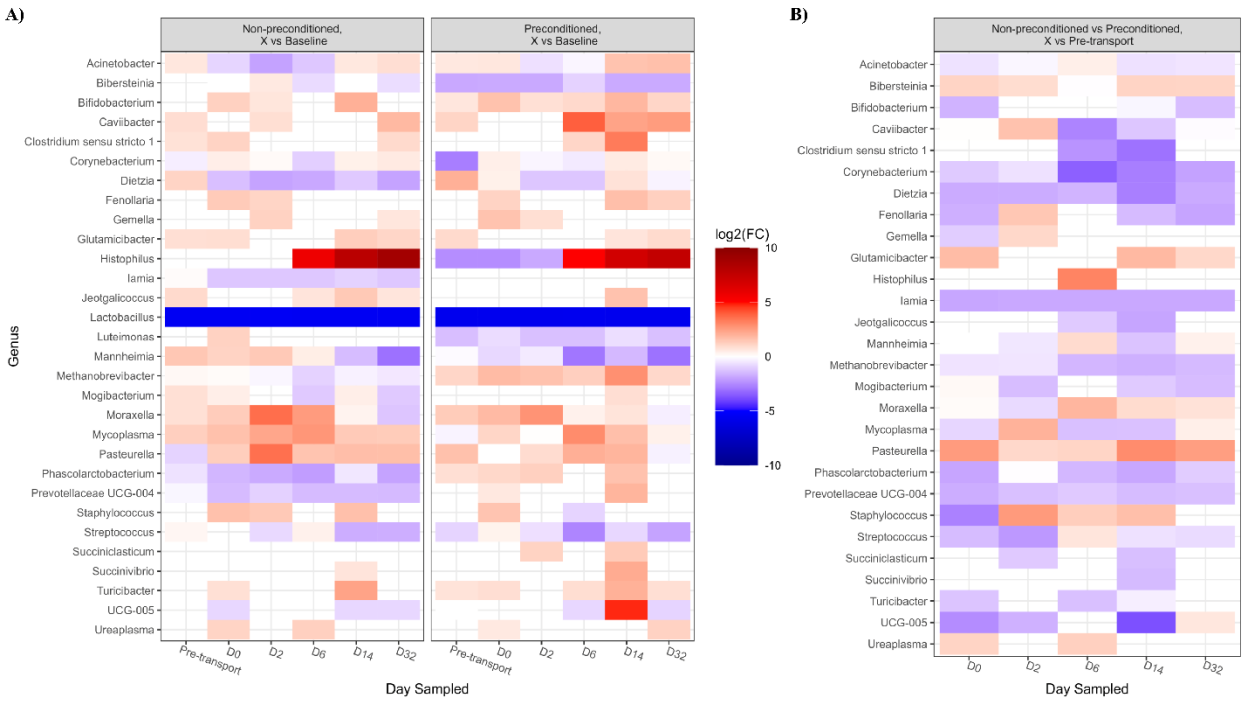


Figure 2.5: Genera of nasopharyngeal swabs that showed a significant change (P < 0.05) between noted treatments. Significant change between noted treatments, with respect to each sampling time against the baseline time, six weeks before transportation (A), and with respect to each sampling time at feedlot against the Pre-transport sampling time (B). The colours displayed represent the average log2(FC) of amplicon sequence variants (ASVs) with a significant change (P < 0.05) within the respective genus at the indicated time. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected six weeks before transportation (Baseline), right before the transportation (Pre-transport), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement.

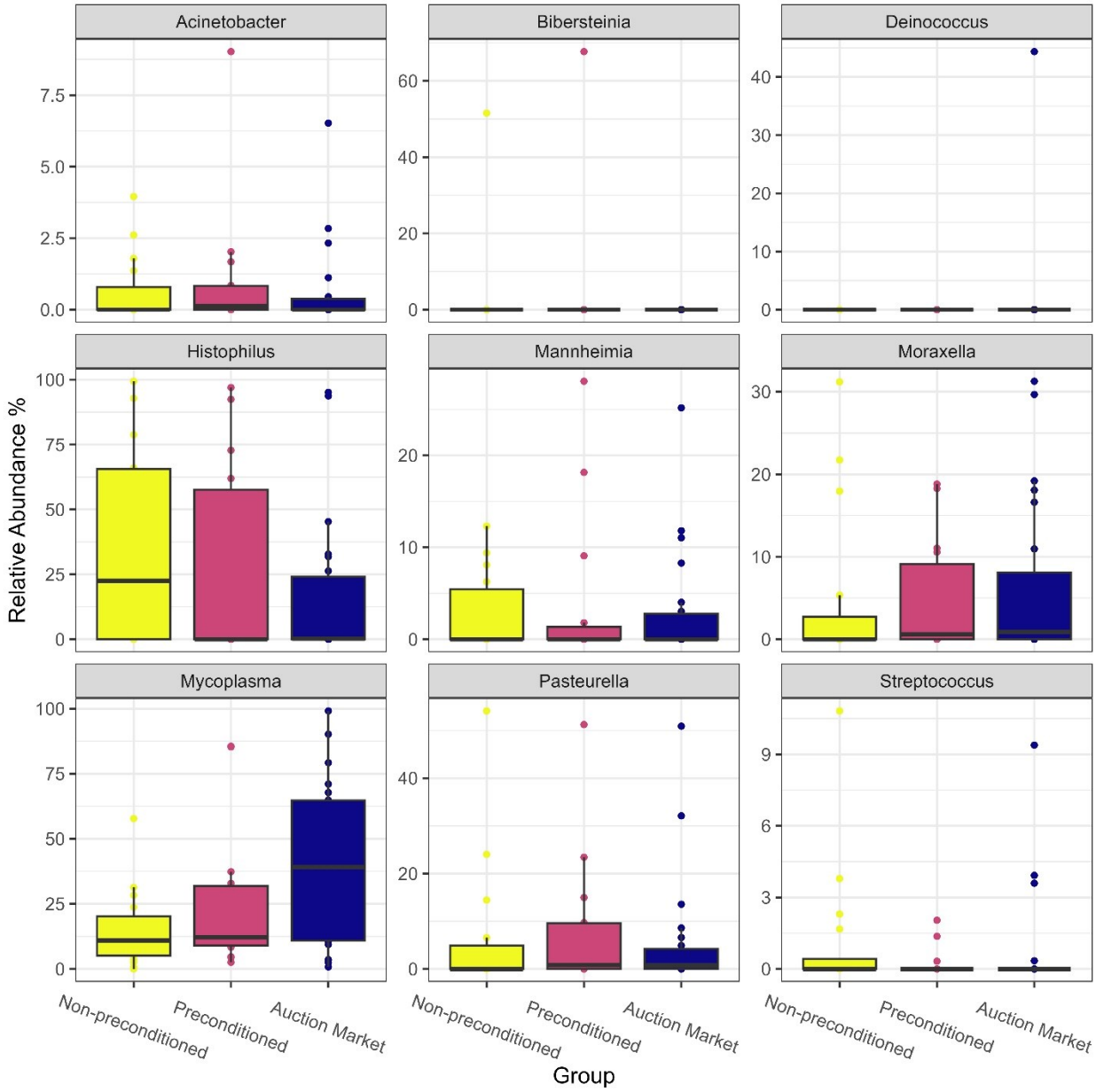


Figure 2.6: Relative abundance of the nine most abundant genera identified in nasopharyngeal swabs from sick animals across treatments. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples were collected from sick calves throughout 32 days of feedlot placement, before antimicrobial treatment. The box in the plots indicates the interquartile range (IQR) (middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.

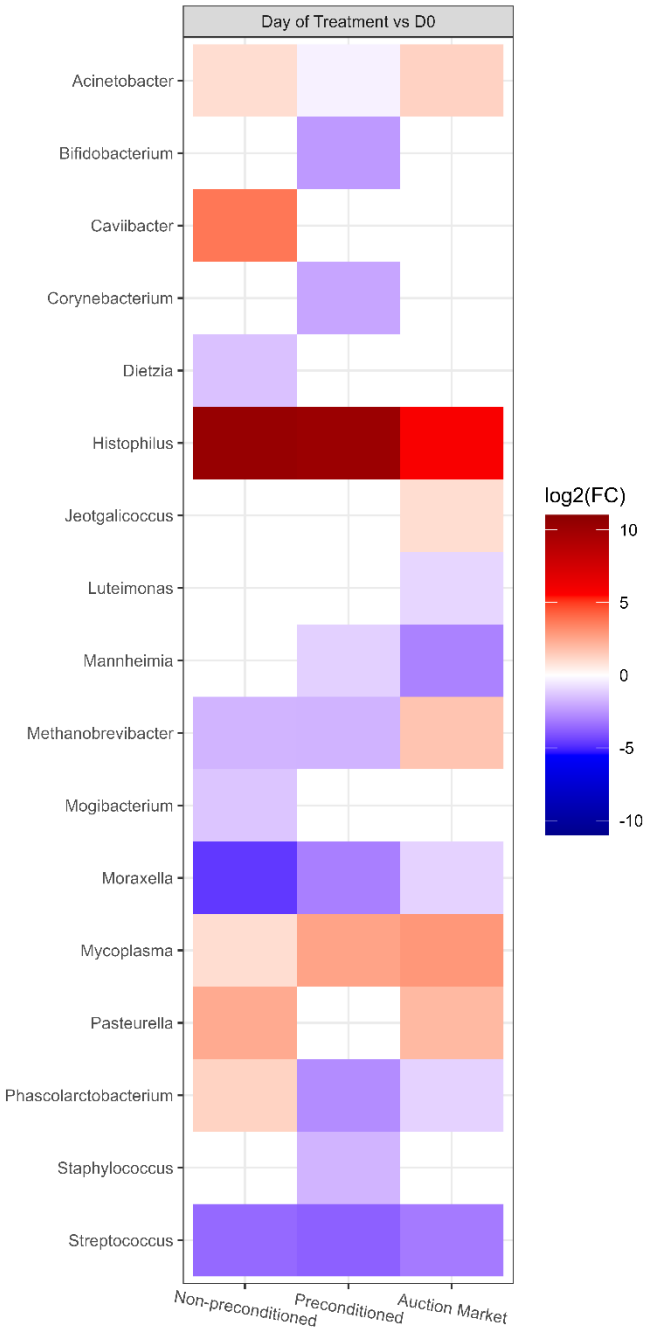


Figure 2.7: Genera of nasopharyngeal swabs from sick animals that showed a significant change (P < 0.05) in the noted treatments, with respect to samples collected when they were sick against their on arrival at feedlot (D0) sampling. The colours displayed represent the average log2(FC) of amplicon sequence variants (ASVs) with a significant change (P < 0.05) within the respective genus at the indicated time. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples from Preconditioned and Non-preconditioned calves were collected right before the transportation (Pre-transport), on arrival to the feedlot (D0), and subsequently on days 2 (D2), 6 (D6), 14 (D14) and 32 (D32) of feedlot placement.

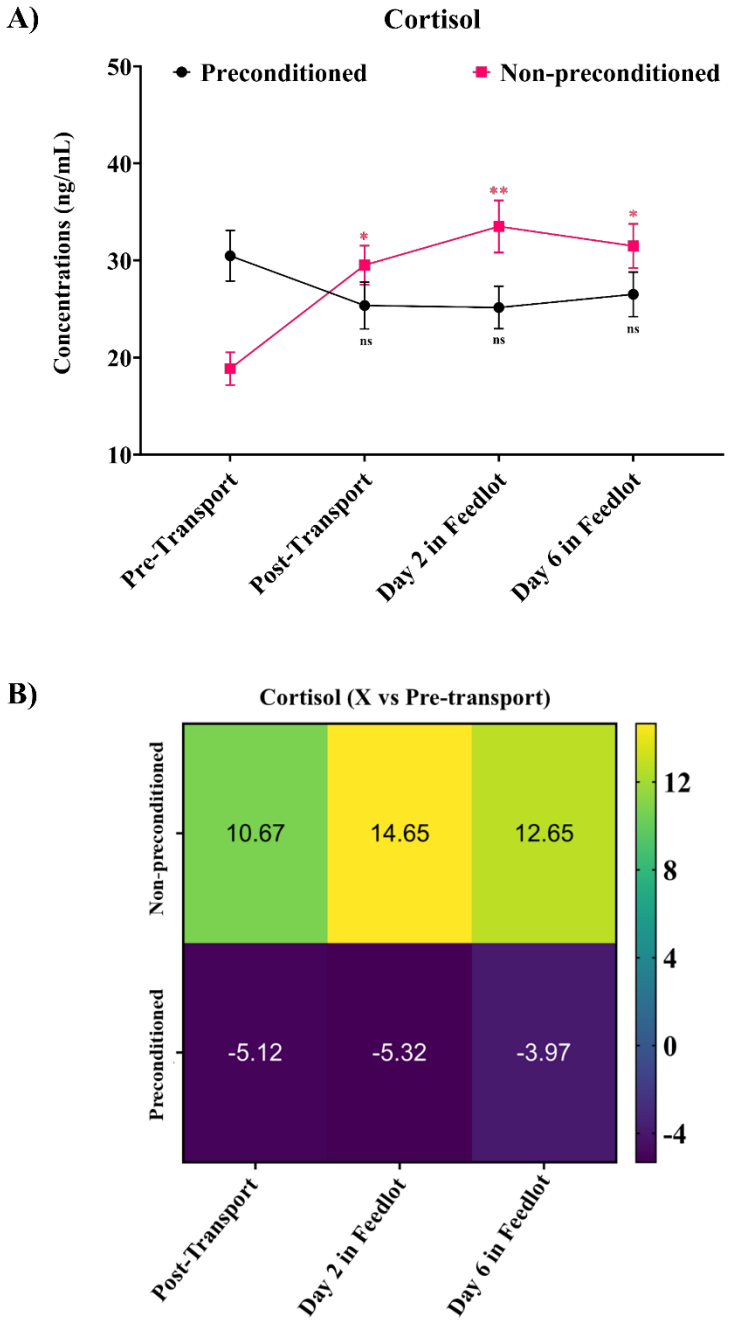


Figure 2.8: Blood cortisol concentrations in preconditioned and non-preconditioned calves following weaning and transportation stress. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). A) Cortisol concentration across time, B) Changes of cortisol level compared to pre-transport time point. ^{ns} not significant, * $p < 0.05$, ** $p < 0.01$ when compared to the Pre-transport timepoint within the same treatment.

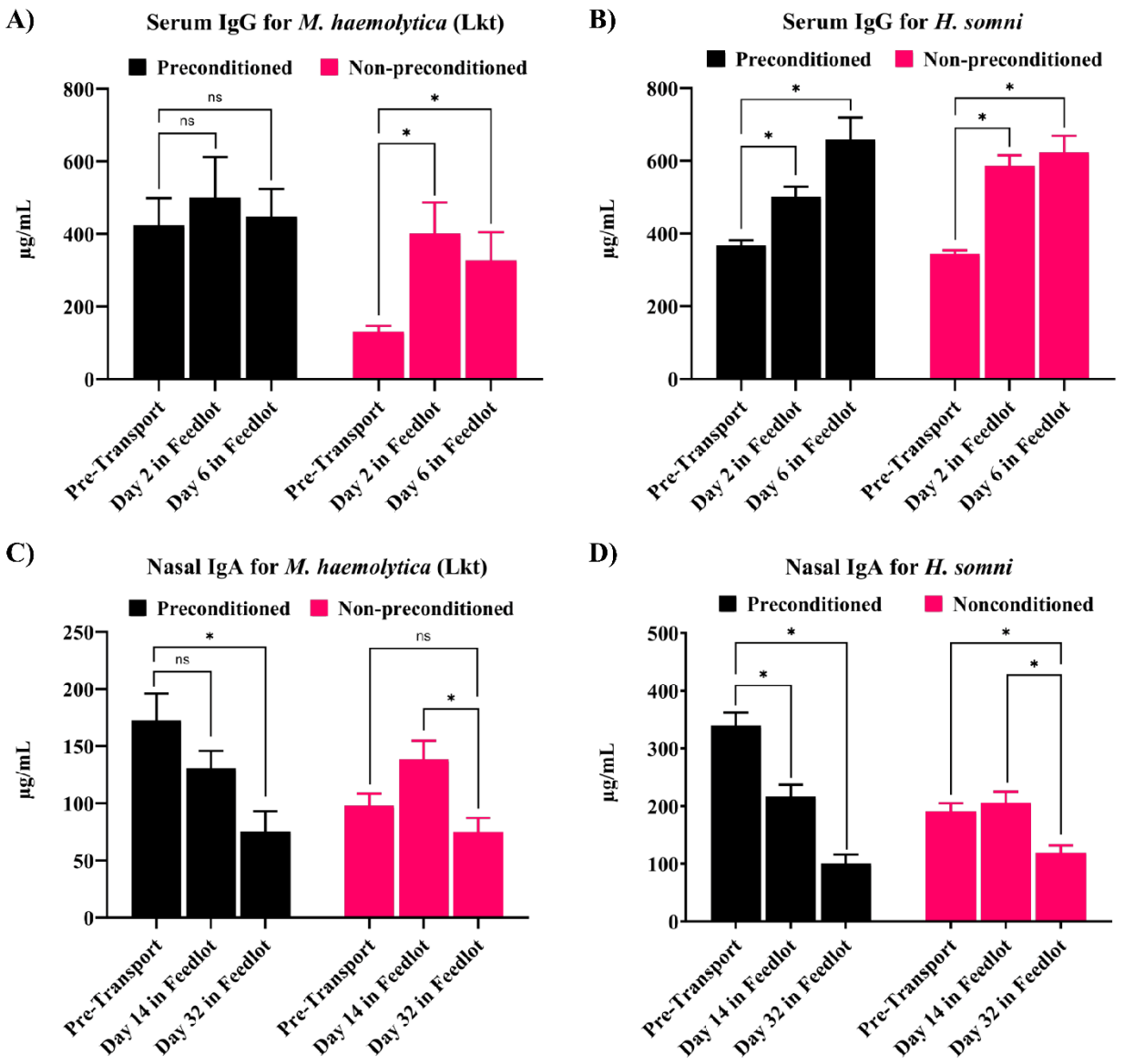


Figure 2.9: Antibody responses to *M. haemolytica* and *H. somni* in preconditioned and non-preconditioned calves across times. Antibody concentrations to *M. haemolytica* were measured using a recombinant leukotoxin (Lkt) and antibody concentrations to *H. somni* were measured using soluble bacterial lysate. Serum IgG against *M. haemolytica* (A) and *H. somni* (B). Secretory IgA from nasal secretions against *M. haemolytica* (C) and *H. somni* (D). Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). ^{ns} not significant, and * $p < 0.05$ when compared to the Pre-transport timepoint within the same treatment.

Chapter 3

Development of a spore-based mucosal vaccine against the bovine respiratory pathogen *Mannheimia haemolytica*[1]

3.1 Introduction

Bovine respiratory disease (BRD), also known as shipping fever, continues to be a challenging health issue resulting in significant economic losses due to morbidity and mortality in North American cattle (Alexander et al., 2013; Duff and Galyean, 2007; Timsit et al., 2017b). Treatment and control of BRD in the beef sector are aimed mainly at bacterial pathogens, through the use of antibiotics and vaccination programs. However, there are public and scientific concerns regarding overuse of antibiotics in livestock production (Alexander et al., 2008), and current vaccines do not afford complete protection against disease (Booker et al., 2008). Novel methods to mitigate BRD-related pathogenic bacteria are therefore greatly needed. *Mannheimia haemolytica* is systematically detected in BRD cases and it is the predominant bacterial agent associated with the disease (Rice et al., 2007; Zecchinon et al., 2005). Because of its recognized importance, development of vaccines against *M. haemolytica* in the form of bacterins, cell culture supernatants, and extracted antigens have been produced (Rice et al., 2007), but have yielded variable results in controlled trials, and limited efficacy in production settings (Larson and Step, 2012). Timing of vaccination (eg. pre- versus post- feedlot arrival), antigen combinations (eg. bacterial and viral antigens mixed), stress factors, and levels of maternal antibodies may affect

[1] Chapter 3 of this thesis has been published as: Uddin MS, Guluarte JO, Abbott DW, Inglis GD, Guan LL, Alexander TW. Development of a spore-based mucosal vaccine against the bovine respiratory pathogen Mannheimia haemolytica. Sci Rep. 2023 Aug 10;13(1):12981. doi: 10.1038/s41598-023-29732-4.

vaccine efficacy (Chamorro and Palomares, 2020a; Richeson and Falkner, 2020a). While this tapered potency may partly be due to vaccines offering narrow protection across strains of bacteria, the systemic immunity targeted by intramuscular injection (Gerdts et al., 2006; Shewen et al., 2009) may also be a factor. Mucosal immunization can be advantageous over parenteral delivery since it induces both mucosal and systemic immunity, providing protection at the site of infection (Holmgren and Czerkinsky, 2005).

The immunization delivery method used plays a key role in the successful transition of chimeric proteins into an effective vaccine (Copland et al., 2018; Cutting et al., 2009). A poor delivery system can lead to loss of biological activity, which often negates antigen efficacy. To date, a wide range of delivery systems, ranging from live microorganisms to nanoparticles, have been proposed as alternative vaccine vehicles (Cutting et al., 2009). Probiotic bacteria have increasingly been studied to deliver antigens and elicit mucosal responses because of their natural ability to interact with mucosa and regulate immune responses. Previously, a non-transgenic application of *Bacillus subtilis* endospores (referred to as spores from here on) that allowed binding of antigens in their native state for presentation to mucosa was evaluated (Huang et al., 2010a). This technology was shown to stimulate mucosal immunity via oral and intranasal administration, providing protection against bacterial and viral infections in mice (Huang et al., 2010a; Song et al., 2012a). There are potential commercial benefits of using this technology for vaccinating cattle against BRD pathogens. Production of *Bacillus subtilis* is well established and has low cost inputs, and *B. subtilis* is used commercially in food/feed products for human beings, poultry, cattle, swine, and fish, as it has generally regarded as safe (GRAS) status (Mazza, 1994; Ricca et al., 2014). Thus, a low-cost and GRAS vaccine component with a history as an adjuvant would facilitate

industry adoption. In addition, oral administration of the vaccine would allow for large-scale administration. These qualities are especially important as livestock management strategies, including vaccination, are cost and ease-of-use dependent. To date, *B. subtilis* spores have not been tested as vaccine delivery systems targeting cattle pathogens.

Immunity against *M. haemolytica* requires generation of antibodies against the virulence factor, leukotoxin (LKT), and to cell surface antigens (Czuprynski, 2009; Shewen and Wilkie, 1988). Multiple studies have demonstrated that outer membrane proteins are immunologically important surface antigens (Confer et al., 2003; Confer et al., 2006; Lee et al., 2008; Pandher et al., 1999). Among them, vaccination of cattle with the 45 kDa, surface-exposed, outer membrane lipoprotein, PlpE, was shown to generate a higher degree of resistance to disease incited by *M. haemolytica* (Confer et al., 2003; Confer et al., 2006; Confer et al., 2009b). In the current study, we used a *M. haemolytica* chimeric protein CTB-PlpE-NLKT-PlpE-NLKT (MhCP), which is composed of truncated Cholera Toxin B subunit (CTB), two copies of the immune-dominant epitope (R2) region of *M. haemolytica* PlpE, and two copies of the neutralizing epitope of LKT (NLKT) (Ayalew et al., 2008). The purified MhCP was adsorbed to *B. subtilis* spores, and the resultant spore-based vaccine termed "Spore-MhCP" was evaluated in mice for its immunogenicity. The objectives of this study were to first, develop a spore-based vaccine targeting *M. haemolytica* and second, evaluate the immunogenicity of this vaccine after delivery via mucosal (intranasal and intragastric) routes.

3.2 Materials and Methods

3.2.1 Preparation of Spores

Lyophilized *B. subtilis* (RK28 strain) spores were obtained from SporeGen Limited (Egham, Surrey, UK). Prior to use, lyophilized spores were washed sequentially with 1M KCl, 0.5 M NaCl, and distilled water (three times) by mixing 0.1 g of spore suspension with 10 ml of each solvent, followed by centrifugation (5,000 x *g*; 15 min) and decanting of the supernatant. The washed spores were heat treated at 65°C for 30 min to kill vegetative cells, and the suspension was diluted in a 10-fold dilution series. A 100 µl aliquot was spread onto Luria Bertani (LB) agar, cultures were incubated overnight at 39°C, and colonies were counted at a dilution that yielded 30-300 colony forming units per plate. After enumeration, washed spores were diluted in water to a final density of 1.0×10^{10} *B. subtilis* spores/ml.

3.2.2 Construction, expression and purification of chimeric protein

The construction of the *M. haemolytica* chimeric protein MhCP was performed as per the modular structure depicted at Figure 3.1A. MhCP was comprised of truncated cholera toxin B subunit (CTB), and two copies each of the immunodominant surface epitope (R2) of *M. haemolytica* PlpE and the neutralizing epitope of leukotoxin (NLKT). The chimeric protein MhCP has a calculated molecular weight of 56.4 kDa and a pI of 9.1. A similar chimeric protein (R2-NLKT-R2-NLKT) was used previously by Ayalew and colleagues with the exception of CTB, which was not appended to their protein structure (Ayalew et al., 2008). Ayalew et al., (2009) reported another chimeric protein (CTB-R2-NLKT) with appended CTB and it differed from the number of constructs used in this current study (Ayalew et al., 2009a). The PlpE and NLKT constructs of MhCP shared identical amino acid sequences to R2 and NLKT used previously (Ayalew et al., 2008; Ayalew et al., 2009a). Sequences corresponding to the chimeric protein, MhCP were commercially synthesized (BioBasic Inc., Markham, Canada), codon optimized for

expression in *E. coli*, and cloned into the pET28a vector. The pET28a constructs were transformed into *E. coli* BL21 Star (DE3), and recombinant chimeric protein were expressed and purified as previously described (Jones et al., 2017). Briefly, cells were grown at 37°C to an optical density (600 nm) of 0.8-1.0 in LB broth containing kanamycin (50 µg/ml). Cultures were cooled to 16°C, and gene expression was induced by addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) with overnight incubation at 200 rpm. Overnight cultures were centrifuged at 6,500 x *g* for 10 min. Cells were resuspended in binding buffer (0.5 M NaCl, 20mM Tris, pH 8.0), and lysed by sonication for 2 min of 1-s intervals of medium intensity sonic pulses (Fisherbrand™ Model 505 Sonic Dismembrator). The cell lysate was clarified by centrifugation at 17,500 x *g* for 45 min, and the pellet was washed sequentially with dH_20, binding buffer with 0.5 % (w/v) Triton X-100, and finally with binding buffer only. The washed pellet was resuspended in binding buffer with 6 M urea, followed byvigorous stirring at ambient temperature. The solubilized pellet was clarified by centrifugation at 20,000 x *g* for 45 min, and the supernatant containing the recombinant protein was passed through a 0.45-µm filter. The filtrate was then loaded onto a nickel nitrilotriacetic acid column, and purified by immobilized metal affinity chromatography. The recombinant protein was eluted via a stepwise gradient of imidazole (5, 10, 30, 100 and 500mM) in binding buffer with 6 M urea. Fractions containing significant amounts of protein were pooled, the denaturant (6 M urea) was removed by step wise dialysis, and buffer exchanged with storage buffer (20 mM Tris-HCl, pH 8.0). Following buffer exchange, samples were concentrated using ultrafiltration cell (Amicon) with a molecular mass cut-off of 10 kDa. The identity, purity, and integrity of purified proteins were determined by SDS-PAGE and confirmed by western blotting using anti-His antibodies (ThermoFisher, Canada).

3.2.3 Spore binding with the chimeric protein

To determine the optimal condition for antigen (MhCP) binding to spores, varying amounts of MhCP (2 µg, 5 µg, and 10 µg) and spores (5 x 10^8 to 4 x 10^9) at different buffer pH (4 and 7) and varied temperature (room temperature and 4°C) were tested (Huang et al., 2010a). Subsequent to each of the conditions employed, the Spore-MhCP mixture was centrifuged, washed, and then analysed for adsorption of MhCP to spores. For all criteria tested, optimal conditions were considered those that resulted in the greatest amount of MhCP binding to the spore coat, as determined by western blotting. The optimized method for adsorbing protein MhCP to endospores, which was used for vaccine preparation in the mice study. An aliquot of *B. subtilis* spores containing 2 x 10^9 spores were centrifuged (5,000 x *g*, 5 min, 4°C), the supernatant was removed, and 10 µg of purified recombinant protein (MhCP) diluted in PBS at pH 4 was added to the spore suspension. The mixture was vortexed, and then mixed in a HulaMixer (Invitrogen, Waltham, MA) for 1 h at 4°C. The binding mixture was centrifuged (5,000 x g, 2 min, 4°C) and the pellet was washed two times with PBS buffer (pH 4). The washed pellet was resuspended in 100 µl of spore coat extraction buffer, incubated at 65°C for 30 min to solubilise spore coat proteins, and one tenth of the above mixture was loaded onto a 12% SDS-PAGE gel (Huang et al., 2010a). Finally, the protein was confirmed by western blotting using anti-His antibodies (ThermoFisher, Canada).

3.2.4 Animals and experimental design

The immunogenicity of recombinant protein MhCP was evaluated in a murine model. Balb/c 6-8-week-old, pathogen-free mice (Charles River Laboratories, Quebec, Canada) were housed in the Lethbridge Research and Development Centre (LeRDC) Vivarium adhering to procedures specified in the Canadian Council on Animal Care guidelines. The project was

approved by the LeRDC Animal Care Committee (Animal Use Protocol # 1712) before commencement of the experiment and the current study was carried out in compliance with the ARRIVE guidelines. Mice were acclimatized to their cages for a week before commencement of the experiment. Mice were maintained on a Laboratory Rodent Diet 5001 (LabDiet, St. Louis, MO), and were allowed free access to the diet and water. They were monitored daily during the entire period of this experiment and a feeding and health log was maintained. A total of 40 mice were divided into the following four experimental groups (N=10 mice per treatment): intramuscular (IM; free MhCP, positive control for reference of antigen immunogenicity); intragastric (IG; spore-bound MhCP); intranasal (IN; spore-bound MhCP); and naïve/control mice (Control; no MhCP used, negative control). In an initial study, spores were analyzed for potential to elicit immune responses in mice (N=6) on day 42, after primary (day 0) and booster (day 21) IN vaccinations of only spores. No detectable IgG (serum) or IgA (BAL) against PlpE or NLKT were observed (data not shown). Therefore, an IN spore only group was not included in the study design.

3.2.5 Immunizations and sample collection

To evaluate the immune response, mice were immunized through respective routes on day 0 followed by a booster immunization on day 21. A total of 5 µg of recombinant protein MhCP was administered to each mouse on each immunization day. For intranasal vaccination, spore-bound antigen (Spore-MhCP) was administered by pipette while mice were under light anaesthesia. Mice were restrained, holding them by the scruff, with thumb and index fingers and pressing the tail with the ring finger against the palm. A half-dose volume of the Spore-MhCP was delivered to each nostril using a micropipette fitted with a tip. For intramuscular immunization,

free antigen MhCP mixed with Freund's incomplete adjuvant (Sigma-Aldrich, Canada) was inoculated into the thigh muscles of anaesthetized mice. The intragastric immunization was performed through oral gavage using a plastic feeding tube (Instech Laboratories, PA, USA) for gastric delivery of Spore-MhCP. Control mice were not administered any antigen. Samples (blood, BAL, fecal pellets, and saliva) were collected on days 21 and 42. At each sampling point, 5 mice from each group were sacrificed under deep anaesthesia. Each animal was transferred into an individual sterile cage and kept there for 3-5 minutes to collect the freshly voided feces. Saliva was collected afterwards using a sterile oral swab (Isohelix, UK). For blood sampling, anaesthesia was induced with 4% isoflurane in an induction chamber and maintained with 2% isoflurane using a somnosuite anaesthesia mask (Kent Scientific Corporation, Torrington, CT). Upon opening of the abdominal cavity, blood was collected from the caudal vena cava under anesthesia and animals were then humanly euthanized by exsanguinating. Immediately after death, BAL was collected by inserting a catheter in the trachea of mice, through which PBS solution was instilled into the bronchioles. The infused liquid was then gently retracted to esure maximum BAL fluid recovery. To determine the effectiveness of the MhCP and the immunogenicity from each vaccination route, each sample was analyzed quantitatively for specific IgG and IgA.

