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Effects of feeding a simulated waste milk on growth, health, fecal microbiota, and antibiotic resistance in dairy heifer calves

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ABSTRACT

Feeding waste milk, a common practice in dairy farming, exposes calves to subtherapeutic levels of antimicrobials, potentially contributing to antibiotic resistance—a growing concern globally. Many dairy farmers, including those in Ireland, continue this practice, feeding waste milk from antibiotic-treated cows to calves. Although previous studies have linked waste milk feeding to changes in calf growth and health during the preweaning period, its effects postweaning remain unclear. This study examined how the duration of antimicrobial exposure at levels equivalent to those found in waste milk influences health and growth outcomes of dairy heifer calves both before and after weaning. It also assessed the prevalence of extended-spectrum β-lactamase (ESBL)-producing antimicrobial-resistant *Escherichia coli* in feces and changes in the fecal microbiota over time. To mimic waste milk, as derived from a cow treated with an intramammary suspension of antibiotics, a simulated waste milk (SWM) was prepared by adding amoxicillin (1.68 mg/L) and neomycin (2.28 mg/L) to a conventional milk replacer (MR). The study employed a randomized block design with 87 dairy heifer calves assigned to 1 of 3 treatments: (1) long-term antibiotic (LTA), with calves fed SWM until weaning at 12 wk; (2) short-term antibiotic (STA), with SWM fed from 3 to 5 wk; and (3) control (CONT), with calves fed antibiotic-free MR. Calves were weighed weekly, and health scores, including fecal scores (tail and hindquarters cleanliness as diarrhea indicator), were recorded twice per week. Fecal and blood samples were collected to analyze microbiome changes and the shedding of antimicrobial resistance. Blood samples were taken to measure systemic inflammation, using serum amyloid A as a biomarker. Results indicated that SWM feeding did not affect average daily gains before or after weaning. However, higher fecal scores were observed in the LTA group during weaning and after weaning in the STA group. Antibiotic-resistant isolates were present in all groups, with the highest prevalence in LTA. Fecal microbiota analysis revealed treatment-specific microbial community variations, with an increase of *Enterococcus faecium* genes resistant to macrolide, aminoglycoside, and tetracycline antibiotics in LTA and STA compared with CONT. In summary, SWM feeding did not significantly affect growth or overall health, but it was associated with increased fecal shedding of resistant bacteria and some changes in the microbiota, indicating potential long-term implications for antimicrobial resistance in dairy herds.

Key words: waste milk, antibiotic, average daily gain, microbiota

INTRODUCTION

When lactating cows are treated with antimicrobials, antibiotic residues may be found in their milk, restricting the milk from entering the food chain. Although dairy cows may be treated for various infections, the most common reason for administering antimicrobials is to treat mastitis (Martin et al., 2020). This is typically administered as intramammary suspensions containing broad-spectrum antibiotics such as amoxicillin and neomycin, which are then present as residues in the milk after treatment (McDougall, 2003; Burmańczuk et al., 2017).

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

This type of milk is considered waste milk (**WM**), and it cannot be sold. Instead farmers may opt to feed it to calves (Gosselin et al., 2022). Waste milk feeding (**WMF**) is generally discouraged due to the potential risks it poses to calf health, as well as its association with the emergence of antimicrobial-resistant (**AMR**) bacteria (EFSA Panel on Biological Hazards, 2017; Firth et al., 2021).

Despite these concerns, WMF remains a common practice. A survey of Irish dairy farms by Barry et al. (2020) found that over half of farmers fed WM to their calves, a practice also common in other countries, including Switzerland (Gosselin et al., 2022), the United States (USDA, 2016), and the UK (Brunton et al., 2012).

Although studies have shown that WMF may negatively affect calf growth, health, and microbiota composition in the short term (Brunton et al., 2014; Firth et al., 2021), little research has explored the long-term effects. Longitudinal research is necessary to understand the effects of long-term WMF, as it potentially alters the microbiota, subsequently affecting digestion, immunity, and disease resistance, which are crucial for overall health and development (Cortese, 2009). Previous studies of the calf microbiota during WMF have typically analyzed only 1 or 2 time points and were conducted on animals in indoor systems with different genetics and rearing methods, compared with pasture-based herds such as those that predominate in Ireland and New Zealand (Godden et al., 2005; Moore et al., 2009; Zou et al., 2017; Maynou et al., 2019; Zhang et al., 2019; Penati et al., 2021; Ma et al., 2022). As a result, these findings may not be fully applicable to calves turned out to pasture following weaning, as the gastrointestinal tract microbiota of ruminants can vary widely based on diet (O'Callaghan et al., 2018).

Several studies have examined the effects of WMF on calf growth performance, but their results conflict. Some studies have suggested that WMF decreases ADG (Penati et al., 2021), whereas others showed that it could increase ADG (Zou et al., 2017; Maynou et al., 2019; Zhang et al., 2019; Ma et al., 2022), or have no effect (Pereira et al., 2014; Li et al., 2019). Variations in the duration of WMF and the nutritional composition of WM fed make it difficult to draw firm conclusions (Zou et al., 2017).

Several studies focusing on the preweaning period suggest a link between WMF and compromised calf health, particularly an increased risk of diarrhea (Calderón-Amor and Gallo, 2020; Penati et al., 2021). Feeding WM containing antibiotic residues can introduce these antibiotics into the calf's gut, potentially disrupting the commensal microbiota, which is essential for the development of mucosal immunity (Amin and Seifert, 2021). Oral antibiotics are known to alter the intestinal microbiota in other animals, leading to dysbiosis and a higher risk of diarrhea (Gomez et al., 2022). This is particularly concerning for calves, as diarrhea is a leading cause of mortality and neonatal enteric immunity relies on stabilizing the gut microbiota (Amin and Seifert, 2021; Gomez et al., 2022). Other studies, however, have found no link between antibiotics in milk or milk replacers (**MR**) and diarrhea (Langford et al., 2003; Thames et al., 2012). Some research suggests that WMF may be linked to poorer health due to increased systemic inflammation (Deng et al., 2017; Zou et al., 2017; Penati et al., 2021), although the extent and duration of this inflammation remain unclear. Serum amyloid A (**SAA**), an acute phase protein, is a key inflammatory biomarker used to assess inflammation (Trela et al., 2022). We measured SAA to monitor potential inflammatory responses associated with WMF and its influence on calf health.

Further research is also needed on the link between WMF and the shedding of AMR bacteria in calves. Already, WMF has been linked to the emergence of antibiotic-resistant pathogens (Langford et al., 2003; Brunton et al., 2012; Maynou et al., 2019; Dupouy et al., 2021; Firth et al., 2021), which can pose risks to both human and animal health (Mathew et al., 2007). Studies suggest dairy calves may serve as reservoirs for AMR on farms (Salerno et al., 2022), but it remains unclear whether this resistance persists in calves' feces as they age.

The objective of this study was to investigate the effects of antibiotic residues in WM following intramammary administration of antibiotics on dairy calf growth, health, fecal microbiota, systemic inflammation, and the emergence of antibiotic-resistant bacteria, while addressing gaps in understanding both pre- and postweaning responses thereof. We hypothesized that feeding simulated waste milk (**SWM**) would not significantly affect growth and health outcomes, but that the antibiotics would alter calves' fecal microbiome and promote emergence of antibiotic-resistant bacteria in a duration-dependent manner.

MATERIALS AND METHODS

Animals and Experimental Treatments

Ethical approval to complete the study was granted by the Teagasc Animal Ethics Committee (TAEC2021-324), and the Health Products Regulatory Authority (AE19132/P152). Experiments were undertaken in accordance with the European Union (Protection of Animals Used for Scientific Purposes) Regulations 2012 (S.I. No. 543 of 2012). This experiment used a randomized block design with 3 experimental treatments and 2 replicates within each treatment group. A total of 87 dairy heifer calves were initially enrolled in the trial as they were born.

Power calculations were conducted using SAS (PROC POWER; version 9.4, SAS Institute Inc., Cary, NC).

The power calculations for calf weight and ADG were based on data from (A. Flynn., 2023, Teagasc Animal and Grassland Research, Fermoy, Co. Cork, Ireland; unpublished data), with SD of 3.34 kg, α of 0.05, and power of 0.811, indicating that 22 calves per treatment were required. The metagenomic calculations employed data from Penati et al. (2021) based on the average number of sequences per treatment and time point, with SD of 72,109 and α of 0.80, this required a sample size of minimum 10 calves per treatment; 29 calves per treatment group were initially enrolled to account for potential dropouts. From these 29 calves, a subset of 10 calves were selected for additional sample collection and analysis, including SAA measurements and metagenomic fecal sequencing.

The experimental treatment groups were as follows:

(1) Long-term antibiotic (**LTA**), whereby calves were fed SWM from 4 d old until weaning at 12 wk of age.
(2) Short-term antibiotic (**STA**), whereby calves were fed SWM for 2 wk from 3 to 5 wk of age. Calves were fed conventional (antibiotic-free) MR from 4 d to 3 wk of age and again from 6 wk of age until weaning at 12 wk of age.
(3) Control (**CONT**), with fed conventional (antibiotic-free) MR from 4 d old until weaning at 12 wk of age.

Calves were randomized and balanced across treatment groups according to breed (LTA replicate 1 = 10 Holstein Friesian [**HF**], 1 Jersey [**JE**], 6 Holstein Friesian × Jersey [**JEX**]; LTA replicate 2 = 9 HF, 2 JEX; STA replicate 1 = 9 HF, 8 JEX; STA replicate 2 = 7 HF, 1 JE, 4 JEX; CONT replicate 1 = 11 HF, 5 JEX; CONT replicate 2 = 7 HF, 1 JE, 3 JEX), birth weight (mean ± SD; replicate 1 = 33.8 ± 5.5 kg; replicate 2 = 33.7 ± 6.2 kg), and birth date (replicate 1 = Jan. 29 ± 7.2 d; replicate 2 = Feb. 16 ± 6.3 d). As the study progressed, 3 calves were removed due to morbidity (abomasal bloat and pneumonia; not treatment related)—1 from the LTA treatment group and 2 from the CONT treatment group. These calves were excluded from the final analysis because their illnesses resulted in incomplete data. For weight and health data analysis, the final sample sizes were LTA n = 28, STA n = 29, and CONT n = 27. For fecal microbiota and SAA analysis, the sample sizes were LTA n = 10, STA n = 10, and CONT n = 10. Throughout the trial, the researchers were not blinded to the calves' experimental treatment.

