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Article

Impact of Parenteral Ceftiofur on Developmental Dynamics of Early Life Fecal Microbiota and Antibiotic Resistome in Neonatal Lambs

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Abstract: Background: Early gut microbiome development is critical for neonatal health, and its dysbiosis may impact long-term animal productivity. This study examined the effects of parenteral Ceftiofur Crystalline Free Acid (CCFA) on the composition and diversity of the neonatal lamb fecal microbiome. Additionally, the emergence of antimicrobial resistance genes associated with CCFA exposure was investigated. **Methods:** Sixteen healthy neonatal lambs were randomly assigned to CCFA-treated (n = 8) or control (n = 8) groups. Fecal samples were collected on days 0, 7, 14, 28, and 56. Genomic DNA was extracted and sequenced using the Illumina MiSeq platform. Microbial composition was analyzed using the MG-RAST pipeline with the RefSeq database. **Results:** There were distinct microbial populations in the CCFA-treated lambs compared to the control group at each time point, with a highly significant decrease in alpha and beta diversity. The CCFA treatment showed a reduction in several key microbial taxa during nursing, but these differences were diminished by day 56. Unlike the control group, CCFA-treated lambs had core microbes potentially carrying multiple antibiotic resistance genes, including those for beta-lactam, fosfomycin, methicillin, and multidrug resistance. **Conclusion:** The early sheep fecal microbiome demonstrated resilience, repopulating after CCFA-induced perturbation despite a temporary reduction in key taxa during the nursing period. This highlights the microbiota's stability following a short-course antibiotic challenge. However, the transient disruption suggests potential negative impacts of antibiotics on the early gut microbiome. Notably, CCFA resistance persisted, raising concerns about its possible spread to the surrounding environment.

Keywords: microbiome; lambs; ceftiofur; antibiotic resistance

1. Introduction

The livestock gut microbiome is an ecosystem with diverse microbial communities living in symbiosis with their host [1]. Homeostasis of this ecosystem is crucial in maintaining the host's immune health and productivity [2]. Gut microbiota represents an innate barrier that protects against pathogen invasion and supplies the host with different metabolic products with nutritional, immune-modulatory, and antimicrobial properties [3]. Several studies have demonstrated that alteration of this microbial community (dysbiosis) may lead to perturbations in metabolism, immune-competence, and chronic disease [4,5]. Therefore, understanding the gut microbiome composition and developmental dynamics is essential for maintaining animal health and production.

The early colonization of the neonatal gut microbiome is critical and influences the host phenotype [6], however its origin is a matter of debate. Regardless of the origin of the first-seeded microbes, their growth generates an anaerobic environment after utilizing the available oxygen, which promotes the development of anaerobic communities such as Bacteroides and Firmicutes [7,8]. This development progresses into niche-specific habitats across different GIT segments, each hosting distinct populations of luminal and mucosal gut microbiota [9,10]. Maturation of the mucosal immune system occurs simultaneously with microbiome development to ensure microbio-mucosal homeostasis [11]. After stabilization, studies suggest that resident microbial populations are stable and resilient to temporary perturbations if there are no dramatic changes in host health and diet. However, it is thought that any hindrance to this classic development pattern during early life will be associated with profound health consequences [12,13].

Several factors, including host genetics, coexistent microbial populations, and environmental events, influence the early colonization and diversity of mucosal microbial communities [14]. Additionally, chemotherapeutics such as antibiotics are important factors that can disrupt microbial composition and promote the development of microbial strains carrying antibiotic resistance genes (ARG) [15]. Unfortunately, antibiotics are heavily used in livestock production systems for disease treatment and prophylaxis. For example, ceftiofur, the only third-generation cephalosporin approved for use in livestock in the US, is widely used to treat mammary gland, uterine and respiratory infections. Multiple lines of evidence indicate that antibiotic administration can disrupt the structure and development of both maternal and neonatal mucosal microbiota [16]. However, knowledge of the early-life impact of perinatal antibiotic administration on the prevalence of the antibiotic resistome and on the establishment of early-life mucosal microbiota in sheep is lacking. Additionally, while the ruminant [4] and pig [17] fecal microbiota has been characterized in various conditions of health and disease, the early development of the gut microbiome in sheep has yet to be fully elucidated.

In this study, whole-genome shotgun metagenomic sequencing (WGSS) is used to characterize the gut microbiota development in two key stages of the early production life cycle in neonatal lambs: the nursing stage and the initial grazing stage during the first two months after lambing. In addition, we assessed the extent of microbiota resilience, microbial diversity, and the emergence of ARG after administration of a single dose of Ceftiofur crystalline-free acid (CCFA).

2. Results

2.1. Impact of CCFA on the Lamb's Body Weight

There was no significant difference in weight gain of the lambs between the control and CCFA-treated groups, nor between males and females or even between single and twins at each time point of the experiment

2.2. Whole Genome Shotgun Sequencing Summary

The entire whole genomic sequence reads from all collected fecal samples was 24,627,178 raw sequence reads (mean number of sequences per sample: 139,803; median: 152,468; and range:

207,217). Amongst these reads, there were 12,222,322 reads for the control group and 12,079,860 for the treated group. The average Phred quality score of all raw sequence reads was 36.7, and less than 1% of these reads were above 22 (medium quality). Across all samples, 28 different phyla and 587 genera were categorized via the MG-RAST® webserver.

