Treatment with 2 commercial antibiotics reduced clinical and systemic signs of pneumonia and the abundance of pathogenic bacteria in the upper respiratory tract of preweaning dairy calves

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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 (BRD) accompanied by fever in calves before weaning compared with diseased and untreated animals. As specific objectives, we evaluated the composition of the bacterial microbiota of the upper respiratory tract (URT) and blood and health parameters of the animals. Preweaning Holstein female calves diagnosed with naturally acquired pneumonia were randomly assigned to one of the following experimental groups on the day of diagnosis (d 0): (1) TLD (n = 36): single subcutaneous injection with 4 mg/kg tildipirosin; (2) FLF (n = 33): single subcutaneous injection with an antimicrobial (40 mg/kg florfenicol) combined with a nonsteroidal anti-inflammatory drug (2.2 mg/kg flunixin meglumine); and (3) NEG (n = 35): no treatment within the first 5 d following enrollment. The NEG treatment group was closely monitored for 5 d, and calves were removed from the study following a standardized late treatment protocol, when necessary, to minimize health concerns. 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 d 0 until d 5 and then weekly until weaning. 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 d after enrollment. In addition, antibiotic-treated calves reached the same serum haptoglobin levels as healthy calves on d 2 after diagnosis, whereas calves in the NEG group had higher haptoglobin levels than the CTR group until at least d 5 after BRD diagnosis. Calves in the TLD and FLF groups had a lower risk of treatment for pneumonia (FLF = 22.8%; TLD = 27.7%) from d 5 to weaning than calves in the NEG group (54.7%). Furthermore, FLF treatment had a significantly lower risk of nasal discharge, otitis media, and treatment failure compared with the NEG group, but did not differ from the TLD group. 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, 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 with healthy and untreated calves.

Key words: bovine respiratory disease (BRD), pneumonia treatment, respiratory tract microbiota, tildipirosin, florfenicol + flunixin meglumine

INTRODUCTION

Bovine respiratory disease (BRD) is a disorder that 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 affects future animal performance (Dubrovsky et al., 2019a, 2020; Buczinski et al., 2021).

A nationwide study evaluated the morbidity and mortality associated with BRD based on the National Animal Health Monitoring System report published in 2014 (Urie et al., 2018). Of animals reported ill, 33.4% had respiratory signs. Furthermore, BRD was responsible for 14.1% of deaths, with a case fatality rate of 6.0%. A prospective study assessing data from dairies in California reported that approximately 22.8% of calves were diagnosed and treated for BRD before weaning, reflecting the high incidence of this disease in dairy operations (Dubrovsky et al., 2019b). 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 infectious diseases such as BRD (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.

The main visual clinical signs of pneumonia include fever, increased respiratory rate, nasal discharge, lacrimation, cough, prostration, dehydration, and anorexia (Ames, 1997; Love et al., 2014; McGuirk and Peek, 2014; Cummings et al., 2022). In addition, lung lesions can be seen on ultrasound. Recent studies have demonstrated that calves affected with pneumonia commonly have lung consolidations (Teixeira et al., 2017b; van Leenen et al., 2020). Furthermore, 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 (Wolfer et al., 2015; Moisá et al., 2019). The release of this inflammatory protein is triggered by proinflammatory cytokines (Higuchi et al., 1994); therefore, calves diagnosed with respiratory diseases have a higher concentration of serum haptoglobin compared with healthy calves (Wittum et al., 1996; Svensson et al., 2007). Additional serum parameters have been evaluated in other studies (Bringhenti et al., 2021a,b), providing valuable information on how animals are systemically affected and respond to the disease.

Another area of increasing scientific interest is the investigation of the composition and dynamics of the upper respiratory tract (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). The microbiome can play an important role in the health of mammals and can be affected by diseases and treatment interventions (Blaser, 2016; Holman et al., 2019). Studies evaluating the composition and dynamics of the microbiome in animals under different management and therapeutic regimens can help to understand the epidemiology of BRD and lead to more effective prevention and control strategies.

