NEGATIVELY CONTROLLED RANDOMIZED FIELD TRIAL EVALUATING THE EFFECT OF TREATMENT OF PNEUMONIA WITH TILDIPIROSIN OR FLORFENICOL + FLUNIXIN MEGLUMINE ON HEALTH AND UPPER RESPIRATORY TRACT MICROBIOTA OF PREWEANED HOLSTEIN DAIRY HEIFERS

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ABSTRACT

As a major economic and health concern in the livestock industry, bovine respiratory disease (BRD) is still highly prevalent on U.S. farms, causing high morbidity and mortality rates in young calves. Controlling BRD can be complicated because it is a multifactorial and polymicrobial disorder that occurs due to complex interactions between the calf's immune system, causative pathogens, and on-farm management systems. The pathogens that cause pneumonia in calves are also commensal inhabitants of the upper respiratory tract (URT), which can cause illness as a consequence of dysbiosis. The best way to prevent the disease is to eliminate the risk factors that can trigger the imbalance of microorganisms in the URT. Among the stressors and risk factors associated with pneumonia are unproper maternity management, comingling, weaning, and poor air and bedding quality. When we fail to prevent pneumonia, the use of antibiotics remains the most effective strategy for controlling the disease. Several drugs are commercially available for treatment of pneumonia, including synthetic antibiotics such as tildipirosin and florfenicol in combination with flunixin meglumine, a nonsteroidal anti-inflammatory drug (NSAID). Studies evaluating the efficacy of therapeutics available on the market can help producers and veterinarians in decision-making for the disease treatment.

Furthermore, in-depth studies evaluating health and blood parameters, and the microbiota of animals with pneumonia can advance our epidemiological knowledge of BRD. In Chapter 1 of this thesis, we summarize some of the most important topics about BRD, including pathogenesis, risk factors, economic impact and harmful consequences, diagnostic tools, and strategies to prevent and treat this disease in dairy and beef operations. The main objective of Chapter 1 is to create a knowledge base about the BRD complex with a focus on infectious pneumonia which served as a reference for the research described in the following chapter.

In Chapter 2, we describe the results of a randomized clinical trial evaluating the effect of therapeutically administered tildipirosin or florfenicol + flunixin meglumine against fever-associated respiratory diseases compared to untreated pneumonic animals. As specific aims, we evaluated the URT bacterial microbiome, blood, and health parameters of the animals. Both drugs were effective in reducing clinical signs of pneumonia and rectal temperature, with florfenicol + flunixin meglumine promoting a greater reduction of rectal temperature. Reduction of systemic inflammation was also confirmed through analyses of blood parameters, such as haptoglobin. Considering untreated calves as reference, both antibiotics reduced pneumonia and/or otitis retreatment. Finally, both drugs were effective in reducing the mean relative abundance (MRA) of important genera associated with pneumonia (*Mannheimia* and *Pasteurella*), although an increase in the *Mycoplasma* MRA was observed in tildipirosin-treated calves.

In Chapter 3, we describe our final thoughts on BRD in dairy calves, the results and implications of our study, and future research directions for this topic. In summary, BRD remains a challenging disease to animal health due to its complexity and multifactorial nature. Farms should focus primarily on prevention strategies such as proper administration of quality

colostrum to newborn calves and reduction of environmental and management stressors that can impair the immunity of calves. In herds at high risk for disease, the use of vaccines can also help to reduce morbidity and mortality in calves. Our research study comparing outcomes from two antibiotic interventions to untreated calves confirmed the importance and benefits of timely diagnosis and proper treatment of group-housed dairy calves during the preweaning period. In addition, we found exciting results in terms of URT composition and dynamics when comparing healthy calves to pneumonic animals untreated or receiving antibiotic interventions.

BIOGRAPHICAL SKETCH

Ana Carolina de Campos Henrique was born in 1989 in Ribeiro Preto a big city in the State of São Paulo, Brazil. She is the first daughter of Aristoteles N. Henrique and Maria Teresa de C. Pinto, and she has one sister, Gabriela de C. Henrique. Despite growing up in a big city, she always enjoyed taking care of farms animals. Her father always encouraged her to have a close relationship with agriculture, taking her whenever possible to farms in small towns in the region, which led Ana to her strong interest about animal production.

In 2010, Ana entered the University of São Paulo, located in the city of Pirassununga, State of São Paulo, Brazil and completed her Animal Science degree in 2016. As part of her appointment, Ana had the privilege to perform two important internships, one at the UNESP (Paulista State University) and the second at Cornell University under supervision of Dr. Rodrigo Bicalho. These two experiences gave Ana the opportunity to carry out scientific studies in dairy cattle, which strongly enhanced her appreciation for research. In 2019, Ana had the good fortune to come back to the United States and begin her Master studies in the Department of Animal Science at Cornell University.

This dissertation is the culmination of that journey, and she hopes that her research will help to improve calf health and performance in dairy farms.

This thesis is dedicated to my husband, Tiago Tomazi, and my mother Maria Teresa for all their support, encouragement, and love.

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Last but not least, I want to thank all the members of our lab at Cornell, the professors, research assistants and internship students for all their hard work throughout the entire process of our research. Without you, none of this would have been possible.

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LIST OF ABBREVIATIONS

BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
CTR	Control
DLSM	Differences of least square means
DNA	Deoxyribonucleic acid
FLF	Florfenicol + flunixin meglumine group
LC	Lung consolidation
LPS	Lipopolysaccharides
LRT	Lower respiratory tract
LSM	Least square means
MRA	Mean relative abundance
NAHMS	National Animal Monitoring System
NC	No lung consolidation
NEG	Negative treatment group
NSAID	Non-steroidal anti-inflammatory drug
OM	Otitis media
PCR	polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SOP	Standard operation procedures
TLD	Tildipirosin group
TP	Total protein
TRT	Treatment
URT	Upper respiratory tract
USA	United States of America

CHAPTER 1: Literature review

BOVINE RESPIRATORY DISEASE COMPLEX AND ITS RELATIONSHIP WITH PNEUMONIA IN CALVES

Bovine respiratory disease (BRD) is a major health and economic concern in the cattle industry. Based on an economical estimation performed in 2015, BRD cost U.S. producers $23.60 per treated calf (Grissett et al., 2015). A study evaluating dairy calves from California reported that approximately 22.8% of calves are diagnosed and treated for BRD before weaning, reflecting the high incidence of this disease in dairy operations (Dubrovsky et al., 2019). A later study reported that 19% of deaths in dairy calves were attributed to BRD and the authors estimated that the cost of treating this disorder is higher now than 10 to 20 years ago and continues to have a substantial economic impact. Despite efforts to control BRD, this multifactorial disease is still one of the major and constant concerns when raising heifers. The significant economic impact of BRD is mostly due to the direct costs associated with calf treatment and mortality. However, long-term effects of the disease on the future performance of the animals, such as reduced milk production and negative impact on the reproductive performance, has also been reported (Buczinski et al., 2021).

Among the conditions associated with the bovine respiratory complex, infectious pneumonia is the most common and detrimental (Kerr and Linnabary, 1989). Pneumonia can develop from infections of the respiratory tract caused by several viruses, including herpesvirus-1 (BHV-1), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPI-3), bovine coronavirus (BC), bovine adenovirus, and bovine viral diarrhea (BVD) (Rice et al., 2007, Fulton, 2009, Taylor et al., 2010). When calves present one of these viral infections and become immune suppressed, commensal, and potentially pathogenic bacteria, such as *Mannheimia haemolytica*,

Pasteurella multocida, *Histophilus somni*, *Trueperella pyogenes* and *Mycoplasma* spp. can increase replication causing disease (Griffin et al., 2010).

One of the most important risk factors predisposing calves to pneumonia is failed colostrum administration. The proper colostrum administration will provide newborn calves with antibodies and increase the neutrophil activity. These components of the immune system are essential for protection of respiratory airways, intestines and other mucosal surfaces against infectious pathogens (Ackermann et al., 2010). Environmental factors such as bedding quality and calf raising facilities can also be a risk factor for pneumonia. Calves housed in comingling pens have an increased risk of disease compared to individual calf hutches due to the close contact between animals (Ollivett, 2020). Other stressful events associated with calf management, such as transportation, dehorning, and weaning, can also increase the risk of pneumonia (Stafford and Mellor, 2011, Hulbert and Moisá, 2016).

Suppression of the immune system associated with the aforementioned factors predisposes calves to viral and bacterial infections causing the appearance of clinical signs of pneumonia such as coughing, nasal discharge, lacrimation, fever, prostration, anorexia, and poor weight gains (Ames, 1997). Although evident, oftentimes the signs of pneumonia are not perceived by the farm workers. The delay of diagnosis and consequently of treatment can result in other pathologic complications, such as tonsilitis, laryngitis, tracheitis, and suppurative to fibrinosuppurative bronchopneumonia (Confer et al., 1996, Dagleish et al., 2010). One noninvasive and efficient method to detect pneumonia is thoracic ultrasonography (Teixeira et al., 2017a), which will be further discussed in this literature review.

Timely diagnosis allows for a fast and adequate treatment of pneumonia. The use of antimicrobials remains the most common strategy to treat pneumonia and several studies have

proven the efficacy of different antimicrobial interventions (Massias et al., 1994, Coetzee et al., 2019, Linhart and Brumbaugh, 2019). However, inappropriate treatment with antibiotics can be the cause of direct and indirect negative effects, such as chronic illness, early culling, euthanasia or death (Booker and Lubbers, 2020). Therefore, performing treatments correctly is important for recovery success and can reduce the impact of the disease on future performance. Another topic that is worth further investigation is the effect of different antimicrobials on the microbiota of the respiratory tract of calves affected by pneumonia.

The objective of this literature review is to summarize the knowledge around the epidemiology and control of BRD and serve as reference for the understanding of the effect of antibiotic interventions on health parameters and the upper respiratory tract microbiota of dairy calves with pneumonia before weaning.

FACTORS AFFECTING THE CALF IMMUNITY AND INFECTION OF THE UPPER RESPIRATORY TRACT

As mentioned previously, BRD is a multifactorial disease occurring from interactions between the host, microbiological agents, and stressors that can predispose the onset of the disease. Some of these factors include management failure in the early life of calves, which increase animal susceptibility to pathogens associated with the BRD complex. Some of these factors are inadequate transfer of passive immunity through colostrum administration, inadequate vaccination protocols, and diet restriction (Van Donkersgoed et al., 1993, Gorden and Plummer, 2010). The inadequate transfer of passive immunity occurs when the absorption of maternal immunoglobulins present in the dam colostrum are reduced by less than the minimum level of IgG in serum of calves (10 mg/mL) in the first 24 hours of life (Pritchett et al., 1991). This management is essential and will

affect the entire life of the calf in terms of immunity and productivity. The lack of adequate passive immune transfer from the dam to the calf can be associated with approximately 40% of BRD cases in calves (Van Donkersgoed et al., 1993). Another study reported that more than 20% of BRD cases could be potentially prevented if quality colostrum were administered in a timely manner (Windeyer et al., 2014). The increased risk of BRD due to poor passive immune transfer also contributes to increased antimicrobial use and antimicrobial resistance (Finch, 2010).

Vaccination, in addition to passive immune transfer from colostrum, is the next step in developing the calf's immune system and reducing BRD incidence. There are several commercially available vaccines claiming to offer protection against viruses (e.g., bovine respiratory syncytial virus [BRSV], bovine viral diarrhea virus [BVDV], bovine herpesvirus type 1[BHV-1]) and bacteria (e.g., *Mannheimia haemolytica*, *Pasturella multocida*). Building an effective immunization program for calves can be difficult due to their immature immune system and the complexity of management (Gorden and Plummer, 2010). Factors to consider in vaccination programs include the effect of colostrum maternal immunity and the age of the animal (Chase et al., 2008). These issues will be further discussed in the next topic. Similarly, inadequate quantity and quality of milk consumed can depress the immune system due to the poor supply of nutrients which indirectly affects the immune system of the animal (Khan et al., 2011).

Later in the calf's life, the most common factors influencing the development of BRD are viral infections that occur during transportation and weaning. Many dairy operations transport calves to an off-site calf raising facility. In the United States, approximately 10% of calves are transported for long distances to be raised in calf ranches and more specialized facilities (Hulbert and Moisá, 2016). Poor transport conditions, such as low air circulation, dehydration, malnutrition, and grouping animals from different sources in a small space increase the risk of BRD (Hulbert

and Moisá, 2016). In order to reduce the risk of infection between transported calves, metaphylactic use of antimicrobials is often used (Celestino et al., 2020). In addition, metaphylaxis is used before the main peak of BRD incidence in high-risk cattle to reduce pathogen load (Nickell and White, 2010). Antibiotics such us tildipirosin, a long-lasting macrolide, is utilized due to the rapid distribution of its active ingredient to the lung tissue and its long half-life. These properties allow sustained concentration of the macrolide in the lower respiratory tract (Goetting et al., 2011). One study carried out by our research group in a single dairy herd in New York state showed a significant decrease in total bacteria load in the URT of animals treated with tildipirosin at 7 ± 7 d of life compared to animals not administered with antimicrobial (Bringhenti et al., 2021b). Another recent study found that tildipirosin used as a metaphylaxis decreased mortality by 60% and improved the inflammatory status in calves transported for approximately 1,700 km from dairies located in Minnesota to a calf raising facility located in New Mexico (Celestino et al., 2020). Although studies showed good results in the metaphylactic use of tildipirosin, concerns have been raised on the use of antimicrobials to prevent infections because of antimicrobial resistance.

Environmental factors including group housing, large group size, poor bedding quality and management, direct contact with older animals, and poor air quality, similarly increase the risk of BRD in dairy calves. From a welfare perspective, group-house facilities are preferable to single-house facilities such as hutches because of the freedom of full social interactions (Webster et al., 1985, Jensen, 1999, Babu et al., 2004). However, calves housed in large-group pens equipped with automatic milk feeders have a higher risk of respiratory disease compared to calves housed in small groups and reared with bucket feeding (Svensson and Liberg, 2006).

Poor bedding and air quality are related to the load of pathogenic microorganisms in the environment (Ollivett, 2020). Calf facilities should have a gap between the roof and the top curtain

to allow continuous air movement which becomes even more important in summer heat. Clean bedding is essential but requires maximum airflow for the dispersal of the dust and dirt. Proper bedding management and air flow will decrease the irritation of the respiratory tract of the calves thereby decreasing the risk of BRD.

Cleanliness of the maternity pen and season of the year can also predispose calves to BRD. Calves born in dirty conditions have increased risk of infectious diarrhea and pneumonia. Furthermore, calves born in the winter had 2.6 times higher odds to present BRD than those born during the summer, and 1.6 times higher odds than those born in the fall (Guterbock, 2014).

All risk factors described in this topic can be generally avoided with good management practices. Avoiding failures such as improper administration of colostrum and therapeutics (e.g., vaccines), and providing calves with comfort and well managed facilities will bring economic gains to the farm. Because BRD is among the major causes of death in dairy calves, practices to reduce these risks factors must be encouraged in dairy farms and calf ranches.

THE USE OF VACCINES TO PREVENT BRD

Some scientists place BRD as one of the most complicated mammalian diseases that exist due to its multifactorial nature that involves stress, immunosuppression, and multi-pathogen involvement (Richeson and Falkner, 2020). The most recognized preventative medical intervention is the administration of vaccines against BRD causative agents. Vaccines can be inactivated (killed) or active (modified live). These vaccines contain antigens against bovine herpesvirus 1 (BHV-1), bovine viral diarrhea virus (BVDV) type 1 and 2, bovine respiratory syncytial virus (BRSV), and parainfluenza virus 3 (PI-3V). There are vaccines against *Mannheimia haemolytica, Pastuerella multocida* and *Histophilus somni* bacteria. Intranasal and

injectable vaccines are commercially available with different pathogen coverage to be used in different conditions. Despite giving good protection to the animal, none of these vaccines are expected to provide full protection to calves against all pathogenic microorganisms. However, the efficacy of BRD vaccines can be improved by optimizing the timing of vaccination and using proper procedures, such as adequate routes of administration, needle changing and good hygiene (Richeson and Falkner, 2020). Optimal timing of when to vaccinate is when calves are in a state of immunologic homeostasis, thereby being free of infection and at least several weeks before typical, natural BRD challenge. Thus, for dairy calves in hutches, calves can be vaccinated with intranasal vaccines after 7 days of age and boosted one to two weeks prior to weaning, before calves are stressed by moving to group housing facilities. For group-housed calves, it is recommended to vaccinate them right after 7 days of age.

Vaccination of very young calves is questionable because the maternal antibodies received from colostrum can prevent the vaccine from being effective (Woolums, 2007). When the maternal antibodies are present at high levels, the calf humoral response to viral antigen will suffer an interference (block) and will not produce antibody response to infection or vaccination (Brar et al., 1978, Van Donkersgoed et al., 1991, Ellis et al., 2001). Viral vaccine studies have shown an induced immunologic memory not susceptible to maternal antibody regulation in calves that were administrated immunologic agents through vaccines (Brar et al., 1978, Menanteau-Horta et al., 1985). In addition, to overcome interference between parenteral vaccines and maternal derived immunity, intranasal vaccination strategies using modified live vaccines for respiratory diseases have been developed and used widely for many years (Windeyer and Gamsjäger, 2019, Masset et al., 2020). Intranasal vaccination induces protective immunity in newborn calves regardless of the presence of antibodies from colostrum by priming mucosal immunization of the upper respiratory

tract, whereas protective immunity is inconsistent after parenteral vaccinations (Osman et al., 2018).

Overall, vaccines seem to be the most effective strategy to prevent BRD in addition to proper cattle management. However, further studies evaluating the effectiveness of early vaccination of dairy calves using different vaccine compositions and environmental conditions must be conducted, especially regarding the effect of vaccines on the calf respiratory tract microbiota.

THE USE OF ANTIMICROBIALS TO TREAT BRD

Prevention remains the best way to control BRD. However, if we fail to prevent the disease, the most common and efficacious treatment is antibiotic therapy. According to the National Animal Health Monitoring System, 94.8% of pneumonia cases identified in cattle are treated with antibiotics in U.S. farms (NAHMS, 2016).

