EXPERIMENTAL INFECTION OF SPECIFIC PATHOGEN-FREE DOMESTIC LAMBS WITH MYCOPLASMA OVIPNEUMONIAE

by

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ABSTRACT

Mycoplasma ovipneumoniae (*M. ovi*) is a respiratory pathogen commonly found in sheep and goats. It is associated with mild to moderate respiratory disease in domestic lambs, but severe pneumonia outbreaks in wild ruminants, specifically bighorn sheep. The goal of our study was to better understand the role of *M. ovi* as a respiratory pathogen in domestic sheep and to explore potential antibiotic treatment approaches. We first established a flock of specific pathogen-free (SPF) lambs through supervised lambing and motherless rearing in a Large Animal BSL-2 facility. Lambs were fed a colostrum replacer that yielded low mortality, steady weight gain and serum IgG and protein concentrations comparable to those of lambs raised on ewe colostrum. We inoculated the SPF lambs with field isolates of *M. ovi* and monitored the lambs for eight weeks for colonization with the bacteria, *M. ovi*-specific antibodies, clinical symptoms, and cellular and molecular correlates of lung inflammation. After eight weeks, lambs were treated with the macrolide antibiotic gamithromycin and observed for an additional four weeks. Stable colonization of the upper respiratory tract with *M. ovi* was established in all four *M. ovi*-inoculated, but in none of the four mock-infected lambs. All *M. ovi*-infected lambs developed a robust antibody response to *M. ovi* within 2 weeks. However, we did not observe significant clinical symptoms, evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with gamithromycin failed to reduce *M. ovi* colonization. These observations indicate that, in the absence of co-factors, *M. ovi* causes asymptomatic colonization of the upper respiratory tract of that is resistant to clearance by the host immune response as well as by gamithromycin treatment in domestic lambs.

CHAPTER 1

INTRODUCTION

Mycoplasma ovipneumoniae (*M. ovi*) is a gram-negative bacterium that commonly colonizes the nasopharyngeal mucosae of sheep and goats. It is present in 88% of domestic sheep operations in the US (Manlove et al., 2019b) where it causes a wide range of symptoms. *M. ovi* infections can present anywhere from asymptomatic (Parham et al., 2006; Prats-van der Ham et al., 2017) to severe pneumonia (Handeland et al., 2014a). Infections can lead to an increase in stillborns, lower birth weights, and an overall descrease in the likelihood of lambs surviving past weaning; overall this leads to a 4.3% decrease in production nationwide (Manlove et al., 2019b).

While *M. ovi* infections have serious consequences in domestic sheep, the ramifications are significantly greater in bighorn sheep. *M. ovi* has upwards of a 90% mortality rate in bighorn sheep (Besser et al., 2012a). Once *M. ovi* infection has been established, it is incredibly difficult to eliminate. The bighorn sheep that do survive infection become carriers and can transmit *M. ovi* to new lambs in subsequent years. In some instances, this exposure can wipe out that entire population (Wood et al., 2017). In other instances, carrier status decreases the number of lambs delivered, thus minimizing recruitment rates (Butler et al., 2018). Herd outcomes can partially be attributed to the age at which the bighorn sheep are exposed (Plowright et al., 2017b). Sheep between the ages of 4 and 14 years old tend to be more effective at clearing *M. ovi* infections, whereas young lambs and older sheep are unable to clear the infection. It has been shown that removal of *M. ovi* carrier bighorn sheep from a population subsequently eliminates

pneumonia from that population (Garwood et al., 2020). While there is minimal evidence that *M. ovi* on its own is lethal, it is often associated with co-infections and can predispose bighorn sheep to other respiratory infections, such as *Mannheimia haemolytica* pneumonia. This happens because *M. ovi* damages mucociliary defense, allowing itself and other pathogens to travel into the lung (Dassanayake et al., 2010a).

In order to address these issues, we must better understand *M. ovi* infections and the pathogenic mechanisms that lead to the wide range of symptoms and disease severity. One explanation for the difference in severity of *M. ovi* cases in domestic sheep and bighorn sheep could be the lack of previous exposure. With previous exposure come protective antibodies. Since domestic sheep are often chronic carriers, they can pass *M. ovi* specific antibodies to their offspring in their colostrum. When the lambs are exposed to *M. ovi*, they are already equipped with the correct antibodies, and thus able to fight infection. Since bighorn sheep do not survive *M. ovi* infections as commonly as domestic sheep and are less likely to be carriers, we can hypothesize that it would be less likely for a bighorn sheep to pass on protective antibodies to their offspring in colostrum.

In this study, we hypothesize that protective colostral antibodies play a role in minimizing the wide range of symptoms associated with *M. ovi* infection. Many *M. ovi* exposures happen at birth or at a fairly young age (Besser et al., 2019), when colostral antibodies are still present (Niewiesk, 2014). To test this hypothesis, we established a flock of specific pathogen free (SPF) and immunologically naïve sheep and conducted an *M. ovi* infection experiment. In order to establish an SPF and immunologically naïve flock, lambs were separated from ewes upon birth and raised in a Large Animal Biosafety

Level-2 (ABSL-2) facility and were prevented from consuming colostrum from the ewe to prevent transfer of pathogens, as well as protective antibodies.

The first aim of this study (Chapter 2) was to establish a flock of SPF and immunologically naïve sheep in order to conduct subsequent infection experiments. The greatest consideration for this project was determining what colostrum or colostrum replacer to feed the lambs. Colostrum intake is vitally important for passive protection of lambs from infectious diseases. There are many scenarios, however, where ewe colostrum cannot be used. These situations include, but are not limited to, the death of the ewe, birth of multiple lambs, the generation of specific pathogen free animals, and disease eradication programs. In these situations, it is critical to have a colostrum replacer available. While colostrum replacers in the past have provided nutrients, they did not contain antibodies and have thus led to poor outcomes with high lamb morbidity and mortality. Rescue Lamb & Kid Colostrum Replacer is a new colostrum replacer which contains bovine serum as an antibody source. Using bovine colostrum as a replacement to ewe colostrum is a common practice and has shown to be a viable source of protective antibodies for lambs since sheep and cattle are often exposed to similar pathogens (Logan et al., 1978; Moretti et al., 2010). We tested the Rescue Lamb & Kid Colostrum Replacer which contains bovine serum as a source of immunoglobulins. All lambs received the replacer for the first 24 hours of life. Lamb performance during the first 8 weeks after birth was assessed based on survival rate, health status, and average daily gains. Serum antibody ELISA performed on day 1 and day 14 revealed immunoglobulin levels of 17.9 ± 2.8 mg/mL and 27.5 ± 2.5 mg/mL, respectively. None of the lambs developed serious

infectious disease conditions and maintained weight gain up until and throughout weaning. Based on these results, we conclude that Lifeline Lamb & Kid Colostrum Replacer is a viable substitute to ewe colostrum in situations where ewe colostrum is unavailable.

The second aim of our study (Chapter 3) was to address the hypothesis that lack of colostral *M. ovi* specific antibodies leads to more severe disease following *M. ovi* challenge. Therefore, we inoculated four-month-old, specific-pathogen-free and immunologically naïve lambs with field isolates of *M. ovi* and monitored the lambs for eight weeks for colonization with the bacteria, *M. ovi*-specific antibodies, clinical symptoms, and cellular and molecular correlates of lung inflammation. After eight weeks, lambs were treated with the macrolide antibiotic gamithromycin and observed for an additional four weeks. Stable colonization of the upper respiratory tract with *M. ovi* was established in all four *M. ovi*-inoculated, but in none of the four mock-infected lambs. All *M. ovi*-infected lambs developed a robust antibody response to *M. ovi* within two weeks. However, we did not observe significant clinical symptoms or evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with gamithromycin failed to reduce *M. ovi* colonization. These observations indicate that, in the absence of co-factors, *M. ovi* causes asymptomatic colonization of the upper respiratory tract of that is resistant to clearance by the host immune response as well as by gamithromycin treatment in domestic lambs.

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CHAPTER TWO

IMMUNOGLOBULIN TRANSFER, SURVIVAL, AND GROWTH IN MOTHERLESS LAMBS FED A BOVINE SERUM-BASED COLOSTRUM REPLACER

Author Contributions

DB, TJ, and KJ designed the study; TJ, KJ, CM, SJ SK, CB, and AS performed the experiments; TJ, BTJ and DB analyzed the data; DB and TJ interpreted the data; DB obtained funding for the study; DB and TJ wrote the manuscript; all authors have read and approved the final manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abstract

Colostrum intake is vitally important for passive protection of lambs from infectious diseases. There are many scenarios, however, where ewe colostrum cannot be used. These situations include, but are not limited to, the death of the ewe, birth of multiple lambs, the generation of specific pathogen free animals, and disease eradication programs. In these situations, it is critical to have a colostrum replacer available. While colostrum replacers in the past have provided nutrients, they did not contain antibodies

and have thus led to poor outcomes with high lamb morbidity and mortality. Rescue Lamb & Kid Colostrum Replacer is a new colostrum replacer which contains bovine serum as an antibody source. We tested the Rescue Lamb & Kid Colostrum Replacer on newborn lambs that were separated from ewes at birth and reared in a Large Animal Biosafety Level 2 (ABSL-2). All lambs received the replacer for the first 24 hours of life. Lamb performance during the first 8 weeks after birth was assessed based on survival rate, health status, and average daily gains. Serum antibody ELISA performed on day 1 and day 14 revealed immunoglobulin levels of 17.9 ± 2.8 mg/mL and 27.5 ± 2.5 mg/mL, respectively. None of the lambs developed serious infectious disease conditions and maintained weight gain up until and throughout weaning. Based on these results, we conclude that Lifeline Lamb & Kid Colostrum Replacer is a viable substitute to ewe colostrum in situations where ewe colostrum is unavailable.

Introduction

Colostrum is the milk produced by a mother for the first 24 hours after giving birth. It contains protective antibodies, as well as high levels of nutrients (Aitken, 2007) and maternal immune cells (Reber et al., 2008). Because ruminants have a synepitheliochorial placenta that is impermeable to macromolecules, such as immunoglobulins, colostrum is the only source of antibodies in newborn lambs since lambs are not producing their own antibodies yet (Hernández-Castellano et al., 2015). High quality colostrum is linked to higher average daily gains as well as higher concentrations of IgG, superoxide dismutase, serum complement factor 3 (C3), and complement factor 4 (C4) in the serum. Consumption of quality colostrum also is

associated with development of greater villus length and width, crypt depth, villus height/crypt depth ratio, and mucosal thickness in the small intestine. (Yang et al., 2015). All these features contribute to improved lamb health and protection from infectious diseases. Without adequate colostrum uptake, newborns are less likely to survive until weaning (Devillers et al., 2011). This is in part because without sufficient colostrum uptake, failure of passive transfer (FPT) of immunoglobulins can occur. FPT leads to an increase in risk of morbidity and mortality, and decreased long-term productivity (Beam et al., 2009). This is because newborns lack the protective antibodies required to clear pathogens until their immune system has time to develop.

Colostrum replacers are critical for a productive livestock operation. If a ewe dies in labor or has multiple offspring and cannot produce enough colostrum to feed all of them, a rancher may turn to colostrum replacer to feed the newborn. This is important especially where raising lambs as twins, triplets, and even quadruplets is common. Oftentimes ranchers will use bovine colostrum as a substitute for ewe colostrum, but this is problematic because of potential contamination with bovine pathogens including rotavirus, *Cryptosporidium parvum*, bovine leukosis virus, and *Mycobacterium avium paratuberculosis* to which sheep are also susceptible (Munoz et al., 1996; Florins et al., 2007; Gollnick et al., 2007).

Up until recently, colostrum replacers were designed to replicate the nutrient content and composition of natural colostrum but failed to contain an antibody source. Because of this, any newborn given colostrum replacer lacked all protective maternal antibodies, leaving it susceptible to disease (GÖKÇE et al., 2013). Lambs that received

maternal colostrum, as opposed to colostrum replacer, demonstrated higher average daily gains and required fewer treatments for various illnesses (Lago et al., 2018).

Rescue Lamb & Kid Colostrum Replacer is a new colostrum replacer that contains bovine serum. The serum provides protective antibodies that previously were overlooked when designing colostrum replacers. While the antibodies are specific to pathogens encountered by cows, there is a significant amount of overlap between the pathogens to which cows and sheep are both exposed. Moreover, immunoglobulin genes and immunoglobulin function are highly conserved between cattle and sheep (Sun et al., 2012). This is why bovine colostrum is a common substitute for ewe colostrum. Therefore, colostrum replacers containing bovine antibodies are expected to achieve significant protection from common and environmental pathogens in lambs.