Pre-Experimental Management

All births were supervised, and calves were immediately separated from their dam. Calves were then weighed (Tru-Test XR 3000, Tru-Test Ltd., Auckland, New Zealand), tagged, and moved to an individual pen. All calves were fed 3 L of colostrum from a single cow, but not necessarily their dam (Barry et al., 2019). Colostrum was given as a single feed via bottle and teat. An esophageal tube was used if the calf refused to drink its colostrum voluntarily. Colostrum quality was tested using a Brix refractometer (HI96801, Hanna Instruments); calves were only fed colostrum of 22% Brix or greater (equivalent to >50 mg/mL IgG; Bielmann et al., 2010).

Calves were fed five 3-L feeds of transition milk twice daily using individual buckets equipped with teats. This transition milk was combined second and third milkings of recently calved cows. At 4 d old, calves were moved and penned in groups according to their respective treatment. In the group pens, calves were fed their respective MR-based diets (Heiferlac, 26% CP content, Volac International Ltd., Hertfordshire, UK) using a VARIOsmart automatic feeding system (**AFS**; FörsterTechnik, Engen, Germany). Calves were taught how to use the AFS over 24 h. The AFS read the calf's radio-frequency identification ear tag and dispensed MR or SWM based on the individual's treatment and age.

Housing and Biosecurity

Calves were accommodated in straw-bedded pens (depth of 15 cm), with a space allocation of >1.7 m^2 per calf. These pens were thoroughly cleaned out once a week and replenished with fresh straw twice weekly. Before cleaning, all straw from the pens was treated with hydrated lime (calcium hydroxide) and then removed and transported to the farm dung stead. Pen floors were then disinfected using FAM 30 (iodophor disinfectant, Evans, Livestock Protection, Lancashire, UK), after which fresh straw was introduced. Additionally, the floors of the calf pens, walkways, and preparation areas within the calf house underwent disinfection twice daily in the morning and evening. To prevent cross-contamination, the LTA and STA calves were housed separately in a similar calf house adjacent to the CONT group. Each treatment group was penned separately, and calves did not have direct contact with animals from other pens. A boot wash station was established outside the calf shed, where boots were disinfected, and gloves were changed when transitioning between treatment groups.

Simulated Waste Milk Formulation

A SWM was created by incorporating antibiotics into a conventional MR. This approach aimed to eliminate any potential confounding effects due to variations in the nutritional composition of raw WM. Amoxicillin and neomycin were added at concentrations of 1.68 mg/L and 2.28 mg/L, respectively. These concentrations were

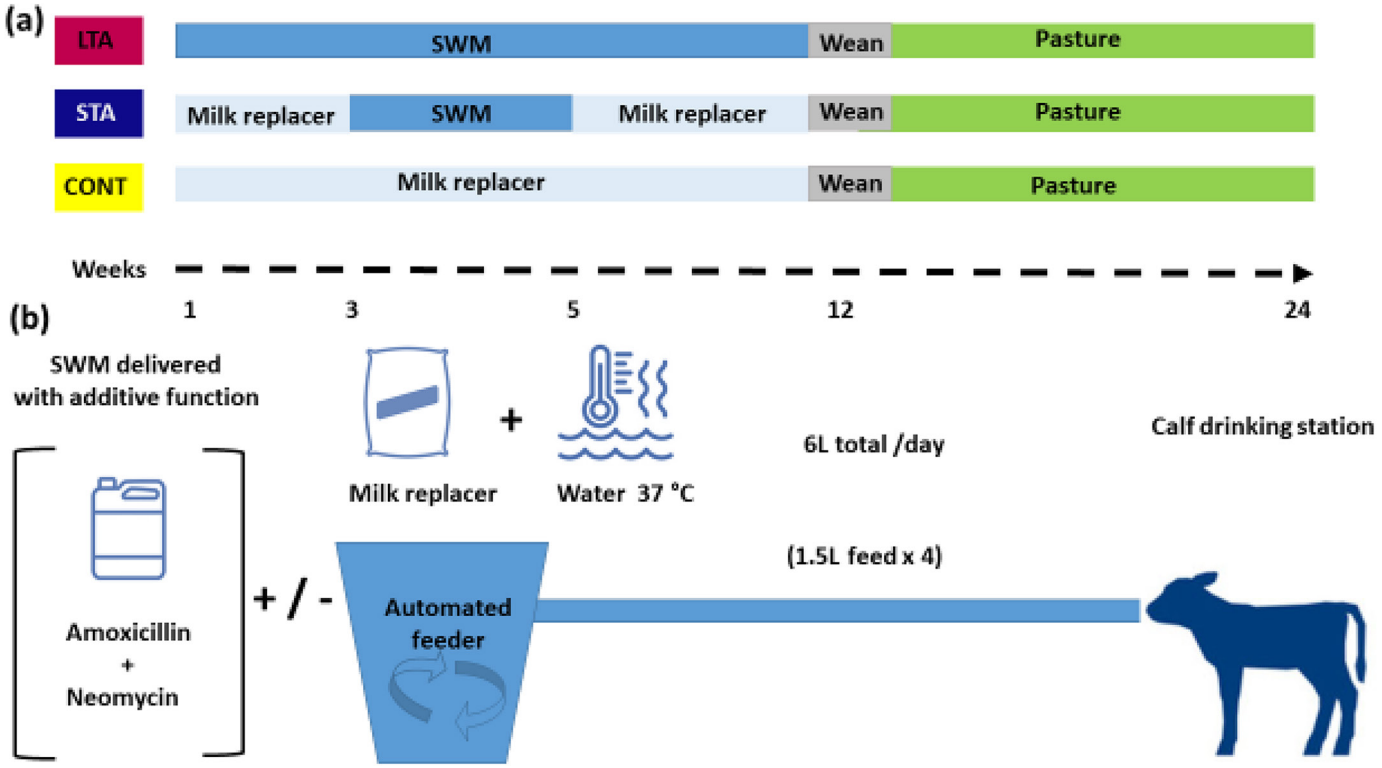

Figure 1. Delivery and preparation of simulated waste milk (SWM) via automated feeder, along with the feeding plans for each treatment group. Preweaning occurred from wk 1–11, weaning took place at wk 12, and postweaning occurred from wk 13–24. Note: all calves were gradually weaned between wk 9–12 and stopped receiving milk completely at 12 wk of age. The long-term antibiotic (LTA) group was fed the SWM formulation containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin from 3 d to 12 wk of age. The short-term antibiotic (STA) group was fed the same SWM formulation for 2 wk, from 3 to 5 wk of age. The control (CONT) group was fed a conventional milk replacer with no antibiotics.

akin to levels observed in the milk of mastitic cows 2 d after intramammary antibiotic treatment and lower than those typically required for bactericidal or bacteriostatic effects (Moretain and Boisseau, 1989, 1993). A 4-L drum containing the antibiotic solution was prepared and connected to the AFS every 2 d. This antibiotic solution could then be integrated into the freshly mixed MR using the additive feature of the AFS (Förster-Technik, Engen, Germany; Figure 1). During their SWM feeding period (i.e., 12 wk for LTA and 2 wk for STA), the calves in LTA and STA received a total daily dose of 10.08 mg of amoxicillin and 13.68 mg of neomycin. These doses were provided in the first 1.5-L feeding of the day. Samples of MR and SWM were collected from the AFS at the drinking station teats every 2 d and tested to verify the absence or presence of antibiotics using the Delvo test (Delvotest DSM, Heerlen, the Netherlands).

Feeding Plans

Each calf received the same amount of MR to ensure consistency across treatments. The AFS mixed MR at a rate of 125 g/L at 37°C (Figure 1). The MR was prepared first, and then the appropriate antibiotic solution was added according to the calf's treatment (Figure 1). Starting at 4 d old, calves were fed 5 L of MR per day, gradually increasing to 6 L by 7 d. They were each given 4 feedings of 1.5 L each, spaced evenly throughout 24 h. Once a calf finished a 1.5-L feeding, they had to wait 4 h before access to the next feeding was granted. From 9 to 12 wk, the MR allocation was reduced gradually from 6 L to 0, with full weaning completed by 12 wk. From 3 d of age, calves had ad libitum access to water, forage (perennial ryegrass hay), and concentrates (18% CP; Prime Elite Krispi Kaf, Dairygold Agri Business, Cork, Ireland; ingredients: barley, soy meal, sugar beet pulp, distillers grains, rape seed meal, and maize). Concentrates were fed ad libitum in troughs, and all calves consumed at least 1 kg of concentrates per day during the weaning period. At 1 to 2 wk after weaning (when calves were 13–14 wk of age), they were moved outside to pasture. The calves grazed on perennial ryegrass and white clover or multispecies swards (perennial ryegrass, white clover, plantain, and timothy grass). They were offered fresh pasture every 2 d. A pregrazing yield between 1,400 and 1,600 kg DM/ha (>4 cm) was targeted. While at grass, calves were

supplemented with hay during the first month to ensure adequate fiber in their diet. Concentrate supplementation at pasture was based on the availability of grass.

Animal Measurements

Weighing. Calves were weighed weekly prior to weaning and fortnightly after weaning (Tru-Test XR 3000, Tru-Test Ltd., Auckland, New Zealand) to calculate the calves' individual ADG as the experiment proceeded.

Blood Sampling. A 10-mL blood sample was obtained using a 20-gauge needle and a serum red top tube (Vacutainer, Vaud, Switzerland) from the jugular vein of a subset of calves at exactly 24 h following birth. The blood samples were tested with a Brix refractometer (Hernandez et al., 2016) to estimate IgG in calf serum at 24 h of age and assess rates of passive transfer of immunity. Additionally, to assess SAA concentrations, serum samples were also collected at 6 time points (age ± SD) during the experiment, when the calves were at an average age of 1 wk (±0.3), 3 wk (±0.5), 5 wk (±1.3), 10 wk (±1.4), 15 wk (±1.8), and 21 wk (±2.3). After collection, all blood samples were refrigerated at 0 to 4°C and left to coagulate for 24 h. They were centrifuged at 10, 062 × *g* at ~17°C for 15 min and then frozen at −20°C until analysis.

Health Scoring. Calves were health-scored twice weekly from birth until 20 wk of age using a modified calf health scoring system, adapted from Barry et al. (2020; Supplemental Table S1, see Notes). This scoring system involved a thorough evaluation of various health parameters, including respiratory rate, coughing, fecal cleanliness, characteristics of the navel tract, nasal and ocular discharge, ear position, mobility, dehydration, demeanor, and interest in surroundings. Each parameter was scored from 0 to 3, with 0 indicating normal (optimal) and 3 indicating severe impairment. Calves were scored on 2 nonconsecutive days per week. All data were categorized into preweaning, weaning, and postweaning stages. The same 2 researchers scored the animals each week to ensure consistency.