Several commonly used antibiotics in the past for BRD treatment include penicillin, oxytetracycline, sulfonamides, spectinomycin, erythromycin, and the unrecommendable use of parenteral aminoglycosides and chloramphenicol. Oftentimes, these compounds were used in an extra-label fashion. Over the past decades, pathogen-targeting antibiotics were developed for BRD treatment, which helped to eliminate the need for extra-label use of antibiotics. These products have different active ingredients such as ceftiofur, enrofloxacin, florfenicol, danofloxacin, gamithromycin, tildipirosin, and tulathromycin. These antimicrobials are called the new generation of antimicrobials, which changed BRD treatment protocols considerably. Most of these products avoid the need for daily injections. A single injection of compounds such as tildipirosin, florfenicol, and tulathromycin are sufficient for most cases of BRD. This is because these

antimicrobials have a long-lasting effect on the lungs of injected animals. Of these new-generation antibiotics, florfenicol, gamithromycin, enrofloxacin, and tulathromycin are labeled for treatment of *Mycoplasma bovis*, an important bacterium associated with the BRD complex. Some of these antibiotics are also labeled for prevention of BRD, which was reported as an effective strategy to reduce the incidence of the disease in cattle operations (Smith et al., 2020).

One of the greatest global concerns these days is the resistance to antibiotics of pathogenic bacteria causing diseases in human and animals. Despite the value of antibiotics for prevention and treatment of BRD, the risk of bacterial resistance is high. Multidrug resistance have been reported for important bacteria associated with BRD, such as *Mannheimia haemolytica, Pasteurella multocida* and *Histophilus somni* (Booker and Lubbers, 2020). Among the strategies used by pneumonia causing bacteria to resist the effect of antimicrobials is the formation of biofilms, which consequently increases the rate of treatment failure (Boukahil and Czuprynski, 2016).

Two "next-generation" antimicrobials highly used in the U.S. to control BRD in beef and dairy cattle are tildipirosin and florfenicol. Both products have demonstrated efficacy against the most common pathogens known to be associated with BRD. The evaluation of their effect on health parameters and on microbiome composition compared to a group of sick but untreated calves is a main objective of this dissertation.

Tildipirosin

Tildipirosin is a semi-synthetic long-acting macrolide drug that acts against *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* (Rose et al., 2012, Zeng et al., 2018). Tildipirosin acts by inhibiting the biosynthesis of an essential bacterial protein with selective binding to ribosomal subunits (Andersen et al., 2012). This drug is derived from the naturally

occurring 16-membered ring macrolide tylosin and has a membrane penetration capability with both hydrophobic and basic properties (Poehlsgaard et al., 2012). Studies have evaluated the effectiveness of this drug against pneumonia and otitis. A single site randomized clinical trial conducted by our research group demonstrated that calves diagnosed with pneumonia had a reduction of rectal temperature, ear scores, leukocyte counts, and relative abundance of the genera *Mannheimia*, *Pasteurella*, and *Moraxella* in the URT (Bringhenti et al., 2021a). As described above, because of its long-lasting action in the lungs, tildipirosin has been used metaphylactically in dairy herds and feedlots. A single herd study conducted in a group-housing facility showed a significant reduction in hazard (hazard ratio = 0.7) of developing BRD and/or otitis in Holstein preweaned calves after tildipirosin administration in comparison to the control group (Teixeira et al., 2017b). Another study showed a significant reduction on the incidence of otitis (CTR = 47.03%; TRT = 37.55%) and a tendency to reduce pneumonia (CTR = 20.71%; TRT = 17.38%) in calves administered with tildipirosin as a metaphylactic method (Bringhenti et al., 2021b) compared to a control group.

Florfenicol

Florfenicol is synthetic drug, derivative of thiamphenicol with the same mechanism of action as chloramphenicol (inhibition of protein synthesis). However, it is more active than either chloramphenicol or thiamphenicol, and may be more bactericidal than previously thought against some pathogens (e.g., BRD bacteria). This broad-spectrum antibiotic belongs to the amphenicol class and can be found on the market individually or combined with flunixin meglumine, a non-steroidal anti-inflammatory drug (NSAID). In addition to treating the infection, the presence of an anti-inflammatory in the drug composition allows the product to control inflammatory signs such as fever (Kleinhenz et al., 2016). Florfenicol has broad-spectrum activity, acting against Gram-

negative and Gram-positive pathogens, has good penetration into most body tissues including the lungs, and has a bacteriostatic and bactericidal concentration-dependent killing action (Adams et al., 1987, De Haas et al., 2002). Published research showed efficacy of florfenicol against important pathogens associated with pneumonia, such as *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* (Varma et al., 1998, Catry et al., 2008, Illambas et al., 2013). Similar to tildipirosin, florfenicol associated with flunixin meglumine was effective in reducing clinical signs of pneumonia but showed faster reduction of rectal temperature (Bringhenti et al., 2021a).

To our knowledge there is no available study evaluating the efficacy of tildipirosin and florfenicol to treat pneumonia in dairy calves in comparison to a group of animals presenting clinical signs of disease, but not treated in the first stages of the disease. Although the importance and efficacy of these drugs are recognized in the field to treat bovine pneumonia, more scientific data about their effect on health parameters and URT microbiota of preweaned dairy calves are needed.

THE UPPER RESPIRATORY TRACT MICROBIOTA OF CALVES

Respiratory disease involves interactions between the respiratory tract microbiota and the immune system of the calf, which is easily influenced by environmental conditions such as changes in temperature. Despite several epidemiologic studies to understand the dynamics of BRD, this disease remains one of the biggest causes of mortality in calves. The complex anatomy and physiology of the respiratory system, divided into the upper respiratory tract (URT) and the lower respiratory tract (LRT), are a major aspect to this complex disease. Another big factor that contributes to the complexity of this disease is the first defense barrier of the calf, more

specifically, the structural and functional aspects of the mucosa and epithelium of the URT (Zeineldin et al., 2019). With the advent of new methods of molecular microbiology, many researchers believe that the key to understanding some diseases and promoting animal health is to understand the interactions of microbiota from different anatomical regions and tissues of the animal. Most BRD studies have been evaluating the effect of the disease on the dynamic of the URT microbiota.

Before diving deeper into the microbiome of the respiratory tract of calves it is necessary to define some concepts. Microbiota is defined as a community of microorganisms belonging to different kingdoms, that live on or within the host, while their microbial structures, metabolites, mobile genetic elements, and relic DNA become embedded in the environmental conditions of the habitat (Zeineldin et al., 2019, Berg et al., 2020). The term ‘microbiome’ was proposed in 2001 and is defined as 'the ecological community of commensal, symbiotic, and pathogenic microorganisms living within a particular environment' (Lederberg and McCray, 2001). Therefore, the microbiome includes all members of microbiota, including bacteria, archaea, fungi, viruses, and eukaryotes. Each member of the microbiota can colonize different organ sites with a specific niche, for example rumen, intestine, uterus and URT (Lederberg and McCray, 2001).

The term bovine respiratory bacterial microbiota also used in this thesis refer to all bacteria identified in the cattle respiratory tract (mostly the URT), which does not include other members of the total microbiota, such as archaea, fungi, viruses, and eukaryotes. Based on BRD studies of bacterial microbiota conducted in the past 15 years, *Mannheimia haemolytica*, *Pasteurella multocida* and *Mycoplasma bovis* are the most prevalent species in the bovine URT (Czuprynski et al., 2004, Caswell and Archambault, 2007, Rice et al., 2007, Timsit et al., 2018). These bacteria are ubiquitous inhabitants of the URT, which means that the host immune system can tolerate a

certain number of bacteria in the respiratory tract. However, certain events like viral infections can cause a dysbiosis, which is defined as a change or disturbance of microbiota from their steady-homeostatic state within a microbial ecosystem (Zeineldin et al., 2019). Usually, bacterial dysbiosis occurs when one species overgrows and reduces the diversity of the whole bacterial population. For BRD, pathogenic species can overgrow and migrate to the lower respiratory tract and cause pneumonia (Timsit et al., 2016, McMullen et al., 2020a).

In addition to management stressors, primary viral infections of the URT caused by BHV-1, BRSV, and BPIV-3 can also compromise the immune system and cause dysbiosis in the respiratory tract. These viruses can overcome the defenses of the immune system, replicate in the mucosal epithelium causing inflammation and cell destruction, which will facilitate the adhesion and colonization of the aforementioned pathogenic bacteria (Yates et al., 1983).

The bacterial dysbiosis of BRD is being increasingly understood through molecular studies. However, the mantenance procecesses of the immune system and harmonious bacterial population are still not well understood. Based on studies conducted with other species and organs (i.e., gastro-intestinal tract), commensal bacteria living in high diversity in the respiratory tract are essential in modulating the host immune defenses and, therefore, helping to maintain the respiratory homeostasis (Zeineldin et al., 2019).

Studies evaluating the microbiome composition in calves have been performed through the collection of nasal and fecal swabs. A thought-provoking study evaluating the composition of the respiratory tract microbiota of feedlot calves (URT and LRT) showed that the most recommended anatomical location to obtain samples to assess bovine respiratory microbiota is the nasopharynx due to the high similarity between this region and lung bacterial microbiota in healthy cattle (McMullen et al., 2020b). For pneumonia diagnostic purposes, the nasopharynx is also the primary

anatomical region in cattle for identification of pathogenic bacteria (Holman et al., 2015, Noyes et al., 2015).

Normally reported at the phylum and genus levels, the URT microbiota of dairy calves is most commonly composed by Tenericutes, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria at the phylum level; and *Mycoplasma*, *Moraxella*, *Mannheimia*, *Pasteurella*, *Pseudomonas*, *Psychrobacter*, *Acinetobacter* and *Streptococcus* at the genus level (Lima et al., 2016, Gaeta et al., 2017, Holman et al., 2018, Bringhenti et al., 2021a, Bringhenti et al., 2021b). As previously mentioned, all these bacteria are commensal inhabitants of the respiratory tract.

Differences in bacteria composition can be clearly detected between healthy calves and calves diagnosed with respiratory diseases. For example, calves diagnosed with BRD presented lower mean relative abundance (MRA) of the *Actinobacteria* genus in comparison to healthy calves (Holman et al., 2015). Higher MRA of *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida* were observed in the URT bacteria metacommunity of animals with pneumonia compared to their healthy counterparts (Timsit et al., 2018). A later study also reported a higher abundance of certain species such as *Mycoplasma dispar*, *Lactococcus lactis*, and *Lactobacillus casei* in healthy calves compared to calves with pneumonia. A study evaluating 50 Holstein female calves in Wisconsin reported reduced microbiota diversity in animals with pneumonia compared to healthy calves (Raabis et al., 2021) whereas the study conducted by Lima et al., (2016) found higher MRAs of the genera *Moraxella*, *Mannheimia*, *Mycoplasma* and *Pasteurella* in animals that later developed pneumonia and/or otitis compared to calves that remained healthy. The latter two studies also reported a higher bacterial load in calves that developed pneumonia and/or otitis later in life.

MOST PREVALENT BACTERIA ASSOCIATED WITH BRD

Mannheimia haemolytica and *Pasteurella multocida*

These two bacteria are commonly responsible for the classical signs of pneumonia in affected cattle (e.g., fever, increased respiratory rate, purulent nasal discharge). Both bacteria are Gram-negative facultative anaerobes that belong to the family Pasteurellaceae, phylum Proteobacteria. The characteristics that distinguish this family of microorganisms from other families are the coccobacilli form, usually oxidase positive, nitrate reducers, and carbohydrate fermenters. These bacteria are isolated in the mucous membrane of the oropharynx and nasopharynx of healthy and diseased cattle.

Both organisms contain lipopolysaccharides (LPS) in the outer cell wall. This endotoxin is a potent proinflammatory mediator that will trigger the release of cytokines and stimulate the flux of inflammatory cells contributing to the disease pathogenesis (Step et al., 2008). In the case of *Mannheimia haemolytica*, LPS can act synergistically with the leukotoxin, which is an important virulence factor that increases the pathogenicity of the disease and causes common clinical signs of the disease (Zecchinon et al., 2005, Step et al., 2008). Both bacteria have mechanisms to colonize the mucosa surface and evade host immune responses. A series of adhesins allow epithelial cell attachment and prevent phagocytosis by components of the immune system (Confer and Ayalew, 2018).

Mannheimia haemolytica and *Pasteurella multocida* are both extremely contagious and are easily transmitted in facilities with direct contact between calves. Furthermore, antimicrobial resistance is an emerging issue in BRD pathogens, including these two bacteria. General trends suggest a decrease in the susceptibility over time of both pathogens to antimicrobials used to treat BRD (Welsh et al., 2004, Portis et al., 2012, Holschbach et al., 2020). Recent research also

suggests that antimicrobial practices commonly used in cattle operations, such as the metaphylactic use of antimicrobials and mass treatment of calves, might be the primary factor driving selection of resistant clones (Holschbach et al., 2020, Snyder and Credille, 2020).

Mycoplasma bovis

This peculiar bacterium cannot be identified using Gram staining, is intermediate in size between virus and bacteria, and does not have a cell wall. One interesting characteristic of this bacteria is its transmission, which includes contaminated milk from infected cows to calves, close contact between healthy and infected animals, and through the air. Usually, it is the second pathogen often seen in animals previously infected by bacteria or viruses, with a potential to be the primary cause of disease as well (Gille et al., 2016, Calcutt et al., 2018, Register et al., 2018).

Mycoplasma bovis is also a commensal organism of the upper and lower respiratory tract of cattle but can be found in a higher proportion in the lower respiratory tract of infected animals (Timsit et al., 2018). Virulence factors of this bacteria include variable surface proteins (Vsp) that allow the bacteria to evade the host immune system and form biofilm (Song et al., 2012, Gao et al., 2018). *Mycoplasma bovis* modulates the immune responses of the host by altering the expression of cytokines, inhibiting peripheral blood mononuclear cell proliferation, and reducing apoptosis of peripheral blood mononuclear cells, monocytes, and alveolar macrophages (Perez-Casal, 2020). In addition to its capacity to evade the bovine immune system, *Mycoplasma bovis* can be highly resistant to antimicrobials commonly used to treat pneumonia and cases can become chronic despite antibiotic therapy (Ter Laak et al., 1993, Ayling et al., 2000).

Histophilus somni

This bacterium is small, Gram-negative, nonspore-forming, pleomorphic bacillus, a member of the Pasteurellaceae family, and does not have flagella or a polysaccharide capsule (Firehammer, 1959, Kennedy et al., 1960, Shirbroun, 2020). *Histophilus somni* is considered a commensal bacterium of the respiratory tract that can be an opportunistic pathogen, complicating viral infections and increasing the severity of infection by other bacteria.

Histophilus somni has demonstrated an impressive ability to evade the immune response of the host, primarily due to the function of some of the recognized virulence factors. The ability to cause the disease is linked to factors including the stimulation of host IgE response (Shirbroun, 2020), adherence to colonize the mucosal membrane and attaching to cells (Corbeil et al., 1985), and potential accessibility to host defenses conferred by outer membrane proteins (OMPs) (Tagawa et al., 1993).

Studies have demonstrated an association of this bacteria with bovine respiratory syncytial virus (BRSV), presenting a synergic effect in pulmonary alveoli (Szacawa et al., 2016). Respiratory disease caused by these two pathogens together have more severe clinical signs compared with infections caused by other microorganisms or when not combined (Gershwin et al., 2005). The pathogenesis of those cases eventually results in death of calves (Shirbroun, 2020).

Studies assessing the genome of *H. somni* revealed significant genetic differences between strains isolated from the URT of cattle, some of which are associated with presence or absence of virulence factors (Shirbroun, 2020). These genetic differences between strains are problematic not only for the host immune system, but also for the development of effective vaccines to prevent pneumonia caused by this emerging pathogen in dairy herds and feedlots.

GENOMIC TECNOLOGIES USED TO STUDY THE BOVINE UPPER RESPIRATORY TRACT MICROBIOTA

Studies conducted with species other than bovine have demonstrated the relationship between the mucosal microbiota and the host immune system and its importance on the regulation of mucosal immunity and maintenance of metabolic homoeostasis (Dickson et al., 2016). Advanced technologies have allowed us to better understand ecological communities of commensal, symbiotic and pathogenic microorganisms living within a particular environment (Lederberg and McCray, 2001).

Not too long ago, culture-based techniques were the only method to investigate pathogens associated with infectious diseases (Amann et al., 1995). Those techniques only permitted the identification of microbes that grow on the culture media used in the method, limiting the comprehension of uncultured microbes. With the advent of molecular microbiology techniques, research has changed to a more holistic approach (Szacawa et al., 2016).

Techniques such as real-time qPCR became commonly used for amplifying and quantifying specific bacterial DNA or RNA. However, this technique does not provide direct molecular sequence data (Zeineldin et al., 2019). Sequencing of the 16S rRNA gene of bacteria, allows for the identification of the constituents of the microbial community by sequencing the genetic material present in the sample. The Illumina MiSeq platform, which gives robust information on the microbiota functional profiles, is one of the most used sequencers today (Johnson et al., 2019).

Finally, specific mass spectrometry techniques have been used to detect microbiota activity. These techniques include metaproteomic (i.e., assessment of protein products), metatranscriptomics (gene expression), and metabolomics (metabolic profiles). These techniques

combined with sequence information are considered the cornerstone for improving the understanding of the respiratory microbial niche and its association with health and disease in animals and humans (Zeineldin et al., 2019).

RELATIONSHIP BETWEEN BRD AND OTITIS

Otitis is another disease that affects cattle in their early life occurring sporadically or as an outbreak. The definition of this disease is an inflammation in the external, middle, or inner ear caused as a complication of inflammations of the ear skin and epithelium of the external auditory canal (Bruyette and Lorenz, 1993). The signs of otitis media (OM) are unilateral or bilateral ear droop, epiphora, head tilt, and recumbency in severely affected cases (Walz et al., 1997). Some events can evolve to meningitis and abscess (Morin, 2004). Because this disease has less obvious clinical signs than pneumonia, it is less reported by farms. The signs of OM presented by calves are often clinically undetected and the disease can be easily overlooked and consequently not treated causing a considerable economic impact to farms and animal welfare concerns (Morin, 2004). Healthy dairy calves can weigh 5 kg more than calves diagnosed with OM during the first 6 weeks of life (Sockett et al., 2008) and have an increased risk to be culled (Pardon et al., 2013).

Otitis usually starts as an acute infection and, due to lack of treatment, evolves into a chronic infection (Jensen et al., 1983). Pathogens such as *Mycoplasma bovis, Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni, Streptococcus* spp. and *Actinomyces* spp. have been reported as the cause of OM (Jensen et al., 1983, Walz et al., 1997). However, the most common pathogen isolated in OM cases is the *Mycoplasma bovis* that can cause the infection either alone or in association with other bacteria (Walz et al., 1997, Maeda et al., 2003).

