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Antimicrobial Resistance in California Dairy Cattle Populations: Impacts on Microbiota and Pathogenic Bacteria

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Antimicrobial Resistance in California Dairy Cattle Populations: Impacts on Microbiota and Pathogenic Bacteria

By

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DISSERTATION

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They say it takes a village to raise a child, so I can only imagine what it takes to produce a Ph.D. graduate! The dissertation presented below would not have been possible without the support (both financially and emotionally) of so many. If I had to credit everyone, this section would look like the end of a Marvel movie.

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"If I have seen further than others, it is by standing upon the shoulders of giants"

-Isaac Newton

Preface

The following chapters comprising this dissertation are collective works in various stages of the publication process. All chapters have been modified from published form to fulfill formatting requirements.

Chapter 1 is full reprint of the open access publication under the terms of the Creative Commons Attribution License (CC BY): Basbas C, Byrne BA, Chigerwe M, Escobar ED, Hodzic E, Pires AFA and Pereira RV (2021) Detection of Cephalosporin and Fluoroquinolone Resistance Genes via Novel Multiplex qPCR in Fecal *Salmonella* Isolates from Northern Californian Dairy Cattle, 2002–2016. *Front. Microbiol.* 12:601924. https://doi.org/10.3389/fmicb.2021.601924

Chapter 2 is full reprint of the open access publication under the terms of the Creative Commons Attribution License (CC BY): Basbas C, Aly S, Okello E, Karle BM, Lehenbauer T, Williams D, Ganda E, Wiedmann M, Pereira RV. Effect of Intramammary Dry Cow Antimicrobial Treatment on Fresh Cow's Milk Microbiota in California Commercial Dairies. *Antibiotics*. 2022; 11(7):963. https://doi.org/10.3390/antibiotics11070963

Chapter 3 is full reprint of the open access publication under the terms of the Creative Commons Attribution License (CC BY): Basbas, C., Garzon, A., Silva-del-Rio, N. *et al.* Evaluation of antimicrobial resistance and risk factors for recovery of intrauterine *Escherichia coli* from cows with metritis on California commercial dairy farms. *Sci Rep* **12**, 13937 (2022). https://doi.org/10.1038/s41598-022-18347-w

Chapter 4 is being adapted for future publication under the title “Metagenomic analysis of intrauterine microbiome of cows with metritis on California commercial dairy farms”

Carl Basbas, Adriana Garzon, Cory Schlesener, Machteld van Heule, Bart Weimer, Noelia Silva-del-Rio, Barbara A. Byrne, Betsy Karle, Sharif S. Aly, Fabio S. Lima, Richard V. Pereira

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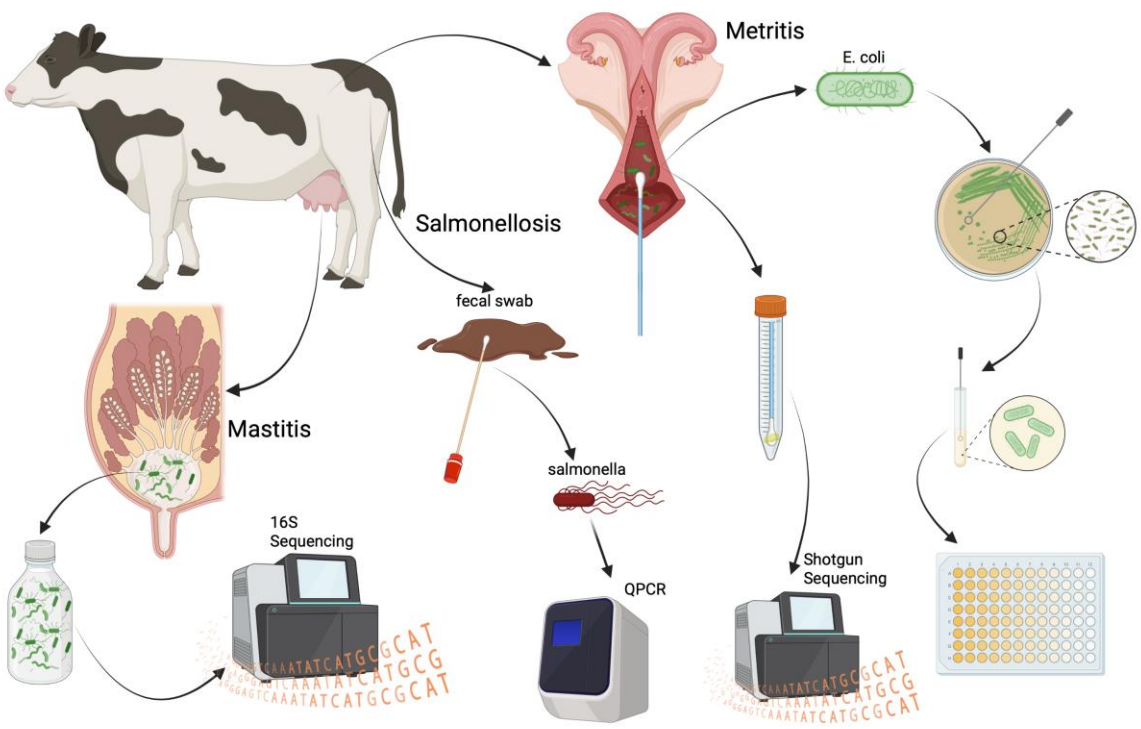
Dissertation Abstract



Credit: Craig Miramontes, created with BioRender.com.

The challenge of new legislation and growing consumer interest in the prudent use of antimicrobials in the dairy industry has spurred research into the state of antimicrobial resistance (AMR) in cattle within the State of California. In addition, the rapid adoption of next generation sequencing has allowed new perspectives into the causes and potential treatments of common diseases of cattle that often require antimicrobial treatment. The data presented in this dissertation focus on three common ailments of cattle: salmonellosis, mastitis, and metritis. Chapter one details the prevalence of extended-spectrum β-lactamase (ESBL) genes, AmpC-type β-lactamase (ACBL) genes, and plasmid-mediated quinolone resistance (PMQR) genes in *Salmonella* isolated from bovine fecal samples at a Veterinary Medical Teaching Hospital microbiology laboratory that were detected using a single, novel multiplex qPCR that was developed. Chapter two uses 16s rRNA

sequencing to evaluate the effects of dry cow antimicrobial therapy (used to prevent mastitis) on the udder milk microbiota by comparing the microbial populations in milk at dry-off (DRY) (~60 days before calving) and post-partum (FRESH) (4-11 days after calving) from cows receiving intramammary antibiotic infusion prior to dry-off (IMT) and cows that did not receive treatment (CTL). Chapter three presents a large cross-sectional study designed to evaluate factors affecting recovery and AMR in intrauterine *E. coli.* In total, 307 cows with and without metritis, from which a single *E. coli* was randomly selected (n = 162), were sampled from 25 farms throughout California. All intrauterine *E. coli* were resistant to ampicillin, with an AMR prevalence of 30.2% and 33.9% observed for chlortetracycline and oxytetracycline, respectively. Only 8.6% of isolates were resistant to ceftiofur, one of the most common drugs used to treat metritic cows on the farms sampled. Lastly, chapter four reveals the microbial ecology and diversity of the microbiota present within the uterus of post-partum dairy cows with and without metritis using shotgun metagenomics, only the second study to do so, on a subset of the sample population analyzed in chapter three. In general, the uterine microbiota from cows with and without metritis were highly diverse, with the top 12 most abundant genera only accounting for roughly 10% of mean relative abundance. The results presented here highlight the need for better clinical testing data used for interpretation of phenotypic antimicrobial susceptibility testing within large animal veterinary medicine and showcase the potential of 16s rRNA and shotgun sequencing to analyze the endogenous and pathogenic bacteria present within the bovine mammary and uterine microbiomes.

Introduction

1. One health and antimicrobial resistance

1.1 Antimicrobial resistance introduction

Antimicrobial resistance (AMR), the ability of pathogenic microorganisms to counter the drugs designed to eliminate them, threatens to send human and veterinary medicine back to a pre-penicillin era. Recent estimates suggest that nearly 3 million Americans are infected with AMR microbes annually—with over 35 thousand deaths as a result [2]. Without intervention, one prediction forecasts 10 million global deaths annually due to AMR by 2050 [3]. In this scenario, by 2050 global gross domestic product was estimated to be 2 to 3.5% lower than it otherwise would be; a loss of 60 to 100 trillion USD in global economic output. While the majority of antimicrobial resistance-related concerns focus on human health, AMR also heavily impacts veterinary medicine and particularly livestock.

1.2 Benefits of a One Health approach

The connectivity of human health, animal health, and the environment is a concept known as One Health [4]. While each discipline has traditionally compartmentalized threats, the emergence of wide-spread antimicrobial resistance along with other key issues has forced collaboration. For example, the 2018 outbreak of Shiga toxin–producing *Escherichia coli* (STEC) that sickened 240 people was traced back to romaine lettuce grown in the Yuma growing region in Arizona [5]. While the definitive cause of the outbreak remains unknown, environmental sampling identified the outbreak strain of *E. coli* O157:H7 in multiple locations of an irrigation canal that ran adjacent to several suspect romaine fields. Cattle were located adjacent to the irrigation canal where the outbreak strain was found, however no direct evidence linking contamination to a particular producer was ever identified. A One Health approach, in this case

assessing the environment around the area of an outbreak, has been a valuable tool in outbreak investigation and research efforts.

1.3 Use of antimicrobials in livestock

While the use of antimicrobials in the production of livestock (and in particular cattle) has continued to garner negative attention, it should be recognized that the use of these drugs has increased animal health, lowered disease incidence, reduced mortality, and allowed for the production of nutritious and economical beef and dairy products [6]. According to the most recent report from the Food and Drug Administration (FDA), domestic sales and distribution of medically important antimicrobials (i.e., antimicrobials important to human medicine including cephalosporins, fluoroquinolones, penicillins, and tetracyclines) have decreased by 38% from peak sales in 2015 through 2020 [7]. Of the roughly six million kilograms of medically important antimicrobials sold in 2020, an estimated 41% (~2.5 million kilograms) were intended for use in cattle; with an estimated breakdown consisting of 80% cephalosporins (e.g., ceftiofur and cephapirin), 57% sulfonamides (e.g., sulfadimethoxine), 54% aminoglycosides (e.g., streptomycin), and 43% tetracyclines (e.g. chlortetracycline and oxytetracycline). It should be noted that there is presently no substantiated or conclusive evidence that antimicrobial use in dairy cattle is directly responsible for AMR in human pathogens [8].

1.4 General public health threats from dairy

While uncommon in the U.S. and industralized nations, human illness from beef and dairy products does occur [9, 10]. Aside from contamination of the environment, as previously mentioned, dairy cattle can also pose a potential threat to public health via the consumption of unpasteurized or “raw” dairy products. Within the U.S. from 2009-2014, unpasteurized dairy products led to 840 times more illnesses and 45 times more hospitalizations than pasteurized

products [11]. Unpasteurized milk has also been found to contain significantly more antimicrobial resistance genes (ARGs), including those capable of moving horizontally between bacterial species, than pasteurized milk [11].

Dairy cattle have also been linked with human illness via the consumption of ground beef processed with a proportion of culled dairy cows [12, 13]. Exact data on the proportion of ground beef made from dairy cows in the US are rare, but one estimate places that number around 18% [14]. AMR prevalence data for *E. coli* and *Salmonella* provided by the National Antimicrobial Resistance Monitoring System (NARMS) from a nationwide sampling of retail ground beef illustrates that resistance to common antimicrobials has either remained relatively stable or decreased since 2016 [15]. However, data from NARMS sampling conducted on dairy cecal contents highlighted a large increase in AMR prevalence within *E. coli* isolates against amoxicillin-clavulanic acid and ampicillin from 2019 to 2020. In contrast, multidrug resistance or MDR (resistance to 3 or greater classes of antimicrobials) has remained stable or decreased in *Salmonella* isolates collected from retail ground beef, beef cecal content, and dairy cecal content samples since 2018. Multidrug resistance in *E. coli* isolates collected from retail ground beef has remained stable. However, MDR has fluctuated considerably in *E. coli* isolates collected from beef and dairy cecal contents.

2. Bovine salmonellosis

2.1 Salmonella in cattle

Bacteria in the genus *Salmonella* are gram-negative, rod-shaped facultative anaerobes which belong to the order Enterobacterales [17]. The species *Salmonella enterica* is further classified into nearly 2,600 serotypes or serovars. Non-typhoidal *Salmonella* (NTS) serovars (e.g.,

Enteritidis, Newport, and Typhimurium) are the primary concern regarding foodborne outbreaks. The increasing prevalence of the cattle-adapted *Salmonella enterica* serovar Dublin or *Salmonella* Dublin has received attention in both human and livestock medicine due to increased virulence, morbidity, and mortality [18]. In a study analyzing demographic, clinical, and AMR characteristics of human *Salmonella* Dublin infections from 1968-2018, 51% of infections occurred in California, with isolation from blood and hospitalization significantly increased compared to other serovars [19].

While cattle can indeed be a potential reservoir for *Salmonella* to enter the food supply, the prevalence within U.S. beef farms and from sampled cows has been observed to be low; approximately 10% of farms and 1% of cows sampled, respectively [19]. Clinical signs of salmonellosis in cattle include fever, diarrhea, anorexia, dehydration, decreased milk production, and abortion [20]. However, cattle can also shed *Salmonella* in fecal matter and milk without displaying signs of infection [21].

2.2 Antimicrobial treatment for Salmonellosis

Most *Salmonella* infections of animals and humans do not require treatment with antimicrobials; however, severe infection can necessitate their use. In adult humans, severe *Salmonella* infections are treated with, among other antimicrobials, 3rd generation cephalosporins (e.g. ceftriaxone) and fluoroquinolones (e.g. ciprofloxacin) [23]. In addition to supportive care, salmonellosis in cattle can also require the administration of antimicrobials including trimethoprim-sulfonamide combinations, ampicillin, or 3rd generation cephalosporins (e.g. Given the shared use of 3rd generation cephalosporins for the treatment of severe *Salmonella* infections in both humans and cattle and the propensity for *Salmonella* to cause foodborne infection; AMR to ceftiofur and ceftriaxone in *Salmonella* poses a one health threat.

2.3 Extended-spectrum beta-lactamases and plasmid mediated quinolone resistance genes

In particular, the presence of extended-spectrum beta-lactamases (ESBLs) and plasmid mediated quinolone resistance (PMQR) genes threaten the efficacy of antimicrobial treatment for salmonellosis in both humans and cattle. Broadly, ESBLs are beta-lactamases (enzymes that inactivate beta-lactam antimicrobials by cleaving the beta-lactam ring) that are capable of conferring bacterial resistance to penicillins, extended-spectrum cephalosporins, and occasionally to carbapenems [25, 26]. Therefore, as ceftriaxone and ceftiofur are common treatments for severe *Salmonella* infections in humans and cattle, respectively, ESBLs pose a danger. Ceftriaxone resistance has increased recently but remains low in *Salmonella* isolates collected from cattle (beef), likely due to the increased prevalence of the *bla*CTX-M-65 gene in *Salmonella* Infantis [27]. A recent study of AMR *E. coli* and *Salmonella* isolates from Texas dairy cow feces confirms that the most common ESBL-encoding gene in cattle remains *bla*CMY-2 [28, 29].

Fluoroquinolones (e.g. ciprofloxacin, levofloxacin) are another class of antimicrobials used to treat severe non-typhoidal *Salmonella* infections in humans, typically in elderly or immunocompromised patients [30]. Plasmid mediated quinolone resistance (PMQR) genes confer resistance to fluoroquinolones in a variety of mechanisms including protection of DNA gyrase and topoisomerase IV (the two mechanisms of action for fluoroquinolones), acetylation, and efflux pumps [31]. However, as indicated in their name, PMQR genes are capable of horizontal transmission via plasmids to other bacteria, thus spreading resistance [32]. These plasmids often also harbor resistance genes to other antimicrobial classes, potentially explaining the strong association between ESBLs and PMQR genes [33]. Therefore, fluoroquinolone treatment may be inadvertently selecting for resistance to cephalosporins, aminoglycosides, sulfonamides, and any other ARGs present on plasmids containing PMQR genes. Notably, from 2018 to 2019 decreased

susceptibility to ciprofloxacin in NTS isolated from humans increased from 9% to 11% [27]. Within food animal production, PMQR genes are present in the U.S. NTS isolates; however, detection from dairy cattle has been limited to two isolates collected from environmental samples at a Texas dairy [34, 35].

3. Bovine Mastitis

3.1 Introduction

Mastitis, broadly defined as mammary gland inflammation, remains a major issue in dairy production worldwide. Signs of clinical mastitis include abnormal milk, an abnormal quarter (i.e. heat, swelling, and pain), and potentially systemic signs (e.g. dehydration, fever, and tachycardia) [36]. In the United States, according to the most recent data available, 99.7% of surveyed dairy operations reported having at least one case of mastitis in 2013; 24.8% of cows in the surveyed operations were diagnosed with clinical mastitis during the same time period [37]. Within this study population, of the 5.6% of all cows that were euthanized or died without assistance, 13.2% were reported to be due to clinical mastitis [38]. Additionally, the economic costs of an average case of clinical mastitis within the first 30 days of lactation have been estimated at $444 [39].

3.2 Bacterial causes

Culture-based methods have traditionally attributed mastitis to various bacteria, typically divided into contagious and environmental pathogens. Contagious mastitis pathogens are classified as bacteria adapted to survive within the mammary gland, capable of establishing sub-clinical infection, mainly spread from cow to cow, and tend to be Gram positive [40]. Major contagious mastitis pathogens include *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Staphylococcus aureus*. In contrast, environmental mastitis pathogens are classified as opportunistic bacteria that are not adapted to survival within the host (e.g. the mammary gland)

and are usually eliminated quickly by host immunity. Major environmental mastitis pathogens include *Streptococcus uberis* and Enterobacterales (in particular *E. coli*).

Traditionally, most cases of clinical mastitis were attributed to contagious pathogens. However, with more stringent milk quality standards and advances in udder health management, the proportion of milk samples sent to diagnostic testing labs culture-positive for these contagious pathogens has declined [41]. A 2013 study of milk samples from clinical mastitis cases from 50 large Wisconsin dairy farms revealed that 35.6% of samples were culture-positive for Gram negative bacteria (typically environmental pathogens), with *E. coli* being the most prevalent pathogen isolated (22.5% of all isolated bacteria) [42].

3.3 Prevention and antimicrobial treatment for Mastitis

According to 2013 data from the United States Department of Agriculture (USDA), 85.6% of cows with clinical mastitis received antimicrobial treatment [38]. Of these cows, 50.5% received 3rd generation cephalosporins (i.e., ceftiofur), 24.6% received lincosamide (i.e., clindamycin), 15.1% received 1st generation cephalosporins (i.e., cephapirin), and 8.7% received penicillin (i.e., penicillin G or cloxacillin). A common practice in preventing clinical mastitis at parturition is the blanket, non-selective intramammary treatment of cows with antimicrobials at dry-off. According to the USDA, in 2013 93% of cows received non-selective dry-cow antimicrobial treatment regardless [37]. Of cows receiving blanket treatment at dry-off, 62% were treated with cephapirin, 12.4% were treated with a combination of penicillin G/dihydrostreptomycin, 6.3% were treated with ceftiofur, 5.3% were treated with a combination of penicillin G/novobiocin, 4.5% were treated with cloxacillin, and 3.4% were treated with penicillin G.

Aside from antimicrobial treatment, prevention of mastitis via management interventions has also helped decrease overall mastitis incidence since the 20th century. In general, the likelihood

of the development of bovine mastitis is affected by: the level of exposure of the teat end to pathogenic microorganisms, the probability of these microbes entering the mammary gland, and the ability of microbes to adapt to the mammary gland and evade host immune defenses [43]. The management practices with the highest impact on mastitis pathogens relate to the cleanliness of milking equipment, the overall environment, and the teat. The ubiquity of milking machines has introduced potential avenues for mammary infections, but correct disinfection practices after every milking and improved design and usage of milking machines have greatly reduced this risk [44]. The correct selection of bedding material is crucial for welfare and reducing the risk of mastitis. Generally, organic bedding materials (e.g. sawdust, straw, and composted manure) should be avoided as they provide a conducive environment for bacterial growth, while sand bedding has been shown to provide a comfortable environment and low bacterial counts [45]. Lastly, the cleanliness and health of the teat have been shown to be improved by the use of both "pre-" and "post-" milking teat disinfectant dips and sprays [46]. In particular, applying post-milking teat disinfectants on every teat of every cow after every milking is one of the most effective ways to reduce bacteria on the teat and minimize the spread between cows [43]. The use of post-milking teat disinfectants not only removes pathogens on the teat surface but may also increase teat skin health with the addition of emollients like lanolin.

3.4 Role of Mammary and Milk Microbiota

A topic of current mastitis research is what role the endogenous microbiota of the mammary gland and milk play in the development of intramammary disease. Due to the availability of nutrients and warm temperature, the intramammary environment provides ideal conditions for the growth of bacteria. Traditionally, any bacterial presence within the mammary gland was believed to be pathogenic because culture-based techniques could not isolate bacteria

from healthy mammary glands and milk [47]. Recently, various studies using 16s rRNA and shotgun metagenomic sequencing have confirmed the presence of microbes in milk samples collected from healthy mammary glands [48, 49]. 16s sequencing of milk microbiota from healthy milk is generally more diverse and has increased species richness compared to milk from mastitis cows [50]. Current research suggests that intramammary infections can be detrimental to udder health and mastitis resistance due to the depletion of the endogenous microbiota by pathogens or antimicrobial treatment [51]. Commensal microbes can inhibit colonization by exogenous pathogens and counter endogenous, opportunistic bacteria via competition over nutrients, production of antimicrobial peptides, changing environmental conditions to suppress pathogens, competitive binding of attachment sites, and regulation of host immune responses [51, 52].