Timely identification and the use of efficacious antibiotics for the treatment of BRD at the onset of clinical signs can improve the cure rates and calf performance during the pre- and postweaning periods. According to a National Animal Health Monitoring System report (NAHMS, 2016), 94.8% of BRD cases are treated with antibiotics, and previous studies have assessed the effectiveness of several antimicrobials, including tilmicosin (Fodor et al., 1993), tulathromycin (Ragbetli et al., 2010), and gamithromycin (Lechtenberg et al., 2011). Despite the value of antibiotics for prevention and treatment of BRD, there is increased global concern about the risk of bacterial resistance to antibiotics used in human and veterinary medicine. Multidrug resistance has been reported for commensal and potentially pathogenic bacteria associated with BRD, including Mannheimia haemolytica and Pasteurella multocida (Booker and Lubbers, 2020). Furthermore, a proportion of animals are given antibiotics unnecessarily as infections caused by viruses alone do not benefit from treatment, which increases the overuse of antibiotics in livestock production. Studies evaluating the efficacy of antibiotics with the inclusion of a negative control group potentially allow for the assessment of natural physiological responses in BRD calves without the interference of antibiotics, promoting further insights into the disease pathophysiology.

Most studies conducted to date evaluating the effectiveness of antibiotics to treat BRD have not assessed the effect of treatments on the respiratory tract microbiota of the animals. A recent study carried out by our group in New York State showed differences in the microbiota composition of calves with pneumonia or otitis compared with healthy calves before weaning (Bringhenti et al., 2021b). That study reported differences in microbiota dynamics in diseased calves treated with tildipirosin or florfenicol combined with flunixin meglumine, and although the results are thought-provoking, the study lacked a negative control group to assess the antibiotic effects. Comparison of health parameters and the microbiota of treated and untreated calves with pneumonia can broaden our epidemiologically understanding of BRD and indicate the efficacy of
commercially available antibiotics used against this complex disease.

The primary 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 with untreated sick animals. As specific objectives, we evaluated the composition of the URT microbiota as well as blood and health parameters of the animals.

**MATERIALS 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 (Ithaca, NY; 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 d before the expected calving date, pregnant cows were moved to pre-maternity pens, where they were monitored 24 h/d. 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 navel disinfection (iodine 7%). 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 low temperatures. Within 3 h of birth, 4 L of heat-treated 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.

Twice daily, newborn calves were moved from the newborn pen to a greenhouse type of barn composed of 27 identical group pens (70 m²) bedded with straw and equipped with a positive pressure ventilation system. Twenty-five calves were placed in each pen; all calves remained in the same pen from d 1 of life until weaning, which occurred at approximately 65 d of life. At weaning, calves were weighed by farm employees using a portable scale (Waypig-15, Vittetoe Inc.) and moved to a different housing facility.

During the preweaning period, calves were fed, ad libitum, acidified milk through automatic feeders. Non-salable milk was acidified in a central stainless-steel tank by adding 20% formic acid until the milk reached a pH of 4.5. The heat-treated and acidified milk was then poured into tanks that were part of the automatic feeder, which heated (37°C) and delivered the milk to calves through 6 nipples per pen. Calves were gradually weaned by reducing the milk availability in the nipples, which started 10 d 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, or injuries. Therapeutic interventions were performed according to farm standard operating procedures 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 2 commercially available products for the treatment of BRD in preweaning dairy female calves. The negative control untreated group was added to this study to advance our understanding of natural physiological and microbiological responses to naturally occurring BRD and to determine the necessity of antibiotic treatment. In addition to the assessment of experimental treatments, healthy animals were enrolled in the study for comparison with diseased animals that were treated or untreated at BRD identification. 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 combined 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 2-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 by a trained farm worker from birth to weaning. The farm worker examination was performed daily, early in the morning, and a list of animals potentially with BRD was generated. For animals with clinical signs of pneumonia, a thorough physical examination was performed by a researcher blinded to the treatment allocation list within 1 h of the examination performed by the farm employee. In this examination, calves had their rectal temperature measured and were visually evaluated for the presence of cough, increased respiratory rate, ocular or nasal discharge (small or heavy amounts of uni- or bilateral discharge), abnormal ear position, and signs of ear pain such as ear flick, head shake, uni- or bilateral ear droop, and head tilt. Calves were defined as having pneumonia if they scored ≥5 according to the Wisconsin Calf Respiratory Scoring System (McGuirk and Peek, 2014). Fever (rectal temperature >39.5°C) and having the first case of pneumonia were selection criteria for this study. Although fever alone is not a requirement for BRD diagnosis by the Wisconsin Calf Respiratory Scoring System, this clinical sign was defined as a selection criterion in our study because it is an early clinical sign of BRD and because of its association with bacterial infection (Gaudino et al., 2022). 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 farm protocols.