2.3. Effects of the Early Ceftiofur Treatment on the Lamb Fecal Microbiome Composition and Diversity

Generally, the configuration and diversity of the fecal microbiota at both phylum and the genus level varied between different time points in both the control and CCFA groups. At the phylum level, *Firmicutes* (52%), *Bacteroidetes* (25%), and *Proteobacteria* (20%) were the most predominant phyla in both the control and CCFA groups (Figure 1). The relative abundance of *Firmicutes* on day 0 represented 59% and 63% of all microbial populations in the control and CCFA-treated groups, respectively. On day 14, the proportion of *Firmicutes* decreased to half in favor of increased *Bacteroidetes* and *Proteobacteria* in both groups. On day 56, the *Firmicutes* proportions were restored and became the most abundant phylum, at 50% of the total in the control and 63% of the total in the CCFA-treated group. When comparing the two groups, this increase in *Firmicutes* on day 56 was only significant (p -value = 0.015) in the CCFA group. Although the other two major phyla showed no significant change between groups at different time points, some minor phyla showed a substantial difference between the control and CCFA groups. These included *Actinobacteria* (p -value = 0.0008) at day 0, *Verrucomicrobia* (p -value = 0.046) at both day 0 and 56, *Fusobacteria* (p -value = 0.035) at day 14, and *Chlorobi* (p -value = 0.035) at day 28. The other bacterial phyla that averaged less than 0.25% of the total are described in Table S1.

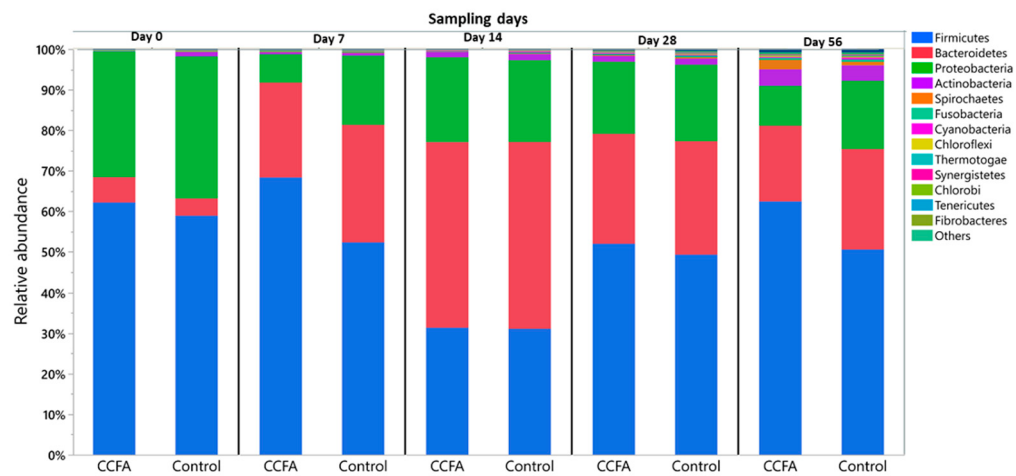


Figure 1. Taxonomic classification at the phylum level for the control and Ceftiofur (CCFA) treated lambs at each sampling time point (day 0, day 7, day 14, day 28, and day 56). This figure shows only the bacterial phyla averaged more than 0.25% of the relative abundance across all samples.

At the genus level, the prevalent bacterial genera that averaged more than 0.25% of total across all samples were *Bacteroides* (22.75%), *Eubacterium* (17.88%), *Streptococcus* (14.04%), *Escherichia* (10.93%), *Clostridium* (7.28%), *Lactobacillus* (1.86%), *Klebsiella* (1.76%), and *Ruminococcus* (1.59%). The distribution of the most abundant bacterial genera in both control and CCFA groups at different time points is illustrated in Figure 2. At all-time points, there was a statistically significant decrease in the abundance of specific taxa in the CCFA-treated group compared to the control group. While there were taxa differences between groups at day 0 (*Campylobacter* (p -value = 0.049), *Klebsiella* (p -value = 0.045), and low abundance genera (p -value = 0.0117), the number and identity of the taxa differences increased over time, from three (*Klebsiella* (p -value = 0.027) , *Clostridiales* (p -value = 0.046), and

Salmonella (p -value = 0.036)) on day 7, to six (*Ruminococcus* (p -value = 0.046), *Bacillus* (p -value = 0.0357), *Erysipelotrichaceae* (p -value = 0.0033), *Alistipes* (p -value = 0.0404), *Treponema* (p -value = 0.0156), and *Holdemania* (p -value = 0.0209)) on day 14. The taxa differences between groups were minimal on day 28 (*Parabacteroides* (p -value = 0.046), and there were no significant differences between both groups on day 56. The other bacterial genera averaged less than 0.25% are in Table S2.

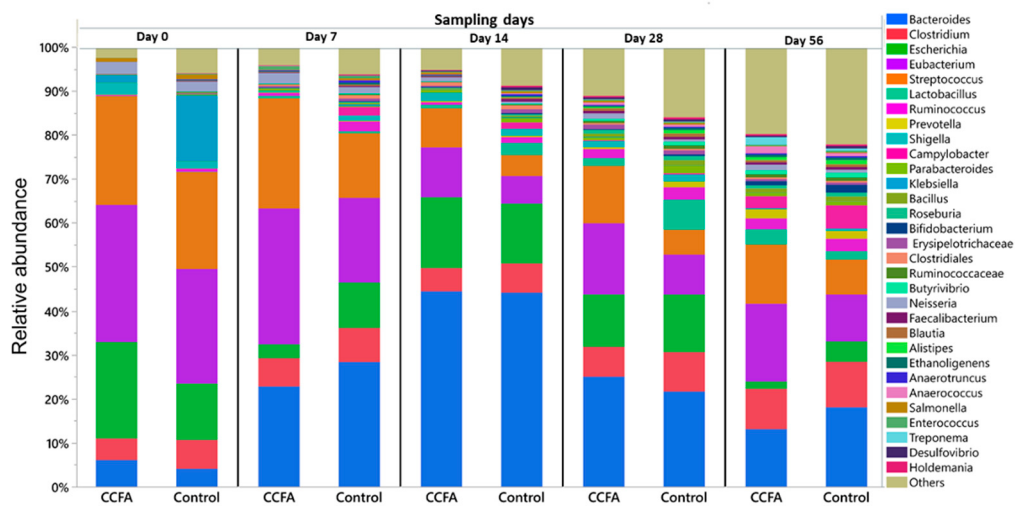


Figure 2. Taxonomic classification of WGSS at the genus level for the control and Ceftiofur (CCFA) treated lambs at each sampling time day (day 0, day 7, day 14, day 28, and day 56). Only those bacterial genera that averaged more than 0.25% of the relative abundance across all samples are displayed.