3.2.6 Quantification of antigen-specific serum IgG

Antigen-specific antibody (IgG) responses in serum were quantified by enzyme-linked immunosorbent assay (ELISA). Purified recombinant antigens, PlpE and NLKT, were used as ligands independently to coat ELISA plates (Nunc-Immuno MaxiSorp) at concentrations of 50 ng/well, and plates were incubated at 4°C overnight. Sera collected from mice were used as primary antibodies. Naïve mice sera were used as control. After being blocked with ELISA

Blocking Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 1 h at 37°C, serum samples were diluted (1:1600 dilution for intramuscular treatment mice, and 1:50 for other treatment mice) in sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20), and 100 µL aliquots of each dilution were dispensed into wells of microtiter plates in duplicate. Plates were incubated for 1 h at room temperature. Horseradish peroxidase-conjugated anti-mouse IgG (Bethyl Laboratories Inc.) was used as secondary antibody. Following the addition of the secondary antibody, plates were incubated for 1 h at room temperature, and then developed with the substrate, TMB (3,3′,5,5′-tetramentylbenzidine; ThermoFisher, Canada). ELISA plates were washed five times with wash buffer. Reactions were stopped by ELISA stop solution (0.2 M H_2SO_4), and fluorescence was measured at 450 nm using a Microtiter Plate Reader (Synergy HTX Microplate Reader, BioTek). Concentrations were determined from a standard curve generated using a Mouse IgG ELISA Quantitation Set protocol (Cat. No. E90-131; Bethyl Laboratories Inc.). Known concentrations of Mouse Reference Serum (RS10-101-6; Bethyl Laboratories Inc.) was used.

3.2.7 Quantification of secretory IgA (sIgA)

BAL, fecal, and oral swab samples were processed for IgA quantification as described previously (Hoang et al., 2008). For BAL, samples were washed with PBS containing 0.1% (wt/vol) BSA and 1 mM of phenyl methyl sulphonyl fluoride (PMSF), followed by centrifugation at 17,000 x g for 10 min. The resultant supernatant was used for IgA analysis. For fecal samples, freshly voided feces were collected and frozen at -80°C. Approximately 0.1 g of feces was thawed in 400 µL PBS containing 1% BSA, 1 mM PMSF, 0.05% Tween 20, and 0.1% Triton X-100 with vortexing to disrupt particulate material. Samples were then centrifuged and supernatants were used for analysis as above. For saliva, oral swabs were incubated in PBS containing 0.1% BSA, 1

mM PMSF, and 0.05% Tween 20 for 1 h at room temperature. Samples were then centrifuged and supernatants were used for analysis. After pre-processing, the concentrations of mucosal secretory IgA (sIgA) were evaluated by ELISA as per the Mouse IgA ELISA Quantitation Set Protocol (Cat. No. E90-103; Bethyl Laboratories Inc.). Briefly, Nunc-Immuno MaxiSorp plates were coated with 50 ng/well of purified recombinant PlpE and NLKT, and incubated at 4°C overnight. After being blocked with ELISA Blocking Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 1 h at room temperature, samples were diluted in sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20), and aliquoted to individual microtitre plate wells in duplicate. Plates were incubated for 1 h at room temperature. After a wash step and the addition of HRP Conjugated anti-Mouse IgA Detection Antibody (A90-103P; Bethyl Laboratories Inc.), plates were further incubated for 1 h at room temperature. Color development was performed with addition of the TMB substrate. Reactions were stopped by adding ELISA stop solution, and fluorescence was measured at 450 nm using the Microtiter Plate Reader.

3.2.8 Complement-mediated serum bactericidal activity assay

Antibodies in mice sera collected on day 42 (N=3 randomly selected sera from each treatment, measured in duplicate) were evaluated for their complement-dependent bactericidal activity against *M. haemolytica* using a serum bactericidal activity (SBA) assay as previously described (Ayalew et al., 2008). Serum from a newborn colostrum-deprived calf was used as the external source of complement and was screened for minimal or no intrinsic bactericidal activity towards *M. haemolytica*. Mouse sera tested in complement-mediated SBA assay were heat-inactivated at 56 °C for 30 min, prior to use, to remove endogenous complement activity. For *M. haemolytica* cell preparation, a serotype 1 strain was grown in brain heart infusion (BHI) broth,

pelleted by centrifugation, washed with PBS, and re-suspended in PBS. To remove the polysaccharide capsule and maximize the exposure of surface epitopes, cells were decapsulated by incubating at 41°C with 100 rpm shaking for 1 h. The decapsulated cells were re-suspended to an optical density (600 nm) of 0.50 in PBS and 1:1000 dilution of this solution was used in the SBA assay. Heat-inactivated mice sera (25 µl), exogenous complement source (25 µl) and decapsulated *M. haemolytica* cells (25 µl) were mixed and spread onto plates at the onset of the experiment (T0) and after 30 min of incubation at 37 °C (T30). Colonies were enumerated after 16 h of incubation at 37°C with 5% CO2. Finally, the percent killing was calculated as: [(T0 growth−T30 growth)/T0 growth]×100% for each replicate. Only complement source without antibodies (mice serum) served as negative control in this SBA assay.

3.2.9 Statistical Analysis

Kruskal-Wallis test was performed to analyze the ELISA results. Significance was tested against the control by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test where a p-value less than 0.05 was considered statistically significant. One-way ANOVA were performed to analyze the SBA assay results. Significance was tested against the control by one-way ANOVA with Tukey's posttest where a p-value less than 0.05 was considered statistically significant. All analyses were performed using Microsoft Excel 2010 and GraphPad Prism version 9.

3.3 Results

3.3.1 Adsorption of MhCP to spores

The modular structure of MhCP is shown in Figure 3.1A. The size, purity, and integrity of recombinant protein MhCP was demonstrated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE; Figure 3.1B) and western blotting (Figure 3.1C). Results from the adsorption experiment revealed that the best binding condition was when 10 μg of antigen, MhCP, was mixed with 2 x 10^9 spores, as indicated by the band intensity from western blotting (Figure 3.2). Among the tested parameters, MhCP was efficiently adsorbed onto the spore coat at pH 4, whereas at pH 7, low levels of binding were observed (Figure 3.3). The temperature also played a key role in binding, and the degree of binding was most stable at 4°C (Figure 3.3). Similar band intensity was observed by western blotting across the tested time points of 60, 90 and 120 min respectively, highlighting stable binding of the Spore-MhCP complex (Figure 3.3).

3.3.2 Spore-MhCP induced antigen-specific antibody production

Immunization of mice with both free MhCP and Spore-MhCP stimulated anti-PlpE and anti-NLKT antibodies indicating that the chimeric antigen MhCP that was used was immunogenic, and retained immunogenicity after spore adsorption (Figure 3.4 and 3.5). On both days 21 (Appendix C) and 42 (Figure 3.4), intramuscular treatment mice exhibited a greater serum IgG immune response as compared to other treatment mice for both the PlpE ($P<0.05$) and NLKT ($P<0.05$). The IgG immune response increased several fold in intramuscular treatment mice by day 42. However, the intranasal group showed numerically higher serum anti-PlpE antibodies as compared to intragastric and control treatment mice at both time points ($P>0.05$). The control and intragastric treatment mice did not show any detectable serum antibody production (detection limit ≥ 7.8 ng/ml) on days 21 or 42.

On day 21, none of the mucosal samples showed any detectable IgA immune responses (detection limit ≥ 7.8 ng/ml; data not shown). On day 42, intranasal vaccination with Spore-MhCP

resulted in specific secretory anti-PlpE and anti-NLKT IgA in bronchoalveolar lavage (BAL) samples (Figure 3.5A and 3.5B). None of the other treatment mice exhibited detectable levels of secretory antibody in BAL fluid. The intranasal treatment mice also had greater amounts of anti-PlpE IgA in both fecal (Figure 3.5C) and oral swab (Figure 3.5E) samples as compared to intragastric and control mice. Intranasal treatment mice also exhibited greater amounts of anti-NLKT IgA in feces as compared to intragastric and control mice ($P<0.05$; Figure 3.5D). The results showed that although PlpE and NLKT were delivered together as a chimera, differential immune responses were observed for each antigen. Anti-PlpE IgA and anti-NLKT IgA were also observed in feces from mice that were vaccinated intramuscularly with free MhCP (Figure 3.5C and 3.5D).

3.3.3 Bactericidal activity of mice sera immunized with MhCP

The ability of vaccine-induced antibodies to mediate complement-dependent killing of *M. haemolytica* was tested using a serum bactericidal activity (SBA) assay. Sera from mice was evaluated for its bactericidal activity in the presence of complement. The newborn calf serum used as a complement source had minimal bactericidal activity, which was also the case for the control treatment (Figure 3.6). Sera from the intramuscular treatment mice demonstrated high bactericidal activity (97.9%) compared to other treatment mice ($P<0.05$; Figure 3.6). The intranasal and intragastric group also showed higher bactericidal activity (51.3% and 11.8%, respectively) compared to controlmice ($P<0.05$; Figure 3.6).

3.4 Discussion

To determine the optimal adsorption parameters, we compared the binding of spores and chimeric protein under varying amounts of each, as well as different buffer pH and reaction

temperatures. Studies have shown that proteins can readily bind to negatively charged bacterial spores when the pH of the aqueous phase falls below the isoelectric point (pI) of the respective protein (Huang et al., 2010a; Song et al., 2012a). Since the pI of the protein, MhCP, was 9.1, we conducted binding experiments at pH 4 and pH 7. MhCP was most efficiently adsorbed onto the spore coat at pH 4, whereas at pH 7, lower levels of binding were observed. This finding is similar to other studies that have reported that the degree of protein adsorption to spores depended on the solution pH as well as the structure of the protein (De Souza et al., 2014; Huang et al., 2010a). It is thought that electrostatic binding and hydrophobic effects are key ways in which proteins interact with spores, resulting in their adsorption (Wiencek et al., 1990). In the current study, we showed that temperature was also an important factor in adsorption, with the highest degree of adsorption occurring at 4°C. It is important to maintain the native conformation of the immunogen to generate protective immunity (Hunter, 2002), and studies have reported that proteins can maintain native conformation when bound to spores (Huang et al., 2010a). The small size of spores (approximately 1 μm in diameter) has its own advantages since it aligns them with particulate adjuvants where an antigen adsorbed on a surface of spores mimics a potential pathogen thereby facilitating antigen presentation and induction of an immune response (Raychaudhuri and Rock, 1998; Singh and O'Hagan, 1999).

In this current study, we developed a spore based vaccine, Spore-MhCP, targeting chimeric protein MhCP of *M. haemolytica*. Chimeric proteins are versatile in nature and can deliver multiple antigens to the immune system, making them ideal candidates for vaccine development (Boucher et al., 1994a; Hertle et al., 2001b). The chimeric protein, MhCP encoded NLKT, which is the predominant virulence factor of *M. haemolytica,* and an epitope (R2) of PlpE, which is an outer

membrane lipoprotein that is conserved in key *M. haemolytica* serotypes (Confer and Ayalew, 2018). Although MhCP was developed as a chimeric protein, both antigens (NLKT and PlpE) resulted in individual seroconversion. A previous study by Ayalew et al. (2008) showed that the components of MhCP are antigenic (Ayalew et al., 2008), which is also evident from the immune responses generated against NLKT and PlpE following intramuscular immunization of mice with free MhCP in the current study. Ayalew et al. (2008) subcutaneously immunized mice with 25-75 μg of a similar chimeric protein (without CTB appended to it), and reported that increasing doses were associated with increasing anti-LKT antibodies (Ayalew et al., 2008). In the current study, anti-NLKT and anti-PlpE antibodies were induced with 5 μg of MhCP after intramuscular administration, perhaps indicating that the CTB in our MhCP protein structure enhanced immunogenicity.

To investigate the effectiveness of the vaccine *in vivo*, three different routes of immunization were compared using a mice model. It was demonstrated that the antigen, MhCP, bound to spores in a manner that retained immunogenicity. Intranasal immunization of mice with Spore-MhCP stimulated mucosal immunity in the form of secretory IgA against *M. haemolytica* NLKT and PlpE. Therefore, *B. subtilis* spores could serve as an effective adjuvant for a mucosal vaccine. While intranasal Spore-MhCP did not elicit as strong an IgG response as did intramuscularly administered free MhCP, it resulted in significantly higher specific secretory IgA at all mucosal sites evaluated. Thus, intranasal Spore-MhCP immunization resulted in both mucosal and systemic immune responses. This suggests that as a vaccine, improved protection may be conferred against *M. haemolytica* colonization in the upper and lower respiratory tract.

Serum bactericidal activity evaluates the complement-mediated functional activity of vaccine-induced antibodies, and their ability to kill the target bacteria of interest (McIntosh et al., 2015). Vaccine-induced antibodies from the intramuscular treatment mice exhibited almost complete killing of *M. haemolytica* in the complement-dependent SBA assay. This result is consistent with bactericidal activity observed after IM vaccination of mice with a similar construct (Ayalew et al., 2008). While sera from the intranasal treatment mice had a lower value of bactericidal activity compared to intramuscular mice, this was likely due to reduced IgG levels resulting from intranasal immunization (Rouphael et al., 2011). Surprisingly, although intragastric mice failed to showcase any detectable range of serum IgG, sera from this group had bactericidal activity. This highlights an important fact that the spore-bound MhCP antigen had the potential to stimulate functional antibodies against *M. haemolytica* whether it was administered in free form via intramuscular route or with spore-bound mucosal routes. Unfortunately, it was not possible to measure bactericidal activities of BAL samples due to the low volumes collected, but measuring this activity in future studies in cattle would provide important insight into whether the spore-bound vaccine could reduce colonization by *M. haemolytica* at mucosal surfaces.

Oral vaccines are appealing for the livestock industry because they can be easily administered to livestock animals without extensive expertise. Although intranasal Spore-MhCP resulted in both mucosal and systemic immunity, intragastric administration only generated a limited immune response in fecal samples. This was likely due in part to antigen dilution in the stomach, and degradation as result of exposure to low gastric pH together with gastric and intestinal proteases (Hoelzer et al., 2018a). Studies have reported induction of systemic and secretory antibodies after intragastric immunization of spore-based vaccines in mice against

Clostridium perfringens (Huang et al., 2010a) and *Bacillus anthracis* (Oh et al., 2020a). However, recombinant *B. subtilis* was used for those studies, and it was engineered to express antigens on the spore surface that were fused to the outer coat protein, which may have provided superior protection. Successful oral immunization with spore-bound antigens is likely related to their stability on the spore surface and enhanced protection from proteolysis. Because spore coat composition is strain-specific, and variation in spore coat material may affect antigen adsorption and protection (Ricca and Cutting, 2003), antigens with pI approaching 2-3 would be favoured to remain adhered and protected while passing through the digestive tract of monogastric. Alternatively, a fibre-rich diet fed to cattle would promote retained adsorption of proteins with higher pI, by promoting a ruminal pH greater than 5.8 (Li et al., 2020). Although oral administration was unsuccessful to generate detectable immune responses in mice, evaluation of spore-based vaccines in ruminants is warranted given anatomical differences and the possibility of rumination stimulating pharyngeal lymphoid tissue as result of antigens in feed (Shewen et al., 2009).

Mucosal vaccination of cattle may be superior to parenteral vaccination for prevention of respiratory disease, as intranasal replication of respiratory pathogens is the first phase in development of BRD (Frank et al., 2003; Lee et al., 2001; Shewen et al., 2009). Ayalew et al. (2009) intranasally vaccinated weaned beef calves with a R2-NLKT-R2-NLKT chimeric protein in the presence and absence of native cholera toxin (Ayalew et al., 2009a). They showed that vaccination with the chimeric protein enhanced resistance against intrabronchial challenge with *M. haemolytica*, as well as stimulating antibody responses to this bacterium (Ayalew et al., 2009a). However, the antibody responses only increased significantly following bacterial challenge. Batra

et al. (2017) recently developed a recombinant BHV-1-vectored vaccine expressing PlpE-LKT chimeric proteins, and they reported production of anti-LKT antibodies after intranasal vaccination of bighorn sheep (Batra et al., 2017). However, the development of antibodies against surface antigen was inconsistent, and the vaccine failed to protect sheep against *M. haemolytica* challenge (Batra et al., 2017). Because intranasally delivered Spore-MhCP produced both mucosal and systemic immune responses in mice, we hypothesise that it may confer effective protection against *M. haemolytica* infection in cattle. Findings from the current study in mice indicate that further studies to determine the efficacy of the *B. subtilis* spore-based mucosal vaccine in cattle to manage BRD are warranted.

3.5 Figures

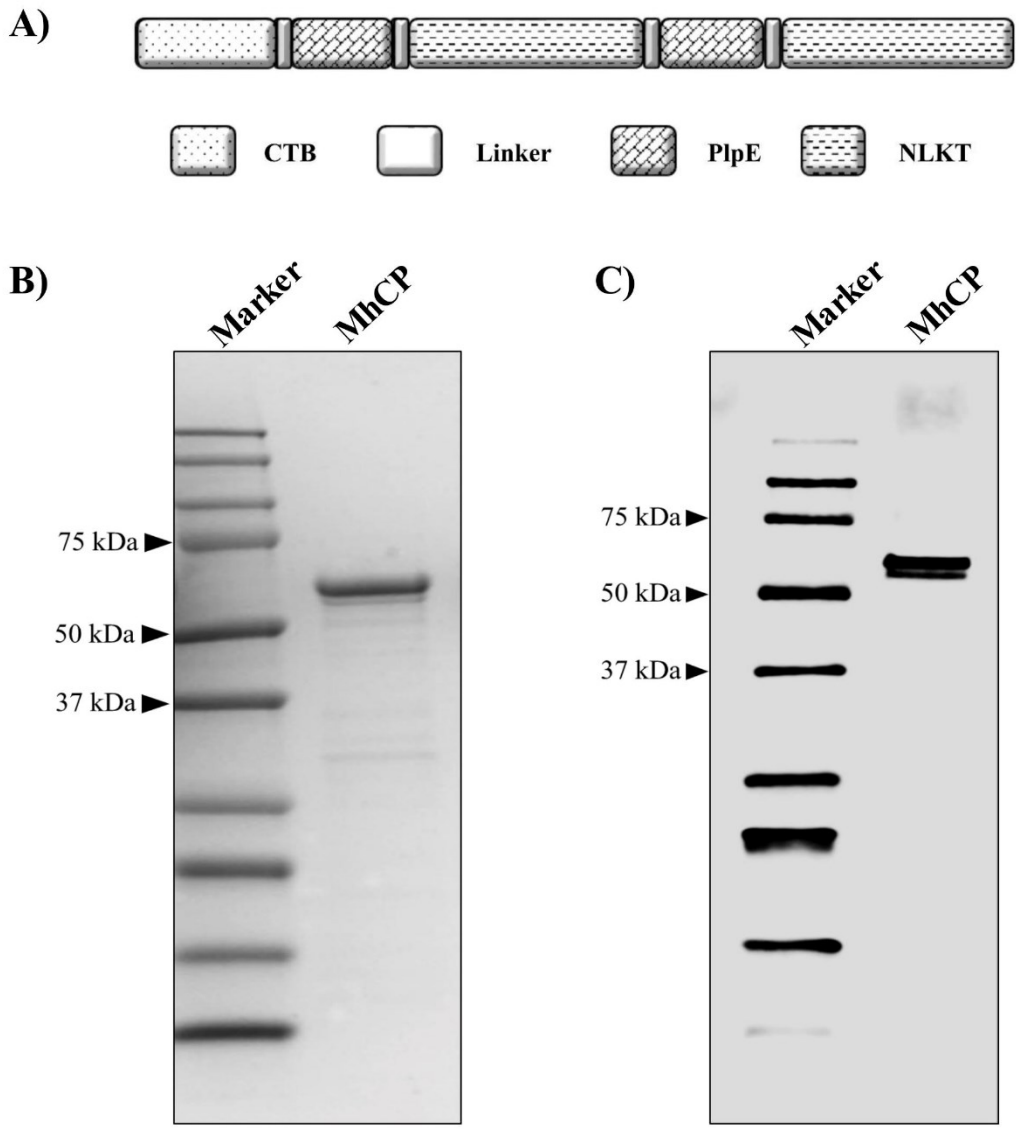


Figure 3.1: Construction and purification of chimeric protein MhCP. A) Modular structure of chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT). MhCP contains truncated cholera toxin subunit B (CTB), immunodominant surface epitope (R2) of *M. haemolytica* outer membrane lipoprotein PlpE, and the neutralizing epitope of leukotoxin (NLKT). MhCP was synthesized and subcloned into the bacterial expression vector, pET28a. The purified plasmids were transformed into *E. coli* BL21 (DE3) protein expression cells. The recombinant chimeric protein MhCP was purified using a NTA-nickel column. B) Purified recombinant chimeric protein MhCP demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Brilliant Blue. C) Western blots were used to confirm the identity of the MhCP protein by using anti-His antibodies.

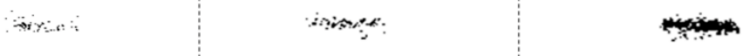
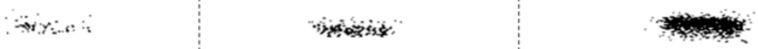
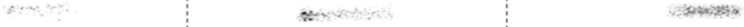
Suspensions of spores	Protein		
	2 µg	5 µg	10 µg
4 x 10^9			
2 x 10^9			
1 x 10^9			
5 x 10^8			

Figure 3.2: Optimization of spore-to-antigen ratio. Chimeric protein, MhCP, of varying amounts (2 µg, 5 µg, 10 µg) were mixed with a range of *Bacillus subtilis* spores (5 x 10^8 to 4 x 10^9) in phosphate buffered saline (PBS), incubated for 1 h, washed, and resuspended in PBS. The supernatant was removed, the pellet containing spore coat antigens was washed and extracted by incubating in extraction buffer for 30 min at 65°C. Quantities of MhCP were determined by Western blotting. Full-length blots, used to create this figure, are available in Appendix D.

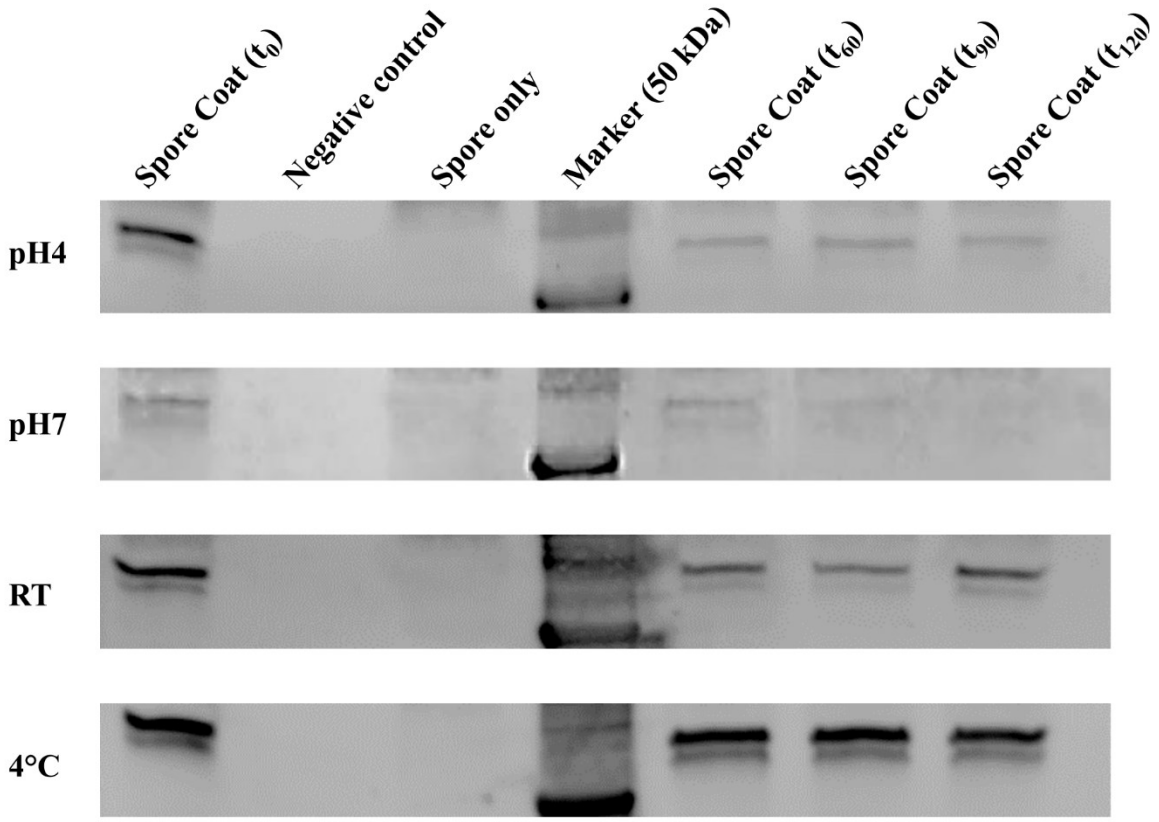


Figure 3.3: Adsorption of chimeric protein MhCP to *Bacillus subtilis* spores. MhCP adsorbtion to spores was evaluated at different pHs and temperatures. 10 µg of MhCP was mixed with 2 x 10^9 spores in phosphate buffered saline (PBS) at pH 4 and PBS at pH 7, incubated 1 h at room temperature (RT). The binding mixture was centrifuged, the supernatant discarded, and the pellet was washed two times with PBS. The washed pellet was next resuspended in 200 µl of PBS at their respective pH. At indicated time points (60, 90 and 120 min), the spore suspensions were centrifuged. Pellets were resuspended in 100 µl of spore coat extraction buffer, incubated at 65 °C for 30 min to remove spore coat proteins from the spores. Using a one in ten dilution of the extraction, western blotting of size-fractionated proteins was used for detection. Equivalent amounts of spores and protein were used for third and fourth panel (pH 4). After the initial 1 h incubation at RT, the binding mixture was incubated at 4 °C (third panel), whereas all reactions and incubations were performed at 4 °C (fourth Panel). The negative control lane displays the corresponding blot that underwent the entire process without addition of any spores or protein. The spore only lane displays the corresponding blot generated from spores that underwent the entire process without the addition of protein. Full-length blots, used to create this figure, are available in Appendix E.

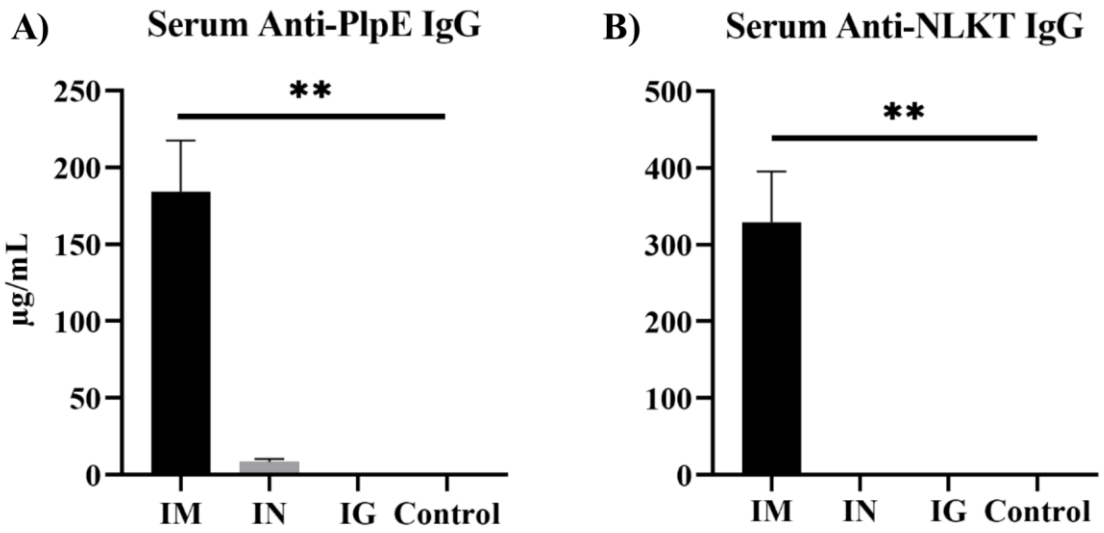


Figure 3.4: Antigen-specific serum IgG antibody responses measured by enzyme linked immunosorbent assay (ELISA) from blood collected on day 42. ELISA plates coated with either recombinant PlpE or NLKT were used to measure anti-PlpE and anti-NLKT antibodies in mice sera. Immune responses from four experimental groups: Intramuscular (IM), Intranasal (IN), Intragastric (IG) and Naïve/control mice (Control) were compared. (A) Levels of serum IgG specific to PlpE (B) Levels of serum IgG specific to NLKT. Results are expressed as mean ± SEM. Significance was tested against the control by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test, **$p < 0.01$.

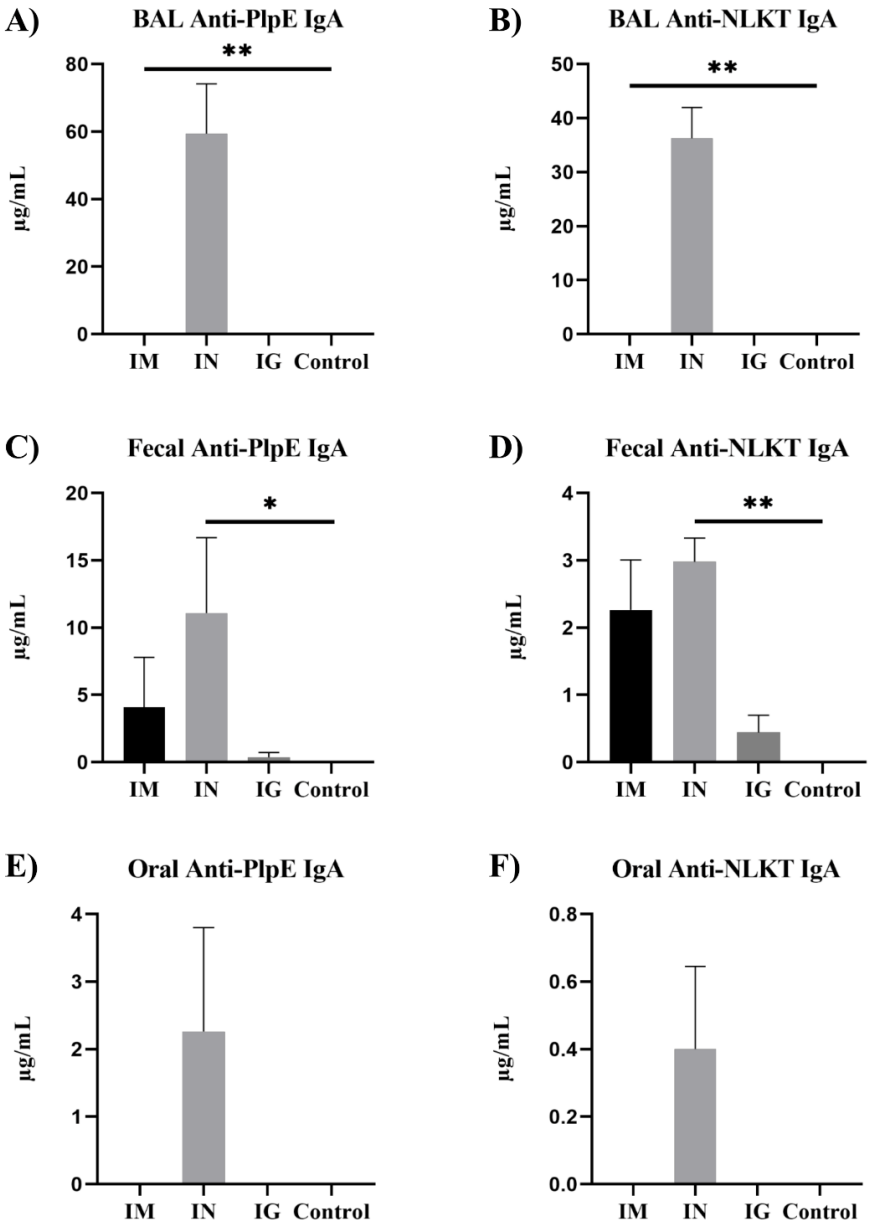


Figure 3.5: Enhanced mucosal immunity induced by Spore-MhCP. Mucosal immune responses measured by ELISA where plates were coated with either recombinant PlpE or NLKT to measure anti-PlpE IgA and anti-NLKT IgA antibodies in bronchoalveolar lavage (BAL), fecal and oral swab samples obtained from mice on day 42. Immune responses from the four experimental treatments, intramuscular (IM), intranasal (IN), intragastric (IG) and naïve/control mice (Control) were compared. Results are expressed as mean ± SEM. Significance was tested against the control by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test, $^{**}p < 0.01$ and $^{*}p < 0.05$.

