Multidrug residues and antimicrobial resistance patterns in waste milk from dairy farms in Central California

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ABSTRACT

Waste milk (WM) is a common source of feed for preweaned calves in US dairy farms. However, limited information is available about characteristics of this product, including concentration of drug residues and potential hazards from antibiotic-resistant bacteria present in the milk. The aims of this cross-sectional study were to (1) identify and measure the concentration of antimicrobial residues in raw WM samples on dairy farms in the Central Valley of California, (2) survey farm management practices for factors associated with the occurrence of specific antimicrobial residues in raw WM, (3) characterize the antimicrobial resistance patterns of *E. coli* cultured from raw WM samples, and (4) evaluate the potential association between WM quality parameter and risk of identifying drug residues in milk. A single raw bulk tank WM sample was collected from dairy farms located in California's Central Valley ($n = 25$). A questionnaire was used to collect information about farm management practices. Waste milk samples were analyzed for a multidrug residue panel using liquid chromatography–tandem mass spectrometry. Bacteria were cultured and antimicrobial resistance was tested using standard techniques; milk quality parameters (fat, protein, lactose, solids-not-fat, somatic cell count, coliform count, and standard plate count) were also measured. Of the 25 samples collected, 15 (60%) contained detectable concentrations of at least 1 antimicrobial. Of the drug residue–positive samples, 44% (11/25) and 16% (4/25) had detectable concentrations of β -lactams and tetracycline, respectively. The most prevalent drug residues were ceftiofur ($n = 7$, 28%), oxytetracycline ($n = 4$, 16%), and cephalixin ($n = 3$, 12%). No significant associations were identified between farm characteristics or management practices

and presence of drug residues in WM. In this study, 20% of farms did not pasteurize WM before feeding to calves. Two of the 10 *Escherichia coli* isolated from WM samples were multidrug resistant. *Streptococcus* spp. ($n = 21$, 84%) was the most common genus cultured from WM samples, followed by *Staphylococcus* spp. ($n = 20$, 80%) and *E. coli* ($n = 10$, 40%). *Mycoplasma* spp. was cultured from 2 WM samples ($n = 2$, 8%). The presence of drug residues in WM at concentrations that increase selection of resistant bacteria indicates the need for additional studies targeting on-farm milk treatments to degrade drug residues before feeding to calves. The presence of multidrug-resistant *E. coli* in WM urges the need for on-farm practices that reduce calf exposure to resistant bacteria, such as pasteurization.

Key words: waste milk, drug residue, ceftiofur, antibiotic resistance

INTRODUCTION

The use of antimicrobials in livestock production and its effect on the development of antimicrobial resistance are a current public health concern. Although prevention approaches can reduce disease and the need for use of antimicrobial drugs to treat animals, drug use in food animals is an important tool to help ensure animal health and welfare. Despite these benefits, there is considerable concern from public health, food safety, and regulatory stakeholders regarding the use of antimicrobials in food animals because of possible selection of antimicrobial-resistant bacteria (Oliver et al., 2011). The selection of resistant bacteria can especially occur when antimicrobials are administered to food animals at regimens outside of labeled dosage and duration (Maron et al., 2013).

In the dairy industry, most antimicrobial drugs used to treat cows result in the milk from these animals being withheld from sale because of the presence of drug residues above the tolerable concentration established by the US Food and Drug Administration (FDA). Milk from cows treated with drugs that require a milk with-

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drawal period and transitional milk (transitioning from colostrum to saleable milk) are considered nonsaleable for human consumption. One-third of dairy farms in the United States use waste milk (WM) to feed preweaned dairy calves (USDA, 2008a). A study examining the effects of feeding pasteurized WM to preweaned calves found that calves consuming pasteurized WM had increased weaning weights and decreased mortality rates compared with calves consuming milk replacer (Godden et al., 2005). Additionally, the total savings of feeding pasteurized WM was \$0.69/calf per day (Godden et al., 2005). However, milk from cows receiving antimicrobial treatment during lactation and colostrum from cows treated with antimicrobials at dry-off may contain concentrations of drug residues, and such milk could lead to increased fecal shedding of antimicrobial-resistant bacteria when fed to preweaned dairy calves (Pereira et al., 2014a; Maynou et al., 2017a; Ricci et al., 2017). Not all dry cow treatments have zero withdrawal milk days (in addition to the minimum dry period length required). As an example, the commercial drug used for dry cow treatment containing cephalixin benzathine (ToMorrow, Boehringer Ingelheim, Ingelheim am Rhein, Germany) states on its label that milk from treated cows must not be used for food during the first 72 h after calving.

In a recent study conducted in New York State, 82% of WM fed to calves contained β -lactam drug residues, including ceftiofur, penicillin G, and ampicillin (Pereira et al., 2014b). A subsequent study evaluating the effect of ingestion of raw milk containing antimicrobial drugs found an increased prevalence of antimicrobial resistance in *Escherichia coli* isolated from the feces of dairy calves (Pereira et al., 2014a). Based on 16S RNA sequencing of fecal samples, feeding milk containing drug residues also affected the developing enteric microbiota of calves, resulting in a microbiota that discriminated at the genus level in their weekly microbial profile (Pereira et al., 2016). A follow-up evaluation using shotgun sequencing to evaluate the effects of feeding WM on the function of the microbiota revealed differences between treatment and control groups for genes linked to stress response, regulation and cell signaling, and nitrogen metabolism (Pereira et al., 2018). For example, calves in the treatment group had a greater abundance of microbial genes related to zinc-regulated enzymes, allowing this microbiota to adapt better to lower concentrations of zinc in feed. Calves in the control group had a greater abundance of microbial genes related to osmotic stress, which could translate into this microbiota being able to withstand greater osmotic pressure variation, such as that caused by feed with high concentration of sugars or salts. Changes in the microbiota functions could directly affect the resilience

of the microbiota to adjust to changes such as variations in nutrient content or TS percentage in milk fed. To the author's knowledge, no studies have evaluated the effects of drug residues at concentrations observed in WM on the gastrointestinal epithelium.