With the expansion of mammary gland microbiome research, one area that has yet to be explored is the application of transcriptomics to the microbiota within the mammary gland and milk samples. This would serve as more direct evidence of a mammary gland microbiome devoid of the potential pitfall of sequencing non-viable microbes.

4. Bovine Metritis

4.1 Diagnosis and causes of metritis in cattle

Metritis, an inflammation or infection of the uterus, remains a detriment to reproduction efficiency in dairy cows. Producers within the United States identified metritis as the fourth most common health issue in cows with an estimated prevalence between 10 and 30% in North America [38, 53]. Metritis is particularly common the first 21 days after calving, likely due to microbial contamination of the uterine lumen during and following parturition, with up to 40% of cows diagnosed with clinical metritis during this period [54]. The economic impact of metritis on producers is significant, with a recent study calculating $511 as the mean cost for a case of metritis,

due to numerous factors, including a decrease in milk production, treatment costs, decreased risk of pregnancy, and replacement costs [55].

Despite significant advances in research of reproductive diseases and the advent of non-culture-based methods to identify bacteria, the diagnosis and treatment of bovine metritis still need to be revised. Currently, metritis is diagnosed based on clinical signs as described in a landmark 2006 publication by Sheldon et al. in which puerperal metritis was described as a cow with "abnormally enlarged uterus and a fetid watery red-brown uterine discharge, associated with signs of systemic illness (decreased milk yield, dullness or other signs of toxemia) and fever >39.5 °C, within 21 days after parturition" [56]. Sheldon et al. also described clinical metritis as "animals that are not systemically ill, but have an abnormally enlarged uterus and a purulent uterine discharge detectable in the vagina, within 21 days post-partum". While a significant improvement to the diagnosis of metritis, the reliance of Sheldon et al. and others on clinical signs predisposes current metritis diagnosis to inconsistencies in the frequency of cows' examination, farm worker training and knowledge, examiner's sense of smell, and other factors [57, 58]. Therefore, research is ongoing to develop a more precise method to diagnose metritis in dairy cows accurately.

Determining the etiology of bovine metritis remains elusive despite years of research. The polymicrobial nature of metritis has complicated the search to define which taxa are either causative of or associated with this uterine disease. Traditionally, it was believed that certain bacteria frequently isolated from uterine swabs collected from metritic cows were the primary cause of uterine disease; in particular, *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica* [59, 60].

In the past 10 to 15 years numerous studies have utilized 16s rRNA gene sequencing to identify metritis-associated bacteria. By using a culture-independent method to identify bacteria,

16s rRNA sequencing studies can capture taxa not easily isolated from uterine samples. Two such studies found that bacteria in the genera *Bacteroides* and *Fusobacterium,* consistent with cultured-based studies, were associated with metritis or decreased uterine health [61, 62]. In contrast with culture-dependent studies, Jeon et al. 2015 observed that bacteria in the genus *Escherichia* were correlated with uterine health [62]. Strain diversity within intrauterine *E. coli* may explain the discrepancy in association with uterine disease from culture-based and genomics-based studies [63].

However, issues with 16s rRNA sequencing can arise from the potential introduction of bias from PCR amplification, library preparation, or database selection leading to low confidence in bacterial identification to the genus level [64]. Shotgun metagenomics eliminates biases created during PCR and allows for microbial identification to at least the genus level [64]. It has also allowed for the reliable identification of virulence factors (VFs), genes associated with increased disease severity, including antimicrobial resistance genes within microbial communities [65].

4.2 Metritis microbiome and virulence factors

In addition identifying specific taxa associated with metritis, research has also noted the discrepancy in the alpha-diversity (i.e., diversity within a sample) of the microbes from uterine samples of healthy and metritis cows. Two common metrics to measure alpha-diversity in the microbiome include species richness or the number of different species present (often estimated using the Chao1 index or the number of OTUs present) and species evenness or how different in abundance various species are in a community (often estimated using the Shanon index) [67]. Some studies have observed increased microbial diversity of uterine samples collected from metritic cows than those from healthy cows [68]. Other studies have observed significant differences in species richness, but no significant differences in species evenness within samples

from metritic and healthy cows [62]; or observed no significant differences in both species richness and evenness within samples [69]. In contrast, other studies have observed significant increases in species richness and evenness from samples of healthy cows' uterine microbiota compared to those of metritic cows [70]. Currently, the consensus in the field is that uterine disease is associated with, if not directly caused by, dysbiosis or an imbalance in the uterine microbiome leading to some imbalance in specific taxa known to be differentially abundant in metritis [57, 71].

The recent proliferation of culture-independent, PCR-free sequencing has continued to advance research into bovine uterine health and all endogenous microbes associated with a healthy uterine environment. This advancement of metagenomic sequencing has allowed the identification of more microbes within the uterine microbiome than previously possible with 16s rRNA sequencing, and VFs present within the uterine metagenome [57]. While detecting VFs associated with metritis initially required bacterial culture and PCR identification [72], metagenomics has allowed the detection of genes of interest from a bacterial community, not just specific species.

One of the initial papers that identified VF genes of *E. coli* associated with metritis highlighted six genes: *fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA*, and *astA* [72]. In this study of 374 cows and 625 *E. coli* isolates, the adhesin-encoding gene *fimH* was the strongest predictor of metritis with cows carrying at least one *E. coli* isolate containing *fimH* having a six-fold greater odds of metritis compared to cows culture-negative for *E. coli*. Moreover, when *fimH* was absent from an *E. coli* isolate the incidence of metritis in that cow was low (<24%) even if one of the other five VF genes was present. The FimH protein, which is encoded by *fimH*, is the receptor-recognizing element of type 1 fimbriae (long, filamentous polymeric protein polymers that allow binding of bacteria to mannosylated surfaces and cell receptors) present on many *E. coli* [73]. Interestingly, type 1 fimbriae have been shown to increase the adhesion and internalization by macrophages of *E. coli*,

therefore promoting extracellular antimicrobial evasion [74]. More recent studies have confirmed a high prevalence of *fimH* from *E. coli* isolated from the vagina and rectoanal junction of cows [75, 76].

Hitherto, a paucity of studies has been conducted using culture-independent methods to analyze VF genes from the bovine intrauterine microbiome and not just certain species such as *E. coli*. Identification of VF genes associated with an increased risk of metritis from a large sample size may lead to the development of bacterial biomarkers for molecular detection of metritis.

4.3 Antimicrobial treatment of metritis and antimicrobial resistance

Given the prevalence of metritis, significant antimicrobial use is associated with treatment. A study quantifying antimicrobial use on 29 U.S. dairies in 2016 and 2017 reported that 1.85 and 1.71 grams of antimicrobials per cow year, respectively, were used to treat metritis during the survey period; second only to dry cow therapy for mastitis [77]. Common antimicrobials used to treat metritis include penicillin, third-generation cephalosporins (i.e., ceftiofur), ampicillin, and oxytetracycline [78] . In particular, studies surveying metritis treatment practices on 45 Californian and 85 Midwestern dairies found ceftiofur was the most frequently used antimicrobial treatment of metritis [79, 80]. The preference for ceftiofur to treat metritis is likely based on its labeled use for the treatment of metritis during lactation and its lack of milk withholding period during treatment [81]. As a third-generation cephalosporin, ceftiofur belongs to the group of the highest priority antimicrobials within the World Health Organization's critically important antimicrobials for human medicine list [82]. Included as a high-priority antimicrobial and another common metritis treatment, ampicillin (a member of the aminopenicillins) is also vital to human medicine. Given their status as critically important antimicrobials, the continued efficacy of these two drugs is vital for not only metritis treatment but also human medicine.

Therefore, continued surveillance for AMR to these treatments within microbes isolated from bovine reproductive tracts is paramount. Due to ease of isolation, availability of clinical breakpoints, and knowledge based on culture-dependent studies, the few studies conducting antimicrobial sensitivity testing via minimum inhibitory concertation (MIC) on metritis pathogens have focused exclusively on *Escherichia coli* and *Trueperella pyogenes.* It should be noted that all determination of phenotypic antimicrobial resistance in these studies was based on guidelines published by the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) at the time of study publication. This common technique can introduce complexities comparing studies from various years as these guidelines are frequently updated and significant changes can alter the classification of a bacteria as susceptible or resistant. In a 2010 study analyzing 72 *T. pyogenes* isolates from uterine lavage samples from ten dairy cows, 86.1%, 62.5%, 54.2%, and 72.3% of isolates were found to be phenotypically resistant to ampicillin, ceftiofur, oxytetracycline, and penicillin, respectively [83]. Another study analyzed the susceptibility of 80 *E. coli* isolates collected from the same sampling as the previously mentioned study [84]. Of the 80 *E. coli,* 33.7%, 1.2%, and 0% of isolates were found to be phenotypically resistant to ampicillin, ceftiofur, and tetracycline, respectively. A larger 2015 study conducted in New Zealand recovered 209 *E. coli* and 35 *T. pyogenes* isolates from 272 cows [85]. Of the 209 *E. coli*, 2.4%, 0%, and 16.8% of isolates were phenotypically intermediate or resistant to ampicillin, ceftiofur, and oxytetracycline, respectively. No determination of resistance was made for the 35 *T. pyogenes* isolates due to a lack of available breakpoints from CLSI and EUCAST at the time of publication. The most recent, large study conducting antimicrobial susceptibility testing of 85 *E. coli* and 37 *T. pyogenes* isolates recovered from 120 cows was conducted in Germany in 2017 [86]. Pohl et al. did not provide phenotypic resistance classification,

leaving the reader to decide what portion of isolates could be classified as resistant. In addition to the challenges requent updates to CLSI or EUCAST MIC breakpoints for *E. coli* may have on antimicrobial sensitivity testing, the lack of any breakpoints for *T. pyogenes* has significantly hampered the identification of resistance to common antimicrobials within this metritis pathogen. Furthermore, a scarce number of studies have been conducted analyzing phenotypic antimicrobial susceptibility testing of other metritis pathogens such as *Fusobacterium necrophorum* and *Prevotella melaninogenica.*

5. Bovine disease and antimicrobial resistance

As previously mentioned, antimicrobial resistance threatens the efficacy of modern human medicine and the efficient production of dairy and beef cattle. Bovine salmonellosis, mastitis, and metritis present vastly different clinical signs and challenges; however, all three diseases rely on antimicrobial treatments, particularly for severe cases. Therefore, molecular surveillance tests, microbiome diversity analysis, and AMR and VF gene analysis were performed to better elucidate the role or presence of specific genetic elements and bacterial taxa with disease or health. In addition, treatment and management factors and antimicrobial susceptibility testing were identified to reveal potential associations with drug resistance and certain practices to increase antimicrobial stewardship efforts. Altogether, the following research projects aim to advance the knowledge of phenotypic antimicrobial resistance, antimicrobial resistance genetic elements, and microbial diversity pertinent to bovine salmonellosis, mastitis, and metritis.

CHAPTER 1. Detection of Cephalosporin and Fluoroquinolone Resistance Genes via Novel Multiplex qPCR in Fecal *Salmonella* Isolates from Northern Californian Dairy Cattle, 2002–2016

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ABSTRACT

The objectives of this study were to evaluate the prevalence of extended spectrum β-lactamase (ESBL) genes, AmpC-type β-lactamase (ACBL) genes, and plasmid mediated quinolone resistance (PMQR) genes in *Salmonella* isolated at a Veterinary Medical Teaching Hospital microbiology laboratory, examine trends in presence of these resistance genes, and to explore the correlation between phenotypic resistance and presence of specific genes. The presence of ESBL, ACBL, and PMQR genes were detected using a single, novel multiplex qPCR. Only the genes $bla_{\text{CMY-2}}$ and bla_{TEM} were detected in the 110 *Salmonella* isolates tested. PMQR genes were not detected in isolates screened. Of 94 third-generation cephalosporin resistant isolates, representing eight serotypes, 48% (n = 45) were positive for $bla_{\text{CMY-2}}$ only and 50% (n = 47) were simultaneously positive for $bla_{\text{CMY-2}}$ and bla_{TEM}. Two third-generation cephalosporin resistant isolates were tested negative for all β-lactamase genes in our qPCR assay and likely house ESBL genes not screened for by our qPCR assay.

A logistic regression model revealed that for serotype Dublin isolates (n = 38) the odds ratio for testing positive for bla_{TEM} when compared to all other serotypes was 51.6 (95% CI:4.01-664.03, p = 0.0029). For serotype Typhimurium (n = 9) the odds ratio for testing positive for bla_{TEM} when compared to all other serotypes was 43.3 (95% CI:1.76-1000, p = 0.0216).Overall, our results suggest that the prevalence of resistance to cephalosporins and fluoroquinolones due to ESBLs, ACBLs, and PMQR genes present in bovine nontyphoidal *Salmonella enterica* isolates has remained relatively constant in the isolates screened over a 14-year period.

1. INTRODUCTION

Globally in 2017, around 91 million cases of human gastrointestinal illness and diarrhea were believed to be caused by nontyphoidal *Salmonella enterica* (**NTS**) [87]. In the United States alone, 1.35 million NTS infections amounted to an estimated $400 million in medical costs annually [2]. In humans, severe infections caused by *Salmonella* usually require treatment with specific recommended antimicrobials, including ciprofloxacin, azithromycin, and ceftriaxone [2]. With 3% and 7% of all human NTS infections in the U.S. classified as either ceftriaxone resistant or ciprofloxacin nonsusceptible, respectively, the U.S. Center for Disease Control and Prevention (CDC) has designated drug resistant NTS as a serious threat [2]. As resistance to third-generation cephalosporins and fluoroquinolones grows, increasing attention is being placed on extended spectrum β-lactamase (ESBL) genes, AmpC-type β-lactamase (ACBL) genes, and plasmid mediated quinolone resistance (PMQR) genes.

Extended spectrum β-lactamase (ESBL) genes encode for enzymes which are able to cleave the β-lactam ring of a wide range of β-lactam antimicrobials (e.g. penicillins and cephalosporins) [26]. They confer β-lactam resistance to the bacteria that produce them, primarily *Klebsiella pneumoniae* and *Escherichia coli*. Worldwide, the most common ESBLs are the SHV, TEM, and CTX-M types. AmpC-type β-lactamase (ACBL) genes also encode for enzymes capable of degrading β-lactam antibiotics, including: extended spectrum cephalosporins (excluding cefepime and cefpirome), cephamycins, and ceftriaxone [88, 89]. $bla_{\text{CMY-2}}$ is the most common plasmid mediated ACBL gene globally [88].

Resistance to the quinolone and fluoroquinolone classes of antimicrobials has generally been attributed to chromosomal mutations in the bacterial enzymes targeted by these classes of antimicrobials: DNA gyrase and DNA topoisomerase IV [90]. Additionally, three types of

plasmid mediated quinolone resistance (PMQR) mechanisms have been identified: *qnr* genes protect DNA gyrase, the *aac(6')-lb-cr* gene acetylates ciprofloxacin, norfloxacin, and certain other quinolones, and *oqxAB* and *qepA* genes produce efflux pumps [31, 32].

Currently, a common method for identification of ESBL-producing bacteria is via culture-based phenotypic methods [91, 92]. Unfortunately, a definitive negative result may take 24-120 hours [93]. Furthermore, as these methods depend on the inhibition of ESBLs by clavulanic acid, the production of additional AmpC or metallo-β-lactamases (which are uninhibited by clavulanic acid) may decrease test sensitivity [94]. To identify particular genes responsible for ESBL production, reference laboratories use molecular analyses, primarily polymerase chain reaction (PCR) [95].

Detection of ACBL-producing bacteria is generally done using phenotypic tests utilizing ACBL inhibitors such as boronic acid and cloxacillin, however these tests are unable to distinguish between chromosomal or plasmid-mediated AmpC-type β-lactamases [96]. Detection of isolates carrying plasmid-mediated ACBL genes may be particularly important as these isolates may appear to be susceptible to cephalosporins *in vitro*, only to fail to respond to treatment [97]. Molecular approaches to identify plasmid-mediated ACBL genes are available, but are typically unavailable in clinical laboratories [98]. Additionally, there is currently no Clinical and Laboratory Standards Institute (CLSI) test for AmpC-type β-lactamases in clinical isolates [99].

While there are multiplex PCR methods available for the detection of either ESBL, ACBL, or PMQR genes, there are few published multiplex PCR methods available for the combined detection of ESBL, ACBL, and PMQR genes relevant to NTS treatment [100, 101]. The goal of this study was to identify trends in resistance of fecal *Salmonella* isolates to cephalosporins

and fluoroquinolones due to the presence of ESBL, ACBL, and PMQR genes from *Salmonella* isolates obtained from cattle fecal samples isolated and tested in the University of California, Davis William R. Pritchard Veterinary Medical Teaching Hospital (VMTH) microbiology laboratory during a 14-year interval using a single, novel multiplex qPCR method.

2. MATERIAL AND METHODS

Isolate collection and selection

A total of 110 *Salmonella* isolates were selected for qPCR analysis from a collection of 242 *Salmonella* isolates recovered from 9,162 bovine fecal samples submitted to the University of California, Davis William R. Pritchard VMTH microbiology laboratory between January 1, 2002 and December 31, 2016 as detailed previously [102]. Sixty-eight isolates were recovered from dairy cattle exhibiting clinical signs of *Salmonella* infection, while 42 isolates were recovered from asymptomatic dairy cattle through the VMTH Infectious Disease Control (IDC) program. All isolates with phenotypic resistance to at least one of the following drugs, nalidixic acid, ceftiofur and/or ceftriaxone, were included in the study (n= 94; Supplemental table 1-1). None of the isolates were phenotypically resistant to ciprofloxacin (MIC $\geq$ 1.0 µg/ml). Only two isolates were resistant to nalidixic acid, and also presented simultaneous phenotypic resistance to ceftriaxone and ceftiofur. All isolates phenotypically resistant to ceftiofur were also resistant to ceftriaxone.

For each year a pan-susceptible *Salmonella* isolate, when available, was selected to serve as a control (n=16) (Supplemental table 1-2). For two years, namely 2002 and 2004, no pan-susceptible isolates were available, and an isolate resistant to streptomycin, and one isolate resistant to ampicillin, streptomycin, and tetracycline, respectively, were selected

(Supplemental table 1-2). These isolates were selected on the criteria that they were susceptible to quinolone and cephalosporin drugs and were the isolates resistant to the fewest number of antimicrobials for that year.

Antimicrobial susceptibility testing

Data from phenotypic antimicrobial susceptibility testing conducted on the same isolates from a previous study were used [102]. Briefly, for that study all isolates were tested using the standardized National Antimicrobial Resistance Monitoring System (NARMS) (Thermo Fisher, Sensititre CMV3AGNF) for aerobic Gram-negative bacteria that included penicillins (ampicillin), β-lactam/ β-lactamase inhibitor combinations (amoxicillin/clavulanic acid), cephalosporins (ceftriaxone, ceftiofur, and cefoxitin), quinolones (ciprofloxacin and nalidixic acid), phenicols (chloramphenicol), sulfa-based drugs (sulfisoxazole and sulfamethoxazole/trimethoprim), tetracyclines (tetracycline), macrolides (azithromycin), and aminoglycosides (gentamicin and streptomycin). Plates were read using the Sensititre Vizion System® (Thermo Fisher) and minimum inhibitory concentrations (MIC) were interpreted using NARMS breakpoints [103].

DNA Extraction

Frozen isolates were streak plated on blood agar plates and incubated overnight at 37°C to check for contamination. Visual inspection did not show any contamination therefore 1.5 mL of autoclaved BHI broth in a 2 mL micro centrifuge tube was inoculated from each isolate in a biological safety cabinet. The DNA was then extracted according to the manufacturer's instructions for the DNeasy Blood and Tissue Kit (Qiagen N.V., Carlsbad, CA). 200 µL of DNA was eluted for each isolate into a sterile 2 mL micro centrifuge tube. The DNA samples were then stored at -80°C until further downstream processing.

Multiplex qPCR development and validation

In collaboration with the UC Davis Real-time PCR Research and Diagnostics Core Facility, a singleplex and several multiplex (duplex and triplex) qPCR assays were developed to facilitate rapid and sensitive analysis of samples. Isolates were analyzed for the presence of β-lactamase encoding genes (*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$, and *bla*$_{\text{CMY-2}}$) and for presence of plasmid mediated quinolone resistance (PMQR) genes (*oqx*A, *oqx*B, *qnr*S, *qnr*B, and *aac(6')-Ib-cr*). q*nr*A was not included in the qPCR assay because it has been rarely identified in *Salmonella* isolates with phenotypic resistance to fluoroquinolones. Cattle studies screening for *qnr* genes have more frequently detected *qnr*B and *qnr*S [104]. Furthermore, other recent studies screening *Salmonella* from isolates originating from broiler chicken and pork products for *qnr* genes did not detect *qnr*A genes [34, 105]. Other recent studies screening human *Salmonella* isolates have also not detected *qnr*A, and noted it as infrequently detected when compared to *qnr*B and *qnr*S [32, 104].