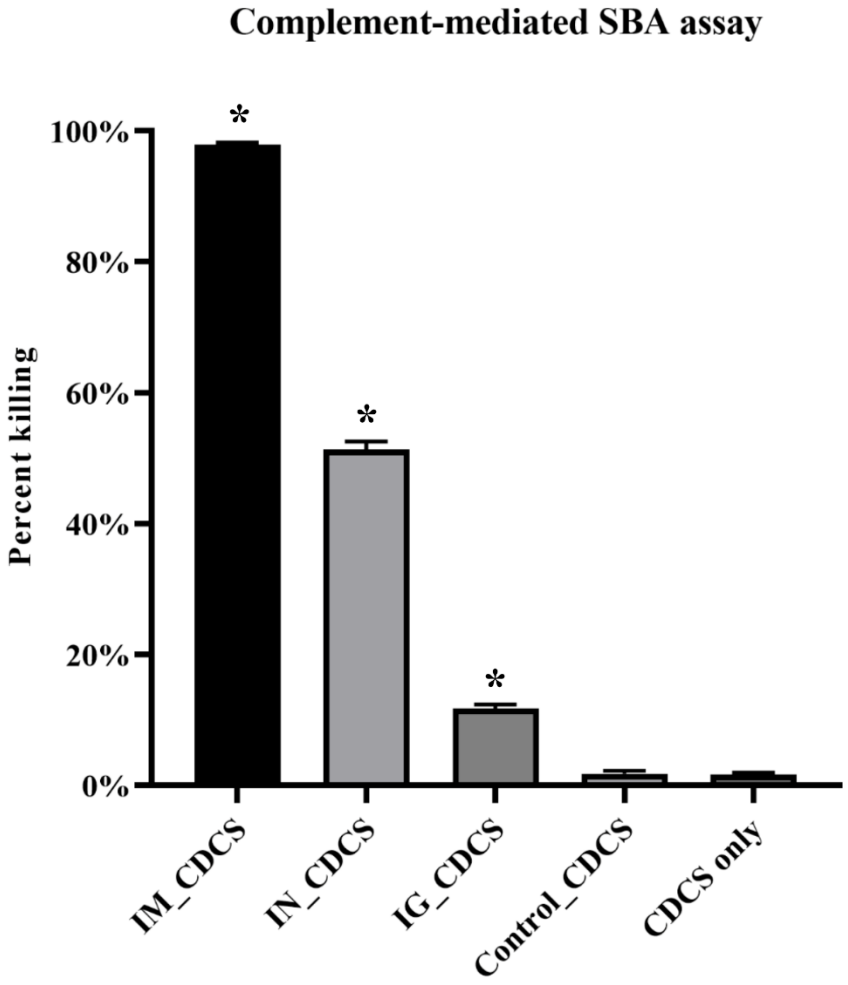


Figure 3.6: Complement-mediated serum bactericidal activity. Antibodies in mice sera collected on day 42 were evaluated for their killing activity against *M. haemolytica.* Bactericidal activity of mice sera collected from the four experimental treatments, intramuscular (IM), intranasal (IN), intragastric (IG) and naïve/control mice (Control) were compared. All mice sera analyzed were undiluted. Colostrum-deprived calf serum (CDCS) collected from a newborn calf was used as external source of complement. Only complement source (CDCS only) without serum/antibodies served as negative control. Results are expressed as mean ± SEM. Significance was tested against the control by one-way ANOVA with Tukey's multiple comparison test, *$p < 0.05$.

Chapter 4

Mucosal immunization with spore-based vaccines against *Mannheimia haemolytica* enhances antigen-specific immunity[2]

4.1 Introduction

Mannheimia haemolytica is a key bacterial pathogen associated with bovine respiratory disease (BRD) (Alexander et al., 2013; Duff and Galyean, 2007; Rice et al., 2007). In North America, the prevention and control of BRD in the beef cattle industry are managed primarily through vaccination and the mass use of antimicrobials at feedlot placement (metaphylaxis). However, current vaccines do not provide complete protection against infection (Larson and Step, 2012; Murray et al., 2016), and the overuse of antimicrobials in livestock production has led to the emergence of antimicrobial resistance (Griffin et al., 2010; Klima et al., 2014a; Rice et al., 2007). Although commercial vaccines for *M. haemolytica* are available, meta-analysis studies have revealed variable outcomes in field trials and demonstrated low efficacy in feedlots (Larson and Step, 2012; O'Connor et al., 2019; Perino and Hunsaker, 1997). Thus, there is a need for the development of novel vaccines against *M. haemolytica* and other BRD pathogens, with increased efficacy.

In an attempt to enhance the effectiveness of vaccines, minimize the time needed to induce immunity, and lessen the stress involved with handling cattle, the development of mucosal BRD vaccines has been investigated (Rice et al., 2007; Shewen et al., 2009). Mucosal immunization can

[2] Chapter 4 of this thesis has been published as: Uddin MS, Kaldis A, Menassa R, Ortiz Guluarte J, Barreda DR, Guan LL, Alexander TW. Mucosal Immunization with Spore-Based Vaccines against Mannheimia haemolytica Enhances Antigen-Specific Immunity. Vaccines (Basel). 2024 Apr 1;12(4):375. doi: 10.3390/vaccines12040375.

induce both mucosal and systemic immune responses, providing protection at the site of infection in addition to systemic immunity, and thus it can be advantageous over the parenteral delivery method (Holmgren and Czerkinsky, 2005). A delivery system capable of preventing the loss of antigenicity is important for converting chimeric proteins into effective vaccines (Cutting et al., 2009). Over the last decade, numerous delivery systems, including microorganisms and nonliving nanoparticles, have been evaluated as adjuvants (Duc le et al., 2003; Rhee, 2020). This includes probiotic bacteria which have a natural capacity to interact with the host mucosa and stimulate immune responses. Among them, the spore-forming Gram-positive bacterium *Bacillus subtilis* has been evaluated (Duc le et al., 2003; Lee et al., 2020). *B. subtilis* spores have several advantages for use as a vaccine vehicle including the ability to bind and present antigens to the mucosa as adjuvants (Ricca et al., 2014; Zhao et al., 2014) and high survivability in a variety of environmental conditions including the digestive tract. Studies have shown that mucosal immunization of mice with *B. subtilis* spores coated with antigens can induce strong antibody responses and provide protection against bacterial and viral infection (Copland et al., 2018; Huang et al., 2010b; Song et al., 2012b; Zhao et al., 2014). The application of this spore-based technology to vaccinate cattle against BRD pathogens may have commercial benefits due to the low cost of inputs, well-established production systems, and the current use of *Bacillus* in livestock as feed additives. Thus, a low-cost, generally regarded as safe vaccine component with a proven record as an adjuvant would expedite industry adoption.

M. haemolytica virulence factors include outer membrane proteins, capsular polysaccharides, lipopolysaccharides, iron-binding proteins, and ruminant-specific repeats in toxin-leukotoxin (LKT) (Confer, 2009). The significance of these virulence factors in pathogenesis

makes them the prime targets of host immune response and, as a result, potential targets as vaccine components (Confer and Ayalew, 2018; Klima et al., 2014a). Multiple in vivo studies with LKT-deletion mutants have demonstrated that LKT is the primary virulence factor of *M. haemolytica* pathogenesis (Dassanayake et al., 2009; Petras et al., 1995; Tatum et al., 1998) and, thus, LKT is considered a key element for vaccine development (Confer and Ayalew, 2018). Several studies have confirmed outer membrane lipoproteins as immunologically key surface antigens (Confer et al., 2003; Confer et al., 2006; Lee et al., 2008; Pandher et al., 1999). In the current study, two *M. haemolytica* chimeric proteins, MhCP1 and MhCP2, have been evaluated as components of candidate vaccines where LKT was retained as a core constituent. MhCP1 (CTB+PlpE+NLKT) is composed of truncated cholera toxin B subunit (CTB), the immune-dominant epitope (R2) region of the *M. haemolytica* outer membrane lipoprotein, PlpE, and the neutralizing epitope of LKT (NLKT) (Ayalew et al., 2009b). MhCP2 (CTB+LKT+GS60) comprises CTB, LKT, and another surface-exposed *M. haemolytica* outer membrane lipoprotein GS60. The purified chimeric proteins MhCP1 and MhCP2 were then adsorbed to *B. subtilis* spores, and the resultant spore-based vaccines termed "Spore-MhCP1" and "Spore-MhCP2" were evaluated in mice for the induction of immunogenicity. In a proof-of-concept study, we previously showed that *B. subtilis* spores coated with a dimer of *M. haemolytica* antigens, PlpE, and NLKT, can induce antibody responses after mucosal immunization in a mouse model (Uddin et al., 2023a). The objectives of this current study were to develop two potential spore-based candidate vaccines, Spore-MhCP1 and Spore-MhCP2, against *M. haemolytica,* then assess the immunogenicity of these vaccines after delivery through mucosal (intranasal and intragastric) routes using mice models.

4.2 Materials and Methods

4.2.1 Spore Preparation

B. subtilis (RK28 strain) spores were used in this study and processed for antigen binding, as described previously (Uddin et al., 2023a). Spores were enumerated by first suspending them in water and heating at 65 °C for 30 min to eliminate vegetative cells. The resulting suspension was then plated onto Luria–Bertani (LB) agar and incubated overnight, followed by the counting of colony-forming units to estimate spore counts.

4.2.2 Chimeric Protein Purification

M. haemolytica chimeric proteins MhCP1 and MhCP2 were constructed as per the modular structure depicted in Figure 4.1A. MhCP1 comprised truncated cholera toxin B subunit (CTB), the immunodominant surface epitope (R2) region of the *M. haemolytica* outer membrane lipoprotein PlpE, and the neutralizing epitope of leukotoxin (NLKT). The chimeric protein MhCP1 had 305 amino acid (AA) residues, a calculated molecular weight of 34.01 kDa, and an isoelectric point (pI) of 9.0. Ayalew and colleagues previously used a comparable chimeric protein (CTB-R2-NLKT) similar to MhCP1, differing solely in the location of the His-tag, which was on the C-terminus end of their protein structure (Ayalew et al., 2009b). MhCP2 was constructed with CTB, LKT, and the surface-exposed outer membrane lipoprotein GS60. The LKT fragment used in MhCP2 consisted of the nucleotides encoding AA 705-953 of the *M. haemolytica* LktA gene (GenBank: M20730.1). The chimeric protein MhCP2 comprised 953 AA residues and had a calculated molecular weight of 104.83 kDa and a pI of 5.1. Sequences corresponding to the chimeric proteins, MhCP1 and MhCP2, were commercially synthesized (BioBasic Inc., Markham, ON, Canada), codon optimized for expression in *Escherichia coli*, and cloned into the pET28a vector. A polyhistidine tag (6 x His) was added at the N-terminal end of both sequences to facilitate purification and detection. The pET28a construct was transformed into *E. coli* BL21 Star (DE3), and the expression and purification of the recombinant chimeric protein MhCP1 was carried out according to previously described procedure

(Uddin et al., 2023a). The chimeric protein MhCP2 was purified under native conditions, without using any denaturant, as previously described (Jones et al., 2017). After purification, the fractions containing the recombinant proteins were pooled and dialysed, and the buffer was exchanged with a storage buffer containing 20 mM Tris-HCl, pH 8.0. Subsequently, the proteins were concentrated by centrifugation using an Amicon Ultra centrifugal filter. The quality and identity of the purified proteins were assessed through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed via Western blot analysis employing anti-His antibodies (ThermoFisher, Toronto, ON, Canada).

4.2.3 Chimeric Protein Adsorption to Spores

The conditions for optimizing spore adsorption with the chimeric proteins MhCP1 and MhCP2 were determined as described previously [17,30]. Briefly, varying amounts of antigen MhCP1 and MhCP2 (5 µg, 10 µg, and 50 µg), spores (5×10^8 to 4×10^9), buffer (citrate, phosphate buffered saline [PBS]), buffer pH (4 and 7), and temperature (4 °C, room temperature) were investigated to optimize antigen adsorption to spores. Following each set of conditions employed, the Spore-MhCP1 and Spore-MhCP2 mixtures were centrifuged and antigen binding to the spore coat, or remaining in supernatant, were evaluated by Western blotting using anti-His antibodies [30]. Conditions that resulted in the greatest amount of antigen adsorption to spore coats were considered optimal. For MhCP1, the following method was identified as optimal for spore adsorption and used in mice Experiment 1 (below): 2×10^9 spores in water were centrifuged (5000× *g*; 5 min; 4 °C). The pellet was resuspended in PBS (pH 4) containing a total of 10 µg of purified MhCP1, and then mixed by inversion (1 h, 4°C). For the MhCP2 antigen used in mice Experiment 2, the optimal binding conditions were similar, except 50 µg of MhCP2 was adsorbed to 2×10^9 spores and citrate buffer (pH 4) was used.

4.2.4. Animals and Experimental Design

Animal experiments were carried out in strict accordance with the recommendations established in the Canadian Council on Animal Care Guidelines [32]. The experiments were reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol # 1712) before commencement. Two separate studies were conducted to evaluate the immunogenicity of MhCP1 and MhCP2 in BALB/c mice (6 to 8 weeks old; Charles River Laboratories [Quebec, QC, Canada]). Prior to initiating the study, mice were acclimated to their cages and were housed in ambient conditions at 22–25 °C with a 12 h light/12 h dark cycle. Mice had free access to water and feed (Laboratory Rodent Diet 5001 [LabDiet, St. Louis, MO, USA]). A feeding and health log was maintained and the daily monitoring of animal health occurred throughout the experiments. In each experiment, mice were randomly assigned to the following six treatment groups (N = 12 per treatment): intramuscular free MhCP (IM:MhCP; positive control to measure the systemic immunity of antigens in free form); intranasal spore-bound MhCP (IN:Spore+MhCP); intranasal free MhCP without spore (IN:MhCP); intranasal spore only without any MhCP (IN:Spore); intragastric spore-bound MhCP (IG:Spore+MhCP); and control/naïve mice (Control; no MhCP administered, negative control). Details of the experimental design for both mice experiments are listed in Table 1.

4.2.5. Immunizations

Experiment 1: Mice were immunized through respective routes on day 0 and then received a booster immunization on day 21. Mice in the control treatment did not receive spores or antigen. Intramuscular immunization was performed on anaesthetized mice by mixing (1:1) purified antigen with Freund's incomplete adjuvant (Sigma-Aldrich, Oakville, ON, Canada) and administering the mixture into the thigh muscle. For intranasal groups, mice were lightly

anaesthetized, and then the respective vaccine formulations were administered in half doses by pipette tip to each nostril. The intragastric mice group was delivered the vaccine by oral gavage using a feeding tube (Instech Laboratories, Plymouth Meeting, PA, USA). The details of vaccine formulations, including the amount of recombinant protein MhCP1 and spore count (where applicable) per immunization time points, can be found in Table 1.

Experiment 2: Antigen MhCP2 was evaluated, where the inoculation procedures were identical to Experiment 1. For the treatment groups receiving MhCP2, either in free form (IM:MhCP2, IN:MhCP2) or in spore-bound antigens (IN:Spore+MhCP2, IG:Spore+MhCP2), a total of 50 µg of MhCP2 was administered at each vaccination time point as this was the optimal amount determined for spore adsorption.

4.2.6. Sample Collection

On days 21 and 42, blood, bronchoalveolar lavage (BAL), feces, and saliva were collected for both mice experiments. At each timepoint, six mice were sacrificed under deep anaesthesia. To collect freshly voided feces, each animal was placed into a separate cage for 3–5 min, or until feces was passed. Sterile oral swabs (Isohelix, Harrietsham, Kent, UK) were used for saliva collection thereafter. To obtain blood samples, anaesthesia was induced using isoflurane (4%) and maintained (2% isoflurane) using a SomnoSuite mask (Kent Scientific Corporation, Torrington, CT, USA). Blood was drawn from the caudal vena cava subsequent to opening the abdominal cavity. Mice were then euthanized humanly by exsanguination. The BAL samples were collected immediately after death by introducing a catheter into the trachea and instilling PBS into the bronchioles, followed by the recovery of BAL fluid. Antigen-specific IgG (blood) and IgA (feces, saliva, BAL) were quantified from samples to assess the immune response resulting from each vaccination route.

4.2.7. Antigen-Specific Serum IgG Quantification

Enzyme-linked immunosorbent assay (ELISA) was performed to quantify antigen-specific antibody (IgG) responses in serum from both mice studies as previously described [30]. Sequences corresponding to individual components of the chimeric fusion constructs (i.e., PlpE, NLKT, LKT, and GS60) were synthesized commercially (BioBasic Inc., Markham, ON, Canada), codon optimized for expression in *E. coli*, and then purified by immobilized metal affinity chromatography. Purified recombinant antigen components PlpE, NLKT, LKT, and GS60 were utilized as ligands to coat ELISA plates individually at a concentration of 50 ng/well, and the plates were incubated overnight at 4 °C. After a wash step the next morning and the addition of Blocking Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0), the plates were incubated for1 h at room temperature. Serum samples were diluted (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20), and aliquots (100 µL) of each dilution were pipetted into wells in duplicate, followed by incubation for1 h at room temperature. Horseradish peroxidase-conjugated anti-mouse IgG (Cat. No. A90-131P; Bethyl Laboratories Inc., Montgomery, Texas, USA) was used as the secondary antibody. After introducing the secondary antibody, plates were incubated at room temperature for 1 h, followed by colour development using TMB (3,3′,5,5’-tetramentylbenzidine; ThermoFisher, Toronto, ON, Canada). Reactions were stopped by ELISA stop solution (0.2 M H_2SO_4), and optical densities of the reactions were measured at a wavelength of 450 nm on a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA) with Gen5 analysis software version 3.05 (BioTek Instruments Inc., Winooski, VT, USA). Concentrations were determined using a standard curve established with a Mouse IgG ELISA Quantitation Kit (Cat. No. E90-131; Bethyl Laboratories Inc.,

Montgomery, Texas, USA), utilizing Mouse Reference Serum (RS10-101-6; Bethyl Laboratories Inc.) as the standard.

4.2.8. Antigen-Specific Secretory IgA (sIgA) Quantification

BAL, feces, and oral swab samples were pre-processed for IgA quantification, as described previously [33]. After pre-processing, ELISA was used to measure the concentrations of mucosal secretory IgA (sIgA) as previously described [30]. Briefly, 96-well ELISA plates were coated with 50 ng/well of purified recombinant antigen components NLKT, PlpE, LKT, and GS60 independently and incubated at 4 °C overnight. Incubation in blocking solution was conducted, as described for IgG, and then duplicate samples were pipetted into individual plate wells, followed by incubation at room temperature for 1 h. Plates were washed, and the HRP conjugated anti-Mouse IgA Detection Antibody (A90-103P; Bethyl Laboratories Inc., Montgomery, Texas, USA) was added, followed by an additional incubation period for 1 h at room temperature. TMB substrate was added, and after colour development, the reaction was stopped by the addition of stop solution. The plates were read at 450 nm to determine optical density on a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

4.2.9. Complement-Mediated Serum Bactericidal Activity (SBA)

Sera collected on day 42 were evaluated for SBA against *M. haemolytica*, as described previously [34]. The SBA assay utilized neonatal colostrum-deprived calf serum (CDCS) as an external complement source, which was previously determined to have limited bactericidal activity against *M. haemolytica*. The CDCS and mice sera were tested in triplicate for bactericidal activity in the presence of complement. Endogenous complement activity of sera was eliminated by heat inactivation (56 °C, 30 min) prior to analysis. A serotype 1 strain of *M. haemolytica* was used for

the assay. To enhance surface epitope exposure by eliminating the polysaccharide capsule, *M. haemolytica* cells underwent decapsulation through incubation at 41 °C with gentle shaking at 100 rpm for 1 h. The decapsulated *M. haemolytica* were suspended in PBS to an optical density of 0.50 at 600 nm and a 1:1000 dilution was then used for SBA. For this, mice sera (25 µL; heat-inactivated), exogenous complement (25 µL), and decapsulated *M. haemolytica* (25 µL) were combined and plated in triplicate, followed by incubation for 30 min (37 °C). Suspensions from the beginning of the experiment (T0) and after 30 min of incubation (T30) were plated and cultured overnight (37 °C with 5% CO_2), followed by bacterial enumeration. Bactericidal activity was determined as the percentage of cells killed and calculated as follows: [(T0 growth-T30 growth)/T0 growth] × 100%. Negative controls in each SBA assay consisted of the complement source alone, without antibodies (mice serum).

4.2.10 Statistical Analysis

The analysis of ELISA and SBA assay was conducted using one-way ANOVA. Statistical significance was assessed against the control through one-way ANOVA with Tukey's post-test. Statistical significance was considered when a *p*-value was less than 0.05. Microsoft Excel 2010 and GraphPad Prism version 10.1.0 were utilized for all analyses.

4.3 Results

4.3.1 Adsorption of Chimeric Proteins to Spores

The modular structures of MhCP1 and MhCP2 are shown in Figure 4.1A. Coomassie-stained SDS-PAGE demonstrated the size, purity, and integrity of the recombinant proteins MhCP1 (Figure 4.1B,C) and MhCP2 (Figure 4.1D). For Spore-MhCP1, the best spore-binding condition was achieved when 10 µg of antigen MhCP1 was mixed with 2×10^9 spores in PBS at

pH 4, as indicated by the presence and absence of a band corresponding to MhCP1 from the spore coat and supernatant in Western blots (Appendix F-A). Spore-MhCP2 displayed the best binding condition when 50 µg of antigen MhCP2 was mixed with 2×10^9 spores in citrate buffer at pH 4, as indicated by the band from Western blots (Appendix F-B). Among the tested parameters, both MhCP1 and MhCP2 were efficiently adsorbed onto the spore coat at pH 4 and the binding remained most stable at 4 °C compared to room temperature, whereas at pH 7, low levels of binding were observed. Freshly prepared Spore-MhCP1 and Spore-MhCP2 (within one hour of their preparation) using optimized binding conditions were used for the immunization of mice.

4.3.2 Experiment 1. Antigen-Specific Antibody Production from Spore-MhCP1

The vaccination of mice with free MhCP1 and Spore-MhCP1 induced anti-PlpE and anti-NLKT antibodies (Figures 4.2 and 4.3). This indicated that MhCP1 was immunogenic and retained immunogenicity after binding to spores. On day 21, after receiving a single immunization, IM:MhCP1 treatment mice had a greater serum IgG immune response for PlpE ($p < 0.05$; Appendix G-A) and NLKT ($p < 0.05$; Appendix G-B), when compared to other treatments. On day 42, subsequent to the booster vaccination, concentrations of IgG increased several fold in IM:MhCP1 mice (Figure 4.2). Apart from IM:MhCP1 mice, the spore-bound intranasal group (IN:Spore+MhCP1) also followed a similar trend and showed numerically greater serum anti-PlpE antibody concentrations, as compared to other mice treatments on day 21 ($p > 0.05$; Appendix G-A) and day 42 ($p > 0.05$; Figure 4.2A). No other treatment group showed serum antibody production on days 21 or 42 that was above the ELISA limit of detection (≥7.8 ng/mL).

On day 21, with a single immunization, all the intranasal treatment groups showed secretory IgA immune responses in BAL samples against PlpE (Appendix G-C) and NLKT (Appendix G-D). However, on day 21, IgA immune responses were not observed for feces and oral swab samples

(limit of detection ≥ 7.8 ng/mL) from any treatment groups. On day 42, BAL samples from IN:Spore+MhCP1 mice had greater secretory anti-PlpE and anti-NLKT IgA concentrations compared to other treatment groups ($p < 0.05$; Figure 4.3A,B). IN:MhCP1 treatment mice exhibited numerically higher anti-PlpE ($p > 0.05$; Figure 4.3A) and anti-NLKT IgA ($p < 0.05$; Figure 4.3B) in BAL samples as compared to control mice. Interestingly, one out of six mice from the IN:Spore only treatment group showed minimal levels of anti-PlpE and anti-NLKT IgA in BAL samples. None of the mice from the IM:MhCP1, IG:Spore+MhCP1, and control groups had detectable secretory antibodies in BAL fluid. Compared to the controls, IN:Spore+MhCP1 treatment mice had increased levels of anti-PlpE IgA in fecal samples ($p < 0.05$; Figure 4.3E) and numerically higher levels of anti-PlpE IgA in oral swabs ($p > 0.05$; Figure 4.3C). The IM:MhCP1, IN:MhCP1, and intragastric Spore+MhCP1 group showed minimal levels of anti-PlpE IgA in feces (Figure 4.3E). The IN:Spore+MhCP1 treatment mice group was the only one that showed anti-PlpE and anti-NLKT IgA in all the mucosal samples evaluated. Despite NLKT and PlpE being delivered to mice as a chimera protein, immune responses to each antigen component were differential.

4.3.3 Experiment 2. Antigen-Specific Antibody Production from Spore-MhCP2

Vaccinating mice with both unbound MhCP2 and Spore-MhCP2 resulted in the generation of antibodies against GS60 and LKT, demonstrating that the chimeric antigen MhCP2 retained its immunogenicity following adsorption onto spores (Figures 4.4 and 4.5).

After receiving a single immunization, on day 21, IM:MhCP2 treatment mice exhibited a greater serum IgG immune response as compared to other treatment mice against GS60 ($p < 0.05$; Appendix H-A) and LKT ($p < 0.05$; Appendix H-B). Although limited in titres, IN:Spore+MhCP2 treatment mice also stimulated serum anti-GS60 and anti-LKT IgG with a single dose of vaccination (Appendix H-A,B). After receiving the booster dose, IgG immune responses increased

several fold both in IM:MhCP2 and IN:Spore+MhCP2 treatment mice by day 42 and remained higher compared to other treatment mice ($p < 0.05$; Figure 4.4). The IN:MhCP2 treatment mice also exhibited serum anti-GS60 and anti-LKT IgG, although to a lesser extent as compared to IN:Spore+MhCP2 treatment mice on day 42 ($p > 0.05$; Figure 4.4). IN:Spore and control treatment mice did not show any detectable serum antibody production (detection limit ≥ 7.8 ng/mL) on day 42.

The IN:Spore+MhCP2 treatment group showed a greater secretory IgA immune response compared to other treatments in BAL samples against GS60 ($p < 0.05$; Appendix H-C) and LKT ($p < 0.05$; Appendix H-D) on day 21, after a single immunization. Compared to control mice, the IN:Spore+MhCP2 treatment group also exhibited a greater anti-GS60 IgA in saliva and feces ($p < 0.05$; Appendix H-E,G). Although the IN:MhCP2 group also stimulated anti-GS60 IgA in BAL, saliva, and feces on day 21, this response was less than that observed in IN:Spore+MhCP2 treatment mice (Appendix H-E,G). On day 42, after receiving the booster dose, immune responses increased several fold, compared to day 21, both in IN:Spore+MhCP2 and IN:MhCP2 treatment mice (Figure 4.5). The IN:Spore+MhCP2 resulted in greater secretory anti-GS60 and anti-LKT IgA compared to other treatment groups in all the mucosal samples evaluated ($p < 0.05$; Figures 4.5A–F). Apart from the IN:Spore+MhCP2 treatment mice, IN:MhCP2 also exhibited a production of anti-GS60 IgA in BAL and saliva ($p < 0.05$; Figure 4.5A,C) and anti-LKT IgA in BAL samples as compared to control mice ($p < 0.05$; Figure 4.5B). IN:Spore and control mice did not show any detectable IgA antibody levels in any of the samples evaluated (detection limit ≥ 7.8 ng/mL) at any time point. IN:Spore+MhCP2 and IN:MhCP2 treatment groups showed anti-GS60 and anti-LKT IgA in all the mucosal samples evaluated, although the IN:Spore+MhCP2 treatment group consistently had greater mucosal titres compared to the IN:MhCP2 treatment group for each

sample evaluated ($p < 0.05$; Figure 4.5). This finding demonstrated that despite the fact that GS60 and LKT were administered as a chimera, each antigen construct elicited distinct immune responses and the spore enhanced immunogenicity.

4.3.4 Complement-Mediated SBA

Control and IN:Spore treatment mice in both experiments, and CDCS used as complement, had minimal bactericidal activity (Figure 4.6). In Experiment 1, IM:MhCP1 mice sera killed more *M. haemolytica* (99.1%), compared to other treatments ($p < 0.05$; Figure 4.6A). Both IN:Spore+MhCP1 and IN:MhCP1 treatment mice showed greater bactericidal activity (54.5% and 29.9%, respectively) than control mice ($p < 0.05$; Figure 4.6A). Interestingly, the IG:Spore+MhCP1 treatment group also showed a level (14.8%) of bactericidal activity. In Experiment 2, sera from IM:MhCP2 treatment mice displayed higher bactericidal activity (99.1%), followed by IN:Spore+MhCP2 (88.0%) and IN:MhCP2 (80.1%) treatment mice in comparison to control mice ($p < 0.05$; Figure 4.6B). The IG:Spore+MhCP2 treatment group also showed limited but significant bactericidal activity (15.1%) versus sera from control mice ($p < 0.05$; Figure 4.6B). Compared to the intranasal administration of free antigen, the intranasal spore-bound antigen group showed greater bactericidal activity against *M. haemolytica* ($p < 0.05$) in both experiments.

4.4 Discussion

In this study, two spore-based vaccine candidates were developed, using *M. haemolytica* chimeric proteins MhCP1 (CTB+PlpE+NLKT) and MhCP2 (CTB+LKT+GS60). Both vaccines were able to stimulate antigen-specific immune responses in immunized mice. In support of this, we previously showed that a larger dimer repeat of NLKT and PlpE (56.4 kDa, pI of 9.1) could bind to spores and remain immunogenic, similar to MhCP1 (Uddin et al., 2023a). The versatility

of chimeric proteins in delivering multiple antigens to the immune system is advantageous in vaccine development (Hertle et al., 2001a). The chimeric protein MhCP1 encoded the neutralizing epitope of LKT, which is the principal virulence factor of *M. haemolytica* and an immunodominant epitope region (R2) of the outer membrane lipoprotein, PlpE (Ayalew et al., 2009b). A truncated CTB (lacking signal peptide), which can also be found in approved human mucosal vaccines (Lavelle and Ward, 2022) was fused to the N-terminus as a mucosal adjuvant. When administered through intranasal or rectal routes, it has been demonstrated in human studies that CTB effectively triggers the induction of antigen-specific local IgA and systemic IgG responses (Bergquist et al., 1997; Lavelle and Ward, 2022). Similarly, MhCP2 consisted of the truncated CTB fused to LKT and a surface-exposed *M. haemolytica* outer membrane lipoprotein, GS60. Both of these outer membrane proteins, PlpE and GS60, are reported to be strongly immunogenic (Confer and Ayalew, 2018) and conserved across *M. haemolytica* serotypes including serotypes 1 and 6, which are most often associated with BRD morbidity and mortality (Confer and Ayalew, 2018; Klima et al., 2014a). Keeping CTB and LKT as core components, two established immunogenic constructs (PlpE and GS60) were chosen to formulate the chimeric proteins. This resulted in two chimeric proteins that differed both in size and amino acid composition.