In this study, we analyzed the performance of lambs raised artificially using a colostrum replacer that contained bovine serum as an antibody source. Lambs demonstrated sufficient IgG and total serum protein concentrations, maintained steady weight gain, and had low incidences of disease and a low mortality rate.

Methods

Study site

All lambs were born and raised at the Johnson Family Livestock Facility at Montana State University in Bozeman, MT. Ewes were housed outside, and once the lambs were born they were transported inside the Large Animal Biosafety Level (ABSL)-2 Facility where they were housed inside for the remainder of the study. Lamb rooms had a combination of concrete and slatted Dura Trac Calf Flooring (ADA Enterprises, INC,

Northwood, IA). Rooms were maintained at a temperature of 16°C via a controlled heating, ventilation, and air conditioning (HVAC) system. Air within the rooms was exchanged 16 times an hour via the HVAC system to reduce humidity. The rooms were on a natural light cycle, with the exception that they were turned on at feeding times. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Montana State University, protocol #2019-95.

Study cohort

We acquired 15 mixed breed (Rambouillet, Suffolk, Targhee, Colombia, and Hampshire) pregnant ewes from a local rancher. All ewes were closely monitored for signs of labor prior to lambing. Once the ewes began to display signs of labor, they were isolated in small catch pens. Two trained staff members assisted with each birth. All staff wore sterile scrubs and gloves for the delivery. Lambs were delivered onto sterile towels places on clean plastic sheets instead. Immediately after birth, the lambs were separated from the ewes and transferred to the ABSL-2 facility where they were raised isolated from other sheep and livestock as part of an ongoing study that involved the derivation of a specific-pathogen-free research flock. Raising the lambs in the ABSL-2 facility was critical to prevent the lambs from being exposed to naturally occurring environmental pathogens and *M. ovi*. All staff members showered and changed into sterile scrubs before entering the lamb rooms. All supplies were sterilized before entering the lamb room with the exception of feed. Between March and April of 2020, 30 live lambs were born and were included in this study.

Administration of colostrum replacer

For the first 24 hours of life, lambs were fed Rescue Lamb & Kid Colostrum Replacer (Lifeline Nutrition Solutions/APC, Ankeny, IA) according to the manufacturer's instructions. Colostrum replacer nutritional components provided by the manufacturer are listed in **Table 1.** Lambs were bottle fed every 4 hours for the first 24 hours of life, yielding 6 meals of colostrum replacer. Ideal colostrum consumption was calculated based on the brand's recommendation, 40 grams of powder per 1 kg of body weight over 24 hours. At each of the six feedings, the staff aimed to feed at least one sixth of the recommended total amount of colostrum to each lamb. Colostrum consumption was recorded after every meal.

Lamb diet and weaning regimen

After receiving colostrum replacer for 24 hours, lambs were switched to SuperLamb Milk Replacer (Milk Specialties Global, New Holstein, WI). Nutritional components provided by the manufacturer are listed in **Table 1**. Lambs were bottle fed for the first week of life before they were trained to use feeding buckets that provided the milk replacer until weaning (**Fig. 1**). Lambs were weaned around day 45 onto a mixture of corn, oats, and barley with molasses as well as Lamb Starter Pellet (Payback, Sioux Falls, SD) and ground hay. A gradual weaning approach that involved a 50% reduction in milk replacer volume for two weeks was used, as described previously (Bimczok et al., 2005b). Lambs were offered water and a salt and mineral supplement ad libitum.

Lamb body weights

Lambs were weighed immediately post birth before their first feeding. Subsequently, lambs were weighed once a week after feeding. A household bathroom scale was used to weigh the lambs. A staff member would stand on the scale holding the lamb, and the weight of the staff member was subtracted from the total weight to obtain the weight of the lamb.

Scoring of lamb health

To assess lamb health during the study period, detailed records of all the lambs were maintained, including how much each lamb consumed at every feeding, and any abnormalities including fever, signs of scours, treatments, and why those treatments were administered. Written records were analyzed using a computational linguistics approach. The raw observations of the sheep health were added to a SQL database and processed with an automated Python 3.8 script that used predefined keywords or conditions to assign scores to 4 different categories for each observation. The categories included Behavior, Appetite, Digestive Symptoms, Medication, and Body Temperature. The keywords used by the program to assign points are listed in **Table 4**. The keyword for the Medication category flagged the entries for manual review. Preventative treatments, such as vitamins at birth and routine vaccines, were excluded from the analyses. All other treatments resulted in a score of 1. The temperature score was calculated by assigning a score of 1 if the rectal temperature was more than 104.0°F. These scores were added together -to calculate a "Total Health Score" for each week.

Measurement of total serum protein and total antigen-specific IgG

Total serum protein concentrations one day and 14 days post- birth were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltman, MA). Because the colostrum replacer contained bovine serum, serum IgG concentrations in the lambs were measured one day and 14 days post-birth using a Bovine Immunoglobulin G ELISA Kit (MyBioSource, San Diego, CA). To analyze antigen-specific IgG levels, parainfluenza 3 virus neutralization titers in lamb serum were measured on days 14, 30, 60, 90, and 180 post-birth by Washington Animal Disease Diagnostic Lab (Pullman, WA).

Results

Lamb characteristics at birth.

The 15 bred ewes delivered 32 live lambs. Of these lambs, 30 survived until weaning and were used to collect data for this study. All birth characteristics are detailed in **Table 2**. Of the 30 lambs that survived throughout the study, 19 were female and 11 were male. Twins were most common, followed by triplets, then quadruplets, then singles. Smaller litter size correlated with higher birth weights in both females (**Fig. 2A**) and males (**Fig. 2B**). Birth weights of males and females of the same litter size were analyzed using a Student's T test, showing there was no significance.

Colostrum replacer yielded high lamb survival rates.

Out of the 32 lambs born during this study, 30 survived the first 8 weeks. This corresponds to a 93.7% survival rate (**Table 2**). Of the two lambs that were euthanized, one lamb was born with neurological issues and consequentially euthanized 6 days after birth. The second lamb was euthanized 2 days after birth due to low levels of colostrum consumption and lethargy even after multiple treatments and tube feedings.

Lambs gained and maintained healthy weights.

All lambs demonstrated steady weight gain up to, and throughout, weaning as shown in **Table 3**. All lambs had 8-week average daily gains (ADG) between 0.29 and 0.45 kg/day. There was no significant difference in ADG between females (**Fig. 2C**) or males (**Fig. 2D**) of varying litter sizes. ADG of males and females of the same litter size were analyzed using a Student's T test, which also revealed no significant differences.

Lambs displayed sufficient IgG and total serum protein concentrations.

Serum antibody ELISA performed on day 1 found immunoglobulin levels of 17.9 ± 2.8 mg/mL. On day 14, immunoglobulin levels were significantly higher at 27.5 ± 2.5 mg/mL (**Fig. 3A**). BCA assay performed on serum from days 1 and 14 revealed 97.58 ± 17.6 mg/mL and 68.12 ± 20.01 mg/mL, respectively, as detailed in **Fig. 3B**. In contrast to total IgG, total serum protein was significantly decreased on day 14 compared to 24 h after birth.

Colostrum consumption did not correlate with IgG or total serum protein concentrations.

As shown in **Fig. 3C** and **Fig. 3D**, there was no significant correlation between colostrum consumption and either IgG uptake or total serum protein concentrations. In addition, there was no correlation between IgG uptake and total serum protein concentrations (**Fig. 3E**).

Antigen-specific IgG from colostrum replacer was maintained for two months.

Parainfluenza 3 (PI3) virus neutralization titers decreased steadily over time until they were measured at zero on day 180 post birth (**Fig. 3F**). Because lambs were raised in the ABSL-2 facility, there was no exposure of lambs to PI3 virus during the experimental period.

Lambs maintained good health status.

To assess lamb health, all animals were monitored multiple times per day, and each lamb was assigned scores at each feeding in categories including Behavior, Appetite, Digestive, Medication, and Body Temperature using an unbiased coding system that assigned points based on key words pulled from the detailed records kept. As shown in **Table 5**, lambs received the highest scores in the Behavior category, followed by Medication and Appetite. Lambs received lower scores in the Digestive and Body Temperature category. Appetite and Digestive scores were highest at week 1 and decreased after that. Body temperatures were highest at week 2 and decreased continually over the 8-week period. Total weekly score averages ranged from 0.10 at week 8 to 4.63

at week 1. Overall, these scores indicate no serious infectious diseases occurred in our experimental flock.

Discussion

In this study, we have shown that colostrum replacer containing bovine serum as an antibody source is a viable replacement to ewe colostrum. Thirty-two lambs were born during this study, thirty of which survived past the first eight weeks and were used to collect data for this study. This yielded a 93.7% survival rate. This percentage is at the high end of the survival rate for lambs, which ranges from 93.4-86.1% (USDA, 2014). While the survival rate was high, that could partially be due to the intensive monitoring and care of the lambs. Range lambs are not monitored around the clock, and subsequently illnesses can go untreated. This can result in increased disease and death. Another factor that may play a role in their high survival rates is that they were raised in an ABSL-2 facility where they were not exposed to as many pathogens as range lambs.

All lambs all demonstrated steady weight gain up to, and throughout, weaning. Average daily gain for the first 8 weeks of life was 0.36 kg/day. It is expected that healthy lambs gain between 0.18 and 0.41 kg per day (Sayed, 2009). Weight gain of lambs in this study fell within the expected range for lambs, indicating they were healthy.

Serum IgG concentrations of 17.9 ± 2.8 mg/mL 24 hours post birth verified the presence of passive transfer of antibodies found in the colostrum replacer. This value is only slightly lower than 19.56 mg/mL (Barta, 1993) and 24.6 ± 17.5 mg/mL (Massimini et al., 2006) which are typical for IgG levels found in 24 hour old lambs. Adequate passive transfer immune transfer is considered an IgG concentration of 15 mg/mL or

higher (Alves et al., 2015). IgG concentrations increased significantly to 27.5 ± 2.5 mg/mL by day 14. This increase is consistent with IgG levels in other livestock, which show an increase to between 21.6 mg/mL and 45.2 mg/mL between 2 and 8 days of age (Waldner and Rosengren, 2009) and between 15.2mg/mL and 22.5 mg/mL at three weeks of age (Erhard et al., 1999). Correlation between serum IgG concentrations and health status of lambs at weaning has also been shown in a study where goat kids that survived until weaking had statistically significantly higher concentrations of serum IgG as opposed to goat kids that did not survive (O'brien and Sherman, 1993). While the IgG present 24 hours after birth is from the colostrum replacer, the increase at day 14 is likely due to the production of antibodies by the lambs' immune systems in response to antigen stimulation. Since a bovine IgG ELISA was used, this indicates the cross reactivity of ovine and bovine antibodies, which has been previously established (Henning and Nielsen, 1992).

Total protein concentrations were also established for days one and 14 post birth and were 97.58 ± 17.6 mg/mL and 68.12 ± 20.01 mg/mL, respectively. This is higher than total serum protein levels of 76.05±1.97 mg/mL in 24 hour old lambs who suckled colostrum from the ewe (Ahmad et al., 2000). Adequate passive transfer immune transfer is considered a serum protein concentration of 45 mg/mL or higher (Alves et al., 2015). By two weeks of age, the total serum protein levels in livestock has dropped to a value around 61.4±1.7 mg/mL (Nagy et al., 2014). This value is comparable to the total serum protein levels seen in our experiment.

Parainfluenza 3 virus neutralization titers were measured on days 14, 30, 60, 90, and 180 post-birth as a way to measure antigen-specific IgG in the lambs. Titer levels were highest on day 14 and decreased steadily at each timepoint until they were non-existent at day 180. Parainfluenza 3 virus is a common bovine pathogen, and antibodies were likely found in the bovine serum used in the colostrum replacer. This data shows that colostral antibodies persist in lamb serum for up to 3 months after birth. This is consistent with other findings that show the duration of maternal antibodies in animals is 3-6 months (Niewiesk, 2014).

There was no significant correlation between amount of colostrum consumption and either IgG uptake or total serum protein concentrations. In addition, there was no correlation between IgG uptake and total serum protein concentrations. This is surprising because IgG and total protein concentrations are commonly used to determine the quality of colostrum (Gulliksen et al., 2008) and to determine passive transfer (Berge et al., 2018). However, it has been previously shown that restricted colostrum consumption in Canary Caprine kids has no significant impact on serum IgG concentrations (Argüello et al., 2004). From this data, it was concluded that testing total serum protein concentration is not an accurate way to measure IgG.