Daily monitoring was also performed by the 2 researchers, along with suitably trained farm staff responsible for the calves' husbandry, to detect any changes in demeanor or abnormalities such as poor condition, ill thrift, diarrhea, nasal or ocular discharge, or reduced milk intake. Instances of diarrhea were managed through oral rehydration therapy throughout the study. As the study progressed, 3 calves had to be removed due to illnesses or morbidity (abomasal bloat and pneumonia): 1 from the LTA treatment and 2 from the CONT treatment. Due to incomplete data for these animals, they were excluded from the final analysis. Treatment records were kept for all animals, and any illnesses necessitating additional therapeutic antibiotics were considered "veterinary interventions" (**VI**).

Fecal Sampling. Naturally voided individual fecal samples were collected from all the calves in 70-mL specimen containers (Sarstedt, Nümbrecht, Germany) approximately once per week from birth to 25 wk of age. Upon collection, the samples were refrigerated at temperatures between 1°C and 5°C. They were stored for up to 7 d, and samples were then split before undergoing microbiological analysis. The remaining fecal matter was frozen at −80°C and stored for DNA extraction. A subset of 10 calves per treatment with the most appropriate spread of sampling frequency was then selected for DNA extraction and metagenomic sequencing analysis.

Environmental Monitoring. Throughout the preweaning and weaning periods (when calves were housed) indoor temperature and relative humidity were monitored using the Tiny Tag system (Gemini Data Loggers Ltd., Chichester, West Sussex, England). Environmental sponge swabs (Medray, Dublin, Ireland) of the pen floors, AFS drinking stations, and the boots and gloved hands of personnel feeding the calves were taken weekly throughout the preweaning period to determine total putative *Escherichia coli* and *E. coli* that produce extended-spectrum β-lactamase (**ESBL**). Sponges were dampened with PBS (product BM1360, E&O Laboratories, Bonnybridge, UK) before swabs were taken. After swabbing, the sponges were stored in 10 mL of PBS solution and refrigerated at 1°C and 5°C (E&O Laboratories, Bonnybridge, UK) for up to 24 h before plating.

Laboratory Analysis. Serum amyloid A levels were measured using an ELISA kit (Life Diagnostics Inc., West Chester, PA). Serum samples were diluted 400-fold, and the subsequent assays were prepared according to the kit standard procedure. Plate washing was carried out on a BioTek ELx405 select CW microplate washer (Agilent Technologies Inc., Santa Clara, CA), assays were read at an absorbency of 450 nm using a BioTekEL808 microplate plate reader, standard curves were prepared, and sample concentrations calculated using the BioTek Gen5 version 3.12 software (Agilent Technologies Inc., Santa Clara, CA).

All calf fecal samples and environmental swabs underwent screening using chromogenic agar plates to identify putative *E. coli* colonies and ESBL-producing *E. coli*. The testing protocol was adapted from the EURL-AR protocols to isolate and quantify ESBL/Amp C-producing *E. coli* (Bortolaia and Hendriksen, 2017). For plating, 1 g of feces was suspended in 9 mL of PBS (E&O Laboratories, Bonnybridge, UK) and vortexed for 5 s. The suspension was prepared at a rate of 1 in 10 (wt/vol), where 9 mL of the solution was added to 1 g of feces to create a 10-mL suspension in PBS. For the environmental swabs, 1 mL of the sample suspension solution was used. The sample

was then serially diluted, and 100 μL of the dilution 10^6 was spread plated onto the surface of Type III Mac Conkey Agar and ESBL chromogenic agars (E&O Laboratories, Bonnybridge, UK). Each sample was plated on both types of agar in duplicate. These plates were then incubated at 44°C for 22 h ± 2 h.

Putative *E. coli* colonies were identified by their characteristic red/purple morphology on the MacConkey or ESBL agars and counted. The final count for each sample was determined as the arithmetic mean of the counts for each replicate. The percentage of resistant *E. coli* (ESBL producers) was calculated by dividing the mean count from the selective plates by the mean count from the nonselective plates.

Microbiota DNA Extraction and Data Generation and Analysis. Shotgun metagenomic sequencing was performed to analyze the fecal microbiota. First, fecal samples were thawed and DNA was extracted and purified using the QIAamp PowerFecal Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA was quantified with the Qubit Fluorometer system using the Qubit dsDNA Broad Range Assay Kit (Life Technologies). DNA libraries were prepared with the Illumina DNA Prep Kit, following the corresponding protocol (Illumina Inc., San Diego, CA; https://support.illumina.com/sequencing/sequencing_kits/illumina-dna-prep/documentation.html). Briefly, this workflow entailed the tagmentation of the genomic DNA, a post-tagmentation cleanup, incorporation of unique dual indices (UDISl, Integrated DNA Technologies), and amplification of the tagmented DNA using PCR, and a final library cleanup after PCR. Following library preparation, samples were run on the Agilent bio analyzer (Agilent) to determine the average size of each sample and quantified using the Qubit Fluorometer System with Qubit dsDNA High Sensitivity Assay Kit (Life Technologies). Libraries were pooled equimolarly and sequenced with 2 × 150 cycles on the Illumina NovaSeq 6000 System according to the manufacturer's instructions.

Bioinformatic Processing and Analysis of Sequencing Data. Raw sequencing data were prepared for analysis by undergoing quality control with Kneaddata (v0.10; https://huttenhower.sph.harvard.edu/kneaddata/), which removed poor-quality and contaminant reads (sourced from the *Homo sapiens* and *Bos taurus* genomes; Supplemental Files: Materials and Methods, Bioinformatic Processing and Analysis of Sequencing Data [continued]; see Notes). Processing of raw sequence data produced a total of 2.64 billion filtered reads with a mean read count of 6,849,861 (±9,134,524 SD) per each of the 200 samples. These refined reads were then assigned taxonomy and genetic function. The taxonomic profile was generated from Kraken2 (version 2.1.1; https://zenodo.org/records/3520272; Lu et al., 2022), and the profile of functional features (e.g., microbial metabolic pathways) was generated with the HUMAnN software pipeline (version 3.6; Supplemental Files: Materials and Methods, Bioinformatic Processing and Analysis of Sequencing Data [continued]; Abubucker et al., 2012; Lu et al., 2022). HUMAnN was further used to estimate AMR by mapping quantified UniRef90 gene families to Kyoto Encyclopedia of Genes and Genomes orthogroups (**KO**), of which AMR-related KO were subset for targeted analysis. Additional organization of metabolic pathways within a functional ontology was performed with in-house scripts. Data in these profiles was represented as estimated read abundance (count). The data were then imported to R (version 4.3.2, R Core Team), where the remainder of microbiota-related data processing and analysis occurred. Before analysis, the abundance data were filtered to exclude values <1,000 and with prevalence across all samples <0.25; subsequently, empty samples and variables with totals of 0.0 were removed. All microbiota data were statistically analyzed in the R software environment.

Microbial Community Dynamics Analysis. Measurements of diversity, including α diversity with Shannon diversity index and feature count, as well as β diversity using nonmetric multidimensional scaling (**NMDS**), were conducted to assess the overall variation within and between the treatment groups. These were calculated with the Vegan R package (version 2.5-6; Supplemental Files: Materials and Methods, Microbial Community Dynamics Analysis [continued]; see Notes). Taxonomic profiles were subset for accompanying comparisons according to phylogenetic level (e.g., phylum and all species belonging to *Archaea*). Graphical elements were generated using the ggplot2 package (version 3.31; Wickham, 2016).

Statistical Analysis

All health and weight data were categorized into 3 distinct calf development stages: preweaning (wk 1–11), weaning (wk 12; note that weaning weight measurement was taken immediately before the cessation of milk feeding in wk 12), and postweaning (wk 13–20). Statistical animal health and weight data analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary, NC). Calf was considered as the experimental unit.

Linear mixed models (PROC MIXED) were used to assess the effects of treatment on growth (BW and ADG) and SAA concentrations (in ng/mL) at each developmental stage. Normality was evaluated using PROC UNIVARIATE. Both BW and ADG variables exhibited a normal distribution pattern, but SAA concentrations were skewed, so they were log-transformed [$\log_{10}$ (SAA concentration, ng/mL + 1)]. Significant associations were determined at $P < 0.05$, with least squares means evalu-

Table 1. Least squares means of BW across all weeks, categorized into stages[1]

	Body weight (kg) by treatment							
Stage	STA	(n)	LTA	(n)	CONT	(n)	SE	*P*-value
Preweaning (wk 1–11)	54.4	27	56.1	28	54.9	27	1.93	0.366
Weaning (wk 12)	86.9	27	90.4	28	87.3	27		0.186
Postweaning (wk 13–24)	112.9	27	113.6	28	109.1	27		0.206

[1]All calves were gradually weaned from wk 9–12 and fully stopped receiving milk at 12 wk of age. LTA = long-term antibiotic group, fed the simulated waste milk (SWM) containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin from 3 d to 12 wk of age. STA = short-term antibiotic group, fed SWM containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin for 2 wk, from 3 to 5 wk of age. CONT = control, fed a conventional milk replacer containing no antibiotics.

ated. Treatment, breed, and replicate were considered as categorical variables. The models' fixed effects included treatment and breed, with birth weight centered by breed included as a covariate. The individual calf was included as a random effect. To accommodate the varying ages of calves at each sampling event for SAA concentrations (mg/mL), the individual calf's age in days was added as an additional covariate in this model.

To simplify the health data for analysis, health scores for each factor were condensed into 2 categories: a binary overall health (**OH**) score. Calves with scores of 0 or 1 in all health factors were classified as having "good/satisfactory" health (OH = 0), and those with a score of 2 or 3 in at least one factor were classified as having "poor" health (OH = 1) on that day. Logistic regression (PROC LOGISTIC) was then used to model the association between treatment and the probability of calves having a "poor" OH score.

In this analysis, "good/satisfactory" health (OH = 0) was considered the reference category for overall health, and the CONT treatment group was used as the reference category for treatment. Treatment, breed, and replicate were the main effects, and age (at scoring) and birth weight centered by breed were included as covariates. Logistic regression (PROC LOGISTIC) was also used to predict the likelihood of a calf needing VI or not during each stage of calf development. We developed a second model to examine the association between treatment and the probability of calves requiring VI; this model used the same structure as the first, with outcome changed to VI (1) or not (0)

Fecal microbiota data were categorized into 6 distinct periods based on days of age (**DOA**) of the calf at collection of the fecal sample (period 1: 0–10 DOA; period 2: 25–45 DOA; period 3: 60–80 DOA; period 4: 95–115 DOA; period 5: 130–150 DOA; and period 6: 165–185 DOA). Nonparametric statistical tests were used to analyze microbiota data. An analysis of similarities (**ANOSIM**, a nonparametric statistical test) using the Bray-Curtis dissimilarity matrix served as the input for NMDS plots. The Kruskal–Wallis (**KW**) test was used when comparing all treatment groups, and Wilcoxon rank sum and signed rank tests were used appropriately for paired and pairwise comparisons. *P*-value correction for multiple comparisons was used throughout the Benjimini-Hochberg procedure, and a predicted false discovery rate (**pFDR**) <0.05 was used to determine significance, all performed in R.