It is interesting that most pathogens causing OM are the same organisms causing BRD, so it is common to identify OM in calves after identification of pneumonia. This can be explaned by the straight conection between the inner ear and URT through an anatomical canal called the Eustachian tub (auditory tub). This conection occurs between the ear canal and the nasopharynx, which is the anatomical region of the URT from where bacteria associated with BRD are commonly isolated (Morin, 2004, Maunsell et al., 2012, Wilson, 2012). The conduit for potential ear infection through the eustachian tub was confirmed by Maunsell et al. (2012) by inoculating calves orally with *Mycoplasma bovis*. The authors found that animals developing OM had the infection ascend to the tonsils and eustachian tubes. Therefore, calves with a respiratory infection are at higher risk for the development of OM as a secondary infection ascending from the nasopharyngeal region.

Otitis media is also treated with antimicrobials but lack standardized treatment with few studies available to support the topic. One retrospective study performed in Canada evaluated 15 otitis cases in calves in which enrofloxacin was the most common antimicrobial used (Francoz et al., 2015). Although this drug is licensed for treatment of respiratory disease and is effective against *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida* with properties that allow its penetration to the middle ear, it is not licensed for otitis media in the U.S. (Massias et al., 1994). Another study conducted in Japan reported oxytetracycline hydrochloride as the main antimicrobial used for treatment of OM on four different farms (Maeda et al., 2003).

Bringhenti et al. (2021a) observed that 92.9% and 95.1% of the group-housed calves that were first treated for pneumonia with florfenicol + flunixin meglumine and tildipirosin, respectively, developed OM later within the preweaning period. On the other hand, 69.0% of calves in the florfenicol group and 69.7% of calves in the tildipirosin group were diagnosed with

pneumonia after being treated for otitis. Although this farm had an extremely high risk of OM and pneumonia, this data also suggests an increased risk of OM following pneumonia in preweaning calves. The study also reported similar risk for retreatment between the two antimicrobials, but a lower proportion of febrile calves was observed in the florfenicol group in the first two days after OM diagnosis in comparison to the tildipirosin-treated calves. Both florfenicol and tildipirosin groups decreased the proportion of calves with abnormal ear scores and increased the proportion of calves with normal ear positions after treatment, although the proportion of calves with more severe ear scores was lower in the florfenicol group (Bringhenti et al., 2021a).

Our group also evaluated the effect of metaphylactic use of tildipirosin at approximately 7 days of life for prevention of pneumonia and otitis (Bringhenti et al., 2021b). The study was conducted at the same farm as in the previous study. Calves treated with tildipirosin had 20.2% reduction in otitis risk compared to control calves (i.e., calves that did not receive the metaphylaxis).

The epidemiology and microbiology of OM still deserves further investigation so that we can develop more targeted and successful strategies for the prevention and treatment of this infectious disease in dairy and beef calves.

ECONOMICAL IMPACT OF BRD TO THE CATTLE INDUSTRY

Despite several studies and extension efforts focusing on understanding the epidemiology of BRD over the years, the disease remains one of the main causes of economic loss to the cattle industry in the U.S. (Gorden and Plummer, 2010). Despite improving cattle management and veterinary medicine, the incidence of respiratory disease in dairy in calves has not changed much since the early 1990s (NAHMS, 2016). On the other hand, the cost to treat the disease increased

substantially over the last decade (Dubrovsky et al., 2019). Although several studies evaluated the economic losses associated with BRD, the comparison between them are difficult due to the variation between farms in terms of production system, risk factors, and consequences of the disease (Van der Fels-Klerx et al., 2001).

The economic impact of BRD include labor costs related to the prevention of the disease and care of sick animals, treatments, veterinary fees, performance losses, and replacement of dead animals (Kaneene and Hurd, 1990, Gorden and Plummer, 2010). From the prevention standpoint, studies conducted in the past estimated a range of $9.84 to $16.35 per calf during the preweaning period and $2.05 to $2.22 per head after weaning (Kaneene and Hurd, 1990, Sischo et al., 1990). Although the cost to prevent BRD can be considered high to some farmers, failing to prevent the disease can significantly increase the treatment costs, reduce the survivability of animals on the farm, and affect their long-term performance and productive life (Dubrovsky et al., 2019).

A recent study estimated the cost of BRD based on longitudinal treatment data from a cohort of 11,470 preweaned dairy calves in California (Dubrovsky et al., 2020). The authors reported a short-term cost of $42.15 per treatment including the use of anti-inflammatory medications in the treatment protocols across all management conditions. The study also performed a cost-benefit analysis suggesting producers with high rates of BRD may benefit financially from implementing preventative measures such as vaccination and increasing milk fed to the calves, whereas these preventative measures may not be cost effective to implement on dairy farms with very low cumulative incidences of BRD.

Regarding treatment protocols, an interesting study evaluating the performance of calves in a feedlot reported that the return of calves treated once, twice and ≥3 times was $40.64, $58.35, and $291.93 less, respectively, compared with untreated calves (Fulton et al., 2002). Pneumonia

within the first 6 months of life caused consequences such as slow growth rates, decreased productivity, and increased mortality risk (Sischo et al., 1990, Gorden and Plummer, 2010).

Bovine respiratory disease during the calfhood of dairy cows can have a remarkable negative impact on their productive life. A recent study reviewed the effects of calfhood BRD on health and performance of dairy cattle and summarized the research findings using a meta-analysis approach (Buczinski et al., 2021). The models created in the study indicated that heifers diagnosed with BRD during calfhood had 2.85 times higher odds of dying and 2.30 times higher odds of herd culling before the first calving compared with heifers not diagnosed with this disease. Heifers experiencing calfhood BRD also had an average daily gain reduced by approximately 0.07 kg/d and they produced 121.2 kg less milk during their first lactation (Buczinski et al., 2021).

Based on the research described within this topic, preventive management strategies are the key to decrease the incidence of BRD in dairy calves. Furthermore, in the event of failure to prevent BRD, rapid diagnosis and proper treatment of this condition can dramatically reduce direct and indirect costs to the farm.

CONCLUSIONS

Bovine respiratory disease is a multifactorial disease with high prevalence in dairy cattle, responsible for significant economic losses associated with treatment, increased labor, and mortality. Among the conditions associated with the BRD complex, infectious pneumonia is the most common form of this disease in dairy operations. BRD is the main cause of morbidity and mortality in preweaning dairy calves along with infectious diarrhea. In addition to its multifactorial nature, BRD is also a polymicrobial disease, as its pathogenesis can be caused by viruses and bacteria that act together in the development of the disease. All these factors complicate BRD's

control strategies on dairy farms. In addition to the proper handling of animals and the administration of high-quality colostrum, the use of vaccines can help prevent BRD. Despite the use of adequate preventive strategies against BRD, some calves are more susceptible to infections of URT and develop bacterial pneumonia. For these calves, timely diagnosis and treatment with effective antibiotics reduce the mortality rate and improve animal welfare. Finally, state-of-the-art methods allowed us to identify the composition of the microbiome present in the respiratory tract of calves and better understand the role of microorganisms in healthy and sick animals. Studies conducted to assess the effect of treatments on the respiratory tract microbiota compared calves treated with antibiotics with healthy counterparts, but never with sick and untreated calves. Such a study can help us to better understand the dynamics of the microbiota after therapeutic interventions, improving our understanding of the epidemiology and pathogenesis of BRD.

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CHAPTER 2: Negatively controlled randomized field trial evaluating the effect of treatment of pneumonia with tildipirosin or florfenicol + flunixin meglumine on the health and upper respiratory tract microbiota of preweaned Holstein dairy heifers

ABSTRACT

The aim of this study was to evaluate the effect of therapeutically administered tildipirosin or florfenicol + flunixin meglumine for the treatment of bovine respiratory disease accompanied by fever in calves before weaning compared to pneumonic and untreated animals. As specific objectives, we evaluated the composition of the microbiota of the upper respiratory tract **(URT)** as well as blood and health parameters of the animals. Preweaned Holstein calves diagnosed with pneumonia were assigned to one of the following experimental groups: (**TLD**; n = 36) single subcutaneous injection with 4 mg/kg of tildipirosin; (**FLF**; n = 33) single subcutaneous injection with an antimicrobial (40 mg/kg of florfenicol) combined with a non-steroidal anti-inflammatory drug (2.2 mg/kg of flunixin meglumine); and (**NEG**; n = 35) no treatment within the first 5 days following enrollment. Healthy untreated calves (**CTR**; n = 31) were also selected for the study and used as controls. Blood samples used for biochemical analysis and nasopharyngeal swabs used for evaluation of URT microbiota were collected daily from day 0 (diagnostic) until day 5 and then weekly until weaning (~65 days of age). Next-generation sequencing of the 16S rRNA gene was used to assess the URT microbiota at the phylum and genus levels. Clinical signs associated with pneumonia and otitis media were assessed daily, as was the need for antibiotic interventions. Calves in the TLD and FLF groups had faster recovery from fever within the first 5 days after enrollment and a lower risk of fever at day 5 than calves in the NEG group (FLF = 8.4%; TLD = 20.4%; NEG = 49.9%). In addition, antibiotic-treated calves had a lower risk of treatment for pneumonia (FLF = 22.8%; TLD = 27.7%) from day 5 to weaning than calves in the NEG group (54.7%). During the same period, the FLF (53.8%) and TLD (53.6%) groups had a lower risk of any treatment (pneumonia and/or otitis) than the NEG group (90.6%). Furthermore, FLF treatment had a significantly lower risk of nasal discharge, treatment failure, and otitis media compared to the NEG

group, but no difference was observed when comparing the two treatment groups of FLF and TLD for these factors. No differences were observed among pneumonic calves in these three groups in terms of lung consolidation at weaning (based on thoracic ultrasound) and average daily gain. Differences in the composition of the URT microbiota were found between groups, and the genus *Mycoplasma* was the most abundant in samples collected from the URT of calves with and without pneumonia. Both drugs were effective in reducing the mean relative abundance **(MRA)** of important genera associated with pneumonia (*Mannheimia* and *Pasteurella*), although an increase in *Mycoplasma* MRA was observed for tildipirosin-treated calves. In conclusion, compared to untreated calves, both drugs were effective in reducing the inflammatory signs of pneumonia and the need for antimicrobial treatment after enrollment. In addition, both TLD and FLF were effective in reducing the MRA of important bacterial genera associated with pneumonia; however, TLD treatment was associated with increased *Mycoplasma* MRA compared to healthy and untreated calves.

Keywords: BRD, pneumonia treatment, respiratory tract microbiota, tildipirosin, florfenicol + flunixin meglumine.

INTRODUCTION

Bovine respiratory disease **(BRD)** is a disorder which can affect both the upper and lower respiratory tracts of cattle. This multifactorial disease is a major concern when raising replacement heifers, as it causes high morbidity and mortality rates, increases farm costs related to treatment and prevention, and potentially impacts future animal performance (Zeineldin et al., 2019, Buczinski et al., 2021).

In the United States, the herd-level incidence of BRD in preweaning dairy calves is reported to be around 23%, causing 30% of all calf mortalities (Chigerwe et al., 2015, Dubrovsky et al., 2019). The high incidence of BRD is commonly associated with poor management practices, which can affect the immunity of calves. The immune defenses of newborn calves are highly dependent on absorption of antibodies acquired through the dam's colostrum, which is time dependent. In addition, calves must quickly adapt to an intensive growth system under constant challenge from pathogens and stressors that can suppress their immunity and increase their susceptibility to infections such as infectious pneumonia (McGill and Sacco, 2020). Controlling BRD in the herd is a difficult task, as it is considered a multifactorial and polymicrobial disease with several risk factors capable of triggering its development.

Typically, BRD occurs when an animal acquires a viral infection such as caused by herpesvirus-1 (BHV-1), respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV3), bovine coronavirus, bovine adenovirus, or bovine viral diarrhea virus (BVDV). These viruses can damage cells of the respiratory tract and suppress the immune defenses of calves, which facilitates the over-replication of commensal bacteria present in the respiratory tract, such as *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*, causing the appearance of clinical signs of the disease. Many factors can facilitate bacterial replication and increase the risk of BRD in dairy

and beef calves. For example, poor colostrum management in terms of quality and quantity can cause failure of passive transfer of immunity (Donovan et al., 1998). Other risk factors associated with BRD include commingling (Svensson et al., 2003), poor bedding management (Lago et al., 2006), stress from transportation (Arthington et al., 2003), and inadequate weaning management (Jasper et al., 2008).

In terms of housing, the use of individual hutches is considered the traditional system in the United States for dairy calves. This system minimizes the direct contact between calves, limiting the transmission of infectious diseases. However, this system has been replaced in some dairy operations by collective pens, where animals are raised in groups of 15 to 25 calves. The group housing system allows calves to express more natural behavior compared to hutches, while reducing labor costs. Unfortunately, environmental factors such as nose-to-nose contact and the stress of competition between calves can cause immune suppression, thus facilitating the animals' susceptibility to infections by viruses and bacteria, which makes group housing a risk factor for respiratory diseases. (Svensson and Liberg, 2006).

The main visual clinical signs of pneumonia include fever, nasal discharge, lacrimation, cough, prostration, dehydration, and anorexia (Ames, 1997). In addition, lung lesions can be seen on thoracic ultrasound. Recent studies have demonstrated that calves affected with pneumonia commonly have lung consolidations (Teixeira et al., 2017b, van Leenen et al., 2020). Timely identification and proper treatment of BRD at the beginning of clinical signs can improve the cure rates and calf performance during the pre- and post-weaning periods.

Some blood parameters have been reported as predictors or biomarkers of pneumonia in calves and heifers. Haptoglobin, which is an acute phase protein produced by hepatocytes, is increased in calves with pneumonia (Wolfger et al., 2015, Moisá et al., 2018). The release of this

inflammatory protein is triggered by pro-inflammatory cytokines (Higuchi et al., 1994); therefore, calves diagnosed with respiratory diseases have a higher concentration of serum haptoglobin compared to healthy calves (Wittum et al., 1996, Svensson et al., 2007). Additional serum parameters have been evaluated in other studies (Bringhenti et al., 2021a, Bringhenti et al., 2021b), providing valuable information on how animals are systemically affected and respond to the disease.

Another area of great scientific interest is the investigation of the composition and dynamics of the URT microbiome of calves (Lima et al., 2016). The microbiome can be defined as a characteristic microbial community that occupies a specific habitat with distinct physicochemical properties and activities that form specific ecological niches (Berg et al., 2020). Therefore, the microbiome can play an important role in the health of animals. Studies evaluating the composition and dynamics of the microbiome in animals under different management and therapeutic regimes can help to understand the epidemiology of BRD and lead to more effective prevention and control strategies.

According to a National Animal Health Monitoring System report (NAHMS, 2016), 94.8% of bovine pneumonia cases are treated with antibiotics, and previous studies have reported the effectiveness of several antimicrobials, including tilmicosin (Fodor et al., 1993), tulathromycin (Ragbetli et al., 2010), and gamithromycin (Lechtenberg et al., 2011). These studies did not assess the effect of the antibiotic treatment on the respiratory tract microbiota of the animals. On the other hand, a recent study carried out in New York State showed differences in the upper respiratory tract **(URT)** microbiota composition of calves with pneumonia and/or otitis compared to healthy calves before weaning (Bringhenti et al., 2021b). That study reported differences in the microbiota dynamics in diseased calves treated with tildipirosin or florfenicol associated with flunixin meglumine, and although the results are thought-provoking, the study lacked a group of diseased

untreated animals. Comparison of health parameters and the microbiota of treated and untreated calves with pneumonia can broaden our epidemiological understanding of BRD and indicate which treatment is more efficacious against this complex disease.

The main objective of this study was to evaluate the effect of administering tildipirosin or florfenicol + flunixin meglumine used therapeutically for the treatment of BRD accompanied by fever in dairy calves before weaning compared to untreated sick animals. As specific objectives, we evaluated the composition of the URT microbiota as well as blood and health parameters of the animals.

MATERIAL AND METHODS

Ethics statement

Samples and data from calves were collected in a commercial dairy farm in strict accordance with the recommendations of the Animal Welfare Act of 1985 (P.L. 99–198). The farm owner authorized the sample collections and was aware of animal handling performed by the researchers. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University (protocol number: 2013-0078). The methods were carried out in accordance with the approved guidelines.

Farm and management

The study was conducted on a commercial dairy farm located in Scipio, New York. Sampling and data collection were performed from September 2020 to April 2021. This farm had approximately 4,300 Holstein cows milked 3 times a day in a 100-stall rotary parlor. Approximately 30 days prior the expected calving date, pregnant cows were moved to pre-maternity pens where

they were monitored 24 hours a day. At the first signs of calving, cows were moved to the maternity pen, which was an indoor open area deep-bedded with straw. Immediately after birth, the newborn calves were cleaned and received naval disinfection (iodine 10%). Subsequently, the calves were weighed and moved to another pen to minimize contact with the dam. The newborn pen was bedded with dry-wood shavings and heated with heating lamps during cold temperatures. Within three hours of birth, four liters of pasteurized pooled colostrum was administered to every newborn calf by an esophageal feeder (Oral Calf Feeder Bag with Probe, Jorvet). This procedure was performed by trained farm workers according to the farm standard operating procedures **(SOP)**.

Twice daily, newborn calves were moved from the newborn pen to a greenhouse type of barn composed of 27 identical group-pens (70 m^2) bedded with straw and having positive ventilation. Twenty-five calves were placed in each pen; all calves remained in the same pen from day one of life until weaning, which occurred at approximately 65 days of the calf's life. At weaning, calves were weighed by the farm employees using a portable scale (Waypig-15, Vittetoe Inc., Keota, IA) and moved to a different housing facility.

During the preweaning period, calves were fed *ad libitum* acidified milk through automatic feeders. The acidification of non-salable milk was performed in a central stainless-steel tank by adding 20% formic acid until reaching a pH of 4.5. The milk was then distributed to smaller tanks located next to the calf pens responsible for heating the milk (37° C) and supplying it through six nipples per pen. Calves were gradually weaned by reducing the milk availability in the nipples, which started 10 days before the moving date (i.e., complete weaning).

Daily, a trained farm worker performed a health check on all calves as part of the normal farm routine. In this check, they monitored the animals for any adverse health event, such as the

presence of respiratory disease, otitis, diarrhea, and injuries. Therapeutic interventions were performed according to farm SOP upon identification of any health disorder.

Study design and sample size determination

This was a negatively controlled randomized clinical trial conducted to evaluate the efficacy of two popular commercially available products for the treatment of BRD in preweaned dairy calves. In addition to the assessment of experimental treatments, healthy animals were enrolled in the study for comparison between diseased and non-treated healthy animals. The study was designed to assess the following hypotheses: (1) the use of antimicrobials is more effective than nontreatment to reduce the health impact of BRD; and (2) there is no difference in the efficacy of antimicrobial interventions against BRD when comparing a protocol using tildipirosin with a protocol using florfenicol associated with an anti-inflammatory (flunixin meglumine). Outcomes such as rectal temperature, blood and health parameters, need for retreatment of pneumonia, risk of otitis, and presence of lung lesions were compared among groups from identification of disease to weaning.