**Randomization and Treatment Protocols**

Randomization was performed before the beginning of the study using the random function in Excel (Microsoft Corp.). Based on the randomization list, eligible calves were allocated into 1 of 3 treatment groups described below.

The treatment groups consisted of 2 different commercial drugs (tildipirosin or florfenicol + flunixin meglumine) labeled for treatment of pneumonia in nonlactating dairy cattle of ≤20 mo of age and a negative control untreated group. Calves assigned to the tildipirosin group (TLD) received a single subcutaneous injection of tildipirosin (4 mg/kg; Zuprevo, Merck Animal Health), 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 florfenicol) combined with a nonsteroidal anti-inflammatory (2.2 mg/kg flunixin meglumine; Resflor-Gold, Merck Animal Health). Antibiotic treatments were administered by a different researcher than the person performing the physical examination. The negative control untreated group (NEG) did not receive any antibiotic intervention up to d 5 after diagnosis. An assessment of health status was performed by the researchers on d 5 following enrollment using the physical examination steps outlined in the Data Collection section. A therapeutic intervention was performed by a farm employee if a calf remained with clinical signs of pneumonia, regardless of the experimental group. Treatments were carried out after sample collection. If necessary, animals in the NEG group received either tildipirosin or florfenicol + flunixin meglumine subcutaneously and according to the dosage described on the product label for therapeutic intervention.

In addition, upon enrollment of 1 calf into each of the experimental groups, 1 to 2 healthy calves (depending on availability on 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 visual health condition (i.e., alert, hydrated, with bright eyes, ears up, laying in sternal recumbency, and without signs of disease), without fever at enrollment and no history of previous diseases treated with antibiotics. Calves in the CTR group 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 (d 0), samples were collected immediately before treatment administration for calves in the TLD and FLF groups or at enrollment for calves in the NEG and CTR groups. After enrollment, calves were followed for samples and data collection daily for 5 d and then weekly until weaning (~65 d of life). Physical examinations are described below and were recorded daily from enrollment to d 5 and once again on d 10 (wk 1). 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 or nasal discharge (small or heavy amounts of uni- or bilateral discharge), and abnormal ear position 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, BW measured by farm personnel...
on the day of calf birth and at weaning were used for estimation of ADG, which was extracted from the farm management software (Dairy Comp 305; Valley Agricultural Software). Events such as death for any reason or occurrence of other diseases (e.g., otitis media) were extracted from Dairy Comp 305.

**Clinical Scores**

Clinical observations were performed daily from d 0 to d 5 and at the first week after diagnosis (wk 1) by evaluation of nasal, eye, and ear scores according to the Wisconsin Calf Respiratory Scoring System (McGuirk and Peek, 2014). Nasal discharge was scored as follows: 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 follows: 0 = no abnormalities; 1 = ear flick or head shake; 2 = slight unilateral droop; and 3 = head tilt or bilateral droop.

**Health Parameters**

Several dichotomized health parameters were assessed from calves identified with pneumonia, including the risk of fever on d 5 of the study, treatment failure, need for antimicrobial use from d 5 to weaning, and risk of otitis treatment after enrollment. Calves were considered to have fever on d 5 if the rectal temperature remained ≥39.5°C. Experimental treatment failure was defined as the need for antimicrobial intervention against pneumonia from d 5 to 10, or in the case of death during the same timeframe. The need for antimicrobial use for treatment of pneumonia, otitis, or both was also evaluated from d 5 of the study until weaning (approximately at 65 d of age). It is important to highlight that the NEG group was considered an experimental group in the analysis of treatment failure and need for antimicrobial use for treatment of pneumonia, otitis, or both; therefore, it was considered in these evaluations even though NEG group calves did not receive antibiotic intervention at diagnosis.