Sequences from GenBank (*bla*$_{\text{TEM}}$ (LT985387), *bla*$_{\text{CTX-M}}$ (CP025146), *bla*$_{\text{CMY-2}}$ (KY612500), *oqx*A (CP019074), *oqx*B (CP019074), *qnr*S (CP026578), *qnr*B (KP012539), and *aac(6')-Ib-cr* (NG_056043)) were aligned using Sequence Analysis and Molecular Biology Data Management software Vector NTI AdvanceTM11 (Thermo Fisher Scientific, Carlsbad, CA). The alignment was used to design primers specific to target for singleplex and multiplex qPCRs assays (Table 1-1). The specificity of the primers and probes was confirmed by BLAST searching against the non-redundant database of GenBank (NCBI). The primers for detecting *bla*$_{\text{TEM}}$ in our qPCR assay were designed to be more general and capable of annealing to both TEM non-extended spectrum β-lactamases and TEM-type ESBLs. This was done in the context of our assay being used as a screening tool.

Primers/Probe	Sequence 5'-3'	Amplicon size (bp) and %GC
***bla*$_{TEM}$ -97f**	GATGCTGAAGATCAGTTGGGTG	71bp, 50.7%
***bla*$_{TEM}$ -168r**	CTCAAGGATCTTACCGCTGTTGA	
***bla*$_{TEM}$ -123p**	FAM- AGTGGGTTACATCGAAC - MGB	
***oqx*A-1079f**	ATAGCGTCATCGTCGACGG	73bp, 49.3%
***oqx*A-1152r**	CATGGCAACGGTTTTGGC	
***oqx*A-1114p**	VIC-ATGCCGGGTATGCC-MGB	
***qnr*S-523f**	GTTGACGAATGTCGTATCACGC	73bp, 50.1%
***qnr*S-596r**	TCACCTTCACCGCTTGCAC	
***qnr*S-553p**	TET-ACGTCGAAAGTCGCTG-MGB	
***bla*$_{CTX-M}$ -792f**	TTACTTCACCCAGCCTCAACCT	59bp, 57.6%
***bla*$_{CTX-M}$ -851r**	GCCGCCGACGCTAATACA	
***bla*$_{CTX-M}$ -816p**	FAM-GGCAGAAAGCCGTCG-MGB	
***bla*$_{CMY-2}$-884f**	CCGATATCGTTAATCGCACCAT	63bp, 55.5%
***bla*$_{CMY-2}$-947r**	ACGGCCATACCCGGAATAG	
***bla*$_{CMY-2}$-911p**	VIC-CGTTGATGCAGGAGC-MGB	
***oqx*B-1361f**	TTCCGTCCGTTTAACCGCT	61bp, 55.7%
***oqx*B-1422r**	TTGCCTACCAGTCCCTGATAGC	
***oqx*B-1385p**	TET-CTGCGCAGCTCGAA-MGB	
***aac*6-lb-59f**	GCGATGCTCTATGAGTGGCTAA	73bp, 56.1%
***aac*6-lb-132r**	AGTGTCGGGCGTGCTTCTT	
***aac*6-lb-90p**	FAM-ATATCGTCGAGTGGTGGG-MGB	
***qnr*B-276f**	TTCAGATCTCTCCGGCGG	72bp, 54.2%
***qnr*B-348r**	GGTCAGATCGCAATGTGTGAAG	
***qnr*B-304p**	VIC-ACTTTCGACTGGCGAGC-MGB	

Table 1-1. Primer and probe sequences for qPCR assays. Fluorophores (FAM, TET, VIC) were specific to each probe, and amplicon lengths are also provided.

Different fluorophores for each multiplex: *bla*$_{TEM}$, *bla*$_{CTX-M}$, *aac(6')-Ib-cr* all used the fluorescent probe 6-carboxy-fluorescein (FAM); *oqx*A, *bla*$_{CMY-2}$, and *qnr*B used the VIC probe; whereas, *qnr*S and *oqx*B used tetrachlorofluorescein (TET). All the probes utilized a 3'

minor groove binding quencher (Table 1-1). All qPCR assays were designed using Primer Express (Thermo Fisher Scientific) following the guidelines for multiplex qPCR assays. Amplicon lengths were ranging from 59 bp to 73 bp with each multiplex having similar lengths and GC percentage (Table 1-1). Primers and probes were synthesized by Life Technologies (Grand Island, NY).

To test the efficiency of each primer/probe combination, singleplex mixes were prepared by combining 20 µL of 100 pmol/µL forward primer, 20 µL of 100 pmol/µL reverse primer, and 4 µL each of the 100 pmol/µL probes individually, in a final volume of 240 µL water. The qPCR multiplex primer/probe mix was prepared by mixing 40µL for duplex and 60 µL for triplex of 100 pmol/µL forward primer, 40µL for duplex and 60 µL for triplex of 100 pmol/µL reverse primer, and 4 µL each of the two or three 100 pmol/µL probes in a final volume of 240 µL water.

The singleplex and multiplex qPCR for each target contained 0.42 µL water, 0.58 µL primer/probe mix (final concentration 400 nM of each primer and 80 nM probe), 6 µL of commercially available TaqMan™ Universal Master Mix (UMM) (Thermo Fisher Scientific) for singleplex or Gene Expression Master Mix (Qiagen) for multiplex, and 5 µL of the DNA in a final volume of 12 µL

All samples were placed in a 384-well plate and amplified in a 7900HT FAST Real-time PCR system (Thermo Fisher Scientific) using the manufacturer's standard amplification conditions (2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 60 s at 60°C). Fluorescent signals were collected during annealing and quantitative cycle (Cq) was calculated and exported with a threshold of 0.15 and a baseline of 3–10 for FAM labeled assays, 0.20 and a baseline of 3-10 for VIC assays, and 0.10 and a baseline of 3-15 for TET assays. The Cq was

defined as the cycle in which there was a significant increase in reporter signal of the amount of PCR product detected during the exponential phase, above the threshold.

qPCR Assay Validation.

To assess and validate the efficiency of singleplex and multiplex qPCR assays for all assays, endpoint analysis of DNA using 10-fold dilutions was performed for each assay. In the singleplex qPCR mixtures (*qnr*S), only one target positive control was conducted using a nucleic acid template of known copy number. In multiplex qPCR mixtures, each of the two *(bla*$_{\text{TEM}}$ and *oqx*A, *aac(6')-Ib-cr*, and *qnr*B) or three (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY-2}}$, and *oqx*B) target positive controls was combined in a single amplification tube. Standard curves were generated for each set of 10-fold serial dilutions of target. We calculated the amplification efficiency (E) of all assays from the slope (S) of the standard curves, using the formula $E = 10^{1/-s}-1$ (Table S1-3).

Sensitivity log (Sl) = (40-y intercept)/S. Sensitivity copy number (CN) = 10^{Sl}

The multiplexes were very similar in sensitivity when all three targets were compared in the reaction. The sensitivity of each assay run as a single or multiplex was ~10 or ~100 gene copies. Such high similarity between the assays' efficiency, sensitivity, amplicon length, and melting temperature assured the same competition efficiency during multiplex qPCR reaction.

Statistical Analysis

Descriptive analysis for the distribution of *Salmonella* by year, phenotype, and resistance genes detected was conducted in JMP (SAS Institute Inc., Cary, NC). To evaluate the reproducibility of C_q measurements and their associated error of the mean, a histogram was generated to visually evaluate the data (Figure 1-1) for each gene detected by qPCR, and mean

and standard error for C_q values [106]. A 99% confidence interval of the standard error of the mean for C_q values was used for each gene to select the cut-off value for C_q values to classify an isolate as being positive for carrying that gene.

Logistic regression models using the GLIMMIX function in SAS using the logit link function were used to evaluate the population of *bla*$_{\text{CMY-2}}$ positive, ceftriaxone resistant *Salmonella* isolates (n=92) for the effect of explanatory variables sex, serotype, submission type (IDC vs Suspect), and year group (calf vs adult) on the risk of detecting an isolate positive for the gene *bla*$_{\text{TEM}}$. Three models were constructed that differed by the presence of a binomial variable that allowed the evaluation of the effect of each of these three serotypes: *S.* Dublin, *S.* Typhimurium, and *S.* Newport when compared against any other serotype. This was a binomial variable that compared one of the three serotypes to all other serotypes combined (e.g., Dublin vs Typhimurium, Newport, and any other serotype present in the study dataset). These three serotypes were selected because they represented the top three serotypes for isolates selected (92% of all isolates). Year group (2002–2009 vs 2010–2016) when isolates were collected was included and maintained in all models as an independent variable to evaluate temporal changes on antimicrobial resistance. These two multi-year periods were chosen because they represent two halves of the time period evaluated. Additionally, these time periods were selected because of legislation related to antimicrobial use that occurred after 2009, such as changes on how ceftiofur could be used in an extra-label manner in livestock [107]. Any explanatory variable that was not significant was removed from the model after evaluating if it negatively affected the model by evaluating the Akaike Information Criterion (AIC) value and overdispersion was evaluated using the Pearson chi-square divided by the degrees of freedom value. For all models, a *P* value of < 0.05 was considered a significant difference.

3. RESULTS

Cycle quantification (C_q) value distribution for *bla*$_{CMY-2}$ and *bla*$_{TEM}$ genes

Figure 1-1 contains two histograms of the C_q values for each of the two genes detected in our study: *bla*$_{CMY-2}$ and *bla*$_{TEM}$ (Fig. 1-1A and 1-1B). Only two genes were detected in the 110 *Salmonella* isolates tested, namely *bla*$_{CMY-2}$ and *bla*$_{TEM}$. The mean and 99% confidence interval for the C_q values of 23.0 (99% C.I: 21.7 to 24.2) and 26.4 (99% C.I: 24.9 to 27.7) were determined for *bla*$_{CMY-2}$ and *bla*$_{TEM}$, respectively. Figure 1-1A, which depicts the C_q value distribution for *bla*$_{CMY-2}$, indicates that isolates below the cut-off of 23 were phenotypically resistant to ceftriaxone (Figure 1-1, blue color).

The opposite is primarily also true as most isolates above the cut-off of 23 are phenotypically susceptible to ceftriaxone (Figure 1-1, orange color). Results for logistic regression evaluating the effect of explanatory variables on the odds of isolating *bla*$_{CMY-2}$-positive, ceftriaxone resistant *Salmonella* isolates also carrying *bla*$_{TEM}$ is depicted in table 1-2.

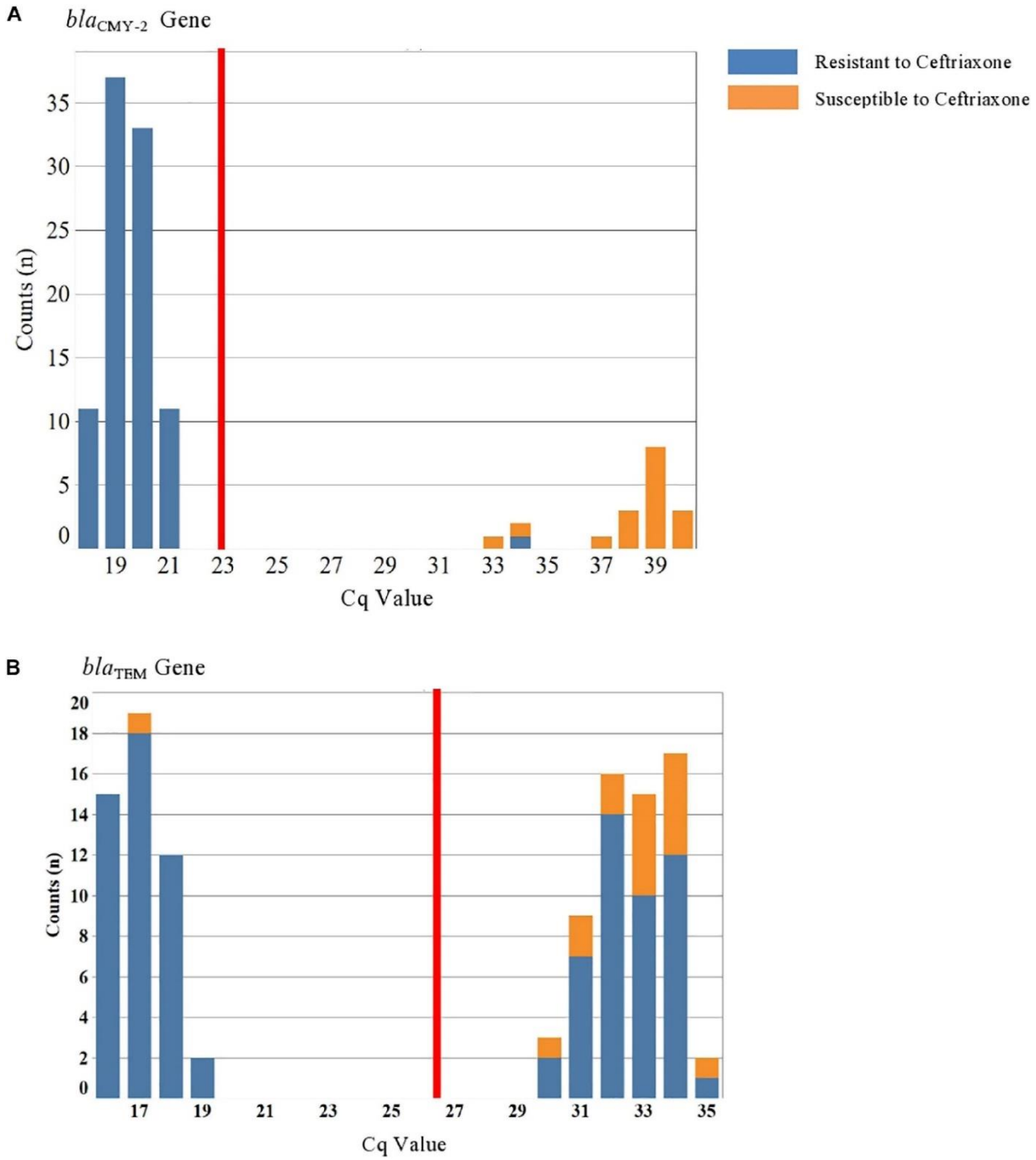

Figure 1-1. Histogram with cycle quantification (Cq) value distribution for *Salmonella* isolates screened for *bla*CMY-2 and *bla*TEM using multiplex qPCR. Blue represents isolates phenotypically resistant to ceftriaxone and orange represents isolates phenotypically susceptible to ceftriaxone. The red line indicated the cut-off Cq values for *bla*CMY-2 and *bla*TEM. Graph A depicts the Cq value distribution for *bla*CMY-2, and graph B depicts the Cq value distribution for *bla*TEM.

Variable	Coefficient	SE	%(n)**	OR	OR (95% Confidence interval) Lower	Upper	*P* value
Intercept	-2.06	1.38					
Serotype[1]							
Dublin	3.94	1.28	89 (38)	51.6	4.01	664.03	**0.0029**
Typhimurium	3.77	1.61	78 (9)	43.3	1.76	1000	**0.0216**
Newport	-0.04	1.21	14 (42)	0.96	0.087	10.5	0.97
Year Group[2]							
2002 to 2009	-0.13	0.84	46 (86)	0.88	0.17	4.66	0.88
2010 to 2016	Reference*		33 (24)				
Submission Type[3]							
Suspect	0.73	0.76	57 (68)	2.09	0.46	9.4	0.34
IDC	Reference*		21 (42)				

Table 1-2. Summary of the logistic regression model evaluating the effect of the explanatory variables serotype, year group, and submission type on the odds ratio of isolating a *bla*$_{\text{CMY-2-}}$positive, ceftriaxone resistant *Salmonella* isolate also carrying *bla*$_{\text{TEM}}$. Statistically significant *P* values are in bold.

OR = odds ratio; SE = standard error.
* Reference group for the odds ratio.
** Percent of isolates that fall within each category described and total number of isolates within that category in parenthesis (n).
1. Binomial variable for the odds ratio of and a serotype isolate carrying resistant gene *bla*$_{\text{TEM}}$ when compared to all other serotypes combined. e.g. Serotype Dublin when compared to Salmonella belonging to any other serotype.
2. Binomial variable for the odds ratio of a *Salmonella* isolated between 2002 to 2009 carrying resistant gene *bla*$_{\text{TEM}}$ when compared to *Salmonella* isolated between 2010 to 2016.
3. Binomial variable for the odds ratio of a *Salmonella* isolated from an animal suspected of salmonellosis carrying resistance gene *bla*$_{\text{TEM}}$ when compared to a *Salmonella* isolated from an animal as part of an infectious disease control protocols (IDC)

Temporal distribution for *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{TEM}}$ genes

Isolates categorized as resistant to at least one third-generation cephalosporin (3GC) via MIC testing and were positive for either *bla*$_{\text{CMY-2}}$ or *bla*$_{\text{TEM}}$ were analyzed by year (Table 1-3). Out of 242 *Salmonella* isolates, 94 (39%) were phenotypically resistant to at least one 3GC. Of these 94 3GC resistant isolates, 48% (n = 45) were positive for *bla*$_{\text{CMY-2}}$ only and 50% (n = 47) were simultaneously positive for both *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{TEM}}$. Two third-generation cephalosporin resistant isolates, which were negative for both *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{TEM}}$, likely house ESBL genes not screened for by our qPCR assay.

Year	Total Nº isolates[1]	Nº isolates 3GC[2](%)	Nº of 3GC and *bla*$_{\text{CMY-2}}$ + [3]	Nº of 3GC and *bla*$_{\text{TEM}}$ + [4]	% of 3GC and[5] *bla*$_{\text{CMY-2}}$ +	% of 3GC and[5] *bla*$_{\text{TEM}}$ +
2002	15	11 (73)	11	11	100%	100%
2003	9	1 (11)	1	1	100%	100%
2004	17	8 (47)	8	6	100%	75%
2005	19	12 (63)	12	4	100%	33%
2006	11	5 (45)	5	4	100%	80%
2007	43	22 (51)	22	0	100%	0%
2008	49	17 (35)	16	13	94%	76%
2009	19	1 (5)	1	0	100%	0%
2010	20	6 (30)	6	0	100%	0%
2011	5	1 (20)	1	0	100%	0%
2012	3	1 (33)	1	1	100%	100%
2013	7	2 (29)	2	1	100%	50%
2014	11	5 (45)	4	4	80%	80%
2015	5	2 (40)	2	2	100%	100%
2016	6	0 (0)	0	0	-	-
TOTAL	239	94	92	47	98%	50%

Table 1-3. Summary of the logistic regression model evaluating the effect of the explanatory variables serotype, year group, and submission type on the odds ratio of isolating a *bla*$_{\text{CMY-2-}}$positive, ceftriaxone resistant *Salmonella* isolate also carrying *bla*$_{\text{TEM}}$. Statistically significant *P* values are in bold.

1. Total number of *Salmonella* isolates recovered from 9,162 bovine fecal samples submitted to a Veterinary Medical Teaching Hospital (VMTH) microbiology laboratory between 2002 and 2016.

2. Number of isolates resistant to at least one third-generation cephalosporin antimicrobial
3. Number of isolates resistant to at least one third-generation cephalosporin antimicrobial and positive for presence of a *bla*$_{\text{CMY-2}}$ gene.
4. Number of isolates resistant to at least one third-generation cephalosporin antimicrobial and positive for presence of a *bla*$_{\text{TEM}}$ gene.
5. Percent of isolates resistant to at least one third-generation cephalosporin antimicrobial and positive for presence of a *bla*$_{\text{CMY-2}}$ or *bla*$_{\text{TEM}}$ gene.

Despite fluctuations between years, there were no significant differences between individual years (Table 1-3). In addition, year group (2002-2009 v. 2010-2016) was found to have no significant effect on the odds of isolating a *bla*$_{\text{CMY-2}}$-positive, ceftriaxone resistant isolate positive for *bla*$_{\text{TEM}}$ (Table 1-2). For all years with isolates resistant to at least one 3GC, nearly all of these isolates were positive for *bla*$_{\text{CMY-2}}$. Three years (2002, 2003, and 2012) reported 100% of third-generation cephalosporin (3GC) resistant isolates (Table S1-1: Isolates 1-12, 85) were positive for both *bla*$_{\text{TEM}}$ and *bla*$_{\text{CMY-2}}$.

Risk factors for presence of *bla*$_{\text{TEM}}$

A logistic regression model revealed that certain serotypes of *Salmonella* and submission type impacted the odds ratio of isolating a *bla*$_{\text{CMY-2}}$ positive, ceftriaxone resistant isolate positive for *bla*$_{\text{TEM}}$ (Table 1-2). A similar analysis for calculating the odds ratio of isolating a ceftriaxone resistant isolate positive for only *bla*$_{\text{CMY-2}}$ could not be conducted due to almost all isolates testing positive for *bla*$_{\text{CMY-2.}}$ For serotype Dublin, which accounted for 38% of all isolates, the odds ratio for testing positive for *bla*$_{\text{TEM}}$ when compared to all other serotypes was 51.6 (95% CI:4.01-664.03, $p = 0.0029$). For serotype Typhimurium, which accounted for 9% of all isolates, the odds ratio for testing positive for *bla*$_{\text{TEM}}$ when compared to all other serotypes was 43.3 (95% CI:1.76-1000, $p = 0.0216$). For serotype Newport, which accounted for 42% of all isolates, the odds ratio for testing positive for *bla*$_{\text{TEM}}$ when compared to all other serotypes was 0.96 (95% CI:0.087-10.5, $p = 0.97$). For isolates in year group 2002 to 2009

(86% of total isolates) the odds ratio of carrying *bla*$_{TEM}$ was 0.88 when compared to *Salmonella* isolated between 2010 to 2016 (24% of total isolates) (95% CI: 0.17-4.66, $p = 0.88$). Although not significant, the odds ratio of having *bla*$_{TEM}$ for isolates collected from animals suspected of salmonellosis (68% of total isolates) was 2.09 when compared to isolates collected as part of an infectious disease control protocol (42% of total isolates)(95% CI:0.46-9.7, $p = 0.34$).