All health data was analyzed using code that assigned points based on key words from the detailed lamb records. This allowed for the rapid and unbiased scoring of the health metrics for the sheep and for easy access to the compiled dataset. Our results showed low incidences of abnormal behavior, decreased appetite, scours, need for

medical intervention, and fever. While there are no papers with comparable data since the method for analysis was unique, these results on their own indicate a healthy flock.

One limitation of our study was that it did not include a control group that was fed ewe colostrum. The rationale for this approach was that all lambs were part of a study to derive an SPF research flock; therefore, all contact with ewes and their body secretions was prevented in order to avoid pathogen transmission. While having a group of lambs fed colostrum from the ewe would have improved this study, there are many other papers that showed that the LifeLine colostrum replacer used here yielded similar ADGs, serum IgG concentrations, and total serum protein concentrations to lambs fed ewe colostrum (Henning and Nielsen, 1992; Barta, 1993; Erhard et al., 1999; Massimini et al., 2006; Sayed, 2009; Waldner and Rosengren, 2009; Nagy et al., 2014).

The data from our study have important implications for sheep ranchers because having a viable replacement to ewe colostrum can save the lives of many lambs. Using old colostrum replacers that do not contain any antibodies, ranchers would lose lambs due to disease and inadequate weight gain. If a ewe is lost or cannot produce enough colostrum for all of her lambs, a rancher can now keep the lambs healthy and alive by feeding colostrum replacer containing bovine serum as an antibody source.

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Tables

Table 1: Nutritional composition of colostrum/milk replacer

	Colostrum Replacer	Milk Replacer
Crude protein	44.0%	24.0%
Crude fat	24.0%	30.0%
Crude fiber	0.5%	0.10%
Calcium	0.4-0.9%	1-0.5%
Phosphorus	0.4%	0.65%
Salt	2-2.5%	Trace amounts
Sodium	1.1-1.6%	Trace amounts
Selenium	0.3 ppm	0.3 ppm
Vitamin A	111,111 IU/kg	66,666 IU/kg
Globulin protein	24.0%	NA

Table 2: Lamb characteristics

	Number	Percentage
Ewes	15	
Lambs	30	
Males	11/30	36.6%
Females	19/30	63.3%
Singles	2/30	6.6%
Twins[1]	12/30	40.0%
Triplets[1]	10/30	33.3%
Quadruplets[1]	6/30	20.0%
Survival	30/32	93.8%

[1]Litter size refers to number of lambs born including still born lambs.

Table 3: Average daily rate of gain, kg

Week	Average daily rate of gain, kg/d
1	0.24 ± 0.06
2	0.34 ± 0.09
3	0.38 ± 0.06
4	0.48 ± 0.10
5	0.36 ± 0.16
6[1]	0.40 ± 0.18
7[1]	0.25 ± 0.11
8	0.39 ± 0.16
Average	**0.36 ± 0.04**

[1]Lambs were weaned between weeks 6 and 7.

Table 4: Health scoring key words

Parameter	Key words
Behavior	Pretty, Fairly, AR, Lethargic, Looks off, Acts off, Abnormal, Uncomfortable, Droopy, Weak, Laid down
Appetite	No appetite, Poor appetite, Did not eat, Did not consume, Did not go to concentrate, Did not go to hay, Decreased appetite
Digestive symptoms	Scours, Diarrhea, Runny, Liquid
Medication[1]	Trmt
Body temperature	>104.0°F

[1]Medication key term flagged records for manual review.

Table 5: Clinical scores by week

Week	Behavior	Appetite	Digestive	Medication	Body Temp	Total
1	2.07 ± 1.26	1.17 ± 0.99	0.63 ± 0.81	0.77 ± 1.04	0.00 ± 0.00	4.63 ± 2.76
2	0.20 ± 0.41	0.10 ± 0.31	0.27 ± 0.64	0.27 ± 0.64	0.13 ± 0.35	0.97 ± 1.61
3	0.27 ± 0.64	0.17 ± 0.59	0.03 ± 0.18	0.40 ± 0.97	0.03 ± 0.18	0.90 ± 2.19
4	0.77 ± 0.77	0.47 ± 0.63	0.17 ± 0.38	1.33 ± 1.37	0.10 ± 0.31	2.83 ± 2.21
5	0.53 ± 0.90	0.37 ± 0.84	0.30 ± 0.84	0.77 ± 1.52	0.10 ± 0.31	2.07 ± 2.70
6	0.33 ± 0.71	0.33 ± 1.03	0.07 ± 0.25	0.23 ± 0.73	0.00 ± 0.00	0.97 ± 1.99
7	0.07 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.37	0.00 ± 0.00	0.13 ± 0.43
8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.40	0.00 ± 0.00	0.10 ± 0.40
Average	**0.53 ± 0.67**	**0.33 ± 0.38**	**0.18 ± 0.22**	**0.49 ± 0.43**	**0.05 ± 0.06**	**1.58 ± 1.54**

Figures

Figure 1: Image of lambs drinking milk replacer from a bucket feeder.

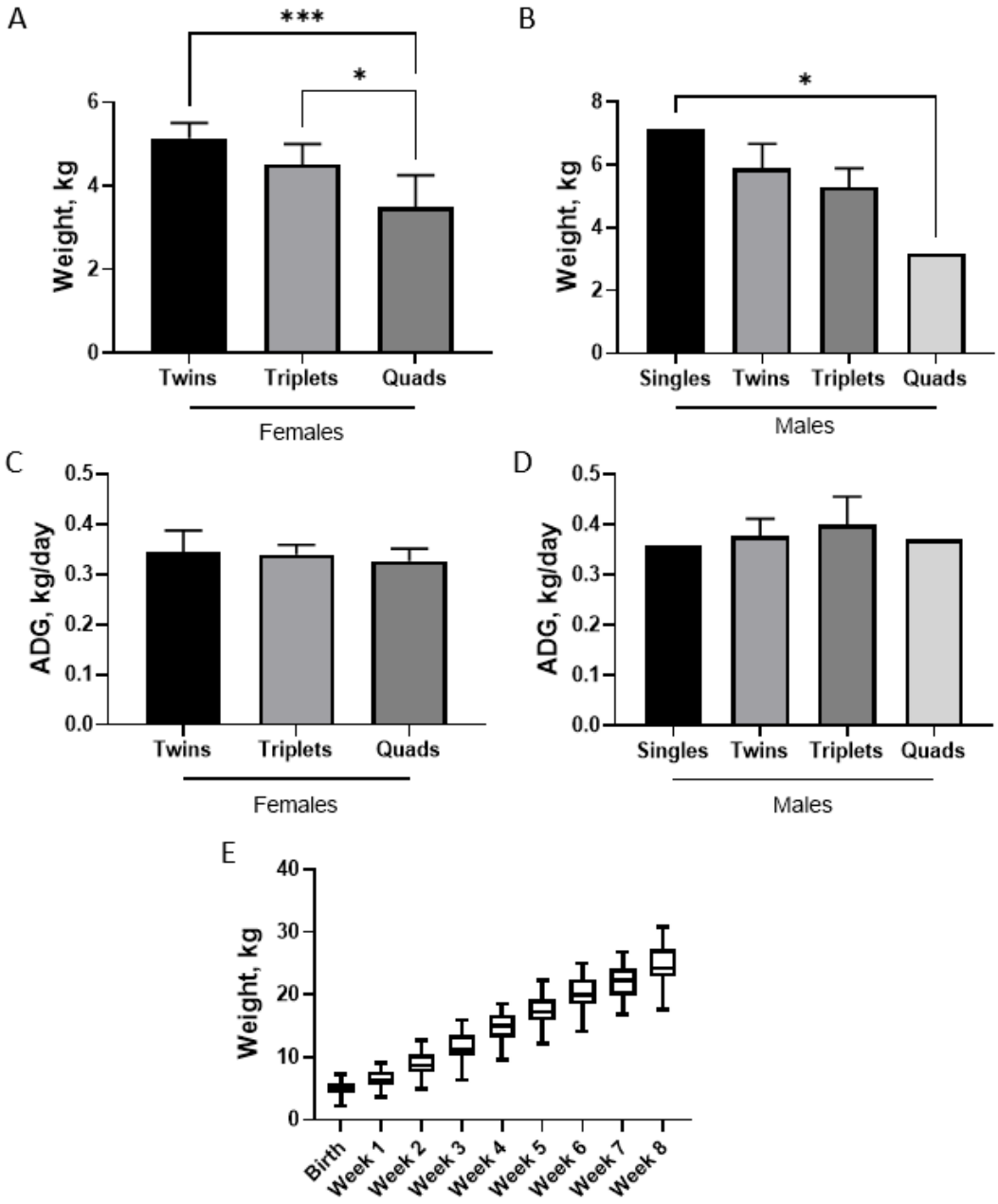

Figure 2: Lambs weights and weight gains. **(A,B)** Birth weights of **(A)** female and **(B)** male lambs by litter size. **(C, D)** Average daily gains of **(C)** female and **(D)** male lambs during the first 8 weeks after birth by litter size. **(A,B,C,D)** Data were analyzed by ordinary one-way ANOVA and are shown as mean ± SD; *$P \leq 0.05$, ***$P \leq 0.001$. **(E)** Development of lamb weights (n=30) over the first 8 weeks, whiskers show min and max, end of boxes show upper and lower quartiles, median is marked by horizontal line inside of box.

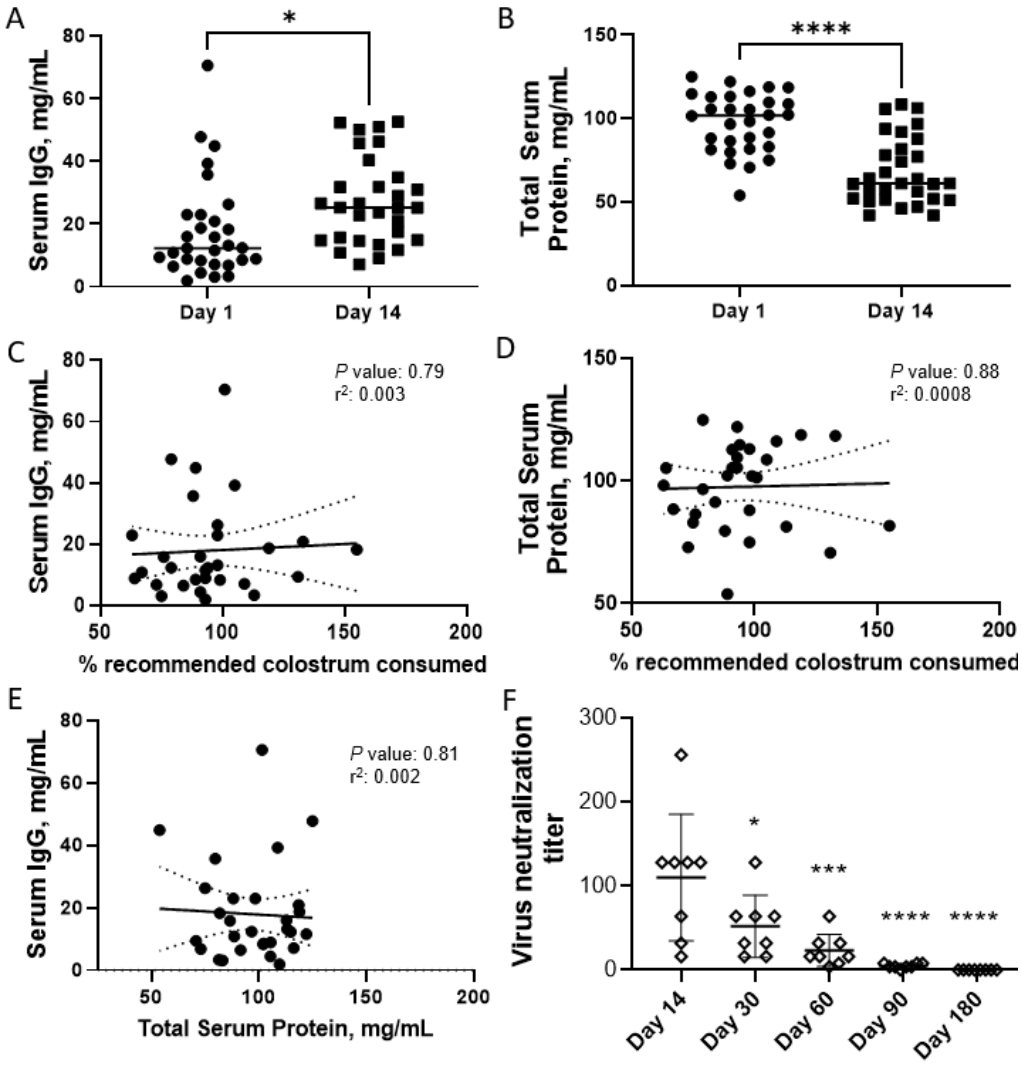

Figure 3: Serum concentrations of IgG, total protein, and Parainfluenza 3 virus neutralizing titers following consumption of colostrum replacer. **(A)** Concentration of total bovine IgG in lamb serum on days 1 and 14 post birth as determined by ELISA. **(B)** Total protein concentrations in lamb serum on days 1 and 14 post birth as determined by BCA assay. **(A,B)** Individual data points, mean ± SD, paired Student's t test, $*P \leq 0.05$, $****P \leq 0.0001$. **(C)** Relationship between concentration of total bovine IgG in lamb serum on day 1 post birth and percentage of recommended colostrum consumed. **(D)** Relationship between total serum protein and percentage of recommended colostrum consumed. **(E)** Relationship between total bovine IgG and protein concentration in lamb serum. **(C,D,E)** Solid line indicates simple linear regression, dotted lines indicate 90% confidence interval. **(F)** Virus neutralization titers for Parainfluenza 3 on days 14, 30, 60, 90, and 180 post birth. Days 30, 60, 90, and 180 are compared to day 14 using ordinary one-way ANOVA. $*P \leq 0.05$, $*P \leq 0.001$, $****P \leq 0.0001$.