Assuming a desired type I error rate of 5%, a power of 80% and a two-sided statistical test, a sample size of 30 calves per group was calculated to detect a difference of 35 percentage units in the need for pneumonia retreatment before weaning when comparing treated (30%) and untreated animals (65%).

Case definition and selection of calves

A clinical examination of all calves in the calf-housing barn was completed daily by a trained farm worker from birth to weaning. In case of clinical signs of pneumonia, a physical examination was performed by researchers, which were blinded to the treatment allocation list. In

this examination, calves were defined as having pneumonia if they presented a respiratory rate >40 breaths/min and fever (rectal temperature >39.5 °C). Only calves with a first case of pneumonia were eligible for the study. Calves that received any antibiotic intervention before pneumonia diagnosis (regardless of disease) were not eligible for enrollment. In addition, calves with poor health condition (e.g., prostrated animals presenting severe dehydration) were not enrolled in the study and were treated according to the farm protocol.

Randomization and treatment protocols

The randomization was performed prior to the beginning of the study using the random function in Excel (Microsoft Corp., Redmond, WA). Based on the randomization list, eligible calves were allocated into one of three treatment groups described below.

The treatment groups consisted of two different commercial drugs (tildipirosin or florfenicol + flunixin meglumine) labeled for treatment of pneumonia in non-lactating dairy cattle of 20 months of age or younger, and a negative treatment group. Calves assigned to the tildipirosin group **(TLD)** received a single subcutaneous injection of tildipirosin (4 mg/kg; Zuprevo®, Merck Animal Health, Millsboro, DE), a synthetic long-acting macrolide. Calves enrolled in the florfenicol + flunixin meglumine group **(FLF)** received a single subcutaneous injection of a cocktail containing an antimicrobial (40 mg/kg of florfenicol) combined with a non-steroidal anti-inflammatory (2.2 mg/kg of flunixin meglumine; Resflor-Gold®, Merck Animal Health, Millsboro, DE). The negative treatment group of untreated calves **(NEG)** did not receive any antibiotic intervention up to the fifth day after diagnosis. An assessment of the health status was performed on the fifth day following enrollment and a therapeutic intervention was performed if a calf remained with clinical signs of pneumonia, regardless of the experimental group. Treatment after day 5 for calves demonstrating

clinical signs was performed after sample collection and at the discretion of farm management. Animals in the NEG group received either tildipirosin or florfenicol + flunixin meglumine, as needed, for therapeutic intervention.

In addition, upon enrollment of one calf into each of the experimental groups, one to two healthy calves (depending on the availability at the day) were enrolled in the study by matching their age with the age of calves with pneumonia. Healthy calves **(CTR)** were defined as animals having good health condition, without fever at enrollment and no history of previous diseases treated with antibiotics. CTR calves diagnosed with pneumonia or otitis media after enrollment to weaning were excluded from the study.

Data Collection

All enrolled animals had the same sampling schedule. On the day of pneumonia diagnosis (day 0), samples were collected right before treatment administration for calves in the TLD and FLF groups, or right after enrollment for calves in the NEG and CTR groups. After enrollment, calves were followed for samples and data collection daily for 5 days and then weekly until weaning (≈ 65 days of life). Physical examinations were recorded daily from enrollment to day 5 and once again on day 10 (W1). The physical exam consisted of an adaptation of a previously reported calf scoring system (McGuirk, 2008). Researchers carefully examined each animal by checking rectal temperature, presence of cough (repeated spontaneous cough), eye and/or nasal discharge (small or heavy amounts of uni- or bilateral discharge), and abnormal ear disposition characterized by ear flick, head shake, or uni- or bilateral ear droop with or without signs of head tilt (Bringhenti et al., 2021b). In addition, body weights measured by farm personnel on the day of calf birth and at weaning were used for estimation of average daily gain **(ADG)**, which was extracted from the farm

management software (Dairy Comp 305; Valley Agricultural Software, Tulare, CA). Events such as death for any reason or occurrence of other diseases (e.g., otitis media) were extracted from Dairy Comp 305.

Blood sample collection and analysis of serum parameters

Blood samples were collected daily from day 0 to day 5 and then weekly until weaning via jugular venipuncture using an 18-gauge by 3.8-cm needle in a 10-mL plastic vacuum tube (Becton, Dickinson and Co.) without anticoagulant. Serum was harvested within 2 hours of collection after centrifugation of the blood tube at 2,000 × *g* for 15 min and stored at -80°C.

Serum concentrations of total protein, glucose, BHB, calcium, lactate, cholesterol, urea, alkaline phosphatase **(ALP)**, aspartate aminotransferase **(AST)**, and alanine aminotransferase **(ALT)** were determined using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd.) using reagents provided by Randox Laboratories. Serum concentration of haptoglobin was determined using a colorimetric assay as described elsewhere (Bicalho et al., 2014).

Clinical scores

Clinical observations were performed daily from day 0 to day 5 and at the first week after diagnosis (W1) by evaluation of nasal, eye and ear scores as described by Bringhenti et al. (2021b). Nasal discharge was scored as: 0 = normal serous discharge; 1 = small amount of unilateral cloudy discharge; 2 = bilateral, cloudy or excessive mucus discharge; and 3 = copious bilateral mucopurulent discharge. For eye scores: 0 = normal with no discharge; 1 = small amount of ocular discharge; 2 = moderate amount of bilateral discharge; and 3 = heavy ocular discharge. Ears were scored as: 0 = no abnormalities; 1 = ear flick or head shake; 2 = slight unilateral droop; and 3 = head

tilt or bilateral droop. To reduce the subjectivity of the diagnosis, the three researchers responsible for conducting the clinical examinations practiced the diagnostic protocol together before the beginning of the study.

Nasopharyngeal swabs

Nasopharyngeal swabs were collected daily from day 0 to day 5 and then weekly until weaning (63 ± 3 days of life). A 20-cm DNA-free sterile swab (BBL™ CultureSwab™) was aseptically introduced approximately 15 cm into one nostril to reach the nasopharynx cavity and a rotation of 360° was performed in contact with the nasopharynx mucosa. Nasopharyngeal swabs were collected in duplicate from the same nostril at each time point; however, the nostril side was not controlled between time points because a previous study did not report significant differences in the composition of microbiota collected from different calf nostrils (McMullen et al., 2020). After collection, swabs were placed on ice for transportation to the laboratory and stored at -80°C until further processing.

Analysis of the nasal microbiome

DNA extraction

The isolation of DNA from all swabs of the URT was performed from each swab using the DNeasy PowerFood Microbial kit (Qiagen) according to the manufacturer's instructions, with the following modifications. After collection the swab was cut with sterile scissors and stored in a 1.5-mL sterile tube at -80º C until further analysis. On the day of DNA extraction, the swabs were thawed for 15 min and 450 µL of MBL solution (provided in the kit) was added to the tube containing the swab and vortexed for 15 min, which is not part of the kit protocol. Subsequently, the entire solution

contained in the tube was pipetted into an extraction tube containing beads and the DNA extraction proceeded according to the kit guidelines.

16s rRNA gene sequencing and bioinformatics

The 16S rRNA gene was amplified by PCR using barcoded primers. Amplification of the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene with barcoded primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) was performed as previously described (Caporaso et al., 2012) using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). The Earth Microbiome Project (http://www.earthmicrobiome.org) was used to select 280 different 12-bp error-correcting Golay barcodes for the 16S rRNA PCR. All DNA samples were amplified using 10 μM of each primer, EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI), 10 ng–100 ng of individual DNA, and UltraPureTM distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) to bring the final reaction volume to 50 μL. PCR conditions for the 16S rRNA gene were: initial denaturing step at 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 90 s; and final elongation at 72 °C for 10 min. Afterwards, PCR amplicons were visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL ethidium bromide. Amplicons were purified using Mag-Bind® Total Pure NGS (Omega Bio-Tek Inc., Norcross, GA) according to the manufacturer's instructions. Samples were standardized to the same concentration and pooled into a run for library preparation and sequencing, which was performed using the MiSeq Reagent Kit v2 (300 cycles) on the MiSeq platform (Illumina Inc., San Diego, CA).

Representative sequences for each Operational Taxonomic Unit **(OTU)** were compared against the Greengenes database (http://greengenes.lbl.gov/) for taxonomy assignment, and only full-length, high-quality reads (r = 0) were used for analysis. The MiSeq reporter classification was

based on the Greengenes database, and the output used from this workflow was a classification of reads at the phylum and genus levels.

Health parameters

Several dichotomized health parameters were assessed from calves identified with pneumonia, including the risk of fever on day 5 of the study, treatment failure, need for antimicrobial use from day 5 to weaning, and risk of otitis treatment after enrollment. Calves were considered to have fever on day 5 if the rectal temperature remained ≥39.5 ºC. Experimental treatment failure was defined as the need for antimicrobial intervention against pneumonia from day 5 to day 10, or in the case of death during the same timeframe. The need for antimicrobial use for treatment of pneumonia, otitis or otitis and/or pneumonia was also evaluated from day 5 of the study until the weaning day (approximately at 65 days of age).

Lung ultrasounds

Lung ultrasound was performed at the week of calf weaning by two researchers, as described by Teixeira et al. (2017a) with the following modifications. The procedure was performed by screening dorsal to ventral intercostal spaces from the right 2nd through 10th intercostal spaces. The examinations were carried out using an Ibex-pro device with a 6.2-MHz linear transducer (E.I. Medical Imaging, Loveland, CO). Calves were not shaved in any area on the thorax and 70% isopropyl alcohol was used to improve the probe contact and imaging quality. The same researcher performed the ultrasonography in all calves, while the other researcher was responsible for restraining the calf. The ultrasonography was performed in the same pen where the calves were housed. Lung ultrasound was not performed in calves in the CTR group. Although the lung

ultrasound in CTR calves would give us an extra outcome to confirm that the calves remained healthy during the study period, it was not performed to minimize stress in accordance with the farm owner.

No lung consolidation **(NC)** was defined as the absence of abnormalities on thoracic ultrasound (i.e., well-ventilated peripheral lung tissue). On the other hand, a calf was defined as having lung consolidation **(LC)** if a detectable consolidation was observed, regardless of the size of the consolidated area (Teixeira et al., 2017a).

Statistics

The statistical software JMP PRO 13 (SAS Institute Inc., Cary, NC) was used for descriptive statistical analyses using the ANOVA function for continuous data and chi-squared and Fisher's tests for categorical data. The same software was used to explore outliers in the blood parameter results. Longitudinal changes in the microbial profile were compared between treatment groups by describing the mean relative abundance **(MRA)** of the 6 most abundant phyla and 12 most abundant genera. The MRA values of all the remaining phyla and genera were combined into a single cluster, defined as "Other." The assessment of treatment effect on the MRA over time of most abundant phyla and genera is described below.

The effect of treatments on dichotomized outcomes, such as the occurrence of clinical signs, lung consolidation at weaning, treatment failure, need for pneumonia retreatment after day 5, and risk of otitis, after enrollment was assessed using multivariate logistic regression models with binary distribution of the GLIMMIX procedure (SAS version 9.4). Based on clinical scores described above, a calf was considered as positive for a clinical sign category (nasal, eye or ear score) if it presented a score greater than 0 in at least one examination performed from day 1 to day 5 (and once

again on day 10). For example, if a calf was identified with nasal discharge (nasal score >0) in at least one time point during the period of physical examination, it was defined as having nasal discharge. Models included the fixed effects of treatment (TLD, FLF and NEG), body weight at birth (kg), dam parity (1, 2 or ≥3 lactations), parturition difficulty (assisted or unassisted), diarrhea before enrollment (yes or no), age at pneumonia (days), and biologically plausible two-way interaction terms between the fixed effects. Differences in the least square means (LSM) were considered for multiple comparisons between treatments.

Repeated measurements, such as rectal temperature and blood parameters, were analyzed using general mixed linear models with the MIXED procedure of SAS (version 9.4; SAS/STAT, SAS Institute Inc.). The independent variables offered to the models were treatment (CTR, TLD, FLF and NEG), body weight at birth (kg), dam parity (1, 2 or ≥3 lactations), parturition difficulty (assisted or unassisted), diarrhea before enrollment (yes or no), and age at enrollment (days). In addition, biologically plausible 2-way interaction terms between independent variables were added to the models. Treatment was the only variable forced into the models. Normality of residuals was assessed using residual plots, and continuous data were log10-transformed when residuals did not follow a normal distribution. The effect of time relative to each outcome variable was included in the repeated statement of SAS, using the cow as subject. First-order autoregressive covariance structure had the best model fit based on Akaike information criterion and was selected for the analyses.

The MRA of the most abundant bacterial phyla and genera identified in the nasopharyngeal swabs was evaluated as continuous and dependent variables using general mixed linear models as described above. The independent variables offered to the models were treatment (CTR, TLD, FLF and NEG), time (study days 0, 1, 2, 3, 4, and 5) and the interaction between treatment and time. The

aim of this analysis was to evaluate the effect of treatments on the URT microbiota composition over time. Therefore, samples collected after day 5 were not assessed because 65% of calves diagnosed with pneumonia received an antimicrobial after day 5. The effect of time relative to each outcome variable was included in the repeated statement of SAS, using the cow as subject. First-order autoregressive covariance structure had the best model fit based on Akaike information criterion and was selected for the analyses.

For all data analyses used to assess continuous and dichotomized outcomes, final multivariable models were reached after performing a manual backward stepwise elimination procedure. After each run, variables and their respective interaction terms with the highest *P*-value were excluded from the model until all variables had $P \leq 0.10$. Potential confounders were monitored by the change in the coefficient of a variable after removing another variable from the model. Variables were considered statistically significant when a *P*-value ≤ 0.05 was detected. A tendency to significance was considered if the *P*-value was between 0.05 and 0.10.

Discriminant analysis using JMP Pro 14 was also performed to investigate possible differences in the microbial composition of the URT microbiota between groups. The 50 most prevalent bacterial genera were used as covariates, and the interaction of time with treatment group (FLF, TLD, NEG and CTR) as a categorical variable. For this analysis, the microbial composition of day 0 was compared with days 1, 3, 5, and 10. A stepwise backward elimination approach was performed and variables with the highest *P*-values were removed until only variables with $P < 0.1$ remained in the model. Total canonical values were used to create the screening graphic.

RESULTS

Descriptive data

In total,104 calves diagnosed with their first case of pneumonia were enrolled in this study: 33 in the FLF group, 36 in the TLD group, and 35 in the NEG group (untreated at diagnosis). In addition, 48 healthy calves were enrolled as controls (CTR group). Of the calves enrolled in the CTR group, 17 had to be treated with antibiotics after enrollment because of infectious diseases (e.g., pneumonia, otitis, diarrhea) and were excluded from the study. Descriptive data about the animals that remained in the study are presented in Table 1. There were no differences among groups in terms of dam parity ($P = 0.51$), calf birth weight ($P = 0.41$), age at enrollment ($P = 0.95$) and incidence of diarrhea before enrollment ($P = 0.69$). The CTR group was not included in the comparison of diarrhea incidence because the absence of previous diseases was an inclusion criterion for this group. As expected, calves in the CTR group had significantly lower rectal temperature at enrollment than animals diagnosed with pneumonia (FLF, TLD and NEG), and no significant difference was observed among these latter groups (Table 1).

Table 1. Descriptive statistics of calves enrolled in the study and their distribution according to experimental groups.

Item	Experimental groups[1]				*P*-value
	FLF (n = 33)	TLD (n = 36)	NEG (n = 35)	CTR (n = 31)	
Dam parity (n)[2]	1.7 (0.17)	1.5 (0.16)	1.5 (0.16)	1.4 (0.17)	0.51
Birth weight (Kg)[2]	41.4 (0.66)	41.2 (0.64)	41.4 (0.66)	40.0 (0.69)	0.41
Incidence of diarrhea (%)[2,3]	30.3 (10)	25.0 (9)	34.3 (12)	0.0 (0)	0.69
Age at enrollment (d)[2]	26.5 (1.68)	26.3 (1.61)	27.5 (1.63)	27.4 (1.73)	0.95
RT at diagnosis (ºC)[2]	40.1 (0.06)[A]	40.0 (0.06)[A]	40.0 (0.06)[A]	38.8 (0.07)[B]	<0.0001

[1]FLF = single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine (Resflor-Gold®, Merck Animal Health, Millsboro, DE); TLD = single subcutaneous injection of tildipirosin (4 mg/kg; Zuprevo®, Merck Animal Health, Millsboro, DE); NEG = calves did not receive any antimicrobial injection up to the fifth day after diagnosis; CTR = healthy calves, defined as animals with good health condition, without fever at enrollment, and no history of previous diseases.
[2]Variables are presented as mean and standard error of the mean within parentheses. RT = rectal temperature.
[3]Incidence of diarrhea is presented as percentage and number of cases within parentheses.
*Different uppercase letters indicate significant differences between treatments ($P < 0.05$).

Only two calves enrolled died during the study period. One of them was 4 days old at enrollment and was euthanized on the third day following assignment to the TLD group. The other was assigned to the NEG group at 23 days of life and was euthanized 4 days after enrollment.

Health parameters and performance

Rectal temperature was evaluated daily from enrollment to day 5 and once again on day 10 (W1). Our regression model evaluating rectal temperature showed an effect of treatment (P <0.0001), time point (P <0.0001) and an interaction between treatment and time point (P <0.0001; Figure 1) as significantly associated with various treatments. In addition, the model included the effect of calf age at enrollment (P = 0.008). Based on differences of LSM, calves in the CTR group had lower rectal temperature (38.8 ºC) than calves in the NEG (39.4 ºC; P <0.0001) and TLD groups

(39.1 ºC; *P* <0.0001). There was no difference in rectal temperature between CTR and FLF (38.9 ºC; *P* = 0.17). In addition, calves in the NEG group had significantly higher rectal temperature than those assigned to the FLF (*P* <0.0001) and TLD (*P* <0.0001) groups. Finally, calves in the FLF group had significantly higher rectal temperature than calves in the TLD group (*P* = 0.006). The interaction effect between treatment and time relative to enrollment is illustrated in Figure 1.