RESULTS

Calf Weight and Average Daily Gain

Treatment did not affect calf weight. Calf weights were not statistically different across treatments before weaning (55.1 kg), at weaning (88.2 kg), or after weaning (111.9 kg; Table 1). There were no differences in ADG between treatments before weaning (wk 1–11: 0.68 kg/d, $P = 0.389$; Figure 2), at weaning (wk 12: 0.99 kg/d, $P = 0.751$; Figure 2), or after weaning (wk 13–24: 0.55 kg/d, $P = 0.180$; Figure 1).

Calf Health

Transfer of passive immunity was similar across all treatments ($P > 0.05$). Average serum Brix percentage in the sampled subsets of calves was 10.7% in the STA group (n = 19), 10.4% in the LTA group (n = 19), and 10.1% in the CONT group (n = 17). Health scores were generally good, with the majority of animals (>75%) in all 3 treatments having OH score = 0, considered indicative of "good/satisfactory" health, at all 3 stages: before, during, and after weaning (Supplemental Table S2, see Notes).

During the preweaning period, LTA calves were less likely to have a "poor" OH score (score ≥2 in at least one individual health factor) compared with CONT calves (odds ratio [**OR**] = 0.65; 95% CI = 0.47–0.89). The odds ratios of a calf having a "poor" OH score were similar between the LTA and STA groups (OR = 1.15; 95% CI = 0.82–1.60) during the preweaning period; the STA and CONT groups also had a similar odds ratios of a calf having a "poor" OH score (OR = 0.74; 95% CI = 0.54–1.02).

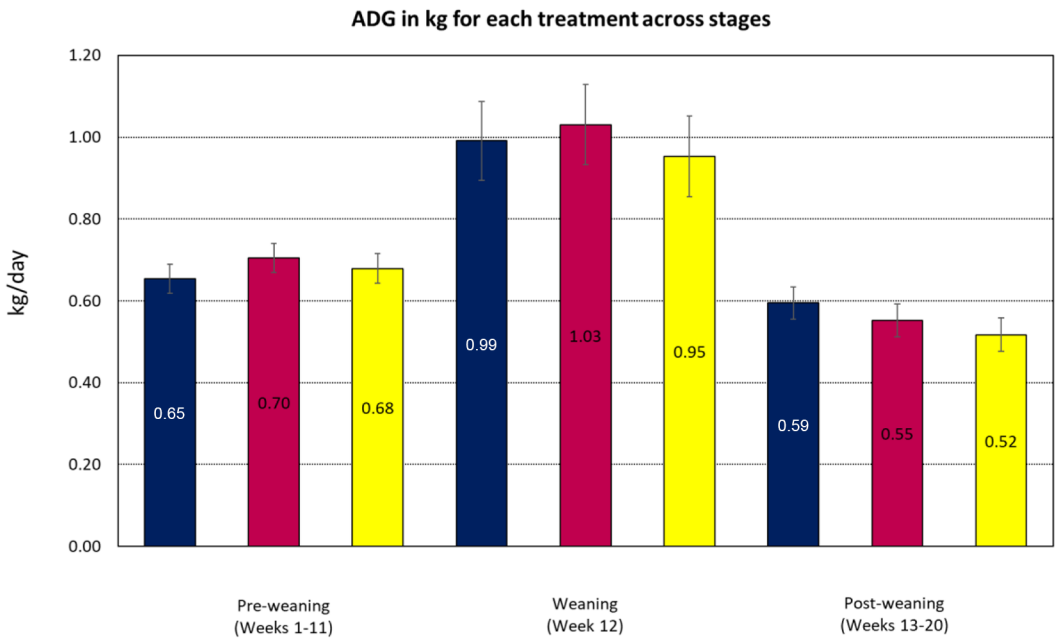

Figure 2. Least squares means of ADG (kg) across all weeks, categorized into stages. The LSM is represented by the height of the columns, and error bars represent SE. Preweaning = wk 1–11, weaning = wk 12, postweaning = wk 13–24. Note: all calves were gradually weaned from wk 9–12 and fully stopped receiving milk at 12 wk of age. The long-term antibiotic group (LTA) was fed the simulated waste milk (SWM) formulation containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin from 3 d to 12 wk of age. The short-term antibiotic group (STA) was fed the SWM formulation containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin for 2 wk from 3 to 5 wk of age. The control group (CONT) was fed a conventional milk replacer containing no antibiotics. Weaning weight measurement was taken immediately before cessation of milk feeding in wk 12.

All treatment groups had similar OH scores at weaning ($P = 0.948$) and after weaning ($P = 0.227$; Supplemental Table S2). In terms of individual health parameters, all treatments had similar fecal scores during the preweaning stage (wk 1–11; $P = 0.395$). During weaning (wk 12), treatment approached significance in affecting the odds ratio of calves exhibiting a fecal score ≥2 ($P = 0.051$). The LTA calves were more likely to have fecal scores ≥2 compared with the CONT group (OR = 5.4; 95% CI = 1.09–27.08), but fecal scores were similar between the STA and LTA groups (OR = 0.28; 95% CI = 0.07–1.15), as well as between the STA and CONT groups (OR = 1.5; 95% CI = 0.24–9.87). Following weaning, treatment significantly affected the odds ratio of calves exhibiting a fecal score ≥2 ($P = 0.010$). After weaning (wk 13–20), the STA group was more likely to have a fecal score ≥2 compared with both the CONT group (OR = 2.3; 95% CI = 1.23–4.47) and the LTA group (OR = 2.2; 95% CI = 1.19–4.14); the STA and CONT fecal scores were similar (OR = 1.1; 95% CI = 0.53–2.12). We found no significant differences between treatment groups for nasal discharge, ocular discharge, or cough scores during the weaning and postweaning periods ($P > 0.05$). However, before weaning, the STA and LTA groups had significantly lower odds of having a navel tract score ≥2 compared with the CONT group ($P = 0.002$). Both the STA and LTA groups also had significantly lower odds of having a cough score ≥2 ($P = 0.002$) during the preweaning period, but these differences were not observed later (see Supplemental Files: Results, Calf Health [continued]; see Notes).

Prior to weaning (wk 1–11), 31.0% of the STA calves, 39.3% of the LTA claves, and 29.6% of the CONT calves received at least 1 VI. No VI were required during weaning (wk 12). After weaning (wk 13–24), 21.2% of the STA calves, 23.0% of the LTA claves, and 19.3% of the CONT calves received at least 1 VI. We detected no before weaning ($P = 0.720$) or after weaning ($P = 0.234$).

Serum amyloid A levels were higher in the LTA (10.6 ± 0.1 ng/mL) than the CONT group (7.4 ± 0.1 ng/mL; $P = 0.044$) prior to weaning, whereas STA (8.6 ± 0.1 ng/mL) and LTA ($P = 0.342$) were similar, as were STA and CONT ($P = 0.594$). Following weaning, treatment had no effect on SAA levels; all treatments were similar, with an average SAA concentration of 1.7 ± 0.1 ng/mL ($P = 0.50$).

Environment and ESBL Shedding

Swabs taken from the boots and gloved hands of personnel involved in calf feeding and screened for ESBL-producing *E. coli* via selective plating revealed the presence of ESBL-producing isolates only on staff boots when the calves were 10.5 ± 1.94 wk old. No ESBL-producing

Table 2. Prevalence of ESBL-producing *Escherichia coli* isolated on ESBL chromogenic agar, expressed as a percentage of total putative *E. coli* isolated on type III MacConkey agar from calf fecal samples[1]

		Prevalence of ESBL-producing *E. coli* (%) in feces by treatment								
		STA			LTA			CONT		
Month	Age, wk ±SE	% ESBL[2]	± SE	n[3]	% ESBL	± SE	n	% ESBL	± SE	n
February	2.8 ± 1.30	10.9%	35.31	39	9.7%	19.05	27	1.99%	8.87	32
March	6.5 ± 2.10	0.95%	3.952	45	1.1%	7.45	45	1.2%	5.54	46
April	10.5 ± 1.94	0.00%	0.00	51	0.00%	0.00	52	0.01%	0.076	46
May	14.2 ± 2.27	0.00%	0.00	46	0.00%	0.00	48	0.00%	0.00	30
June	17.4 ± 1.46	0.00%	0.00	8	0.00%	0.00	15	0.00%	0.00	12

[1]LTA = long-term antibiotic group, fed the simulated waste milk (SWM) containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin from 3 d to 12 wk of age. STA = short-term antibiotic group, fed SWM containing 2.28 mg/L of neomycin and 1.68 mg/L of amoxicillin for 2 wk, from 3 to 5 wk of age. CONT = control, fed a conventional milk replacer containing no antibiotics.

[2]% ESBL = percentage of total fecal samples screened as positive for ESBL-producing *E. coli*.

[3]n = total fecal samples collected per treatment per month.

isolates were detected on staff gloved hands at any time point. Environmental swabs collected from calf pens indicated the presence of ESBL-producing isolates on the AFS teat of the LTA treatment on 2 occasions: once when calves were 2.8 (±1.30) wk old and again when calves were 10.5 (±1.94) wk old. However, ESBL-producing isolates were not found on the AFS teat of the STA and CONT groups. No ESBL-producing isolates were found on the pen floor of any treatment. Plate agar screening confirmed the presence of ESBL-producing isolates in the feces of all treatment groups (Table 2), with the highest prevalence noted when the calves were ~2.8 ± 1.30 wk old (Table 2); also, a higher number of ESBL-producing fecal isolates were observed in the STA and LTA calves compared with the CONT group (Table 2).