The two-way clustering analysis comparing the predominant genera between the control and the CCFA-treated groups at different time points revealed overlapping clades at days 0, 7, and 14. However, the clustering pattern diminished on day 28 but reappeared on day 56, as shown in the heatmap (Figure 3).

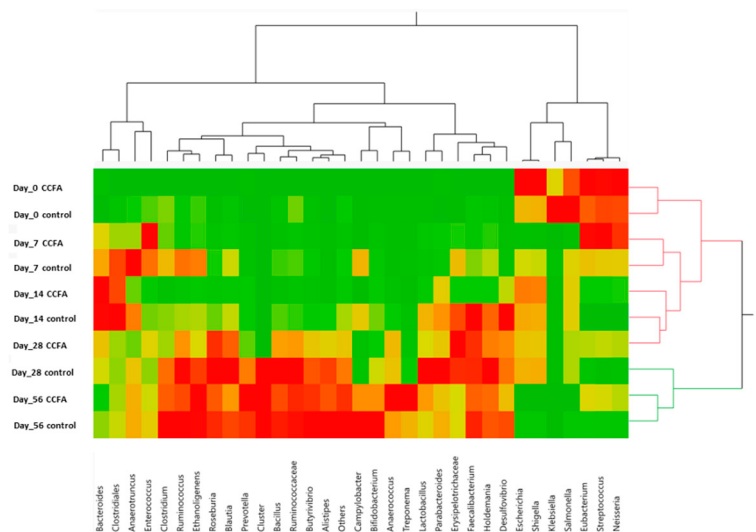


Figure 3. A heat map showing the two-way clustering of the predominant genera between the control and the CCFA group at different time points.

The core microbiome heatmap showed the relative abundance of the richest genera in both control and CCFA-treated groups at each time point (Figure 4). It showed a clearer delay in

Ruminococcus and *Lactobacillus* development in the CCFA-treated group than in the control group. In addition, the CCFA-treated group core microbiota encountered an exclusive enrichment of some genera, such as *Prevotella*, *Treponema*, and *Anaerococcus*, on day 56.

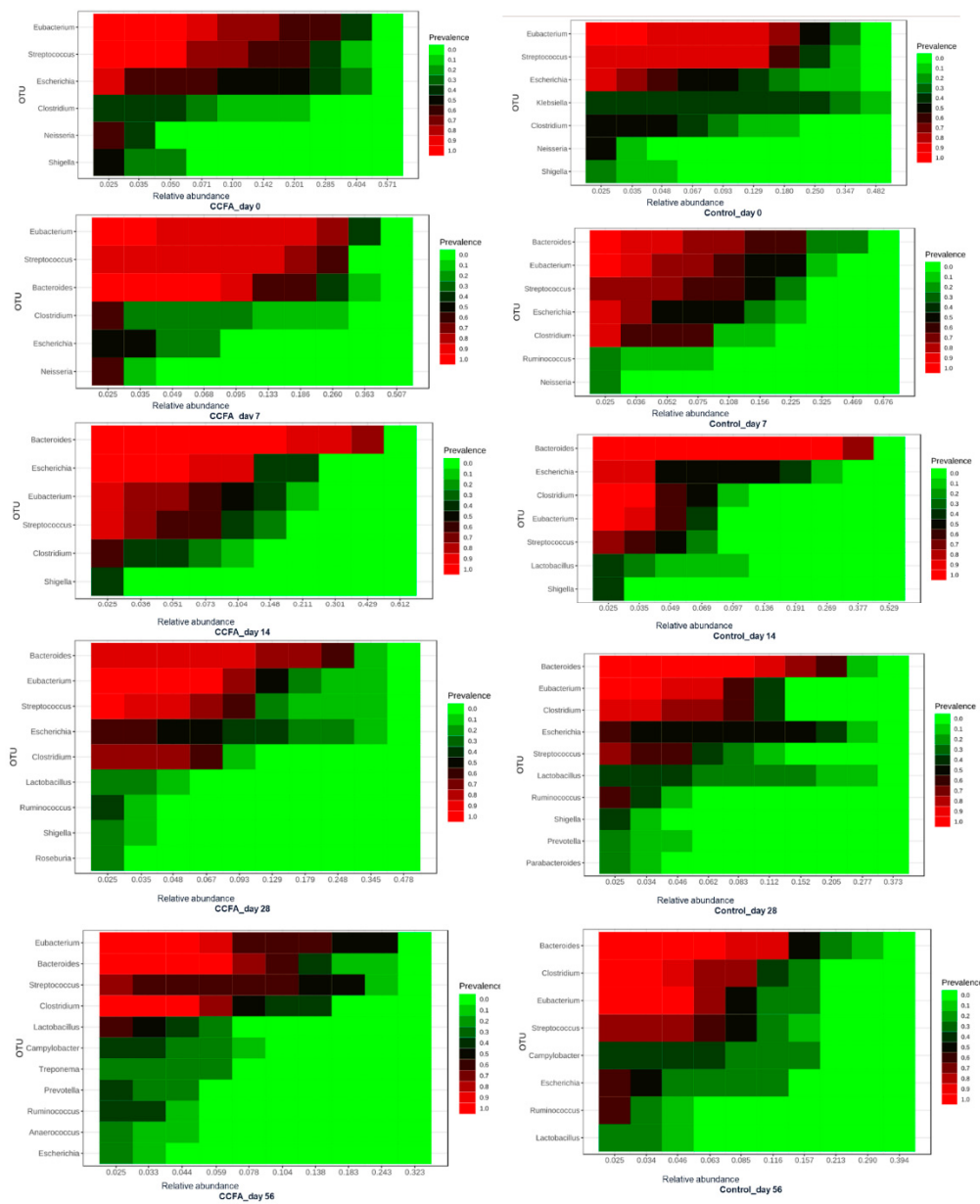


Figure 4. Heatmap of the relative abundance of the core microbiome in the CCFA-treated and the control group at different time points.