**Blood Sample Collection and Analysis of Serum Parameters**

Blood samples were collected daily from d 0 to 5 and then weekly until weaning via jugular venipuncture using an 18-gauge, 3.8-cm needle in a 10-mL plastic vacuum tube (Becton, Dickinson and Co.) without anticoagulant. Serum was harvested within 2 h of collection after centrifugation of the blood tube at 2,000 × g for 15 min at 4°C and stored at −80°C.

Serum concentrations of total protein, glucose, BHB, calcium, lactate, cholesterol, urea, alkaline phosphatase (ALP), aspartate aminotransferase, and alanine aminotransferase 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).

**Nasopharyngeal Swabs**

Nasopharyngeal swabs were collected daily from d 0 to 5 and then weekly until weaning. 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 URT Microbiome**

**DNA Extraction.** DNA was isolated from all swabs of the URT 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 −80°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 (GTGYCAGCMGGCGGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) was performed as previously described (Caporaso et al., 2012) using the Illumina MiSeq platform.
No lung consolidation was defined as the absence of abnormalities on thoracic ultrasound (i.e., well-ventilated peripheral lung tissue). A calf was defined as having lung consolidation if a detectable consolidation was observed, regardless of the size of the consolidated area (Teixeira et al., 2017a).

**Statistical Analysis**

The statistical software JMP PRO 13 (SAS Institute Inc.) was used for descriptive statistical analyses using the ANOVA function for continuous data, and the 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 the most abundant phyla and genera is described below.

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), BW 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 log_{10}-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 the 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 d 0, 1, 2, 3, 4, and 5), and their interaction. The aim of this analysis was to evaluate the effect of first treatment on the URT microbiota composition after the initial treatment. Therefore, samples collected after d 5 were not assessed because 65% of calves diagnosed with pneumonia received an antimicrobial after d 5. The effect of time relative to each outcome variable was included in the repeated statement of SAS, using the calf as...
First-order autoregressive covariance structure had the best model fit based on the Akaike information criterion and was selected for the analyses. 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 d 5, and risk of otitis after enrollment was assessed using multivariable logistic regression models with binary distribution of the GLIMMIX procedure (SAS version 9.4). Based on clinical scores described above for nasal and ocular discharge and ear positioning (i.e., scores 0 to 3), a calf was considered positive if it presented a score >0 in at least one examination performed from d 1 to 5, and at d 10. For example, if a calf was identified with nasal discharge (nasal score >0) on at least one time point during the period of physical examination, it was defined as having nasal discharge in the statistical analysis. Models included the fixed effects of treatment (TLD, FLF, and NEG), BW 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 2-way interaction terms between the fixed effects. Differences in the least squares means (LSM) were considered for multiple comparisons between treatments.

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 $P$-values 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 significant when a $P$-value $\leq 0.05$ was detected. A tendency to significance was considered if the $P$-value was between 0.05 and 0.10.

**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 (i.e., 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 ($P = 0.95$), or 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).

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLF (n = 33)</td>
</tr>
<tr>
<td>Dam parity$^a$ (no.)</td>
<td>1.7 (0.17)</td>
</tr>
<tr>
<td>Birth weight$^a$ (kg)</td>
<td>41.4 (0.66)</td>
</tr>
<tr>
<td>Incidence of diarrhea$^b$ (%)</td>
<td>30.3 (10)</td>
</tr>
<tr>
<td>Age at enrollment$^b$ (d)</td>
<td>26.5 (1.68)</td>
</tr>
<tr>
<td>RT at diagnosis$^b$ (°C)</td>
<td>40.1 (0.06)$^A$</td>
</tr>
</tbody>
</table>

$^a$A,BDifferent uppercase letters indicate significant differences between treatments based on LSM ($P \leq 0.05$).

$^b$FLF = single subcutaneous injection of a product containing 40 mg/kg florfenicol combined with 2.2 mg/kg flunixin meglumine (Resflor-Gold, Merck Animal Health); TLD = single subcutaneous injection of tildipirosin (4 mg/kg; Zuprevo, Merck Animal Health); 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.

$^c$Variables are presented as mean (SE). RT = rectal temperature.

$^d$Incidence of diarrhea is presented as percentage and number of cases within parentheses.