While the three serotypes previously mentioned accounted for a majority (39%, 8.5%, and 44.7%) of the 94 3GC resistant isolates, five other serotypes were also detected (Supplemental table 1-1). These serotypes are Reading (Isolate 19), Meleagridis (Isolates 46, 50, and 52), Montevideo (Isolate 69), 9,12:nonmotile (Isolate 86), and Give (Isolate 93).

Lack of *bla*$_{CTX-M}$ and PMQR genes

The ESBL gene *bla*$_{CTX-M}$ was not detected in any of the 110 NTS isolates screened. Additionally, none of the 110 NTS isolates, including two isolates phenotypically resistant to nalidixic acid, were positive for the PMQR genes screened in our assay (*oqx*A, *oqx*B, *qnr*S, *qnr*B, and *aac(6')-lb-cr*). It should be noted that the methods used in our study did not allow for the delineation between *aac(6')-lb-cr* and other highly-similar variants like *aac(6')-lb*. Likewise, the methods used in our study did not allow for differentiation between *bla*$_{CTX-M}$ groups.

4. DISCUSSION

Of the 242 NTS isolates tested, 39% of isolates (n = 94) were phenotypically resistant to a 3GC and 98% (n = 92) and 50% (n = 47) of these resistant isolates were PCR-positive for *bla*$_{CMY-2}$ and *bla*$_{TEM}$, respectively (Table 1-3). In the United States, ceftriaxone resistant NTS has primarily been observed to carry the gene *bla*$_{CMY-2}$ encoding the AmpC-type β-lactamase

(ACBL) CMY-2 [103]. The high prevalence of *bla*$_{\text{CMY-2}}$ in 3GC resistant NTS in our study was similarly observed in a 2007 USDA study in which 81.6% of a subsample of ceftiofur resistant *Salmonella* isolates collected from 34,000 *Salmonella* isolates from the NARMS between 1999 and 2003 were positive for *bla*$_{\text{CMY-2}}$ [108]. More recently, a 2017 study which focused primarily on NTS isolated from beef cattle fecal samples detected *bla*$_{\text{CMY-2}}$ in 8% of 571 isolates [109]. Analysis of these CMY-2-positive isolates revealed 90% homology within serotypes, highlighting the clonal dissemination of *bla*$_{\text{CMY-2}}$ within the cattle populations sampled in this study. Future work analyzing the homology within serotypes of the 92 isolates positive for *bla*$_{\text{CMY-2}}$ in our study may be warranted given the number of isolates within the same serotype to be positive for *bla*$_{\text{CMY-2.}}$ *bla*$_{\text{CMY-2}}$ is a very common resistance gene present when phenotypic resistance to ceftriaxone is observed, as shown in table 1-3 and observed in other studies [28], and represents a potential gene to focus future diagnostic approaches to classify an isolate as resistant to ceftriaxone without the need for use of phenotypic, culture-based methods. It should be noted that the methods used in our study did not allow for the delineation between *bla*$_{\text{CMY-2}}$ and other highly-similar variants like *bla*$_{\text{CMY-4}}$.

The β-lactamase encoding gene *bla*$_{\text{TEM}}$ was present in 50% (n = 47) of 3GC resistant NTS isolates in our study and which were also simultaneously positive for *bla*$_{\text{CMY-2}}$. TEM-1, discovered in 1965, is one of the most ubiquitous β-lactamases among *Enterobacteriales* [110, 111]. TEM-1 is not an ESBL and generally only degrades penicillins and the earliest developed cephalosporins. The first reported TEM-type ESBL, TEM-3, was discovered in 1989 [112]. With TEM variants now numbering greater than 200 and with many belonging to the ESBL subclass, a significant diversity exists within this resistance mechanism [113]. A 2012 French study of 204 ESBL-producing *E. coli* isolates collected from sick cattle between 2006 and

2010 revealed only 7/204 (3.4%) expressed ESBL-type TEM-52 [114]. In the context of human medicine, the presence of TEM-type ESBLs in NTS in Bangladesh poses a public health concern [115]. The 2014 Bangladesh study of 2,120 *Salmonella* isolates from 128,000 human stool samples collected between 2005 and 2013 revealed that 88% (7/8) ceftriaxone resistant strains were positive for bla_{TEM}. It should be noted that a limitation of our study was that all isolates positive for bla_{TEM} were also positive for $bla_{\text{CMY-2}}$ and that the methods used in our study did not allow for the delineation between the beta-lactamase gene $bla_{\text{TEM-1}}$ and other highly-similar ESBL variants like $bla_{\text{TEM-52}}$.

In the U.S., the first reported *Enterobacteriales* carrying $bla_{\text{CTX-M}}$ in dairy cattle was an *E. coli* strain in a study by Wittum and others from Ohio in 2009 [116]. Identification of CTX-M-producing NTS in the U.S. has been relatively rare, but recent detection of such isolates in both livestock and retail chicken meat in the U.S. poses a potential threat to food safety [95, 117]. None of the *Salmonella* isolates screened in our study were positive for $bla_{\text{CTX-M}}$.

Our multiplex qPCR assay, while originally developed for use in an epidemiological or microbiological research setting, has potential advantages over traditional phenotypic testing common in a clinical setting. While research PCR assays tend to be low throughput and prioritize the ability to detect the lowest number of gene target copies, clinical PCR assays have additional requirements including high throughput and minimizing the chance of either false positive or negative results [118]. Frequently used in a clinical setting, phenotypic antimicrobial susceptibility testing (e.g. broth microdilution or Kirby-Bauer test) relies on multiple incubations of the microorganism and requires a minimum of 12 hours [119]. A multiplex qPCR assay could be clinically relevant when performing culture-based antibiotic resistance testing. A multiplex qPCR assay could serve as a complementary, rapid screening

test for antimicrobial resistance genes while phenotypic tests are being conducted. Our qPCR assay (not including initial isolation) can be completed in about 6 hours. Unlike phenotypic antimicrobial susceptibility tests, our assay requires DNA extraction and a qPCR run, but does not necessitate the bacteria to be incubated twice.

Only two serotypes were shown to significantly increase the odds ratio of isolating a *bla*$_{\text{CMY-2}}$-positive, ceftriaxone resistant isolate also positive for *bla*$_{\text{TEM}}$, namely Dublin and Typhimurium (Table 1-2). For our study, 98% of isolates resistant to ceftriaxone were also positive for *bla*$_{\text{CMY-2}}$ (Table 1-3); because of that we cannout indicate causation of resistance to ceftriaxone as originating from *bla*$_{\text{TEM}}$ or *bla*$_{\text{CMY-2}}$ gene (the latter being the most probable). A previous study conducted on colostrum fed to dairy calves screened cephalosporin resistant *E. coli* for β-lactamase resistance genes and observed, similarly to our study, that none of the isolates were positive for *bla*$_{\text{CTX-M}}$; they also observed that 45% and 35% of these isolates were positive for *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{TEM}}$, respectively [120]. The higher prevalence of *bla*$_{\text{TEM}}$ observed in our study compared to the colostrum study, in addition to increased odds for detection of *bla*$_{\text{TEM}}$ in *S.* Dublin isolates, is of critical importance as *bla*$_{\text{TEM}}$ has been linked to resistance to cephalosporins and various other β-lactam antibiotics; reducing the potential effective antimicrobial treatment options for infections caused by pathogens [110].

While there is little research on the effect of serotype on the odds of isolating a ceftriaxone resistant and *bla*$_{\text{TEM}}$-positive NTS isolate in cattle; a previous study has demonstrated both serotypes to possess high levels of ceftiofur (3GC) resistance, and the driver of resistance is most probably being driven by another antimicrobial gene [121]. The most recent NARMS data of human NTS isolates revealed that 66.7% of serotype Dublin and 4.7% of serotype Typhimurium isolates were resistant to ceftriaxone. Despite being a cattle-adapted serotype,

Dublin causes increased hospitalization and mortality in human infections when compared to other NTS serotypes [19]. Typhimurium is also one of the most common serotypes to cause human infection in both the US and globally [122].

Our prior study evaluating phenotypic resistance of *Salmonella* isolates from cattle observed a 13.7 higher odds (*P* value = 0.0004) for isolating a multidrug resistant *Salmonella* from suspect clinical salmonellosis cases when compared to isolates originating from the VMTH IDC protocol sampling [102] . Our current study further evaluated specific resistance mechanisms for cephalosporin and fluoroquinolone resistance genes. We did not detect a significant difference in the odds ratio for isolating *Salmonella* from animals suspected of salmonellosis when compared to isolates originating from the IDC program for the resistance genes screened. This result could indicate that cephalosporin and fluoroquinolone resistance genes were not the main factors increasing the risk for MDR isolates between these two different sources of *Salmonella* isolates. Although antimicrobial resistance is not in itself a virulence factor, it is a key factor in development of infection, and may be considered a virulence-like factor in specific ecological niches which antibiotic resistant bacteria are able to colonize [123]. This is especially consistent in a hospital environment where, if an opportunistic pathogen is drug resistant, it can cause disease more readily. Mutations increasing antimicrobial resistance have a range of effects on bacterial fitness during infection including decreased or increased pathogenic potential. Future studies should further elucidate the determinants of altered virulence potential in resistant pathogens and illuminate the mechanisms by which resistance traits modulate the outcome of disease in veterinary hospitals [124]. A limitation of our study was that the sample population were animals from a VMTH, and may not necessarily be extrapolated to other populations that may not be under similar

circumstances and also explain a wider confidence interval for some of the variables evaluated in the model. Another limitation is that *qnr*A was not included as one of the PMQR genes screened in the qPCR assay; this was due to the very low risk of detecting qnrA in *Salmonella* of cattle origin [104].

5. CONCLUSION

Out of 242 *Salmonella* isolates, 39% (n = 94) were resistant to at least one 3GC. Of these 3GC resistant isolates, 98% (n = 92) were positive for $bla_{\text{CMY-2}}$ and 50% (n = 47) were positive for bla_{TEM} and $bla_{\text{CMY-2}}$. The consistently high prevalence of $bla_{\text{CMY-2}}$ over time in isolates resistant to ceftriaxone suggests this gene may be a potential target for rapid molecular screening to identify isolates resistant to 3GC when compared to culture-based methods. The lack of isolates positive for $bla_{\text{CTX-M}}$ or PMQR genes screened suggest that the cattle population evaluated continued to be low risk group for carrier of these important resistance genes. There was also no significant association between the odds ratio of isolating a $bla_{\text{CMY-2}}$-positive, ceftriaxone resistant isolate also positive for bla_{TEM} and the year or year-group the isolates were collected. The higher odds for NTS serotype Dublin, ceftriaxone resistant isolate being positive for bla_{TEM} highlight the need for continued monitoring of this important cattle host-adapted strain. Overall, our study suggests that the prevalence of resistance to cephalosporins due to ESBL and ACBL genes present in bovine NTS isolates has remained relatively constant in this hospital population in Northern California from 2002 to 2016.

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CHAPTER 1 SUPPLEMENTAL

Supplemental Table 1-1. Descriptive information for isolates selected for PCR testing for screening of cephalosporin resistance genes.

N	Year[1]	Serotype	Submission Type[2]	Gender	Age Group	Antibiotic Resistances [3]	*bla*$_{TEM}$ only	*bla*$_{TEM}$ + *bla*$_{CMY-2}$	*bla*$_{CMY-2}$ only
1	2002	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
2	2002	*S.*DUBLIN	IDC	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
3	2002	*S.* DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
4	2002	*S.* DUBLIN	Disease	Male	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
5	2002	*S.* DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
6	2002	*S.* DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
7	2002	*S.* DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
8	2002	*S.* DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
9	2002	*S.*TYPHIMURIUM	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
10	2002	*S.* DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
11	2002	*S.* DUBLIN	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
12	2003	*S.*NEWPORT	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
13	2004	*S.* DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
14	2004	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
15	2004	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
16	2004	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
17	2004	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
18	2004	*S.*DUBLIN	Disease	Male	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
19	2004	*S.*READING	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
20	2004	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroStr	0	0	1
21	2005	*S.*DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
22	2005	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
23	2005	*S.*DUBLIN	Disease	Male	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	0	1
24	2005	*S.*DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenTet	0	1	0
25	2005	*S.*NEWPORT	Disease	Female	Adult	AmxAmpFoxCtfCroStrTet	0	1	0

26	2005	*S.NEWPORT*	Disease	Male	Calf	AmxAmpFoxCtfCroStrTet	0	0	1
27	2005	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
28	2005	*S.NEWPORT*	Disease	Male	Calf	AmxAmpFoxCtfCroStrTet	0	0	1
29	2005	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
30	2005	*S.NEWPORT*	Disease	Male	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
31	2005	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
32	2005	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
33	2006	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
34	2006	*S.NEWPORT*	IDC	Male	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
35	2006	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
36	2006	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
37	2006	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
38	2007	*S.NEWPORT*	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
39	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
40	2007	*S.NEWPORT*	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
41	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
42	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
43	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
44	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
45	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
46	2007	*S.MELEAGRIDIS*	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
47	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
48	2007	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
49	2007	*S.NEWPORT*	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
50	2007	*S.MELEAGRIDIS*	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
51	2007	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
52	2007	*S.MELEAGRIDIS*	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
53	2007	*S.NEWPORT*	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
54	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
55	2007	*S.NEWPORT*	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
56	2007	*S.NEWPORT*	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1

57	2007	*S*.NEWPORT	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
58	2007	*S*.NEWPORT	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
59	2007	*S*.NEWPORT	IDC	Female	Adult	AmxAmpFoxCtfCroStrTet	0	0	1
60	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
61	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
62	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
63	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
64	2008	*S*.DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	1	0
65	2008	*S*.DUBLIN	IDC	Female	Adult	AmxAmpFoxCtfCroChlGenStrTet	0	0	1
66	2008	*S*.DUBLIN	IDC	Female	Adult	AmxAmpFoxCtfCroChlGenTet	0	1	0
67	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenTet	0	1	0
68	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenTet	0	1	0
69	2008	*S*.MONTEVIDEO	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenTet	0	0	0
70	2008	*S*.TYPHIMURIUM	IDC	Male	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
71	2008	*S*.TYPHIMURIUM	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
72	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
73	2008	*S*.TYPHIMURIUM	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
74	2008	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
75	2008	*S*.NEWPORT	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
76	2008	*S*.NEWPORT	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
77	2009	*S*.TYPHIMURIUM	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
78	2010	*S*.NEWPORT	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
79	2010	*S*.NEWPORT	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
80	2010	*S*.NEWPORT	IDC	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
81	2010	*S*.NEWPORT	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
82	2010	*S*.NEWPORT	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	1
83	2010	*S*.NEWPORT	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
84	2011	*S*.NEWPORT	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	0	1
85	2012	*S*.TYPHIMURIUM	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
86	2013	*S*. SP. 9,12:NONMOTILE	Disease	Female	Calf	AmxAmpFoxCtfCroChlNalStrTet	0	0	1

87	2013	*S*.DUBLIN	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
88	2014	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlGenTet	0	1	0
89	2014	*S*.TYPHIMURIUM	IDC	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
90	2014	*S*.DUBLIN	Disease	Female	Calf	AmxAmpFoxCtfCroChlStrTet	0	1	0
91	2014	*S*.DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0
92	2014	*S*.TYPHIMURIUM	Disease	Male	Calf	AmxAmpFoxCtfCroChlStrTet	0	0	0
93	2015	*S*.GIVE	Disease	Male	Calf	AmxAmpFoxCtfCroChlNalStrTet	0	1	0
94	2015	*S*.DUBLIN	Disease	Female	Adult	AmxAmpFoxCtfCroChlStrTet	0	1	0

1. Year in which *Salmonella* was isolated from fecal sample.

2. Submitted as part of a veterinary hospital infectious disease control (IDC) program or due to animals suspected of having salmonellosis.

3. Amx: Amoxicillin, Amp: Ampicillin, Fox: Cefoxitin, Ctf: Ceftiofur, Cro: Ceftriaxone, Chl: Chloramphenicol, Nal: nalidixic acid, Gen: Gentamicin, Str: Streptomycin, and Tet: Tetracycline

Supplemental Table 1-2. Descriptive information for *Salmonella* isolates from cattle selected as controls for PCR testing. Isolates were primarily selected based on pan-susceptible phenotype, however for 2002 and 2004 no pan-susceptible isolate was available. Isolates susceptible to ceftriaxone and resistant to the lowest number of drug classes were selected. Two control isolates were selected for year 2007 because a greater number of isolates for that year was included in the study. None of the control isolates were positive for bla_{CMY-2}.

N	Year*	Serotype	Submission Type**	Gender	Age Group	Antibiotic Resistances ***	bla_{TEM} ***
95	2002	*S.*DUBLIN	IDC	Female	Adult	Str	0
96	2003	*S.* HEIDELBERG	Suspect	Female	Adult		0
97	2004	*S.*TYPHIMURIUM	Suspect	Female	Adult	AmpStrTet	1
98	2005	*S.* SENFTENBERG	Suspect	Female	Adult		0
99	2006	*S.*MONTEVIDEO	IDC	Female	Adult		0
100	2007	*S.* INFANTIS	Suspect	Female	Calf		0
101	2007	*S.* MELEAGRIDIS	IDC	Female	Adult		0
102	2008	*S.* LEXINGTON	IDC	Female	Adult		0
103	2009	*S.* UGANDA	IDC	Female	Adult		0
104	2010	*S.* BARRANQUILLA	IDC	Female	Adult		0
105	2011	*S.* MBANDAKA	IDC	Female	Adult		0
106	2012	*S.* ENTERIDITIS	IDC	Female	Adult		0
107	2013	*S.*MONTEVIDEO	IDC	Female	Calf		0
108	2014	*S.* UGANDA	IDC	Female	Adult		0
109	2015	*S.*MONTEVIDEO	IDC	Female	Adult		0
110	2016	*S.*MONTEVIDEO	Suspect	Female	Calf		0

* Year in which *Salmonella* was isolated from fecal sample
** Submitted as part of a Veterinary Medical Teaching Hospital Infectious Disease Control (IDC) program or due to animals suspected of having salmonellosis.
*** Amp: Ampicillin, Str: Streptomycin, and Tet: Tetracycline.
**** Isolates positive for only bla_{TEM}.

Supplemental Table 1-3. Efficiencies, y-intercepts, and sensitivities of singleplex and multiplex assays using Universal Master Mix (Applied Biosystems). *qnr*S was tested separately via singleplex qPCR.

Sensitivity log (Sl) = (40-y intercept)/S. Sensitivity copy number (CN) = 10^{Sl}.

	Singleplex (Universal MM)			Multiplex (Gene Expression MM)		
Gene	**Efficiency**	**Y-intercept**	**Sensitivity**	**Efficiency**	**Y-intercept**	**Sensitivity**
***bla*TEM**	97%	37.2	<10	91.3%	39.5	<10
***oqx*A**	**95.7%**	**36.99**	**<10**	**92.3%**	**38.2**	**<10**
***qnr*S**	**93.8%**	**37.7**	**<10**			
***bla*CTX-M**	**92.3%**	**35.5**	**<100**	**99.3%**	**39.7**	**<10**
***bla*CMY-2**	**97.2%**	**37.9**	**<10**	**95.7%**	**38.3**	**<10**
***oqx*B**	**93.8%**	**37.3**	**<10**	**96.5%**	**38.4**	**<10**
aac(6')-lb-cr	**99.7%**	**38.5**	**<10**	**99.7%**	**38.5**	**<10**
***qnr*B**	**98.4%**	**38.3**	**<10**	**94.2%**	**37.2**	**<10**

CHAPTER 2. Effect of Intramammary Dry Cow Antimicrobial Treatment on Fresh Cow's Milk Microbiota in California Commercial Dairies

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ABSTRACT

This study used 16S rRNA sequencing to evaluate the effects of dry cow antimicrobial therapy on the udder milk microbiota by comparing the microbial populations in milk at dry-off (DRY)(~60 days before calving) and post-partum (FRESH)(4-11 days after calving) from cows receiving intramammary antibiotic infusion prior to dry-off (IMT) and cows that did not receive treatment (CTL). Milk was collected from 23 cows from IMT group and 27 cows from the CTL group. IMT&DRY samples had a greater correlation with genera *Brevibacterium* and *Amaricoccus*, and the family Micrococcaceae when compared to IMT&FRESH samples. CTL group samples collected at DRY had a greater correlation with genera *Akkermansia* and *Syntrophus* when compared to FRESH samples; no bacterial taxa were observed to have a significant correlation with FRESH samples in the CTL group. DRY samples collected from CTL group had a greater correlation with the genus *Mogibacterium* when compared to IMT&CTL samples. For DRY samples collected from IMT group, a greater correlation with the genus *Alkalibacterium* when compared to DRY&CTL samples was observed. The lack of a correlation for FRESH samples between CTL and IMT treatment groups indicating intramammary antimicrobial dry cow therapy had no significant effect on the udder milk microbiota post-partum.