In order to establish the most effective adsorption conditions, a comparative analysis of spore and chimeric protein binding was conducted with various quantities of each component, under varied pH levels and reaction temperatures. Previous research has demonstrated that proteins exhibit enhanced binding for negatively charged bacterial spores when the pH of the aqueous environment drops below the isoelectric point (pI) of the protein (Huang et al., 2010b; Song et al., 2012b). Given that the pI values for MhCP1 and MhCP2 were 9.0 and 5.1, respectively, binding experiments were conducted at both pH 4 and pH 7. MhCP1 and MhCP2 exhibited optimal

adsorption onto the spore coat at pH 4, while reduced binding levels were observed at pH 7. This observation supports findings from our earlier study involving a dimer of a PlpE-NLKT construct (Uddin et al., 2023a) and is consistent with other studies (Huang et al., 2010b; Zhao et al., 2014). These findings suggest that the extent of protein adsorption to spores is reliant upon both solution pH and protein structure. While the complete molecular mechanisms underlying spore adsorption remain elusive, the current understanding suggests that the isoelectric point, electric charge, and the relative hydrophobicity of the antigen play pivotal roles in facilitating efficient adsorption (Huang et al., 2010b; Saggese et al., 2023). Despite the efficient adsorption of both MhCP1 and MhCP2 onto the spore coat at pH 4, notable distinctions were observed in the reaction buffers used. Specifically, MhCP1 demonstrated the highest adsorption efficacy when employing PBS buffer (pH 4), whereas MhCP2 exhibited optimal binding using citrate buffer (pH 4) in the binding experiment. The differences between the protein structures and their pI values necessitated two different buffers to be used to establish optimal adsorption conditions. It was also necessary to use a higher amount of MhCP2 (five times compared to MhCP1), to achieve optimal adsorption.

Previously, Ayalew et al. (2009) conducted a study with the same chimeric protein as MhCP1 and revealed that the components were immunogenic (Ayalew et al., 2009b). This supports our study, where the generation of immune responses against NLKT and PlpE were observed in mice, following intramuscular immunization with free MhCP1. Despite MhCP1 being developed as a chimeric protein, NLKT and PlpE components yielded seroconversion independently. When we tested three different routes either with free or spore-bound MhCP1 in mice, our results demonstrated that the MhCP1 antigen adsorbed to spores in a manner that preserved its immunogenicity. While the IM:MhCP1 treatment group was used as a positive control and showed strong seroconversion, the main goal of this study was to develop Spore-MhCP1 as a mucosal

vaccine candidate and to investigate if this spore-bound vaccine could enhance the immune response compared to the free form of this antigen. Mice immunized via the intranasal route with Spore-MhCP1 induced mucosal immunity, as evidenced by the production of secretory IgA against *M. haemolytica* NLKT and PlpE. Although IN:Spore+MhCP1 did not produce an IgG response as strong as that induced by intramuscularly administered free MhCP1, it was the only group other than IM:MhCP1 to elicit an IgG response. In addition, IN:Spore+MhCP1 was the sole group to result in specific IgA responses against both PlpE and NLKT antigens at all mucosal sites evaluated and with a significantly greater IgA levels in BAL and fecal samples. Thus, IN:Spore+MhCP1 immunization resulted in both mucosal and systemic immune responses. Intranasal immunization with free MhCP1 (IN:MhCP1) also stimulated anti-PlpE IgA in all the mucosal samples evaluated, but to a lesser extent than IN:Spore+MhCP1. This indicated that binding MhCP1 to *Bacillus* spores did result in an increased immune response and highlighted that the spores were an effective adjuvant. Mucosal immunity is shared between different mucosae (Shewen et al., 2009), and our study showed that intranasal immunization can result in secretory antibodies in both the oral cavity and gut.

Batra and colleagues formulated a BHV-1-vectored vaccine expressing PlpE-LKT chimeric proteins and showed the generation of antibodies in bighorn sheep following intranasal vaccination (Batra et al., 2017). However, antibody production against surface antigen was inconsistent, and the vaccine was not effective in protecting against *M. haemolytica* challenge. An intranasal vaccination of cattle with the same MhCP1 chimeric protein used in our study stimulated serum and nasal antibodies in vaccinated calves, and intrabronchial challenge in these calves displayed fewer clinical symptoms than unvaccinated animals (Ayalew et al., 2009b). Given the enhanced immune responses from IN:Spore+MhCP1 compared to IN:MhCP1 in our study, we hypothesise that the intranasal

immunization of this Spore-MhCP1 vaccine may confer increased protection against *M. haemolytica* infection in cattle. The intragastric administration of spore-bound MhCP1 was able to generate a limited IgA immune response against PlpE in fecal samples, and this finding is consistent with a previous report where an immune response was reported in fecal samples after oral immunization with a spore-bound antigen (Uddin et al., 2023a). Dilution of antigens in the stomach and degradation due to the low gastric pH and the presence of proteases in the gastrointestinal tract are possible contributing factors that reduce the antigenicity of intragastric vaccines (Hoelzer et al., 2018b). Interestingly, one out of six mice from the IN:Spore group showed anti-PlpE IgA and anti-NLKT IgA in BAL samples, though not in other samples. While difficult to explain, it is noteworthy that this outcome is not unique, as other studies have reported similar findings where antigen-specific antibody production has been reported from spore-only immunization (Katsande et al., 2023; Oh et al., 2020b). Studies have also reported that spores alone can provide protection against H5N1 virus challenge (Song et al., 2012b) and lethal *B. anthracis* challenge (Oh et al., 2020b) in mouse models. The reported underlying mechanism was the involvement of innate immunity via TLR-mediated NF-κB expression, the recruitment of NK cells to the lungs, and dendritic cell maturation (James et al., 2022; Song et al., 2012b).

Similar to Spore-MhCP1, immunization with Spore-MhCP2 stimulated detectable anti-LKT and anti-GS60 antibodies, demonstrating that the chimeric antigen MhCP2 also retained immunogenicity after spore adsorption. While the intramuscular immunization of MhCP2 demonstrated a stronger serum IgG response compared to other groups, the intranasal immunization of spore-bound and free MhCP2 also induced seroconversion against both antigen constructs, LKT and GS60, independently. Between these two intranasal groups, the spore-bound MhCP2 more efficiently elicited a systemic immune response, yielding a higher IgG antibody titre.

GS60 has previously been targeted for vaccine development in the form of transgenic alfalfa expressing GS60 in rabbits (Lee et al., 2008) and recombinant GS60 in calves (Orouji et al., 2012) and was reported to be immunogenic. Although GS60 is expressed *in vivo* during bronchopneumonia disease progression and is conserved among several BRD pathogens (Lee et al., 2008), there are very few reported vaccination trials testing this antigen (Lee et al., 2008; Orouji et al., 2012). Orouji et al. (2012) reported that the elevated IgG antibody titre against GS60 correlated with a reduction in the percentage of pneumonic tissue, a potential association between protection and anti-GS60 IgG (Orouji et al., 2012). However, none of these studies reported any antibody levels at mucosal sites.

The intranasal Spore-MhCP2 in immunized mice displayed the strongest anti-LKT and anti-GS60 IgA in all mucosal samples (BAL, feces, and saliva) evaluated. The intranasal immunization of free MhCP2 also generated IgA antibodies in all samples, albeit at lower levels than the spore-bound MhCP2. This indicates that binding MhCP2 to *Bacillus* spores did increase the immune response, again highlighting that the spores were effective as an adjuvant. Also similar to Experiment 1, it was shown that intranasal immunization resulted in mucosal immunity in the oral cavity and gastrointestinal tract. None of the spore-only treatment mice in Experiment 2 exhibited detectable antibody responses, in contrast to Experiment 1. The intragastric administration of spore-bound MhCP2 induced limited levels of anti-LKT IgA in BAL, saliva, and fecal samples. This was in contrast to Experiment 1, where IG:Spore+MhCP1 did not elicit detectable mucosal immunity in BAL or oral samples. It was also apparent that MhCP2 in Experiment 2 resulted in greater antigen-specific IgA at all mucosal sites. While this may have been partly due to the antigen itself, the differences in the amount of antigen administered between the two experiments (10 µg of MhCP1 versus 50 µg of MhCP2) likely also contributed to the observed differences. Recently,

our group has developed a plant-based oral vaccine and reported the induction of both systemic and mucosal immune responses in mice after five vaccinations (Kaldis et al., 2023). It is important to consider the differences in vaccine delivery methods, antigen concentration, and the number of doses administered when evaluating vaccines, as these factors can impact immunity outcome. Since intranasally delivered Spore-MhCP2 elicited both mucosal and systemic immune responses in mice, it could provide substantial protection against *M. haemolytica* infection in cattle, particularly given the stronger response observed compared to Spore-MhCP1.

Complement-mediated serum bactericidal activity assay is considered a standard method for evaluating the capability of antibodies induced by a vaccine to kill the targeted pathogen (McIntosh et al., 2015). In both mouse experiments, vaccine-induced antibodies from the intramuscular treatment almost completely killed *M. haemolytica* in the complement-dependent SBA assay. This outcome aligns with previous findings where bactericidal activity was noted following the intramuscular vaccination of mice using a comparable construct to MhCP1 (Ayalew et al., 2008; Uddin et al., 2023a). Although the sera from intranasal Spore-MhCP1 and free MhCP1 treatment mice exhibited a lower level of bactericidal activity in comparison to intramuscular treated mice in Experiment 1, this was likely attributed to lower IgG levels following intranasal immunization (Rouphael et al., 2011). In Experiment 2, this was also evident as we observed an elevated level of bactericidal activity from these two intranasal treatment mice groups, which also possessed a better IgG titre compared to Experiment 1. Surprisingly, although intragastric administration failed to demonstrate any detectable range of serum IgG for spore-bound MhCP1 and limited IgG for spore-bound MhCP2, sera from these groups had bactericidal activity. This finding features an important observation that the spore-bound MhCP1 and MhCP2 antigens had the capability to elicit functional antibodies against *M. haemolytica* whether it was delivered intranasally or

intragastrically. Even though oral administration failed to elicit appreciable immune responses in mice, it is justifiable to explore the assessment of oral spore-based vaccines in ruminants due to anatomical distinctions between mice and cattle and the prospect of rumination. This process could lead to the activation of pharyngeal lymphoid tissue by the exposure of antigens via regurgitation (Shewen et al., 2009). If similar IgA and IgG production occurs in cattle after vaccination with Spore-MhCP1/MhCP2, this would be advantageous by limiting the colonization and proliferation of *M. haemolytica* in the upper respiratory tract and potentially reducing infection in the lungs (Rice et al., 2007; Shewen et al., 2009).

4.5 Conclusions

In summary, the intranasal immunization of mice with both Spore-MhCP1 and Spore-MhCP2 resulted in mucosal and systemic antibody production. These responses were notably heightened in comparison to the intranasally delivered free antigen, demonstrating that binding antigens to *Bacillus* spores did increases immune responses. This observation emphasizes the effectiveness of spores as an adjuvant in augmenting the immune response and highlights their potential use in the development of novel immunization strategies against infectious diseases. It is interesting to note that the intragastric delivery of spore-bound vaccines conferred partial systemic protection, as evidenced by SBA, despite limited IgG responses. The outcomes from the mouse experiments in this study suggest that the employment of a similar approach could provide substantial protection against *M. haemolytica* infection in cattle and warrant further investigations to assess the effectiveness of the *B. subtilis* spore-based mucosal vaccines in cattle.

4.6 Tables and Figures

Table 4.1 Design of mice experiments (Exp) in which BALB/c mice were immunized with *Mannheimia haemolytica* chimeric protein MhCP1 (CTB+PlpE+NLKT; Exp. 1) or MhCP2 (CTB+LKT+GS60; Exp. 2) in free form or bound to *B. subtilis* spores.

Exp. No.	Treatment Group	No. of Mice	Route of Administration	Vaccine Formulations (Amount per Dose)	Vaccination Days	Samples Collected
Exp. 1	IM:MhCP1	12	Intramuscular	MhCP1 (10 µg) mixed with IFA *; No Spore	Days 0 and 21	Blood, BAL #, feces, saliva
	IN:Spore+MhCP1	12	Intranasal	Spore (2×10^9)-bound MhCP1 (10 µg)	Days 0 and 21	Blood, BAL, feces, saliva
	IN:MhCP1	12	Intranasal	MhCP1 (10 µg) only; No Spore	Days 0 and 21	Blood, BAL, feces, saliva
	IN:Spore	12	Intranasal	Spore (2×10^9) only; No MhCP1	Days 0 and 21	Blood, BAL, feces, saliva
	IG:Spore+MhCP1	12	Intragastric	Spore (2×10^9)-bound MhCP1 (10 µg)	Days 0 and 21	Blood, BAL, feces, saliva
	Control	12	N/A	No MhCP1; No Spore	Days 0 and 21	Blood, BAL, feces, saliva
Exp. 2	IM:MhCP2	12	Intramuscular	MhCP2 (50 µg) mixed with IFA; No Spore	Days 0 and 21	Blood, BAL, feces, saliva
	IN:Spore+MhCP2	12	Intranasal	Spore (2×10^9)-bound MhCP2 (50 µg)	Days 0 and 21	Blood, BAL, feces, saliva
	IN:MhCP2	12	Intranasal	MhCP2 (50 µg) only; No Spore	Days 0 and 21	Blood, BAL, feces, saliva
	IN:Spore	12	Intranasal	Spore (2×10^9) only; No MhCP2	Days 0 and 21	Blood, BAL, feces, saliva
	IG:Spore+MhCP2	12	Intragastric	Spore (2×10^9)-bound MhCP2 (50 µg)	Days 0 and 21	Blood, BAL, feces, saliva
	Control	12	N/A	No MhCP2; No Spore	Days 0 and 21	Blood, BAL, feces, saliva

* IFA: Incomplete Freund′s adjuvant, # BAL: bronchoalveolar lavage.

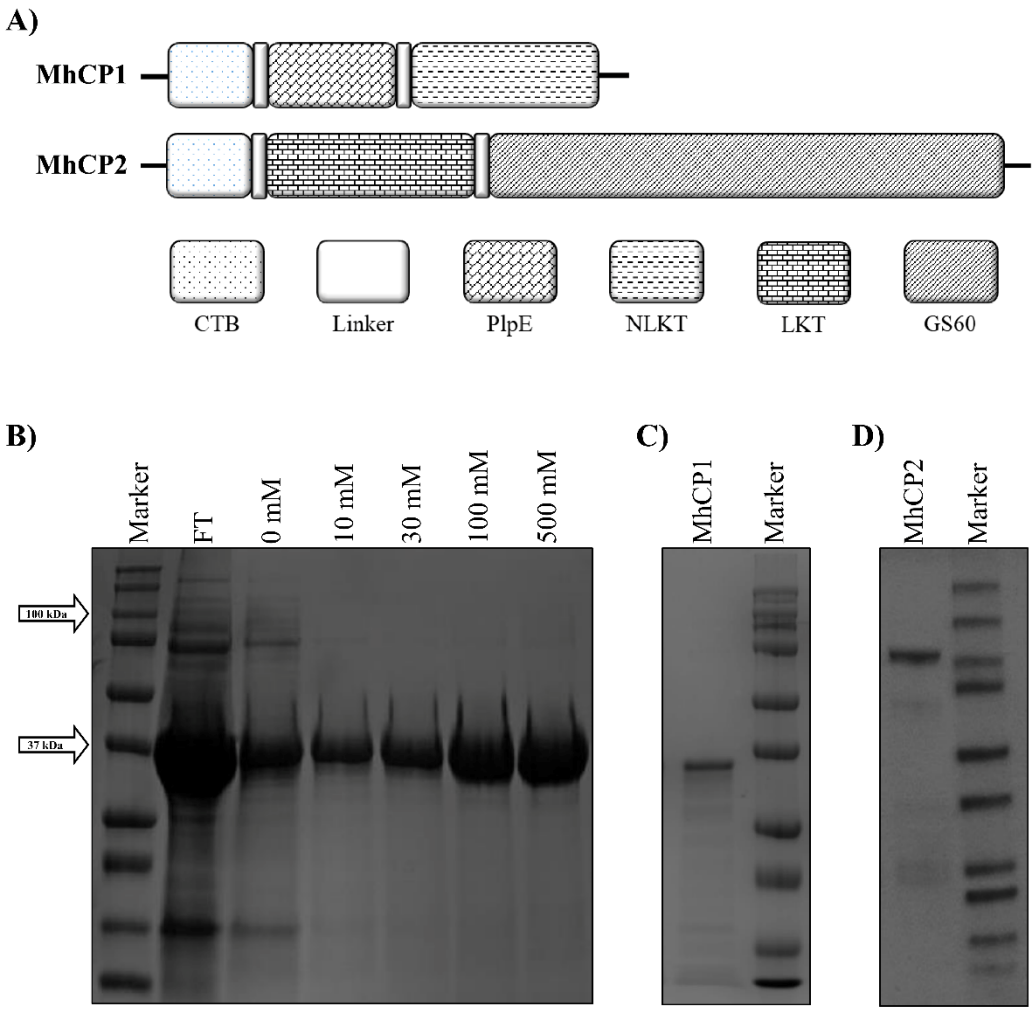


Figure 4.1: The construction and purification of chimeric proteins MhCP1 and MhCP2. (**A**) The modular architecture of MhCP1 and MhCP2. Both protein structures included truncated cholera toxin subunit B (CTB). MhCP1 contained epitopes of lipoprotein PlpE and neutralizing leukotoxin (NLKT); MhCP2 comprised a larger leukotoxin segment (LKT) and the surface-exposed *M. haemolytica* outer membrane lipoprotein GS60. (**B**) Eluted fractions of MhCP1, demonstrated by SDS-PAGE. (**C**) The purified recombinant chimeric protein MhCP1 demonstrated by Coomassie-stained SDS-PAGE. (**D**) The Coomassie-stained SDS-PAGE illustrated the purified chimeric protein MhCP2.

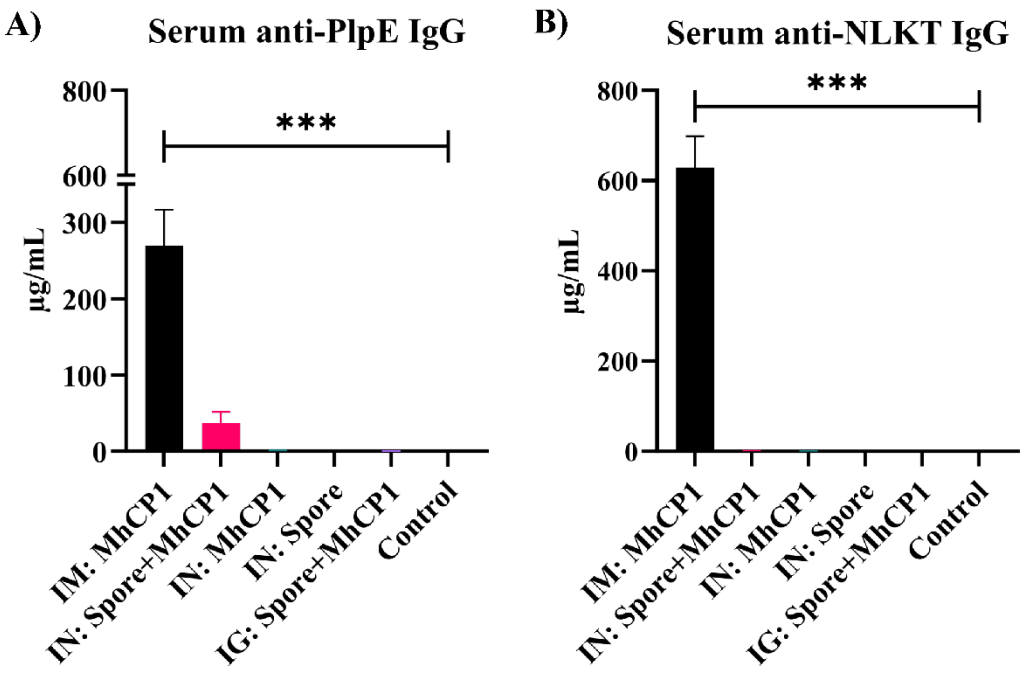


Figure 4.2: Experiment 1—Antigen-specific IgG concentrations in mice sera on day 42. Mice were vaccinated on day 0 and then received a booster immunization on day 21. The treatment groups were: intramuscular (IM:MhCP1), intranasal spore-bound antigen (IN:Spore+MhCP1), intranasal antigen only (IN:MhCP1), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP1), and control/naïve mice (Control). IgG specific to PlpE (**A**) and NLKT (**B**) are shown (mean ± SEM). *** $p < 0.001$.

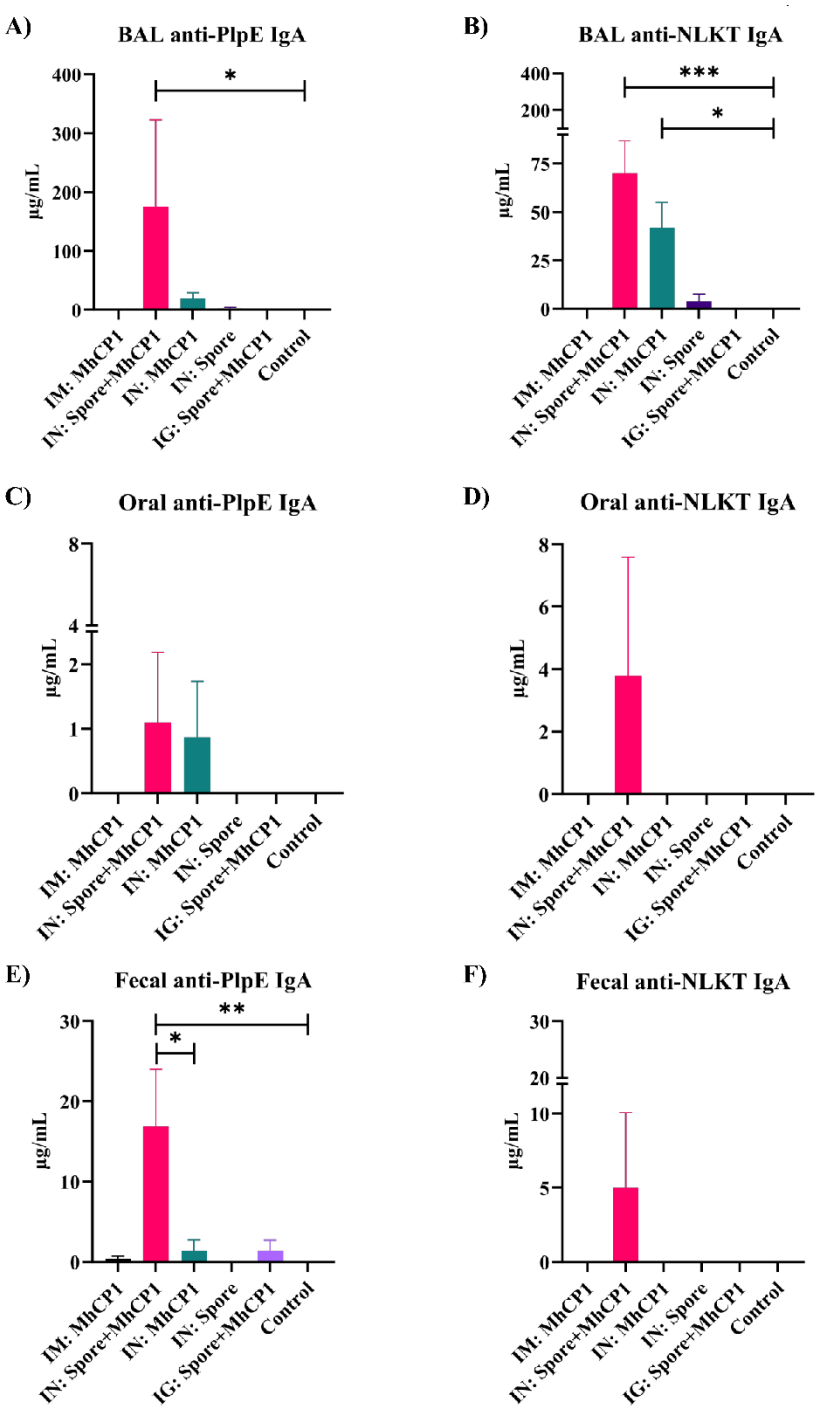


Figure 4.3: Experiment 1—Enhanced mucosal immunity induced by Spore-MhCP1 in mice on day 42. Anti-PlpE IgA and anti-NLKT IgA antibody concentrations in mucosa samples from mice on day 42 are shown for the following treatment groups: intramuscular (IM:MhCP1), intranasal spore-bound antigen (IN:Spore+MhCP1), intranasal antigen only (IN:MhCP1), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP1), and control/naïve mice (Control). Mice were vaccinated on day 0 and then received a booster immunization on day 21. IgA was measured in bronchoalveolar lavage (BAL), feces, and oral swab samples (mean ± SEM). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

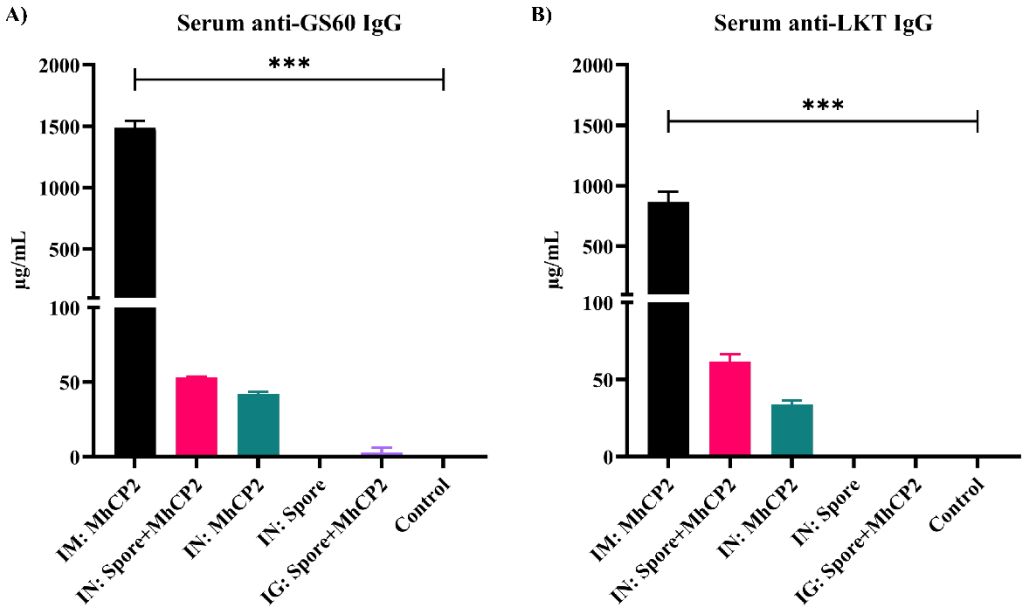


Figure 4.4: Experiment 2—Antigen-specific IgG concentrations in mice sera on day 42. Mice were vaccinated on day 0 and then received a booster immunization on day 21. The treatment groups were: intramuscular (IM:MhCP2), intranasal spore-bound antigen (IN:Spore+MhCP2), intranasal antigen only (IN:MhCP2), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP2), and control/naïve mice (Control). IgG specific to GS60 (**A**) and LKT (**B**) are shown (mean ± SEM). *** $p < 0.001$.

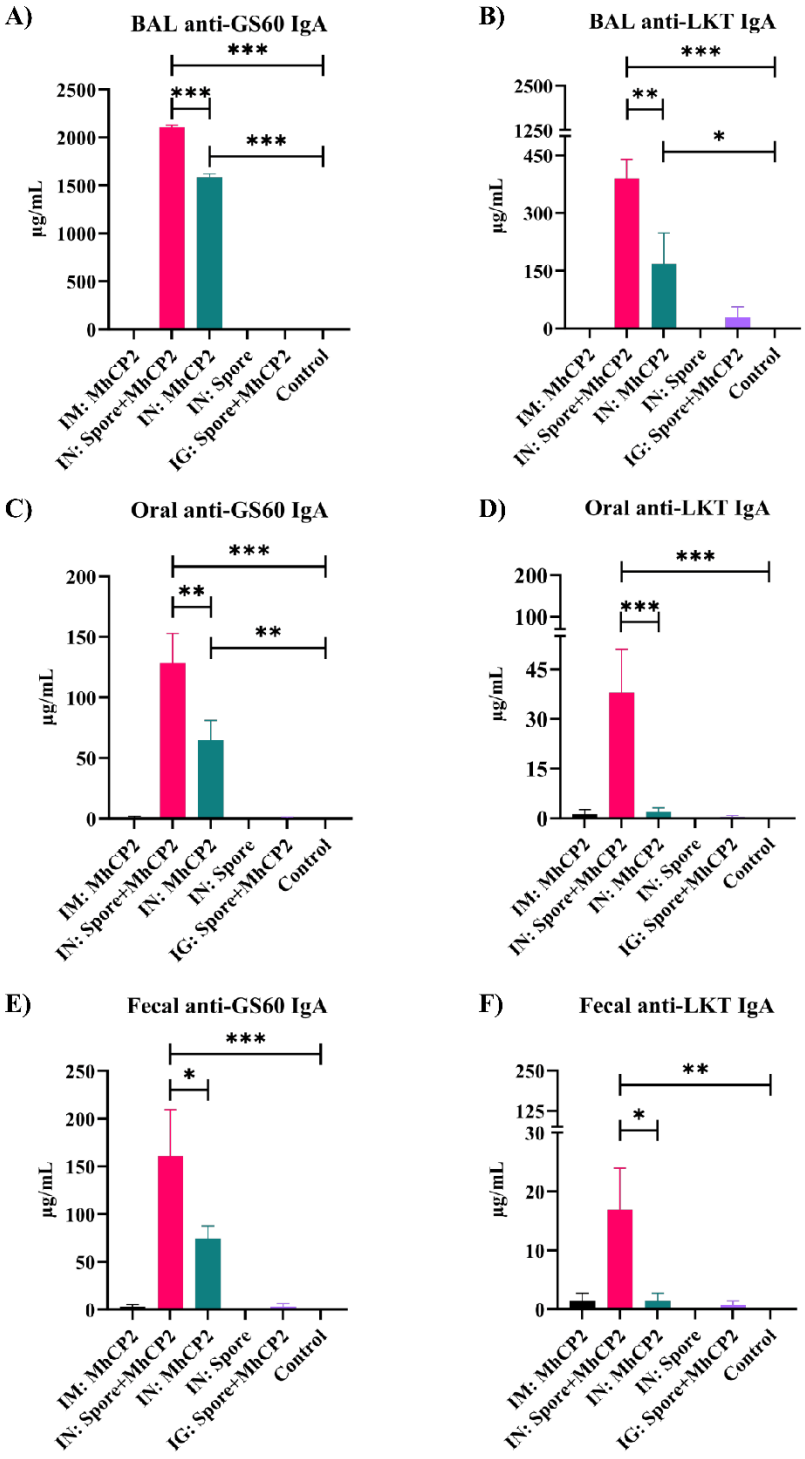


Figure 4.5: Experiment 2—Enhanced mucosal immunity induced by Spore-MhCP2 in mice on day 42. Anti-GS60 IgA and anti-LKT IgA antibody concentrations in mucosa samples from mice on day 42 are shown for the following treatment groups: intramuscular (IM:MhCP2), intranasal spore-bound antigen (IN:Spore+MhCP2), intranasal antigen only (IN:MhCP2), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP2), and control/naïve mice (Control). Mice were vaccinated on day 0 and then received a booster immunization on day 21. IgA was measured in bronchoalveolar lavage (BAL), feces, and oral swab samples (mean ± SEM). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

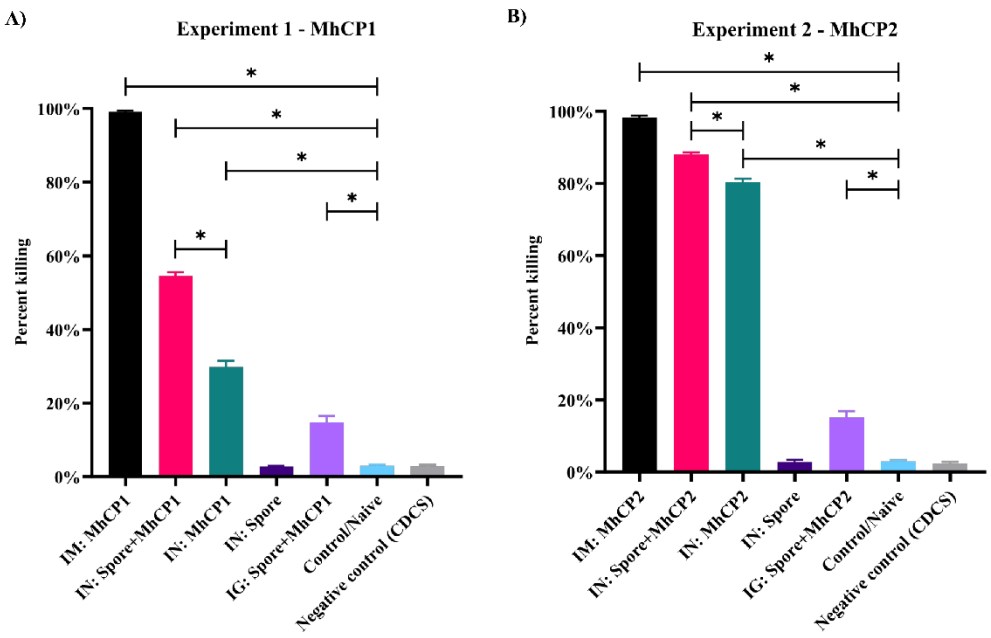


Figure 4.6: Serum bactericidal activity (SBA) assay, from samples collected on day 42. Antibodies present in the mice sera from Experiment 1 (**A**) and Experiment 2 (**B**) were assessed for their bactericidal activity against *M. haemolytica* through the complement-dependent SBA assay. Bactericidal activity from both experiments are shown for the following treatment groups: intramuscular (IM:MhCP), intranasal spore-bound antigen (IN:Spore+MhCP), intranasal antigen only (IN:MhCP), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP), and control/naïve mice (Control). The negative control in each SBA assay involved using only the complement source without the addition of antibodies (mice serum). * $p < 0.05$.