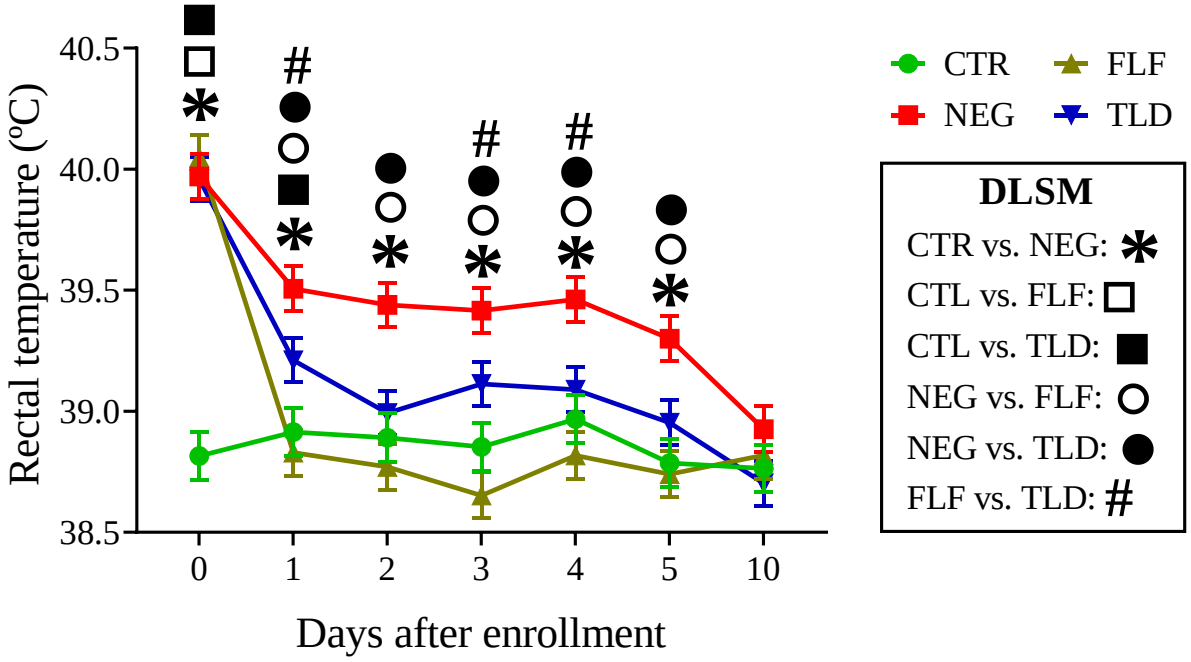

Figure 1. Rectal temperature of 135 calves over the first 10 days after enrollment in the study. FLF (n = 33) single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 36): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis; CTR (n = 31): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. DLSM = differences of least square means (LSM) between treatment groups. Of calves in the NEG group, one died on day 4, and 27 (79.4%) required antimicrobial therapy after day 5 of the study. Results are presented as LSM ± SEM.

Data regarding clinical scores and risks of fever on day 5, treatment failure, and pneumonia and/or otitis retreatment after enrollment are presented in Table 2. No differences were observed among groups in terms of eye discharge ($P = 0.71$) and dropped ears ($P = 0.29$). For the assessment of nasal discharge, only the effect of treatment ($P = 0.04$) remained in the final regression model. Based on adjusted incidences (LSM), calves in the NEG group had a higher risk of nasal discharge (52.9%) than calves assigned to the FLF group (21.2%), and no significant differences were observed when comparing calves in the TLD group to those assigned to the NEG and FLF groups. In addition, the data analysis showed that the odds of a calf presenting nasal discharge was 0.24 times higher in the NEG group compared to the FLF group, with no treatment association observed for nasal discharge when the TLD group was compared with the NEG group (Table 2).

Table 2. Outcomes from the logistic regression models evaluating the effects of experimental treatments on binary variables assessed in 104 calves diagnosed with pneumonia before weaning.

Item	Adjusted incidence[1]			Odds ratio (95% CI)		*P*-value
	TLD	FLF	NEG	TLD	FLF	
Nasal discharge	40.0^{AB}	21.2^{B}	52.9^{A}	0.59 (0.23, 1.56)	0.24 (0.08, 0.71)	0.04
Eye discharge	11.4	18.2	17.1	0.62 (0.16, 2.50)	1.07 (0.30, 3.80)	0.71
Dropped ears	8.6	3.0	14.7	0.54 (0.12, 2.53)	0.18 (0.02, 1.69)	0.29
Fever at day five[2]	20.4^{B}	8.4^{B}	49.9^{A}	0.26 (0.08, 0.78)	0.09 (0.02, 0.39)	0.002
Treatment failure[3]	24.9^{AB}	16.9^{B}	46.5^{A}	0.38 (0.13, 1.10)	0.23 (0.07, 0.76)	0.04
Pneum. retreatment[4]	27.7^{B}	22.8^{B}	54.7^{A}	0.33 (0.11, 0.97)	0.25 (0.08, 0.77)	0.03
Otitis risk	41.7^{AB}	24.4^{B}	58.4^{A}	0.51 (0.17, 1.52)	0.23 (0.07, 0.76)	0.05
Any treatment[5]	56.3^{B}	53.8^{B}	90.6^{A}	0.13 (0.03, 0.57)	0.12 (0.03, 0.54)	0.01

[1]Adjusted incidence based on LSM multiplied by 100. TLD = single subcutaneous injection of tildipirosin (4 mg/kg; Zuprevo®, Merck Animal Health, Millsboro, DE). FLF = single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine (Resflor-Gold®, Merck Animal Health, Millsboro, DE). NEG = calves did not receive any antimicrobial injection up to the fifth day after diagnosis.

[2]Rectal temperature ≥39.5 ºC (>103 ºF) at day 5 after enrollment.

[3]Need for pneumonia treatment from day 5 to day 10 of the study.

[4]Need for pneumonia treatment from day 5 of the study to weaning, approximately 65 days of calf life.

[5]Need for antimicrobial use to treat pneumonia or otitis from day 5 of the study to weaning.

*Different uppercase letters indicate significant differences between treatments ($P < 0.05$).

A treatment effect ($P = 0.002$) was also found on risk of fever at day 5. Calves in the NEG group had a higher risk of fever at day 5 (49.9%) than calves in the FLF (8.4%) and TLD (20.4%) groups, and no statistical difference was found between antibiotic-treated groups according to differences in LSM ($P = 0.18$).

Treatment failure was defined as the need of antimicrobial use for treatment of pneumonia signs according to farm personnel observations performed from day 5 to day 10 of the study. Calves in the NEG group had a higher risk of treatment failure (46.5%) than calves in the FLF group (16.9%; $P = 0.04$). No statistical difference was observed when comparing FLF to TLD (24.9%; $P = 0.42$), although calves in the TLD group tended ($P = 0.07$) to have a lower risk of treatment failure than calves in the NEG group (Table 2).

Pneumonia retreatment was defined as the need of antimicrobial use for treatment of pneumonia from day 5 of the study to the day that calves completed weaning and were moved to the other facility. The NEG group was considered an experimental group in this analysis; therefore, it was considered in this evaluation even though NEG group calves did not receive antibiotic intervention at diagnosis. Calves in the NEG group had a higher risk of treatment (54.7%; $P = 0.03$) from day 5 to the completed weaning day than calves in the FLF (22.8%) and TLD (27.7%) groups. According to the differences in LSM, no statistical difference was found between antibiotic-treated groups ($P = 0.18$).

The logistic regression analysis also showed an association ($P = 0.05$) between treatment and risk of otitis in calves from day 5 of the study to weaning. In addition to the treatment effect, the final model included the effects of age at pneumonia diagnosis ($P = 0.01$) and need for assisted calving ($P = 0.09$). The risk of otitis was higher in the NEG group (58.4%) compared to FLF (24.4%), whereas there was no significant difference observed when comparing the TLD group

(41.7%) with the FLF group or the NEG group. In addition, calves in the NEG group (90.6%) had a higher risk of treatment of pneumonia and/or otitis clinical signs from day 5 of the study to weaning than calves in the FLF (53.8%) and TLD (56.3%) groups ($P = 0.01$), and no difference was detected between antimicrobial-treated calves (Table 2).

In addition to the dichotomous variables mentioned above, calf performance was assessed based on average daily gain (ADG) considering two body weight measurements: one at birth and the second at approximately 60 days of age. Four of the 135 calves enrolled in the study (i.e., including controls) did not have the ADG estimated because the farm workers did not weigh them on the day of birth. Of these, two were from the NEG group, one from TLD group and one from the CTR group. Calves diagnosed with pneumonia had significantly lower ADG ($P = 0.04$) than calves in the CTR group (0.81 kg/d), and no differences were observed among calves in the TLD (0.72 kg/d), FLF (0.72 kg/d) and NEG (0.72 kg/d) groups.

Blood parameters

Serum concentrations of alanine aminotransferase, aspartate aminotransferase, urea, calcium, cholesterol, and BHB were not affected by treatment (Table 3, Figures 2 and 3). However, calves in the NEG group had the highest concentrations of ALP ($P < 0.0001$), total protein ($P = 0.01$) and haptoglobin ($P = 0.002$) among the treatment groups, and no differences were observed between calves in the CTR, TLD, and FLF groups for those blood parameters. In addition, healthy calves (i.e., CTR group) had higher serum concentrations of glucose ($P = 0.003$) and lactate ($P = 0.0009$) than calves diagnosed with pneumonia and enrolled in the NEG, TLD, and FLF groups (Table 3). The interaction effects between treatment and time points after enrollment are presented in Figures 2 and 3.

Table 3. Treatment effects on the serum biochemical concentrations of 135 calves enrolled in the study.

Item[2]	Treatment group[1]				*P*-value
	CTR	TLD	FLF	NEG	
ALT, U/L	7.85 ± 0.44	8.18 ± 0.43	8.59 ± 0.42	7.19 ± 0.42	0.12
ALP, U/L	210.13 ± 7.17^{A}	194.42 ± 6.79^{A}	196.79 ± 7.01^{A}	163.18 ± 6.85^{B}	< 0.0001
AST, U/L	38.85 ± 1.54	42.38 ± 1.65	41.43 ± 1.77	41.04 ± 1.67	0.43
Urea, mg/dL	26.62 ± 0.78	26.63 ± 0.69	25.78 ± 0.69	27.64 ± 0.67	0.27
Calcium, mg/dL	10.70 ± 0.10	10.67 ± 0.08	10.60 ± 0.08	10.58 ± 0.08	0.70
Cholesterol, mg/dL	78.31 ± 3.08	77.25 ± 2.94	73.87 ± 2.95	82.13 ± 2.94	0.26
Glucose, mg/dL	116.76 ± 1.92^{A}	111.97 ± 1.74^{B}	111.04 ± 1.72^{B}	107.80 ± 1.66^{B}	0.003
BHB, mmol/L	0.091 ± 0.003	0.089 ± 0.003	0.093 ± 0.003	0.089 ± 0.003	0.83
TP, mg/dL	6.21 ± 0.08^{B}	6.28 ± 0.07^{B}	6.16 ± 0.07^{B}	6.49 ± 0.07^{A}	0.01
Lactate, mg/dL	21.54 ± 0.60^{A}	19.20 ± 0.60^{B}	19.21 ± 0.59^{B}	18.26 ± 0.58^{B}	0.0009
Haptoglobin, OD_{450}[3]	0.027 ± 0.002^{B}	0.030 ± 0.002^{B}	0.031 ± 0.002^{B}	0.039 ± 0.002^{A}	0.002

[1]FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 36): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis; CTR (n = 31): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases.

[2]ALT = alanine aminotransferase. ALP = alkaline phosphatase. AST = aspartate aminotransferase. BHB = beta-hydroxybutyrate. TP = total protein.

[3]Haptoglobin was Log_{10} back-transformed to normalize the data distribution.

*Variables are presented as least square means ± standard error of the mean. Different uppercase letters indicate significant differences between treatments ($P < 0.05$).

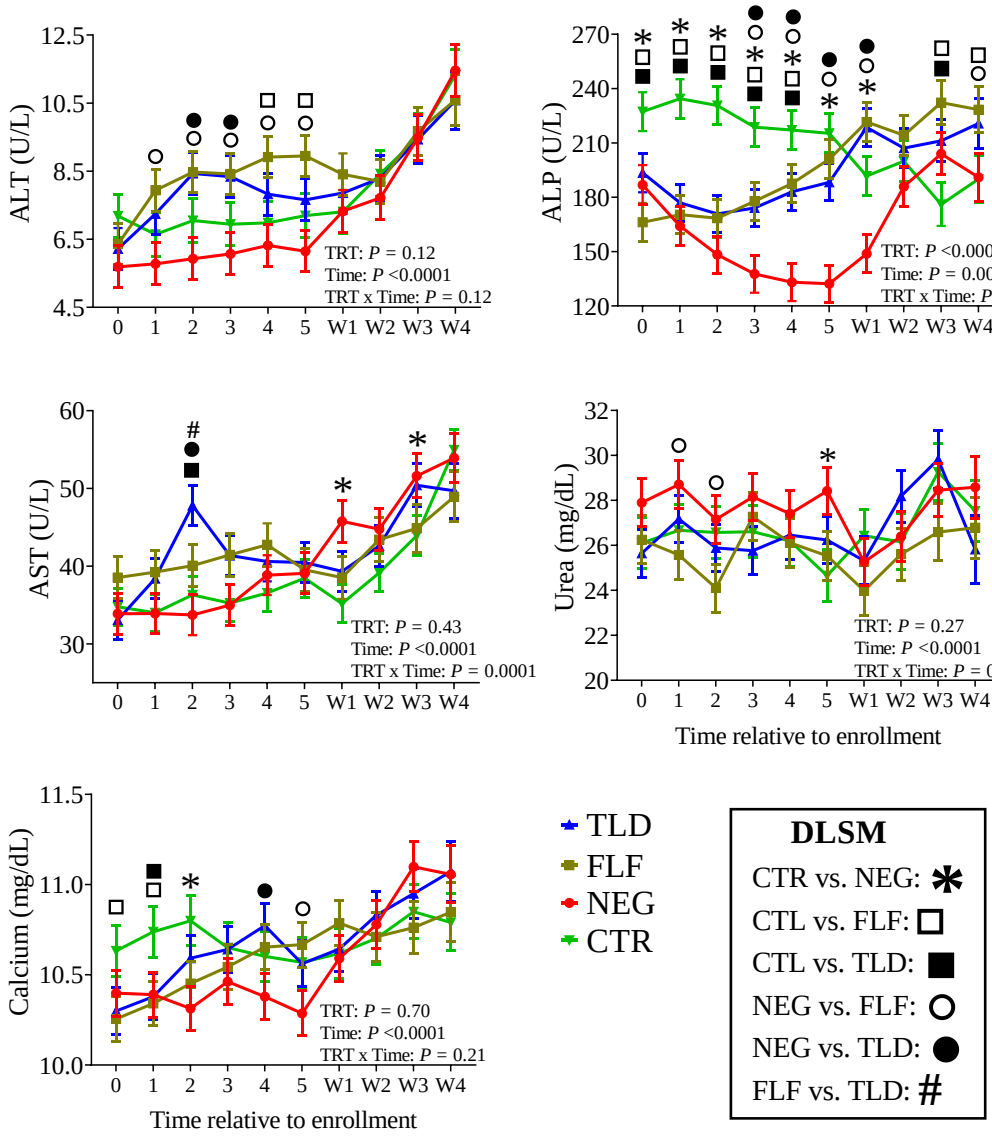

Figure 2. Serum concentrations of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), urea and calcium of 135 calves submitted to blood sample collection from the day of pneumonia diagnosis to the fourth week (W) after enrollment. FLF (n = 33) single subcutaneous injection of 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 36): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. CTR (n = 31): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. DLSM = differences of least square means (LSM) defined as P <0.05. Of the calves in the NEG group, one died on day 4, and 27 (79.4%) required antimicrobial therapy after day 5 of the study. Results are presented as LSM ± SEM.

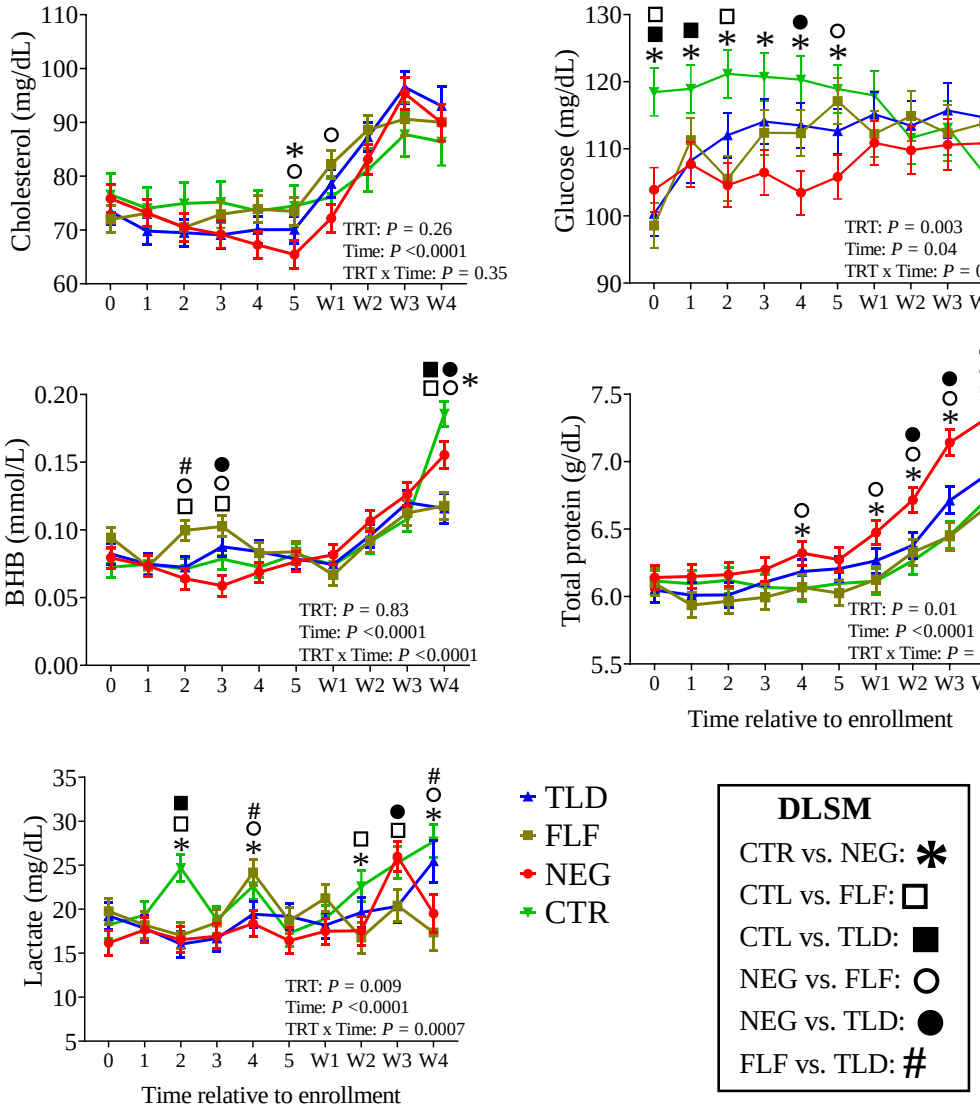

Figure 3. Serum concentrations of cholesterol, glucose, beta- hydroxybutyrate (BHB), total protein, and lactate of 135 calves submitted to blood sample collection from the day of pneumonia diagnosis to the fourth week (W) after enrollment. FLF (n = 33) single subcutaneous injection of 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 36): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. CTR (n = 31): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. DLSM = differences of least square means (LSM) defined as P <0.05. Of calves in the NEG group, one died on day 4, and 27 (79.4%) required antimicrobial therapy after day 5 of the study. Results are presented as LSM ± SEM.