The fecal microbiota alpha-diversity for the predominant genera was evaluated using Chao1 and Shannon metrics and the non-parametric tests. Both metrics showed a significant increase in diversity and richness (p -value = 0.001) over time in both groups. However, the Shannon index revealed that the genus richness was significantly less in the CCFA-treated group than in the control group on day 7 (p -value = 0.03) and day 14 (p -value = 0.01) (Figure 5). Discriminant analysis showed a significant (p -value = 0.001) and similar change in beta diversity in both the CCFA and control groups over time, but with an apparent pattern of clustering between groups at each time point (Figure 6).

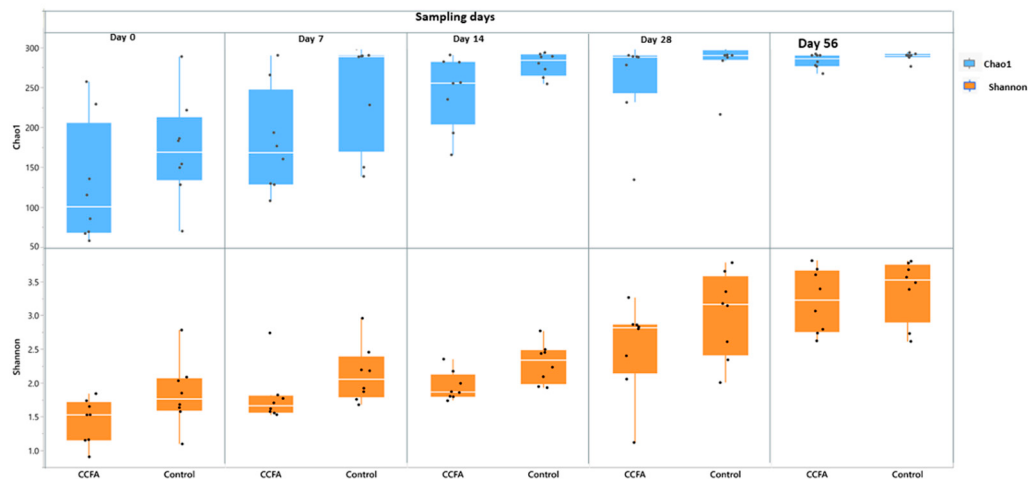


Figure 5. A box plot showing the alpha metrics, Chao1 and Shannon, between the CCFA-treated and the control group at each time point.

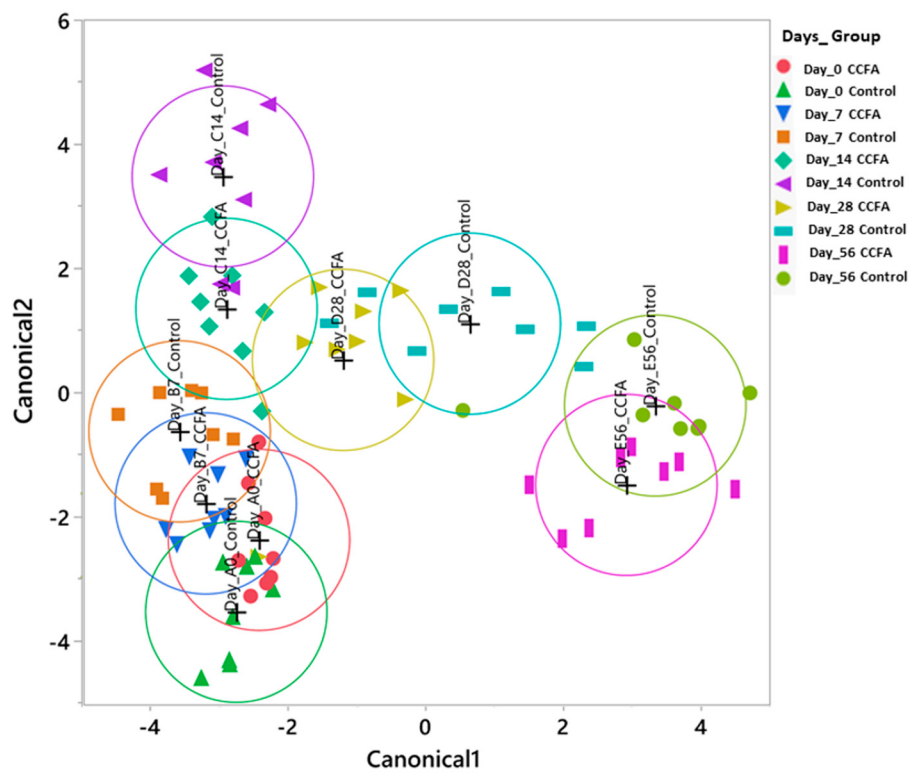


Figure 6. Discriminant analysis of beta diversity between the CCFA-treated and the control group at different time points.

2.4. Normal Fecal Microbiota Development

Development of the normal lamb fecal microbiota over time was depicted in the control group using canonical discriminant analysis (CDA), which showed the overall microbial population at the different time points of the experiment (Figure 7A). Using LEfSe algorithm, the most enriched genera with relative abundance averaging more than 0.25 were *Eubacterium*, *Streptococcus*, *Klebsiella*, *Nisseria*,

Salmonella, *Propionibacterium*, *Anaerotruncus*, *Bacterioidete*, *Colstridiales*, *Desulfovibrio*, *Veillonella*, and *Faecalibacterium* in the nursing stage (Figure 7 B). The most enriched genera during the early phase after weaning comprises of *Lactobacillus*, *Parabacteriodes*, *Bacillus*, *Rosiburia*, *Erysipelotrichaceae*, *Blautia*, *Ruminococcaceae*, *Holdemania*, *campylobacter*, *Ruminococcus*, *Prevotella*, *Bifidobacterium*, *Butyrivibrio*, *Alistipes*, *Prophyromonas*, *Anaerococcus*, *Treponema*, *Helicobacter*, *Lachnospiraceae*, and *Alkaliphilus*).