Chapter 5

Effect of a spore-based *Mannheimia haemolytica* vaccine on immune response and respiratory microbiota in sheep

5.1 Introduction

Mannheimia haemolytica is a predominant bacterial pathogen associated with severe pneumonia in ruminants, affecting cattle, goats, and sheep (Bkiri et al., 2023; Confer and Ayalew, 2018; Dassanayake et al., 2009; Singh et al., 2011). This bacterium is a primary opportunistic pathogen in bovine respiratory disease (BRD), a significant contributor to cattle morbidity and mortality (Rice et al., 2007), and also causes mastitis in ewes and septicemia in lambs (Confer and Ayalew, 2018; Singh et al., 2011). While various serotypes of *M. haemolytica*, including A1 and A2, can be found in the upper respiratory tract of healthy ruminants, serotype A1 is mainly responsible for bovine bronchopneumonia, whereas serotype A2 is primarily associated with pneumonia in sheep (Confer and Ayalew, 2018; Rice et al., 2007). Current commercial *M. haemolytica* vaccines have exhibited variable efficacy in field trials measuring BRD incidence (Larson and Step, 2012; O'Connor et al., 2019; Perino and Hunsaker, 1997), highlighting the need for novel vaccines with improved efficacy against this pathogen. An ideal vaccine would be capable of eliciting both mucosal and systemic immune responses, providing localized protection at the infection site in addition to systemic immunity.

M. haemolytica virulence factors, including leukotoxin (LKT), outer membrane proteins, lipopolysaccharides, iron-binding proteins, and capsular polysaccharides, are key for pathogenesis

and are prime targets of the host immune response, making them potential vaccine components (Confer, 2009; Confer and Ayalew, 2018; Klima et al., 2014a). Several *in-vivo* studies with LKT-deletion mutants have identified LKT as the dominant virulence factor (Dassanayake et al., 2009; Petras et al., 1995; Tatum et al., 1998), underscoring its significance in pathogenesis and importance as a preferred antigen for vaccine development (Confer and Ayalew, 2018). Additionally, several outer membrane lipoproteins have been reported as immunologically key surface antigens (Confer et al., 2003; Confer et al., 2006; Lee et al., 2008; Pandher et al., 1999). We previously developed a spore-based mucosal vaccine designated as "Spore-MhCP" against *M. haemolytica* and demonstrated its ability to stimulate antibody responses following mucosal immunization in a mouse model (Uddin et al., 2023a). This vaccine was prepared by purifying a chimeric protein, comprising repeated neutralizing antigenic epitopes from *M. haemolytica* leukotoxin A (NLKT) and outer membrane protein PlpE, which was subsequently adsorbed to *Bacillus subtilis* spores. Effective delivery systems are essential for maintaining the antigenicity of chimeric protein vaccines (Cutting et al., 2009). *Bacillus subtilis* spores, known for their ability to interact with host mucosa and survive in diverse environmental conditions (Ricca et al., 2014; Zhao et al., 2014), have been investigated as a vaccine vehicle. Several studies have demonstrated that mucosal immunization with *B. subtilis* spores coated with antigens can elicit strong antibody responses, thereby conferring protection against various infections (Copland et al., 2018; Huang et al., 2010b; Song et al., 2012b; Zhao et al., 2014). In this study, the Spore-MhCP vaccine was further evaluated in sheep, as a ruminant model, due to their natural colonization by *M. haemolytica*, their similarities in rumination to cattle, and their use as a more cost-effective model compared to cattle.

Interactions between host microbiota and immune cells can impact vaccine effectiveness (Brotman et al., 2014; Valdez et al., 2014a). However, the direct or indirect effects of vaccination on respiratory tract microbiota are still underexplored. Therefore, we evaluated the upper respiratory microbiota from sheep nasopharyngeal samples to assess the impact of this mucosal vaccine on bacterial microbiota. Based on the evidence that intranasal administration of Spore-MhCP induced both mucosal and systemic immune responses in mice, we hypothesized that immunization with intranasally delivered Spore-MhCP would elicit similar immune responses in sheep and effectively reduce the relative abundance of *Mannheimia* in the upper respiratory tract. Therefore, the objectives of this study were to firstly, evaluate the immunogenicity of Spore-MhCP vaccine in sheep after delivery through oral or intranasal routes and secondly, explore the effect of Spore-MhCP vaccine on the sheep respiratory microbiota.

5.2 Materials and Methods

5.2.1 Vaccine (Spore-MhCP) preparation

The *M. haemolytica* chimeric protein MhCP (CTB-PlpE-NLKT-PlpE-NLKT), used in vaccine formulation was comprised of a truncated cholera toxin B subunit (CTB) along with two copies each of the immunodominant surface epitope (R2) from *M. haemolytica* PlpE and the neutralizing epitope of leukotoxin (NLKT) (Uddin et al., 2023a). MhCP had a calculated molecular weight of 56.4 kDa and a pI of 9.1. The chimeric protein MhCP was synthesized, purified and then adsorbed to *B. subtilis* spores, to use as vaccine (Spore-MhCP). The preparation of spores along with the construction, expression and purification of MhCP have been described previously (Uddin et al., 2023a). The adsorption of the chimeric protein MhCP to *B. subtilis* spores was conducted following previously established optimized conditions (Uddin et al., 2023a), where 100 µg of

MhCP was adsorbed to 2×10^{10} spores in phosphate buffered saline (PBS; pH 4). The resultant spore-bound vaccine Spore-MhCP was evaluated in sheep.

5.2.2 Animals and experimental design

The work described in this study was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol # 1906) before commencement of the trial, and was carried out in strict accordance with the recommendations established in the Canadian Council on Animal Care Guidelines (CCAC, 2009). Forty-eight male sheep, approximately 4 months old and of similar body weight (37.2 ± 1.9 kg), were sourced from the LeRDC herd, and housed in individual pens at the sheep barn. The sheep were randomly assigned to six experimental groups (N=8 per treatment): intramuscular free MhCP (IM:MhCP; positive control to measure systemic immunity of MhCP in native form, without spores); intranasal spore-bound MhCP (IN:Spore+MhCP); intranasal free MhCP without spore (IN:MhCP); intranasal spore without any MhCP (IN:Spore); intragastric spore-bound MhCP delivered in feed (IG:Spore+MhCP); and control sheep (Control; no MhCP administered, negative control, received PBS intranasally). Details of the experimental design are listed in Table 1.

5.2.3 Immunizations and sample collection

Sheep were immunized on days 0 and 14 with the exception of IG:Spore+MhCP (in-feed) group which were immunized on days 1, 2, 14, 15, 28 and 29. A total of 100 µg of antigen MhCP was administered per sheep per immunization time point. The IM:MhCP treatment group received antigen MhCP mixed with Incomplete Freund's adjuvant (IFA) in a 1:1 ratio. For intranasal vaccinations, the treatments were delivered as a liquid spray, with spore-bound antigen, free

antigen, and spores suspended in 2 ml of PBS, loaded into a LMA® MADgic® Laryngo-Tracheal Mucosal Atomization Device, and 1 mL was sprayed into each nasal cavity. For IG:Spore+MhCP (in-feed) vaccination, the spore-bound antigen was suspended in PBS and sprayed onto 500 g of alfalfa pellet and offered to sheep prior to the standard diet, to ensure complete consumption. The Control group received 2 ml of PBS by intra-nasal spray, with 1 ml applied to each nasal cavity. Blood, deep nasal swab (left nostril), and fecal samples were collected on days 0 (prior to vaccination), 7, 14 (prior to vaccination), 21, 28, 35, and 42 from all treatment groups. Bronchoalveolar lavage (BAL) samples were also collected on day 0, 14, 28 and 42. To determine the immunogenicity of vaccination routes, samples were analyzed quantitatively for antigen-specific IgG and IgA. An additional deep nasal swab from the right nostril was collected from IN:Spore+MhCP and Control sheep on days 0, 7, 14, 21, 28, and 35 to evaluate the effects of vaccine on the sheep respiratory bacterial microbiota.

5.2.4 Quantification of antigen-specific serum IgG

Antigen-specific IgG in sheep serum was quantified using enzyme-linked immunosorbent assay (ELISA). Individual components of the chimeric constructs (i.e., PlpE and NLKT) were synthesized commercially (BioBasic Inc., Markham, Canada). The sequences were optimized for expression in *Escherichia coli* and purified using immobilized metal affinity chromatography. The purified recombinant antigens, PlpE and NLKT, were suspended in coating buffer and used as ligands to coat ELISA plates at a concentration of 50 ng per well. The plates were then incubated overnight at 4°C. After washing the plates, blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) was added and incubated for an hour at room temperature. Serum samples were diluted in sample buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) and 100 µL aliquots of

each dilution were then added to wells in duplicate, followed by another hour-long incubation at room temperature. After the addition of horseradish peroxidase-conjugated secondary antibody (A50-104P-24; Bethyl Laboratories Inc.), the plates were incubated at room temperature for an hour. The color development was carried out using TMB (3,3′,5,5′-tetramethylbenzidine; ThermoFisher, Canada). The reactions were halted by adding ELISA stop solution (0.2 M H2SO4), and the optical densities of the reactions were measured at 450 nm using a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA) and analyzed with Gen5 software (BioTek Instruments Inc., Winooski, Vermont, USA). Concentrations were determined using a standard curve generated from a reference serum (RS10-108-2; Bethyl Laboratories Inc.).

5.2.5 Quantification of antigen-specific secretory IgA

BAL and fecal samples were pre-processed to quantify IgA as previously described (Hoang et al., 2008). The nasal swabs were pre-processed by incubating in PBS containing 0.1% BSA, 0.05% Tween 20, and 1 mM phenylmethylsulphonyl fluoride (PMSF) at normal room temperature for 1 h, followed by centrifugation at 13,000 Xg for 10 min. The resulting supernatant obtained from these processes was utilized to measure mucosal secretory IgA (sIgA) concentrations by ELISA. For the determination of IgA, 96-well ELISA plates were coated with 50 ng/well of purified recombinant antigens NLKT and PlpE separately, and incubated at 4 °C overnight. After a blocking solution was added, as described for IgG, samples were pipetted into individual wells in duplicate, followed by incubation at room temperature for 1 h. The plates were then washed and a HRP-conjugated secondary antibody (AHP949P; BioRad, Canada) was added, followed by an additional incubation period of 1 h. TMB substrate was added, and after color development, the

reaction was stopped by the addition of a stop solution. The plates were read at 450 nm using a Synergy HTX multi-detection microplate reader (BioTek Instruments Inc, Winooski, Vermont, USA) to determine optical density. Concentrations were determined using a standard curve generated from a sheep IgA standard (CLFA06, Cedarlane, Canada).

The ELISA data were analyzed using SAS PROC GLIMMIX (SAS version 9.4, SAS Inst. Inc., Cary, NC). For each generalized linear model, a distribution from the exponential family (gamma, inverse Gaussian, log-normal, normal, exponential, or shifted t) was selected based on the respective values of the Bayesian information criterion (BIC). A lower value of the BIC associated with the model indicated superior model fit. Statistical significance was indicated by p-values less than 0.05. Microsoft Excel 2010 and GraphPad Prism version 10.1.0 were utilized for visual representation of all ELISA analyses.

5.2.6 DNA extraction, amplicon sequencing and analysis

Nucleic acids were extracted from nasopharyngeal (NP) swabs using the method previously described (Holman et al. 2017). DNA was amplified using primers 515-F (5′-GTGYCAGCMGCCGCGGTAA-′3) and 806-R (5′-GGACTACNVGGGTWTCTAAT-′3) targeting the V4 region of the 16S rRNA gene, as described earlier (Holman et al., 2019). The amplicon was sequenced on a MiSeq instrument (PE250) using the MiSeq Reagent Kit v2, generating 25,000 read coverage at Genome Quebec in Montreal, Quebec, Canada.

Data quality assessment of the unprocessed reads was executed using FastQC v0.11.9 and MultiQC v1.12 (Ewels et al., 2016). Sequence trimming was conducted using Trimmomatic v0.39

(Bolger et al., 2014). Trimmomatic eliminated primer sequences and low-quality reads, employing the following parameters: HEADCROP:10, SLIDINGWINDOW:5:20, LEADING:20, and TRAILING:20. Subsequent statistical analyses were carried out using R version 4.2.2 (Team, 2013). Following sequence trimming, the paired reads underwent additional filtering utilizing the DADA2 v1.22.0 'filterAndTrim' function with default parameters and subsequently merged. Bimeric sequences were excised from the merged dataset using the DADA2 'removeBimeraDenovo' function. Taxonomic classification of the 16S sequences was achieved utilizing DADA2's 'IdTaxa' function and the SILVA 138 database (Quast et al., 2013), which created the amplicon sequence variants (ASVs) table. Data manipulation was conducted using Phyloseq v1.42.0 (McMurdie and Holmes, 2013), and figures were generated with ggplot2 v3.4.1 (Wickham, 2011). The α-diversities were computed via 'vegan' v2.6-4 (Oksanen et al., 2019) in the form of the Shannon diversity index and richness (Chao1), and corresponding plots were generated. To reduce noise in downstream analysis, the ASV table was filtered to retain ASVs present with a count of at least 2 in a minimum of 1% of the samples. One-way analysis of variance (ANOVA) was employed to assess α-diversity with respect to treatment and time. The package 'vegan' was used to calculate β-dispersion and permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations to examine the effects of treatment at each sampling time on the structure of the microbial community. β-diversity was calculated from the filtered ASV counts after they were subjected to size factor normalization using 'GMPR' within DESeq2 v1.40.2 (Love et al., 2014). Sample-to-sample distances were determined with the Bray-Curtis metric through Phyloseq ordination and plotted as a detrended correspondence analysis (DCA). Significant [$p < 0.01$] differentially abundant genera were identified with DESeq2 by fitting a negative binomial model to the equation sampling time X vs. Day 0 with respect to treatments

IN:Spore+MhCP vs Control ("~Treatment + Time + Time:Treatment"), and visualized with heatmaps.

5.3 Results

5.3.1 Antigen-specific antibody production in serum

On day 0, all sheep had low but detectable concentrations of serum IgG against *M. haemolytica* antigens NLKT and PlpE (Figure 5.1). All the treatments carrying antigen MhCP had higher concentrations of serum anti-PlpE and anti-NLKT IgG on at least one timepoint, compared to Control treatment (Figure 5.1). The serum IgG in Control and IN:Spore groups were not different at any time point. Intramuscular immunization with MhCP on day 0, resulted in increased serum antibody concentrations in the IM:MhCP group against both NLKT and PlpE by day 7 ($P<0.05$; Figure 5.1). A booster dose on day 14 further enhanced immune responses in the IM:MhCP treatment group such that maximum antibody concentrations were detected on day 21 against both NLKT and PlpE. The immune responses in the IM:MhCP group remained significantly higher until end of the study period (day 42), as compared to all other treatment groups ($P<0.05$). The IN:Spore+MhCP group reached maximum IgG antibody concentrations on day 21 against PlpE and NLKT after receiving the booster dose on day 14, and remained higher ($P<0.05$) until study end as compared to the Control treatment. After receiving the booster immunization, the IN:MhCP treatment group also showed higher anti-PlpE and anti-NLKT IgG antibody responses on day 21 ($P<0.05$), compared to the Control treatment. When comparing these two antigen carrying intranasal treatment groups, IN:Spore+MhCP induced higher anti-PlpE IgG concentrations ($P<0.05$) at most evaluated time points. Notably, intragastric (in-feed) vaccination resulted an increased anti-PlpE IgG concentrations on days 28 and 35 ($P<0.05$), when compared

to Control treatment. Overall, among the treatment groups immunized via oral or intranasal routes, the IN:Spore+MhCP group exhibited a tendency to increase serum IgG concentrations more rapidly than other groups and maintained higher levels compared to the Control treatment.

5.3.2 MhCP induced production of antigen-specific secretory antibodies

In nasal samples, all the treatment groups showed detectable concentrations of PlpE- and NLKT-specific IgA on day 0, indicating that sheep in this study had an immune response to *M. haemolytica* colonization prior to study initiation (Figure 5.2). Both of the spore-bound groups, IN:Spore+MhCP and IG:Spore+MhCP, showed a significant increase in anti-PlpE IgA concentrations over time compared to their pre-immunization levels (Figure 5.2A). Compared to day 0, IN:Spore+MhCP group exhibited increased concentrations across all the time points ($P<0.05$), and IG:Spore+MhCP group showed higher anti-PlpE IgA on days 7, 21 and 28 ($P<0.05$). IM:MhCP and IN:MhCP groups also showed a limited increase of anti-PlpE IgA concentrations ($P>0.05$), after receiving the booster immunization. However, unlike the IN:Spore+MhCP group, the antibody concentrations of these groups did not remain consistent until study completion and almost returned to pre-booster levels over time. No other treatment groups showed a significant increase in their anti-PlpE IgA concentrations compared to their initial levels. After receiving the initial immunization on day 0 and booster dose on day 14, the IN:Spore+MhCP group reached the highest anti-PlpE IgA concentrations on day 28, and it was the only group that showed higher anti-PlpE IgA at two time points when compared to Control treatment ($P<0.05$; Figure 5.2A). After receiving the booster immunization, both the IN:Spore+MhCP and IN:MhCP groups exhibited higher anti-NLKT IgA on days 21 to 42, when compared to the Control group ($P<0.05$; Figure 5.2B). No other treatment groups demonstrated a significant increase in their anti-NLKT IgA

concentrations compared to the control treatments or their initial levels. Overall, the IN:Spore+MhCP group exhibited a tendency to enhance the immune response faster than other treatments and maintained a higher PlpE- and NLKT-specific IgA until study end compared to the Control treatment.

Similar to nasal samples, sheep from all treatment groups had quantifiable PlpE- and NLKT-specific IgA in BAL samples on day 0, indicative of an immune response to *M. haemolytica* before study initiation (Figure 5.3). With the exception of the Control group, all the other treatment groups showed an increased anti-PlpE IgA concentration on days 28-42, as compared to their pre-immunization levels ($P<0.05$; Figure 5.3A). However, IN:Spore+MhCP was the only treatment group that showed higher anti-PlpE IgA on day 42, when compared to Control treatment ($P<0.05$). None of the other treatment groups showed increased anti-PlpE IgA concentrations compared to the control treatment at any time points ($P>0.05$; Figure 5.3A). Similar trends were observed while measuring the NLKT-specific IgA from BAL samples (Figure 5.3B). All the antigen MhCP carrying groups showed an increased anti-NLKT IgA concentrations on day 42, when compared to their pre-immunization levels ($P<0.05$; Figure 5.3B). IN:Spore and Control treatment groups did not increase anti-NLKT IgA concentrations compared to their levels on day 0. Apart from the IN:Spore treatment group, all the other groups carrying MhCP showed a greater anti-NLKT IgA levels on day 42 compared to the Control group ($P<0.05$; Figure 5.3B). However, immune responses in the IN:Spore+MhCP group was greatest both on days 28 and 42, indicating an earlier immune response in this group. Notably, the IG:Spore+MhCP group showed a sharp spike of anti-NLKT IgA on day 42, as compared to Control treatment or to the earlier time points of this group ($P<0.05$).

In fecal samples, both IN:Spore+MhCP and IN:MhCP groups reached their maximum anti-PlpE IgA titres on day 21 after receiving the booster immunization, and remained higher until day 35 as compared to their levels on day 0 ($P<0.05$; Figure 5.4A). However, antibody concentrations for both of these groups did not remain elevated until the end of the study, and stayed numerically higher compared to their initial levels ($P>0.05$). Compared to the pre-immunization levels, IG:Spore+MhCP group showed a higher anti-PlpE IgA by day 21 ($P<0.05$), and remained higher until end of the study (day 42). Compared to Control treatment, IN:Spore+MhCP and IN:MhCP groups showed higher anti-PlpE IgA concentrations only at one time point on day 21 ($P<0.05$; Figure 5.4A). Compared to day 0, the three intranasal treatment groups and the IG:Spore+MhCP group showed higher anti-NLKT IgA concentrations by day 21 ($P<0.05$) and remained elevated until the end of the study (Figure 5.4B). However, when compared to Control treatment, IN:Spore+MhCP and IN:MhCP groups showed higher anti-NLKT IgA concentrations on day 21 ($P<0.05$; Figure 5.4B). None of the other treatment groups demonstrated increased immune responses compared to the control treatment at any time points.

5.3.3 16S rRNA gene sequencing overview of sheep respiratory microbiota

The upper respiratory tract microbiota of sheep was analyzed in control and intranasal Spore-MhCP groups across time, by sequencing the 16S rRNA gene of nasal samples. Pre-processing steps produced 1,901 ASVs in 84 samples from 1,912,730 merged paired reads. At this stage the median number of sequences per sample was 21902.5 ± 7229, with a minimum of 9,599 and a maximum of 41,001. After removing the ASVs present in less than 1% of the samples, 432

ASVs comprised of 1,843,341 reads remained. The final median number of sequences was 20,732 ± 7382 with a minimum of 6,483 and a maximum of 40,772 sequences per sample.

5.3.3.1 The community structure of sheep respiratory microbiota

ANOVA testing of alpha diversity revealed that sampling time had a significant effect on richness and Shannon index ($P < 0.001$). A further TurkeyHSD analysis on richness and Shannon index with respect to the sampling time revealed the significant pairwise comparisons of times. The diversity on day 28 significantly decreased compared to days 0, 7, and 14, whereas day 35 was significantly lower compared to day 14 ($P < 0.05$; Figure 5.5). Overall the diversity decreased across time. These differences over time were observable in the plotted richness and Shannon index metrics (Figure 5.5A and 5.5B). IN:Spore+MhCP was also determined to significantly lower mean Shannon diversity index when compared to the control ($P < 0.05$).

PERMDISP testing with respect to time and treatment variables determined that only time was significant ($P < 0.001$), suggesting that sampling times do not have homogenous dispersion of microbial communities. Further testing with PERMANOVA identified that sampling time was significant ($R2 = 0.361$, $P < 0.001$), but treatment and the interaction of treatment and time of was not. These results further verified that there were significant differences in microbial communities between sampling times. These trends were observed within the DCA plots as the samples within each treatment clustered differently with time (Figure 5.6).

5.3.3.2 Composition of sheep respiratory microbiota

Across treatment groups and sampling time, a total of 20 different bacterial phyla were identified, and unidentifiable reads were classified as "Unclassified". The percentage of reads of the ten most abundant phyla were: Proteobacteria, 69.84%; Bacteroidota, 11.38%; Firmicutes, 11.05%; Actinobacteriota, 6.63%; Euryarchaeota, 0.12%; Planctomycetota, 0.05%; Chloroflexi, 0.04%; Spirochaetota, 0.01%; Deinococcota, 0.01%; Verrucomicrobiota, 0.01%; and Unclassified, 0.83%. The relative abundance of Proteobacteria increased in both treatments at days D28 and D35 compared to their initial time points (Appendix I). When evaluated across sampling time and treatment groups, a total of 195 genera were identified. The top 9 most abundant classified genera and their relative abundance in the dataset were: *Mycoplasma*, 7.03%; *Moraxella*, 5.17%; *Mannheimia*, 3.89%; *Corynebacterium*, 1.62%; *Dietzia*, 1.34%; *Brevibacterium*, 1.03%; *Brachybacterium*, 0.98%; *Staphylococcus*, 0.94%; and *Jeotgalicoccus*, 0.61% (Figure 5.7).

5.3.3.3 Changes in sheep respiratory microbiota across sampling time and within the treatments

A total of 27 genera were identified that showed a significant change ($P < 0.01$, log2(FC) > 2 or log2(FC) < -2) from baseline (day 0) in the IN:Spore+MhCP treatment above and beyond any changes in the Control sheep over the course of 35 days (Figure 5.8). For the IN:Spore+MhCP group, 7 genera increased at more than a single time point, while 8 genera decreased for more than one time point and almost all of them remained lower until day 35. Notably, *Mannheimia* were consistently decreased over time in IN:Spore+MhCP group, compared to Control. The relative abundance of *Aliicoccus*, *Brachybacterium, Dietzia, Jeotgalicoccus, Methanobrevibacter, Mycoplasma, and Staphylococcus* also decreased at most time points. In contrast, *Acinetobacter*, *Bifidobacterium*, *Brevibacterium*, *Corynebacterium,* and *Facklamia* increased at most time points.

5.4 Discussion:

In this study, immunization of sheep with both unbound MhCP and Spore-MhCP resulted in the production of specific antibodies against NLKT and PlpE, indicating that the chimeric antigen MhCP maintained its immunogenicity even after being adsorbed onto spores, and that the antigen constructs individually induced seroconversion. Chimeric proteins possess versatility, facilitating the delivery of multiple antigens to the immune system, rendering them promising candidates for vaccine development (Boucher et al., 1994b; Hertle et al., 2001a). Previous studies established that components of the chimeric protein MhCP, i.e., NLKT and PlpE, were immunogenic (Ayalew et al., 2008; Ayalew et al., 2009b; Uddin et al., 2023a). This study further validates this finding, as evidenced by the induction of immune responses against NLKT and PlpE in sheep following intramuscular immunization with unbound MhCP. Sheep are naturally colonized by *M. haemolytica*, with serotype 2 being commonly pathogenic in this species. In this study, elevated levels of both PlpE- and NLKT-specific IgA were observed on day 0 (before first immunization), in all mucosal samples across all treatment groups, suggesting pre-existing immune responses to *M. haemolytica* colonization before the study commenced. This was supported by sequencing analysis of the nasal samples from IN:Spore+MhCP and Control groups, where *Mannheimia* was evident on day 0. Although the initial immunization on day 0 did not induce a significant mucosal immune response in treatment groups, the booster dose elicited a sharp spike of the antibody concentrations. The delayed immune response observed during the initial two weeks could be attributed to the heightened levels of early IgA specific to *M. haemolytica* antigens. This could have, potentially hindered the mucosal antibody responses

following the first immunization. The finding of an initial delayed immune response in the current study aligns with previous research findings (Ayalew et al., 2009b; Shewen et al., 2009).

All treatments containing the MhCP antigen resulted in increased systemic immune response, with IN:Spore+MhCP group showing the earliest increase on day 7 among mucosal routes, as compared to Control indicating a rapid immune response. In addition, intranasal administration of Spore-MhCP induced greater PlpE-specific IgG compared to unbound MhCP, though this was not the case for NLKT-specific IgG. The IN:Spore-MhCP group resulted in stronger secretory IgA immune responses across all evaluated mucosal samples, including nasal, BAL and fecal samples, demonstrating a robust mucosal and systemic immune responses. Between the two intranasal groups carrying MhCP, the IN:Spore+MhCP group exhibited a tendency to enhance the immune response faster and maintained higher levels of PlpE- and NLKT-specific IgA until the end of the study in most cases. This suggests that the binding of MhCP to Bacillus spores led to an augmented immune response, emphasizing spores as effective adjuvants. Ayalew and colleagues (2009) conducted an animal trial where weaned beef calves were immunized intranasally, on days 0 and 14, with the R2-NLKT-R2-NLKT chimeric protein, mixed with and without native cholera toxin (Ayalew et al., 2009b). The same study also included another treatment group where calves were vaccinated intranasally with CTB appended chimeric protein, CTB-R2-NLKT (Ayalew et al., 2009b). Their study revealed that vaccination with these chimeric proteins enhanced resistance to intrabronchial challenge with *M. haemolytica* and induced antibody responses against the bacterium. However, significant increases in antibody responses against individual constructs were observed only following bacterial challenge (Ayalew et al., 2009b). Although our study did not involve challenging sheep with *M. haemolytica*, the significant

increase in immune responses observed following booster immunization of sheep with intranasal Spore-MhCP features the effectiveness of the spore-based technology utilized in this study. A recent attempt has been made to develop a recombinant bovine herpes virus-1 vectored vaccine expressing PlpE-LKT chimeric proteins (Batra et al., 2017). Although anti-LKT antibodies were produced following intranasal vaccination of bighorn sheep, inconsistent development of antibodies against surface antigens and failure of the vaccine to protect against *M. haemolytica* challenge emphasize the importance of antigen delivery in immunization (Batra et al., 2017). The notable enhancements in immune responses observed in the intranasal spore-bound group, following booster immunization in our study highlights the effectiveness of the *B. subtilis* spores in augmenting immune responses as an adjuvant.

In-feed or oral vaccines hold significant appeal for the livestock industry due to their ease of administration, requiring minimal expertise and time to administer to livestock. Despite the limited effectiveness of intragastric delivery of Spore-MhCP in mice (Uddin et al., 2023a), we explored its in-feed delivery in sheep. The rationale was that prolonged rumination in sheep could potentially allow antigens to remain in the upper digestive tract long enough to stimulate laryngeal lymphoid tissue (Shewen et al., 2009), which could result in immunity through the upper respiratory tract instead of the lower gastrointestinal tract. In this regard, we fed sheep a high-fiber alfalfa-based diet to promote prolonged rumination. Compared to the pre-immunization titres, IG:Spore+MhCP group resulted in significantly higher PlpE- and NLKT-specific IgA in BAL and fecal samples after receiving the third round of immunization, and remained elevated until end of the study. The immune response generated from the in-feed immunization of Spore-MhCP vaccine is a noteworthy finding. This indicates that, when bound to spores, the recombinant protein resisted

degradation sufficiently for both LktA and PlpE antigenic components to be taken up by M cells, activating immunocompetent cells in the mucosa-associated lymphoid tissue (Taghavian et al., 2013). Additionally, the presence of antigen-specific secretory antibodies in the lungs of sheep from the IG:Spore+MhCP group suggests the possible trafficking of stimulated immune cells from the gut, while antigen-specific IgA in feces may indicates a local response (Shewen et al., 2009). While it was not possible to determine whether the immune activation of the IG:Spore+MhCP group occurred from laryngeal activation or trafficking from the gut, it could have been a combination of both. Unlike IN:Spore+MhCP group, in-feed group required three rounds of immunizations. One of the limitations of this study was the absence of in-feed unbound antigen group. However, the strong increase in immune responses in the lungs after the third in-feed Spore+MhCP vaccination suggests that it may be effective in reducing the severity of lung infection by *M. haemolytica*.