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CHAPTER 3

EXPERIMENTAL INFECTION OF SPECIFIC-PATHOGEN-FREE DOMESTIC LAMBS WITH MYCOPLASMA OVIPNEUMONIAE CAUSES ASYMPTOMATIC COLONIZATION OF THE UPPER AIRWAYS THAT IS RESISTANT TO ANTIBIOTIC TREATMENT

Author Contributions

DB, MJ, ARA, TJ, KJ and TB designed the study; TJ, KJ, BTJ and JS performed the experiments; TJ, BTJ and DB analyzed the data; DB, TJ, ARA and MJ interpreted the data; DB obtained funding for the study; DB and TJ wrote the manuscript; all authors have read and approved the final manuscript.

Abstract

Mycoplasma ovipneumoniae (*M. ovipneumoniae*) is a respiratory pathogen associated with the development of mild to moderate respiratory disease in domestic lambs and severe pneumonia outbreaks in wild ruminants such as bighorn sheep. However, whether *M. ovipneumoniae* by itself causes clinical respiratory disease in domestic sheep in the absence of secondary bacterial pathogens is still a matter of debate. The goal of our study was to better understand the role of *M. ovipneumoniae* as a respiratory pathogen in domestic sheep and to explore potential antibiotic treatment approaches. Therefore, we inoculated four-month-old, specific-pathogen-free lambs with field isolates of *M. ovipneumoniae* and monitored the lambs for eight weeks for colonization with the bacteria, *M. ovipneumoniae*-specific antibodies, clinical symptoms,

and cellular and molecular correlates of lung inflammation. After eight weeks, lambs were treated with the macrolide antibiotic gamithromycin and observed for an additional four weeks. Stable colonization of the upper respiratory tract with *M. ovipneumoniae* was established in all four *M. ovipneumoniae*-inoculated, but in none of the four mock-infected lambs. All *M. ovipneumoniae*-infected lambs developed a robust antibody response to *M. ovipneumoniae* within 2 weeks. However, we did not observe significant clinical symptoms or evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with gamithromycin failed to reduce *M. ovipneumoniae* colonization. These observations indicate that, in the absence of co-factors, *M. ovipneumoniae* causes asymptomatic colonization of the upper respiratory tract of that is resistant to clearance by the host immune response as well as by gamithromycin treatment in domestic lambs.

Introduction

Mycoplasma ovipneumoniae (M. ovipneumoniae) is a highly prevalent respiratory pathogen associated with atypical, chronic non-progressive pneumonia in sheep and goats. Colonization of the upper respiratory tract of adult sheep with *M. ovipneumoniae* is increasingly recognized across the world, with infections reported in Europe, North America, Africa, Asia, Australia and New Zealand (Ionas et al., 1991; Mohan et al., 1992; USDA, 2013; Cheng et al., 2015; Jay et al., 2020). In the United States, active *M. ovipneumoniae* infection was detected in 88.5% of commercial sheep flocks in a 2011 study (USDA, 2013). While most cases of *M. ovipneumoniae* infection in adult sheep are thought to be asymptomatic or mild, significant production losses may occur due to

reduced weight gains, lower carcass quality and increased mortality in lambs (Cheng et al., 2015; Besser et al., 2019; Manlove et al., 2019a). Moreover, *M. ovipneumoniae*-infected domestic sheep pose a significant threat to wild ruminant populations such as bighorn sheep (*Ovis canadensis*) (Besser et al., 2008; Besser et al., 2013), Dall's sheep (*Ovis dalli dalli*) (Black et al., 1988), Argali sheep (Li et al., 2020), and Norwegian Muskox (*Ovibos moschatus*) (Handeland et al., 2014b), where *M. ovipneumoniae* infection causes severe pneumonia outbreaks with up to 100% mortality.

However, it is still unclear to what extent *M. ovipneumoniae* causes clinical respiratory disease in sheep in the absence of other pathogenic. Several studies have demonstrated that *M. ovipneumoniae* greatly increases the susceptibility for infection with other opportunistic pathogens such as *Mannheimia haemolytica* and *Bibersteinia trehalosi*, which then leads to proliferative interstitial pneumonia with severe clinical symptoms and significant mortality (Jones et al., 1982a; Dassanayake et al., 2010b; Besser et al., 2013; Dassanayake et al., 2013). However, infection studies with *M. ovipneumoniae* alone have yielded conflicting results. Thus, respiratory symptoms and lung pathology were observed in a subset of sheep following endobronchial application of *M. ovipneumoniae* in two early studies (Foggie et al., 1976; Jones et al., 1982b), and more recently, Du et al. described coughing, wheezing and increased body temperatures in Bashbay lambs experimentally inoculated with *M. ovipneumoniae* (Du et al., 2020). In contrast, Buddle et al. (Buddle et al., 1984) did not find evidence of pneumonia in colostrum-deprived lambs after experimental *M. ovipneumoniae* infection. In bighorn sheep, inoculation with cultured strains of *M. ovipneumoniae* did not result in clinical

respiratory disease (Besser et al., 2008), whereas natural transmission of *M. ovipneumoniae* between animals led to severe bronchopneumonia (Besser et al., 2014). These conflicting findings point to a need for further experimental investigations.

While the contribution of *M. ovipneumoniae* to sheep respiratory disease is widely acknowledged and elimination of *M. ovipneumoniae* colonization in domestic sheep could prevent severe disease outbreaks in bighorn sheep and other wild ruminants, no vaccines or treatments to combat the infection are currently approved. While experiments have demonstrated the *in vitro* susceptibility of most *M. ovipneumoniae* isolates to a wide range of macrolide, tetracycline and fluoroquinolone antibiotics (Jay et al., 2020; Maksimovic et al., 2020), no studies on antibiotic treatment of *M. ovipneumoniae* infection *in vivo* have been published.

To better understand the role of *M. ovipneumoniae* as a respiratory pathogen in domestic sheep and to explore potential antibiotic treatment approaches, we performed an *M. ovipneumoniae* infection and antibiotic treatment study in four-month-old specific-pathogen-free lambs. Intranasal inoculation of lambs with a native field isolate of *M. ovipneumoniae* resulted in consistent colonization of the upper respiratory tract and robust *M. ovipneumoniae*-specific humoral immunity. However, we did not observe clinical symptoms or evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with the macrolide antibiotic gamithromycin, which has been used successfully to treat clinical *Mycoplasma*-associated pneumonia in cattle, goats and pigs (Baggott et al., 2011a; Kacar et al., 2018a; Maes et al., 2020a), failed to reduce *M. ovipneumoniae* colonization in the infected lambs. Overall, these data suggest that, in

the absence of other opportunistic pathogens, *M. ovipneumoniae* behaves like an upper respiratory tract commensal that is not affected by the host antibody response or a macrolide antibiotic gamithromycin, which is commonly used to treat respiratory disease in ruminants.

Methods

Animals and husbandry

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Montana State University, protocol #2019-95. To generate specific-pathogen-free lambs, 15 mixed-breed ewes (Rambouillet, Suffolk, Targhee, Columbia, and/or Hampshire) 4-5 years of age were purchased several weeks before the projected lambing date from a local sheep farmer. Pregnancy was confirmed using abdominal ultrasound, and *M. ovipneumoniae* exposure was determined by serology and nasal swab PCR, as described below. Prior to lambing, ewes were fed hay from the Johnson Family Livestock Facility farm, grain, and an appropriate vitamin/mineral supplement.

To derive lambs free from *M. ovipneumoniae* and other facultative respiratory pathogens, we used supervised lambing and motherless rearing, as previously described (Bimczok et al., 2005a; Voigt et al., 2007). Around the projected lambing date, ewes were monitored around the clock for signs of imminent delivery. Ewes developing signs of labor were separated from the flock and moved into a designated lambing pen, and lambs were manually delivered onto sterile towels placed on top of a clean plastic sheet and then were transferred into a separate, heated nursery area within the Johnson Family

Livestock Facility (JFLF) ABSL-2 laboratory. All animal care personnel showered and changed into sterile PPE prior to entering the nursery, and personnel responsible for the lambs did not have any contact with other non-SPF sheep for the duration of lambing. Lambs were housed in heated animal rooms (15.5-16.8 °C) inside the JFLF in groups of 5 – 10 animals, with siblings and lambs of similar ages grouped together. During the first 24 h, lambs were bottle-fed a commercial colostrum replacer (Rescue Lamb & Kid Colostrum Replacer, Lifeline Nutrition Solutions/APC, Ankeny, IA), which contains bovine serum as an antibody source. Animals were trained to use bucket feeders and were fed a commercial lamb milk replacer diet (Hubbard Feeds, Mankato, MN) for 5 weeks, with ad libitum access to hay and water. Lambs were gradually weaned onto pelleted lamb food starting at 36-48 days of age days by decreasing the concentration of milk replacer to 50% for two weeks as previously described (Bimczok et al., 2005a).

Health monitoring

Approximately 2 months prior to lambing, ewes were screened for *M. ovipneumoniae, Coxiella burnetii* and *Mycobacterium avium paratuberculosis* (MAP) exposure by serum ELISA and for parainfluenza 3 (PI3) exposure by serum-based virus neutralization assay. Nasal swabs from the ewes were also analyzed for *M. ovipneumoniae* by PCR.

SPF lambs at the JFLF were screened daily for signs of illness or discomfort. In addition, SPF-status of the lambs was confirmed by testing nasal swab samples for *M. ovipneumoniae* infection by quantitative PCR at birth, at the age of 1 month, and seven days prior to the start of the experiment. At 3 months of age, nasal swabs from a subset

of lambs were analyzed for *M. ovipneumoniae* by PCR and for the presence of Pasteurellaceae by conventional aerobic culture. All laboratory assays were performed at the Washington Animal Disease Diagnostic Laboratory (WADDL, accredited by the American Association of Veterinary Laboratory Diagnosticians).

Following experimental *M. ovipneumoniae* challenge or mock treatment, lamb health status was assessed twice daily by trained JFLF personnel. The following health parameters were assessed and were used to calculate a clinical disease score: (1) general behavior, (2) appetite, (3) rectal temperature, (4) respiratory symptoms, and (5) any medications administered exclusive of the experimental treatment provided at 8 weeks p.i. , as detailed in **Table 1**. Daily scores represent the sum of the two values obtained for each day, and weekly scores represent the sum of all 14 values collected over a 7-day period. In addition, lamb body weights were determined on days 20, 32 and 55 post challenge.