Limited information is available on antimicrobial drug residues and resistant bacteria in WM fed to preweaned dairy calves. California is the leading dairy state in the United States, with 1.7 million lactating cows and contributing almost 20% of the country's milk supply in 2015 (Monson et al., 2015), so the California dairy industry is particularly poised to address this research need. Public awareness and increasing concerns about the development of antimicrobial resistance associated with the use of antimicrobials in livestock production and the possible spread of resistance to human medicine require field data to provide scientific information for stakeholders.

The aims and hypotheses for this study were as follows. First, we aimed to identify and measure the concentration of antimicrobial residues in raw WM samples on dairy farms in the Central Valley of California; our hypothesis was that more than 50% of dairy farms in California would have drug residues in bulk tank WM and that ceftiofur would be the most common drug residues detected in WM based on previous reports screening drug residues in WM (Pereira et al., 2014a; USDA, 2016). Second, we aimed to survey farm management practices for factors associated with the occurrence of specific antimicrobial residues in raw WM; our hypothesis was that use of specific antimicrobial drugs or management practices that affect the frequency of antimicrobial drug use would result in higher prevalence of specific drugs in the WM bulk tank. Third, we aimed to evaluate the association between WM quality parameters and identification of drug residues in milk; our hypothesis was that milk quality parameters could be associated with prevalence of drug residues. It was also an objective of the study to characterize the antimicrobial resistance patterns of *E. coli* cultured from raw WM samples. To our knowledge, this is the first study to focus on addressing all the aims and objectives on WM from dairy farms in California.

MATERIALS AND METHODS

Study Population and Design

Raw hospital WM samples from 25 dairy farms were collected from herds in the following counties in the Central Valley in California: Tulare ($n = 12$), Kings ($n = 2$), Fresno ($n = 2$), Madera ($n = 1$), Solano ($n = 1$), Placer ($n = 1$), Glenn ($n = 5$), and Butte ($n = 1$). Samples were collected from August 2016 to February

2017. Study farms were identified through collaboration with private practitioners in Kings, Fresno, Madera, and Tulare counties or conveniently selected based on their working relationship with the University of California, Davis School of Veterinary Medicine, including the Veterinary Medicine Teaching and Research Center in Tulare, California, and a University of California Agriculture and Natural Resources Cooperative Extension dairy advisor. As part of a cross-sectional study design, each participating farm was visited once between September 2016 and March 2017. During the farm visit, a WM sample was collected and a questionnaire was completed through an in-person interview with the farm owner or manager. Sample size was calculated using a simple random sampling approach, adjusting for a finite population (Thrusfield, 2005). An estimated sample size of 25 farms was obtained when the following parameters were offered to the sample size formula: target population of 1,300 dairy farms (CDFA, 2017), an expected prevalence of 82% (Pereira et al., 2014b), 90% confidence interval, and a 12.5% desired absolute precision.

The current study did not fall within the criteria that would require the University of California Institutional Animal Care and Use Committee protocol and was therefore exempted. The study was reviewed by the University of California, Davis Institutional Review Board and granted exemption status.

Milk Sample Collection and Processing

Approximately 650 mL of raw, pooled milk with no additives or preservatives was collected from the WM bulk tank and placed into sterile milk tubes. The contents of the tank were thoroughly mixed before sample collection. For dairies that milked fresh and treated cows separately, only the bulk tank for the treated cows was sampled. All samples were collected by the authors or by farm managers. On the farm, the milk sample was divided into two 200-mL sterile containers and four 60-mL sterile containers. Immediately after collection, the containers were stored on ice and transported to the nearest study laboratory (Pereira Lab, Davis, CA, or Aly Lab, Tulare, CA), where they were refrigerated at 4°C. Samples were then transported or shipped overnight in cold packaging to Sierra Dairy Laboratory (Tulare, CA) for milk quality parameter determination and to Eurofins Central Analytical Laboratories (CAL; New Orleans, LA) for drug residue quantification using liquid chromatography–tandem MS (LC-MS/MS). The concentrations of the stock solutions used in the LC-MS/MS corresponded to the active drug compound, and the amounts weighed were adjusted to take into account purity and any counter-

ions. Last, 1 aliquot of each WM sample was frozen at –20°C for subsequent bacterial culture at the Milk Quality Laboratory (Veterinary Medicine Teaching and Research Center, Tulare, CA).

Questionnaire

To determine farm characteristics, management practices, and antimicrobial drug usage, data were collected from interviewees using a structured questionnaire with 18 closed-ended questions and options for comments. Interviews were conducted face to face, and the interviewee was provided a copy of the questionnaire to follow during the interview process, allowing them to read the questions being asked. Interviews were conducted during the milk sample collection visit with the exception of 1 dairy, where the manager collected the samples and stored them in a refrigerator overnight until pick-up the following day. The questionnaire addressed general information about farm characteristics, management practices, and antimicrobial drug use. The information from the questionnaire is available in Supplemental File S1 (<https://doi.org/10.3168/jds.2018-14398>). Based on approaches used by the USDA National Animal Health Monitoring System (NAHMS) reports and expected number of animals in dairy farms in California, multiple-choice questions for dairy herd size and number of preweaned calves were formatted into categories (USDA, 2016; CDFA, 2017). Questions were based on information from NAHMS on diseases in dairy cattle that result in higher use of antimicrobial drug use (USDA, 2008b).

Milk Quality Parameters

An aliquot of all WM samples was sent to Sierra Dairy Laboratory (Tulare, CA) to measure the following milk quality parameters: fat, protein, lactose, SNF, SCC, coliform count, and SPC. Milk quality parameters were measured using standard procedures.