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**Health Parameters and Performance**

Rectal temperature was evaluated daily from enrollment to d 5 and once again on d 10 (wk 1). 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°C) than calves in the NEG (39.4°C; \( P < 0.0001 \)) and TLD groups (39.1°C; \( P < 0.0001 \)). There was no difference in rectal temperature between CTR and FLF (38.9°C; \( P = 0.17 \)). 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 lower 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. Briefly, calves treated with FLF reached the temperature observed in the CTR group on the first day after drug administration, whereas calves in the TLD group took one extra day to do so. Calves in the NEG group required at least 10 d after enrollment to reach the same body temperature as the other groups.

Data regarding clinical scores and risks of fever on d 5, treatment failure, and pneumonia or otitis retreatment after enrollment are presented in Table 2. No differences were observed among groups in terms of ocular discharge \( (P = 0.71) \) and drooped 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, data analysis showed that the odds of a calf presenting nasal discharge was 0.24 times higher in the NEG group compared with the FLF group, with no treatment association observed for nasal discharge when the TLD group was compared with the NEG group (Table 2).

Treatment effect \( (P = 0.002) \) was also found on risk of fever at d 5. Calves in the NEG group had a higher risk of fever at d 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 for antimicrobial use for treatment of pneumonia signs from d 5 to 10 of the study according to examinations performed by farm personnel. 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 d 5 of the study to the day that calves completed weaning and were moved offsite. Calves in the NEG group had a higher risk of treatment failure (54.7%) from d 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 \( (P = 0.18) \). In addition, calves in the NEG group (90.6%) had a higher risk of treatment of pneumonia or otitis clinical signs from d 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.

The logistic regression analysis also showed an association \( (P = 0.05) \) between treatment and risk of otitis in calves from d 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

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**Figure 1.** Rectal temperature of 135 calves over the first 10 d after enrollment in the study. FLF (n = 33) = single subcutaneous injection of a product containing 40 mg/kg florfenicol combined with 2.2 mg/kg flumixin meglumine; TLD (n = 36) = single subcutaneous injection of tildipirosin (4 mg/kg); NEG (n = 35) = calves did not receive any antimicrobial injection up to d 5 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 LSM between treatment groups. Symbols above the time points represent statistical differences \( (P \leq 0.05) \) between treatments based on the Tukey’s significance test for multiple comparisons. Of calves in the NEG group, one died on d 4, and 27 (79.4%) required antimicrobial therapy after d 5 of the study. Results are presented as LSM ± SEM.
need for assisted calving ($P = 0.09$). The risk of otitis was higher in the NEG group (58.4%) compared with FLF (24.4%), whereas no significant difference was observed when comparing the TLD group (41.7%) with the FLF group or the NEG group (Table 2).

In addition to the dichotomous variables mentioned above, calf performance was assessed based on ADG considering 2 BW measurements: one at birth and the second at approximately 60 d of age. Four of the 135 calves enrolled in the study (i.e., including controls) did not have ADG estimated because the farm workers did not weigh them on the day of birth. Of these, 2 were from the NEG group, 1 from TLD group, and 1 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 total protein ($P = 0.01$) and haptoglobin ($P = 0.002$), and the lowest concentration of ALP ($P < 0.0001$) among the treatment groups; 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.

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 d 5 after BRD diagnosis (Figure 4).

### Lung Consolidation

Calves diagnosed with pneumonia underwent a thoracic ultrasonography in 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 ($P = 0.32$).

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

The MRA values of the 6 most prevalent phyla were compared among treatments using the nasopharyngeal swabs collected daily from diagnosis until d 5. The other time points (i.e., after d 5) were not assessed because a high proportion of calves were treated for either pneumonia or otitis after d 5 as described above. 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 with the NEG group, calves in the TLD group had a higher MRA from d 1 to d 5. Finally, calves in the TLD group had lower Proteobacteria MRA than calves in the CTR and FLF groups from d 1 to d 5, whereas TLD calves had lower MRA on d 1 to 3 compared with the FLF group (Figure 6).

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

In total, 899 genera were identified from nasopharyngeal swabs collected from enrollment to weaning, and the 12 most prevalent genera are shown in Figure 7. The MRA of the 12 most prevalent genera was compared between treatments using the nasopharyngeal swabs collected daily from diagnosis up to d 5. Differences between treatments were observed for 8 genera (Mycoplasma, Mannheimia, Pasturella, Ureaplasma, Sneathia, Gallibacterium, Brenneria, and Bacteroides; Table 5).