Nasopharyngeal microbiota profiling showed the most prevalent phyla identified in the nasopharynx of sheep were Proteobacteria, Bacteroidota, Firmicutes, and Actinobacteriota. This finding aligns with previous studies that have also observed Proteobacteria, Bacteroidota, and Firmicutes as predominant phyla in the nasopharynx (Ding et al., 2023) and lungs (Miao et al., 2023) of sheep. The predominance of *Mycoplasma*, *Moraxella*, and *Mannheimia* as the most abundant genera in our study is also consistent with findings from previous reports in sheep (Ding et al., 2023; Miao et al., 2023). The composition and structure of the microbial communities in the nasopharynx exhibited changes over time, likely influenced by the vaccination process. The intranasal vaccination of Spore-MhCP exhibited significant alterations in a total of 27 genera. Among them 8 genera showed a decrease in abundance, while 7 showed increase in abundance for

more than a single time point, compared to Control treatment. Most notable was a consistent decrease of *Mannheimia* in the IN:Spore+MhCP group compared to the Control group, which could be attributed to the heightened antibody levels induced by intranasal immunization with spore-MhCP. While it is difficult to explain, this spore-bound intranasal vaccine also demonstrated a reduction in the abundance of important pathogenic genera, including *Moraxella*, *Mycoplasma*, and *Staphylococcus*, at one or more time points. The probiotic *Bacillus* spores utilized in the Spore-MhCP vaccine formulation may potentially have a contributory effect. *Bacillus* is used as a gastrointestinal probiotic in both human and livestock, and is known for its potential health benefits (Cutting, 2011). Probiotics are generally believed to positively influence the microbiota in a way that promotes host well-being. While *Bacillus* has been used as probiotic to decrease pathogens (Miljaković et al., 2020), it did not increase in abundance after intranasal administration of Spore-MhCP and spore did not germinate in the sheep upper respiratory tract. Therefore, it is likely that changes observed in microbiota were due to specific (i.e. *Mannheimia*) and general immune responses from host interaction with this Spore-MhCP vaccine. The spore-based technology presents an opportunity to integrate immunologically significant antigens from additional pneumonia or BRD-causing pathogens like *Pasteurella multocida* and *Histophilus somni*. This strategy holds potential for creating a comprehensive multivalent vaccine for ruminants, providing immunity against a wider range of respiratory diseases.

5.5 Conclusion

Intranasal vaccination with spore-bound antigen resulted in mucosal and systemic antigen-specific antibody production. Combined with the analysis of the sheep nasopharyngeal microbiota, secretory IgA to *M. haemolytica* resulting from IN:Spore+MhCP showed potential to reduce

colonization by this pathogen. In addition, in-feed spore-bound antigen shows promising results and can be considered as a potent oral vaccine that would allow for large-scale administration. These qualities are especially important as livestock management strategies, including vaccination, are cost and ease-of-use dependent. Since the proliferation of *M. haemolytica* in the upper respiratory tract is a prerequisite to lung infection, this spore-bound vaccine may offer protection against proliferation and subsequent infection. Future challenge studies are warranted to fully evaluate the efficacy of spore-based vaccines against *M. haemolytica*. Furthermore, while immune responses can be hindered by passive or pre-existing immunity (Windeyer and Gamsjäger, 2019), this current study demonstrated that intranasal immunization of Spore-MhCP still elicited a strong immune response despite sheep having detectable levels of antibodies at the beginning of the study. This suggests that *Bacillus* spores present a promising vaccination technology and warrant further investigation in cattle with passive immunity, such as newborn calves, or that may have been exposed naturally to *M. haemolytica*, such as those transported to feedlots.

5.7 Tables and figures

Table 5.1 Experimental design in which sheep were immunized with *Mannheimia haemolytica* chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT) via different routes either in free form or bound to *B. subtilis* spores [a]

Treatment group	No. of Sheep	Route of administration	Vaccine formulations (amount per dose)	Samples collected
IM: MhCP	8	Intra-muscular	MhCP (100 µg) mixed with IFA; No Spore	Blood, BAL, NS, Feces
IN: Spore+MhCP	8	Intra-nasal	Spore (2×10^{10}) bound MhCP (100 µg)	Blood, BAL, NS, Feces
IN: MhCP	8	Intra-nasal	MhCP (100 µg) only; No Spore	Blood, BAL, NS, Feces
IN: Spore	8	Intra-nasal	Spore (2×10^{10}) only; No MhCP	Blood, BAL, NS, Feces
IG: Spore+MhCP	8	Intra-gastric/In-feed	Spore (2×10^{10}) bound MhCP (100 µg)	Blood, BAL, NS, Feces
Control	8	Intra-nasal	PBS; No MhCP; No Spore	Blood, BAL, NS, Feces

[a] Sheep were immunized on days 0 and 14 with the exception of the Intra-gastric/In-feed group which were immunized on days 1, 2, 14, 15, 28 and 29. A total of 100 µg antigen was administered per sheep per immunization time point. Blood, nasal secretions (NS), and fecal samples were collected on days 0 (prior to vaccination), 7, 14 (prior to vaccination), 21, 28, 35, and 42. Bronchoalveolar lavage (BAL) samples were also collected on days 0, 14, 28 and 42.

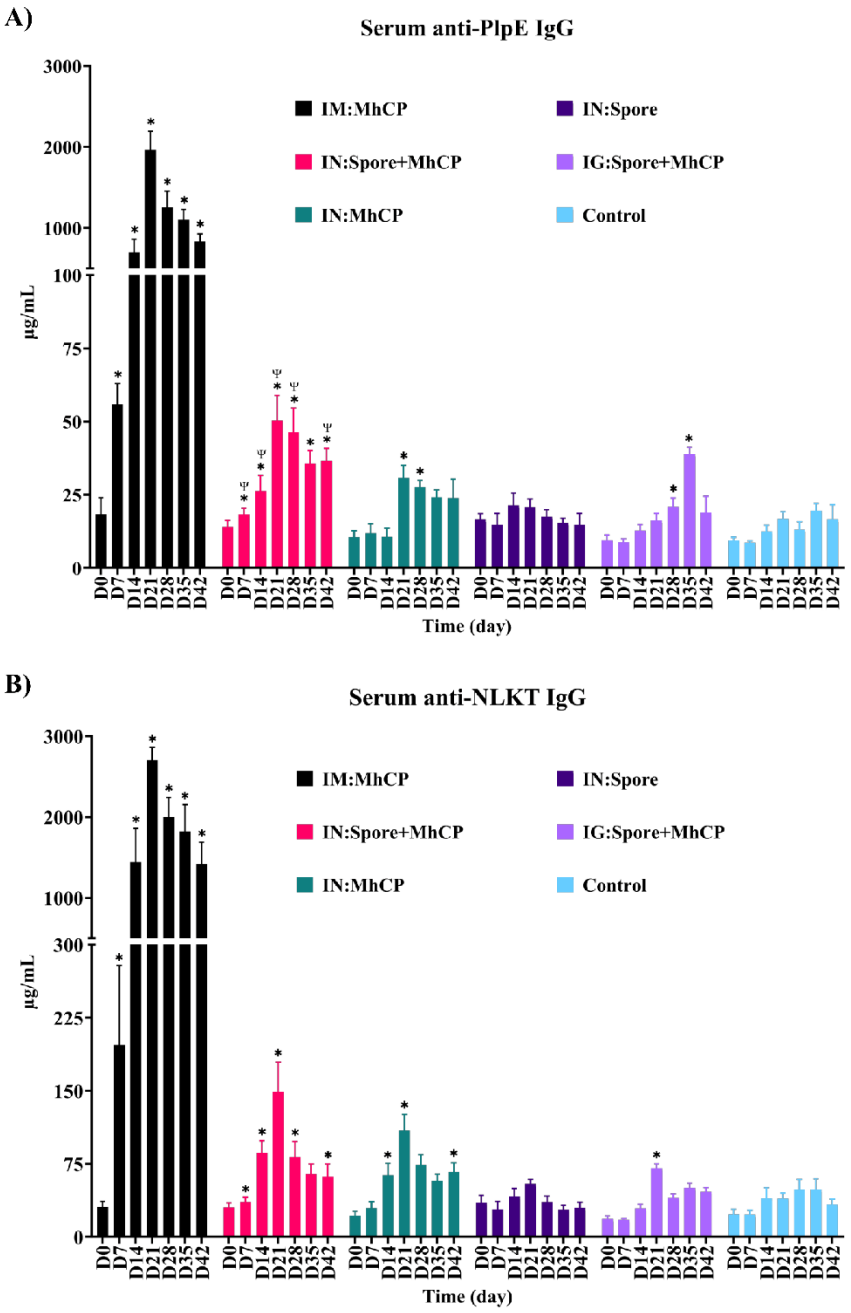


Figure 5.1: Antigen-specific serum IgG responses in sheep. *Mannheimia haemolytica* chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT) was used to immunize sheep via different routes either in free form or after bound to *B. subtilis* spores. A total of 48 sheep were divided into six experimental groups (N=8 sheep per treatment): Intramuscular free antigen (IM:MhCP), Intranasal spore-bound antigen (IN:Spore+MhCP), Intranasal free antigen only (IN:MhCP), Intranasal spore only (IN:Spore), Intragastric spore-bound antigen delivered in feed (IG:Spore+MhCP) and Control/Naïve sheep (Control; no antigen used, negative control). Sheep were immunized on days 0 and 14, with the exception of the IG:Spore+MhCP (in-feed) group, which was vaccinated on days 1, 2, 14, 15, 28 and 29. Blood samples were collected on days 0, 7, 14, 21, 28, 35, and 42 and sera was isolated and analyzed by enzyme linked immunosorbent assay (ELISA) to measure anti-PlpE and anti-NLKT antibodies. IgG specific to PlpE (A) and NLKT (B) are shown (mean ± SEM). * $p < 0.05$ when compared to the same timepoint of Control treatment. $^{\Psi}$ $p < 0.05$ when compared between two intranasal treatment groups (IN:Spore+MhCP vs IN:MhCP).

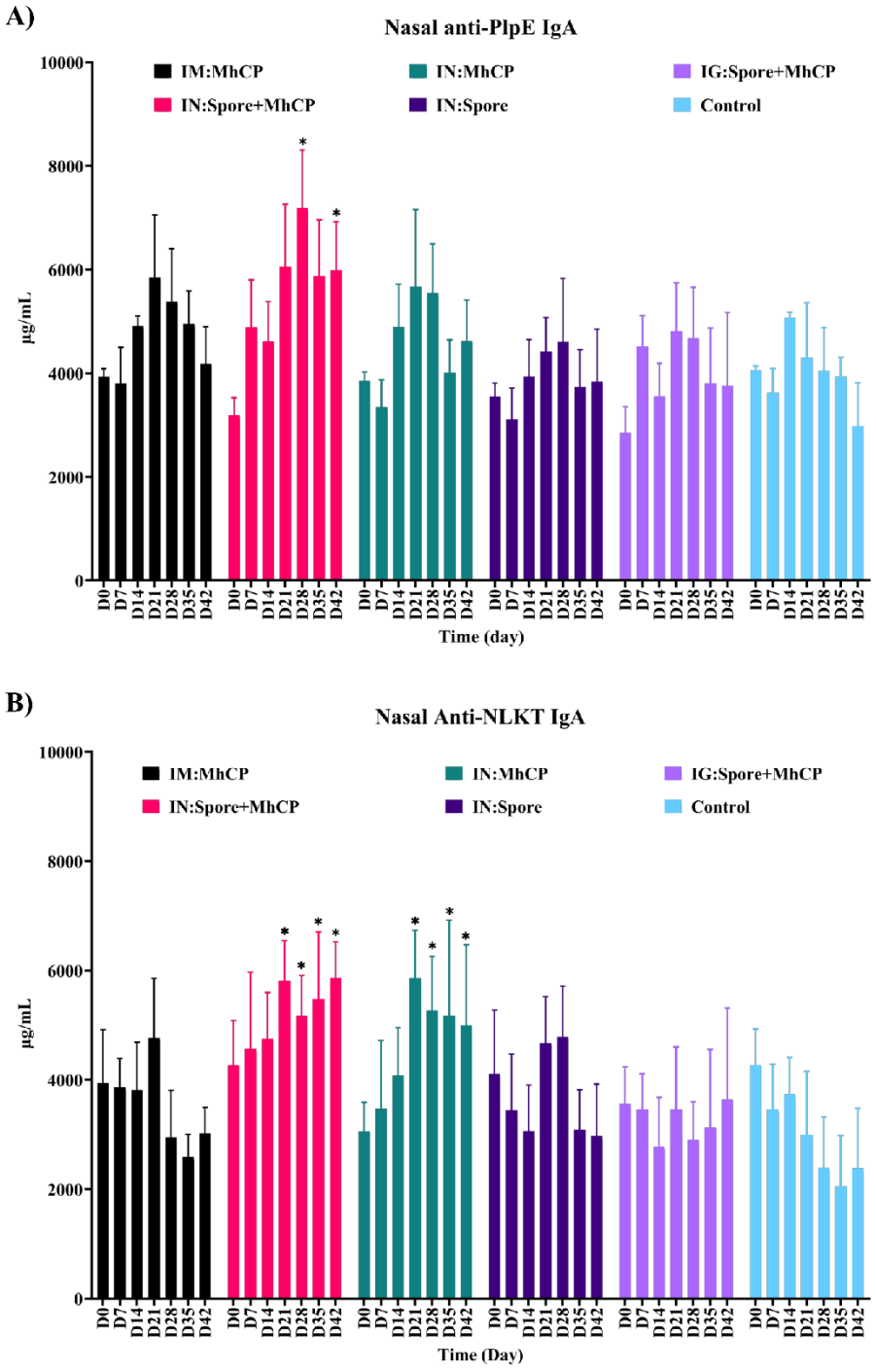


Figure 5.2: Mucosal IgA immune responses from sheep nasal secretions. *Mannheimia haemolytica* chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT) was used to immunize sheep via different routes either in free form or after bound to *B. subtilis* spores. A total of 48 sheep were divided into six experimental groups (N=8 sheep per treatment): Intramuscular free antigen (IM:MhCP), Intranasal spore-bound antigen (IN:Spore+MhCP), Intranasal free antigen only (IN:MhCP), Intranasal spore only (IN:Spore), Intragastric spore-bound antigen delivered in feed (IG:Spore+MhCP) and Control/Naïve sheep (Control; no antigen used, negative control). Sheep were immunized on days 0 and 14, with the exception of the IG:Spore+MhCP (in-feed) group, which was vaccinated on days 1, 2, 14, 15, 28 and 29. Nasal samples were collected on days 0, 7, 14, 21, 28, 35, and 42 and analyzed by ELISA to measure anti-PlpE and anti-NLKT antibodies. IgA specific to PlpE (A) and NLKT (B) are shown (mean ± SEM). * $p < 0.05$ when compared to the same timepoint of Control treatment.

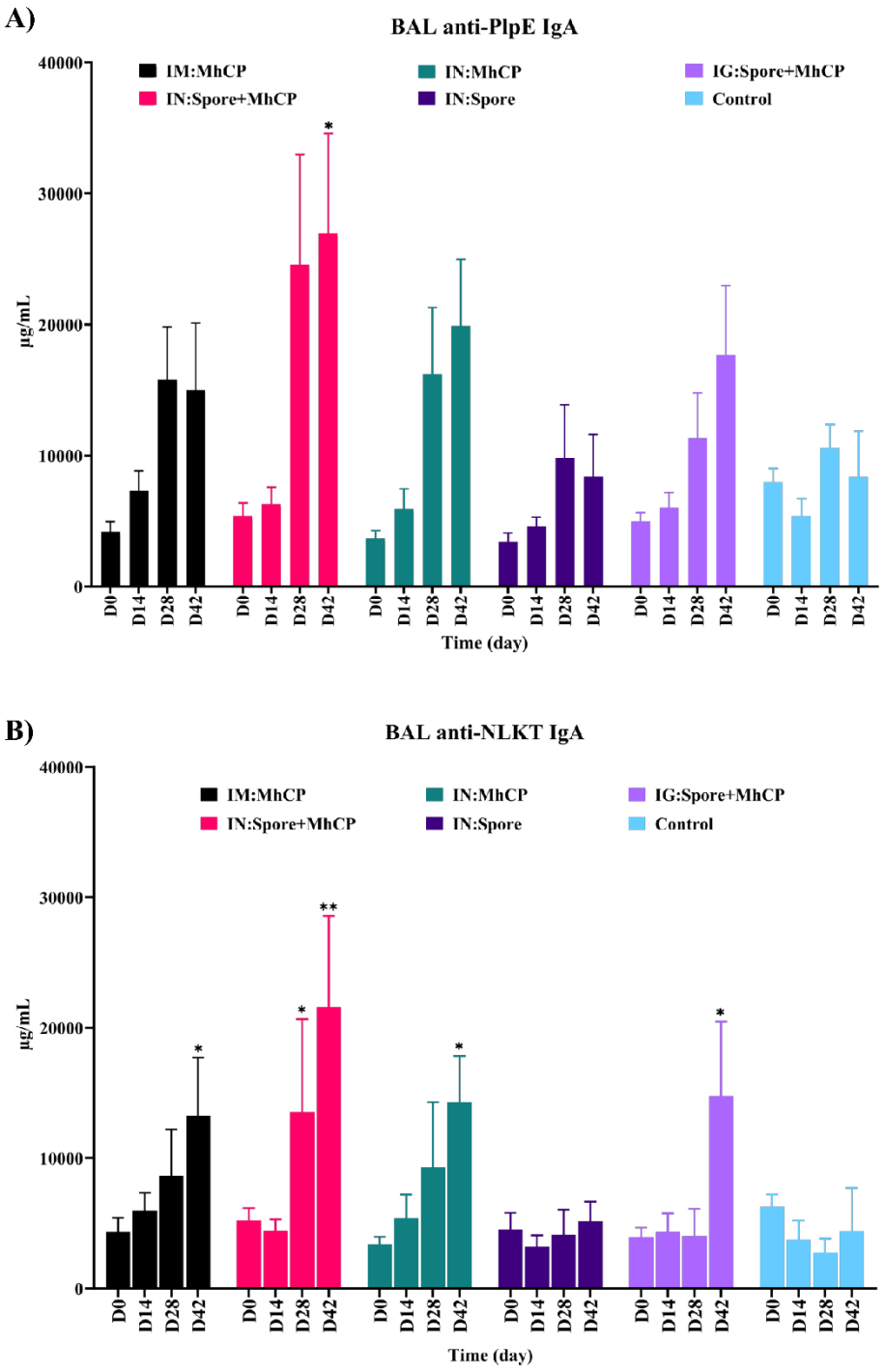


Figure 5.3: Mucosal IgA immune responses from sheep Bronchoalveolar lavage (BAL). *Mannheimia haemolytica* chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT) were immunized via different routes either in free form or after bound to *B. subtilis* spores. A total of 48 sheep were divided into six experimental groups (N=8 sheep per treatment): Intramuscular free antigen (IM:MhCP), Intranasal spore-bound antigen (IN:Spore+MhCP), Intranasal free antigen only (IN:MhCP), Intranasal spore only (IN:Spore), Intragastric spore-bound antigen delivered in feed (IG:Spore+MhCP) and Control/Naïve sheep (Control; no antigen used, negative control). Sheep were immunized on days 0 and 14, with the exception of the IG:Spore+MhCP (in-feed) group, which was vaccinated on days 1, 2, 14, 15, 28 and 29. BAL samples were collected on days 0, 14, 28 and 42 and analyzed by ELISA to measure anti-PlpE and anti-NLKT antibodies. IgA specific to PlpE (A) and NLKT (B) are shown (mean ± SEM). * $p < 0.05$, ** $p < 0.01$ when compared to the same timepoint of Control treatment.

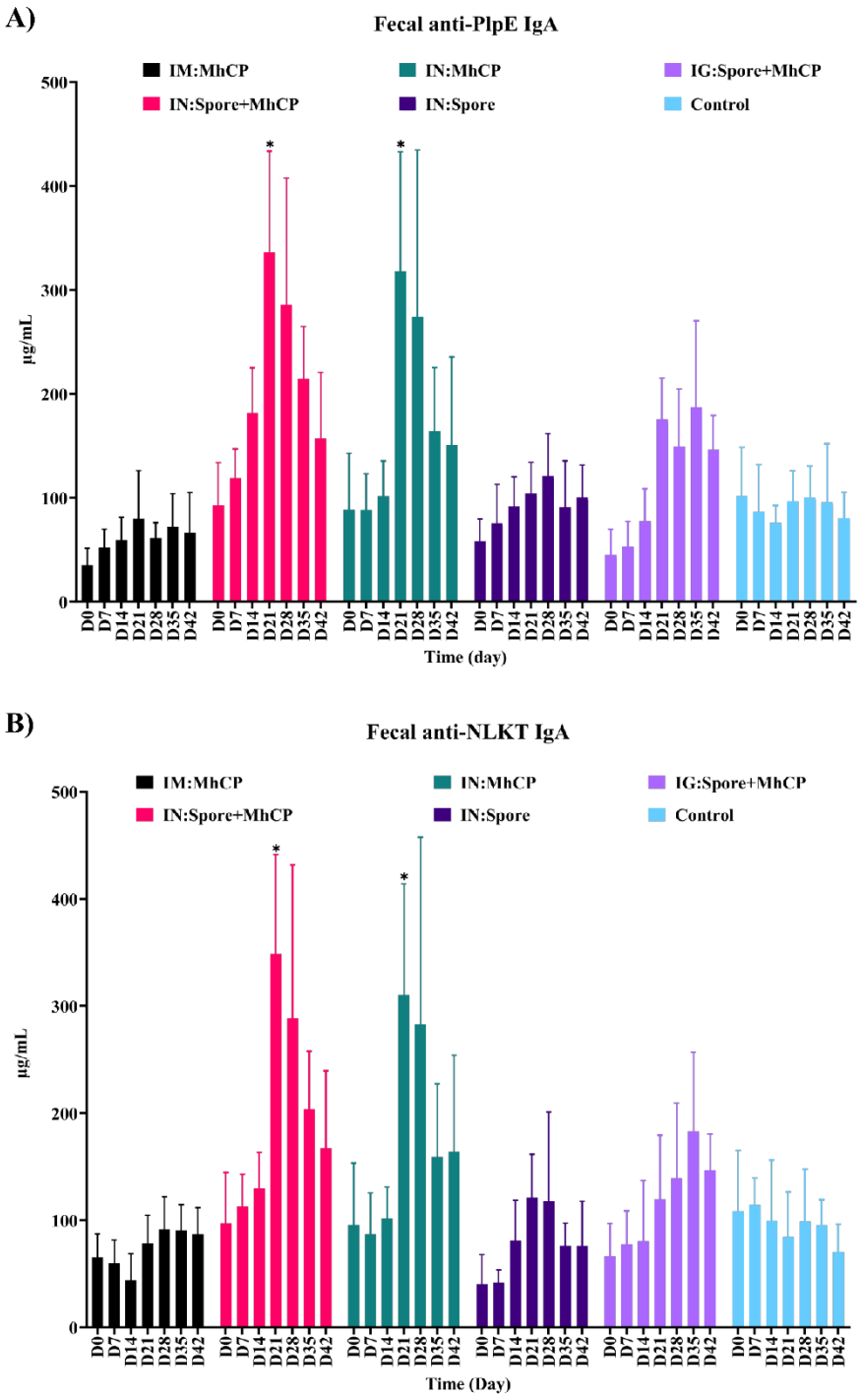


Figure 5.4: Mucosal IgA immune responses from sheep feces. *Mannheimia haemolytica* chimeric protein MhCP (CTB+PlpE+NLKT+PlpE+NLKT) were immunized via different routes either in free form or after bound to *B. subtilis* spores. A total of 48 sheep were divided into six experimental groups (N=8 sheep per treatment): Intramuscular free antigen (IM:MhCP), Intranasal spore-bound antigen (IN:Spore+MhCP), Intranasal free antigen only (IN:MhCP), Intranasal spore only (IN:Spore), Intragastric spore-bound antigen delivered in feed (IG:Spore+MhCP) and Control/Naïve sheep (Control; no antigen used, negative control). Sheep were immunized on days 0 and 14, with the exception of the IG:Spore+MhCP (in-feed) group, which was vaccinated on days 1, 2, 14, 15, 28 and 29. Fecal samples were collected on days 0, 7, 14, 21, 28, 35, and 42 and analyzed by ELISA to measure anti-PlpE and anti-NLKT antibodies. IgA specific to PlpE (A) and NLKT (B) are shown (mean ± SEM). * $p < 0.05$ when compared to the same timepoint of Control treatment.

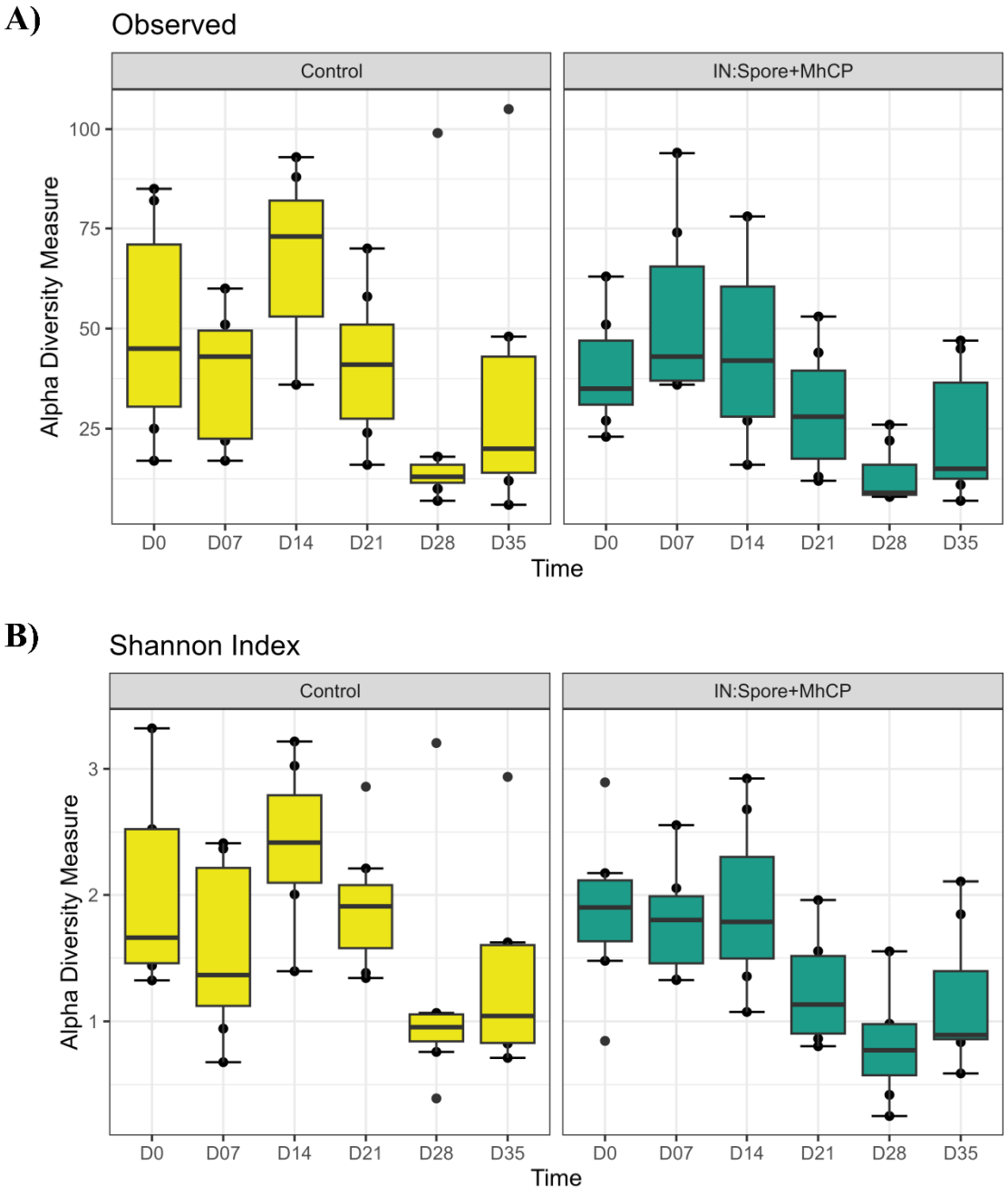


Figure 5.5: Alpha diversity of nasopharyngeal bacteria from vaccinated sheep. Upper plot shows the bacterial richness or counts of taxa observed (A), and lower plot shows the Shannon diversity index (B). The box in the plots indicates the interquartile range (IQR; middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR. Sheep were immunized on days 0 and 14. Deep nasal swabs (from right nostril) were collected from Intranasal spore-bound antigen (IN:Spore+MhCP) and Control/ Naïve (Control; administered intranasal saline) treatment groups on days 0, 7, 14, 21, 28, and 35 and analyzed by sequencing of the 16S rRNA gene.

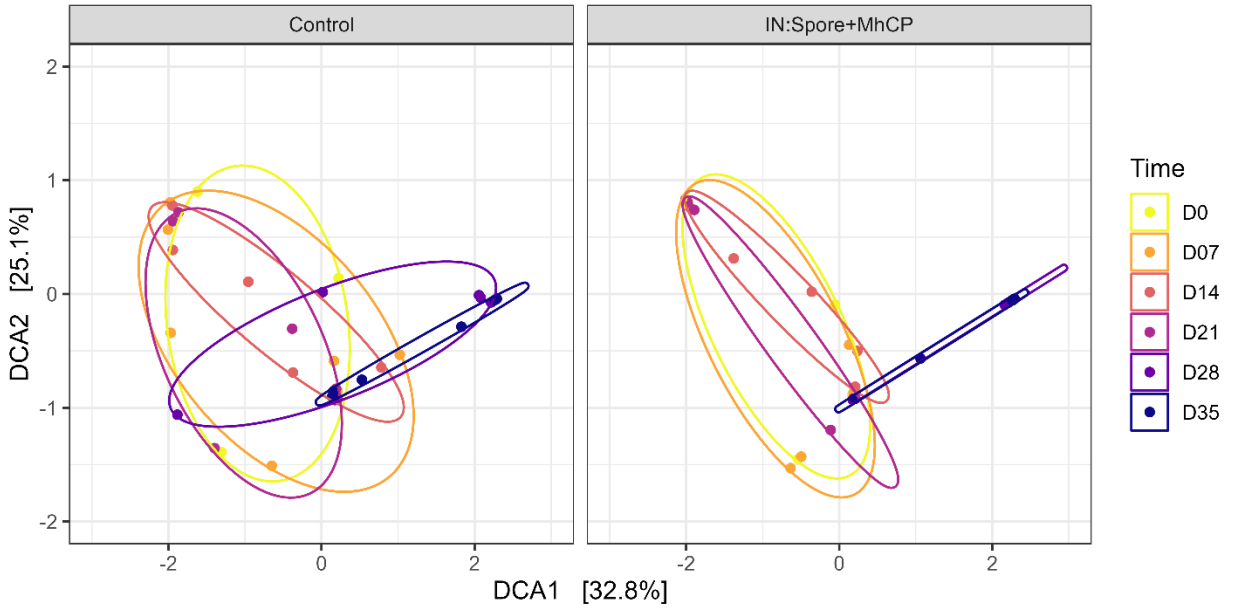


Figure 5.6: Detrended correspondence analysis (DCA) plots of the Bray-Curtis metric of nasopharyngeal samples from vaccinated sheep. Sheep were immunized on days 0 and 14. Deep nasal swabs (from right nostril) were collected from Intranasal spore-bound antigen (IN:Spore+MhCP) and Control/ Naïve (Control; administered intranasal saline) treatment groups on days 0, 7, 14, 21, 28, and 35 and analyzed by sequencing of the 16S rRNA gene.