Experimental infection with *Mycoplasma ovipneumoniae*

Out of thirty live lambs born in our flock, four SPF lambs aged between 15 and 16 weeks were selected for experimental infection with *M. ovipneumoniae,* and four additional lambs, matched for age and sex, were selected as a control group. Animal details are listed in **Table 2**. The SPF lambs were infected using pooled nasal wash fluids from lambs previously determined to be infected with *M. ovipneumoniae*, following published protocols (Besser et al., 2014; Ziegler et al., 2014; Besser et al., 2017). Animals housed at MSU's Red Bluff Research Ranch, aged 11-13 weeks, were used as donor lambs for nasal washes. We selected eight lambs (4 ewes and 4 wethers)

with mild respiratory disease symptoms that were confirmed to be infected with *M. ovipneumoniae* based on PCR analysis of nasal swabs. To obtain nasal wash fluids, the *M. ovipneumoniae*-infected lambs were restrained using halters, with the heads kept in a slightly lowered position. Nasal washes were performed by squirting 2 x 15 mL of sterile PBS into each nostril and collecting the flush fluid into a clean polyethylene bag. Nasal wash fluids were transferred to the laboratory and were immediately pooled, diluted 1 : 1 with tris-buffered saline, and then were treated with ceftiofur (100 µg/mL, MWI Animal Health, Boise, ID) for 2 h at 37°C to reduce contamination with *Pasteurellacea.* Microbiological analysis of the pooled nasal wash fluid confirmed that both *Mannheimia haemolytica* and *Bibersteinia trehalosi* were present before ceftiofur treatment, but were undetectable after the treatment. We then inoculated sheep with 50 mL of the treated nasal wash fluid (*M. ovipneumoniae* group) or PBS (control group) by infusing 15 mL into each nostril, 10 mL into the oral cavity and 5 mL into each conjunctival sac. Following inoculation, the *M. ovipneumoniae* group and the control group were housed in separate rooms of the JFLF to prevent aerosol transmission of *M. ovipneumoniae*. Each room had its own set of equipment so that no equipment was shared between the rooms. For the duration of the infection experiment, staff first performed all necessary husbandry procedures on the control animals before entering the room with the infected animals and showered and changed immediately after leaving the room with the infected lambs.

Antibiotic treatment

Eight weeks after experimental challenge with *M. ovipneumoniae* or mock challenge with PBS, all lambs were treated with 6 mg/kg BW gamithromycin (Zactran®, Boehringer Ingelheim, Ridgefield, CT) two times over 5 days by subcutaneous injection.

Collection and analysis of bronchoalveolar lavage fluids

To collect bronchoalveolar lavage fluid (BAL), a flexible fiber-optic endoscope measuring 6.6 mm x 100 cm (VFS-2B VetVu, Swiss Precision Products, Inc, Oxford, MA) was introduced into the trachea under local anesthesia with lidocaine. Lavage was performed by instilling 60 mL of sterile saline into the lungs and then aspirating the liquid again; approximately 20 mL of fluid were routinely recovered from the sheep. BAL fluid was stored on ice until transfer to the laboratory, where cells were harvested by centrifugation at 400 *g* for 10 min. Supernatants were then analyzed for evidence of lung damage using the Lactate Dehydrogenase Assay Kit (Abcam, Cambridge, UK). Cell pellets were processed for absolute cell counts using a hemocytometer and for differential cell counts using cytospin preparations stained with a DippKwik stain (ThermoFisher, Waltham, MA). At least 300 cells from each sample were classified by a scientist blinded to the treatment of the animals.

Detection of *Mycoplasma ovipneumoniae* infection

PCR detection of *M. ovipneumoniae* infection was performed at the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, WA) using nasal swab samples and standard protocols (Lawrence et al., 2010). Briefly, probe based quantitative

PCRs were performed with the following primers and probe: forward: 59-GGG GTG CGC AAC ATT AGT TA-39; reverse: 59-CTT ACT GCT GCC TCC CGT AG-39; and probe: 59-6-FAM-TTA GCG GGG CCA AGA GGC TGT A-BHQ-1-39 derived from GenBank sequences EU290066 and NR_025989 of *M. ovipneumoniae.* Samples with cT values <40 were considered positive. Data are shown as 40 minus the measured cT value to enable semi-quantitative comparison between samples.

M. ovipneumoniae serology

Analysis of serum samples for the presence of *M. ovipneumoniae*-reactive antibodies was performed at WADDL using the laboratory's monoclonal antibody-based competitive enzyme-linked immunosorbent assay (cELISA) test, which has a diagnostic sensitivity of 88% and a diagnostic specificity of 99.3%. Validation data for the assays may be obtained directly from the laboratory at http://www.vetmed.wsu.edu/depts_waddl/). Data are shown as % inhibition and represent the reduction in binding of the labelled monoclonal antibody to the *M. ovipneumoniae* test antigen caused by competitive binding of serum antibodies from the diagnostic samples. Inhibition of >50% was considered a positive result, and inhibition between 40% and 50% was considered indeterminate.

Statistical analyses

Data were analyzed by GraphPad Prism, version 9.0 (San Diego, CA) and are shown as mean ± standard deviation (SD). Differences between groups were analyzed by 2-way ANOVA with Sidak's or Dunnett's multiple comparisons test or by Student's *t* test and were considered significant at $P \leq 0.05$.

Results

Supervised lambing and motherless rearing successfully prevent colonization of domestic lambs with *M. ovipneumoniae* and Pasteurellaceae.

Specific-pathogen-free (SPF) lambs were derived by supervised lambing and artificial rearing from a domestic sheep flock with a history of *M. ovipneumoniae* infection. Pathogen exposure of the ewes was determined by serological analysis prior to lambing. The ewes were free from *C. burnetii* and *M. avium ssp. paratuberculosis*, but had variable serological responses to *M. ovipneumoniae* and parainfluenza virus (PI-3) (**Table 3**). Notably, nasal swabs collected at the same time as the serum samples tested negative for *M. ovipneumoniae* by PCR, indicating previous exposure of the sheep, but no active pathogen shedding.

All thirty lambs born from our ewe flock including the eight experimental lambs selected for our study were free from *M. ovipneumoniae* on days 0 and 30 after birth and one week prior to the experimental inoculation (**Table 4**). We also confirmed the absence of upper respiratory tract colonization by Pasteurellaceae, which include the facultative respiratory pathogens *Mannheimia haemolytica* and *Bibersteinia trehalosi*, in all eight experimental lambs at 3 months of age.

Application of nasal wash fluids from *M. ovipneumoniae*-infected lambs leads to successful colonization of specific-pathogen free lambs and induces *M. ovipneumoniae*-specific serum antibodies.

Experimental infection of SPF-lambs aged 103-109 days (3-4 months) with *M. ovipneumoniae* was performed by inoculating four SPF lambs with nasal wash fluids from *M. ovipneumoniae* carriers, while a control group was inoculated with PBS. To achieve a monoinfection with *M. ovipneumoniae*, nasal washes were treated with ceftiofur before experimental inoculation. Microbiological analyses of the nasal washes demonstrated that this treatment successfully eliminated *Bibersteinia trehalosi* and *Mannheimia haemolytica*, which were present at low to moderate levels in the nasal washes prior to antibiotic treatment (data not shown). *M. ovipneumoniae* was detected in pooled nasal wash fluid both before and after ceftiofur treatment. Following inoculation, lambs were monitored for clinical symptoms for twelve weeks, and nasal swabs, serum and BALs for laboratory analyses were collected as shown in **Figure 1A**. All lambs in the *M. ovipneumoniae* group, but none of the lambs in the control group, showed positive PCR results for *M. ovipneumoniae* at two weeks post infection (p.i., **Fig. 1B**). *M. ovipneumoniae* levels peaked at 2–4 weeks, declined at 6 weeks and then plateaued. Colonization with *M. ovipneumoniae* was confirmed by successful culture of viable mycoplasma in nasal swab samples from two of the experimental lambs at 4 weeks post inoculation. We did not detect Pasteurellaceae in any nasal swab samples collected on days 0, 56, and 84 of the infection experiment using standard microbiology techniques (**Table 4**).

All infected lambs developed a strong *M. ovipneumoniae*-specific antibody response that peaked at 4 weeks p.i. and remained high throughout the experimental period (**Fig. 1C**), whereas no significant *M. ovipneumoniae*-reactive antibodies were detected in the control group. Likewise, no *M. ovipneumoniae*-reactive antibodies were present in any of the animals one day after birth, demonstrating that the colostrum replacer used did not contain any cross-reactive antibodies that might have contributed to *M. ovipneumoniae*-resistance through passive antibody transfer.

M. ovipneumoniae colonization levels detected at different time points throughout the experiment in the experimentally infected lambs by qPCR resembled those seen in lambs of similar ages that were naturally infected with *M. ovipneumoniae* and that served as donor lambs for the nasal wash inocula (**Fig. 2A**). Likewise, antibody levels detected by ELISA in the experimentally infected lambs at multiple time points did not differ significantly from antibody levels detected in the ewes (**Fig. 2B**). These observations suggest that our experimental *M. ovipneumoniae* infection closely replicated natural infection with regards to pathogen load and immune response.

M. ovipneumoniae monoinfection in domestic SPF lambs does not cause clinical disease.

Following experimental inoculation, all lambs were closely monitored for symptoms of respiratory disease by clinical scoring (**Table 1**). Interestingly, no significant increase in clinical symptoms was seen in the *M. ovipneumoniae*-infected compared to the uninfected lambs throughout the 12-week experiment (**Fig. 3A-F**). While increased respiratory rates, panting and labored breathing were detected in some of the *M. ovipneumoniae*-inoculated lambs two to three weeks after infection, these

observations did not reach significance (**Fig. 3E**). Appetite and body temperatures varied widely in both groups, and significantly decreased appetite was seen in the control group at several time points (**Fig. 3C,D**). As described in more detail below (Fig. 6), lambs in both groups had increased clinical scores and received anti-inflammatory treatment following administration of the antibiotic gamithromycin (**Fig. 3A,F**). Lamb body weights and daily gains measured between days 20 and 55 of the experiment, prior to administration of antibiotics, also did not differ significantly between *M. ovipneumoniae*-infected and control lambs (**Fig. 4A, B**). These data indicate that *M. ovipneumoniae* alone did not cause respiratory symptoms in domestic SPF-lambs under controlled laboratory conditions.

In addition, we analyzed BAL samples collected at 14 and 42 days post infection for evidence of pulmonary inflammation and damage by performing differential cell counts and a lactate dehydrogenase assay. As shown in **Fig. 5A-C**, >90% of cells present in BAL had a typical alveolar macrophage morphology, with oval or round nuclei and cytoplasmic vacuoles. A small number of lymphocytes, neutrophils and eosinophils were also detected (**Fig. 5C**). Neither cell composition nor total cell counts differed significantly between the *M. ovipneumoniae*-infected and non-infected lambs (**Fig. 5C, D**). We also analyzed the cell-free supernatants for the presence of lactate dehydrogenase (LDH) as a marker of lung injury (Drent et al., 1996). Although there was a trend for increased LDH release upon *M. ovipneumoniae* infection 14 days after experimental inoculation, no significant differences between the two groups were found at either of the time points analyzed (**Fig. 5E**). These data suggest that the experimental infection did

not cause pneumonia in the SPF lambs, consistent with the clinical findings presented above.

High dose treatment with the macrolide antibiotic gamithromycin fails to eliminate *M. ovipneumoniae* colonization.

We next sought to determine whether subclinical *M. ovipneumoniae* infection could be eliminated by antibiotic treatment with gamithromycin (Zactran®), which is currently used therapeutically to treat *M. bovis* infection in cattle and *M. hyopneumoniae* infection in swine (Baggott et al., 2011a; Maes et al., 2020a). Eight weeks after initiation of experimental *M. ovipneumoniae* infection, all lambs were treated with two doses of gamithromycin on days 56 and 61 post infection (**Fig. 6A**). However, the antibiotic treatment neither eliminated nor significantly decreased the level of *M. ovipneumoniae* infection as determined by qPCR in any of the lambs either at 2 or at 4 weeks after the treatment (**Fig. 6B**). Notably, significant side effects of the gamithromycin treatment including changes in behavior, decreased appetite, and increased body temperatures, were observed after treatment in both the *M. ovipneumoniae*-infected lambs and the control group (**Fig. 6C**). To counteract these side effects, anti-inflammatory treatment was administered to some of the animals (**Fig. 3F**). These data strongly indicate that gamithromycin is unsuitable for treatment of *M. ovipneumoniae* infection in domestic lambs, since it does not eliminate infection, but has considerable adverse effects.