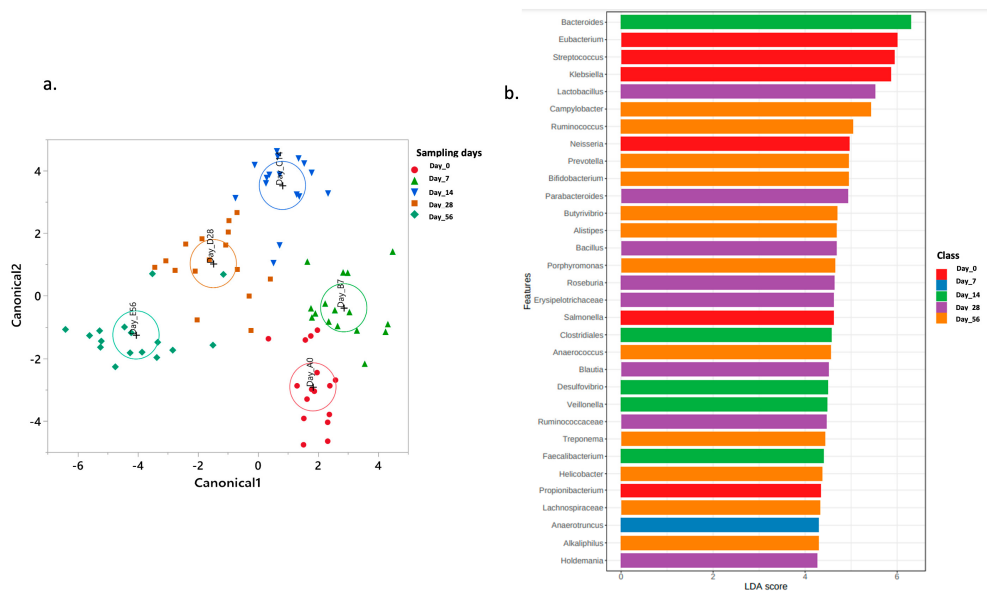


Figure 7. (a) Discriminant analysis of all genera in the control group at different time points. (b) linear discriminant analysis of the relative abundance of genera averaged more than 0.25 over the different time points.

2.5. Effect of Ceftiofur on the Microbial Functional Resistant Genes

The annotated functional profile reads indicated that the correlation between the number of potential antibiotic-resistant genes and the relative abundance of some core microbiome taxa was higher in the CCFA group than in the control group (Figure 8). We further investigated this correlation in the CCFA group at different time points (Figure 8 B). Beta-lactamase-resistant genes had the highest abundance in the CCFA group, especially on day 28. The number of resistance genes was significantly correlated with the relative abundance of *Bacteroides*, *Bacillus*, *Ruminococcus*, *Roseburia*, *Treponema*, *Butyrivibrio*, *Desulfovibrio*, *Faecalibacterium*, *Anaerotruncus*, and the low abundance genera (p -value ≤ 0.05). In addition, there was a significant increase in the relative abundance of other antibiotic-resistant genes with specific taxa at different time points, and they were more concentrated on day 28 (Figure 9).

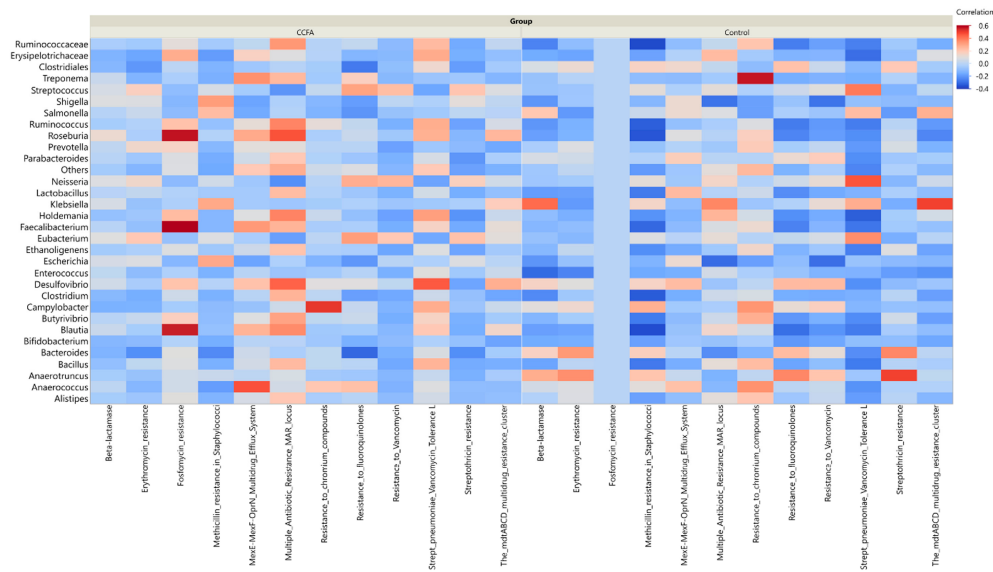


Figure 8. Heat map illustrating the correlation between the relative abundances of the enriched taxa in the CCFA-treated and the control group to the relative abundance of the potential functionally resistant genes.