Among the 12 most prevalent genera, Mycoplasma, Morazella, Mannheimia, and Pasturella had the highest abundance 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 their interaction (Figure 8). No statistical effects were observed for the genus *Moraxella*. For *Mycoplasma*, the TLD group had a statistically higher MRA than the FLF and NEG groups from d 1 to 5. Compared with healthy calves, the TLD group had a higher *Mycoplasma* MRA on d 1, 3, and 4. When comparing FLF to CTR calves, FLF had a lower *Mycoplasma* MRA on d 1 and 2. Differences in the MRA of *Mycoplasma* between the CTR and NEG groups were observed on d 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 *Mannheimia* MRA was observed between calves in the TLD and FLF groups or between calves in the NEG and CTR groups, based on nasal swabs collected during the first 5 d after enrollment (Table 5; Figure 8).

A treatment effect was 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).

**DISCUSSION**

The use of antibiotics remains the most effective strategy for treatment of pneumonia (Booker and Lubbers, 2020; Cummings et al., 2022). 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 with...
a group of untreated calves. 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 our 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. Compared with animals in 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 retreatment 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 with 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 on 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, whereas calves in the TLD group took 1 extra day to reach the same body temperature as the CTR calves. On the other hand, calves in the NEG group required at least 10 d after enrollment to reach the same body temperature as the other groups. Furthermore, based on the logistic regression analysis performed at d 5 of the study, the risk of fever was 83.2 and 59.1% lower in the FLF and TLD groups, respectively, compared with the NEG group. These results demonstrate the importance of antimicrobials to reduce 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 with the TLD group, which is likely due to the presence of flunixin meglumine in the former. Nonsteroidal anti-inflammatory drugs 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 temperature was observed in both groups, a faster reduction in the florfenicol + flunixin formulation was reported from pretreatment to 6 h posttreatment (Thiry et al., 2014). Similar results 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 compared with TLD at 1, 2, and 3 d after infection treatment (Bringhenti et al., 2021b). The latter study included calves with pneumonia and calves with otitis media. In addition, the presence of fever (i.e., body temperature ≥39.5°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
Table 4. Effect of treatments on mean relative abundance [%; LSM (SEM)] of the 6 most prevalent bacterial phyla identified in the upper respiratory tract of 133 calves during the first 5 d after enrollment

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Treatment group</th>
<th>CTR</th>
<th>FLF</th>
<th>TLD</th>
<th>NEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>58.3 (3.2)B</td>
<td>52.9 (2.8)B</td>
<td>68.2 (2.8)A</td>
<td>53.0 (2.7)B</td>
</tr>
<tr>
<td><em>Tenericutes</em></td>
<td></td>
<td>33.0 (2.9)AB</td>
<td>30.0 (2.5)B</td>
<td>21.5 (2.5)C</td>
<td>37.9 (2.5)A</td>
</tr>
<tr>
<td><em>Proteobacteria</em></td>
<td></td>
<td>1.9 (0.3)B</td>
<td>3.5 (0.3)A</td>
<td>1.9 (0.3)B</td>
<td>1.7 (0.3)B</td>
</tr>
<tr>
<td><em>Firmicutes</em></td>
<td></td>
<td>3.3 (0.6)B</td>
<td>6.4 (0.6)A</td>
<td>3.0 (0.6)B</td>
<td>1.6 (0.6)C</td>
</tr>
<tr>
<td><em>Bacteroidetes</em></td>
<td></td>
<td>1.8 (0.4)B</td>
<td>4.7 (0.4)A</td>
<td>2.6 (0.4)B</td>
<td>1.6 (0.4)B</td>
</tr>
<tr>
<td><em>Fusobacteria</em></td>
<td></td>
<td>0.8 (0.6)B</td>
<td>0.8 (0.6)B</td>
<td>2.3 (0.5)A</td>
<td>3.1 (0.6)A</td>
</tr>
</tbody>
</table>

A–C Different uppercase letters indicate significant differences between treatments based on LSM (P ≤ 0.05).

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 florfenicol combined with 2.2 mg/kg 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 d 5 after diagnosis.