1. INTRODUCTION

Mastitis, characterized by abnormal milk, is an inflammation of the mammary gland which is responsible for significant economic loses. The great majority of mastitis cases are of bacterial origin, being one of the most prevalent infections in dairy cows, which results in the use of antimicrobial drugs [125, 126]. Aside from its effects on the mammary gland, mastitis has a detrimental effect on welfare, reproduction, and productivity of dairy cows [127, 128]. A common practice at dairy farms for treating existing or chronic cases of mastitis or preventing new mastitis cases during the dry period is the wide use of intramammary antibiotics at the time of dry-off. In 2014, 93% of all U.S. dairy cows received intramammary antimicrobials at dry-off, with 80.3% of all dairy operations choosing to use intramammary antimicrobials non-selectively on all cows at dry-off [37]. This non-selective administration of intramammary antimicrobials at dry-off, also known as blanket therapy, blanket dry cow therapy, or total dry cow therapy, is thought to account for a sizeable portion of the 15,645 kilograms of medically important antibiotics administered intramammary annually in the U.S. [129]. The antimicrobial drugs most commonly used during the dry-off period in the U.S. are the first-generation cephalosporin cephapirin (58% of all operations and 32% of all cows) and the third-generation cephalosporin ceftiofur (28% of all operations and 22% of all cows) [37]. Cephalosporin drugs, especially third generation, have been listed on the World Health Organization's list of critically important antimicrobials for human medicine due to their role in treating important infections caused by Gram negative bacteria [130]. Therefore, identifying alternatives to reduce the need for therapeutic use of third generation cephalosporin drugs, especially for prevention of infection, has become an important area for efforts in livestock medicine.

For decades, the National Mastitis Council has recommended that all quarters of all cows be treated with intramammary antimicrobials at dry-off [43]. The rationale being that blanket therapy is more effective in preventing new infections and does not require any type of screening procedure, as compared to selective therapy. Researchers have continued to debate the effectiveness of blanket treatment, and the introduction and use of commercial non-antibiotic internal teat sealant has only added to this discussion [131-134]. However, decreased cost is a reason some producers consider the use of selective antibiotic treatment over blanket treatment at the time of dry-off. A mathematical model from a 2007 study suggested the choice between blanket or selective treatment is highly farm specific, while a more recent model concluded that selective treatment was more economically beneficial [135, 136]. Furthermore, one study has proposed the use of an algorithm to selectively treat only cows at high risk of developing new cases of mastitis, and by using this approach they estimated a reduction in dry cow antibiotic use by approximately 60% without any adverse effects in animal health [137].

A concern with intramammary antimicrobial dry cow therapy is the potential for disruption of the endogenous microbiota present in the bovine mammary gland. Additionally, there is a risk of inadvertent introduction of environmental organisms into the udder due to contamination during the administration process of the intramammary antimicrobials. Advances in culture-independent methods of microbial analysis of the mammary gland, often via milk samples, has challenged the idea that this environment is sterile [138, 139]. A recent longitudinal cohort study noted that while both Chao richness and Shannon diversity were greater in healthy compared to inflamed mammary glands, a low sequencing success rate suggests that the milk microbiota may not be especially abundant [48, 140]. Nevertheless, it is broadly accepted that a healthy microbiome contributes positively to host health, and

conversely, any disruption of the microbiota may detrimentally impact the host [141]. Therefore, treatment with intramammary antimicrobial dry cow therapy could potentially decrease colonization resistance against pathogens of the mammary gland [142, 143]. This has led researchers to debate whether mastitis, traditionally viewed as a host-pathogen interaction, may actually be a result of dysbiosis of the mammary microbiota [51].

Given the high prevalence and cost of mastitis in the dairy industry and the controversy of blanket intramammary antimicrobial dry cow therapy, more research is needed to fully understand the impacts and benefits of this practice to the microbiota. To address this aim, a subset of a repository of milk samples and its respective data from a statewide dry cow therapy trial were employed [144]. We proposed to evaluate the effect of dry cow antimicrobial therapy on the udder milk microbiota by comparing the microbial populations in milk at dry-off (~60 days before calving) and post-partum from cows receiving intramammary antibiotic infusion and cows that did not receive therapy.

DNA was extracted using a standardized approach [145]. Due to the small quantities of DNA present in each sample, an additional step was required to amplify DNA to a necessary concentration for 16s sequencing [146-148]. Taxonomy was assigned using Greengenes version 13.8 at 99% match [149, 150].

2. MATERIAL AND METHODS

Collection of milk samples for the current study was approved by the University of California, Davis Institutional Animal Care and Use Committee (protocol number 19761).

Milk samples

A random subset of 100 milk samples from 50 cows on 3 of the 8 study herds in the original trial described below were selected [144]. Choice of the 3 herds was based on geographic representation of herds from California's San Joaquin Valley and to minimize the number of freeze-thaw cycles. Specifically, the three herds were in Stanislaus (1,600 milking Holsteins; bulk tank somatic cell count (BTSCC) 200,000 cells/ mL), San Joaquin (1,800 milking Holsteins; BTSCC 145,000 cells/ mL), and Tulare counties (1,100 milking Jerseys; BTSCC 250,000 cells/ mL). At dry off, no cows with clinical signs of mastitis, health events, body condition score < 2.5, lameness or non-functional quarters were enrolled. In the larger study, cows were randomized to one of four groups at dry off and received either intramammary infusion (IMT), internal teat sealant or both. For the current study, 50 cows were randomly selected that either did not receive any treatment at dry off or received only intramammary antibiotic infusion. The decision to utilize the dry off (DRY) and post calving (FRESH) milk samples from each of the 50 randomly selected cows, which yielded 100 milk samples, was based on budgetary reasons.

The decision to use cephapirin or cloxacillin was at the farm level, with 2 farms enrolled in the study using cephapirin and one farm using cloxacillin. Of the 50 cows, 12 from the San Joaquin herd and 4 from the Tulare herd received intramammary infusion (IMT) with cephapirin benzathine (ToMORROW®, Boehringer Ingelheim Animal Health USA Inc., Duluth, GA) and 7 from the Stanislaus herd received cloxacillin benzathine (Dry-Clox®, Boehringer Ingelheim Animal Health USA Inc., Duluth, GA) at the time of dry-off following manufacturers'manufacturers label instructions. The remaining 27 cows from San Joaquin (n=13), Tulare (n=8) and Stanislaus (n=6) received no intramammary infusion (CTL) at the time of dry off.

DNA Extraction

DNA was extracted using a standardized approach [145]. A total of 6 mL of thawed milk from each sample was centrifuged at 8500 rpm for 5 minutes in a sterile 15 mL conical centrifuge tube to pellet bacteria. Whey and fat were then discarded. 500 µL of PowerTube buffer was added to conical tubes and then vortexed briefly to loosen pellet. A 10-minute incubation at 65°C of the sample then followed to increase DNA output. DNA extraction then continued as recommended by the DNeasy PowerSoil Kit (Qiagen N.V., Carlsbad, CA). A total of 100 µL of DNA was eluted for each sample into a sterile 2 mL micro centrifuge tube. DNA samples were stored in -80°C until further processing.

PCR Amplification of 16S rRNA and DNA Sequencing

Due to the small quantities of DNA present in each sample, an additional step was required to amplify DNA to a necessary concentration for 16s sequencing [146]. In this additional step, primers 27F-YM+4 and 1492R were used to amplify the nearly full length 16s rRNA. The 27F-YM+4 primer mix is an eightfold-degenerate primer containing four parts 27F-YM (AGAGTTTGATYMTGGCTCAG), plus one part each of primers specific for the amplification of *Atopobium* (AGAGTTCGATCCTGGCTCAG), *Bifidobacteriaceae* (AGGGTTCGATTCTGGCTCAG), *Borrelia* (AGAGTTTGATCCTGGCTTAG), and *Chlamydiales* (AGAATTTGATCTTGGTTCAG). Each 25 µL PCR reaction contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, Inc., Wilmington, MA), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 µM final concentration of each primer and 2 µL of DNA for each sample. PCR conditions were: an initial incubation at 95°C for 3 min, followed by 25 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 3 min.

Following amplication of the full length 16s rRNA, primers 319F and 806R were used to specifically amplify the V3-V4 domain of the 16S rRNA. Each 25 μL PCR reaction contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, Inc., Wilmington, MA), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 μM final concentration of each primer and 1 μL of DNA for each sample. An initial incubation at 95°C for 3 min, followed by 25 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 3 min comprised the PCR conditions.

In the final PCR run, each sample was barcoded with a unique forward and reverse barcode combination. The PCR reaction in step three contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, Inc., Wilmington, MA), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 μM final concentration of each uniquely barcoded primer and 1ul of the product from the PCR reaction in step two. PCR conditions were: an initial incubation at 95°C for 3 min, followed by 8 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension of 72°C for 3 min. The product of this final PCR reaction was quantified on the Qubit instrument using the Qubit Broad Range DNA kit (Invitrogen/Life Technologies Inc., Carlsbad, CA,).

Individual amplicons were pooled in equal concentrations and the pooled library was cleaned utilizing Ampure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN). The library was quantified via qPCR followed by 300-bp paired-end sequencing using an Illumina MiSeq platform. Forward and reverse reads were trimmed to 260bp and 200bp respectively before proceeding with the DADA2 portion of the QIIME2 analysis pipeline.

Bioinformatics

The 16S rRNA sequencing data was demultiplexed with dbcAmplicons (Matt Settles, UC Davis Bioinformatics Core Facility) and processed through the Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2018.6 utilizing the DADA2 pipeline [147, 148]. Taxonomy was assigned using Greengenes version 13.8 at 99% match [149]. All sample libraries were rarefied at an equal depth of 16,000 reads using QIIME2 prior to generating Shannon diversity indices.

Statistical Analysis

Descriptive data was analyzed using JMP Pro 14.0. Distribution of amplicon sequence variants (ASVs) in milk samples from cows by treatment group and time point was visualized using a Venn diagram. Shannon diversity was displayed using a quantile-box plot.

Relative abundances of different bacterial taxa in each sample were used as covariates in stepwise discriminant analysis models built in JMP Pro 14.0. Each variable was removed in a stepwise manner until only variables with a P value < 0.05 were retained in the final model. Groups used in the analysis were treatment group and sampling time point combinations. Analyses were conducted both independent of specific antibiotic treatment being used, as well as by stratifying the dataset according to antibiotic treatment the interactions between treatment and time points. Two separate models were built to evaluate bacterial taxa. The first model did not consider the type of antimicrobial used at dry-off. In the second model, data were stratified by antimicrobial treatment (cephapirin and cloxacillin). For models not differentiating between antibiotics used for IMT, canonical values for these analyses were used to create a graphical display of the taxonomical results. A canonical cut-off value of ± 0.3 was used.

For taxa identified as having a significant effect on the microbial composition for each treatment group and time interaction, a linear regression was used to evaluate potential

significant differences in relative abundance for taxa of interest. Multilinear regression models were generated, where the relative abundance for each taxa with a canonical value of ± 0.3 was used as the dependent variable, and the treatment groups, time points, and interaction were included as explanatory variables. For each model, animal individual identifier was nested within farm where cow was located and inserted in the model as a random effect. If the time and treatment interaction between sampling time points and treatment groups were significant, a pairwise comparison analysis was conducted using Tukey's Honest Significant Difference (HSD) [150]. Differences were considered significant when a *P* value <0.05 was observed.

Relative mean abundances for six genera associated with mastitis (*Staphylococcus, Bacillus, Streptococcus, Mycoplasma, Escherichia,* and *Trueperella*) were compared at DRY and FRESH time points for both IMT and CTL (Supplementary Figure 2-1). For this analysis a linear regression was used, where the dependent variable was the bacteria genus relative abundance, and the independent variable was the time point and treatment group variable, as well as its interaction. The individual identifier of the cow was nested by the farm it was sampled and entered in the model as a random effect.

3. RESULTS

Descriptive Data and Microbial Diversity Data

A descriptive analysis of the 16s data revealed the top 5 most abundant phyla. In order, these include: Firmicutes, Proteobacteria, Bacteroidetes, Actineobacteria, and Tenericutes (Figure 2-1). For DRY samples, the mean values for phyla were as follows: Firmicutes (56.6%), Proteobacteria (25.3%), Bacteroidetes (6.4%), Actineobacteria (6.6%), and Tenericutes (0.7%). For FRESH samples, the mean values for phyla were as follows: Firmicutes (50.7%), Proteobacteria (32.5%), Bacteroidetes (7.1%), Actineobacteria (5.1%), and Tenericutes (1.9%).

A Venn Diagram of ASVs from treatment groups for each time point displaying ASVs shared between groups is presented in Figure 2-2. A total of 106 ASVs were shared between DRY&CTL and DRY&IMT, while only 1 ASV was shared between FRESH&CTL and FRESH&IMT. A total of 17 ASVs were shared between DRY&CTL and FRESH&CTL, while 3 ASVs were shared between DRY&IMT and FRESH&IMT. A total of 132 ASVs were shared among all four treatment and time point combinations.

No significant differences were found in mean relative abundance for the six genera commonly associated with mastitis (*Staphylococcus* spp, *Bacillus* spp, *Streptococcus* spp, *Mycoplasma* spp, *Escherichia* spp, and *Trueperella* spp.) for IMT and CTL at DRY and FRESH (Supplemental Figure 2-1).

Relative mean abundances for the top 20 taxa ranging from the order to genus level were tabulated to identify differences in relative abundance among the four experimental groups (Table 2-1). The genus *Staphylococcus* was the most abundant taxa for DRY&CTL and FRESH&IMT. The genus *Delftia* was the most abundant taxa for DRY&IMT and FRESH&CTL.

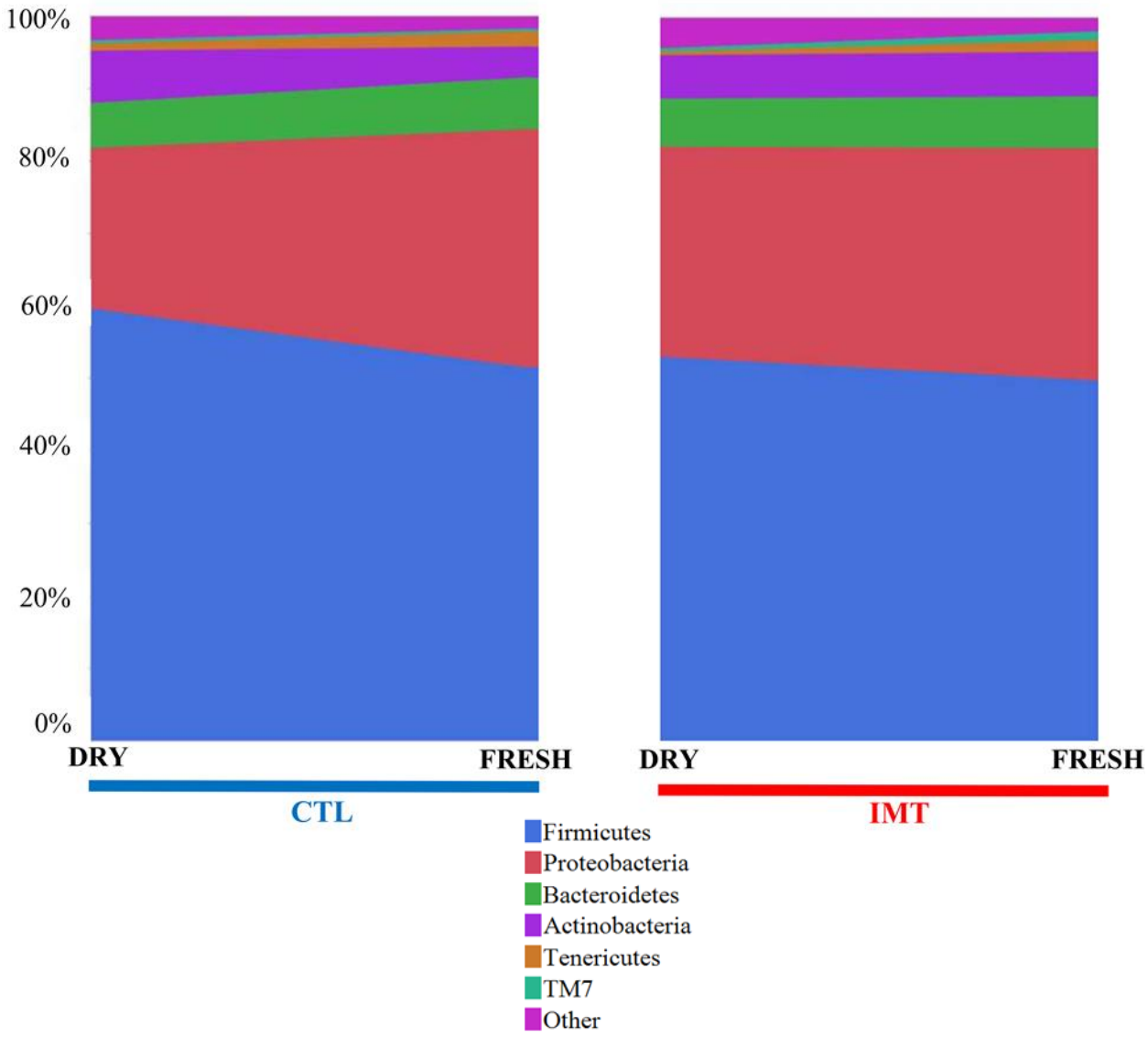


Figure 2-1. Mean percentage distribution for the top 5 most prevalent phyla for each sampling point by treatment group.

IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off; **DRY**: samples collected at the time of dry-off; **FRESH** samples: samples collected from post-partum cows between 4 and 11 days in milk; **CTL**: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off

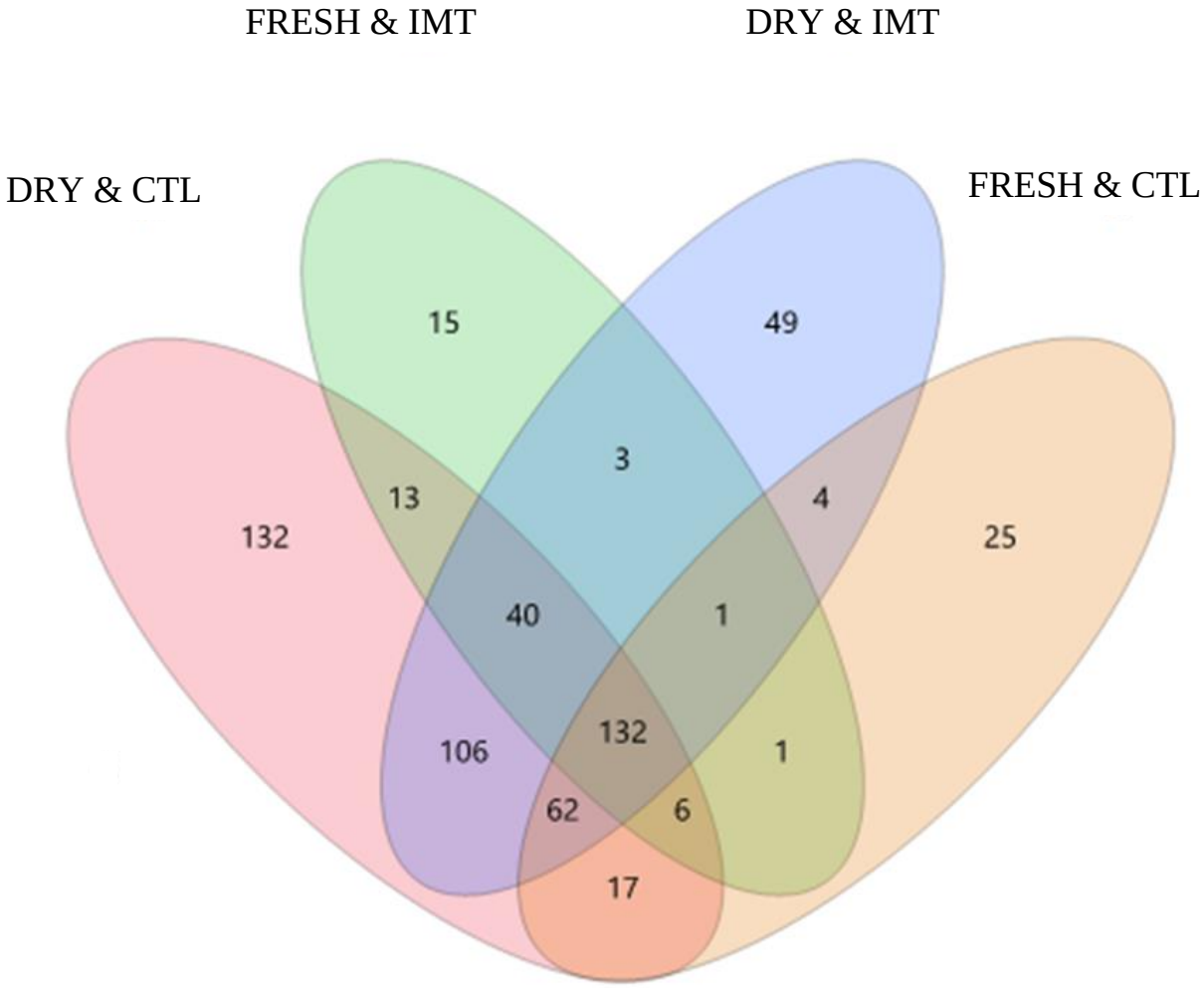


Figure 2-2. Venn diagram of the amplicon sequence variants found in milk samples from cows receiving intramammary antimicrobial treatment at dry-off (DRY & IMT), cows not receiving IMT at dry-off (DRY & CTL) and the follow up sample for these cows when fresh (FRESH & IMT, and FRESH & CTL, respectively).

1. IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off
2. DRY: samples collected at the time of dry-off
3. FRESH: samples collected from post-partum cows between 4 and 11 days in milk.
4. CTL: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off.

	Treatment / Time, % (SE)			
	CTL [1]		IMT[2]	
TAXON[3]	**DRY[4]**	**FRESH[5]**	**DRY[4]**	**FRESH[5]**
g__Staphylococcus	21.3 (4.7)	19.2 (5.6)	13.7 (3.7)	15.7 (3.9)
g__Delftia	11.4 (3.8)	19.7 (5.2)	19.9 (6.3)	20 (5)
f__Peptostreptococcaceae	7.6 (1.7)	5.1 (1.2)	6.2 (0.9)	3.0 (1.0)
f__Ruminococcaceae	4.8 (1.2)	5.3 (1.2)	5.7 (1.8)	4.7 (1.3)
g__Corynebacterium	3.9 (1.3)	2.2 (0.9)	3.1 (1.2)	2.5 (0.5)
g__Turicibacter	3.3 (0.8)	1.9 (0.6)	2.8 (0.6)	3.3 (1.2)
o__Clostridiales	2.7 (0.6)	1.2 (0.3)	3.1 (0.9)	1.8 (0.6)
f__Lachnospiraceae	2.6 (0.6)	1.4 (0.4)	1.7 (0.6)	2.1 (0.7)
g__Serratia	0.7 (0.7)	2.5 (2.5)	1.6 (1.6)	1.5 (1.5)
o__Bacteroidales	1.1 (0.4)	1.2 (0.5)	1.2 (0.5)	2.3 (1.4)
g__Epulopiscium	0.6 (3.4)	4 (3.6)	0.1 (0)	0.1 (0.1)
g__Streptococcus	1 (0.4)	1.2 (0.9)	0.7 (0.3)	2.2 (2.1)
g__Acinetobacter	1.3 (1.0)	0.8 (0.5)	1.1 (0.4)	1.7 (0.6)
f__Clostridiaceae	1.5 (0.4)	0.9 (0.3)	1 (0.3)	1.3 (0.6)
g__5-7N15	1.3 (0.5)	1.1 (0.4)	1.2 (0.5)	1.0 (3.7)
g__Salinicoccus	1.1 (0.6)	0.4 (0.2)	1.4 (0.6)	1.7 (1.2)
g__Herbaspirillum	0.7 (0.6)	0.4 (0.2)	0 (0)	3.5 (2.4)
f__Neisseriaceae	0.9 (0.5)	1.0 (0.7)	1.1 (7.6)	0.8 (0.7)
g__Bacillus	0.8 (0.3)	0.7 (0.3)	1.2 (0.4)	0.8 (0.5)
f__Aerococcaceae	0.5 (0.2)	0.5 (0.2)	1.2 (0.4)	1.2 (0.7)

Table 2-1. Relative abundances for the top 20 taxa observed by treatment group and sampling point.

1. CTL: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off
2. IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off
3. Taxon: refers to the various levels of classification used to describe sequence data (g-genus, f-family, and o-order).
4. DRY: samples collected at the time of dry-off
5. FRESH samples: samples collected from post-partum cows between 4 and 11 days in milk

Quantile box plots were generated to illustrate changes in Shannon diversity between DRY and FRESH time points for IMT and CTL groups (Figure 2-3). A Wilcoxon sum rank test was performed to access the significance of differences between experimental groups. The only Shannon diversity values deemed significantly different (P value < 0.05) were those from DRY&CTL and FRESH&IMT.

Canonical Coefficients and Linear Regression

A canonical cut-off loading value of ± 0.3 was used to identify taxa for different treatment groups and time points within those groups as previously reported [62, 151, 152]. For the canonical analysis comparing time points by treatment, we observed that IMT group samples collected at dry-off had a greater correlation with genera *Brevibacterium* and *Amaricoccus*, and the family Micrococcaceae when compared to FRESH samples; no samples were indicated to have a greater correlation with FRESH samples (Figure 2-4A). We observed that CTL group samples collected at DRY had a greater correlation with genera *Akkermansia* and *Syntrophus* when compared to FRESH samples. No samples were observed to have a significant correlation with FRESH samples in the CTL treatment group (Figure 2-4B).

For the canonical analysis comparing treatment by time points, we observed that DRY samples collected from CTL group had a greater correlation with the genus *Mogibacterium* when compared to IMT samples. For DRY samples collected from IMT group, a greater correlation with the genus *Alkalibacterium* when compared to CTL samples was observed (Figure 2-5A). No correlations were observed for FRESH samples comparing CTL and IMT treatment groups (Figure 2-5B).

No significant difference in the relative abundance of bacteria was observed between treatment by time points or between time points for different treatment groups for taxon identified as having a significant effect on the microbial composition (canonical ± 0.3), except for *Brevibacterium* (Supplemental Table 2-1). Bacterial taxa evaluated included f_Micrococcaceae, g_Amaricoccus, g_Brevibacterium, g_Akkermansia, g_syntrophus, g_Alkalibacterium, and g_Mogibacterium. Further analysis using Tukey pairwise analysis for

the genus *Brevibacterium* revealed no significant difference between different treatment groups by time points (Supplemental Table 2-2).

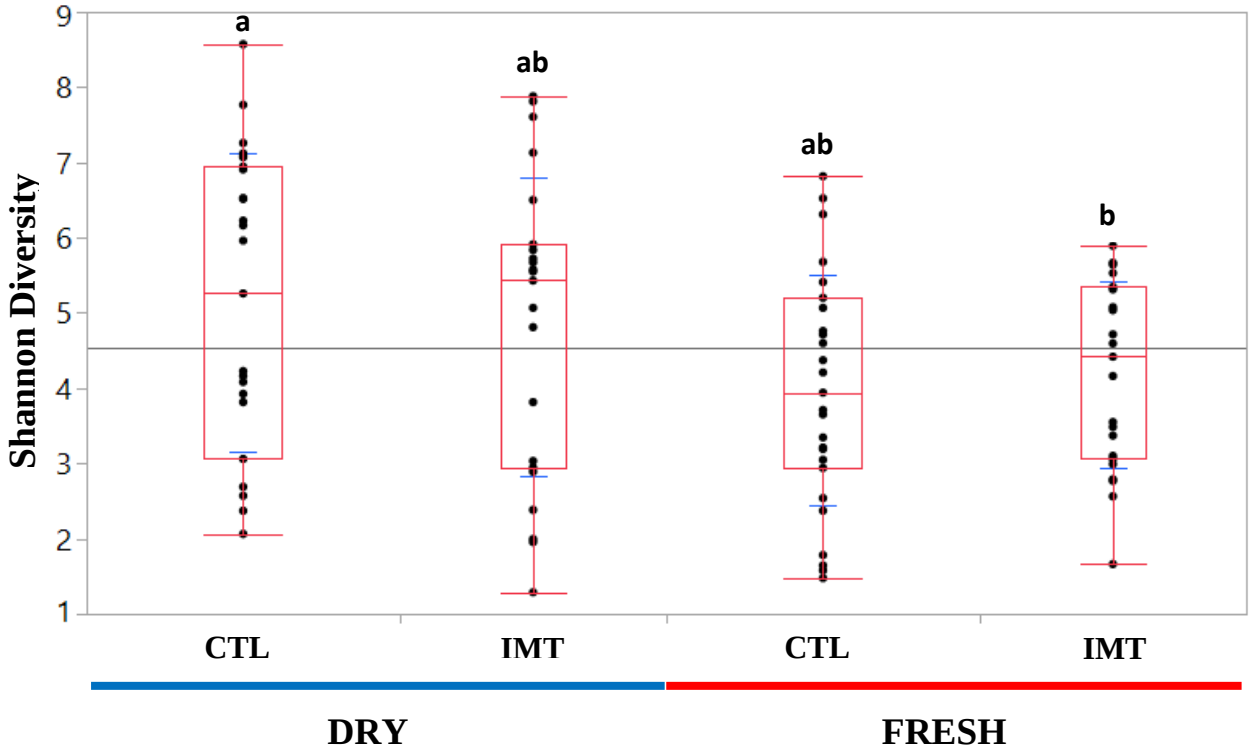
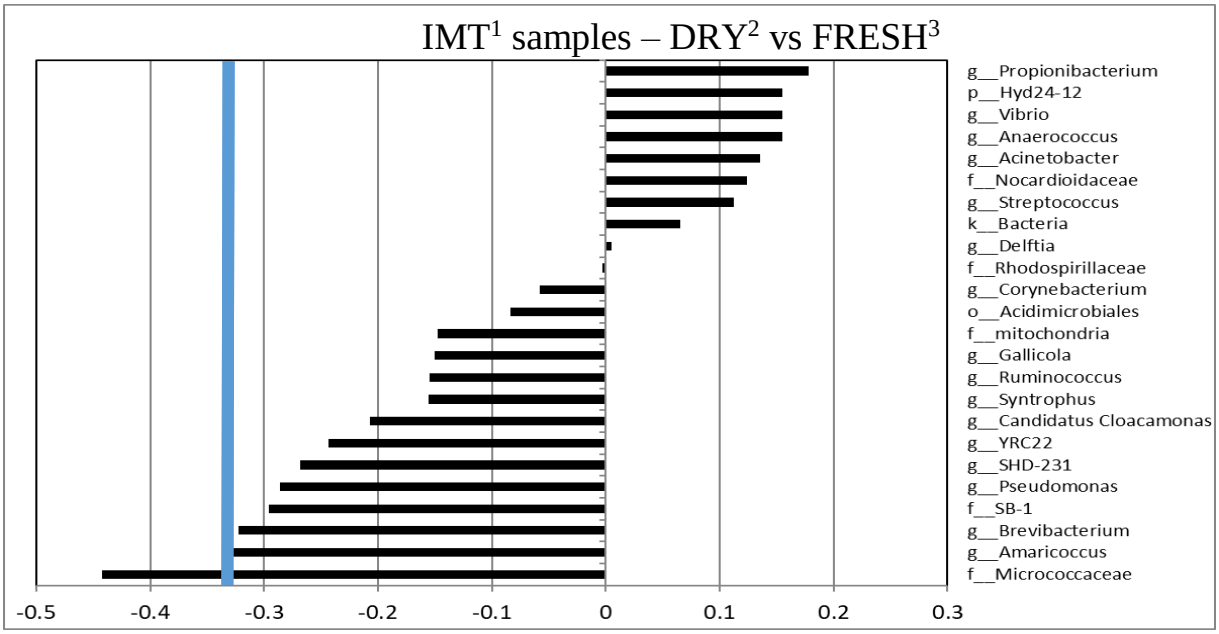


Figure 2-3. Shannon diversity quantile box plots for each treatment group for each sampling time point. Data was not normally distributed (Shapiro Wilks *P* value= 0.044). Different letters (a and b) indicated treatment groups that had significantly different values based on Wilcoxon Sum Rank Test (*P* value < 0.05).

Fresh: samples collected from post-partum cows between 4 and 11 days in milk; **CTL**: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off; **IMT**: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off; **DRY**: samples collected at the time of dry-off.

A.



B.

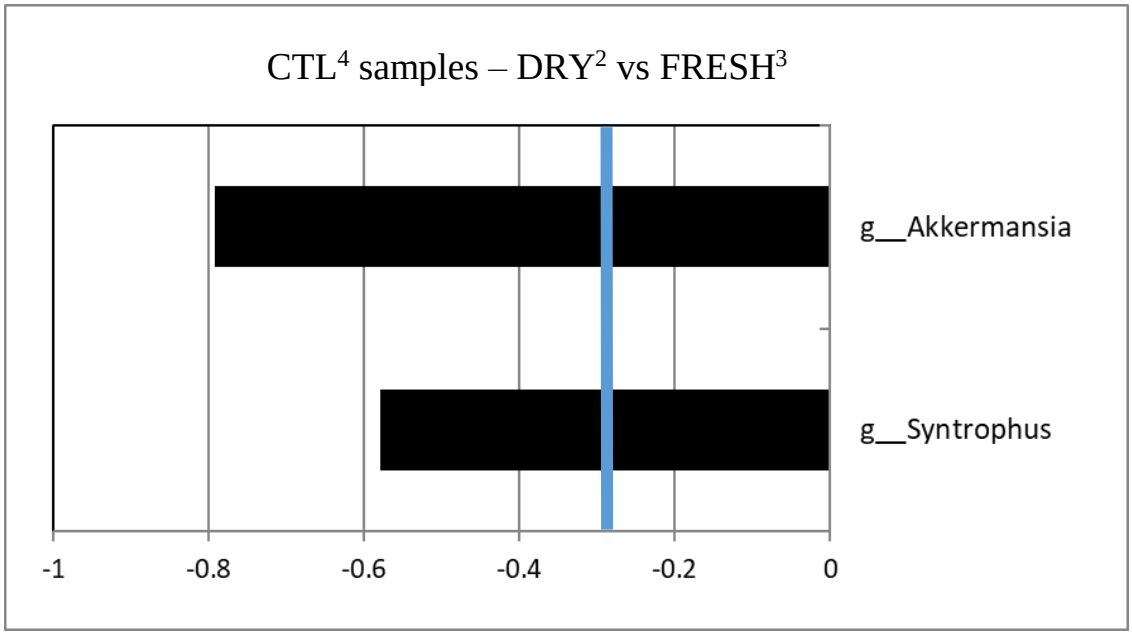
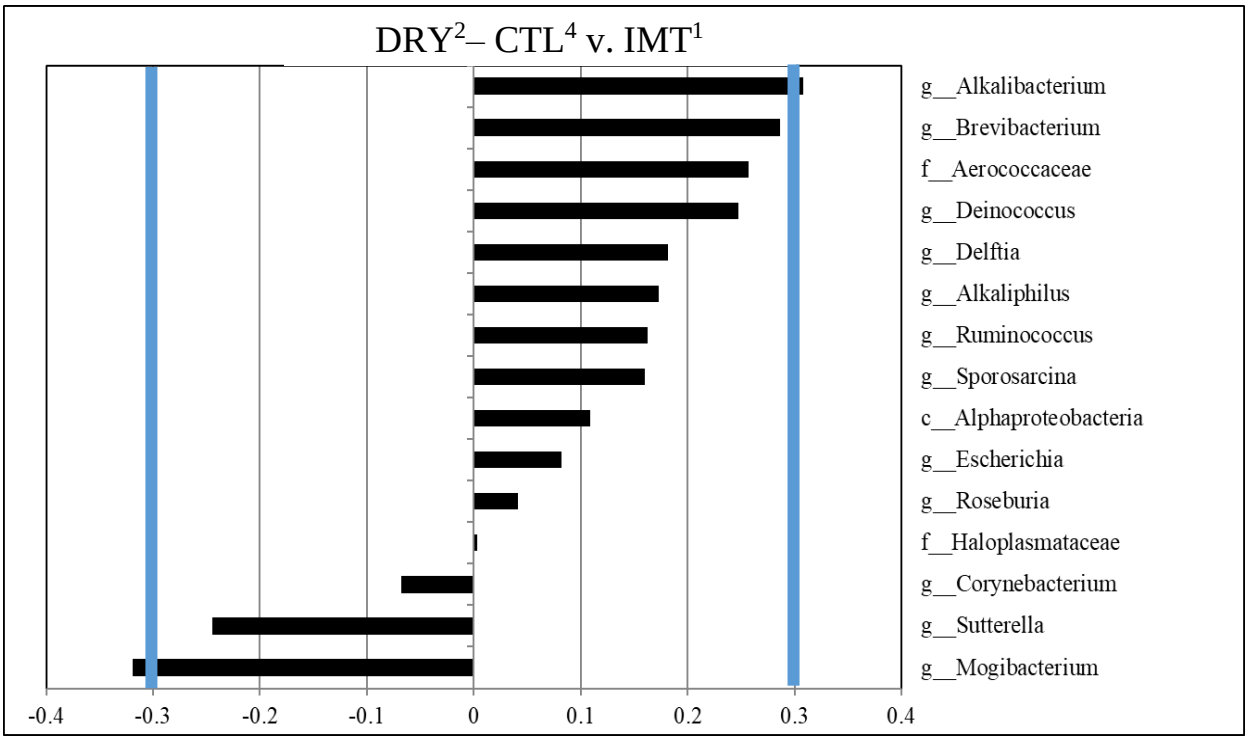


Figure 2-4. Canonical structure coefficients comparing sampling microbiota for milk collection sampling points (DRY and FRESH) by treatment group—namely cows either receiving intramammary antimicrobial treatment (IMT) at dry-off and cows not receiving IMT at dry-off (CTL). **(A)** Correlation between microbial taxa and the discriminant function for Dry v. Fresh sampling points of IMT cows. Bacterial taxa with canonical structure coefficients ≤ -0.3 or ≥ 0.3

(Blue line) are considered important when distinguishing sampling times (Dry v. Fresh) from IMT and CTL cows. **(B)** Correlation between microbial taxa and the discriminant function for DRY v. FRESH sampling points of CTL cows.

1. IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off
2. DRY: samples collected at the time of dry-off
3. FRESH: samples collected from post-partum cows between 4 and 11 days in milk.
4. CTL: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off

A.


DRY[2]– CTL[4] v. IMT[1]
g__Alkalibacterium
g__Brevibacterium
f__Aerococcaceae
g__Deinococcus
g__Delftia
g__Alkaliphilus
g__Ruminococcus
g__Sporosarcina
c__Alphaproteobacteria
g__Escherichia
g__Roseburia
f__Haloplasmataceae
g__Corynebacterium
g__Sutterella
g__Mogibacterium
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4

B.

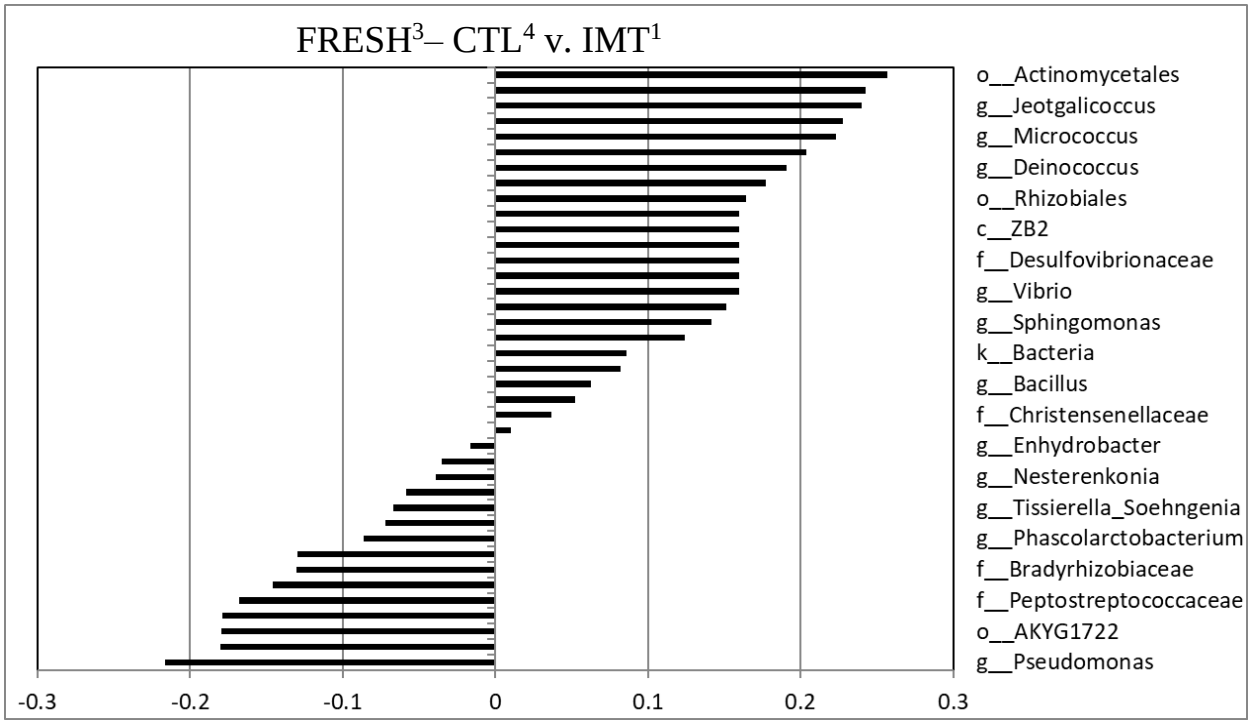

FRESH[3]– CTL[4] v. IMT[1]
o__Actinomycetales
g__Jeotgalicoccus
g__Micrococcus
g__Deinococcus
o__Rhizobiales
c__ZB2
f__Desulfovibrionaceae
g__Vibrio
g__Sphingomonas
k__Bacteria
g__Bacillus
f__Christensenellaceae
g__Enhydrobacter
g__Nesterenkonia
g__Tissierella_Soehngenia
g__Phascolarctobacterium
f__Bradyrhizobiaceae
f__Peptostreptococcaceae
o__AKYG1722
g__Pseudomonas
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

Figure 2-5. Canonical structure coefficients comparing sampling microbiota for cows receiving intramammary antimicrobial treatment (IMT) at dry-off and cows not receiving intramammary antimicrobial treatment (CTL) at dry-off. **(A)** Correlation between microbial taxa and the discriminant function for DRY comparing CTL versus IMT samples. **(B)** Correlation between microbial taxa and the discriminant function for FRESH comparing CTL versus IMT samples. Bacterial taxa with canonical structure coefficients ≤ -0.3 or ≥ 0.3 (Blue lines) are considered important when distinguishing between CTL and IMT samples.

1. IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off
2. DRY: samples collected at the time of dry-off
3. FRESH: samples collected from post-partum cows between 4 and 11 days in milk.
4. CTL: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off

Linear discriminant analysis of milk samples for each treatment group by time points is displayed in Figure 2-6. This analysis was stratified by antimicrobial drug used for IMT infusion (cephapirin vs cloxacillin) and is also displayed (Figure 2-6B and 2-6C, respectively). Samples from FRESH, regardless of whether collected from IMT or CTL cows, were highly similar despite stratifying by individual drug used for IMT. Analysis stratified by cephapirin only (Figure 2-6B) more closely resembled that of the combination of cephapirin and cloxacillin (Figure 2-6A). Analysis stratified by cloxacillin only (Figure 2-6C) revealed similarities of the DRY&CTL samples with FRESH cows, independent of treatment. This similarity may have been driven by the aforementioned greater diversity in DRY sample time points in conjunction with a limited sample size, that may not had been sufficiently large to appropriately represent the abundance of individual taxa. Together, these factors could have resulted in the lack of differentiation that we observed.

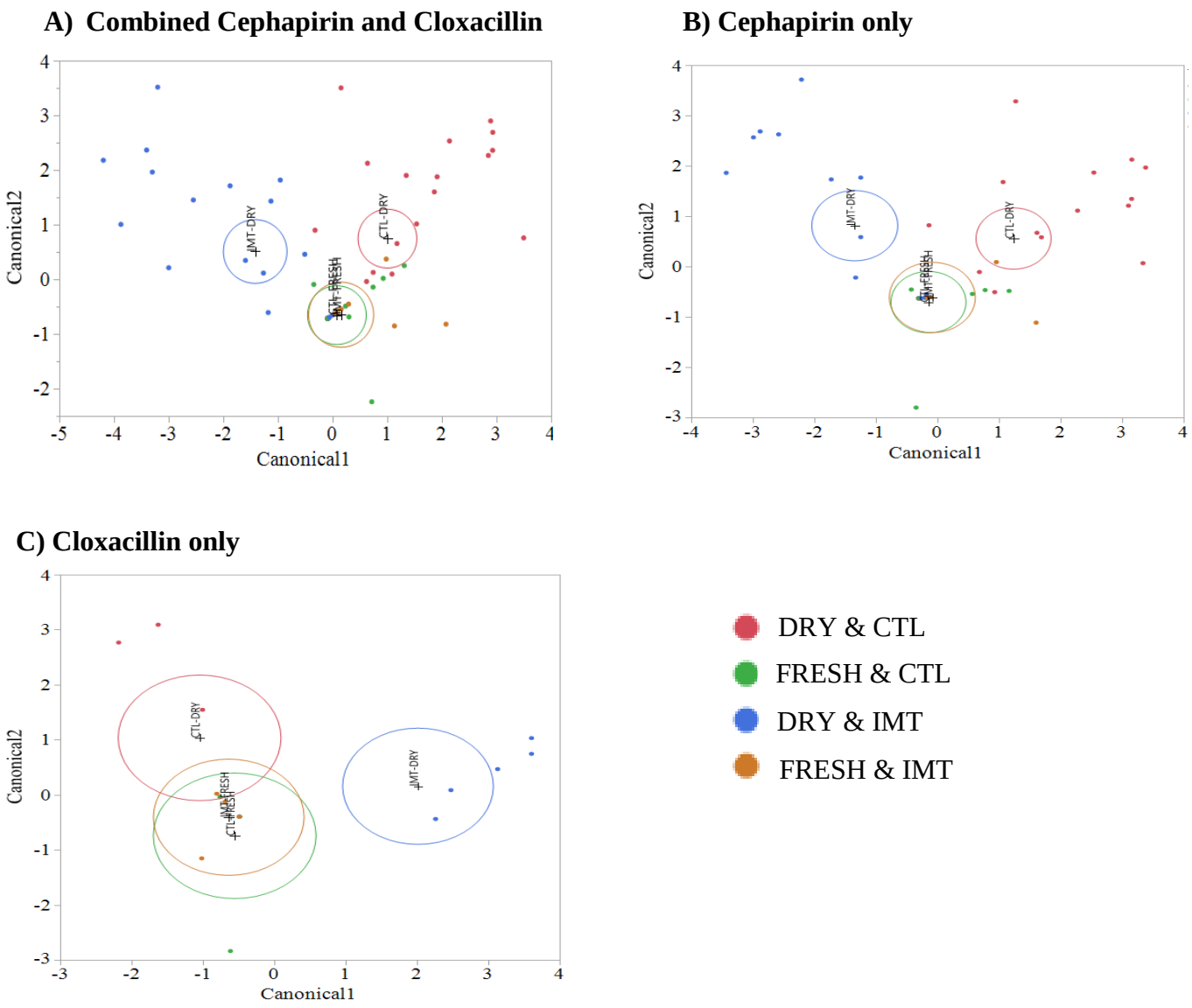


Figure 2-6. Discriminant analyses of milk sample microbiomes for each treatment group by sampling point for IMT cows treated with either: **(A)** cephapirin and cloxacillin **(B)** cephapirin only **(C)** cloxacillin only. Bacterial relative abundance was used as covariates and treatment group as the categorical variable (NR = red dots, DR = blue dots). An ellipse indicates the 95% confidence region to contain the true mean of the group, and a plus symbol indicates the center (centroid) of each group.

1. IMT: treatment group representing cows receiving intramammary antimicrobial drug treatment at dry-off
2. DRY: samples collected at the time of dry-off
3. FRESH samples: samples collected from post-partum cows between 4 and 11 days in milk.
4. CTL: control group representing cows not receiving intramammary antimicrobial drug treatment at dry-off

4. DISCUSSION

Lack of microbiota differentiation of FRESH samples from CTL and IMT cows

No genera were deemed significant in differentiating FRESH samples from either CTL or IMT cows, indicating intramammary antimicrobial dry cow therapy had no significant effect on the udder milk microbiome post-partum (Figure 2-5B). A study by Derakhshani et al. (2018) qualitatively evaluated the microbiota of teat canal and mammary secretions of healthy udder quarters subjected to dry cow therapy using a long-acting antimicrobial product containing penicillin G and novobiocin, in combination with internal teat sealant. Although shifts in the bacterial genera and phyla abundance were observed in their study, further analysis indicated a commonality between pre-IMT and postpartum microbiota of both teat canal and mammary secretions, indicating limited effect of IMT on microbiota. Another study by Ganda et al. (2016) evaluated the impact of intramammary antimicrobial treatment on the milk microbiome of healthy cows and from cows presenting with clinical mastitis. Treatment with the third-generation cephalosporin ceftiofur had no significant effect on clinical cure, bacteriological cure, pathogen clearance, or bacterial load. Although this study focused on clinical cases of mastitis, they also observed similar results where antimicrobial drugs had little to no effect on milk microbiome and bacterial load. Our study did not include a third-generation cephalosporin treatment in the IMT group so our results are not directly comparable to this particular finding.

Microbiological differentiation of DRY samples from IMT and CTL cows compared to FRESH samples

We observed that DRY cows, independent of treatment, had a greater microbial diversity when compared to milk from cows at FRESH (Figure 2-3). Similar findings for greater diversity of milk microbiota at dry-off was also observed by Derakhshani et al. (2018). For

both analyses evaluating microbial differences driving differentiation between time points by treatment, we only observed differentiated microbes in the DRY cow samples independent of treatment (Figure 2-4). A possible explanation for this is that cows in FRESH maintained a similar core microbiota as DRY cows, but with a less diverse composition, leading to the observed difference in individual microbes in DRY.

The genera *Brevibacterium* and *Amaricoccus*, and the family Micrococcaceae were observed to have a significant canonical score for the discriminant analysis between DRY samples from IMT cows (Figure 2-4A). Bacteria in the genus *Brevibacterium* are Gram positive, non-endospore forming, nonmotile, obligate aerobes, halotolerant, proteolytic, peptidolytic, esterolytic, and lipolytic in nature [155]. It has been isolated from human skin, marine, and terrestrial environments [156]. *Brevibacterium* is also found in the microbial communities present in raw milk and cheese [157]. A study on the microbial composition of Dutch "Danbo", a surface ripened semi-hard cheese, revealed that during ripening *Brevibacterium* was the third most abundant genera on the cheese surface [158]. Research on udder cleft dermatitis found *Brevibacterium* was present in a samples taken from three mild and one severe case, with 49.2% of classified reads belonging to *Brevibacterium* in the severe sample [159].

Although not reaching the ± 0.3 canonical cut-off loading value, *Brevibacterium* was also observed for IMT discriminant analysis comparing CTL within DRY cows (Figure 2-5A). An explanation as to why *Brevibacterium* seems to be of greater relevance in the microbial composition of milk in DRY cows has yet to be determined.

The genus *Amaricoccus* was discovered in 1997 in activated sludge biomass from wastewater treatment plants around the world [160]. Bacteria in this genus are Gram negative

aerobic cocci that can form tetrads, a grouping of four cells. *Amaricoccus* species are able to store polyhydroxyalkanoates (PHA), a biologically produced polymer similar to the plastics polyethylene and polypropylene [161].

The family Micrococcaceae includes Gram positive cocci bacteria found in dairy products and cured meats. Micrococcaceae includes the genus *Micrococcus* which, along with other members in the family, may reduce ripening times in cheese [162]. Given the locations where bacteria in the family Micrococcaceae have been found, it seems likely these bacteria are commensal milk bacteria.

Differentiated bacteria from DRY and FRESH samples collected from CTL Cows

The genus *Amaricoccus* was discovered in 1997 in activated sludge biomass from wastewater treatment plants around the world [160]. Bacteria in this genus are Gram negative aerobic cocci that can form tetrads. *Amaricoccus* species are able to store polyhydroxyalkanoates (PHA), a biologically produced polymer similar to the plastics polyethylene and polypropylene [161]. Bacteria belonging to the genus may be able to degrade the antibacterial tricoslan [163]. *Amaricoccus* has also been detected in the microbiome of colostrum from human mothers who delivered via C-section [164]. It is uncertain why *Amaricoccus* was observed at a great abundance in IMT cows.

The family Micrococcaceae includes Gram positive cocci bacteria found dairy products and cured meats. Micrococcaceae includes the genus *Micrococcus* which, along with other members in the family, may reduce ripening times in cheese [162]. Micrococcaceae seem to be especially abundant in raw sheep's milk cheeses including the Spanish semi-soft "Casar de Cáceres" and soft "Tetilla" [165, 166]. Micrococcaceae were more abundant on the surface of Tetilla cheese than the interior and may contribute to ripening [165]. Aside from dairy,

Micrococcaceae are found in starter cultures for fermented meats and may prevent colonization of pathogenic bacteria by lowering the pH [167]. Micrococcaceae may also play a role in aroma development in the Spanish, fermented sausage "Chorizo" [168]. It has also been detected in human breast milk of mothers living in mainland China and Taiwan and in porcine breast milk samples collected from sows in various stages of pregnancy [169, 170]. Given the locations where bacteria in the family Micrococcaceae have been found, it seems likely these bacteria are commensal milk bacteria.

Differentiated bacteria from DRY and FRESH samples collected from CTL cows

The genera *Akkermansia* and *Syntrophus* were observed to have a significant canonical score for the discriminant analysis between DRY and FRESH samples from CTL cows (Figure 2-4B). Bacteria in the genus *Akkermansia* are Gram negative, obligate anaerobic, non-motile, and non-sporulating [171]. *Akkermansia muciniphila* plays an important role in the human gut microbiome as it is able to breakdown mucin in mucus as a carbon and nitrogen source [172]. Accounting for 1-4% of the bacteria in the adult intestine, *Akkermansia muciniphila* is inversely associated with diabetes, obesity, and other metabolic issues [173]. This health benefit suggests *Akkermansia muciniphila* potential as a future probiotic. Analysis of mice intestinal microbiota has also shown that *Akkermansia* growth can be affected by consumption of cow and goat's milk, as goat milk consumption had a positive effect on growth [174]. Although typically found in the intestine, the unique mucin-degrading ability of *Akkermansia muciniphila* may allow it to create a specific niche among the mammary gland microbiota. While the presence of *Akkermansia* in cow milk has not been previously reported, a recent studies have detected the genus in human milk samples from subjects in Ghana and in the feces of lactating dairy cows [175, 176].

Bacteria in the genus *Syntrophus* are Gram negative and strictly anaerobic. These bacteria get their energy from breaking down chemicals that might kill other bacteria such as phenol, benzoate, and fatty acids [177, 178]. Bacteria in this genus are generally syntrophic, meaning they rely on partner organisms for key metabolites. *Syntrophus* has been identified in a study in which 16s analysis of anaerobic digesters fed manure from cows was conducted [179]. The lack of data on *Syntrophus* in studies evaluating microbiota of milk limit interpretation of the relevance of this bacterium.

Differentiated bacteria from DRY samples collected from IMT and CTL Cows

An unexpected finding of our study was that the milk microbiota of cows at DRY sampled before administration of treatment differed in their microbial composition between the two treatment groups. As previously mentioned, a greater diversity of milk microbiota was observed for cows at DRY, and a greater individual milk microbiota diversity could have resulted in the observed findings. Nevertheless, only two genera were observed to significantly discriminate between DRY sampling points when comparing CTL and IMT, namely the genus *Alkalibacterium*, for DRY samples from IMT cows, and the genus *Mogibacterium* for DRY samples from CTL cows (Figure 2-5A). Bacteria in the genus *Alkalibacterium* are Gram positive, non-sporulating, and found in various basic environments [180]. In addition to the previously mentioned *Brevibacterium*, *Alkalibacterium* can also be found on the surface of Dutch "Danbo" cheese—accounting for about 1.3% of total ASVs [158]. It was hypothesized that *Alkalibacterium* was introduced to the cheese via the sea salt used in brining. However, given the presence of *Alkalibacterium* in our milk samples, it is possible these bacteria were instead selected for by the saline brine used in the cheese making process. It was also found on the rind of a blue-veined, raw milk cheese from the UK made in the style of Blue Stilton cheese

[181]. In this case, it is believed that the alkaline pH found in the mature rind could have selected for *Alkalibacterium.* Aside from cheese, *Alkalibacterium* has also been found in the fermentation of Spanish-style green table olives and indigo dye [182, 183]. It has also been discovered to have the potential to recover up to 52% of the copper present in waste produced by the burning of solid waste via a process called bioleaching [184]. Lastly, the species *Alkalibacterium kapii* has been found to inhibit the growth of *Listeria* when present on the surface of cheese [185].

Bacteria in the genus *Mogibacterium* are Gram positive, non-spore forming, obligate anaerobes. Interestingly, research has shown that *Mogibacterium* are significantly more abundant in the rumen of high methane producing cattle [186]. *Mogibacterium* has also been shown to decrease in abundance in dairy cows fed a high-grain diet designed to induce subacute ruminal acidosis [187]. The authors concluded that use of a high-grain diet may increase the risk of mastitis.

5. CONCLUSION

IMT group samples collected at dry-off had a greater correlation with the genera *Brevibacterium* and *Amaricoccus,* and the family Micrococcaceae when compared to CTL for the FRESH samples. Furthermore, CTL group samples collected at DRY had a greater correlation with genera *Akkermansia* and *Syntrophus* when compared to FRESH samples. For DRY samples collected from IMT group, a greater correlation for the genus *Alkalibacterium* was observed when compared to CTL samples. Future research to evaluate the impacts of the findings related to prevalence of different taxa on individual animal health and production are needed. No correlations between taxa were observed for FRESH samples comparing CTL and IMT treatment groups. Taken together, the lack of genera deemed significant in differentiating FRESH samples from either CTL or IMT cows, indicated intramammary antimicrobial dry

cow therapy based on the drugs used in our study had no significant effect on the udder milk microbiota post-partum.

ACKNOWLEDGMENTS

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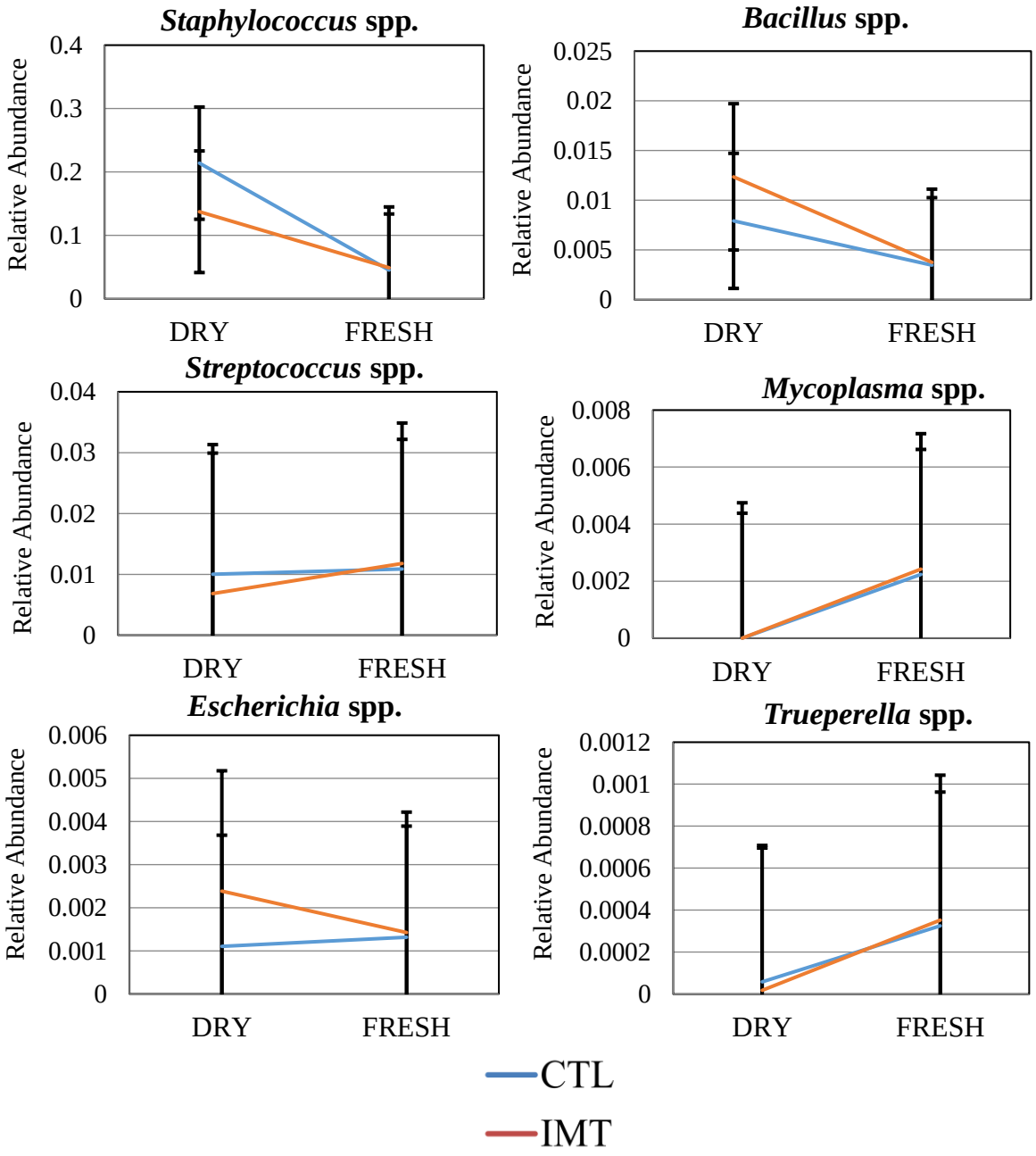
CHAPTER 2 SUPPLEMENTAL

Supplemental Figure 2-1. Relative mean abundance of the genus *Staphylococcus sp*, *Bacillus sp*, *Streptococcus* spp, *Mycoplasma* spp, *Escherichia* spp, and *Trueperella* spp. by treatment group (CTL and IMT) for both DRY and FRESH time points. Error bars correspond to 95% confidence interval.



Supplemental Table 2-1. Results from multivariate models for each taxon with a canonical value of ± 0.3 for analysis in figures 2-2 and 2-3. Results are only displayed for the interaction variable between sampling time points and treatment group.

Taxon[1]	Estimate[2]	Std Error[3]	*P* value[4]
g_*Syntrophus*	-0.001253	0.001517	0.4127
f_Micrococcaceae	-0.000253	0.000384	0.5135
g_*Amaricoccus*	-0.000594	0.000523	0.2618
g_*Proprionibacterium*	0.0005021	0.000587	0.3963
g_*Pseudomonas*	-0.003282	0.00187	0.0857
g_*Akkermansia*	2.21E-06	8.49E-05	0.9793
g_*Brevibacterium*	-0.000656	0.000294	**0.0303**

1. Taxon: refers to the various levels of classification used to describe sequence data (g-genus and f-family)
2. Parameter estimate for the multivariate model evaluating interaction between sampling time points and treatment group.
3. Standard error for the model estimate for the variable representing interaction between treatment group and sampling time point
4. *P*-value for the variable representing interaction between treatment groups and sampling time point. A *P*-value < 0.05 indicates that at least one sampling time and treatment interactions was significantly different.