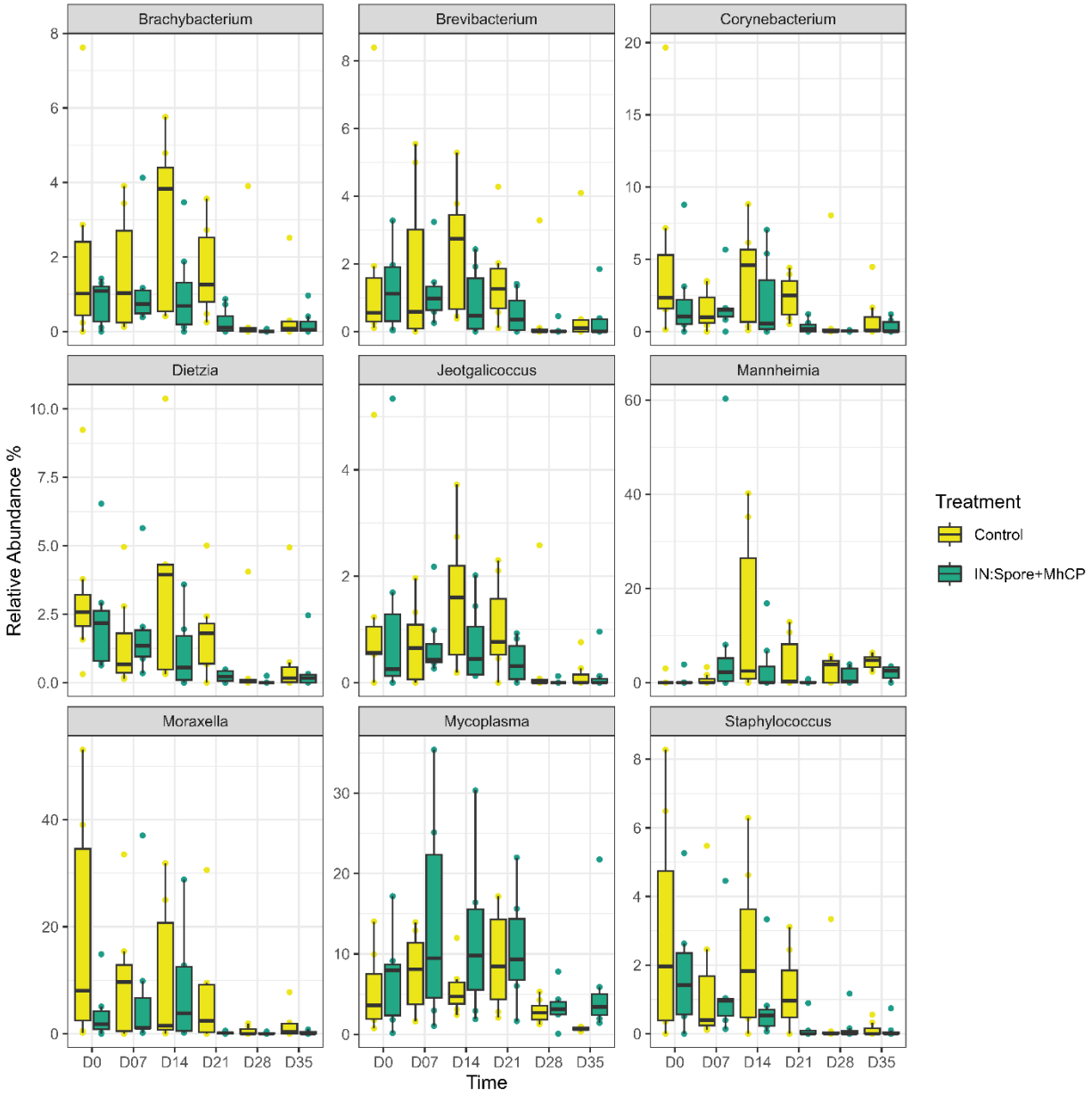


Figure 5.7: Relative abundance of the top nine most abundant genera identified in nasopharyngeal samples from vaccinated sheep. Sheep were immunized on days 0 and 14. Deep nasal swabs (from right nostril) were collected from Intranasal spore-bound antigen (IN:Spore+MhCP) and Control/ Naïve (Control; administered intranasal saline) treatment groups on days 0, 7, 14, 21, 28, and 35 and analyzed by sequencing of the 16S rRNA gene. The box in the plots indicates the interquartile range (IQR; middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.

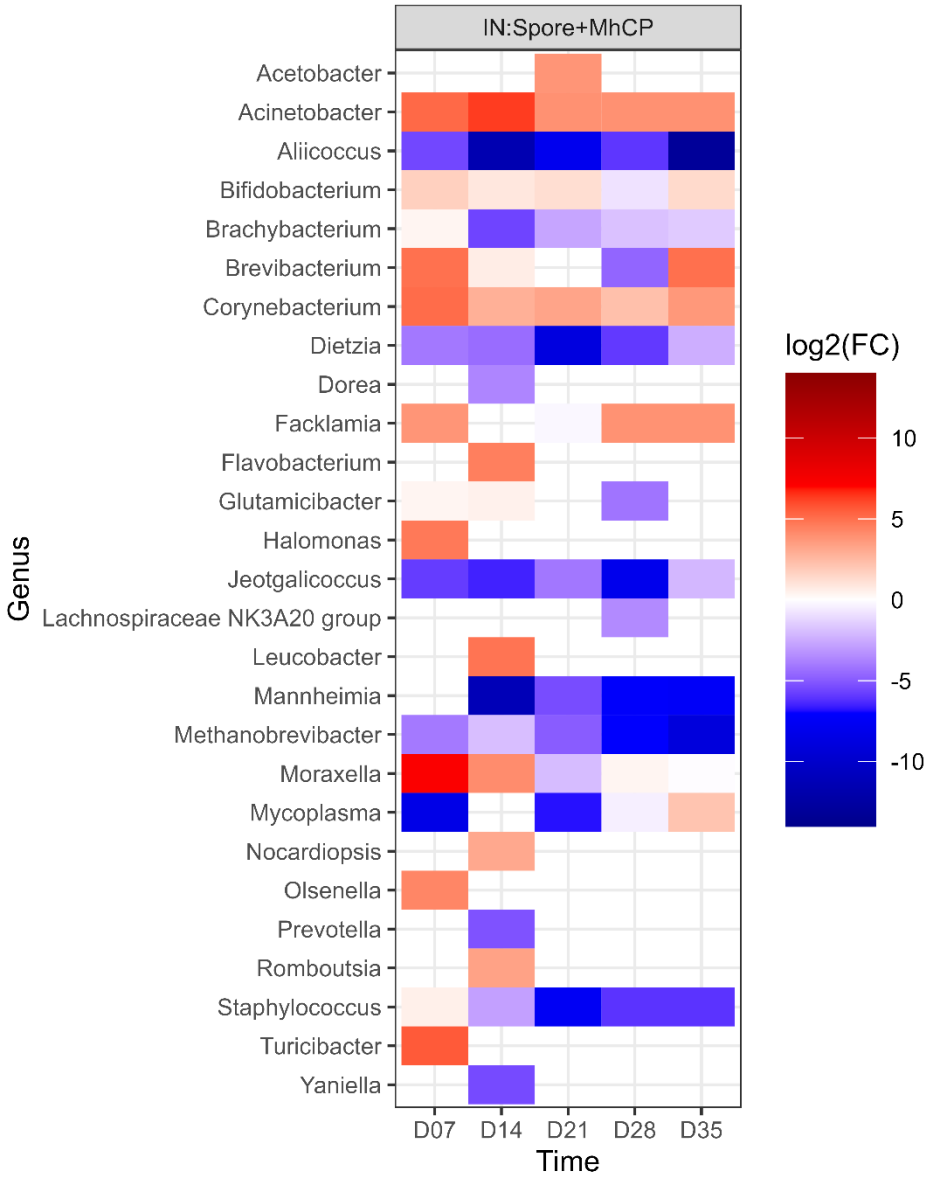


Figure 5.8: Genera that showed a significant change (P < 0.01, log2(FC) > 2 or log2(FC) < -2) from baseline (day 0) in IN:Spore+MhCP treatment group above and beyond any changes in the Control sheep over the course of 35 days. Sheep were immunized on days 0 and 14. Deep nasal swabs (from right nostril) were collected from Intranasal spore-bound antigen (IN:Spore+MhCP) and Control/ Naïve (Control; administered intranasal saline) treatment groups on days 0, 7, 14, 21, 28, and 35 and analyzed by sequencing of the 16S rRNA gene.

Chapter Six

General discussion, future directions, and conclusions

6.1 General discussion

Bovine respiratory disease (BRD) is the most financially burdensome illness for beef cattle in North America. Decades of research on BRD have not led to significant changes in its morbidity and mortality rates (Taylor et al., 2010). Transitioning to feedlots exposes cattle to stressors that make them vulnerable to respiratory infections (Nickell and White, 2010). In North American feedlots, antimicrobials are mainly used to address the economic and welfare impacts of BRD by broadly targeting bacterial pathogens (Duff and Galyean, 2007; Hilton, 2014). However, the growing concerns over antimicrobial use in livestock and increasing antimicrobial resistance in key BRD and zoonotic pathogens (Catry et al., 2016), highlight the need for alternative strategies to reduce antimicrobial dependency for BRD prevention in feedlot cattle. Preconditioning programs are designed to reduce stress, enhance host health, and better prepare beef calves for subsequent production stages, though they are partially dependent on the efficacy of vaccines used. This thesis aimed to evaluate the impact of preconditioning on stress, immune responses, and respiratory bacterial communities in beef cattle, as well as to develop a spore-based mucosal vaccine against a BRD pathogen, using *M. haemolytica* as a model.

At feedlot arrival, BRD pathogens can proliferate in the upper respiratory tract of cattle and migrate to the lungs, causing bronchopneumonia (Caswell, 2014). A central premise of this thesis postulated that implementing a preconditioning program would enhance respiratory health by increasing vaccine-enhanced immunity against BRD pathogens and lowering stress and subsequent pathogen colonization. In Chapter 2, a preconditioning program was evaluated that

included three vaccinations against viral and bacterial respiratory pathogens, low-stress weaning, and introduction to a feedlot bunk for feeding prior to transportation. It was shown that preconditioning reduced physiological stress response in preconditioned calves, as indicated by quantifying serum cortisol. After transportation, the cortisol levels in non-preconditioned calves increased, which is supported by findings from other studies (Cooke et al., 2013b; Grandin, 1994). These elevated levels persisted for up to six days after the calves arrived at the feedlot and were higher compared to preconditioned calves. This was likely due to the combined stress of abrupt weaning and inexperience in a feedlot pen for nonpreconditioned calves, as cortisol is associated with psychological stress (Mellor et al., 2002). Elevated cortisol levels have been linked to imbalances in the oral (Duran-Pinedo et al., 2018), nasal (Zhao et al., 2020), and gut microbiota (Mudd et al., 2017). A recent study reported a positive correlation between blood cortisol levels and certain respiratory bacterial genera such as *Mannheimia*, *Moraxella*, and *Streptococcus* (Uddin et al., 2023b). In Chapter 2, it was observed that *Pasteurella* and *Moraxella* were reduced in preconditioned calves, despite animals not being vaccinated against these BRD-associated genera as part of preconditioning. Thus it is tempting to speculate that reduced stress from preconditioning may have benefited the upper respiratory tract microbiota by enhancing resilience against certain pathogens.

Vaccinating calves upon arrival at the feedlot is a common practice (Edwards, 2010; Taylor et al., 2010a). A recent meta-analysis of feedlot studies revealed that vaccination at feedlot arrival did not reduce BRD and the commercial products used were not effective (O'Connor et al., 2019). This may be due to several reasons potentially relating to the timing of vaccination, including adverse immune response from stress, pathogen exposure, and immunosuppression (Perino and Hunsaker, 1997; Richeson et al., 2008). To ensure the optimal health and well-being of newly-

weaned calves upon their arrival to feedlots, it is important to vaccinate them against disease-causing agents several weeks before they depart from the farm or ranch, or before they are naturally exposed to disease challenges (Chamorro et al., 2020; Richeson et al., 2020). However, at the time of this research, there was a limited number of studies evaluating how on-farm vaccination affects the respiratory microbiota and health of cattle.

The subcutaneous vaccines used in Chapter 2 included bacterin-toxoid from *H. somni* and *M. haemolytica*. Subcutaneous vaccines primarily generate serum IgG antibodies, with mucosal spill resulting in secretory IgA antibodies. A main finding of this thesis was that immune response varied between vaccine targets. Preconditioned calves exhibited higher levels of systemic IgG and secretory IgA against *M. haemolytica* before transportation, compared to nonpreconditioned calves, but this difference diminished after feedlot vaccination. However, despite receiving two additional doses of the *H. somni* vaccine, preconditioned calves showed no difference in serum IgG concentrations at any point compared to the non-preconditioned animals, and in both groups, *H. somni*-specific IgA levels declined in the feedlot implying limited protection at respiratory mucosa. Interestingly, *Histophilus* was the most dominant taxon associated with BRD cases in both preconditioned and nonpreconditioned groups, which also exhibited similar BRD morbidity rates up to 32 days after feedlot placement. While only the upper respiratory tract microbiota were investigated, strong concordance has been shown for BRD pathogens in nasopharyngeal and lung samples from BRD cases (Timsit et al., 2018). Therefore, it can be concluded that the *H. somni* vaccine did not protect against infection by this pathogen. The benefits achieved from preconditioning programs that include on-farm vaccination, in relation to stress and increased antibody response, can therefore be negated without proper vaccine efficacy. The *H. somni* vaccine may have had limited efficacy due to lack of immunity or narrow scope of protection. It is possible

that the *H. somni* spread between cattle was a strain that evaded protection from vaccination. With these results, the focus of this thesis shifted, to develop a novel vaccine that would confer mucosal protection at the site of pathogen colonization.

Efficient mucosal vaccine development relies on appropriate vectors. Several approaches utilizing live microorganisms (phages, bacteria) and nanoparticles have been proposed for delivering antigens to mucosal surfaces (Isticato, 2023). *Bacillus* spores, in particular, have been studied for this purpose and have been shown to elicit mucosal responses (Copland et al., 2018; Duc le et al., 2003) and resist degradation in the intestinal tract (Koopman et al., 2022), making them an ideal candidate for ruminants. Antigens displayed on spores via recombinant or non-recombinant methods were shown to stimulate mucosal immunity via oral and intranasal administration, providing protection against bacterial and viral infection in animals (Huang et al., 2010; Saggese et al., 2023). The small size of spores (about 1 μm) makes them well-suited as particulate adjuvants, mimicking pathogens and enhancing antigen presentation and immune response (Saggese et al., 2023). Although the *H. somni* vaccine in Chapter 2 was observed to have low efficacy, few antigen candidates have been characterized for this pathogen. Among the common bacterial pathogens causing BRD, *M. haemolytica* is perhaps the best-characterized species with numerous antigenic epitopes being identified over decades of research. Therefore, *M. haemolytica* was used as a model pathogen for proof-of-concept development of a spore-based vaccine. Three vaccines were developed, by adsorbing *M. haemolytica* chimeric proteins MhCP, MhCP1, and MhCP2 to *B. subtilis* spores. Keeping leukotoxin (LKT), the principal virulence factor of *M. haemolytica*, as core component, two *M. haemolytica* outer membrane proteins, PlpE and GS60, were chosen to formulate the chimeric proteins. Both PlpE and GS60, are highly immunogenic (Confer and Ayalew, 2018), and conserved across *M. haemolytica* serotypes,

including serotypes 1 and 6, which are most frequently associated with BRD morbidity and mortality (Klima et al., 2014a). In Chapters 3 and 4, comparative analyses were conducted to determine the optimal spore adsorption conditions of the different chimeric proteins by varying concentration, pH, and temperature. Consistent with previous studies (Huang et al., 2010; Song et al., 2012), all three chimeric proteins were found to adsorb to negatively charged bacterial spores at pH 4, which is below their isoelectric points (pI). This highlights that protein adsorption to spores depends on solution pH and protein structure, with isoelectric point, electric charge, and hydrophobicity all having important roles (Saggese et al., 2023).

Preserving the native conformation of an immunogen is essential for eliciting protective immunity (Huang et al., 2010). To investigate the immunogenicity of the developed spore-based vaccines *in vivo*, three immunization routes-intranasal, intramuscular, and intragastric were compared using mice models. Although MhCP, MhCP1, and MhCP2 were developed as chimeric proteins, each construct (NLKT, PlpE and/or GS60) resulted in seroconversion individually. In Chapter 3, anti-NLKT and anti-PlpE antibodies were induced with just 5 μg of MhCP after intramuscular administration, suggesting that CTB in our MhCP structure enhanced immunogenicity. Intranasal immunization of mice with Spore-MhCP, compared to the intragastric route, significantly enhanced mucosal immunity, generating higher levels of secretory IgA against *M. haemolytica* NLKT and PlpE at all evaluated mucosal sites. In Chapter 4, Spore-MhCP1 and Spore-MhCP2 were developed and screened for their immunogenicity in mice. Both vaccines were able to stimulate antigen-specific immune responses after the immunization of mice, demonstrating that the chimeric antigens also retained immunogenicity after spore adsorption. In two separate trials, mice were immunized with spore-bound vaccines (Spore-MhCP1 and Spore-MhCP2) via intranasal and intragastric routes, while free MhCP1 and MhCP2 were administered

intranasally and intramuscularly. Intramuscular immunization, serving as the positive control, produced the strongest serum IgG antibody response in both mouse experiments, whereas intranasal administration of Spore-MhCP1 and Spore-MhCP2 induced appreciable amount of antigen-specific IgG. Notably, intranasal administration of Spore-MhCP1 and Spore-MhCP2 generated the highest secretory IgA-specific response against LKT, PlpE, and GS60 in bronchoalveolar lavage, saliva, and feces. Thus, intranasal spore-bound vaccines confer both systemic and mucosal immunity. Intranasal immunization with free MhCP1 and MhCP2 also generated IgA antibodies in all samples, though at lower levels compared to the spore-bound antigens. This implied that binding antigens to *Bacillus* spores enhanced the immune response, demonstrating the spores' effectiveness as an adjuvant.

In all three mice trials, intragastric administration of spore-bound vaccines led to minimal mucosal immunity, likely due to antigen dilution and degradation from stomach acid and digestive enzymes. In addition, complement-mediated serum bactericidal activity (SBA) assay was utilized in Chapters 3 and 4 to evaluate the efficacy of vaccine-induced antibodies in eradicating the targeted pathogen. In all three mouse studies, antibodies from the intramuscularly administered vaccines demonstrated almost complete killing of *M. haemolytica* in the complement-dependent SBA assay. The sera from mice immunized intranasally with spore-bound vaccines showed lower bactericidal activity than intramuscular treatments that received unbound antigens, likely due to lower IgG levels from intranasal immunizations (Rouphael et al., 2011). Despite the limited or undetectable serum IgG levels observed following intragastric administration of these spore-bound vaccines, sera from intragastric treatment mice still exhibited bactericidal activity. This indicated that these spore-based vaccines can generate functional antibodies against *M. haemolytica* in mice regardless of the delivery route. Given the enhanced immune responses from intranasal spore-

bound vaccine compared to free antigen in our study, we hypothesise that the intranasal immunization of this spore-based vaccine may confer increased protection against *M. haemolytica* infection in cattle.

Among the three spore-based vaccines evaluated in Chapters 3 and 4, Spore-MhCP was chosen for further characterization in sheep over other candidates for the following reasons: i) its components are known to stimulate immunity in both sheep (Batra et al., 2017) and calves (Ayalew et al., 2009), ii) it encodes repeated NLKT and PlpE antigens, potentially providing additional protection, and iii) it effectively generated both systemic and mucosal immunity in mice with just 5 μg of MhCP, compared to 10 μg and 50 μg used for the other candidates. In Chapter 5, the Spore-MhCP vaccine was further tested in sheep, as a ruminant model, due to their natural colonization by *M. haemolytica*, their similarities in rumination to cattle. Spore-MhCP was administered to sheep through two mucosal routes, intranasal and intragastric, while the unbound MhCP antigen was administered intranasally and intramuscularly. Unbound spores were also evaluated intranasally, and the intranasal administration of saline was used as the control treatment. When compared to control, intranasal immunization with Spore-MhCP generated the strongest secretory IgA-specific response against both PlpE and NLKT in all samples (nasal swab, bronchoalveolar lavage, and feces), and exhibited a tendency to enhance immune responses more rapidly than intranasally delivered unbound MhCP. Ayalew and colleagues (2009) immunized weaned beef calves intranasally with the same R2-NLKT-R2-NLKT chimeric protein, with and without cholera toxin, and also with a CTB-appended chimeric protein, CTB-R2-NLKT (Ayalew et al., 2009b). The vaccination enhanced resistance to M. *haemolytica* and induced antibody responses, but significant increases were seen only after bacterial challenge (Ayalew et al., 2009b). In contrast, it was observed in Chapter 5 that intranasal vaccination of sheep with Spore-MhCP resulted in

significant immune response after booster administration, demonstrating the effectiveness of the spore-based vaccine technology in sheep.

The convenience of in-feed or oral vaccines for livestock, requiring minimal expertise and time for administration, makes them an attractive option for managing disease. Despite poor immune responses from oral administration in mice, the potential of an oral spore-based vaccine was explored in sheep. This investigation was prompted by anatomical differences between the species and the possibility that rumination in sheep could activate pharyngeal lymphoid tissue through antigen exposure via regurgitation (Shewen et al., 2009). Sheep were fed a high-fiber alfalfa diet in Chapter 5, to promote rumen retention and regurgitation of ingested Spore-MhCP. The intragastric group showed increasingly higher PlpE- and NLKT-specific IgA in BAL and fecal samples after sequential rounds of immunization. This suggests that ingested spore-bound recombinant protein resisted degradation to an extent that allowed antigen uptake by M cells and activation of mucosa-associated lymphoid tissue. The presence of antigen-specific antibodies in the lungs indicates possible immune cell trafficking from the gut or pharyngeal tissue (Taghavian et al., 2013), while antigen-specific IgA in feces indicated a local response.

In Chapter 5, the upper respiratory tract microbiota of sheep in control and intranasal Spore-MhCP groups were analyzed over time by sequencing the 16S rRNA gene from nasal samples. The composition and structure of the nasopharyngeal microbial communities changed over time, likely influenced by the vaccination method. The sequencing analysis of the nasopharyngeal samples showed that the abundance of 27 genera was altered in the intranasal Spore-MhCP group compared to the control group across time. There was a reduction in the abundance of pathogenic genera like *Moraxella*, *Mycoplasma*, and *Staphylococcus* at various time points. The changes in these genera were proposed to result from the spore interacting with host mucosa, possibly eliciting

general immune response locally. Aligned with our findings, a recent study demonstrated that intranasal administration of heat-killed *B. subtilis* spores regulated both innate and adaptive immunity, providing heterologous protection against three major respiratory pathogens (Xu et al., 2024). A notable decrease in *Mannheimia* was observed consistently in the intranasal Spore-MhCP group from days 14 to 35, possibly resulting from vaccine-induced antibodies. The analysis of the sheep nasopharyngeal microbiota, combined with the secretory IgA response to *M. haemolytica* from intranasal Spore-MhCP vaccination, indicated a potential reduction in colonization by this pathogen. Additionally, the results from the in-feed Spore-MhCP vaccine suggest it could be an effective oral vaccine for large-scale administration. These characteristics are particularly valuable given the cost and ease-of-use considerations in livestock management. Since *M. haemolytica* proliferation in the upper respiratory tract is necessary for lung infection, this Spore-MhCP vaccine may provide protection against both proliferation and subsequent infection. In total, the spore-based vaccine developed elicited both mucosal and systemic immune response when administered orally or intranasally in sheep.

6.2 Limitations:

One of the limitations of this thesis was the lack of a challenge study to fully evaluate the efficacy of this spore-based vaccine technology. Because *M. haemolytica* is classified as a risk group-2 pathogen, a specialized agriculture facility would have been required for a challenge study, thus it was not possible to complete. However, the *in vitro* complement-mediated bactericidal assay provided important insight into the potential effectiveness of the vaccine. The

vaccine elicited bactericidal activity against *M. haemolytica,* thus highlighting a protective response.

A second limitation was that antigen-specific lymphocytes were not characterized, which would have provided information on immune protection and complemented the assessment of systemic and mucosal antibody responses. Identifying and characterizing antigen-specific lymphocytes, which are subpopulations of T and B cells, is important for understanding immunity from vaccines and pathogens (Newell et al., 2022). Various methods exist for detecting T and B cells, including enzyme-linked immunospot (ELISPOT), flow cytometry, peptide/HLA staining, and activation-induced marker analysis (Newell et al., 2022; Saade et al., 2012; Slota et al., 2011). A simultaneous approach to identify and characterize antigen-specific B cells, CD4+ T cells, and CD8+ T cells would require small sample and enhance assessments of cells targeting the same antigen (Newell et al., 2022). Activation-Induced Marker (AIM) assays provide a more comprehensive approach to measuring vaccine-induced T cell responses by identifying antigen-specific T cells through surface markers (Bowyer et al., 2018). Unlike traditional cytokine-based methods such as ELISPOT and intracellular cytokine staining (ICS), AIM assays have shown higher frequencies of antigen-specific CD4+ and CD8+ T cells after vaccination (Bowyer et al., 2018), indicating that AIM assays enhance the evaluation of vaccine immunogenicity in clinical trials.

Lastly, while this thesis evaluated antigen-specific total IgG in serum, assessing IgG subclasses would offer more insight into antibody protection. Each IgG subclass has unique roles, including antigen binding, immune complex formation, complement activation, phagocytosis, and

differences in half-life (Vidarsson et al., 2014). IgG1 and IgG3 typically respond to protein antigens, while IgG2 is more associated with polysaccharides. IgG3, with its pro-inflammatory properties, is crucial in viral infections, while IgG4 tends to arise from prolonged antigen exposure, such as in allergy or parasitic infections (Vidarsson et al., 2014). Deficiencies in IgG2 and IgG3 are linked to recurrent infections (Ferrante et al., 1990). Thus, overall defining the IgG subclasses would have provided detailed information into the mechanism of immune response after vaccination.

6.3 Future directions

The findings of this thesis provide new knowledge regarding preconditioning and how it affects host immune, stress, and respiratory microbiota, in the presence of stressors including weaning, transportation and commingling. While both treatment groups exhibited similar levels of morbidity in Chapter 2, the preconditioned animals demonstrated better stress response, immunity, and reduced abundances of several BRD-associated genera compared to non-preconditioned calves. However, the effectiveness of host immunity upon arriving at the feedlot varied against different pathogens the animals were vaccinated against. This indicates a potential to enhance protection against BRD by developing new mucosal vaccines with improved efficacy to replace current less effective bacterial vaccines. This thesis also introduced new insights into developing a mucosal vaccine using spore adsorption technology, with *M. haemolytica* as the model organism. The effective response of the Spore-MhCP vaccine observed in mice (Chapter 3) carried over to sheep (Chapter 5), where intranasal administration of the Spore-MhCP vaccine led to the production of both mucosal and systemic antigen-specific antibodies. Since the proliferation of *M. haemolytica* in the upper respiratory tract is a prerequisite to lung infection, this spore-bound

vaccine may offer protection against proliferation and subsequent infection in cattle. Future challenge studies in cattle are warranted to thoroughly assess the efficacy of the Spore-MhCP vaccine against *M. haemolytica.* In addition, *M. haemolytica* is a major respiratory pathogen in sheep, thus the vaccine developed is highly relevant to the sheep industry.

This spore-based technology offers the potential to incorporate immunologically relevant antigens from other pneumonia or BRD pathogens, such as *P. multocida* and *H. somni*. This approach could lead to the development of a broad-spectrum, multivalent vaccine for ruminants, offering protection against a wider array of respiratory infections. Despite the potential impact of passive or pre-existing immunity, Chapter 5 showed that intranasal Spore-MhCP vaccination still triggered a robust immune response in sheep, even with detectable antibody levels at the beginning of the study. This indicates that *Bacillus* spores could be a promising vaccination technology and should be further explored in cattle with passive immunity, like newborn calves, or those exposed to *M. haemolytica*, such as feedlot-bound animals. While research has demonstrated the advantages of mucosal vaccines over parenteral vaccines for respiratory infections, it is important to note that current commercially available intranasal vaccines are made from an avirulent live culture of *M. haemolytica* and *P. multocida*. It is also essential to consider that live attenuated or bacterin-based vaccines may have potential adverse effects, and may not be effective in new emerging pathogen strains. In contrast, recombinant subunit vaccines bound to a probiotic *Bacillus* spore may offer enhanced safety. The production of *B. subtilis* is well established and involves low-cost inputs. Commercially used in food and feed products for various animals, *B. subtilis* is recognized as a probiotic with generally regarded as safe (GRAS) status. Therefore, a low-cost and GRAS vaccine component with a history as an adjuvant would facilitate industry adoption.

Moreover, oral administration of the vaccine would enable large-scale usage. These attributes are particularly crucial as livestock management strategies, including vaccination, rely on cost-effectiveness and ease of use.

6.4 Conclusions

Through further development of the presented mucosal spore-based vaccine technology, using *M. haemolytica* as a model organism, the beef industry will have an additional tool for improving preconditioning programs, mitigating BRD and reducing antimicrobial use. This project has laid the groundwork for the discovery of effective mucosal vaccines against BRD pathogens. While additional evaluation of the developed spore-based vaccine is needed, it is hypothesized that this technology can be applied to other bacterial or viral vaccines, resulting in improved efficacy that may replace current ineffective vaccines. Ultimately, this could lead to the replacement of metaphylactic antimicrobials currently used for BRD prevention in conventional feedlot systems. Overall, the research presented here supports the idea that this spore-based mucosal vaccine technology has the potential to improve bovine respiratory health, and be incorporated into management strategies aimed at reducing BRD in the beef industry.