Quantification of Drug Residues Using LC-MS/MS

A simple SNAP test for the identification of β -lactam drug residues (SNAP Beta-Lactam ST test; Idexx Laboratories Inc., Westbrook, ME) was performed on all WM samples in the study laboratories. Identification and quantification of drug residues by LC-MS/MS were performed at Eurofins CAL using a protocol that can detect and quantify up to 27 drugs (26 antimicrobials and 1 nonsteroidal anti-inflammatory drug, 5-hydroxy-flunixin). The protocol for LC-MS/MS at Eurofins CAL followed a modified laboratory information bulletin by the FDA (FDA, 2011a), which was modified and inter-

nally validated by Eurofins CAL to include testing for ceftiofur. Briefly, the reagents and materials used for LC-MS/MS were deionized water (18.2 M Ω /cm), high-purity chromatographic- and spectrophotometric-grade acetonitrile and methanol, and formic acid at 96% purity. The limits of quantification for the 27 drugs screened using LC-MS/MS are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-14398>).

Milk Cultures and Antimicrobial Susceptibility Test

All microbiological testing was performed at the Milk Quality Laboratory (Veterinary Medicine Teaching and Research Center, Tulare, CA). Waste milk samples were thawed, plated by streaking 0.01 mL on bovine blood agar, and incubated at 37°C for 48 h. Bacteria isolated on culture medium were identified according to colony morphology, hemolytic characteristics, Gram stain reaction, and catalase and coagulase tests. Estimation of number of viable bacteria at the genus level to detect the most common mastitis pathogens was performed using the serial dilution method. To detect *Mycoplasma* spp., WM samples were directly cultured on modified Hayflick agar as well as in an enriched *Mycoplasma*-specific broth and incubated at 37°C in 4% CO₂ for 7 d. To discriminate between *Acholeplasma* spp. and *Mycoplasma* spp., a digitonin disk inhibition test was performed.

Escherichia coli colonies isolated from WM samples were tested for antimicrobial susceptibility using a Kirby Bauer disk diffusion agar assay in accordance with the guidelines published by the Clinical and Laboratory Standards Institute (CLSI, 2015). We chose to test *E. coli* isolates because it is a commonly used model for evaluating effects of antimicrobials on gram-negative bacteria, and several studies have examined the effect of feeding WM to calves on the occurrence of resistance in fecal *E. coli* (Pereira et al., 2014a; Maynou et al., 2017b). Antimicrobial susceptibility for all *E. coli* isolates was initially assessed using a standard panel used at the Milk Quality Laboratory for mastitis pathogens containing 8 antimicrobials (10 μ g of ampicillin, 30 μ g of cephalothin, 15 μ g of erythromycin, 10 units of penicillin, 2 μ g of pirlimycin, 30 μ g of tetracycline, 10 units/30 μ g of penicillin/novobiocin, and 30 μ g of ceftiofur) to which a 250 μ g sulfisoxazole disk was added. Penicillin, penicillin/novobiocin, and pirlimycin were excluded from the analysis because of the weak spectrum of action against gram-negative bacteria, whereas erythromycin was excluded because of its previously reported weak activity against *E. coli* (Maynou et al., 2017b). Inclusion of these drugs in the analysis may have resulted in a false increase in the prevalence of antimicrobial resistance and would serve as an addi-

tional source of bias. Susceptibility of the isolates to antimicrobial drugs was categorized as susceptible, intermediate, or resistant by measuring the inhibition zone according to interpretive criteria and breakpoints established by CLSI guidelines (CLSI, 2013, 2015; Supplemental Table S2, <https://doi.org/10.3168/jds.2018-14398>). Multidrug-resistant isolates were defined as having resistance to 3 or more classes of antimicrobials (Pereira et al., 2014a).

Statistical Analysis

Answers to the questionnaire were transferred to Excel (Microsoft Corp., Redmond, WA) and coded by the type of response. Descriptive and statistical analyses were conducted using JMP software version 4.0 and SAS 9.4 (SAS Institute, Cary, NC). For all analyses, a *P*-value of <0.05 was considered to be the threshold of significance.

Multiple Pearson chi-squared tests were first used to evaluate the association between the categorical variable of a WM sample being positive or negative for at least 1 drug residue and the various categorical variables of farm management practices and dairy characteristics generated through the on-farm questionnaire. These variables included antimicrobial drug used to treat cows for dry treatment, mastitis, reproductive disease, lameness and pneumonia, how antimicrobial treatments are recorded, range of number of cows milked into the WM bulk tank, and whether cows not treated with antimicrobials are milked into the WM bulk tank. Variables with a significant *P*-value were added to multiple logistic regression models to investigate associations between farm management practices and dairy characteristics as the independent variables and the presence of drug residues in WM as the dependent dichotomous variable. A Pearson chi-squared test was used to assess goodness of fit of the logistic regression models.

A 1-way ANOVA was used to evaluate associations between mean values of milk quality parameters and the presence of drug residues in WM. The independent dichotomous variables were the presence or not of at least 1 drug residue, or only cephalosporin drug residues, or only tetracycline drugs residues. The dependent variables of interest were the continuous variables from milk quality testing and included fat (%), protein (%), lactose (%), SNF (%), SCC (cells/mL), coliform count (cfu/mL), and SPC (cells/mL). Levene's test was used to test the equal variances assumption, and a Wilcoxon rank-sum test was used to compare group means in place of any linear model that violated this assumption.

Multiple Pearson chi-squared tests were used to evaluate the association between the categorical variable of a WM sample being positive or negative for

at least 1 drug residue, 1 cephalosporin drug residue, and 1 oxytetracycline drug residue and the categorical variables for presence or not of culture-positive results for each microbe cultured from milk. Variables with a significant *P*-value were reported using relative risk for the risk of WM samples positive for a residue being culture positive when compared with the risk of the WM sample positive for this same drug residue being culture negative for the referred microbe.