A significant interaction effect between treatment and time of blood sample collection was observed for serum haptoglobin. Calves in the TLD and FLF groups reached the same haptoglobin level of calves in the CTR group on the second day after enrollment, whereas calves in the NEG group remained with higher levels of haptoglobin than CTR calves at least until the fifth day after BRD diagnosis (Figure 4).

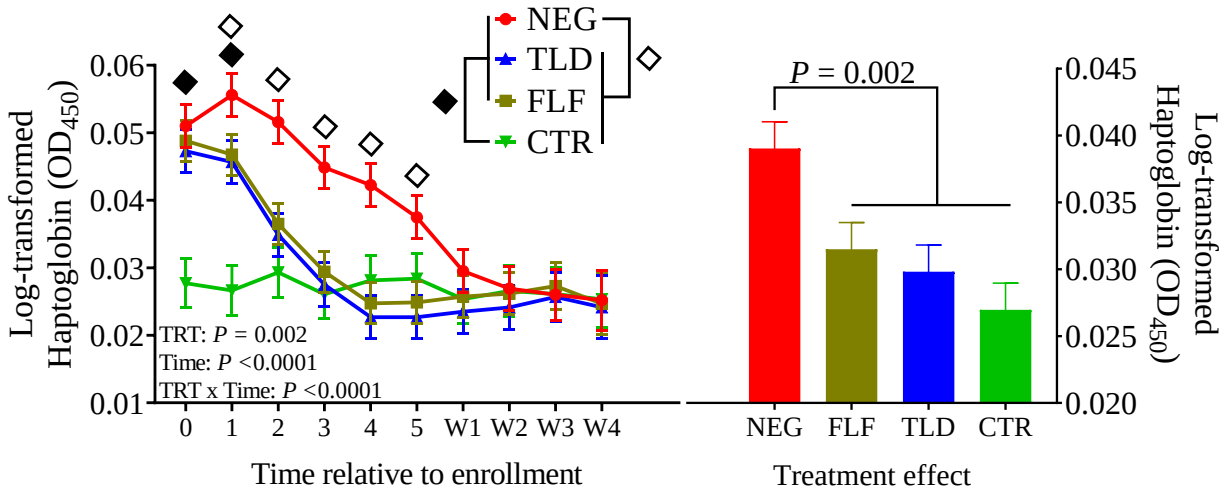

Figure 4. Serum concentrations of haptoglobin of 135 calves submitted to blood sample collection from the day of pneumonia diagnosis (day 0) to the fourth week (W) after enrollment. FLF (n = 33) single subcutaneous injection of 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 36): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. CTR (n = 31): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. Of calves in the NEG group, one died on day 4, and 27 (79.4%) required antimicrobial therapy after day 5 of the study. Results are presented as LSM ± SEM.

We compared the blood parameters between calves in the NEG group that were treated after day 5 (n = 27) to those that recovered from pneumonia clinical signs without treatment (n = 7). Treatment effects were observed for AST (P = 0.008), in which NEG untreated calves had lower AST (34.9 U/L) than NEG treated calves (44.6 U/L), mainly because of differences from day 4 to week 4 of the study. On the other hand, NEG untreated calves had a higher average level of glucose (112 mg/dL) than NEG treated calves (101.1 mg/dL). According to the assessment over time, calves in the NEG group that did not require treatment had higher serum glucose on days 0, 1, and 4 of the study than those that required treatment after day 5.

Lung consolidation

Calves diagnosed with pneumonia underwent a thoracic ultrasonography on the weaning week to evaluate the presence of lung consolidation. Thoracic ultrasound was carried out in 100 of 104 calves diagnosed with pneumonia. Four calves (FLF = 1, TLD = 2, NEG = 1) did not have results of thoracic ultrasound because of missing data (n = 2) or death before weaning (n = 2). Overall, lung consolidation was observed in 18 calves (18.0%); 3 in the FLF group (9.4%), 8 in the TLD group (23.5%), and 7 in the NEG group (20.6%). The logistic regression model showed no significant effect of treatment on lung consolidation (P = 0.32).

Upper respiratory tract microbiota

In total, 1,330 nasopharyngeal swabs collected from 133 calves were assessed for composition of the URT microbiota using next-generation sequencing of the 16S rRNA gene region. Of these calves, 30 belonged to the CTR group, 33 to FLF, 35 to TLD, and 35 to NEG. Evaluations were carried out at the phylum and genus levels. With regard to the sequencing results, 1,117 samples

were sequenced in 5 runs, quality filtered reads were de-multiplexed, and the total number of reads was 36,587,510. The average coverage was 3,698.68 and the standard deviation was 7,529.88 reads per sample.

In total, 28 phyla were identified from the sampled calf population. The most prevalent phyla regardless of treatment group was Tenericutes, followed by Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Fusobacteria, respectively (Figure 5).

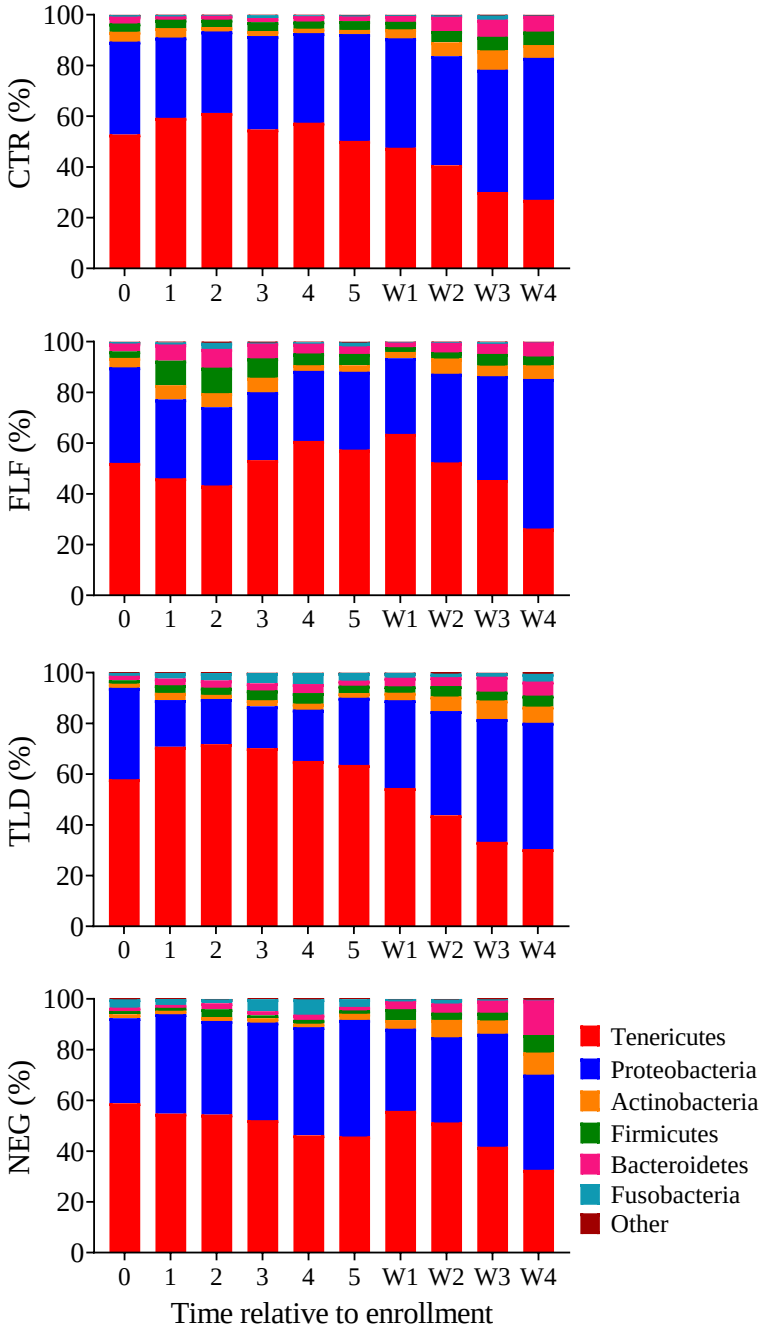

Figure 5. Mean relative abundance (%) of the 6 most prevalent bacterial phyla identified in upper respiratory tract samples of untreated healthy calves (CTR; n = 30), calves treated for pneumonia with florfenicol + flunixin meglumine (FLF; n= 33) or tildipirosin (TLD; n=35), and pneumonic untreated calves (NEG; n = 35). The x-axis presents the time relative to enrollment; after day 5, calves in each group had samples collected on a weekly (W) basis.

The MRA values of the 6 most prevalent phyla were compared among treatments using the nasopharyngeal swabs collected daily from diagnosis up to day 5. The other time points (i.e., after day 5) were not assessed because a high proportion of calves were treated for either pneumonia or otitis after day 5. Treatment effects were observed in all 6 phyla (Table 4). The TLD group had the highest MRA of Tenericutes and no treatment differences were observed between calves in the FLF, CTR and NEG groups. Compared to the NEG group, calves in the TLD group had a higher MRA from day 1 to day 5, whereas compared to the healthy untreated calves, differences in the LSM were observed only on day 3 and day 5. Differences in the Tenericutes MRA between the TLD and FLF groups were observed from day 1 to day 3 (Figure 6).

Table 4. Effect of treatments on mean relative abundance (%) of the 6 most prevalent bacterial phyla identified in the upper respiratory tract of 133 calves during the first 5 days after enrollment.

	Treatment group[1]			
Genus	CTR (LSM ± SEM)	FLF (LSM ± SEM)	TLD (LSM ± SEM)	NEG (LSM ± SEM)
Tenericutes	58.3 (3.2)^{B}	52.9 (2.8)^{B}	68.2 (2.8)^{A}	53.0 (2.7)^{B}
Proteobacteria	33.0 (2.9)^{AB}	30.0 (2.5)^{B}	21.5 (2.5)^{C}	37.9 (2.5)^{A}
Actinobacteria	1.9 (0.3)^{B}	3.5 (0.3)^{A}	1.9 (0.3)^{B}	1.7 (0.3)^{B}
Firmicutes	3.3 (0.6)^{B}	6.4 (0.6)^{A}	3.0 (0.6)^{B}	1.6 (0.6)^{C}
Bacteroidetes	1.8 (0.4)^{B}	4.7 (0.4)^{A}	2.6 (0.4)^{B}	1.6 (0.4)^{B}
Fusobacteria	0.8 (0.6)^{B}	0.8 (0.6)^{B}	2.3 (0.5)^{A}	3.1 (0.6)^{A}

[1]CTR (n = 30): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 35): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis.

*Variables are presented as least square means ± standard error of the mean. Different uppercase letters indicate significant differences ($P < 0.05$) between treatments based on least square means (LSM).

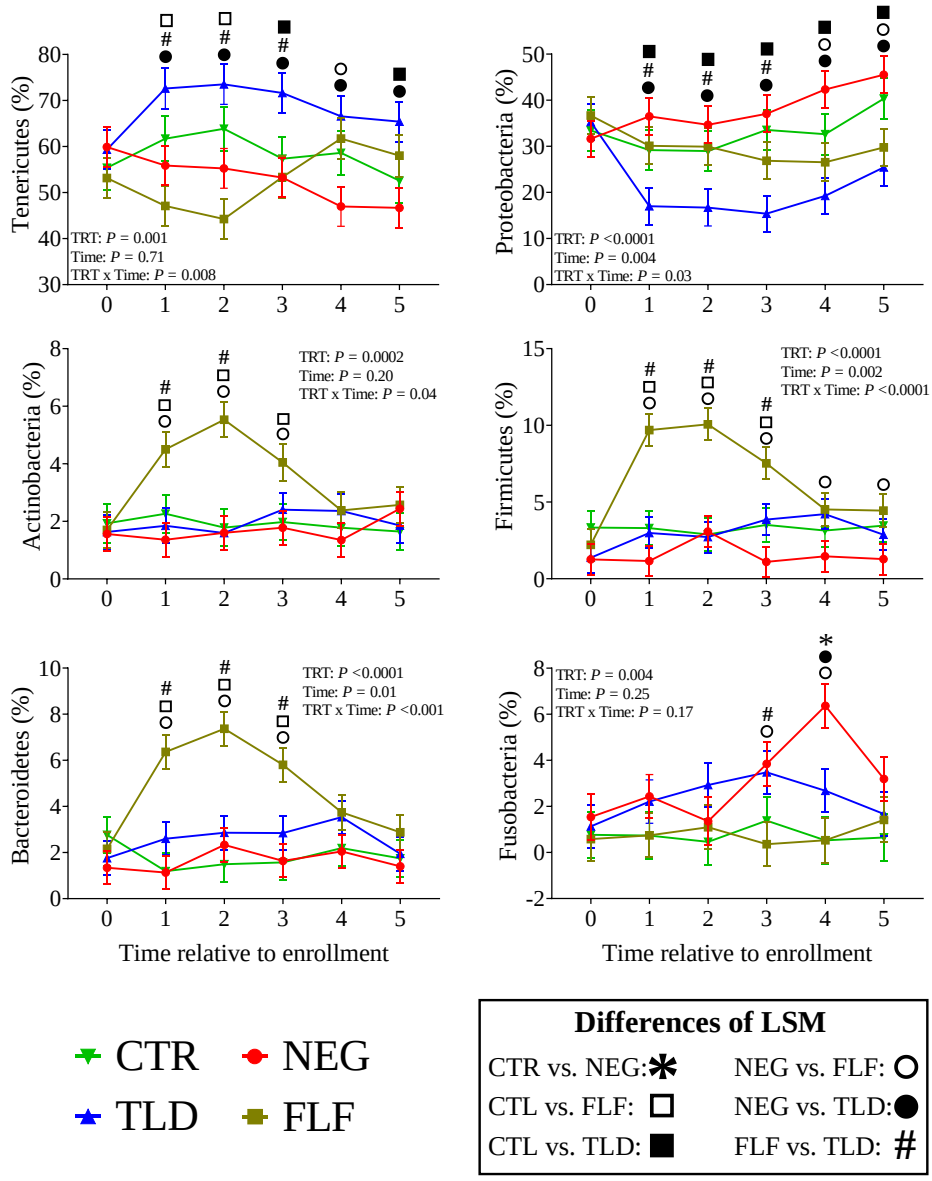

Figure 6. Mean relative abundance (%) of the 6 most prevalent bacterial phyla identified in the upper respiratory tract over the first 5 days sampled from 133 calves enrolled in the study. CTR (n = 30): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 35): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. Results are presented as least square means (LSM) ± SEM. Symbols above the timepoints represent differences ($P \leq 0.05$) between treatments based on the Tukey's significance test for multiple comparisons.

For the Proteobacteria phylum, the TLD group had the lowest MRA followed by the FLF group. Both antibiotic groups had significantly lower Proteobacteria MRA compared to the NEG group. Surprisingly, the MRA of the Proteobacteria phylum in the CTR group did not differ from that of the NEG group, and no significant difference was observed between the CTR and FLF groups (Table 4). Calves in the TLD group had lower Proteobacteria MRA than calves in the CTR and NEG groups from day 1 to day 5, whereas when compared to the FLF group, TLD calves had lower MRA on days 1 to 3 (Figure 6).

For the evaluations performed for the Actinobacteria, Firmicutes and Bacteroidetes phyla, the FLF group had the highest MRA among groups, which was mostly due to higher abundances from days 1 to 3. Finally, calves in the TLD and NEG groups had higher MRA of the Fusobacteria phylum than calves in the CTR and FLF groups. Differences in the Fusobacteria MRA was mainly due to differences in the LSM found on days 3 and 4 (Table 4; Figure 6).

In total, 899 genera were identified from the nasopharyngeal swabs collected from enrollment to weaning, and the 12 most prevalent genera are shown in Figure 7.