Taxonomic Response to Treatment

Diversity measures of the fecal microbiota demonstrated divergence in the communities associated with each treatment across the study's duration. Changes in α diversity were captured with the Shannon H-index and species count (Figure 3A). The trajectories of Shannon diversity initially showed no significant differences between the groups, with all groups demonstrating a gradual increase until approximately d 84. After this point, however, a distinct divergence occurred: the LTA group exhibited relatively higher Shannon diversity after weaning than both the STA and CONT groups (Figure 3B). Comparisons of α diversity between subsets of phylogeny according to higher ranks indicated that bacteria were the main drivers of diversity dynamics (Supplemental Figure S1, see Notes). Beta diversity was shown with NMDS plots and demonstrated significant differences between the treatment groups at periods 2 and 3 (Figure 3B and C, respectively; ANOSIM $P < 0.05$). In period 2, distinct coordination or overlap between the LTA and STA samples was observed, as visualized in the NMDS plots. However, in period 3, the STA samples began moving closer to the CONT samples (Figure 3C and D). When comparing ordination plots of each period, samples began as similar and again returned to similarity after period 3, although complete coordination with CONT was never observed (Supplemental Figure S2, see Notes). Within-group ordination illustrates significant and distinct clustering by period in all groups for both species and metabolic categories (metabolic categories referring to a set of genes for a particular metabolic pathway, typically found downstream of one another on the chromosome; Supplemental Figures S3, S4, S5, and S6, see Notes; ANOSIM $P < 0.05$). Taxonomic differences at the phylum level were nonsignificant but showed deviating patterns from CONT in the STA and LTA groups for both high- and low-abundance taxa (Figure 3D). Overall, from period 1 to 6, a trend emerged of a gradually greater proportion of *Pseudomonadota* and a lower proportion of *Bacteroidota* across all 3 groups; the bacterium *Enterococcus faecium* was significantly altered between treatments (Figure 3A).

Antibiotic Resistance and Metabolic Categories

Several AMR genes showed significant variation in the numbers of mapped reads between treatments (Figure 4). In CONT, higher levels of mapped reads were observed for the ribosomal protection tetracycline resistance protein and the major facilitator superfamily (**MFS**) DHA3 family macrolide efflux protein in *Ruminococcus torques* during period 3 (Figure 4). Additionally, metabolic categories such as pyruvate degradation, valine biosynthesis, pyruvate acetate fermentation, and pyruvate lactate fermentation were significantly more abundant in the CONT group during period 3 (Figure 4). In contrast, higher levels of mapped reads for AMR genes, includ-

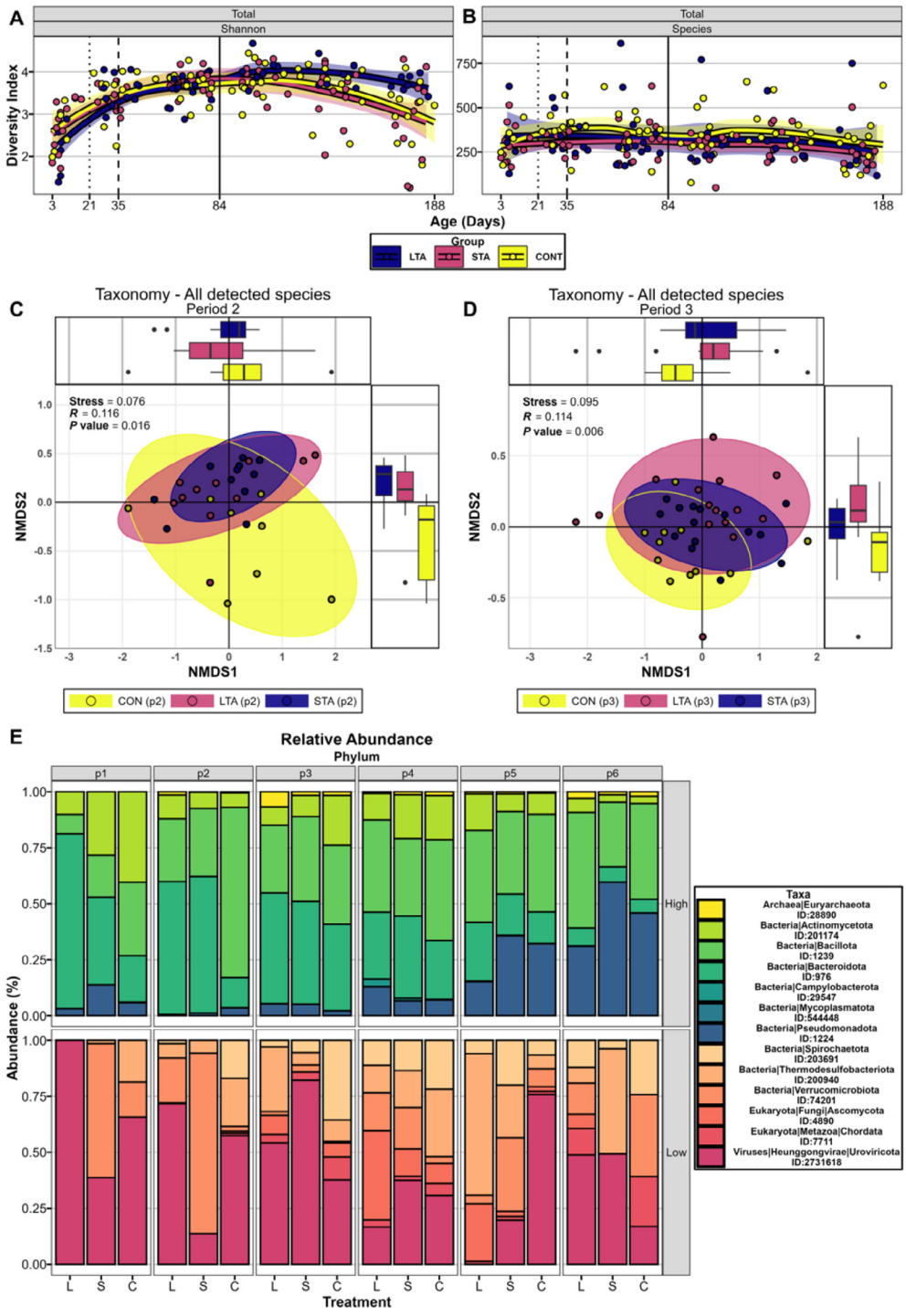

Figure 3. Diversity dynamics of the fecal microbiota change in response to time and treatment. (A) Shannon H-index and (B) species counts over the course of observation show differences in α diversity between the treatment groups. Shaded regions refer to 95% CI. The dotted vertical line indicates d 21 (i.e., 3 wk of age), when the feeding of the SWM to the STA treatment group began. The dashed vertical line indicates d 35 (i.e., 5 wk of age), when the feeding of SWM to the STA treatment group ceased. The solid vertical line indicates d 84, when calves across all treatment groups were weaned, defining the preweaning period (before d 84) and the postweaning period (after d 84). (C, D) Beta diversity, as demonstrated by NMDS for samples taken at periods 2 and 3, illustrates significant dissimilarity between groups and distinction between LTA and STA vs. CONT (ANOSIM *P*-value <0.05). Each boxplot shows the median (midline), interquartile range (IQR; box edges), NMDS value with whiskers extending to 1.5 × IQR, and outliers plotted as individual points. (E) Phylum-level scaled relative abundances of taxa show changes in proportions between the groups over time for both high- and low-abundance taxa. Periods are indicated as P1–P6; L (LTA) = long-term antibiotic group; S (STA) = short-term antibiotic group; C (CONT) = control group. Fecal microbiota data were categorized into 6 distinct periods based on calf days of age (DOA) at collection of the fecal sample (P1: 0–10 DOA, P2: 25–45 DOA, P3: 60–80 DOA, P4: 95–115 DOA, P5: 130–150 DOA, and P6: 165–185 DOA).

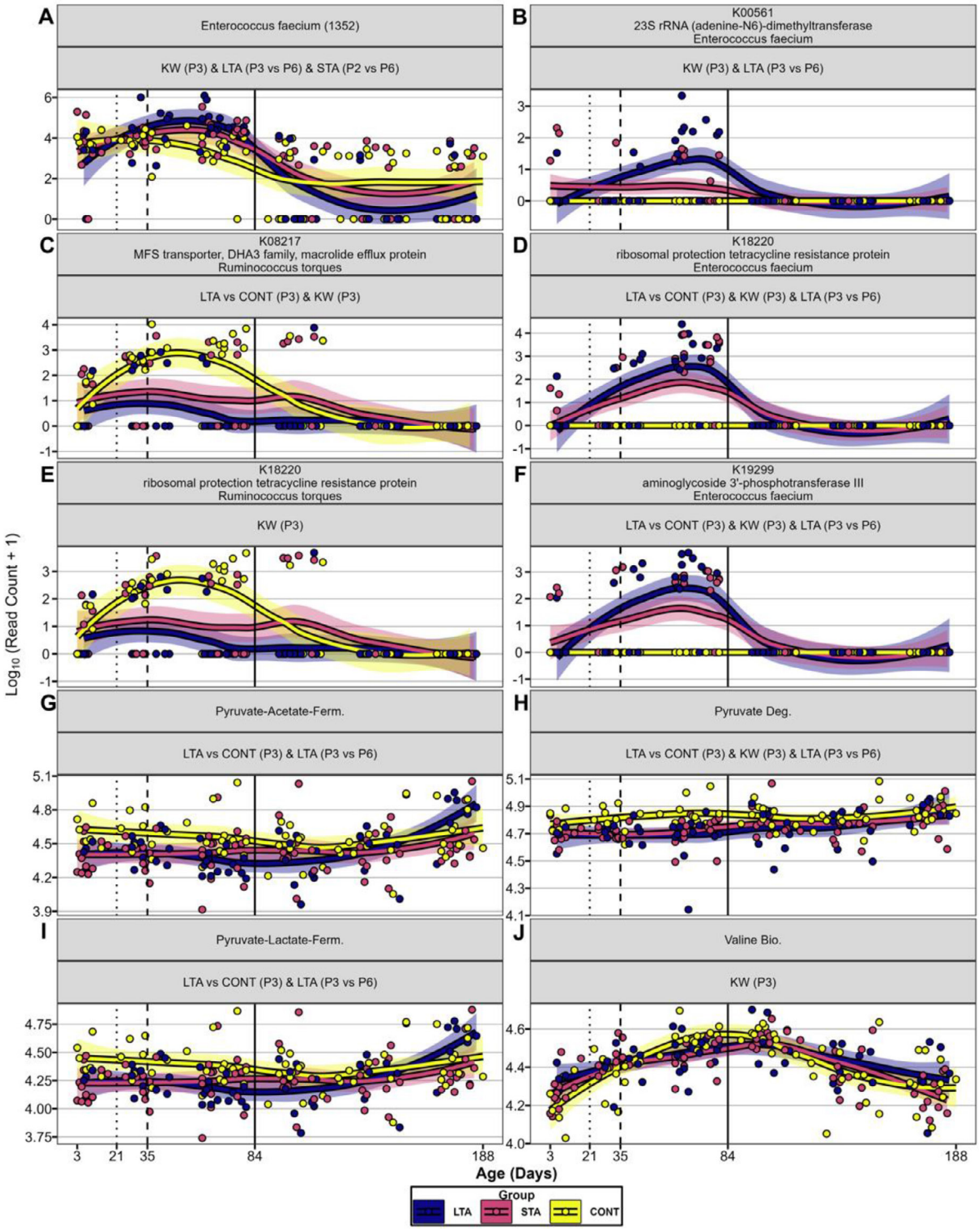

Figure 4. Treatment-associated changes in microbiota features. Significantly varied features of the fecal microbiota include (A) *Enterococcus faecium*, (B–F) KO terms related to AMR, and (G–J) metabolic categories. All statistical tests are described in panels, and unless Kruskal–Wallis (KW), are Wilcoxon, both with pFDR <0.05. Shaded regions refer to 95% CI. The dotted vertical line indicates d 21 (i.e., 3 wk of age), when the feeding of the SWM to STA treatment group began. The dashed vertical line indicates d 35 (i.e 5 wk of age), when the feeding of SWM to the STA treatment group ceased. The solid vertical line indicates d 84, when calves across all treatment groups were weaned, defining the preweaning period (before d 84) and the postweaning period (after d 84). Periods are indicated as P1–P6; LTA = long-term antibiotic group; STA = short-term antibiotic group; CONT = control group; Ferm = fermentation; Bio = biosynthesis; Deg = degradation. Fecal microbiota data were categorized into 6 distinct periods based on calf days of age (DOA) at collection of the fecal sample (P1: 0–10 DOA, P2: 25–45 DOA, P3: 60–80 DOA, P4: 95–115 DOA, P5: 130–150 DOA, and P6: 165–185 DOA).