RESULTS

Farm Characteristics

Farms were categorized based on the combined number of lactating and dry cows: 200 to 499 cows (*n* = 2), 500 to 999 cows (*n* = 4), 1,000 to 2,999 cows (*n* = 12), 3,000 to 5,000 cows (*n* = 5), and >5,000 cows (*n* = 2). The number of preweaned dairy calves present on the farm ranged between 40 and 64 calves (*n* = 1), 65 and 199 calves (*n* = 5), 200 and 499 calves (*n* = 6), 500 and 999 calves (*n* = 4), and >1,000 calves (*n* = 3). Of the 25 farms sampled, 5 raised heifer replacements off-site. The combined number of cows milked into the WM bulk tank was 1 to 9 cows (*n* = 5 herds), 10 to 29 cows (*n* = 10), 30 to 59 cows (*n* = 6), 60 to 89 cows (*n* = 3), and 90 to 119 cows (*n* = 1). The majority of the farms (*n* = 18, 72%) milked both treated and fresh cows into the WM bulk tank, whereas the remaining farms (*n* = 7, 28%) stored WM from treated cows and fresh cows separately.

Questionnaire

A total of 23 dairy farms (92%) reported that all antibiotic treatments were recorded for each individual animal. Of the 23 farms that reported keeping a complete record of antibiotic treatments, 16 (70%) recorded antimicrobial treatments using computerized dairy management software and 7 (30%) maintained written records in a book. Distribution of farms using dairy management software versus a written record by number of lactating cows was, respectively, 0% versus 38% for 200 to 499 cows, 6% versus 38% for 500 to 999 cows, 56% versus 28% for 1,000 to 2,999 cows, 31% versus 0% for 3,000 to 5,000 cows, and 6% versus 14% for >5,000 cows.

Of all the study dairies, 12 (48%) routinely used a teat sealant at dry-off. However, almost all farms used intramammary antimicrobial treatment at dry-off (*n* = 24, 96%). In farms using intramammary antimicrobial treatment at dry-off, 21 farms (87.5%) applied a blanket treatment program and 3 (12.5%) used selective

dry cow treatment. For the farms using selective dry cow treatment, SCC (*n* = 3) and days pregnant (*n* = 1) were the factors considered when choosing which cows to treat. The most common antimicrobial used for intramammary dry-cow treatment was ceftiofur (*n* = 12, 48%) followed by cephalixin (*n* = 5, 20%), penicillin (*n* = 5, 20%), and cloxacillin (*n* = 2, 8%).

The classes of antimicrobials used to treat lactating cows in the study farms were β -lactams, tetracyclines, sulfonamides, and lincosamides (Table 1). Intramammary ceftiofur was the most common drug used to treat mastitis during lactation (68%, *n* = 17). Ceftiofur was also the most common drug used to treat reproductive diseases (76%, *n* = 19), pneumonia (68%, *n* = 17), and lameness (40%, *n* = 10) that warranted systemic antimicrobial treatment. Reproductive disease included metritis, retained placenta, clinical endometritis, or other diseases related to reproduction for which the producer opted to treat an animal using antimicrobial drugs. No significant associations (*P* > 0.05) were identified between farm characteristics, management practices, and presence of drug residues in WM.

Calf Feeding Management Practices

Information regarding calf-rearing practices for those farms was not collected from the 5 farms that raised replacement heifers off-site. For the 20 farms that raised replacement heifers on-site, 19 (95%) used WM as a feed for preweaned calves. The 1 exception sold WM to a nearby calf ranch, where the WM was fed to preweaned dairy calves. A total of 13 (65%) farms fed milk replacer, either mixed with WM or administered alone for specific calf age groups. Of these farms that fed milk replacer, 7 (53.8%) used nonmedicated milk replacer, 3 (23.1%) added neomycin, and 3 (23.1%) added chlortetracycline. A total of 8 farms (40%) fed saleable milk to preweaned dairy calves. Of the dairies raising preweaned calves, 15 (75%) routinely pasteurized the milk before feeding. Methods of pasteurization included low temperature, long time (62°C for 30 min, *n* = 7); high temperature, short time (71°C for 15 s, *n* = 7); and UV light (*n* = 1). The mean age at weaning was 9 wk.

Quantification of Drug Residues Using LC-MS/MS

Of 25 milk samples submitted for LC-MS/MS, 15 (60%) had detectable concentrations of 1 or more of the 27 drug residues screened (Table 2). The classes of antimicrobials found in the WM samples were β -lactams (*n* = 11), tetracyclines (*n* = 4), and sulfonamides (*n* = 1). Only 1 sample of the 15 positive samples con-

Table 1. Responses to an on-farm questionnaire regarding antimicrobial drug use preferences for dry cow treatment, mastitis, reproductive diseases, lameness, and pneumonia on a convenience sample of California dairy farms (n = 25)¹

Antimicrobial agent	Dry cow treatment	Mastitis	Reproductive diseases	Lameness ²	Pneumonia
Amoxicillin		1 ³	7 ³	2 ³	4
Ceftiofur	13	22	19	10	17
Cephapirin	6	10			
Cloxacillin	3	1			
Oxytetracycline			5	2	5
Penicillin G	6	1	9 ³	8 ³	3
Pirlimycin		4			
Sulfadimethoxine				5	3
Tetracycline				19	
Penicillin + sulfadimethoxine				1	
Oxytetracycline + sulfadimethoxine					1
Florfenicol					2
No treatment ⁴					4

¹Some farms reported 2 antimicrobials for treating cows.

²This included a combination of both parenteral and topical antimicrobials used to treat cows diagnosed with lameness disease.

³Antimicrobial drugs that do not have any commercial drug label that includes the referred disease as an indication for use.

⁴Farms that reported not having a drug preference for treating that specific disease.

tained both β -lactams and tetracyclines, whereas the remaining 14 were positive for only 1 class of antibiotic. Ceftiofur was the most frequently detected drug residue [present in 7 (28%) of the WM samples], followed by oxytetracycline [present in 4 (16%) of the samples] and cephapirin [found in 3 (12%) of the samples; Table 3]. The FDA tolerance and safe limit for drug residues in raw milk is displayed in Table 3. One sample had detectable concentrations of penicillin G, 1 had detectable concentrations of sulfadimethoxine, and 1 had detectable concentrations of tetracycline. No samples had detectable concentrations of 5-hydroxyflunixin. Ten (40%) out of 25 milk samples submitted for LC-MS/MS had no detectable concentrations of any of the 27 drug residues screened.