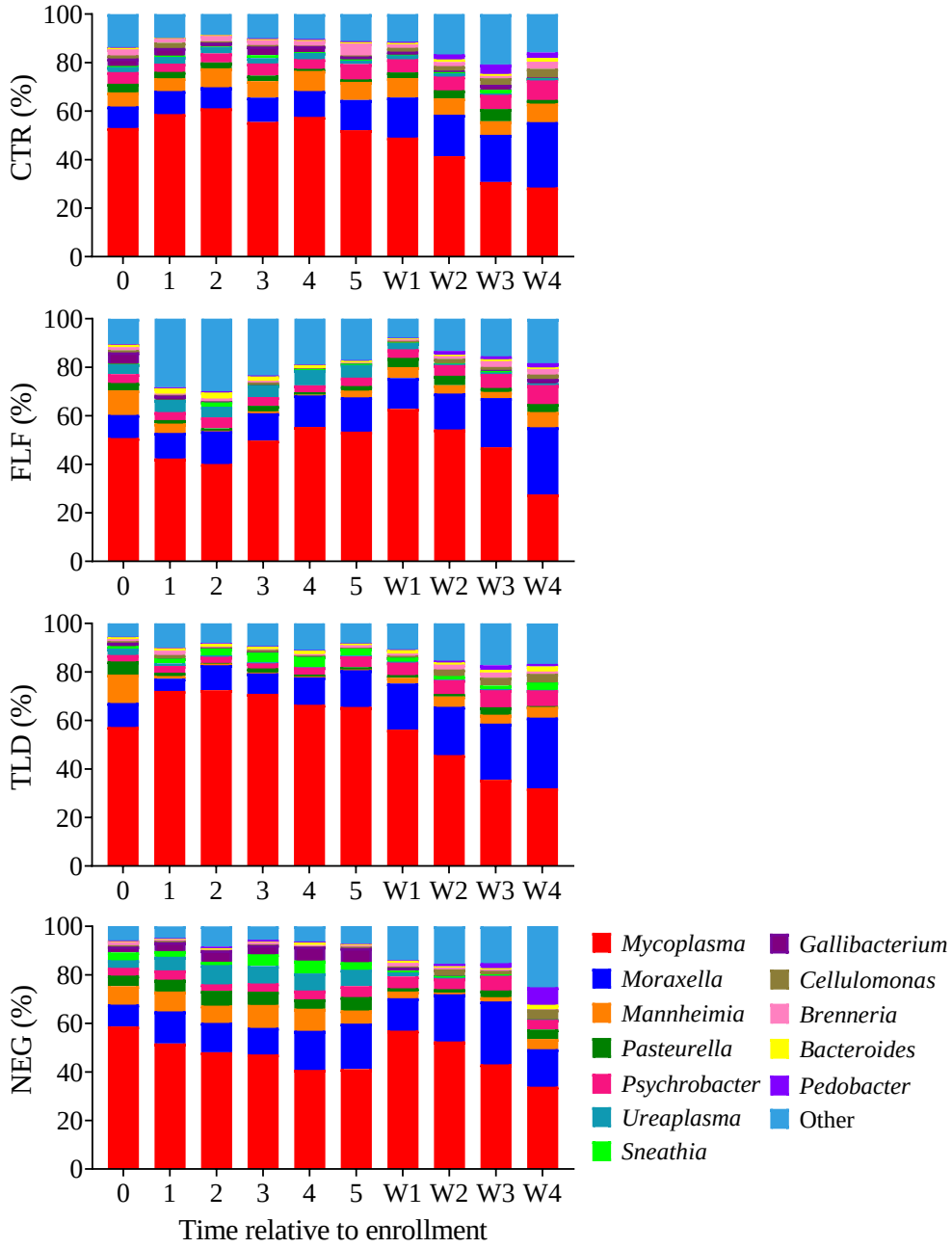

Figure 7. Mean relative abundance (%) of the most prevalent bacterial genera identified in upper respiratory tract samples of untreated healthy calves (CTR; n = 30), calves treated for pneumonia with florfenicol + flunixin meglumine (FLF; n= 33) or tildipirosin (TLD; n=35), and pneumonic untreated calves (NEG; n = 35). The x-axis presents the time relative to enrollment; after day 5, samples collected were on a weekly (W) basis.

The MRA of the 12 most prevalent genera was compared between treatments using the nasopharyngeal swabs collected daily from diagnosis up to day 5. Differences between treatments were observed for 8 genera (*Mycoplasma*, *Mannheimia*, *Pasteurella*, *Ureaplasma*, *Sneathia*, *Gallibacterium*, *Brenneria* and *Bacteroides*; Table 5).

Table 5. Effect of treatments on mean relative abundance (%) of the 12 most prevalent bacterial genera identified in the upper respiratory tract of 133 calves during the first 5 days after enrollment.

	Treatment group[1]			
Genus	CTR (LSM ± SEM)	FLF (LSM ± SEM)	TLD (LSM ± SEM)	NEG (LSM ± SEM)
Mycoplasma	56.8 (2.9)^{B}	48.1 (2.8)^{C}	68.0 (2.7)^{A}	48.3 (2.7)^{C}
Moraxella	13.8 (2.7)	15.6 (2.4)	13.9 (2.6)	17.0 (2.6)
Mannheimia	6.8 (1.3)^{A}	3.0 (1.3)^{B}	2.4 (1.2)^{B}	7.9 (1.2)^{A}
Pasteurella	2.1 (1.0)^{B}	1.7 (0.9)^{B}	1.8 (0.9)^{B}	5.1 (0.9)^{A}
Psychrobacter	4.0 (0.6)	3.3 (0.5)	2.6 (0.5)	3.4 (0.5)
Ureaplasma	2.3 (1.0)^{B}	4.9 (0.9)^{A}	0.9 (0.9)^{B}	6.2 (0.9)^{A}
Sneathia	0.7 (0.7)^{BC}	0.5 (0.6)^{C}	2.2 (0.6)^{AB}	3.5 (0.6)^{A}
Gallibacterium	2.5 (0.8)^{AB}	1.2 (0.8)^{B}	0.3 (0.8)^{B}	4.3 (0.8)^{A}
Cellulomonas	0.6 (0.2)	0.5 (0.2)	0.5 (0.2)	0.4 (0.2)
Brenneria	2.4 (0.4)^{A}	0.5 (0.4)^{B}	0.8 (0.3)^{B}	0.7 (0.3)^{B}
Bacteroides	0.01 (0.3)^{C}	1.3 (0.3)^{A}	0.6 (0.3)^{B}	0.1 (0.3)^{C}
Pedobacter	0.3 (0.1)	0.4 (0.1)	0.2 (0.1)	0.5 (0.1)

[1]CTR (n = 30): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 35): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis.

*Variables are presented as least square means ± standard error of the mean. Different uppercase letters indicate significant differences ($P < 0.05$) between treatments based on least square means (LSM).

Among the 12 most prevalent genera, *Mycoplasma*, *Moraxella*, *Mannheimia* and *Pasteurella* had the greatest abundancy and represented more than 65% of the URT microbiome of calves at enrollment, regardless of treatment group. For these genera, regression models were performed to evaluate the effects of treatment, time and the interaction between treatment and time (Figure 8). No statistical effects were observed for the genus *Moraxella*. For the genus *Mycoplasma*, the TLD group had a statistically higher MRA than the FLF and NEG groups from days 1 to 5. Compared to healthy calves, the TLD group had a higher *Mycoplasma* MRA on days 1, 3 and 4. When comparing FLF to CTR calves, FLF had a lower *Mycoplasma* MRA on days 1 and 2. Differences in the MRA of the *Mycoplasma* genus between the CTR and NEG groups were observed on days 2 and 4 (Figure 8).

For the genus *Mannheimia*, calves in the CTR and NEG groups had higher MRA than calves treated with antibiotics. No difference in the *Mannheimia* MRA was observed between calves in the TLD and FLF groups, nor between calves in the NEG and CTR groups, based on nasal swabs collected during the first 5 days after enrollment (Table 5; Figure 8).

A treatment effect was also observed for the genus *Pasteurella*. The NEG group had the highest *Pasteurella* MRA among the treatment groups, and no statistical differences were observed between calves in the CTR, TLD and FLF groups (Table 5; Figure 8).

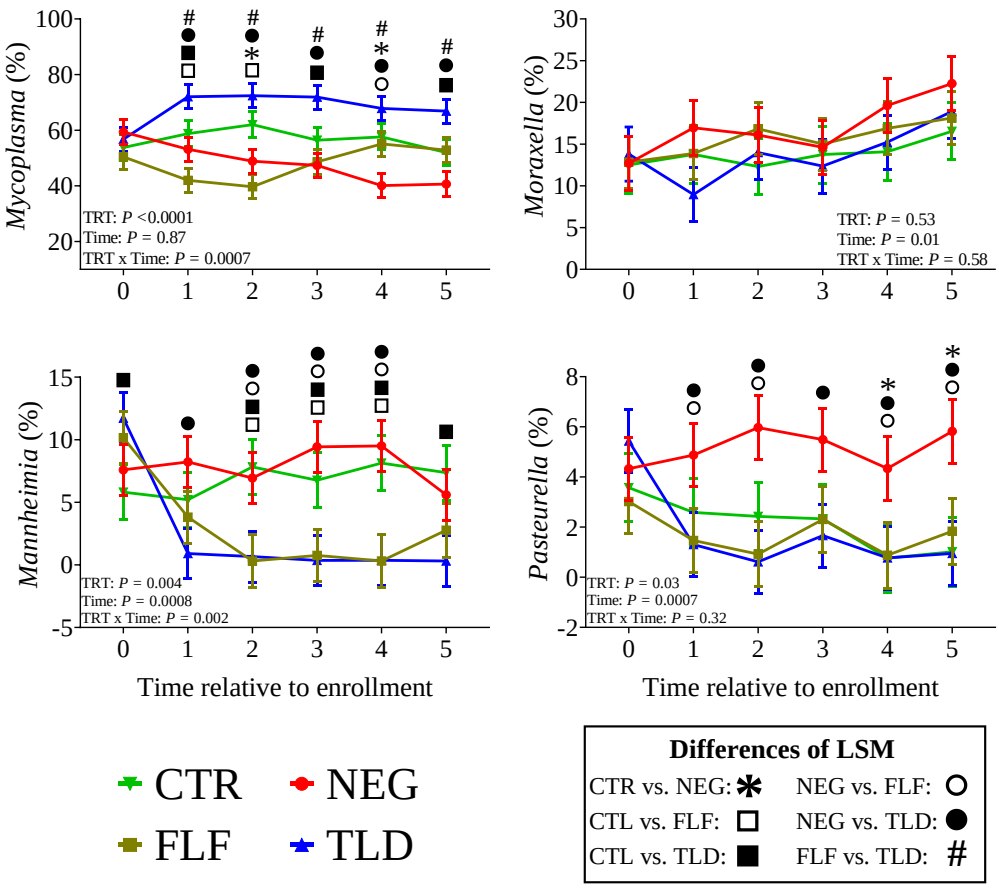

Figure 8. Mean relative abundance (%) of the 4 most prevalent bacterial genera identified in the upper respiratory tract over the first 5 days of 133 calves enrolled in the study. CTR (n = 30): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 35): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. Results are presented as least square means (LSM) ± SEM. Symbols above the timepoints represent differences ($P \leq 0.05$) between treatments based on the Tukey's significance test for multiple comparisons.

The 50 most prevalent bacterial genera found in the URT samples were analyzed using discriminant analyses based on treatment groups and time points of sample collection (Figure 9). No differences in the URT microbiota composition between groups were observed at day 0. However,

at 1 day and 3 days after the antimicrobial treatment administration, the microbial composition significantly differed in the FLF group compared to the other groups.

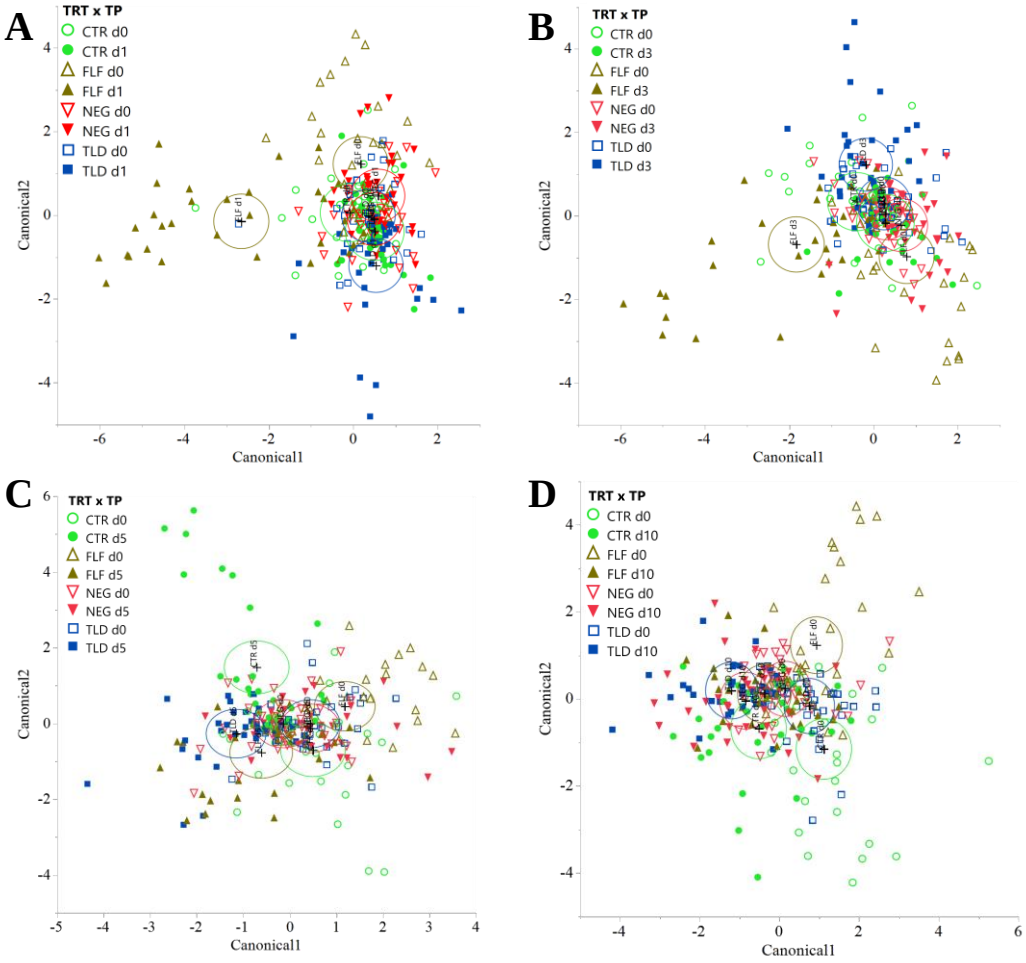

Figure 9. Discriminant analysis of the upper respiratory tract microbial composition in terms of the treatment group and the day relative to enrollment. CTR (n = 30): healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. FLF (n = 33): single subcutaneous injection of a product containing 40 mg/kg of florfenicol combined with 2.2 mg/kg of flunixin meglumine. TLD (n = 35): single subcutaneous injection of tildipirosin (4 mg/kg). NEG (n = 35): calves did not receive any antimicrobial injection up to the fifth day after diagnosis. Graphs A, B, C, and D illustrate the microbial composition at day 0 compared with days 1, 3, 5, and 10, respectively.

DISCUSSION

The use of antibiotics remains the most effective strategy for treatment of pneumonia. Efficacious therapeutics can increase cure rates while reducing the recurrence of infections, which, in turn, reduces antibiotic use by dairy operations. Previous studies by our research group compared the effect of tildipirosin and florfenicol + flunixin meglumine for treatment of calves with pneumonia and otitis and found interesting results in terms of health parameters and microbiome dynamics (Bringhenti et al., 2021b), as will be discussed below. To our knowledge, the present study is the first to report health and blood parameters of calves with pneumonia treated either with tildipirosin or florfenicol plus flunixin meglumine compared to a negative group. This is also the first field trial assessing the effect of commercially available antibiotics on the URT microbiota of calves to include a group of untreated but sick calves. Negative-controlled field trials can improve the understanding of BRD epidemiology as well as the physiological response of animals treated with anti-infective drugs. Similar to the results of Bringhenti et al. (2021b), the present study showed clear benefits of timely diagnosis and treatment of pneumonia in dairy calves before weaning. When compared to the NEG group, animals receiving either of the antimicrobial treatments in our study presented faster reduction of body temperature, faster recovery of inflammatory indicators in serum (e.g., haptoglobin), reduced risk of treatment failure or need for re-treatment against pneumonia, and lower risk of otitis media during the preweaning period. In addition, calves treated with antimicrobials at pneumonia diagnosis had reduced relative abundance of bacteria associated with severe clinical signs of pneumonia in the URT compared to the NEG group.

Our results demonstrate that both antimicrobials evaluated in our study were effective in reducing the body temperature of calves identified with fever at the day of pneumonia diagnosis. The FLF group reached the temperature observed in the CTR group (i.e., healthy animals) on the

first day after drug administration, while calves in the TLD group took one extra day to reach the same body temperature as the CTR calves. On the other hand, calves in the NEG group required 10 days after enrollment to reach the same body temperature as the other groups. Furthermore, based on the logistic regression analysis performed at day 5 of the study, the risk of fever was 83.2% and 59.1% lower in the FLF and TLD groups, respectively, compared to the NEG group. These results demonstrate the importance of antimicrobials to reduce the systemic inflammatory signs of pneumonia, an improvement probably associated with the reduction of bacterial load in the respiratory tract of the calves.

Our study also showed a faster reduction of body temperature in the FLF group compared to the TLD group, which is likely due to the presence of flunixin meglumine in the former. Nonsteroidal anti-inflammatory drugs **(NSAID)** such as flunixin-meglumine inhibit cyclooxygenase activity and block prostaglandin synthesis, which can partially mediate inflammatory signs such as fever (Morteau, 2000). A study performed with different breeds of beef and dairy calves compared the effect of florfenicol with florfenicol plus flunixin meglumine on body temperature of calves with pneumonia. Although a drop in the temperature was observed in both groups, a faster reduction in the florfenicol + flunixin formulation was reported from pretreatment to six hours post-treatment (Thiry et al., 2014). Furthermore, similar results as found in our study were reported in a previous study in which both therapeutic protocols (i.e., FLF and TLD) were effective in reducing rectal temperature; however, statistically lower values were observed for FLF in comparison to TLD at 1, 2, and 3 days after infection treatment (Bringhenti et al., 2021b). The latter study included both calves with pneumonia and calves with otitis media. In addition, the presence of fever (i.e., body temperature ≥39.5 ^{o}F) was not a criterion for animal selection in that study. Herein, only calves with pneumonia associated with fever and without a previous history of antimicrobial treatment were

selected, which allowed us to perform a more precise assessment of treatment effects on body temperature of diseased calves.

Calves treated with FLF also had a lower risk of nasal discharge within the first 10 days after diagnosis than calves in the NEG group. Again, this result may be due to the reduced inflammatory response associated with the flunixin meglumine. A previous study evaluating the efficacy of diclofenac sodium or flunixin meglumine in association with antibiotics for treatment of respiratory disease of Holstein calves also reported improvement of clinical signs in the anti-inflammatory groups based on a clinical index score that included nasal discharge (Guzel et al., 2010). Excessive or abnormal discharge is usually an indication of URT disease that typically appears approximately 24 hours after fever appearance (Timsit et al., 2011). A discharge may change in color and consistency over time depending on the progression of the underlying disease. Unilateral discharge usually indicates local involvement of one nasal passage. Bilateral discharge may indicate systemic disease affecting the URT (Radostits et al., 2006). In our study, although a statistical effect was observed between the FLF and NEG groups, no difference in the risk of nasal discharge was observed between FLF and TLD. The TLD formulation does not have an anti-inflammatory compound, so the lack of difference between the two drugs may be associated with an indirect effect of tildipirosin on inflammation due to reduction of pathogenic organisms. However, that result must be interpreted with caution as no statistical difference on the risk of nasal discharge was observed between the TLD and NEG groups.