Discussion

Mycoplasma ovipneumoniae is a key co-factor in the pathogenesis of chronic, atypical pneumonia in sheep, which has been associated with production losses in domestic lambs and significant deaths in wild sheep populations. In our study, we sought to define whether *M. ovipneumoniae* monoinfection causes clinical respiratory disease in immunologically naïve, specific-pathogen-free domestic lambs. We also tested whether established *M. ovipneumoniae* infection could be eliminated by antibiotic treatment. We found that *M. ovipneumoniae* caused stable, asymptomatic colonization of the upper respiratory tract that induced a significant antibody response, but that could not be cleared by gamithromycin application.

Whether *M. ovipneumoniae* causes respiratory disease in healthy domestic lambs has been a matter of debate (Foggie et al., 1976; Gilmour et al., 1979; Jones et al., 1982b; Buddle et al., 1984; Du et al., 2020). Our tightly controlled experimental conditions ensured that experimental lambs were not exposed to respiratory pathogens other than the *M. ovipneumoniae* used in the infections, leading to true *M. ovipneumoniae* monoinfections. The twice-daily clinical screens and cellular and molecular analyses of the bronchoalveolar lavage fluid demonstrated that active *M. ovipneumoniae* colonization of the upper airways did not lead to clinical disease or lung pathology in our model. In contrast to recent studies, we also did not observe any differences in lamb body weights or daily gains associated with *M. ovipneumoniae* infection (Besser et al., 2019; Manlove et al., 2019a). Multiple different hypotheses have been proposed to explain why *M. ovipneumoniae* causes asymptomatic airway colonization in some instances, but severe

pneumonia in other cases: (1) differences in *M. ovipneumoniae* strain virulence; (2) age-related differences in disease susceptibility; (3) differences in the immune response to *M. ovipneumoniae;* and (4) presence of additional facultative pathogens such as *Mannheimia haemolytica.*

Asymptomatic *M. ovipneumoniae* infections as seen in our study may be caused by experimental inoculation with non-pathogenic *M. ovipneumoniae* variants, since *M. ovipneumoniae* strains are highly diverse and can differ in their virulence (Niang et al., 1998b). Moreover, *Mycoplasma* may lose their virulence upon prolonged culture (Niang et al., 1998b). Such unintended, culture-dependent attenuation of *M. ovipneumoniae* isolates has been discussed as the cause for asymptomatic *M. ovipneumoniae* infection in several previous studies (Buddle et al., 1984; Besser et al., 2014). However, the inocula used in our study were fresh nasal washes obtained from *M. ovipneumoniae*-positive lambs that showed symptoms of respiratory disease, strongly suggesting that the lack of clinical symptoms upon experimental *M. ovipneumoniae* infection were not due to the use of an avirulent or attenuated *M. ovipneumoniae* strain.

With regard to the second hypothesis, it is generally accepted that clinical infections with *M. ovipneumoniae* most commonly occur in lambs under one year of age, while adult sheep serve as asymptomatic *M. ovipneumoniae* carriers (Martin, 1996; USDA, 2013). Plowright et al. showed that, in bighorn sheep, prevalence of *M. ovipneumoniae* was highest in lambs and aged animals, but low in adults, pointing to age-dependent infection dynamics (Plowright et al., 2017a). However, it is unclear whether the increased susceptibility of lambs was due to the lack of established adaptive immune

responses to *M. ovipneumoniae* or other age-related differences in respiratory anatomy, physiology, or immunity. Interestingly, Gilmour et al. found increased lung pathology in seven-month-old lambs compared to five-week-old lambs upon experimental *M. ovipneumoniae* infection (Gilmour et al., 1979). In bighorn sheep, yearlings and older adults with no previous *M. ovipneumoniae* infection were equally susceptible to *M. ovipneumoniae*-induced pneumonia (Besser et al., 2014). Thus, age-dependent mechanisms alone do not explain the variable susceptibility of sheep to *M. ovipneumoniae* disease.

In bighorn sheep, introduction of *M. ovipneumoniae* into herds with no prior exposure to the pathogen leads to particularly devastating disease outbreaks, suggesting that immunologically naïve populations are especially prone to symptomatic infections. *In vitro* studies have shown that *M. ovipneumoniae* specific antibodies can have protective functions by mediating opsonization and phagocytosis of the Mycoplasma (Al-Kaissi and Alley, 1983). Likewise, Niang et al. (Niang et al., 1999) found increased levels of *M. ovipneumoniae*-reactive antibodies in lambs that had recovered from the clinical *M. ovipneumoniae* infection. In our study, lambs had no detectable *M. ovipneumoniae* specific antibodies prior to experimental inoculation but developed a significant, stable antibody response within two weeks after infection, confirming previous studies that have tracked humoral responses to *M. ovipneumoniae* (Thirkell et al., 1990). These observations indicate that lack of existing immunity does not lead to more severe disease in *M. ovipneumoniae* infected sheep. Interestingly, the development of an antibody response was not associated with decreased *M. ovipneumoniae*

colonization in our study. A possible explanation for this phenomenon is that antibody levels measured in serum consist mainly of IgG, which may have protective functions within the lungs, whereas a strong mucosal IgA response may be necessary to eliminate asymptomatic *M. ovipneumoniae* colonization from the upper respiratory tract.

Overall, our findings that *M. ovipneumoniae* application in domestic lambs did not cause respiratory disease were in line with earlier studies that showed that pneumonia develops as a result of *M. ovipneumoniae* co-infections with other facultative bacterial pathogens (Jones et al., 1982a; Buddle et al., 1984; Besser et al., 2013; Shanthalingam et al., 2016). In bighorn sheep, where a large number of studies have been performed, *M. ovipneumoniae* is likely essential for causing severe respiratory disease (Besser et al., 2012b), but consistent clinical disease and lung pathology only developed when additional agents such as *Mannheimia haemolytica, Bibersteinia trehalosi, Pasteurella multocida* or *Fusobacterium necrophorum* (Dassanayake et al., 2010b; Besser et al., 2013; Shanthalingam et al., 2016) also were present. In further support of this hypothesis, the lambs that served as donors for the nasal wash fluids that we used to inoculate the experimental animals in our study showed symptoms of respiratory disease and tested positive for *Mannheimia haemolytica* and *Bibersteinia trehalosi* in addition to the *M. ovipneumoniae.*

Even if *M. ovipneumoniae* alone does not cause clinical respiratory disease in domestic sheep, an effective treatment strategy that eliminates *M. ovipneumoniae* infection from domestic sheep flocks could reduce production losses due to complex respiratory disease and protect bighorn sheep from devastating pneumonia epizootics. To

address this issue, we analyzed whether antibiotic therapy could eliminate *M. ovipneumoniae* colonization in our asymptomatically infected lambs. We chose the macrolide antibiotic gamithromycin (Zactran®), because gamithromycin application in goats infected with *Mycoplasma* and *Mannheimia* was successful in treatment of clinical pneumonia [8], and preventive treatment with gamithromycin in cattle was successful for reducing bovine respiratory disease, which commonly involves *Mycoplasma spp.* [9]. A recent study by Jay et al. on the antibiotic susceptibility of *M. ovipneumoniae* have showed homogenously low minimum inhibitory concentrations for a wide range of antimicrobials including macrolides, with no indication of significant antimicrobial resistance (Jay et al., 2020). Since gamithromycin is currently not approved for use in sheep in the US, the presence of resistant *M. ovipneumoniae* strains in the infected donor sheep was unlikely. However, our data showed no significant reduction in *M. ovipneumoniae* colonization levels in our experimental lambs following two injections of the recommended gamithromycin dose. It remains to be investigated whether failure of the antibiotic treatment was due to bacterial biofilm formation (McAuliffe et al., 2006), intracellular location of the mycoplasma (Einarsdottir et al., 2018) or an alternative antimicrobial resistance mechanism. Notably, all lambs developed significant side effects following gamithromycin administration, including increased body temperatures, decreased appetites and altered behavior as well as local inflammation at the injection sites that warranted the application of analgesics. These side effects were greatly more severe than the transient mild to moderate swellings at the site of injection described for this drug by the European Medicines Agency (EMA, 2018).

Why domestic lambs tolerate *M. ovipneumoniae* infection, while bighorn lambs suffer severe clinical disease may thus be due to host genetics and will require further investigations. The inoculation approach that involved nasal, oral and ocular application of 4-months-old domestic SPF lambs with ceftiofur-treated nasal washes from naturally infected, symptomatic lambs led to 100% successful upper airway colonization with *M. ovipneumoniae,* but not with Pasteurellaceae, confirming earlier studies by Thomas Besser's group (Besser et al., 2014; Ziegler et al., 2014; Besser et al., 2017). Colonization levels as determined based on the cT values in the qPCR reactions was similar in experimentally and naturally infected lambs, and antibody levels measured by *M. ovipneumoniae*-specific ELISA also closely replicated antibody levels seen upon natural infection. Thus, our infection model represents a valid tool to investigate the role of *M. ovipneumoniae* in sheep respiratory disease. In future studies, we plan to utilize this *M. ovipneumoniae* infection model to explore novel preventative or treatment approaches and to define how interactions between *M. ovipneumoniae* and other facultative respiratory pathogens lead to clinical respiratory disease and pulmonary pathology in domestic sheep.

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Tables

Table 1: Parameters for lamb health scoring during challenge experiment

Parameter	0	1	2	3	4	5
Behavior	bright, alert, responsive	small change in attitude/behavior	less active, visibly appears ill	ill, but still responsive	lethargic	unresponsive
Appetite	ate well	ate most of the ration	ate 3/4 feed	ate 1/2 feed	ate <1/2 feed	not eating
Body temperature	100°F-102°F	102.1°F-103°F or below 99.9°F	103.1°F-104°F	104.1°F-104.5°F	104.6°F-105°F	above 105°F
Respiratory symptoms	no nasal or ocular discharge, no cough	slight clear nasal or ocular discharge	clear nasal or ocular discharge, or coughed 1-5 times	moderate nasal or ocular discharge or frequent coughing	mucous nasal or ocular discharge, coughing, lamb visibly unwell	discharge, coughing, wheezing, increased respiratory rate
Medication	no treatments	electrolytes	NSAID	antibiotic	NSAID and antibiotic	multiple NSAIDS and antibiotics

Table 2: Characteristics of experimental animals

Animal #	**Treatment**	**Age (days)***	**Sex**
12	*M. ovipneumoniae*	109	F (twin of 15)
19	*M. ovipneumoniae*	108	F
26	*M. ovipneumoniae*	105	M[#] (twin of 25)
30	*M. ovipneumoniae*	103	M[#]
15	PBS	109	M[#] (twin of 12)
17	PBS	109	M[#]
25	PBS	105	F (twin of 26)
27	PBS	104	F

*age at time of infection; [#] all males were castrated at 11 weeks.

Table 3: Health status of ewe flock

Pathogen	**Diagnostic method**	**Positive animals**	**Response in positive animals** (mean ± SD)
M. ovipneumoniae	Competitive serum ELISA	7/15[#]	60.7 ± 10.4%
	Nasal swab PCR	0/15[#]	N/A
PI3 virus	Serum virus neutralization assay	3/15	1 : 45.3 ± 58.5
MAP*	Serum ELISA	0/15	N/A
C. burnetii	Serum ELISA	0/15	N/A

*MAP = *M. avium ssp. paratuberculosis; [#]*One additional animal had an indeterminate test result.

Table 4: Health status of SPF lambs

Pathogen	**Diagnostic method**	**Positive animals***
M. ovipneumoniae	Nasal swab PCR, 0 and 30 days post birth	0/30
	Nasal swab PCR, 7 days before inoculation	0/8‡
Pasteurellaceae	Nasal swab microbiology, 3 months post birth	0/19#
	Nasal swab microbiology, day 0, 56 and 84 post inoculation	0/8‡

*Second number indicates number of animals that were tested. ‡Only experimental lambs were tested.

#Lambs tested include all eight experimental lambs.