Milk Quality Parameters

The mean fat (%), protein (%), lactose (%), SNF (%), SCC, coliform count, and SPC for WM samples are displayed in Table 4. Significant differences in coliform count ($P = 0.009$) and SPC ($P = 0.01$) were found only between WM samples positive for tetracycline class residues compared with samples negative for tetracycline residues (Table 5).

Milk Microbiology

The most common genera of bacteria isolated from WM samples was *Streptococcus* spp. (n = 21, 84%) followed by *Staphylococcus* spp. (n = 20, 80%) and *E. coli* (n = 10, 40%; Table 6). Other major mastitis pathogens isolated were *Staphylococcus aureus* (n = 5, 20%), *Klebsiella* spp. (n = 4, 16%), and *Mycoplasma* spp. (n

= 2, 8%). Culture-positive *S. aureus* WM samples were significantly associated with samples being positive for at least 1 drug residue in WM ($P = 0.04$) when compared with WM samples negative for any drug residues in WM. The relative risk for a WM sample positive for at least 1 drug residue being culture positive for *S. aureus* was 2.0 (95% CI: 1.2–3.1) compared with being culture negative for *S. aureus*.

Similarly, the probability of a WM sample being positive for *S. aureus* was significantly higher ($P = 0.04$) if the sample was positive for cephalosporin drug residues compared with WM samples negative for cephalosporin residues. In addition, an increased probability for culturing *Mycoplasma* spp. was significantly associated ($P = 0.04$) with WM samples containing drug residues for cephalosporin residues when compared with WM samples negative for cephalosporin residues. The rela-

Table 2. Distribution of antimicrobial drug residues in raw waste milk from a convenience sample of California dairies (n = 25)¹

Description ²	Frequency (%)
Result of commercial LC-MS/MS test (n = 25)	
Positive	15 (60)
Negative	10 (40)
Distribution by drug class (by LC-MS/MS; n = 25)	
Negative	10 (40)
Only β -lactams	10 (40)
Only sulfamethazine	1 (4)
Only tetracyclines	3 (12)
β -Lactams and tetracycline	1 (4)

¹Residues were detected by liquid chromatography–tandem MS (LC-MS/MS).

²Positive = milk samples tested positive for at least 1 of the drugs tested; negative = milk samples tested negative for all of the drugs tested.

Table 3. Antimicrobial drug residues in waste milk from a convenience sample of California dairies (n = 25) as measured by liquid chromatography–tandem MS (LC-MS/MS)

Drug residue	% (no.) ¹	Mean (ng/mL)	SE	LOQ ² (ng/mL)	Tolerance ³ (ng/mL)
β-Lactams	44 (11)				
Ceftiofur	28 (7)	251.14	177.0	<10	100
Cephapirin	12 (3)	39	24.5	<10	20
Penicillin G	4 (1)	10	—	<5	0
Sulfonamides					
Sulfadimethoxine	4 (1)	7	—	<2	10
Tetracyclines	16 (4)				
Tetracycline	4 (1)	590	—	<10	300
Oxytetracycline	12 (3)	378.5	308.7	<10	300

¹Number of milk samples with antimicrobial drug residues at concentrations above the limit of quantification for LC-MS/MS for each individual drug or drug class described.

²Limit of quantification of LC-MS/MS for each drug detected in the milk samples.

³Food and Drug Administration tolerance or safe limit for drug residues in raw milk.

tive risk for a WM sample positive for cephalosporin drug residues being culture positive for *S. aureus* was 2.6 (95% CI: 1.2–5.9) and for *Mycoplasma* was 2.8 (95% CI: 1.6–5.0) when compared with being culture negative for these bacteria.

Antimicrobial Resistance in *E. coli* Isolates

Escherichia coli was isolated from 10 (40%) WM samples. Of the 10 *E. coli* isolates that were tested against selected antimicrobials, 70% were susceptible to β-lactams and tetracyclines and 80% were susceptible to sulfonamides (Table 7). Prevalence of multiple drug resistance was 20%. Among the β-lactams tested, 3 isolates were resistant to ampicillin and 2 were resistant to cephalothin. One isolate was resistant to ceftiofur, and 2 isolates showed intermediate susceptibility. Only 40% of the isolates were sensitive to all antimicrobials tested.

DISCUSSION

Quantification of Drug Residues

Ceftiofur was the most common drug detected in WM and is the only third-generation cephalosporin licensed

to treat food-producing animals in the United States. Parenteral administration of commercially available ceftiofur at the approved label dosage does not result in milk residues at concentrations above the tolerance levels established by the FDA. Ceftiofur residues are likely from the use of an intramammary treatment of a cow for mastitis. According to the label, intramammary ceftiofur has a withdrawal period of 72 h after the last treatment before milk from a treated cow may be sold for human consumption.

In a study by Pereira et al. (2014a), feeding pre-weaned calves milk containing a very low concentration of antimicrobials for 6 wk resulted in selection of multidrug-resistant *E. coli* in their feces when compared with the control group fed milk without drug residues. In the Pereira et al. (2014a) study, milk fed to calves in the treatment group was spiked with ceftiofur, penicillin, ampicillin, and oxytetracycline at concentrations of 100, 5, 10, and 300 ng/mL, respectively. Except for ampicillin, all these drugs were detected in WM of dairy farms in California at similar concentrations. Furthermore, the Pereira et al. (2014a) study supports the hypothesis that, on average, if calves in California are fed WM, we can expect to observe a higher prevalence of antimicrobial resistant (**AMR**) enteric bacteria in

Table 4. Milk quality parameters for waste milk samples collected from a convenience sample of California dairies (n = 25)

Variable	Mean	SD	95% CI
Milk fat (%)	4.24	1.41	3.66, 4.82
Milk protein (%)	3.74	0.43	3.56, 3.92
Lactose (%)	4.4	0.22	4.31, 4.49
SNF (%)	8.77	0.45	8.59, 8.96
SCC ($\times 10^3$ cells/mL)	2,133.60	1,260.14	1,613.44, 2,653.76
Coliforms (cfu/mL)	702.4	691.12	417.12, 987.68
SPC ($\times 10^3$ cfu/mL)	116.27	101.19	74.50, 158.04