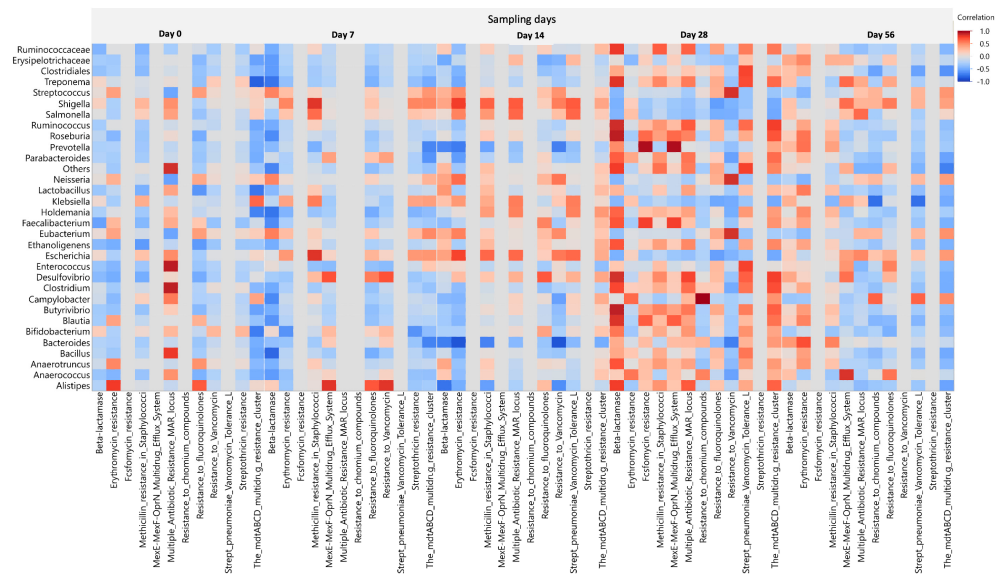


Figure 9. Heat map illustrating the correlation between the relative abundance of the enriched taxa across the CCFA-treated group only at the different time points to the relative abundances of the potential functionally resistant genes.

3. Discussion

In this study, we used WGSS to analyze early gut microbiome development in lambs and the microbial community perturbations that occurred after a single intramuscular injection of CCFA. We also examined the potential emergence of antimicrobial resistance genes in these early-life microbial communities. The initial fecal microbial populations comprised three main phyla: *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, similar to those described in the developing gastrointestinal tract of other monogastric and herbivorous animals [18]. These taxa have been reported to be present in colostrum and are consistent with the notion that colostrum contributes to the seeding of such microbial communities in the neonatal intestine [19]. The apparent variation in the relative abundance of these three taxa over time in our study aligns with the results reported in calves [7]. The other prevalent bacteria were *Bacteroides*, *Eubacterium*, *Streptococcus*, *Escherichia*, *Clostridium*,

Lactobacillus, *Klebsiella*, and *Ruminococcus*. Each of these genera has been shown to create anaerobic conditions that are essential for the growth of other beneficial anaerobes in the ruminant gastrointestinal tract [20,21]. The fact that our data showed a progressive and highly significant progression of diversity and richness in these taxa with the increasing age of the lambs is strongly indicative of the importance of these organisms in the temporal development of the alimentary system [22].

One unique aspect of this study was the evaluation of microbial changes with lamb age over two stages of husbandry and development: nursing and grazing. The presence of specific microbial taxa during each of these health management stages has been shown to be critical for the maturation and regulation of various physio-immunological processes [23,24]. In addition, a dysbiosis of these pioneer taxa has been associated with the occurrence of gastrointestinal diseases, growth disruption, and potential immune dysregulation [8,25]. It was notable that several of the predominant taxa observed in our experimental lambs have been shown to be affected by nutrition, and maturity and associated with immune status and health outcomes in other ruminant species. For instance, a high abundance of *Prevotella* and *Ruminococcus* has been linked to forage intake [22,26]. In pre-weaned calves, the increased relative abundance of *Prevotella* was positively correlated with age [27]. Lastly, high proportions of *Propionibacterium* and *Faecalibacterium* taxa have been linked to immune regulation, increased weight gain, and a reduction in the incidence of diarrhea in young calves and poultry [8].

Another key finding in this study was the transient impact of a single injection of CCFA on the microbiota of the neonatal lamb gastrointestinal tract. Ceftiofur (CCFA) was selected as the antibiotic of interest in our study because of its widespread use in the livestock industry and its importance as a human therapeutic. This finding is compatible with those seen in other species, such as mice, where antibiotic exposure during the pre- or post-natal periods shifted and increased the development of neonatal intestinal microbiota [28,29]. The longevity of low-level exposure of the gastrointestinal microbial community to CCFA following a single parenteral injection matches the temporal pattern of change observed in the treated lambs in our study.

An important aspect of the design of this study was to ensure that any observed differences in microbial community composition between experimental groups were due to the treatment effect and not a result of individual variation. The presence of a confounding difference in pre-treatment and the initial differences in the microbial relative abundances between the CCFA-treated and the control groups at day 0 within a few phyla or genera did not significantly affect or even became a permanent signature at subsequent time points. These small differences in microbiome composition at this early age could be attributed to variations in maternal influence or could be a result of secondary influences from factors such as colostrum intake [30,31].

In our study, differences in the configuration and diversity of the fecal microbiota between experimental groups were observed at both the phylum and the genus level. The extent and nature of these changes varied over time, but after two months, there was almost no significant difference between control and antimicrobial-treated groups. These findings show both concordance and discordance with existing literature in some phyla and/or taxa; for details, see (Table S3). The overall trajectory of CCFA-associated change was a decrease in abundance (*Salmonella* and unclassified derived *Clostridiales*; *Bacillus*, *Ruminococcus*, *Holdemania*, and *Alistipes*). The only taxa with a significant increase was in *Firmicutes*, and this change was only observable after two months from the antimicrobial exposure. The abundance of this taxa has been correlated with health and growth in growing pigs [32]. The observation of short-term changes in *Salmonella*, unclassified derived *Clostridiales*, and *Bacillus* is compatible with their *in vitro* sensitivity to ceftiofur [33]. There is public health concern regarding the former two taxa since they can degrade and build resistance to cephalosporins [34]. Early life and short-term declines in the *Ruminococcus*, *Holdemania*, and *Alistipes* taxa are unlikely to be biologically significant since the lambs are still in the nursing stage and at an early stage of gastrointestinal development. These taxa are involved in cellulose digestion and rumen development and are important for rumen development in the grazing period [24,35].