Figures

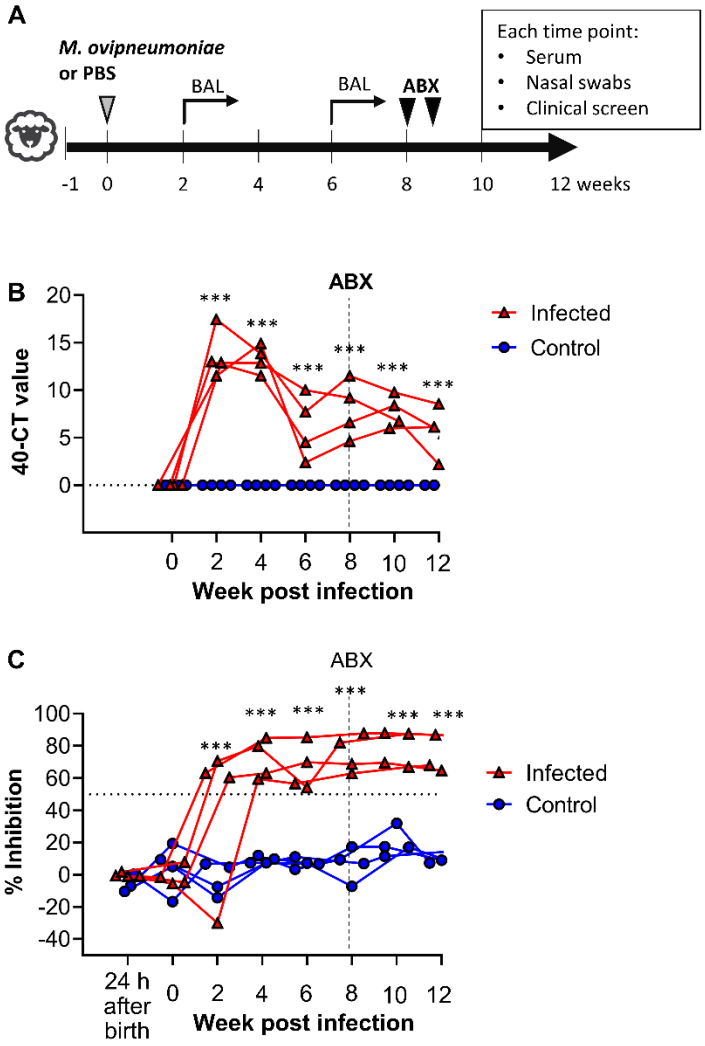

Figure 1: *M. ovipneumoniae* colonization and antibody responses in experimentally infected lambs. (A) Experimental schedule. Four SPF lambs aged 3 – 4 months were inoculated with *M. ovipneumoniae*-positive nasal washes, and four control lambs were mock-infected with PBS. All lambs received an antibiotic treatment (gamithromycin) after 8 weeks and were monitored for a total of 12 weeks. **(B)** *M. ovipneumoniae* infection levels in nasal swab samples were determined by qPCR. Data are shown as 40 minus cT value. Triangles represent individual lambs from *M. ovipneumoniae* infection group, circles represent lambs from control group. Data were analyzed by 2-way ANOVA with Dunnett's multiple comparisons test for differences between week 0 and other time points. ***$P \leq 0.001$ for the *M. ovipneumoniae*-infected lambs. (**C**) *M. ovipneumoniae*-specific antibody levels were determined by competitive ELISA analysis

of serum samples. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. Data were analyzed by 2-way ANOVA with Dunnett's multiple comparisons test for differences between week 0 and other time points. ***$P \leq 0.001$ for the *M. ovipneumoniae*-infected lambs.

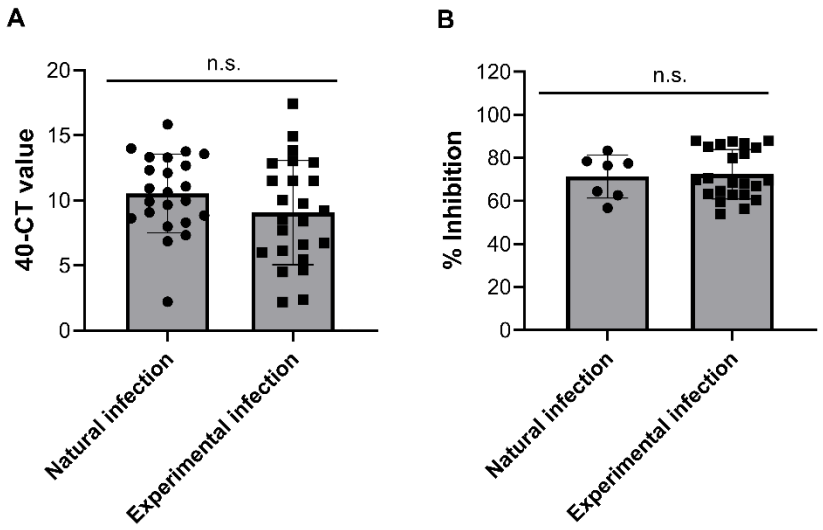

Figure 2: Comparison of *M. ovipneumoniae* levels and serum antibody response to *M. ovipneumoniae* in experimentally and naturally infected domestic sheep. (A) *M. ovipneumoniae* infection levels in nasal swab samples were determined by qPCR. Naturally infected animals were lambs of similar ages that tested positive for *M. ovipneumoniae* and that served as donors for the nasal washes. Experimentally infected lambs were the SPF lambs shown in Fig. 1B; symbols represent pooled individual samples from multiple different lambs and time points. Differences between groups were analyzed by Student's *t* test. **(B)** Antibody levels in the ewes (mothers) compared to the experimentally infected lambs from this study. Only data from animals with positive detection were included. Data from experimental lambs represent multiple animals and time points. Differences between groups were analyzed by Student's *t* test.

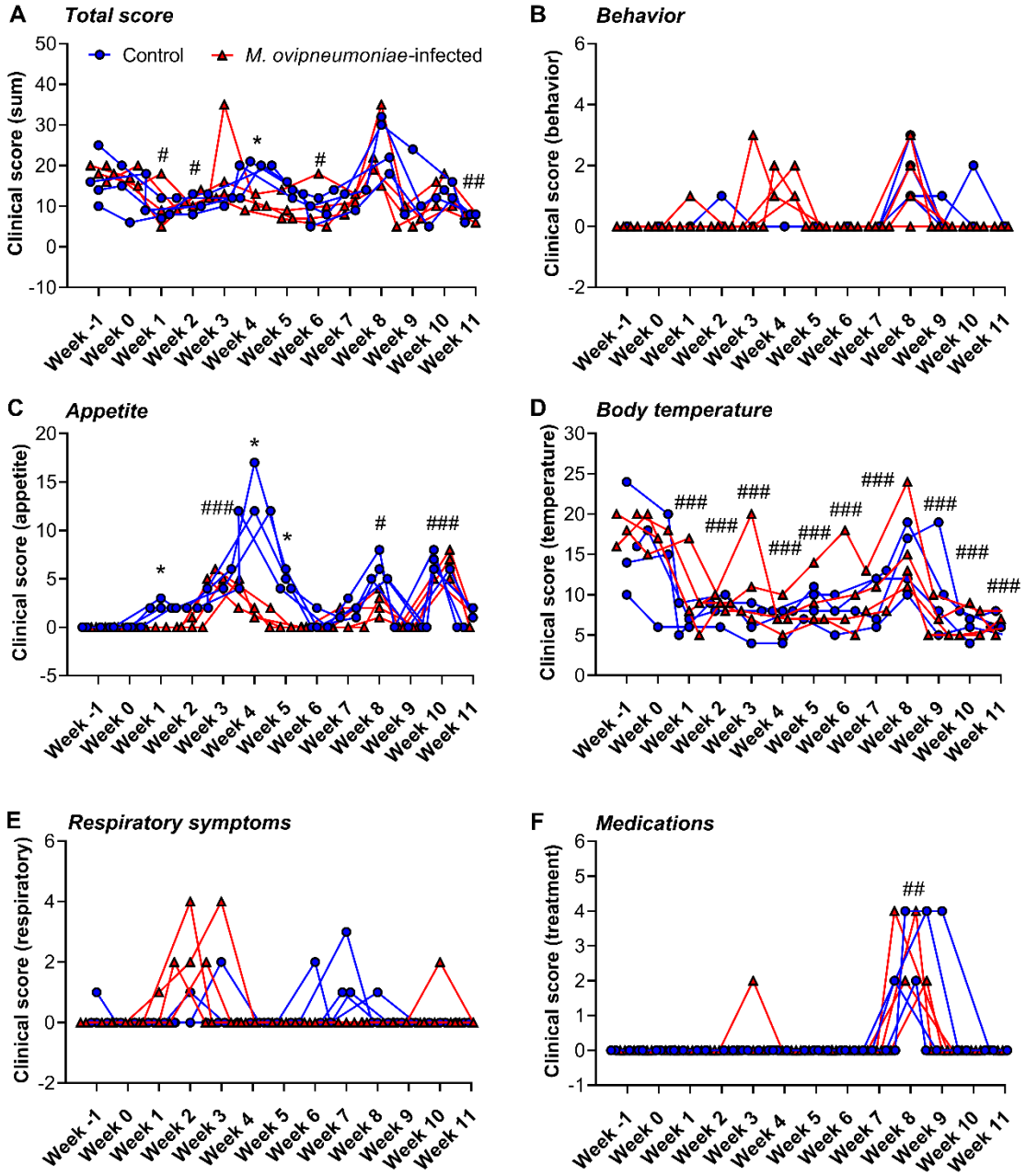

Figure 3: SPF lambs with *M. ovipneumoniae* monoinfection do not develop clinical symptoms of respiratory disease. All lambs were screened twice daily for changes in behavior and appetite and for symptoms of respiratory disease; body temperatures and administered medications were also recorded. Scores were determined using the criteria listed in **Table 1**. Weekly scores are sums of all 14 scores obtained for each lamb per week and per category. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. (**A**) Total clinical scores, sum of B-F; (**B**) Behavior scores, (**C**) Appetite scores, (**D**) Body temperature

scores; (**E**) Respiratory score; (**F**) Medication score. Data were analyzed by 2-way ANOVA with Sidak's multiple comparisons test for differences between the *M. ovipneumoniae*-infected and control groups. **P*≤0.05; ***P*≤0.01; ****P*≤0.001. Differences between individual time points and baseline values (week -1 p.i.) for all lambs are indicated by $^{\#}P\leq0.05$; $^{\#\#}P\leq0.01$; $^{\#\#\#}P\leq0.001$.

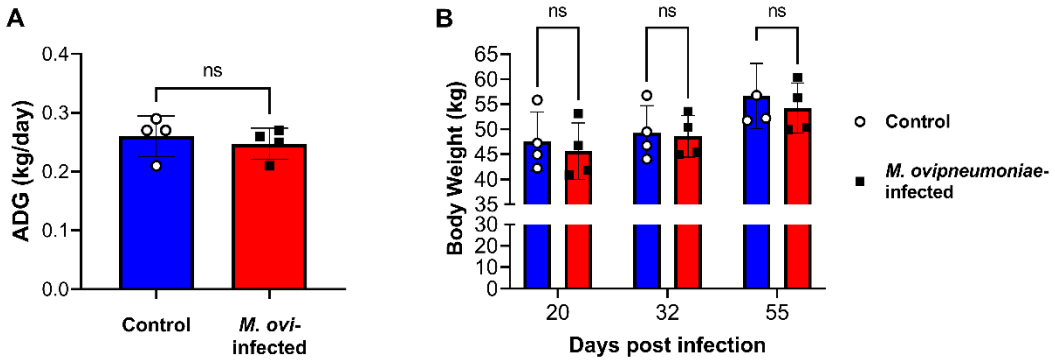

Figure 4: *M. ovipneumoniae* infection does not alter lamb body weights or average daily gains. (**A**) Average daily gains in *M. ovipneumoniae*-infected and control lambs between days 20 and 55 post infection. Differences between groups were analyzed by Student's *t* test. (**B**). Lamb body weights on days 20, 32 and 55 post infection. Data were analyzed by 2-way ANOVA with Sidak's multiple comparisons test for differences between the *M. ovipneumoniae*-infected and control groups.