Table 5. Comparison of milk quality parameters for waste milk samples from a convenience sample of California dairies (n = 25) stratified by test status for at least 1 antimicrobial drug residue, ceftiofur drug residues, and oxytetracycline drug residues

Parameter	Negative		Positive	
	Mean	95% CI	Mean	95% CI
At least 1 antimicrobial residue				
Fat (%)	4.39	3.78, 5.00	4.14	3.20, 5.08
Protein (%)	3.65	3.49, -3.82	3.80	3.51, 4.09
Lactose (%)	4.42	4.3, 4.55	4.39	4.25, 4.53
SNF (%)	8.71	8.51, 8.90	8.82	8.52, 9.12
SCC ($\times 10^3$ cells/mL)	2,452.0	1,583.0, 3,320.9	1,921.3	1,209.3, 2,633.3
Coliforms (cfu/mL)	563.00	86.05, 1,040.0	795.33	399.8, 1,190.8
SPC ($\times 10^3$ cells/mL)	117.00	49.4, 184.6	115.78	60.5, 170.9
Cephalosporin class residues				
Fat (%)	4.24	3.60, 4.87	4.22	2.51, 5.94
Protein (%)	3.67	3.49, 3.84	4.24	3.76, 4.71
Lactose (%)	4.39	4.29, 4.49	4.47	4.21, 4.74
SNF (%)	8.69	8.51, 8.87	9.33	8.84, 9.81
SCC ($\times 10^3$ cells/mL)	2,080.0	1,516.2, 2,643.7	2,526.6	999.9, 4,053.4
Coliforms (cfu/mL)	593.6	313.3, 874.0	1,500.0	740.7, 2,593.2
SPC ($\times 10^3$ cells/mL)	109.9	65.0, 154.9	163.0	41.4, 284.5
Tetracycline class residues				
Fat (%)	4.02	3.41, 4.62	5.38	3.9, 6.7
Protein (%)	3.68	3.49, 3.68	4.05	3.62, 4.48
Lactose (%)	4.41	4.31, 4.51	4.33	4.10, 4.56
SNF (%)	8.72	8.52, 8.91	9.04	8.58, 9.50
SCC ($\times 10^3$ cells/mL)	2,220.9	1,647.5, 2,794.3	1,675.0	361.1, 2,988.8
Coliforms (cfu/mL)	832.3 ¹	546.2, 1,114.9	20.0 ¹	7.0, 33.0
SPC ($\times 10^3$ cells/mL)	134.2 ¹	91.8, 176.7	21.8 ¹	-43.1, 86.7

¹Mean value and 95% confidence interval for the 1-way ANOVA used to evaluate samples positive or negative for presence of a specific drug residue.

their feces compared with calves fed milk not containing antimicrobial residues.

Compared with 2 other studies that evaluated drug residues in milk, the first conducted in the Central Valley of California (Selim and Cullor, 1997) and the second conducted in New York State (Pereira et al., 2014b), our results were within the range expected for observing drug residues in WM. From these 2 studies, the first showed an overall prevalence of drug residues in WM of 63%, whereas the second showed that 82% of

WM samples had at least 1 drug residue at detectable concentrations. In both studies, the overall prevalence of antimicrobial drug residues in WM was calculated using commercial SNAP tests. In the New York study, all the positive samples on SNAP tests were submitted for drug identification and quantification using LC-MS/MS. In our study, all the WM samples collected were tested with LC-MS/MS, and we found an overall prevalence of 60% of WM samples positive for at least 1 of the antimicrobials tested. The lower prevalence

Table 6. Culture results for waste milk samples collected from a convenience sample of California dairies (n = 25)

Bacteria	No. (%) culture positive	Mean cfu ($\times 10^3$ /mL)	SD ¹
<i>Streptococcus</i> spp.	21 (84)	51.01	89.96
<i>Staphylococcus</i> spp.	20 (80)	8.63	22.09
<i>Escherichia coli</i>	10 (40)	5.87	7.83
Nonfermentative gram negative	8 (32)	9.63	8.73
<i>Staphylococcus aureus</i>	5 (20)	121.40	267.55
<i>Bacillus</i> spp.	5 (20)	0.86	1.26
<i>Klebsiella</i> spp.	4 (16)	78.75	147.54
<i>Trueperella pyogenes</i>	3 (12)	20.40	26.01
<i>Mycoplasma</i> spp. ²	2 (8)	—	—
<i>Enterobacter</i> spp.	1 (4)	10	—
<i>Prototheca</i> spp.	1 (4)	3	—
Yeasts	1 (4)	10	—

¹Standard deviation of the mean for milk colony-forming units per milliliter.

²No quantification of *Mycoplasma* culture-positive samples was conducted.

Table 7. Distribution of antimicrobial susceptibility testing of *Escherichia coli* isolated from waste milk samples (n = 10) from a convenience sample of California dairies (n = 25)

Antimicrobial	% (no.) susceptible	% (no.) intermediate	% (no.) resistant
β-Lactams			
Ampicillin	70 (7)	0 (0)	30 (3)
Cephalothin	80 (8)	0 (0)	20 (2)
Ceftiofur	70 (7)	20 (2)	10 (1)
Tetracycline			
Tetracycline	70 (7)	0 (0)	30 (3)
Sulfonamides			
Sulfisoxazole	80 (8)	0 (0)	20 (2)

of positive samples may have been due to the higher limit of detection of the LC-MS/MS compared with the commercial SNAP tests. In both previous studies, the most common classes of antimicrobial residues were β -lactams followed by tetracyclines. Similarly, in the present study, the β -lactam class was the most common antimicrobial class and the tetracycline class was the second most common. Common use of these drugs on dairy farms may be the reason for the higher prevalence of residues of these drugs.