Further evaluation of the fecal microbial diversity between the CCFA-treated and control lambs revealed apparent microbial community changes with potential antibiotic resistance development. Although previous studies showed a significant decrease in both alpha and beta diversity after ceftiofur treatment [36], our study revealed insignificant differences between the two groups using the Bray-Curtis beta diversity index. However, there was a tendency (P -value = 0.14) for a complete microbial dissimilarity between the two groups on day 14. This pattern effect of the CCFA over time was also clear in the two-way clustering comparison between the CCFA and the control groups. Additionally, assessment of the core microbiome between the CCFA-treated and control group at each time point showed a delay in the development of *Ruminococcus* and *Lactobacillus* in the CCFA group, which might have a temporary adverse effect on lamb's health [2]. Moreover, on day 28 and day 56, the CCFA-treated lambs showed enrichment of *Roseburia*, *Treponema*, and *Anaerococcus*. Although they were members of the normal microbiome development in the control lambs, the LDA revealed their enrichment in the CCFA-treated lambs, which may be associated with the rapid adaptation and antibiotic resistance development by these organisms [37]. The low exposure of the gastrointestinal microbial community to CCFA certainly raises concern regarding the possible emergence of antimicrobial resistance following a single injection [29].

The most important finding in this study was that the CCFA-associated changes were relatively small in scope and of short duration. While it has been established that the reduction in microbial taxa in the early developmental stages might leave a permanent change in the adulthood microbial communities with an increased risk of reduced production or immunological diseases[38], there is growing evidence that host genetics can homeostatically modulate this disturbance with increasing age [39–41]. Besides the recent report by Liu et al. (2022), who described that the gut microbiome could develop resilience to antibiotic perturbation [42]. This might explain the minimal differences between the CCFA- treated lambs and the control group at day 28 and the non-significant changes at day 56. Overall, these findings highlight the complex interplay between antibiotic treatment and microbial community dynamics, underscoring the importance of considering both short and long-term effects when assessing the impact of antimicrobial interventions on gut microbiota.

Unlike the transient effect of the CCFA on the differences in the microbial relative abundances and diversities between the CCFA-treated group and the control group, the development of antibiotic resistome, including both specific and multidrug-resistant genes, can persist for a long time. This persistence is due to the horizontal transfer of these genes within and between hosts, as well as into their environment, resulting in a significant concern for both animal and human health [43–45]. In this study, the correlation between the relative abundance of potential resistant genes from the functional analysis and the relative abundance of prevalent taxa was most pronounced in ceftiofur-resistant bacteria. This correlation was notable on day 14 and became more pronounced by day 28, likely due to the time required for bacteria to express resistant genes. For instance, it has been shown that it takes about 17 days for calves exposed to a therapeutic dose of ceftiofur to express these genes [46,47]. In contrast to the findings of Weinorth et al. (2018), who reported no association of ceftiofur treatment with beta-lactamase-resistance in cattle [48], our results showed that the beta-lactam-resistant genes were obvious for most of the prevalent taxa. This observation aligns with the findings by Dong et al. (2022) [49]. Moreover, ceftiofur-resistant genes are known to be transferrable within and between bacterial genera, which could explain their spread among the predominant genera in our study [47]. Consistent with previous research, our results indicate that the presence of potentially resistant genes varies over time [50]. Lastly, we anticipate that specific and multidrug-resistant genes within the enriched gut antibiotic resistome could persist for extended periods, facilitated by horizontal gene transfer to other hosts and their environment [45,51]. These findings highlight the critical need for ongoing monitoring and management of antibiotic practices to mitigate the long-term impacts of antibiotic use on both animal and human health.

4. Materials and Methods

4.1. Experimental Design and Sample Collection

4.1.1. Animals

A total of 24 healthy neonatal lambs were included in this study, assigned into two groups: CCFA-treated (n=12) and control (n=12). Within the CCFA-treated group, there were five males and seven females, while the control group had four males and eight females. The average body weight (Kg) of the CCFA-treated and the control groups was 4.1 ± 0.62 and 3.8 ± 0.88 , respectively.

4.1.2. Challenge

The treated lambs have received a single intramuscular dose of ceftiofur crystalline free acid (CCFA^a, Excede®) at a dose of 5.0 mg /kg 24 hrs after birth.

4.1.3. Samples

Deep fecal swabs from both groups were collected at five-time points representing two lamb's developmental stages: the nursing stage that represents day 0 (D0), day 7 (D7), and day 14 (D14), and the start of grazing stage that comprises day 28 (D28), and day 56 (D56).

4.1.4. Ethics Statement of Animal Use

All procedures in this study were conducted in the university research sheep farm under the agreement and protocol guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois, Urbana-Champaign.

4.2. DNA Extraction

DNA was extracted from each sample using the QIAGEN QIAamp power fecal DNA extraction kit (Cat. No. 12830, MO BIO Laboratories, Inc., Carlsbad, CA, United States) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were evaluated using a NanodropTM spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 260 and 280 nm. The DNA integrity was assessed through running in gel electrophoresis (Bio-Rad Laboratories, Inc, Hercules, CA, United States) and stored at -20°C until sequencing.

4.3. Whole Genome Shotgun DNA Sequencing (WGSS)

The extracted DNA was sent on dry ice to the W. M. Keck Center for Comparative and Functional Genomics (the University of Illinois at Urbana-Champaign, Urbana, IL, United States), where the WGSS workflow was carried out. The initial step started with the Nextera DNA Flex Library Preparation Kit, which was used to build the DNA libraries (Illumina, Inc., San Diego, CA). Briefly, aliquots of 100 ng of DNA were cleaned by magnetic beads and amplified five times via Illumina Enhanced PCR Mix and Nextera FS dual-indexed primers. The Amplified DNAs were subjected to another cleaning process, and fragments lengths between 250-750 bp were purified using a double-sided bead purification procedure. The final products were measured using the Qubit DNA quantifier (Life Technologies, Grand Island, NY, United States). The mean size was detected using the AATI Fragment Analyzer (Advanced Analytics, Ames, IA, United States). After uniform amalgamates of the libraries, they were cleaned using a 1:1 ratio with AxyPrep Mag PCR Cleanup beads (Axygen, Inc., Union City, CA, United States) and checked for size for a second time using AATI Fragment Analyzer. Then, only 5 nM of the final pool was further quantified using qPCR (Bio-Rad Laboratories, Inc., CA, United States) and exposed for denaturation, then 4% non-indexed PhiX control library was loaded by a concentration of 10 pM into the MiSeq V3 flowcell for cluster formation and sequencing. Finally, DNA sequencing proceeded from both ends of the molecules, forming reads of 250 nt from each end following the manufacturer's guidelines (Illumina, Inc., San Diego, CA, United States).