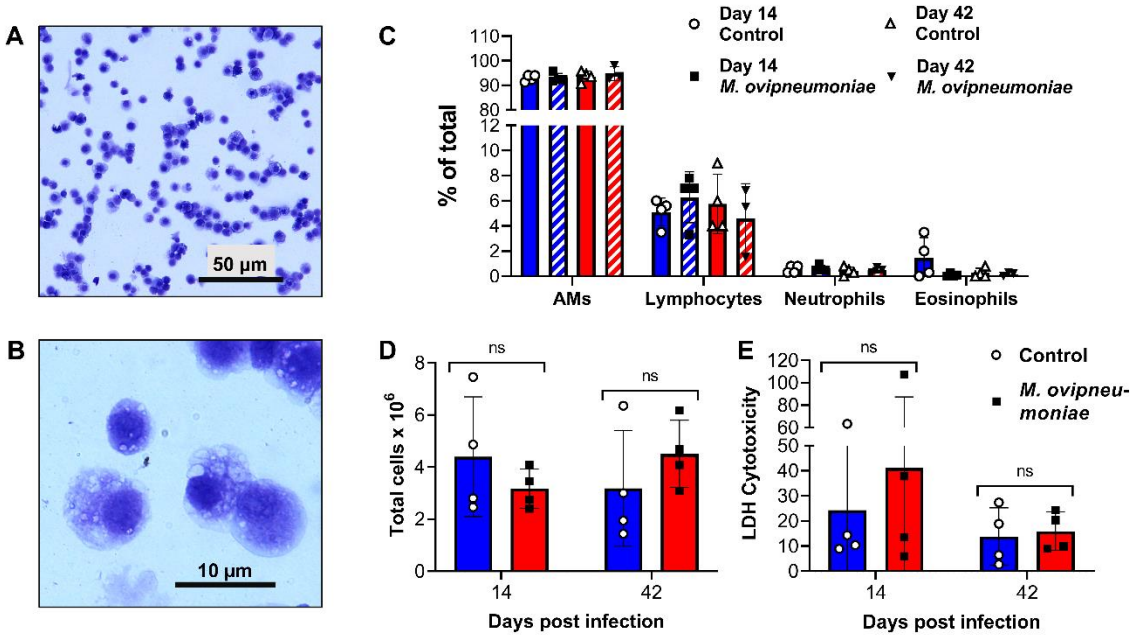

Figure 5: Bronchoalveolar lavage fluid from SPF-lambs experimentally infected with *M. ovipneumoniae* shows no evidence of lung inflammation. (A, B) Cellular composition of BAL from an *M. ovipneumoniae*-infected lamb 14 days after experimental inoculation. Representative image from one of four infected sheep shows typical macrophage morphology of the majority of the cells at (A) low and (B) high magnification. **(C)** Differential cell counts of BAL collected on days 14 and 42 after experimental inoculation from *M. ovipneumoniae*-infected and non-infected lambs (n=4). **(D)** Total cell counts of BAL collected on days 14 and 42 after experimental inoculation from *M. ovipneumoniae*-infected and mock-infected lambs (n=4). **(E)** Analysis of lactate dehydrogenase in cell-free supernatants of BAL collected on days 14 and 42 post inoculation. Individual data points and mean ± SD are shown. Differences between groups were analyzed by Student's *t* test.

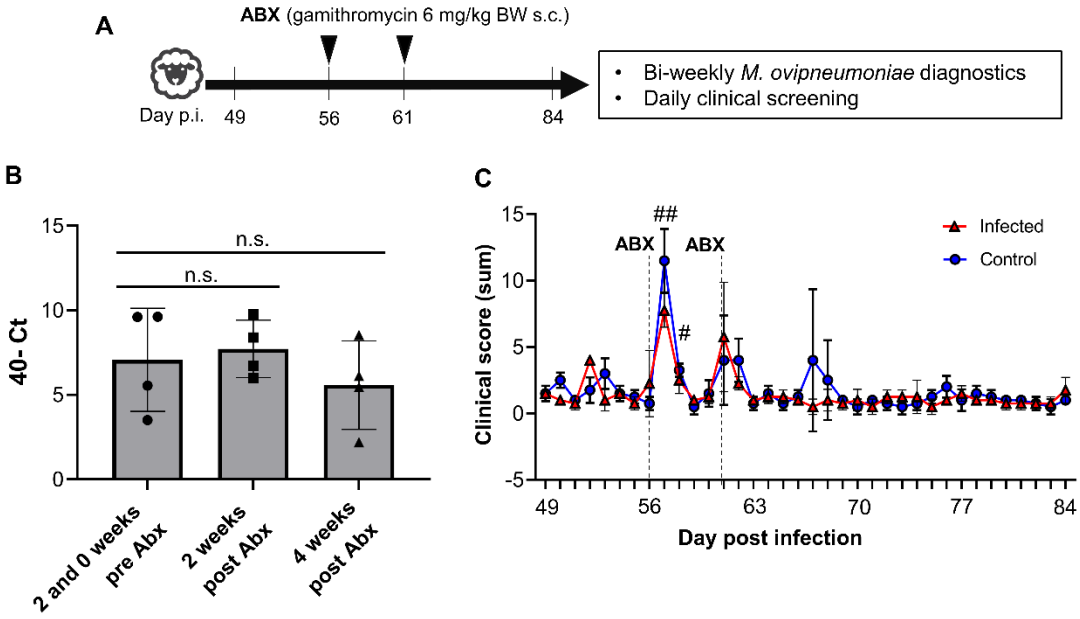

Figure 6: Antibiotic treatment with gamithromycin does not eliminate asymptomatic *M. ovipneumoniae* infection. (A) Detailed timeline for antibiotic administration. (**B**) *M. ovipneumoniae* infection levels in nasal swab samples of lambs inoculated with *M. ovipneumoniae*-positive nasal washes were determined by qPCR at 14 and 0 days before administration of gamithromycin (average of two time points) and at 14 and 28 days post administration of gamithromycin. Data are shown as 40 minus cT value. (**C**) Clinical scores of *M. ovipneumoniae*-positive and negative lambs after gamithromycin treatment show side effects of antibiotic administration. All lambs were screened twice daily for changes in behavior and appetite and for symptoms of respiratory disease; body temperatures and administered medications were also recorded. Scores were determined using the criteria listed in Table 1. Daily scores reflect the sum of two scores per day for all five criteria. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. Significant differences ($P \leq 0.05$) between individual time points and baseline values (day 49 p.i.) for all lambs are indicated by #.

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CHAPTER 4

DISCUSSION

In this study, we investigated the outcome of *M. ovi* infection in SPF, immunologically naïve domestic lambs. The first step of this project was to generate the flock of SPF lambs, that were also immunologically naïve to *M. ovi*, used in this experiment. This was done by separating lambs from their mothers at birth and raising them on colostrum replacer in an ABSL-2 facility. This portion of the study yielded its own set of data, showing how effective colostrum replacer containing bovine serum as an antibody source is at producing healthy lambs.

Our data in Chapter 2 shows that colostrum replacer containing bovine serum as an antibody source is a feasible replacement to ewe colostrum and yields healthy lambs. Thirty lambs were raised on colostrum replacer over the course of the study. By collecting serum samples, weighing the lambs weekly, and documenting lamb health, we were able to perform a comprehensive evaluation. Lambs in this study demonstrated ADGs similar to lambs raised on ewe colostrum up to and throughout weaning (Sayed, 2009). Lambs had serum IgG (Henning and Nielsen, 1992; Barta, 1993; O'brien and Sherman, 1993; Erhard et al., 1999; Massimini et al., 2006; Waldner and Rosengren, 2009; Alves et al., 2015) and total protein concentrations (Ahmad et al., 2000; Nagy et al., 2014; Alves et al., 2015) comparable to lambs and other newborn livestock that are raised naturally with their dams. Overall, lambs had low incidences of poor appetite, scours, fever, and need for treatments.

One limitation of this study was the lack of a control group raised on ewe colostrum. It was crucial for all lambs to be raised on the colostrum replacer to maintain SPF and immunologically naïve status for the infection experiment detailed in Chapter 3. Further sampling of the next generation of SPF lambs will help compensate for the lack of a control group of lambs fed colostrum produced by the ewe. The SPF ewes were bred in winter 2020/2021 and will be delivering lambs in May 2021. Since the ewes are SPF, the lambs will be able to nurse from the ewes, as opposed to requiring bottle feeding of colostrum replacer, while maintaining their SPF and *M. ovi* immunologically naïve status. Serum and weight samples will be collected from the lambs in order to compare their ADGs, serum IgG concentrations, and total serum protein concentrations to the lambs that received colostrum replacer last year. The clean, controlled environment of the ABSL-2 facility may have also contributed to the low mortality and overall good health. The lambs raised this coming season will provide a good control for this condition as well, since they will be raised in the same facility, albeit in outdoor paddocks instead of the heated indoor rooms.

In Chapter 3, we demonstrate that *M. ovi* can establish a chronic, asymptomatic infection in SPF and *M. ovi* immunologically naïve lambs. While this data does not support the hypothesis that pre-existing protective antibodies are the reason *M. ovi* infections lead to a higher mortality rate in bighorn sheep than in domestic sheep, it helps create a better overall understanding of *M. ovi* in domestic sheep. Even domestic lambs that have never been exposed to *M. ovi* and have no previous *M. ovi* specific antibodies presented asymptomatically when inoculated with *M. ovi*. No antibodies cross-reactive to

M. ovi were detected in the lambs fed colostrum replacer. All antibodies that were derived from the colostrum replacer were reduced to zero or close to zero by the time of the experiment.

These asymptomatic infections were unexpected, since they were infected using a strain collected from lambs exhibiting symptoms. There are also many studies where *M. ovi* infections lead to pneumonia (Alley et al., 1999; Lin et al., 2008; Manlove et al., 2019b). The bacteria established a chronic infection in the nasal cavity that persisted despite the immune response mounted by the lambs and despite the antibiotic treatment. Since we concluded that protective antibodies do not play a role in decreasing *M. ovi* infection symptoms, the next step is to conduct another infection experiment looking at the role coinfections play in *M. ovi* pathogenesis. There is little data to suggest that *M. ovi* causes severe pneumonia on its own; however, there are several papers that discuss the effects of *M. ovi* predisposing domestic and bighorn sheep to other infections (Dassanayake et al., 2010a; Fox et al., 2015).

The lack of clinical symptoms in the *M. ovi* lambs could have been associated with the specific strain of *M. ovi* that the lambs were inoculated with. Since experimental lambs were inoculated with pooled nasal wash collected from several lambs, they could have been infected with one or several strains. Different strains of *M. ovi* have different levels of virulence and pathogenicity (Besser et al., 2017), and sheep can be infected with multiple strains concomitantly. The process of genotyping the strain used in our studies has been started following a previously established protocol (Cassirer et al., 2017). The

genotyping data can be used to determine the strain or strains of *M. ovi* used to infect the lambs in this experiment.

The fact that *M. ovi* established a chronic, asymptomatic infection in the SPF lambs despite a robust antibody response points to successful immune evasion strategies. When *M. ovi* enters the lung during pneumonia, the first type of immune cell it encounters is the resident alveolar macrophage (AM). Bronchial alveolar lavages were performed throughout the infection experiment and AMs were isolated. Our lab is in the process of optimizing a phagocytosis assay using AMs and laboratory grown *M. ovi* that can be used to determine if *M. ovi* infection increases or decreases the AMs' ability to clear bacteria. Robust, resistant colonization was demonstrated by the persistence of *M. ovi* infection after multiple treatments with gamithromycin, which has successfully been used to treat *Mycoplasma*-associated infections in several species (Baggott et al., 2011b; Kacar et al., 2018b; Maes et al., 2020b).

The microbiota also influences disease susceptibility and outcome (Malmuthuge and Guan, 2016; Yuan et al., 2020). When the mucociliary escalator is inhibited by hydrogen peroxide produced by *M. ovi* during colonization, a variety of normally commensal upper respiratory and rumen microbes are able to invade the lungs, causing polymicrobial pneumonia (Brogden et al., 1998; Niang et al., 1998a; Besser et al., 2012c; Cassirer et al., 2017). Samples for microbiome characterization from the SPF flock were collected at various timepoints to be used as a comparison to data points collected from lambs that nursed directly from their mothers. Samples included oral swabs, rectal swabs, and rumen fluid and will be analyzed to determine the prominent bacterial taxa present in

each location. Since these lambs did not nurse from their mothers and were raised in a highly controlled environment, we hypothesize that the composition of their microbiome will be significantly different from lambs raised in a natural environment, and that the lambs will lack many of the opportunistic pathogens that are commonly detected in co-infections with *M. ovi*.

Overall, the data produced by these two studies is valuable and useful in their respective fields. By raising healthy, SPF lambs on a colostrum replacer, we demonstrated that colostrum replacer containing bovine serum as an antibody source is a viable replacement when ewe colostrum is not able to be used. Lambs showed average ADGs, average serum IgG and total serum protein concentrations, and minimal incidences of disease. The infection experiment using SPF and immunologically naïve lambs demonstrated that *M. ovi* established a chronic infection in the nasal cavity that peaked at 2 weeks p.i.. In response to *M. ovi* infection, lambs mounted an antibody response that peaked at week 4 before plateauing. Despite establishment of infection, the lambs did not show any clinical symptoms and no damage was found in the lung via LDH assay. Neither the lamb immune systems nor antibiotics were able to clear the *M. ovi* infection. Overall, this data provides insight into *M. ovi* infections upon which other studied can be built.

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