Antimicrobial treatment also had a beneficial effect on blood parameters of calves with pneumonia. Serum haptoglobin was significantly higher in the NEG group compared to the CTR, TLD, and FLF groups. Haptoglobin is an acute phase protein synthesized in hepatocytes of mammals, which has been used as a sensitive and nonspecific biomarker of infection and

inflammation (Tothova et al., 2014). Joshi et al. (2018) reported a rise of 14-fold in the serum concentration of haptoglobin in calves suffering from BRD which was associated with severe tissue injury caused by inflammation. Those authors also reported haptoglobin to be a sensitive indicator of treatment effectiveness (Joshi et al., 2018). Reducing the duration of the acute inflammatory response in cases of BRD treated with antibiotic in a timely fashion can improve cattle welfare and recovery from this disease.

Using the serum haptoglobin level in the CTR group as a baseline to compare differences between treatments over time revealed the benefits of antibiotics for calves with pneumonia. Our results showed that antibiotic-treated calves reached normal levels of serum haptoglobin on the second day after treatment, while calves in the NEG group remained with significantly higher serum haptoglobin until the first week after enrollment (i.e., approximately at day 10 of the study). The marked reduction of haptoglobin in the NEG group beginning after day 5 was probably associated with the high number of calves treated with antibiotics at that point due to continued clinical signs. Our data showed that 24 of 34 calves (70.6%) in the NEG group had to be treated for pneumonia and/or otitis within this time frame. It is important to acknowledge that secondary treatments for pneumonia in our study, as well as the identification and treatment of otitis media, were performed by the farm personnel and were not controlled by the researchers, which can be considered as a limitation of our study.

In addition to haptoglobin, other blood parameters were affected by the experimental treatment in our study. Calves in the NEG group had the lowest ALP serum concentration compared to the other groups. Based on LSM differences over time, CTR calves had higher serum ALP than calves diagnosed with pneumonia from days 0 to 4 of the study. Using calves in the CTR group as a reference, the serum ALP level of antibiotic-treated calves began to recover on the third day after

treatment and had normalized at day 5 of the study. In contrast, calves in the NEG group took until the second week after enrollment to reach the same ALP level as the CTR group. Alkaline phosphatase is an enzyme that is present in many mammalian tissues, but it is primarily found in liver, bone, intestine, and kidney (Sharma et al., 2014). Little has been reported regarding the physiological function of ALP in inflammatory and infectious diseases in cattle. A study evaluating blood parameters in calves with acute bronchopneumonia also reported a significantly lower ALP concentration in the diseased group compared with healthy animals (Basoglu et al., 2016), which indicates a potential role of this enzyme during lung disease in cattle. Bringhenti et al. (2021a) reported higher serum ALP in calves that received a metaphylactic administration of tildipirosin at 7 ± 3 days of life compared to healthy animals, which may suggest an effect of the antibiotic in the calf liver. Based on our results, serum ALP warrants further investigation as a blood indicator of BRD in dairy calves, although this was not within the scope of our study.

With respect to the glucose evaluation in our study, calves that had pneumonia had a lower serum glucose concentration compared to healthy animals. Low glucose levels were also observed in an earlier study of heifers treated for BRD (Montgomery et al., 2009). Moreover, hypoglycemia was found to be related to neonatal calf diarrhea and endotoxemia (Trefz et al., 2016). In cases of sepsis, the hypoglycemia can be attributed to increased peripheral glucose utilization, depletion of hepatic glycogen stores, and inhibition of hepatic glucose production (Lang et al., 1993, Maitra et al., 2000). In addition to the direct effect of inflammation on calf glycemia, reduction of feed consumption may be another factor contributing to lower blood glucose in diseased calves as compared to the CTR calves, although feed intake was not controlled in our study. Support for this speculation can be found by comparing the treatment groups over time: antibiotic-treated calves returned to normal

blood glucose levels within the first three days after enrollment, while those in the NEG group did so only after the first week.

With regards to total protein, calves that did not receive antimicrobial treatment had higher levels of this parameter than the other groups, especially after the first week post treatment. This parameter is used as an indicator of passive immunity in calves within the first weeks after birth (MacFarlane et al., 2014, Hernandez et al., 2016). Elevated total protein can also be associated with a higher release of immunoglobulins, acute phase proteins, and amino acids from muscle into the blood of animals not treated at diagnosis (Chorfi et al., 2004, Tsukano et al., 2015), which may be the case here. As inflammation may be one of several factors affecting total protein, it is reasonable to state that animals not treated with antimicrobials will have a higher concentration of total protein in the blood, although this can also be a consequence of dehydration.

Calves treated with either TLD or FLF had, respectively, 49.4% and 58.3% lower risk of pneumonia retreatment than calves that did not receive antimicrobial treatment within the first 5 d after diagnosis. In addition, compared to the NEG group, the risk of antimicrobial use to treat pneumonia or otitis during the same timeframe was 37.9% lower in the TLD group and 40.6% lower in the FLF group. These results reinforce the importance of early diagnosis and treatment of calves with pneumonia. In addition to the reduced use of antimicrobials, prompt treatment can lessen the severity and duration of clinical signs, which may also be viewed as a way to improve cattle welfare.

Calves diagnosed with pneumonia in our study gained 90 grams/day less weight than healthy calves enrolled in the CTR group, regardless of treatment group. No differences in ADG were observed between treated and untreated calves identified with pneumonia. Similar results were found in the study by Bringhenti et al. (2021b), in which no differences in ADG were observed between preweaning calves treated for pneumonia and/or otitis with TLD or FLF, although healthy calves

gained more weight than diseased and treated calves. Other studies have reported the negative impact of pneumonia on development of calves (Pardon et al., 2013). These results highlight the importance of pneumonia prevention on growth and development of dairy calves during the preweaning period. It is important to state that the evaluation of ADG in our study was based only on two time points (at birth and at moving day to the heifer facility). However, an assessment over time during the preweaning period would provide more accurate results on the dynamics of ADG of treated and untreated calves. In addition, a postweaning ADG assessment would be needed to determine if there is any compensatory effect during the heifer stage of life.

Thoracic ultrasound has been used by veterinarians as a tool to diagnose calves with pneumonia (Ollivett and Buczinski, 2016). This technique allows for identification of lung consolidation, which is one of the consequences of pneumonia associated with a higher risk of euthanasia or death of calves (Rademacher et al., 2014). Furthermore, the presence of lung consolidation during the preweaning period of dairy calves has negative impacts on heifer reproductive performance, increasing the culling risk prior to first lactation (Teixeira et al., 2017b). In our study, although no statistical differences in lung lesions were observed between groups, a thought-provoking numerical difference was observed between calves in the FLF group compared to the other groups. Only 9.4% (3/29) of calves in the FLF group had lung consolidation, while 20.6% (7/27) of calves in the NEG group had lesions identified by thoracic ultrasound. Therefore, it is possible that we lacked statistical power to find differences between groups for this outcome. Although we can speculate that the calves in the FLF group benefited from the NSAID effect, further studies using a larger number of animals should be encouraged to assess the benefits of the FLF treatment on lung consolidation. Such a study should consider performing the technique over time after pneumonia diagnosis and screening the entire lung field on both the right and left sides of the

calf. In our study, we evaluated the calves once during the weaning week, and only the cranial aspect of the right cranial lung lobe was examined. Although this site is considered the most affected anatomical region in cases of bronchopneumonia (Ollivett and Buczinski, 2016), a proportion of lung lesions might have been missed in our study.

Our results showed that calves diagnosed with pneumonia and enrolled in the FLF group had a 58% reduction in the risk of subsequent otitis media treatment, but no difference was observed between calves in the TLD and NEG groups. The risk of otitis media was assessed in our study because this disease can develop as a consequence of respiratory infections (Linhart and Brumbaugh, 2019). Although otitis media can also result from migration of pathogens from the external ear (Oliver Jr et al., 1997), the most common route used by pathogens is through the Eustachian tubes, which provide a direct connection of infections of the nasopharynx to the middle ear (Murphy et al., 2009, Maunsell et al., 2012). Based on our results, it is difficult to say whether the reduced risk of otitis in the FLF group was because of flunixin meglumine, which potentially reduced the clinical signs of otitis media after pneumonia treatment, or because florfenicol was effective in preventing ear infection in calves treated for pneumonia. On the other hand, the TLD treatment did not prevent otitis when we used the NEG group as the reference, and this might be related to the pathogen causing the disease. *Mycoplasma* spp. are the main pathogens associated with otitis in calves up to 18 months of age (Walz et al., 1997) and was the most abundant genus identified from our samples regardless of treatment. Based on our results and previous study, tildipirosin was not effective in reducing the abundance of *Mycoplasma* in the URT, which might increase the risk of ear infection. Further studies evaluating the ear microbiota of calves are encouraged to better understand the effect of antimicrobials on the auditory tract.

The effect of treatments on the URT microbiota of calves was also evaluated in our study. Results from the 16S rRNA gene sequencing were presented at the phylum and genus levels. Although an assessment at the species level could have generated more accurate information about the microbiota dynamics of calves with pneumonia (i.e., treated vs. untreated vs. healthy calves), the next-generation sequencing technique used in our study has limitations for making inferences at the species level. Short segments of the 16S rRNA that include hypervariable regions are sequenced for bacterial classification in the technique we used, and only a minority (30% – 50%) of these sequences can be classified as operational taxonomic units **(OTUs)** beyond the genus level (Timsit et al., 2020).

To our knowledge, this is the first study comparing the URT microbiota between treated and untreated preweaning dairy calves diagnosed with pneumonia. Interestingly, no major differences were observed in the composition of the URT of healthy calves compared to calves in the NEG group based on analysis at the phylum level. However, some differences were observed between the CTR and NEG groups at the genus level (e.g., *Mycoplasma, Pasteurella, Ureaplasma, Sneathia, Brenneria*). These differences may be associated with a dysbiosis facilitated by the increase in abundance of some pathogenic species within specific genera in diseased calves, such as *Mycoplasma* and *Pasteurella*. This is speculative, and a more accurate technique (e.g., whole metagenome sequencing) would be necessary to precisely evaluate the microbiota at the species level; however, such evaluation was beyond of the scope of this study.

Although 28 different phyla were identified from the nasopharyngeal swabs collected in our study, Tenericutes and Proteobacteria represented more than 80% of the URT microbiota, regardless of treatment group. The six most abundant phyla found in our study were also reported by others, albeit with differences in abundances among the studies. For example, while some studies reported Proteobacteria as the most abundant phylum present in the microbiota of healthy and pneumonic

calves (Lima et al., 2016, Amat et al., 2019, Bringhenti et al., 2021b), others corroborate our results and found Tenericutes to be the most prevalent phylum in the respiratory tract of calves (Timsit et al., 2016, Stroebel et al., 2018, Timsit et al., 2018). Comparison between studies can be difficult because several factors can affect the composition and dynamics of the calf microbiota, including the anatomical site used for sample collection (McMullen et al., 2020), calf age, environment, diet, antimicrobials used for treatment or metaphylaxis, commingling, and transportation (Timsit et al., 2020).

Based on treatment effects in our study, the MRA of the phylum Tenericutes was significantly higher in calves that received tildipirosin compared to the other groups. However, when compared to the CTR group over time, differences in the Tenericutes MRA were observed only at two time points (day 3 and day 5). This increase of the Tenericutes MRA in the TLD group occurred mainly due to the increased abundance of *Mycoplasma*, which was the main genus of the Tenericutes phylum in our samples. Several *Mycoplasma* spp. have been identified from pneumonia in cattle; however, *Mycoplasma bovis* is the most reported species associated with BRD (Perez-Casal, 2020). After tildipirosin administration, we observed that *Mycoplasma* became relatively more abundant over the first 5 days of evaluation. Similar results were reported by our group in a previous study (Bringhenti et al., 2021b), which indicates a lack of efficacy of tildipirosin against *Mycoplasma* bacteria. As reported in the latter study, the increased abundance of *Mycoplasma* after tildipirosin injection may be due to reduced microbial competition in the URT as a consequence of reduction of other genera such as *Mannheimia* and *Pasteurella*. It is also important to mention that the commercial product containing tildipirosin is not labeled for use against *Mycoplasma* spp.

Our results showed higher MRA of the phyla Actinobacteria, Firmicutes and Bacteroidetes in the FLF group compared to the other groups from day 1 to day 3 of the study. This result was

unexpected and, to our knowledge, has not been reported previously. Those phyla are composed of bacteria which are not recognized as causes of BRD in cattle. Suggestively, this result may be associated with the greatest reduction of Tenericutes abundance in the FLF group after treatment compared to the other groups in the first 2 days, which increased the relative abundance of commensal bacteria composing those phyla.

In our study, both the TLD and FLF treatments were effective in reducing the MRA of *Mannheimia* and *Pasteurella*, which are important genera associated with pneumonia in cattle. *Mannheimia haemolytica* and *Pasteurella multocida* are the most recognized species in those genera associated with pneumonia clinical signs in beef and dairy calves (Snyder and Credille, 2020). It is also important to note that the MRA of *Mannheimia* in the CTR group did not differ from that of the diseased and untreated group during the first 5 days of the study. This result corroborates previous studies that reported these bacteria as ubiquitous inhabitants of the URT (Lima et al., 2016, Timsit et al., 2020). From another perspective, the abundance of *Mannheimia* in the respiratory tract could be increased in diseased animals before the occurrence of the clinical signs, as previously reported (Lima et al., 2016). In addition, although the *Mannheimia* MRA between healthy and diseased animals was similar at the genus level, differences in composition might have occurred at the species level; however, this was not assessed in our study.

CONCLUSIONS

Compared to the pneumonic and untreated group, calves injected with tildipirosin (TLD) or florfenicol + flunixin meglumine (FLF) had lower rectal temperature within the first 5 days after enrollment and lower risk of fever at day 5. Results on rectal temperature and serum haptoglobin levels showed that both antimicrobials were effective in reducing inflammatory signs of pneumonia, although calves in the FLF group reached the same temperature as healthy calves one day earlier than calves in the TLD group. In addition, antibiotic-treated calves had a lower risk of treatment against pneumonia and/or otitis media during the preweaning period compared to untreated calves. Calves injected with the FLF treatment had lower risks of nasal discharge, treatment failure, and otitis media compared to the NEG group, but no difference was observed when comparing the FLF and TLD groups. Finally, the genus *Mycoplasma* was the most abundant in samples collected from the URT of calves with and without pneumonia in our study. Differences in the composition of URT microbiota were found based on analyses performed at the phylum and genus levels. Both drugs were effective in reducing the abundance of important genera associated with pneumonia (*Mannheimia* and *Pasteurella*), although an increase in the MRA of *Mycoplasma* was observed in tildipirosin-treated calves. Future studies using a more accurate technique to assess the dynamics of the respiratory tract microbiota at the species level are encouraged to extend our understanding of the role of pathogenic and commensal microorganisms in the health of dairy calves.

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CHAPTER 3: Final considerations and future work

This thesis had the general objective of understanding the epidemiology, etiology, and pathogenicity of bovine respiratory disease (BRD) in cattle. More specifically, we aimed to describe the effect of two popular commercially available antibiotic interventions at the diagnosis of pneumonia on health and blood parameters, and the upper respiratory tract (URT) microbiota of preweaning dairy calves.

Based on the literature review in Chapter 1, pneumonia remains one of the most frequent causes of morbidity and mortality in dairy operations, causing substantial economic losses to the dairy industry. The multifactorial and polymicrobial nature of BRD makes it difficult to control and numerous risk factors can predispose calves to this disease. In addition to proper animal handling and management, new commercially available vaccines administered by different routes have shown efficacy for the prevention of BRD. However, when preventative measures fail, the use of antibiotics remains the most effective strategy to treat infectious pneumonia. Several antibiotics of different classes are available in the market to treat bovine pneumonia. Some of these antimicrobials have a single active ingredient in the composition (e.g., tildipirosin), while others can be combined with anti-inflammatory drugs (e.g., florfenicol + flunixin meglumine). These so called “new-generation antibiotics” have a good dispersion and long-lasting effect in the lungs, which increases the cure rates and reduces the need of several injections per treatment of pneumonia. Over the last decade, advances in diagnosis methods for pneumonia and other infectious diseases have been realized. Biomolecular methods such as next-generation sequencing are now used to assess the microbiome composition in cattle and other species, which have increased our understanding of infectious disease epidemiology, including BRD.

In Chapter 2, we conducted a randomized controlled field trial to investigate the effect of two commercially available antibiotics to treat BRD in dairy calves during the preweaning period. We assessed the effect of two drugs, tildipirosin and florfenicol + flunixin meglumine, on several health and blood parameters, and bacterial microbiota of calves presenting clinical signs of pneumonia. The novelty of this study compared to other work evaluating the same antibiotics for treatment of pneumonia was the comparison of treated calves with diseased untreated animals. Previous research only compared those parameters with healthy calves. This approach allowed us to better understand the actual benefit of antimicrobials on health, welfare, and URT microbiota of preweaning calves diagnosed with pneumonia.

Compared with untreated calves at the diagnosis of pneumonia, calves treated with tildipirosin (TLD) or florfenicol + flunixin meglumine (FLF) had a faster recovery from the inflammation caused by the infection, which was determined by differences in the rectal temperature and serum parameters such as haptoglobin, ALP, glucose, and total protein. In addition, antibiotic-treated calves had lower risks of fever at day 5 after enrollment, as well as lower treatment failure and need for antimicrobial retreatment. Furthermore, calves treated with FLF had a faster recovery from fever and lower frequency of nasal discharge than TLD-treated calves, which is probably related to the anti-inflammatory effect (i.e., flunixin meglumine) in the FLF treatment.

Our microbiome analysis found that the bacterial microbiota of the URT was affected by both the disease and antimicrobial treatment. Differences between experimental groups were observed across all of the most prevalent phyla in our study (tenericutes, proteobacteria, actinobacteria, firmicutes and bacteroidetes). At the genus level, *Mycoplasma*, *Moraxella*, *Mannheimia*, and *Pasteurella* were the most prevalent, representing more than 75% of the URT

bacterial microbiota in calves enrolled in this study, regardless of treatment. As observed in previous studies performed by our group, there was a greater relative abundance of the *Mycoplasma* genera in the TLD group in comparison to the other groups. In addition, both treatments were effective in reducing the abundance of other potentially pneumonia-causing genera (*Mannheimia* and *Pasteurella*).

By comparing calves treated with TLD and FLF with untreated calves, we confirmed that the timely diagnosis and use of effective antimicrobials can reduce the morbidity and the URT abundance of pathogenic bacteria associated pneumonia. Future research should focus on the impact of BRD occurring in the early stage of calves' life during their productive stage of life as a cow. A similar approach should be done to evaluate the effect of different therapeutic approaches, such as vaccines and antibiotics used during calfhood on the performance of cows in the future. In addition, studies evaluating the role and dynamics of commensal and less prevalent bacteria of the respiratory tract on prevention and development of BRD are encouraged.