A survey conducted on 113 dairy farms in Pennsylvania (Sawant et al., 2005) found that β -lactams and tetracyclines were the most widely used antibiotics. Despite 14 antimicrobials belonging to 8 different classes having approved use for adult lactating dairy cows in the United States, in the present study we found only 6 antimicrobial drug residues in WM belonging to 3 different classes.

On-Farm Antimicrobial Use

Ceftiofur was the most commonly reported antimicrobial used for all the disease categories evaluated (intramammary dry-off treatment, mastitis during lactation, reproductive disease, pneumonia, and nontopical lameness treatment). This result confirms findings from other surveys on antimicrobial use on dairy farms (Zwald et al., 2004). Although ceftiofur is used only in veterinary medicine, all third-generation cephalosporins have been classified as critically important antimicrobials in human medicine by the World Health Organization (WHO, 2007), and the use of ceftiofur in food animals represents a major public health concern because of the possible development of resistance to other extended-spectrum cephalosporins, such as ceftriaxone and cephamycins, used in human medicine (Dunne et al., 2000). Due to the importance of ceftiofur as a veterinary drug as well as a drug class of importance to human medicine, the extralabel use of ceftiofur in the United States is limited, and according

to the FDA it can only be used extralabel to treat or control an extralabel disease indication as long as this use adheres to a labeled dosage regimen (i.e., dose, route, frequency, and duration of administration) approved for that particular species and production class (FDA, 2018b). Furthermore, unnecessary extralabel use of antimicrobials considered of critical importance such as third-generation cephalosporin drugs, especially when an antimicrobial belonging to a class of lower importance to veterinary and human medicine could be used, can result in unnecessary selection of antimicrobial resistance to important drug classes (FDA, 2003).

Tetracycline was the treatment of choice for topical antimicrobial treatment of lameness in 60% of the farms. The only nonapproved antimicrobial used on the study dairies was florfenicol, which was routinely used by 2 dairy producers to treat pneumonia in adult cows. Florfenicol is not approved for use in dairy cattle ≥ 20 mo of age (Smith et al., 2005); however, it may be used in an extralabel fashion in food-producing species (Payne et al., 1999; Davis et al., 2009) by a licensed veterinarian or a layperson under the supervision of a licensed veterinarian within the scope of a valid veterinary client–patient relationship and with appropriate prescription and labeling.

Keeping adequate records of antimicrobial drug treatment is a very important routine practice for farms to ensure a low risk of cows with milk containing antimicrobial drug residues above tolerance or safe levels being milked into the saleable milk bulk tank. Our study questionnaire reported that 2 out of the 25 farms did not have complete records on cow antimicrobial treatment. The FDA views the failure of a dairy farm to keep adequate drug treatment records as an inadequate control measure that creates unsanitary conditions, and therefore adulterated food, under the US Federal Food, Drug, and Cosmetic Act (FDA, 2018a). Most of the farms in the study kept records using a farm management computer program (70%). This is in agreement with the NAHMS report on dairy cattle management, where 60.8% of medium (100–499 cows) and 98% of large (≥ 500 cows) farms reportedly used a computer system for record keeping, which included use of antimicrobials (USDA, 2016). Keeping adequate drug treatment records using a computerized system can be an effective method to monitor individual animal treatment and generate reports on overall usage of antimicrobials on the farm. A guideline for adequate record keeping can be found on the FDA website and includes keeping a record of the name of the drug used, individual identity of the animal treated, date of each administration of the drug to the animal, dosage used, and route of administration (FDA, 2018a). This information can be a useful tool to monitor disease

incidence over time and improve decision making on investment in preventive measures that could reduce disease risk and antimicrobial drug use on the farm. To the authors' knowledge, the FDA does not currently consider computer records as an inadequate method to record individual animal antimicrobial treatments (FDA, 2018a); the exception is that the FDA requires a farm to have written records after a drug residue violation (FDA, 2011b). Furthermore, digital records can be printed when needed to generate hard copies of that information.

Although our study questionnaire collected relevant information that could result in drug residues being detected in WM, questions were mostly limited to practices directly linked to selection of drugs used to treat the most common diseases observed in dairy cattle. Therefore, there is a need for future research that collects more extensive information about herd management to allow identification of practices that could be addressed in order to reduce unnecessary treatment of cattle with antimicrobial drugs and therefore decrease antimicrobial residues in milk.

Milk Quality Parameters

Most of the study farms housed and milked both treated and postpartum cows together. Therefore, the WM samples were a blend of colostrum, transition milk, and other nonsaleable milk. Consequently, the mean fat, protein, and lactose contents were intermediate between the standard composition of the different types of milk reported in the literature (Godden, 2008). In our study, we found high variability in the SCC in WM samples, likely reflecting different management practices related to hospital and fresh cows between different farms where WM samples were collected. High SCC values and high variability in the composition of WM have been previously reported in the literature (Moore et al., 2009) and suggested to be related to milk from mastitic cows with high SCC increasing the average SCC in the WM bulk tank. A study evaluating SPC and coliform counts in more than 150 colostrum samples from 40 dairy farms observed that SPC and coliform counts exceeded 1,000,000 and 10,000 cfu/mL, respectively, in 85% of the samples tested (McGuirk and Collins, 2004). We also observed high SPC and coliform counts in our study, with mean values above 111,000 and 700 cfu/mL, respectively.

The only drug class for which residues in WM had significant effects on the SPC and coliform counts was tetracycline, which resulted in significantly lower concentrations for both bacteriological measures when compared with WM samples not containing tetracycline residues. A hypothesis for this finding is that the

concentration of tetracycline drugs in WM samples was significantly reducing the replication of culturable bacteria in WM samples. Although the MIC of tetracycline for *E. coli*, a common gram-negative environmental bacteria, is 1,000 ng/mL, exposure of gram-negative bacteria to tetracycline concentrations below the MIC has been shown to affect growth rates as well as the dynamics of selection of antimicrobial-resistant bacteria (Gullberg et al., 2011). A potential explanation for why other drug residues (e.g., cephalosporins) in WM did not similarly affect SPC and coliform counts could be because they were present at lower mean concentrations compared with the tetracycline drugs and because cephalosporin MIC for common gram-negative bacteria such as *E. coli* is 2 times greater (2,000 ng/mL) than that of tetracycline.