Figure 5. Mean relative abundance (%) of the 6 most prevalent bacterial phyla (and “other”) 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 d 5, calves in each group had samples collected on a weekly (W) basis.
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 d after diagnosis than calves in the NEG group. Again, this result may be due to the reduced inflammatory response associated with 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

Figure 6. Mean relative abundance (%) of the 6 most prevalent bacterial phyla identified in the upper respiratory tract over the first 5 d sampled from 133 calves enrolled in the study. FLF (n = 33) = single subcutaneous injection of a product containing 40 mg/kg florfenicol combined with 2.2 mg/kg 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 d 5 after diagnosis; CTR (n = 31) = healthy calves defined as animals with good health condition, without fever at enrollment, and no history of previous diseases. Results are presented as LSM ± SEM. Symbols above the time points represent statistical differences (\( P \leq 0.05 \)) between treatments (TRT) based on the Tukey’s significance test for multiple comparisons.
on a clinical index score that included nasal discharge (Guzel et al., 2010). 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 2 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 with 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 14-fold increase 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...
bin on the second day after treatment, whereas calves in the NEG group remained with significantly higher serum haptoglobin until the first week after enrollment (i.e., approximately at d 10 of the study). The marked reduction of haptoglobin in the NEG group beginning after d 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, otitis, or both 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 farm personnel and were not controlled by the researchers, which could be considered as a limitation of our study.

In addition to haptoglobin, other blood parameters were affected by experimental treatment in our study. Calves in the NEG group had the lowest ALP serum concentration compared with the other groups. Based on LSM differences over time, CTR calves had higher serum ALP than calves diagnosed with pneumonia from d 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 d 3 after treatment and had normalized at d 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 d of life compared with 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 glucose evaluation in our study, calves that had pneumonia had a lower serum glucose concentration compared with healthy animals. Low glucose levels were 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, 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 compared with 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 3 d after enrollment, whereas those in the NEG group did so only after the first week.
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 with 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 g/d 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 or otitis with TLD or FLF, although healthy calves gained more weight than diseased and treated calves. Other studies have reported the negative effect 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 note that the evaluation of ADG in our study was based on only 2 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 whether 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 effects on heifer reproductive performance, increasing culling risk before first lactation (Teixeira et al., 2017b). In our study, although no statistical differences in lung lesions were observed between groups, an interesting numerical difference was observed between calves in the FLF group compared with the other groups. Only 9.4% (3/29) of calves in the FLF group had lung consolidation, whereas 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 calves in the FLF group benefited from the nonsteroidal anti-inflammatory drug (NSAID) effect, further studies using a larger number of animals should be encouraged to assess the benefits of parenteral florfenicol + flunixin meglumine 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.

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 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 with 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 an evaluation was beyond the scope of this study.

Although 28 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 6 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 pneumonia 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, 2018; Stroebel 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 with the other groups. However, compared with the CTR group over time, differences in *Tenericutes* MRA were observed only at 2 time points (d 3 and d 5). This increase in *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 d 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 reductions in other genera such as *Mannheimia* and *Pasteurella*. It is also important to note that the commercial product containing tildipirosin is not labeled for use against *Mycoplasma* spp.

As mentioned above, 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 these 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 d of the study. This result corroborates previous studies that reported these bacteria as ubiquitous inhabitants of the URT (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.

Although differences were observed between antibiotics in some blood parameters and microbiota composition, no significant differences in clinical outcomes were observed between FLF and TLD in our study. On the other hand, the NEG group demonstrated that delaying treatment can affect calf wellbeing and increase the need for retreatment, which emphasizes the importance of early diagnosis and timely treatment of calves with pneumonia.

**CONCLUSIONS**

Calves injected with tildipirosin or florfenicol + flunixin meglumine had lower rectal temperature and serum haptoglobin levels compared with calves in the pneumonia and untreated group. Calves in the FLF group reached the same temperature as healthy calves 1 d earlier than calves in the TLD group. In addition, compared with untreated calves, antibiotic-treated calves had a lower risk of pneumonia retreatment and treatment for otitis media during the preweaning period. The FLF group had lower risks of nasal discharge, treatment failure, and otitis media compared with 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. 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 microbiome research is encouraged to advance our understanding of the role of commensal and pathogenic microorganisms in the health of dairy calves and BRD pathophysiology.

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