ing aminoglycoside 3′-phosphotransferase III, 23s rRNA (adenine-N6)-dimethyltransferase, and the ribosomal protection tetracycline resistance protein in *Enterococcus faecium*, were seen in the LTA and STA groups during period 3 (KW $P < 0.05$; Figure 4). From period 4 onward, the mapped reads for these resistance genes decreased in all treatment groups (Figure 4).

DISCUSSION

Effects of SWM on Growth

Results indicated that feeding SWM had no effect on weaning weight or ADG either before or after weaning. Although in-feed antibiotics, when administered at high levels, have been shown to promote growth (Gaskins et al., 2002), the daily antibiotic dose in our study was low, so as to reflect levels found in milk from cows on the second day of withdrawal following intramammary treatment (Moretain and Boisseau, 1989, 1993). As a result, the potential growth-promoting effects of the antibiotics were likely minimal.

Although some studies have reported improved growth rates in calves fed WM (Brunton et al., 2014; Zou et al., 2017; Maynou et al., 2019; Zhang et al., 2019), these findings stem from research involving higher levels of antibiotics in the WM. For example, Brunton et al. (2014) reported a range of between 0.39 and 1.70 mg/kg of cefquinome residues in the WM fed to calves. Other studies have found that feeding WM is associated with reduced ADG; however, these studies fed authentic WM, of variable nutritional composition (Zou et al., 2017). As such, growth responses in these studies were likely influenced by differences in the nutritive value of dump line WM and MR rather than by the presence of antibiotic residues. In addition, these prior studies typically lasted only a few weeks (Firth et al., 2021). In this study, the SWM was prepared to ensure the treatments' nutrition were carefully balanced, and the study spanned a full 24 wk, allowing a more comprehensive comparison between calves exposed to antibiotics and controls. We found that subclinical antibiotic doses in WM do not affect calf growth before or after weaning. However, in an on-farm setting, real WM may often contain pathogens that could affect weight gain, independent of the antibiotics present.

Effects of SWM on Health and the Fecal Microbiota

We found that feeding SWM did not have overtly adverse effects on physical health or external clinical signs associated with calf health. There were no differences in the requirement for VI between treatments. However, before weaning, calves in the LTA group were less likely to have poor health scores compared with the CONT group, indicating that the antibiotics in the SWM may have helped reduce infectious diseases prior to weaning. This finding is consistent with several other studies that reported improved health outcomes in calves fed WM (Zou et al., 2017; Zhang et al., 2019; Firth et al., 2021). As the calves matured and were weaned off the SWM, these differences disappeared.

Despite minimal differences in OH scores, which were based on several individual health factors, some differences were observed in specific factors. For example, the LTA group showed an increase in fecal scores (looser fecal matter and more fecal matter on the hindquarters) at weaning (wk 12), whereas the STA group showed an increase in fecal scores. Though potentially a stress colitis due to weaning, as these looser fecal scores were not observed in the CONT calves, it may be related to an altered intestinal microbiota in the SWM treatments. If the intestinal microbiota in the LTA and STA calves were less adapted to the metabolic changes needed to digest the increased solid feed intake during weaning, this could also explain the observed increase in diversity in the LTA group after weaning.

It is important to note that, as the calves age and transition to ruminants, less can be inferred about the intestinal microbiota from fecal samples. Other studies have also reported increases in diarrhea when feeding artificially spiked raw milk or MR (Li et al., 2019; Calderón-Amor and Gallo, 2020). Research links WMF to altered microbial diversity and shifts in bacterial populations (Yousif et al., 2018; Zhang et al., 2019). In our study, this was reflected in increased microbial diversity in the LTA group following weaning. Antibiotics can also affect gut immune function, increasing the risk of inflammation and diarrhea (Dickinson and Surawicz, 2014). Further research is needed on the oral bioavailability and localized effects of antibiotics in WM on the gastrointestinal tract of preruminant calves. The observed trend of increased proportions of *Pseudomonadota* and *Bacillota*, and a decreased proportion of *Bacteroidota* across all 3 treatments, is likely driven by a combination of the calves' rumen development, aging, and dietary changes, particularly as the calves were weaned and moved to pasture (O'Callaghan et al., 2018; Maslen et al., 2023; C. Sun, H. Gao, J. He, H. Yao, A. Yu, Y. Xie, W. Zhang, Z. Lei, Gansu Agricultural University, Lanzhou, Gansu, China; H. Wang, J. Hu, Y. Duan, Tianjin Halo Biotechnology Co. Ltd., Tianjin, China; D. Tang, W. Liu, Gansu Agricultural University, Lanzhou, Gansu, China; unpublished data). Although not the focus of this study, this also warrants further investigation. A secondary component of this study, the collection of naturally voided fecal samples allowed us to compare only a subset of calves, which may mean that the study was not powered sufficiently to detect

differences in microbial diversity. Further study of the microbiota responses to WMF is warranted.

In terms of inflammation, the LTA calves showed higher SAA levels prior to weaning, although these levels remained within the range typically seen in clinically healthy calves, as reported in previous studies (Humblet et al., 2006; Kabu et al., 2016; Zou et al., 2017; Peetsalu et al., 2022). Other biomarkers, such as IL-8 and IL-10, were reportedly upregulated in calves fed WM (Zou et al., 2017). This suggests a complex immune response that may be linked to early antibiotic exposure and disruptions in gut microbiota. However, due to the wide variability in SAA concentrations across studies and the limited statistical power of this experiment, these findings should be interpreted with caution (Trela et al., 2022).

Shedding of AMR Bacteria and Altered Metabolic Categories

All treatments showed presence of ESBL-producing *E. coli*, but higher levels were seen in the STA and LTA groups. Calves fed SWM had higher ESBL-producing isolates in their feces at 2.8 wk. The observation that only calves in the CONT group displayed phenotypic resistance at 10.5 wk of age is not fully understood. However, it is possible that this resistance developed naturally in the environment, rather than as a result of selective pressures such as antibiotic exposure. Finally, the wide confidence intervals indicate that individual animals varied in the shedding of resistant *E. coli*. Although ESBL-producing *E. coli* ratios were low compared with previous studies, which have found resistance in as much as 93% of isolates from calves fed WM (Horton et al., 2016), they indicate that the feeding of SWM selected for resistant bacteria, which is consistent with previous research (Maynou et al., 2019).

Levels of ESBL-positive bacteria also decreased with age, consistent with other studies showing a decline in resistant bacteria in WM-fed calves (Brunton et al., 2014; Horton et al., 2016). The decrease in phenotypic resistance may result from lower antibiotic concentrations relative to the calves' BW or changes in antibiotic bioavailability as their diet and digestion evolved, or it may be due to poor plate count sensitivity.

The fecal bacteria contained genes resistant to aminoglycosides (aminoglycoside 3′-phosphotransferase III), macrolides (23S rRNA (adenine2085-N6)-dimethyl transferase), and tetracycline (ribosomal protection tetracycline resistance protein) antibiotics. In addition, higher levels of the macrolide and tetracycline resistance genes (MFS DHA3 family macrolide efflux protein and ribosomal protection tetracycline resistance protein) were found in the commensal species *Ruminococcus torques* in CONT before weaning. Feeding SWM likely contributed to the selection of these resistant bacteria in the calf's gastrointestinal environment. Although resistance to tetracycline and aminoglycosides is relatively widespread, this remains a cause for concern (Mayer, 1986; Stine et al., 2007; Garneau-Tsodikova and Labby, 2016; Gasparrini et al., 2020). As studies have shown how multiple AMR genes can often be found adjacently in gene clusters or within identical plasmids, these genes can be acquired and passed in conjunction with genes for more critically important antibiotics, leading to multidrug resistance (Boerlin and Reid-Smith, 2008; Ziebell et al., 2011). Recent research has highlighted the role commensals may also play in harboring AMR genes in the environment (Brinkac et al., 2017). Additionally, these resistance genes were identified in the bacterium *Enterococcus faecium*, which, although not typically a pathogen in calves, is a well-documented causative agent of nosocomial infections in human healthcare settings and has been found to harbor multiple resistance genes (Spera and Farber, 1994; Zhou et al., 2020). *Enterococcus faecium* is also a potential pathogen involved in both clinical and subclinical mastitis (Kuyucuoğlu, 2011; Erbas et al., 2016), although its role as a pathogen is less common in Ireland than in other countries and more typical of indoor dairy systems (Barrett et al., 2005; Clabby et al., 2023). The reduction in mapped reads for AMR genes from periods 4 to 6 suggests that the AMR induced by WMF is transient and decreases rapidly following weaning. However, the potential for calves to serve as reservoirs for AMR over time on farms has been documented (Salerno et al., 2022; Uyama et al., 2024).

The mapping of higher levels of the metabolic categories for pyruvate degradation, valine biosynthesis, pyruvate acetate fermentation, and pyruvate lactate fermentation in the CONT calves during period 3 suggests a differential response of the rumen metabolome to weaning compared with the LTA and STA calves. As fecal populations cannot accurately imply the activity of the rumen metabolome after weaning, a study to investigate the effects of WMF employing longitudinal rumen sampling is warranted.