Isolation and Identification of Bacteria

Selim and Cullor (1997) found a significantly higher number of bacteria in WM compared with other types of milk. In our study, we found high numbers of bacteria, which may be due to the fact that most farms stored milk in nonrefrigerated tanks before feeding it to calves. Given that WM can originate from cows treated with antimicrobials, the risk of AMR bacteria in WM could be from direct selection of AMR bacteria from the cow as a consequence of drug treatment as well as further selection of AMR bacteria in bulk tank milk due to the selective pressure caused by drug residues present in that environment (Sandegren, 2014; Awosile and Smith, 2017). Although pasteurization may reduce the bacteria load of WM, limited information is available on approaches to degrade drug residues in WM to reduce selection of AMR bacteria postpasteurization due to the presence of drug residues.

Waste milk samples cultured positive for *S. aureus* were significantly more commonly identified in samples positive for cephalosporin drugs compared with negative samples. Similarly, *Mycoplasma* spp.-positive samples had a significantly higher probability of being cultured in samples positive for cephalosporin drugs compared with negative samples. Both *S. aureus* and *Mycoplasma bovis* are contagious bacteria that cause mastitis and result in a significant cost to the dairy industry because of the low cure rate (*S. aureus*) and the lack of effective antimicrobial drugs (*Mycoplasma* spp.) to treat these pathogens, resulting in many cases of culling of animals from the herd (Barkema et al., 2006; Royster and Wagner, 2015). The higher concentration of cephalosporin drugs in milk samples that cultured positive for these 2 bacteria could be due to the spread of mastitis caused by these contagious bacteria, resulting in greater numbers of cows being treated for clinical mastitis, as well

as an increase in chronic cases of mastitis, resulting in larger numbers of cows with cephalosporin drug residues in milk being withheld from human consumption. Use of intramammary antimicrobials to treat mastitis without diagnostic information on the pathogen causing mastitis is a good example of how erroneous or lack of diagnostic information can result in greater cost to the dairy. This increased cost could be from antimicrobial drug costs, milk discard, disease transmission risk, and the potential for transmission of antimicrobial drug-resistant pathogens (e.g., *Mycoplasma* spp.) via WM (Pereira et al., 2014a; Cha et al., 2016). Furthermore, presence of *Mycoplasma* spp. in WM used as a feed source may be detrimental to calf health and may result in higher occurrence of *Mycoplasma* pneumonia and otitis media in calves (Walz et al., 1997).

Antimicrobial Resistance in *E. coli* Isolates

In the present study, we found both detectable concentrations of drug residues in the milk and presence of resistant bacteria. Because we focused our analysis on *E. coli* isolates, these resistant bacteria more likely come from environmental or fecal contamination of the WM during harvest and storage. Even in those cases, *E. coli* isolates that originate from the environment may be exposed to selective pressure in the WM due to antimicrobial residues (Ricci et al., 2017). Although no significant association between the presence of drug residues in WM samples and AMR in the *E. coli* isolates was found, no quantitative methods for evaluating the prevalence of AMR were used in the study, and we could not separate AMR isolates originating from cows being treated with antimicrobials versus selection of AMR bacteria caused by drug residues in the WM bulk tank. The low number of samples that cultured positive for *E. coli* warrants further studies that focus on evaluating risk factors of AMR bacteria selection in WM samples. The presence of multidrug-resistant *E. coli* in WM urges the need for on-farm practices, such as pasteurization, that reduce the risk of exposure to calves and dissemination of resistant bacteria into the environment (Awosile and Smith, 2017).

One of the limitations of the current study is the cross-sectional study design, where data collection occurred only at a single point, limiting causal inference between exposures of interest (on-farm management practices) and outcomes (drug residues in WM). Longitudinal studies are required to study the causal associations between management practices and antimicrobial resistance and provide further information on the effects of feeding preweaned calves WM. A controlled trial was previously conducted in which calves were fed

raw saleable milk from birth to weaning, with half of calves receiving milk spiked with 4 antibiotics, including ceftiofur, at the tolerance levels established by the FDA and the other half fed the same raw saleable milk but without the addition of any antimicrobials. This controlled trial showed a significant increase in the proportion of fecal shedding of *E. coli* resistant to ceftiofur as well as multidrug-resistant bacteria (Pereira et al., 2014a). One limitation of that study was that it did not take into account potential on-farm effects; however, it provides scientific evidence of how low drug residue concentrations can select for antimicrobial-resistant bacteria.

The novel information generated by this study will help guide future studies focused on addressing the issue of feeding milk containing drug residues to calves using data that improved the validity of conclusions made specifically for farms in Central California. Concerns worldwide with this practice can be observed in the recent report by the European Food Safety Authority evaluating the risk for development of antimicrobial resistance due to feeding calves milk that contains residues of antibiotics (EFSA, 2017). With increased concerns about unnecessary exposure of animals to antimicrobial drugs, especially those present in feed, and the current ambiguous legality of feeding calves milk containing drug residues in the United States, scientific information is of critical importance to support future decisions and interventions that may affect the practice of feeding WM to dairy calves.

CONCLUSIONS

Waste milk fed to calves on dairies in California's Central Valley contains residues of antimicrobial drugs that are important to veterinary and human medicine. The presence of drug residues in WM at concentrations that increase the selection of resistant bacteria indicates the need for additional studies targeting on-farm milk treatments to degrade drug residues before feeding to calves. This also highlights the importance of antimicrobial stewardship and proper preventive disease management practices that reduce disease risk and the need for antimicrobial drug use in lactating dairy cows. The presence of multidrug-resistant *E. coli* in WM urges the need for on-farm practices, such as pasteurization, that reduce calf exposure to resistant bacteria.

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