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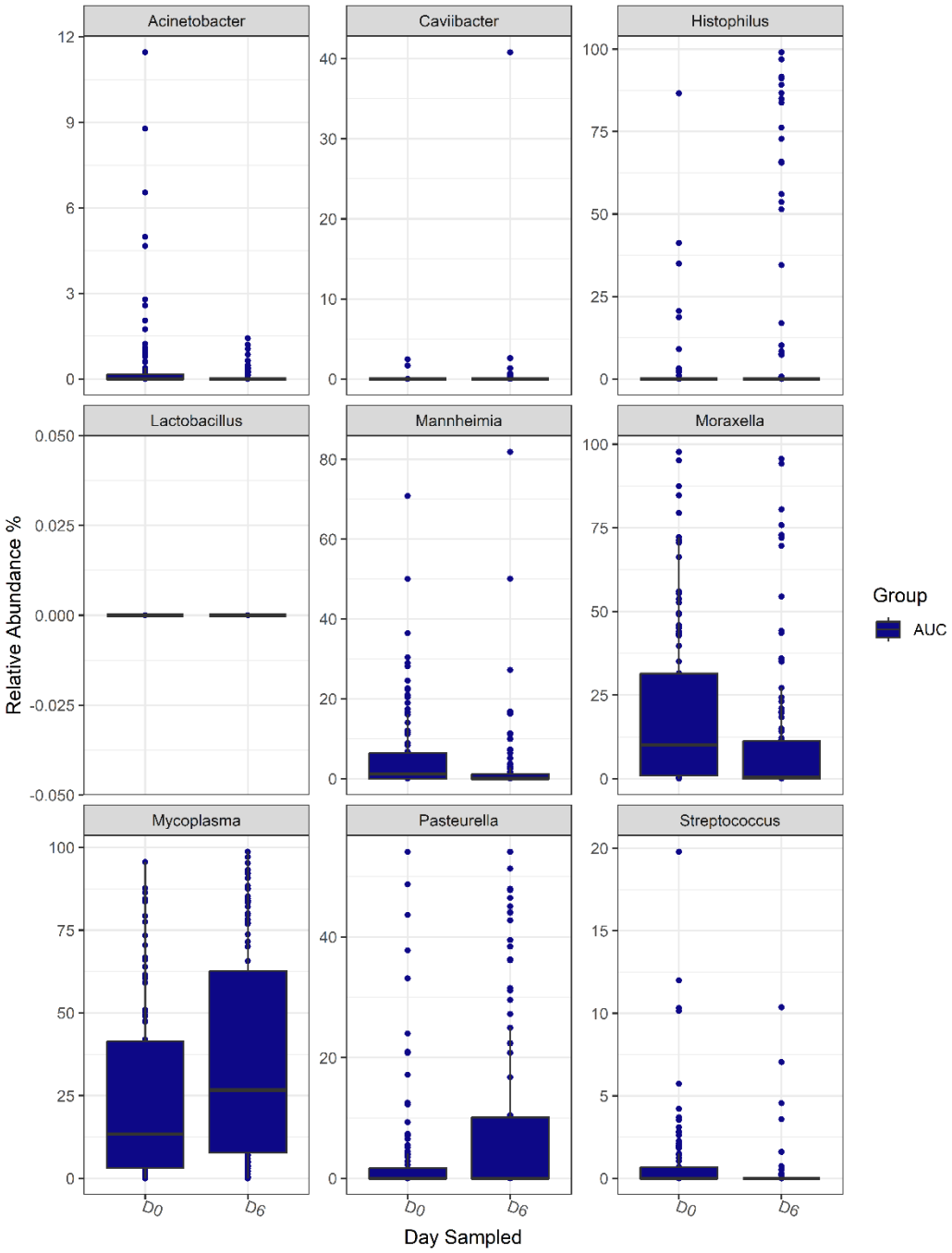
Appendices

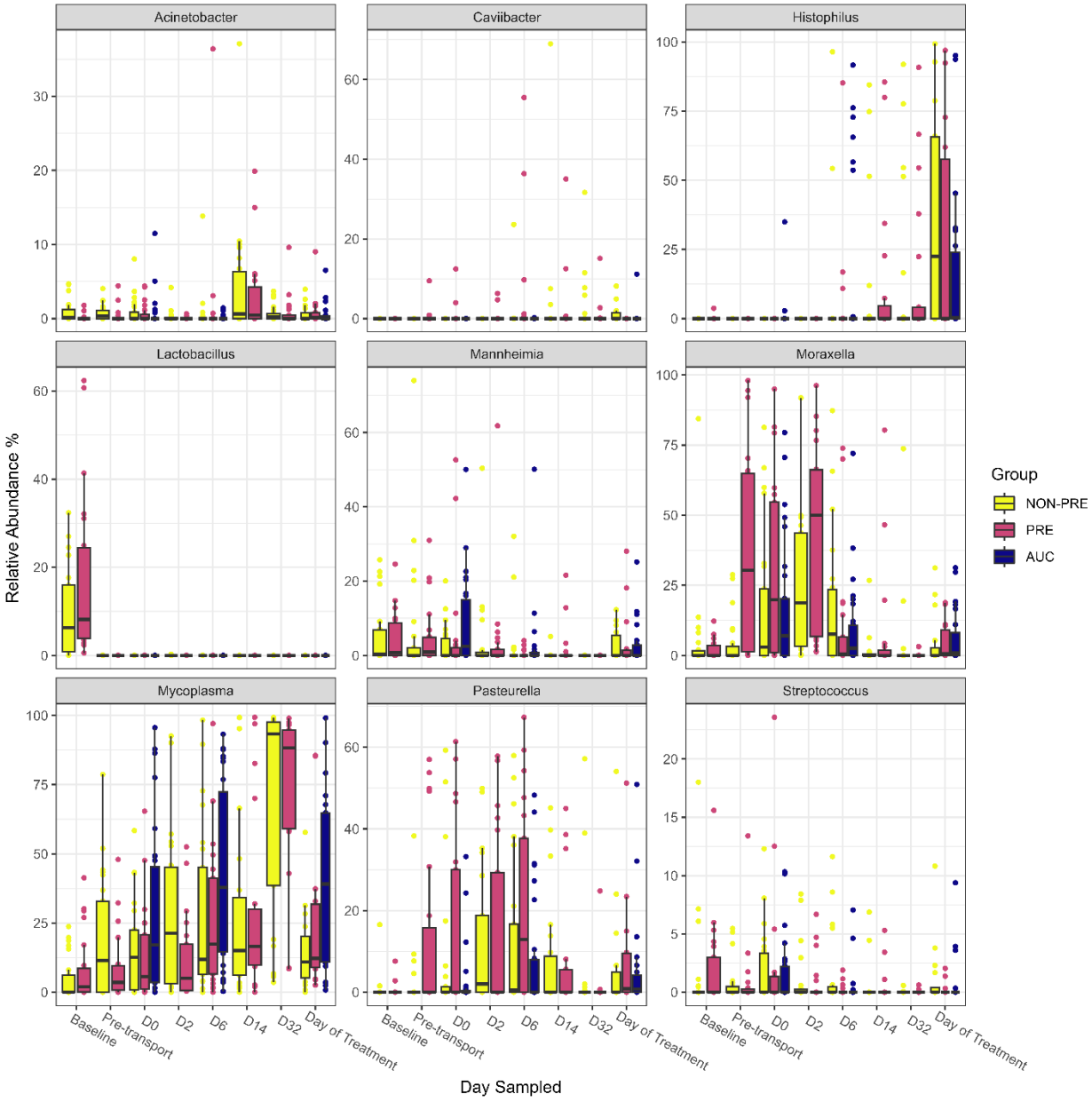
Appendix A:

Relative abundance of the nine most abundant genera identified in nasopharyngeal swabs from auction market calves across time. The Auction Market calves were sampled on arrival to the feedlot (D0), and day 6 (D6) of feedlot placement. Error bars indicate ± standard error of the mean. The box in the plots indicates the interquartile range (IQR) (middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.



Appendix B:

Relative abundance of the nine most abundant genera identified in nasopharyngeal swabs from sick animals across time and treatments. Preconditioned (n=50) and Non-preconditioned (N=50) calves were raised according to their designated on-farm management protocols. Both groups of calves were transported separately for 12 h, prior to feedlot placement. At the feedlot Preconditioned and Non-preconditioned calves were mixed 1:1 with Auction Market-derived calves (n=10 per pen). Nasopharyngeal (NP) samples were collected from sick calves throughout 32 days of feedlot placement, before antimicrobial treatment. The box in the plots indicates the interquartile range (IQR) (middle 50% of the data), the middle line represents the median value, and the whiskers represents 1.5 times the IQR.



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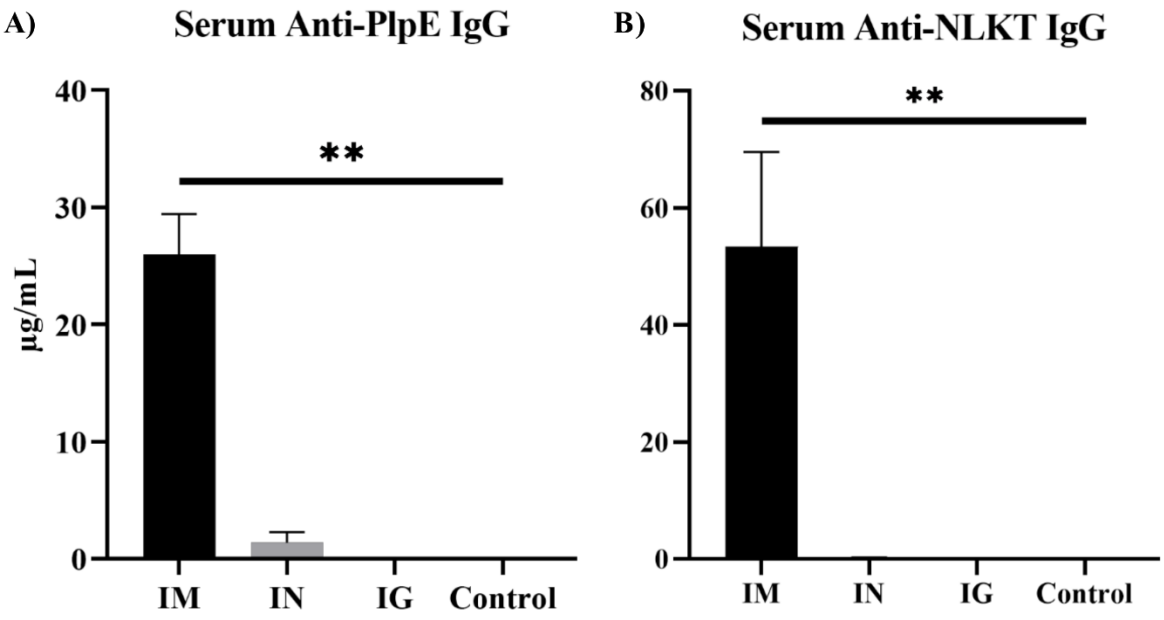
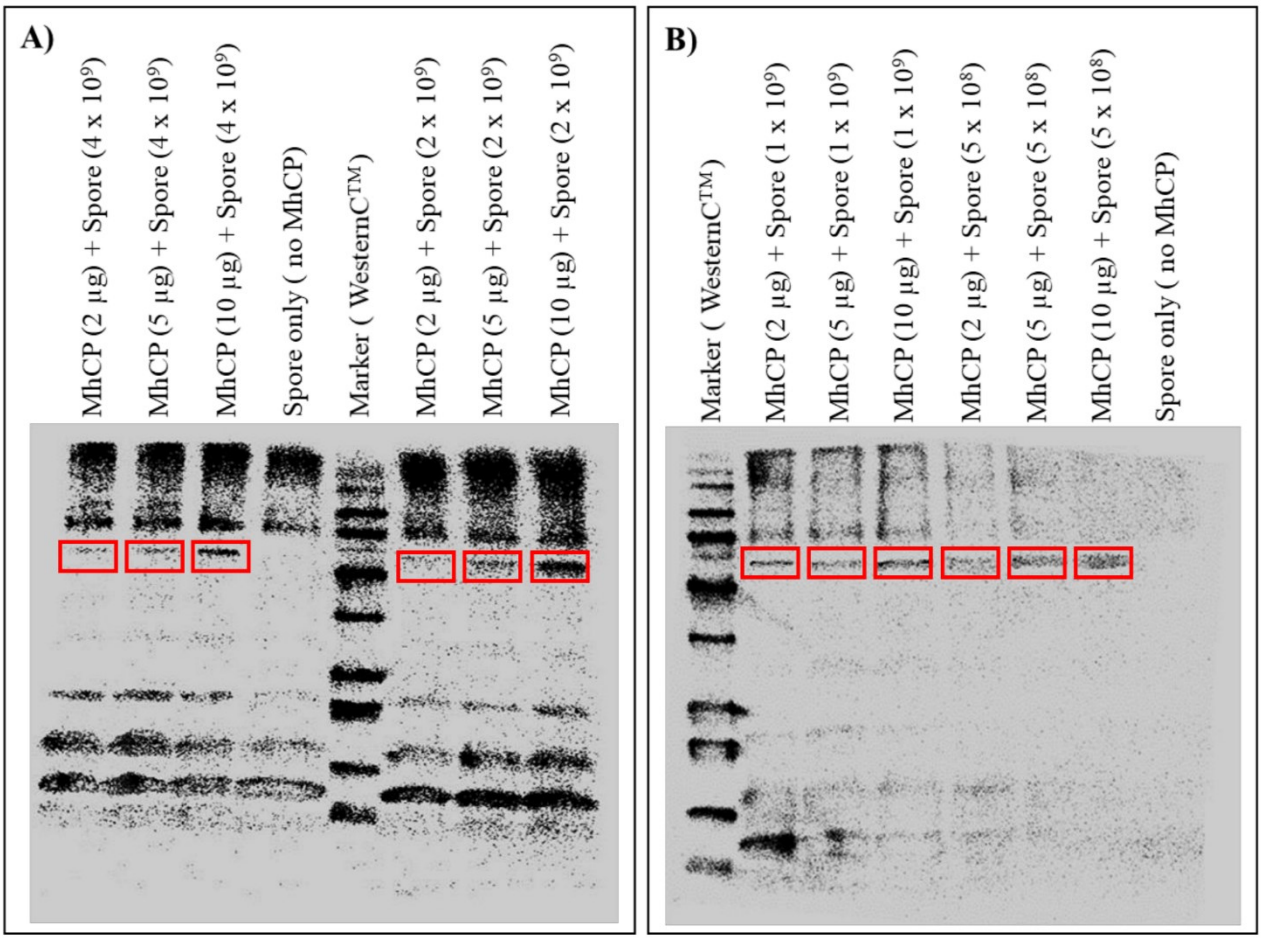
Appendix C:

Antigen-specific serum IgG antibody responses measured by enzyme linked immunosorbent assay (ELISA) from samples collected on day 21. ELISA plates coated with either recombinant PlpE or NLKT were used to measure anti-PlpE and anti-NLKT antibodies in mice sera from day 21. Immune responses from four experimental groups: Intramuscular (IM), Intranasal (IN), Intragastric (IG) and Naïve/control mice (Control) were compared. (A) Levels of serum IgG specific to PlpE (B) Levels of serum IgG specific to NLKT. Results are expressed as mean ± SEM. Significance was tested against the control by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test, **$p < 0.01$.



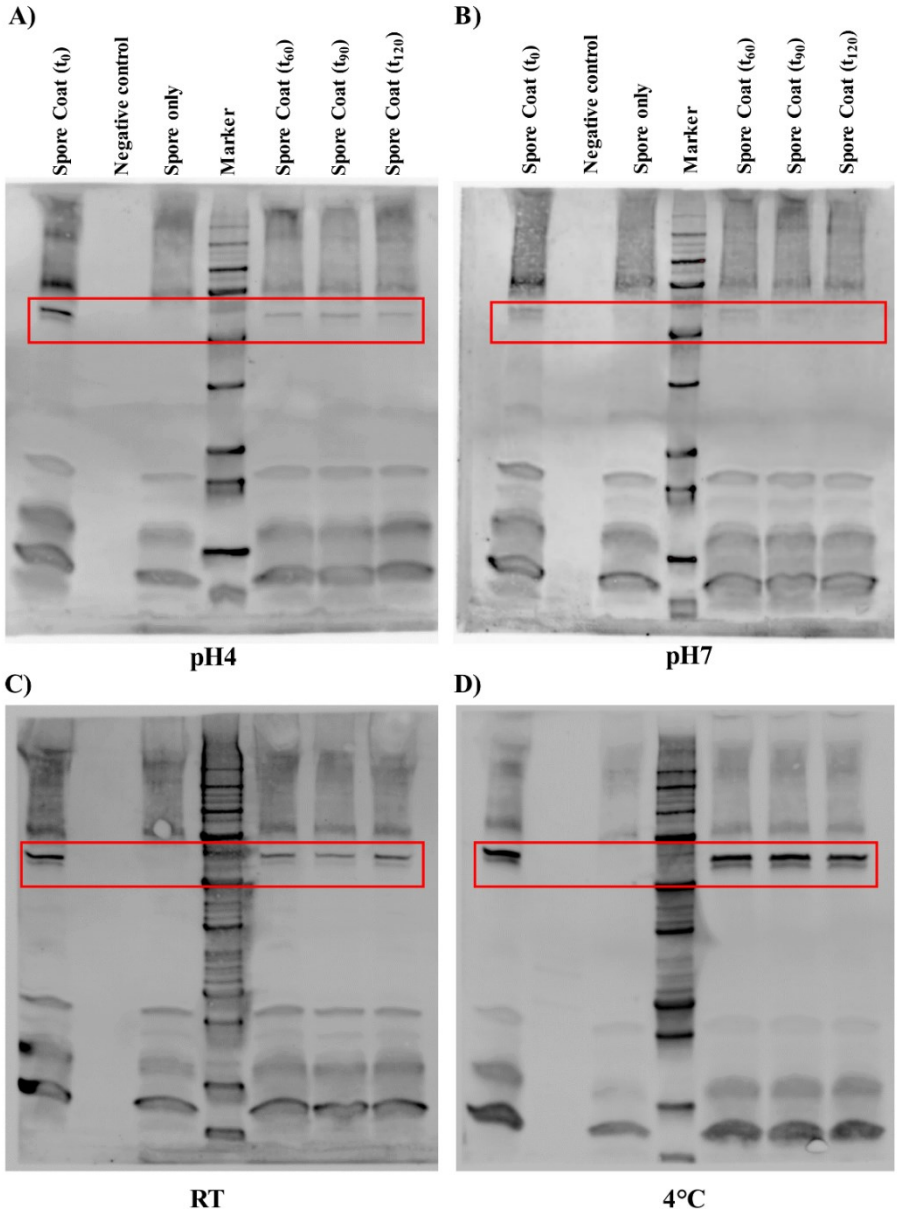
Appendix D:

Optimization of spore-to-antigen ratio. Chimeric protein, MhCP, of varying amounts (2 µg, 5 µg, 10 µg) were mixed with a range of *Bacillus subtilis* spores (4×10^9 to 5×10^8) in phosphate buffered saline (PBS), incubated for 1 h, washed, and resuspended in PBS. The supernatant was removed, the pellet containing spore coat antigens was washed and extracted by incubating in extraction buffer for 30 min at 65°C. Quantities of MhCP were determined by Western blotting. Spore only lane displays the corresponding blot generated from spores that underwent the entire process without the addition of protein MhCP. Outlined red boxes indicate the cropped regions denoted in Figure 3.2.



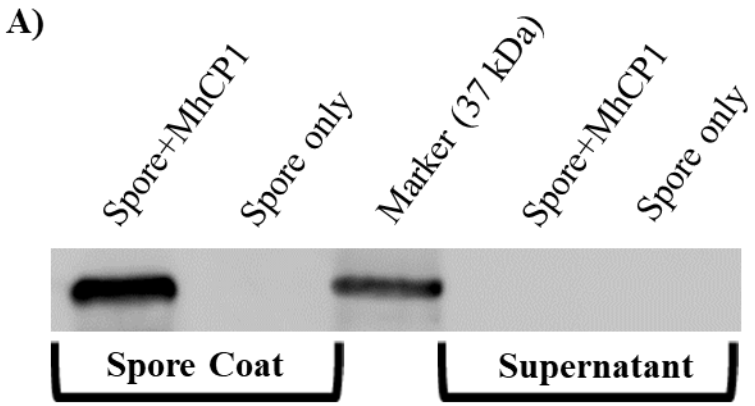
Appendix E:

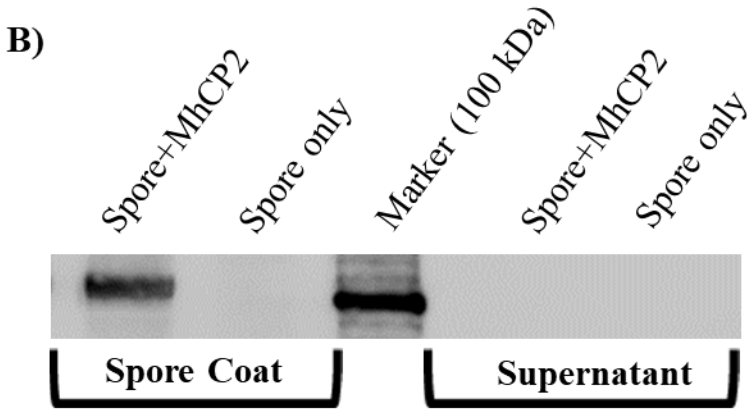
Adsorption of chimeric protein MhCP to *Bacillus subtilis* spores. MhCP adsorbtion to spores was evaluated at different pHs (A, B) and temperatures (C, D). 10 µg of MhCP was mixed with 2 x 10^9 spores in phosphate buffered saline (PBS) at pH 4 (A) and PBS at pH 7 (B), incubated 1 h at room temperature (RT). The binding mixture was centrifuged and the pellet was washed two times with PBS. The washed pellet was next resuspended in 200 µl of PBS at their respective pH. At indicated time points (60, 90 and 120 min), the spore suspensions were centrifuged. Pellets were resuspended in 100 µl of spore coat extraction buffer, incubated at 65 °C for 30 min to remove spore coat proteins from the spores. Using a one in ten dilution of the extraction, western blotting of size-fractionated proteins was used for detection. Equivalent amounts of spores and protein were used at pH 4 (C, D). After the initial 1 h incubation at RT, the binding mixture was incubated at 4 °C (C), whereas all reactions and incubations were performed at 4 °C (D). The negative control lane displays the corresponding blot that underwent the entire process without the addition of any spores or protein. Outlined red boxes indicate the lanes denoted in Figure 3.3.



Appendix F:

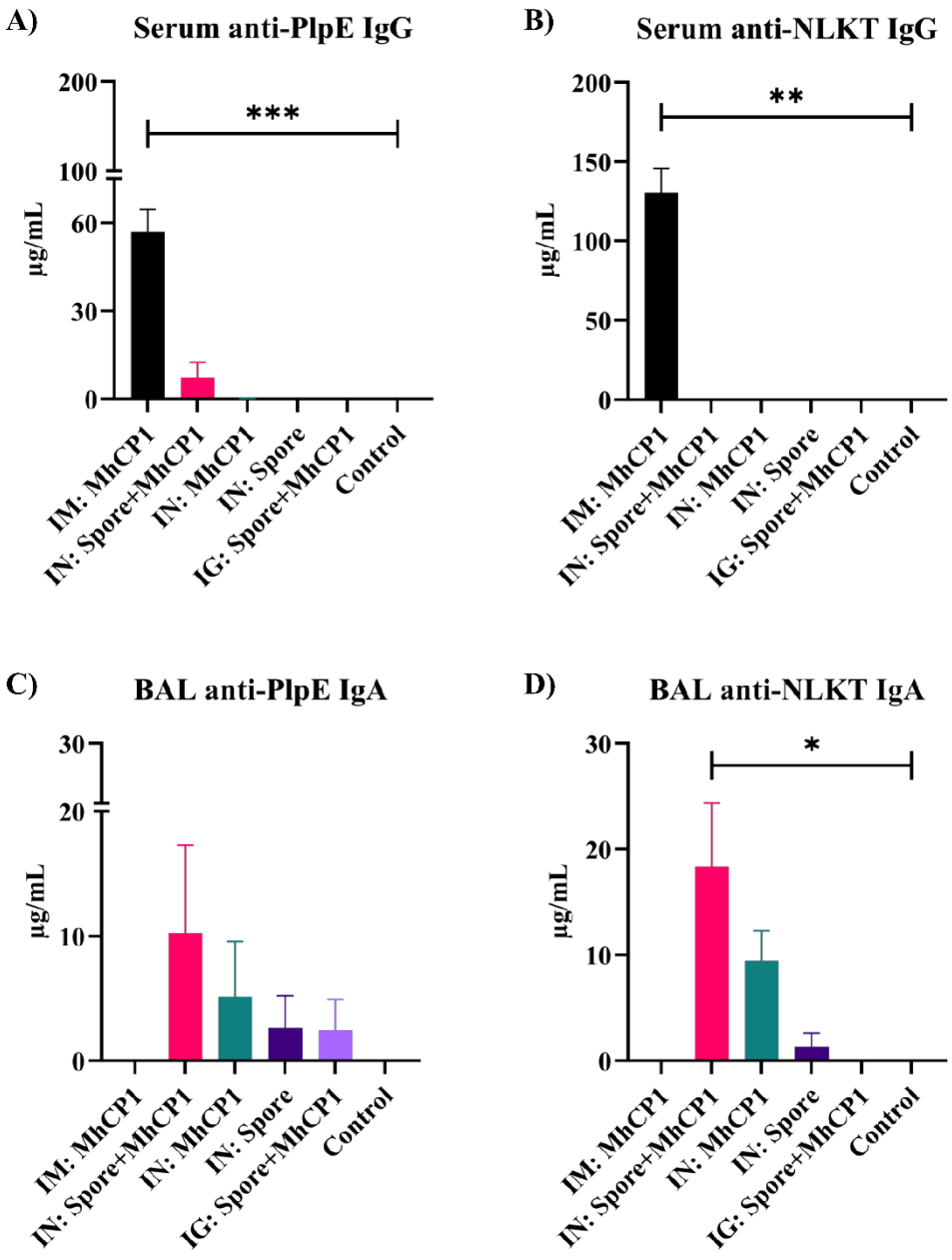
The adsorption of *Mannheimia haemolytica* chimeric proteins MhCP1 and MhCP2 to *Bacillus subtilis* spores using optimized binding conditions. A) The optimized adsorption of MhCP1 to *Bacillus subtilis* spores demonstrated by Western blotting. Briefly, 10 µg of MhCP1 was mixed with 2 x 10^9 spores in phosphate buffered saline (PBS) at pH 4 and incubated for 1 h at 4 °C. The binding mixture was centrifuged, the supernatant collected, and the pellet was washed two times with PBS. Pellets were resuspended in 100 µl of spore coat extraction buffer, then incubated at 65 °C for 30 min to remove spore coat proteins from the spores. Using a one in ten dilution of the extraction, detection was confirmed by the Western blotting of size-fractionated proteins. B) The optimized adsorption of MhCP2 to *B. subtilis* spores demonstrated by Western blotting. Briefly, 50 µg of MhCP2 was mixed with 2 x 10^9 spores in citrate buffer at pH 4 and incubated for 1 h at 4 °C, then the same procedures described above were followed.





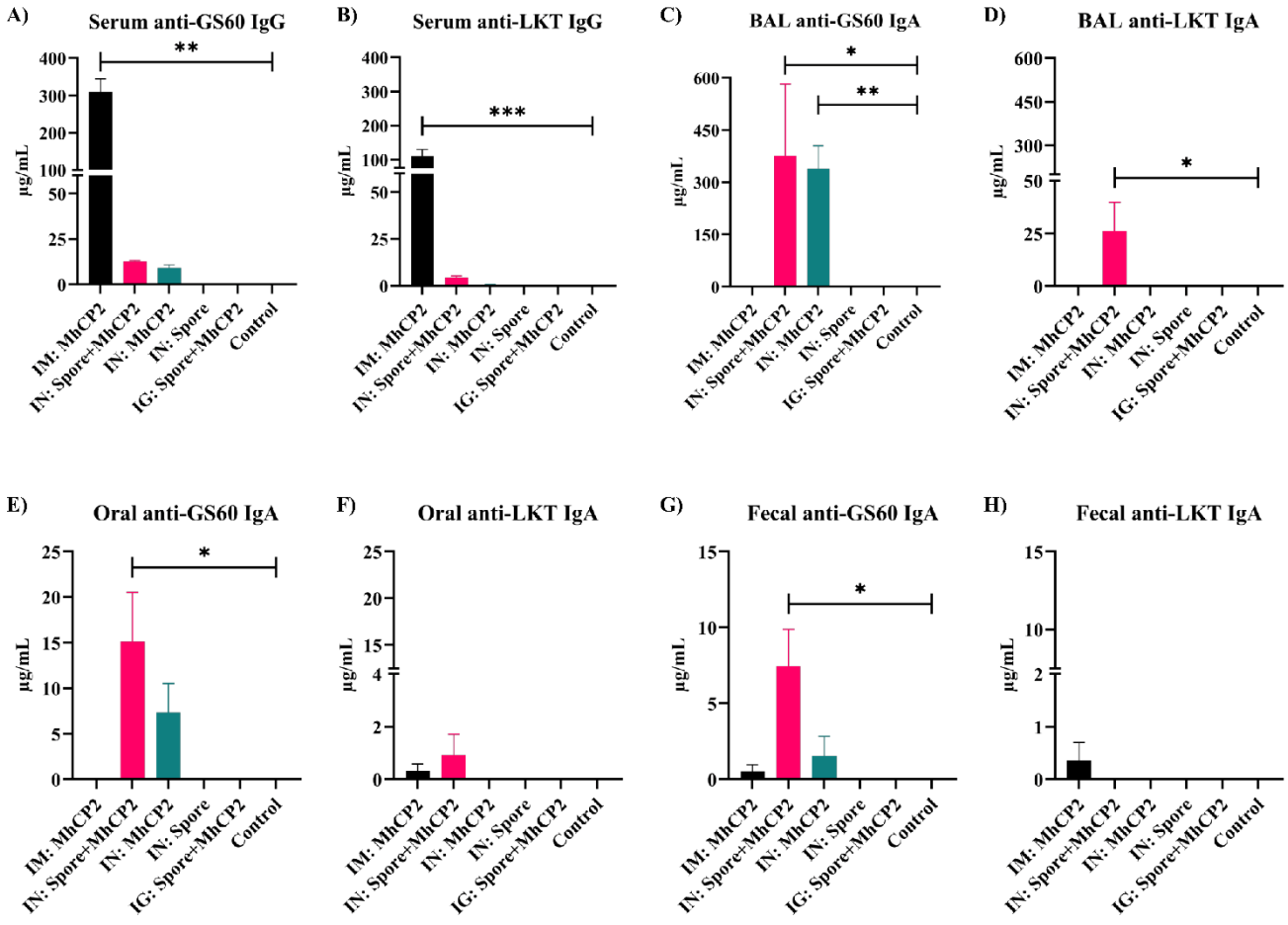
Appendix G:

Experiment 1—Antigen-specific antibody responses measured by enzyme-linked immunosorbent assay (ELISA) from samples collected on day 21. ELISA plates coated with either recombinant PlpE or NLKT were used to measure anti-PlpE and anti-NLKT antibodies in mice sera and bronchoalveolar lavage (BAL). Immune responses from six experimental groups: intramuscular (IM:MhCP1), intranasal spore-bound antigen (IN:Spore+MhCP1), intranasal antigen only (IN:MhCP1), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP1), and control/naïve mice (Control) were compared. (A) Levels of serum IgG specific to PlpE. (B) Levels of serum IgG specific to NLKT. (C) Levels of secretory IgA specific to PlpE from BAL. (D) Levels of secretory IgA specific to NLKT from BAL. Results are expressed as mean ± SEM. Significance was tested against the control by one-way ANOVA with Tukey's multiple comparison test, *p < 0.05, ** p < 0.01, and ***p < 0.001.



Appendix H:

Experiment 2—Antigen-specific antibody responses measured by enzyme-linked immunosorbent assay (ELISA) from samples collected on day 21. ELISA plates coated with either recombinant GS60 or LKT were used to measure anti-GS60 and anti-LKT antibodies in mice sera, bronchoalveolar lavage (BAL), saliva, and fecal samples. Immune responses from six experimental groups: intramuscular (IM:MhCP2), intranasal spore-bound antigen (IN:Spore+MhCP2), intranasal antigen only (IN:MhCP2), intranasal spore only (IN:Spore), oral/intragastric spore-bound antigen (IG:Spore+MhCP2), and control/naïve mice (Control) were compared. (A) Levels of serum IgG specific to GS60. (B) Levels of serum IgG specific to LKT. (C) Levels of secretory IgA specific to GS60 from BAL. (D) Levels of secretory IgA specific to LKT from BAL. (E) Levels of secretory IgA specific to GS60 from saliva. (F) Levels of secretory IgA specific to LKT from saliva. (G) Levels of secretory IgA specific to GS60 from feces. (H) Levels of secretory IgA specific to LKT from feces. Results are expressed as mean ± SEM. Significance was tested against the control by one-way ANOVA with Tukey's multiple comparison test, *$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.



Appendix I:

Relative abundance of the phyla in nasopharyngeal samples from vaccinated sheep. Sheep were immunized on days 0 and 14. Deep nasal swabs (from right nostril) were collected from Intranasal spore-bound antigen (IN:Spore+MhCP) and Control/ Naïve (Control; administered intranasal saline) treatment groups on days 0, 7, 14, 21, 28, and 35 and analyzed by sequencing of the 16S rRNA gene.