Study Limitations

This study has several limitations. First, the delivery of SWM did not account for variations in WM concentration over time, as would occur with real milk from a cow on withdrawal, which should be considered when comparing against studies using authentic WM. The use of Delvotest to detect antibiotics did not allow precise measurement of antibiotic concentrations or differentiation between amoxicillin and neomycin. The use of only amoxicillin and neomycin in the SWM was a limitation of this study, as it does not reflect the broader range of antimicrobi-

als found in real WM, potentially limiting the relevance of AMR findings. Neomycin, in particular, is now less commonly used in mastitis treatments. Classified by the World Health Organization as a critically important antimicrobial, its use is increasingly restricted internationally. However, in Ireland, neomycin-containing intramammary products remain available. A study by Burke and Adley (2021) found that more than 36% of Irish farmers chose products containing neomycin as their first-choice treatment, highlighting its ongoing use. Still, as antimicrobial stewardship advances, including neomycin in experimental models may not be appropriate.

Furthermore, the study was underpowered to assess health scores and SAA concentrations, due to the low incidence of poor health scores on the farm, and the lack of additional inflammatory biomarkers limits the assessment of inflammation status. Finally, refrigeration during fecal sample storage may have caused variability in the microbiota analysis by altering bacterial abundance and composition.

CONCLUSIONS

This study found that feeding SWM, regardless of duration, did not significantly affect calf growth. However, calves fed SWM had higher fecal scores at weaning and after weaning, with the duration of SWM feeding not influencing these outcomes. More concerning is the finding that SWM feeding contributed to antibiotic resistance, as evidenced by the higher prevalence of ESBL-producing bacteria and increased mapping of reads for resistance genes in calves fed SWM. These findings suggest that even short-term WM feeding can contribute to the emergence of AMR. Additionally, WMF may alter the development of the fecal microbiota, although the downstream implications of this remain unclear. In conclusion, our study suggests that farmers should limit the practice of feeding WM to calves when possible.

NOTES

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Nonstandard abbreviations used: AFS = automatic feeding system; AMR = antimicrobial-resistant; ANOSIM = analysis of similarities; Bio = biosynthesis; CONT = control group; Deg = degradation; DOA = days of age; ESBL = extended-spectrum β-lactamase; Ferm = fermentation; HF = Holstein Friesian; JE = Jersey; JEX = Holstein Friesian × Jersey; IQR = interquartile range; KO = Kyoto Encyclopedia of Genes and Genomes orthogroups; KW = Kruskal–Wallis; LTA = long-term antibiotic; MFS = major facilitator superfamily; MR = milk replacer; NMDS = nonmetric multidimensional scaling; OH = overall health; OR = odds ratio; pFDR = predicted false discovery rate; SAA = serum amyloid A; STA = short-term antibiotic; SWM = simulated waste milk; VI = veterinary intervention; WM = waste milk; WMF = waste milk feeding.

REFERENCES

Abubucker, S., N. Segata, J. Goll, A. M. Schubert, J. Izard, B. L. Cantarel, B. Rodriguez-Mueller, J. Zucker, M. Thiagarajan, and B. Henrissat. 2012. Metabolic reconstruction for metagenomic data and its application to the human microbiome. PLoS Computational Biology 8:e1002358. https://doi.org/10.1371/journal.pcbi.1002358.

Amin, N., and J. Seifert. 2021. Dynamic progression of the calf's microbiome and its influence on host health. Comput. Struct. Biotechnol. J. 19:989–1001. https://doi.org/10.1016/j.csbj.2021.01.035.

Barrett, D. J., A. M. Healy, F. C. Leonard, and M. L. Doherty. 2005. Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland. Ir. Vet. J. 58:333. https://doi.org/10.1186/2046-0481-58-6-333.

Barry, J., E. A. Bokkers, D. Berry, I. J. de Boer, J. McClure, and E. Kennedy. 2019. Associations between colostrum management, passive immunity, calf-related hygiene practices, and rates of mortality in preweaning dairy calves. J. Dairy Sci. 102:10266–10276. https://doi.org/10.3168/jds.2019-16815.

Barry, J., E. A. M. Bokkers, I. J. M. de Boer, and E. Kennedy. 2020. Pre-weaning management of calves on commercial dairy farms and its influence on calf welfare and mortality. Animal 14:2580–2587. https://doi.org/10.1017/S1751731120001615.

Bielmann, V., J. Gillan, N. R. Perkins, A. L. Skidmore, S. Godden, and K. E. Leslie. 2010. An evaluation of Brix refractometry instruments for measurement of colostrum quality in dairy cattle. J. Dairy Sci. 93:3713–3721. https://doi.org/10.3168/jds.2009-2943.

Boerlin, P., and R. J. Reid-Smith. 2008. Antimicrobial resistance: Its emergence and transmission. Anim. Health Res. Rev. 9:115–126. https://doi.org/10.1017/S146625230800159X.

Bortolaia, R., and R. Hendriksen. 2017. Laboratory protocol for the quantification of ESBL/AmpC-producing *Escherichia coli* for quantification of ESBL/AmpC-producing *E. coli* in caecum and fresh meat samples. EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). Accessed Aug. 21, 2023. https://www.eurl

-ar.eu/CustomerData/Files/Folders/21-protocols/399_esbl-ampc-quantification-protocol-19-03-2018.pdf.

Brinkac, L., A. Voorhies, A. Gomez, and K. E. Nelson. 2017. The threat of antimicrobial resistance on the human microbiome. Microb. Ecol. 74:1001–1008. https://doi.org/10.1007/s00248-017-0985-z.

Brunton, L., H. Reeves, L. Snow, and J. Jones. 2014. A longitudinal field trial assessing the impact of feeding waste milk containing antibiotic residues on the prevalence of ESBL-producing *Escherichia coli* in calves. Prev. Vet. Med. 117:403–412. https://doi.org/10.1016/j.prevetmed.2014.08.005.

Brunton, L. A., D. Duncan, N. G. Coldham, L. C. Snow, and J. R. Jones. 2012. A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. Vet. Rec. 171:296. https://doi.org/10.1136/vr.100924.

Burke, N., and C. C. Adley. 2021. Prevalence of intramammary antibiotic usage in dairy farming. J. Dairy Res. 88:176–178. https://doi.org/10.1017/S0022029921000431.

Burmańczuk, A., G. Tomasz, M. Gbylik-Sikorska, A. Gajda, and C. Kowalski. 2017. Withdrawal of amoxicillin and penicillin G procaine from milk after intramammary administration in dairy cows with mastitis. J. Vet. Res. 61:37–43. https://doi.org/10.1515/jvetres-2017-0005.

Calderón-Amor, J., and C. Gallo. 2020. Dairy calf welfare and factors associated with diarrhea and respiratory disease among Chilean dairy farms. Animals (Basel) 10:1115. https://doi.org/10.3390/ani10071115.

Clabby, C., A. Valldecabres, P. Dillon, S. McParland, S. Arkins, K. O'Sullivan, J. Flynn, J. Murphy, and P. S. Boloña. 2023. Evaluation of test-day milk somatic cell count to predict intramammary infection in late lactation grazing dairy cows. J. Dairy Sci. 106:4991–5001. https://doi.org/10.3168/jds.2022-22627.

Cortese, V. S. 2009. Neonatal immunology. Vet. Clin. North Am. Food Anim. Pract. 25:221–227. https://doi.org/10.1016/j.cvfa.2008.10.003.

Deng, Y. F., Y. J. Wang, Y. Zou, A. Azarfar, X. L. Wei, S. K. Ji, J. Zhang, Z. H. Wu, S. X. Wang, S. Z. Dong, Y. Xu, D. F. Shao, J. X. Xiao, K. L. Yang, Z. J. Cao, and S. L. Li. 2017. Influence of dairy by-product waste milk on the microbiomes of different gastrointestinal tract components in pre-weaned dairy calves. Sci. Rep. 7:42689. https://doi.org/10.1038/srep42689.

Dickinson, B., and C. M. Surawicz. 2014. Infectious diarrhea: An overview. Curr. Gastroenterol. Rep. 16:399. https://doi.org/10.1007/s11894-014-0399-8.

Dupouy, V., J.-Y. Madec, J. Wucher, N. Arpaillange, V. Métayer, B. Roques, A. Bousquet-Mélou, and M. Haenni. 2021. Selection of ESBL-producing *Escherichia coli* in the gut of calves experimentally fed with milk containing antibiotic residues. Vet. Microbiol. 257:109049. https://doi.org/10.1016/j.vetmic.2021.109049.

EFSA Panel on Biological Hazards. 2017. Risk for the development of antimicrobial resistance (AMR) due to feeding of calves with milk containing residues of antibiotics. EFSA J. 15:e04665. https://doi.org/10.2903/j.efsa.2017.4665.

Erbas, G., U. Parin, S. Turkyilmaz, N. Ucan, M. Ozturk, and O. Kaya. 2016. Distribution of antibiotic resistance genes in *Enterococcus* spp. isolated from mastitis bovine milk. Acta Vet. (Beogr.) 66:336–346. https://doi.org/10.1515/acve-2016-0029.

Firth, C. L., K. Kremer, T. Werner, and A. Käsbohrer. 2021. The effects of feeding waste milk containing antimicrobial residues on dairy calf health. Pathogens 10:112. https://doi.org/10.3390/pathogens10020112.

Garneau-Tsodikova, S., and K. J. Labby. 2016. Mechanisms of resistance to aminoglycoside antibiotics: Overview and perspectives. MedChemComm 7:11–27. https://doi.org/10.1039/C5MD00344J.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants: Mode of action. Anim. Biotechnol. 13:29–42. https://doi.org/10.1081/ABIO-120005768.

Gasparrini, A. J., J. L. Markley, H. Kumar, B. Wang, L. Fang, S. Irum, C. T. Symister, M. Wallace, C.-A. D. Burnham, S. Andleeb, N. H. Tolia, T. A. Wencewicz, and G. Dantas. 2020. Tetracycline-inactivating enzymes from environmental, human commensal, and pathogenic bacteria cause broad-spectrum tetracycline resistance. Commun. Biol. 3:241. https://doi.org/10.1038/s42003-020-0966-5.

Godden, S. M., J. P. Fetrow, J. M. Feirtag, L. R. Green, and S. J. Wells. 2005. Economic analysis of feeding pasteurized nonsaleable milk versus conventional milk replacer to dairy calves. J. Am. Vet. Med. Assoc. 226:1547–1554. https://doi.org/10.2460/javma.2005.226.1547.

Gomez, D. E., L. Li, H. Goetz, J. MacNicol, L. Gamsjaeger, and D. L. Renaud. 2022. Calf diarrhea is associated with a shift from obligated to facultative anaerobes and expansion of lactate-producing bacteria. Front. Vet. Sci. 9:846383. https://doi.org/10.3389/fvets.2022.846383.

Gosselin, V. B., M. Bodmer, G. Schüpbach-Regula, A. Steiner, and M. Meylan. 2022. Survey on the disposal of waste milk containing antimicrobial residues on Swiss dairy farms. J. Dairy Sci. 105:1242–1254. https://doi.org/10.3168/jds.2021-20948.