4.4. Bioinformatic Analysis

4.4.1. Sequence Reads Processing

The raw sequence reads were demultiplexed and transformed into Fastq files using Casava v.1.8.2 (Illumina, Inc. San Diego, CA, United States), then evaluated by the FastQC software [52]. After that, Trimmomatic software [53] was used to snip both low-resolution reads (< 30 on Phred score) and the adaptor sequences. The sequence files were synced via Subsystems Technology (MG-RAST) webserver into the Metagenome Rapid Annotation, where the microbial composition was ready to be analyzed [54]. This web server involves extra refinement processes for the reads, such as length filtering and ambiguous base filtering. These processes ensure retaining all sequences with a standard deviation ≤ 2 and those containing less than five dubious base pairs. In addition, the reads were fine-tuned by removing both host-exclusive species read sequences, and other sequencing-based artifact reads. Eventually, the filtered sequences were subjected to normalization and standardization processes. Normalization was carried out by $\log_2$ -based transformation [$\log_2(x+1)$], and the standardization process was constructed within each sample and linearly between all samples.

4.4.2. Operational Taxonomic Units (OTUs) Analysis

The MG-RAST webserver analyzed the OTUs of the fecal microbiota at the phylum, genus, and species levels as well as metabolic functional gene profiles [55]. It identifies and converts millions of metagenomic sequence reads into discrete microorganisms using the rapid and highly efficient inquisitive algorithms within the genome databases. The microbial classification was accomplished by a nonredundant multisource protein annotation database (M5NR). The relative abundance was analyzed via the best-hit approach with a maximum e value of 1×10^{-5} , a minimum identity cutoff of 60%, and a minimum alignment length cutoff of 15 [56]. Microbiome Analyst computed both alpha and beta diversity indices of the fecal microbiota. Chao 1 and Shannon indices were used to demonstrate alpha diversity. In contrast, beta diversity was determined by principal component analysis (PCA) based on non-phylogenetic built-in Bray-Curtis distance metrics [57]. Lastly, the predicted functional gene profiles were elucidated using the SEED annotation system. All sequence reads could be accessible through the NCBI with the Bio-project ID number PRJNA917185

Beta-lactam resistome identification: the predicted antibiotic-resistant genes (ARGs) to ceftiofur and the relative abundance of beta-lactam resistant genes and other resistant genes in the functional profile was calculated using MG-RAST and then correlated to the relative abundance of the taxa identified in the fecal microbiota using JMP pro13.

4.5. Statistical Analysis

The difference in the overall microbial composition and beta diversity between the control and the CCFA-treated groups were assessed using the non-parametric multivariate analysis of variance (PERMANOVA) with 9999 permutations, and Bonferroni corrected p -values using the microbiome analyst®online platform at each time point (D0, D7, D14, D28, and D56). The difference in fecal microbiota relative abundance and alpha diversity between the two groups were estimated by Mann-Whitney pairwise comparison test with sequential Bonferroni significance at a p -value < 0.05 using the JMP pro 13® software. The overall difference in the relative abundance and diversity of the ARGs between the control and CCFA-treated groups was computed using the same statistical method. The similarities within the overall microbial composition between the two groups at each time point were additionally assessed using linear discriminant analysis effect size (LEfSe) via Galaxy1®[58]. On the other hand, to assess the overall predictive function gene profiles between the control and CCFA-treated groups, the differences were compared using principal component analysis (PCA) and heatmap using JMP pro13 software.

5. Conclusions

This study demonstrated the normal early gut microbiome development in sheep and their ability to withstand antibiotic perturbation over time. A single intramuscular dose of ceftiofur crystalline-free acid in neonatal lambs can cause transitory disturbances to the average microbial profile, posing potential risks to the animal's general health. These risks arise from the reduction of specific important taxa during a critical developmental stage, alongside the likely long-term emergence of antimicrobial resistance.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: taxonomic classification of the fecal microbiome at the phylum level for both control and Ceftiofur (CCFA) treated lambs at each sampling time point (day 0, day 7, day 14, day 28, and day 56). This table summarizes bacterial phyla of relative abundance averaged more than 0.25% across all samples.; Table S2: taxonomic classification of the fecal microbiome at the phylum level for both control and Ceftiofur (CCFA) treated lambs at each sampling time point (day 0, day 7, day 14, day 28, and day 56). All other bacterial phyla of relative abundance averaged less than 0.25% across all samples are included in this table.; Table S3: summary table of the significant microbiome changes in CCFA-treated lambs over time with supporting evidence from the literature.

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Data Availability Statement: the data is available at the NCBI with the Bio-project ID number PRJNA917185.

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Abbreviations

The following abbreviations are used in this manuscript:

CCFA	Ceftiofur Crystalline Free Acid
ARG(s)	Antibiotic Resistance Gene(s)
WGSS	Whole Genome Shotgun Sequencing
DNA	Deoxyribonucleic Acid
OTUs	Operational Taxonomic Units
MG-RAST	Metagenomics Rapid Annotation using Subsystem Technology
RefSeq	Reference Sequence Database
PCA	Principal Component Analysis
CDA	Canonical Discriminant Analysis
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
PCR	Polymerase Chain Reaction

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