Hernandez, D., D. V. Nydam, S. M. Godden, L. S. Bristol, A. Kryzer, J. Ranum, and D. Schaefer. 2016. Brix refractometry in serum as a measure of failure of passive transfer compared to measured immunoglobulin G and total protein by refractometry in serum from dairy calves. Vet. J. 211:82–87. https://doi.org/10.1016/j.tvjl.2015.11.004.

Horton, R., D. Duncan, L. Randall, S. Chappell, L. Brunton, R. Warner, N. Coldham, and C. Teale. 2016. Longitudinal study of CTX-M ESBL-producing *E. coli* strains on a UK dairy farm. Res. Vet. Sci. 109:107–113. https://doi.org/10.1016/j.rvsc.2016.09.018.

Humblet, M.-F., H. Guyot, B. Boudry, F. Mbayahi, C. Hanzen, F. Rollin, and J.-M. Godeau. 2006. Relationship between haptoglobin, serum amyloid A, and clinical status in a survey of dairy herds during a 6-month period. Vet. Clin. Pathol. 35:188–193. https://doi.org/10.1111/j.1939-165x.2006.tb00112.x.

Kabu, M., B. Elitok, and I. Kucukkurt. 2016. Detection of serum amyloid-A concentration in the calf clinically diagnosed with pneumonia, enteritis and pneumoenteritis. Ciência Rural 46:293–299. https://doi.org/10.1590/0103-8478cr20150571.

Kuyucuoğlu, Y. 2011. Antibiotic resistances of enterococci isolated from bovine subclinical mastitis. Eurasian J. Vet. Sci. 27:231–234. https://dergipark.org.tr/en/download/article-file/233016.

Langford, F. M., D. M. Weary, and L. Fisher. 2003. Antibiotic resistance in gut bacteria from dairy calves: A dose response to the level of antibiotics fed in milk. J. Dairy Sci. 86:3963–3966. https://doi.org/10.3168/jds.S0022-0302(03)74006-5.

Li, J. H., M. H. Yousif, Z. Q. Li, Z. H. Wu, S. L. Li, H. J. Yang, Y. J. Wang, and Z. J. Cao. 2019. Effects of antibiotic residues in milk on growth, ruminal fermentation, and microbial community of preweaning dairy calves. J. Dairy Sci. 102:2298–2307. https://doi.org/10.3168/jds.2018-15506.

Lu, J., N. Rincon, D. E. Wood, F. P. Breitwieser, C. Pockrandt, B. Langmead, S. L. Salzberg, and M. Steinegger. 2022. Metagenome analysis using the Kraken software suite. Nat. Protoc. 17:2815–2839. https://doi.org/10.1038/s41596-022-00738-y.

Ma, Y., M. Z. Khan, J. Xiao, G. M. Alugongo, X. Chen, S. Li, Y. Wang, and Z. Cao. 2022. An overview of waste milk feeding effect on growth performance, metabolism, antioxidant status and immunity of dairy calves. Front. Vet. Sci. 9:898295. https://doi.org/10.3389/fvets.2022.898295.

Martin, H., E. G. Manzanilla, S. J. More, L. O'Neill, L. Bradford, C. I. Carty, Á. B. Collins, and C. G. McAloon. 2020. Current antimicrobial use in farm animals in the Republic of Ireland. Ir. Vet. J. 73:11. https://doi.org/10.1186/s13620-020-00165-z.

Maslen, B. N., C. Duff, S. A. Clark, J. Van der Werf, J. D. White, and S. D. Pant. 2023. Increased yearling weight gain is associated with a distinct faecal microbial profile. Animals (Basel) 13:3062. https://doi.org/10.3390/ani13193062.

Mathew, A. G., R. Cissell, and S. Liamthong. 2007. Antibiotic resistance in bacteria associated with food animals: A United States perspective of livestock production. Foodborne Pathog. Dis. 4:115–133. https://doi.org/10.1089/fpd.2006.0066.

Mayer, K. H. 1986. Review of epidemic aminoglycoside resistance worldwide. Am. J. Med. 80:56–64. https://doi.org/10.1016/0002-9343(86)90480-8.

Maynou, G., H. Chester-Jones, A. Bach, and M. Terré. 2019. Feeding pasteurized waste milk to preweaned dairy calves changes fecal and upper respiratory tract microbiota. Front. Vet. Sci. 6:159. https://doi.org/10.3389/fvets.2019.00159.

McDougall, S. 2003. Intramammary treatment of clinical mastitis of dairy cows with a combination of lincomycin and neomycin, or penicillin and dihydrostreptomycin. N. Z. Vet. J. 51:111–116. https://doi.org/10.1080/00480169.2003.36349.

Moore, D. A., J. Taylor, M. L. Hartman, and W. M. Sischo. 2009. Quality assessments of waste milk at a calf ranch. J. Dairy Sci. 92:3503–3509. https://doi.org/10.3168/jds.2008-1623.

Moretain, J. P., and J. Boisseau. 1989. Excretion of penicillins and cephalexin in bovine milk following intramammary administration. Food Addit. Contam. 6:79–89. https://doi.org/10.1080/02652038909373741.

Moretain, J. P., and J. Boisseau. 1993. Elimination of aminoglycoside antibiotics in milk following intramammary administration. Vet. Q. 15:112–117. https://doi.org/10.1080/01652176.1993.9694386.

O'Callaghan, T. F., R. Vázquez-Fresno, A. Serra-Cayuela, E. Dong, R. Mandal, D. Hennessy, S. McAuliffe, P. Dillon, D. S. Wishart, C. Stanton, and R. P. Ross. 2018. Pasture feeding changes the bovine rumen and milk metabolome. Metabolites 8:27. https://doi.org/10.3390/metabo8020027.

Peetsalu, K., T. Niine, M. Loch, E. Dorbek-Kolin, L. Tummeleht, and T. Orro. 2022. Effect of colostrum on the acute-phase response in neonatal dairy calves. J. Dairy Sci. 105:6207–6219. https://doi.org/10.3168/jds.2021-21562.

Penati, M., G. Sala, F. Biscarini, A. Boccardo, V. Bronzo, B. Castiglioni, P. Cremonesi, P. Moroni, D. Pravettoni, and M. F. Addis. 2021. Feeding pre-weaned calves with waste milk containing antibiotic residues is related to a higher incidence of diarrhea and alterations in the fecal microbiota. Front. Vet. Sci. 8:650150. https://doi.org/10.3389/fvets.2021.650150.

Pereira, R. V. V., J. D. Siler, R. C. Bicalho, and L. D. Warnick. 2014. In vivo selection of resistant *E. coli* after ingestion of milk with added drug residues. PLoS One 9:e115223. https://doi.org/10.1371/journal.pone.0115223.

Salerno, B., M. Cornaggia, R. Sabatino, A. Di Cesare, M. Furlan, L. Barco, M. Orsini, B. Cordioli, C. Mantovani, L. Bano, and C. Losasso. 2022. Calves as main reservoir of antibiotic resistance genes in dairy farms. Front. Public Health 10:918658. https://doi.org/10.3389/fpubh.2022.918658.

Spera, R. V. Jr., and B. F. Farber. 1994. Multidrug-resistant *Enterococcus faecium*: An untreatable nosocomial pathogen. Drugs 48:678–688. https://doi.org/10.2165/00003495-199448050-00003.

Stine, O. C., J. A. Johnson, A. Keefer-Norris, K. L. Perry, J. Tigno, S. Qaiyumi, M. S. Stine, and J. G. Morris Jr. 2007. Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. Int. J. Antimicrob. Agents 29:348–352. https://doi.org/10.1016/j.ijantimicag.2006.11.015.

Thames, C. H., A. Pruden, R. E. James, P. P. Ray, and K. F. Knowlton. 2012. Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. Front. Microbiol. 3:139. https://doi.org/10.3389/fmicb.2012.00139.

Trela, M., D. Domańska, and O. Witkowska-Piłaszewicz. 2022. Diagnostic use of serum amyloid A in dairy cattle. Agriculture 12:459. https://doi.org/10.3390/agriculture12040459.

USDA. 2016. Dairy Cattle Management Practices in the United States, 2014. National Animal Health Monitoring System. Accessed May 16, 2024. https://www.aphis.usda.gov/sites/default/files/dairy-trends-hlth-mngmnt-1991-2014.pdf.

Uyama, T., D. F. Kelton, E. I. Morrison, E. de Jong, K. D. McCubbin, H. W. Barkema, S. Dufour, M. Fonseca, J. T. McClure, J. Sanchez, L. C. Heider, and D. L. Renaud. 2024. Associations among antimicrobial use, calf management practices, and antimicrobial resistance in *Escherichia coli* from a pooled fecal sample in calves on Canadian dairy farms: A cross-sectional study. J. Dairy Sci. 107:4961–4972. https://doi.org/10.3168/jds.2023-24262.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York. https://ggplot2.tidyverse.org.

Yousif, M. H., J.-H. Li, Z.-Q. Li, G. Maswayi Alugongo, S.-K. Ji, Y.-X. Li, Y.-J. Wang, S.-L. Li, and Z.-J. Cao. 2018. Low concentration of antibiotics modulates gut microbiota at different levels in preweaning dairy calves. Microorganisms 6:118. https://doi.org/10.3390/microorganisms6040118.

Zhang, R., W. Zhang, Y. Bi, Y. Tu, Y. Beckers, H. Du, and Q.-y. Diao. 2019. Early feeding regime of waste milk, milk, and milk replacer for calves has different effects on rumen fermentation and the bacterial community. Animals (Basel) 9:443. https://doi.org/10.3390/ani9070443.

Zhou, X., R. J. Willems, A. W. Friedrich, J. W. Rossen, and E. Bathoorn. 2020. *Enterococcus faecium*: From microbiological insights to practical recommendations for infection control and diagnostics. Antimicrob. Resist. Infect. Control 9:130. https://doi.org/10.1186/s13756-020-00770-1.

Ziebell, K., R. Johnson, A. Kropinski, R. Reid-Smith, R. Ahmed, V. Gannon, M. Gilmour, and P. Boerlin. 2011. Gene cluster conferring streptomycin, sulfonamide, and tetracycline resistance in *Escherichia coli* O157: H7 phage types 23, 45, and 67. Appl. Environ. Microbiol. 77:1900–1903. https://doi.org/10.1128/AEM.01934-10.

Zou, Y., Y. Wang, Y. Deng, Z. Cao, S. Li, and J. Wang. 2017. Effects of feeding untreated, pasteurized and acidified waste milk and bunk tank milk on the performance, serum metabolic profiles, immunity, and intestinal development in Holstein calves. J. Anim. Sci. Biotechnol. 8:53. https://doi.org/10.1186/s40104-017-0182-4.

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