Supplemental Table 2-2. Results from Tukey pairwise analysis for the genus *Brevibacterium* comparing all treatment groups and sampling time point interactions.

Groups compared [1]		Difference[2]	Std Error[3]	*P* value[4]
DRY-CTL	**DRY-IMT**	-0.001463	0.0008194	0.2927
DRY-CTL	**FRESH-CTL**	-0.000797	0.000797	0.75
DRY-CTL	**FRESH-IMT**	0.000363	0.0008194	0.9708
DRY-IMT	**FRESH-CTL**	0.000666	0.0008194	0.8482
DRY-IMT	**FRESH-IMT**	0.001826	0.0008635	0.1632
FRESH-CTL	**FRESH-IMT**	0.00116	0.0008194	0.4962

1. Group interactions (treatment group and sampling time point) compared using Tukey pairwise analysis.
2. Absolute difference in the means between groups compared as determined by Tukey pairwise analysis.
3. Standard error for difference in the means
4. *P* value for the variable representing a significant difference (*P*-value < 0.05) between the means of the two groups being compared using Tukey pairwise analysis.

CHAPTER 3. Evaluation of antimicrobial resistance and risk factors for recovery of intrauterine *Escherichia coli* from cows with metritis on California commercial dairy farms

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Basbas C, Garzon A, Silva-Del-Rio N, Byrne BA, Karle B, Aly SS, Champagne JD, Williams DR, Lima FS, Machado VS, Pereira RV. Evaluation of antimicrobial resistance and risk factors for recovery of intrauterine Escherichia coli from cows with metritis on California commercial dairy farms. Sci Rep. 2022 Aug 17;12(1):13937. doi: 10.1038/s41598-022-18347-w. PMID: 35978077; PMCID: PMC9386028

ABSTRACT

The goals of this study were to evaluate factors affecting recovery and antimicrobial resistance (AMR) in intrauterine *E. coli* in post-partum dairy cows with and without metritis from commercial California dairy farms. Using a cross-sectional study design, a total of 307 cows were sampled from 25 farms throughout California, from which a total of 162 intrauterine *E. coli* isolates were recovered. During farm visits, cows within 21 days post-partum were categorized in one of three clinical presentation groups before enrollment: metritis (**MET**, n = 86), defined as a cow with watery, red or brown colored, and fetid vaginal discharge; cows with purulent discharge **(PUS**, n = 106), defined as a non-fetid purulent or mucopurulent vaginal discharge; and control cows, (**CTL**, n = 115) defined as cows with either no vaginal discharge or a clear, non-purulent mucus vaginal discharge. Cows diagnosed as MET had significantly higher odds for recovery of *E. coli* compared to cows diagnosed as CTL (OR= 2.16, 95% CI: 1.17 – 3.96), with no significant difference observed between PUS and CTL, and PUS and MET. An increase in days in milk (DIM) at the time of sampling was significantly associated with a decrease in the odds ratio for *E. coli* recovery from intrauterine swabs (OR= 0.94, 95% CI: 0.89 – 0.98). All intrauterine *E. coli* were resistant to ampicillin (AMP), with an AMR prevalence of 30.2% and 33.9% observed for chlortetracycline and oxytetracycline, respectively. Only 8.6% of isolates were resistant to ceftiofur (CEFT), one of the most common drugs used to treat cows on farms sampled. No significant difference in the prevalence of AMR was observed among clinical groups at the individual cow level. At the farm level, a significantly higher odds for isolating intrauterine *E. coli* resistant to chlortetracycline (OR: 2.6; 95% C.I: 3.7 – 58.0) or oxytetracycline (OR: 1.9; 95% C.I: 1.4 – 33.8) was observed at farms that used an intrauterine infusion of oxytetracycline as a treatment for metritis when compared to those farms that did not use this practice. Findings from

this study indicate the need for further research supporting a broader understanding of farm practices driving AMR in cows with metritis, as well as data to increase the accuracy of breakpoints for AMR classification of intrauterine *E. coli* from cattle.

1. INTRODUCTION

Metritis is a major uterine disease in dairy cattle, typically occurring within 21 days post-partum, characterized by an enlarged uterus, fever, and fetid, watery red-brown uterine discharge [56]. In North America, metritis impacts 10 to 30% of post-partum dairy cows [53, 68]. Within the U.S., metritis is the fourth most common health issue in cows, as identified by producers [38]. Metritis has a complex etiology with various bacteria including *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Bacteroides* spp. associated with post-partum uterine infections [60]. Metritis negatively impacts milk production, reproductive performance, and increases the risk of culling [188]. The economic impacts of these production issues cost producers a mean of $511 per case of metritis [55].

The most common systemic antimicrobial treatment for metritis in California is ceftiofur (CEFT), a third-generation cephalosporin with broad-spectrum activity [79]. Ceftiofur is the only antimicrobial approved by the US Food and Drug Administration (U.S. FDA) for the treatment of metritis that does not require milk to be discarded during treatment [81]. The second and third most popular antimicrobials used to treat metritis in California are ampicillin (AMP) and penicillin, respectively [79]. A survey of Midwestern dairy farms also identified CEFT as the preferred treatment for metritis, followed by AMP [80].

Research evaluating minimum inhibitory concentrations (MIC) of *E. coli* from bovine uteri has been conducted in New York, New Zealand, and Germany using samples collected from one to seven commercial dairy farms [84-86]. While there is some research on metritis treatment

preferences and diagnostic practices in California, information on MICs of intrauterine *E. coli* to common antimicrobial drugs (AMDs) used to treat metritis is lacking [79]. To address this knowledge gap, the goals of this study were to evaluate post-partum dairy cattle with and without metritis from commercial dairy farms in California for animal level factors affecting the recovery of intrauterine *E. coli* and to evaluate and identify the animal and farm-level factors affecting the prevalence of antimicrobial resistance (AMR) in intrauterine *E. coli*. Our study hypotheses were that: **1)** dairy cows diagnosed with metritis (watery, reddish or brownish, and fetid vaginal discharge) will have a significantly higher risk for isolation of intrauterine *E. coli* when compared to cows with non-fetid purulent or mucopurulent vaginal discharge (PUS), or cows with clear lochia, clear mucus, or no vaginal discharge (CTL); **2)** dairy cows diagnosed with metritis will have a significantly higher risk for isolation of AMR intrauterine *E. coli* when compared to PUS or CTL cows; **3)** farm management practices related to diagnosis and treatment of metritis will be significantly associated with farm-level prevalence of AMR in intrauterine *E. coli*. This is the first study to report MIC data for intrauterine *E. coli* from post-partum dairy cows with metritis housed on multiple (n = 25) commercial dairy farms in California. Additionally, this is one of the first studies of AMR prevalence within intrauterine *E. coli* recovered from post-partum dairy cows to use the most recently updated Veterinary Clinical & Laboratory Standards Institute (CLSI) MIC breakpoints [1] and CLSI guidelines related to MIC breakpoints for veterinary pathogens [189].

2. MATERIAL AND METHODS

The University of California Institutional Animal Care and Use Committee (IACUC; #20620) approved all experimental procedures conducted with animals for this study. The UC

Davis Institutional Review Board (IRB) Administration granted an exemption (IRB ID 1307716-1) for all experimental procedures for this study.

Study design

A convenience sample of 25 commercial dairy farms from the Sacramento and San Joaquin Valleys in California was recruited with the help of local veterinarians and UC Davis faculty and extension advisors. The study was conducted between September 2018 and November 2019.

Using a cross-sectional study design, intrauterine swabs were collected from post-partum cows between 3 and 21 DIM; cows that were unable to stand were not eligible for enrollment in the study. Three clinical presentation groups were defined based on vaginal discharge (VD) characteristics [190] as metritis discharge (**MET)**: watery, reddish or brownish, and fetid), purulent discharge (**PUS)**: non-fetid purulent or mucopurulent vaginal discharge), and normal discharge (**CTL)**: clear lochia, clear mucus, or no vaginal discharge. Due to sampling time limitations, five cows per clinical group were targeted as the maximum number per dairy.

Researchers visited each of the 25 farms once during the morning lockups of fresh cow pens, while farm employees were performing their own health checks. Researchers (R.V.P. and A.G.) collected vaginal discharge from cows using a Metricheck^{TM} device (Simcrotech, Hamilton, New Zealand) cleaned with 2% chlorhexidine gluconate solution between cows. The VD was evaluated by sight and smell, and cows were assigned to the corresponding clinical presentation group. Evaluation of animals was conducted independent of data from the farm on prior diagnosis of metritis, and were conducted independently of farm employee findings.

For animals selected to be enrolled in the study, rectal temperature was measured using the GLA M900 thermometer (GLA Agricultural Electronics, San Luis Obispo, CA, USA). Prior to intrauterine sample collection, researchers cleaned the vulva using dry paper towels and 70%

isopropyl alcohol. A 30-inch double-guarded sterile culture swab (McCullough; Jorgensen Labs Inc., Loveland, CO, USA) was gently passed through the vulva and cervix until reaching the uterine body. The swab was exposed and rolled against the uterine wall, retracted within the double sheath, removed from the cow, and immediately placed in Amies transport media with charcoal (BBL™ CultureSwab™ Plus; Becton, Dickinson, and Company, Sparks, MD, USA). The swabs were kept on ice until inoculation on to solid medium in the laboratory within 24 hours of sample collection. Individual animal antimicrobial treatment in the last 14 days for animals sampled was recorded.

A sample size calculation was conducted based on our first hypothesis that dairy cows diagnosed with metritis will have a higher risk for isolation of intrauterine *E. coli* when compared to CTL cows. For this purpose, an *a priori* sample size estimation for the proportion of *E. coli* culture positive cows in MET and CTL was made based at a 90% power (σ: 0.1; α: 0.05; $\mu_{MET.}$: 0.7, μ_{CTL}: 0.4) was calculated in JMP Pro 16.0 (SAS Institute Inc., Cary, NC), resulting in a minimum of 53 animals per clinical group.

Survey and treatment records

A survey questionnaire was developed to collect information on farm characteristics, management practices, and antimicrobial treatment regarding metritis. The survey was administered by R.V.P or A.G. and targeted dairy managers and animal handlers. On-farm antimicrobial treatment history for each sampled cow for the last fourteen days was collected via either electronic records or after interviewing workers during farm visits. Data collected from interviews were entered into spreadsheets for analysis (Microsoft Office Excel 2010, Microsoft Corp., Redmond, WA).

Bacterial Isolation and Antimicrobial Susceptibility Testing

Within 24 hours after collection, each uterine swab was used to inoculate a single CHROMagar-*E. coli* selective plate (CHROMagar Microbiology, Paris, France) which was then incubated at 37°C for 24 hours. A single, isolated colony was chosen at random and subcultured in 10 mL of brain heart infusion broth (Difco; Becton, Dickinson, and Company, Sparks, MD, USA) at 37°C for 24 hours. The broth culture (500µL) was mixed with 50% sterile glycerol/50% sterile water solution (500 µL) prior to storage at -80°C.

For all isolates, antimicrobial susceptibility testing was conducted in batches after completion of sample collection using a broth microdilution method following the Clinical Laboratory Standards Institute (CLSI) guidelines [1]. The Sensititre Vet Bovine/Porcine plate (BOPO6F, Trek Diagnostic Systems, Oakwood Village, OH, USA) was used for testing susceptibility to the following antimicrobial drugs: penicillins (penicillin and ampicillin), cephalosporins (ceftiofur), fluoroquinolones (danofloxacin and enrofloxacin), phenicols (florphenicol), sulfas (sulphadimethoxine and sulfamethoxazole/trimethoprim), tetracyclines (chlortetracycline and oxytetracycline), macrolides (tylosin tartrate, tulathromycin, and tilmicosin), aminoglycosides (gentamicin and neomycin), lincosamides (clindamycin), pleuromutilins (tiamulin), and aminocyclitols (spectinomycin). Sensititre plates were read manually, and minimum inhibitory concentrations were interpreted using current CLSI breakpoints when available [1] (supplemental table 3-9).

Prior to the veterinary CLSI guidelines, the only option to define the susceptible, intermediate or resistant (SIR) classification of *E. coli* isolates from cows with metritis was to utilize human-based breakpoints, which have been used for currently available studies in literature. However, as defined by CLSI VET09 guidelines for extrapolating breakpoints for veterinary pathogens, the use of human-based breakpoints result in SIR interpretations that have very low confidence, and are

not recommended [189]. Instead, as per Chapter 8 of the CLSI VET09 document, which focuses on **bovine-specific breakpoints,** the recommendation for defining breakpoints when they are not available for specific bacteria or anatomical locations, is to apply a different bacterial species or infection site from a bovine-specific source. Based on these guidelines by CLSI, we utilized this updated approach to maximize the accuracy of SIR classification of isolates in the study (supplemental table 3-9).

Data Management and Statistical Analysis

CLSI MIC breakpoints were available for only nine of the 18 AMDs tested (ampicillin, ceftiofur, chlortetracycline, oxytetracycline, florfenicol, gentamicin, danofloxacin, spectinomycin, and enrofloxacin) (supplemental table 3-9). The nine drugs tested and later used in the analysis had CLSI breakpoints for either *Enterobacterales* or *Pasturella multocida* in cattle and horses, as recommended by CLSI. Isolates that grew in all dilutions of an antimicrobial assessed were classified as "Growth in all dilutions" (GAD) because their MIC was higher than the highest dilution tested in our study (supplemental figure 3-1). By using GAD, we stratified the data between isolates for which the highest concentration in the plate was the actual MIC (e.g., CEFT for isolates where the MIC = 8) from those that grew at the highest concentration available on the MIC plate (e.g., CEFT for isolates where the MIC >8), for which the actual MIC value is unknown. Antimicrobial drug resistance profiles for intrauterine *E. coli* isolates are presented in supplemental table 3-2.

Risk factors for *E. coli* isolation

A logistic regression model in SAS (SAS Institute Inc., Cary, NC; version 7.15) using PROC GLIMMIX logit function was used to evaluate animal-level risk factors collected at the time of

sampling on the odds of isolating *E. coli* from an intrauterine swab sample. The dependent variable was the binomial variable for culture-positive or negative for *E. coli*, and the independent variables were clinical presentation group (MET, PUS, or CTL), lactation number (1, 2, and 3 or greater), days in milk (DIM), and fever (categorical variable, with 39.5ºC as a fever benchmark) at time of sampling[56]. All interactions were considered in the model. Univariate analysis for each explanatory variable was conducted; all variables with a $p < 0.3$ were selected to be offered to the model using a backward stepwise elimination process. Farm was controlled as a random effect in the model. The quadratic association between DIM and outcomes of interest were evaluated and retained in the model if significant. Pairwise comparisons between the clinical presentation groups were conducted, adjusting for multiple comparisons using the Tukey-Kramer approach. The Akaike information criterion (AIC) was used for model selection and to ensure a more parsimonious model was selected. Clinical presentation group was forced into all models regardless of the *p*-value. A variable was considered a confounder if the coefficient of a significant variable in the model changed ≥ 20% after removal from the model. All models included farm as a random effect.

Evaluation of antimicrobial treatment on AMR prevalence

Univariate analysis was conducted to evaluate the effect of individual animal antimicrobial treatment on AMR in *E. coli* (n=162), independent of clinical presentation group, as well as by stratifying the analysis by treatment group. A binary variable was created for being treated in the preceding 14 days prior to sampling with any antimicrobial drug. Because most animals that had an *E. coli* isolate and received any antimicrobial treatment were treated with ceftiofur or tetracycline (17/18), antimicrobial specific binary variable were created, where animals were either treated with that specific antimicrobial or did not receive that specific antimicrobial (supplemental

table 3-10). Fisher's Exact Test analysis was used to evaluate the effect of individual animal treatment with any antimicrobials (supplemental table 3-4), only ceftiofur (supplemental table 3-5, supplemental table 3-6), or only tetracyclines (supplemental table 3-7, supplemental table 3-8) on AMR in *E. coli* for all antimicrobials tested.

Risk factors for *E. coli* antimicrobial resistance

A logistic regression model using the Logit function in PROC GLIMMIX in SAS (SAS Institute Inc., Cary, NC) was used to evaluate the association between intrauterine *E. coli* AMR and animal-level variables. Univariate analysis between each explanatory variable and the categorical binomial variables for ampicillin, ceftiofur, chlortetracycline, oxytetracycline, florfenicol, gentamicin, danofloxacin, spectinomycin, and enrofloxacin as resistant or susceptible was used to identify tests with a $p < 0.3$; these were selected to be offered to the model using a backward stepwise elimination process. A model was generated for each of the nine AMDs with available breakpoints using a categorical binomial variable to classify an isolate as resistant or susceptible. Independent individual animal-level variables offered to the model were clinical presentation group (retained in all models), lactation number, rectal temperature, and days in milk at the time of sample collection, and antimicrobial treatment in the last 14 days with either ceftiofur or tetracycline drug. Farm was controlled as a random effect in the model. The Akaike information criterion (AIC) was used for model selection and to ensure a more parsimonious model was selected. Clinical presentation group was forced into all models regardless of the *P*-value. Confounding effects were evaluated by examining the effect of the removing variables on the coefficients of the remaining variables. A variable was considered a confounder if the coefficient of a significant variable in the model changed ≥ 20% after removal from the model.

Mixed-effect multinomial logistic regressions were used for the analysis of binomial data for AMR categorization of an isolate for each AMDs with MIC breakpoints using the logit link function in PROC GLIMMIX; in this model the response variable was a proportion using the events/trials syntax, where the events were the number of intrauterine *E. coli* isolates at a farm with AMR to the antimicrobial drug being evaluated (events) out of the total intrauterine *E. coli* isolated from that farm (trials) [191]. More specifically, the dependent variable was the number of isolates with AMR to the antimicrobial drug being evaluated at a farm (events) out of the total intrauterine *E. coli* isolated from that farm (trials). Using this approach, the models assessed the least square means for the prevalence of AMR at the farm level. The explanatory variables offered to the model were farm-level practices, including antimicrobial drugs commonly used as first choice for treatment of metritis on the farm. Using this approach, the models assessed the association between AMR proportion at the farm level and surveyed farm practices.

A model was generated for each of the nine AMDs with MIC breakpoints to evaluate the association of farm-level prevalence of *E. coli* AMR and farm-level management practices as explanatory variables. Individual models were created for ampicillin, ceftiofur, chlortetracycline, oxytetracycline, florfenicol, gentamicin, danofloxacin, spectinomycin, and enrofloxacin. Models were built and evaluated as previously described.

Heat maps for isolate susceptibility to antimicrobials

Heat maps representing each individual isolate and its susceptibility to 12 antimicrobials by clinical presentation group and farm were created using RStudio (Version 1.4.1106) (R Foundation for Statistical Computing, Vienna, Austria) using the heatmap.2 function. Of the eighteen total drugs tested, five drugs were not included (tiamulin, sulfadimethoxine, trimethoprim-sulfamethoxazole, tylosin, and clindamycin) because these drugs had either fewer than two

antimicrobial concentrations tested, or more than 98% of isolates had the same MIC value. The percentile scale for susceptibility to antimicrobials was generated after categorizing MIC dilution ranges available for each antimicrobial in ascending order, representing the percent decrease in susceptibility for the evaluated range. As an example, for oxytetracycline, five antimicrobial dilution concentrations were available in the MIC plate (0.5, 1, 2, 4, and 8 μg/mL), generating a percentile decrease in susceptibility with increments of 25%, assigned a percent category of decreased susceptibility 0%, 25%, 50%, 75%, and 100%.

3. RESULTS

Descriptive Data

The number of *E. coli* samples recovered and information on animal samples by farm is presented in table 3-1. A total of 307 cows were sampled from the 25 enrolled farms. All enrolled farms had at least one cow assigned to each of the three clinical classifications of vaginal discharge, except for two farms where no MET cows were identified during our visit. DIM at time of diagnosis for cows with culture positive results for *E. coli* for MET, PUS, and CTL were 8.1 (95% CI 7.1- 9.1), 10.6 (95% CI 9.1- 12.0), and 10.6 (95% CI 8.9- 12.3), respectively.

Farm	CTL[1] %, (A/B) [2]	MET[1] %, (A/B) [2]	PUS[1] %, (A/B) [2]	TOTAL %, (A/C)[3]
1	20 (1/5)	0 (0/2)	0 (0/4)	9 (1/11)
2	75 (3/4)	75 (3/4)	0 (0/3)	55 (6/11)
3	50 (2/4)	100 (5/5)	67 (2/3)	75 (9/12)
4	0 (0/3)	0 (0/0)	33 (2/6)	22 (2/9)
5	60 (3/5)	50 (1/2)	20 (1/5)	42 (5/12)
6	40 (2/5)	0 (0/0)	100 (1/1)	50 (3/6)
7	75 (3/4)	67 (2/3)	60 (3/5)	67 (8/12)
8	20 (1/5)	50 (1/2)	0 (0/3)	20 (2/10)
9	50 (2/4)	33 (1/3)	50 (2/4)	45 (5/11)
10	60 (3/5)	50 (1/2)	40 (2/5)	50 (6/12)
11	0 (0/5)	40 (2/5)	40 (2/5)	27 (4/15)
12	20 (1/5)	60 (3/5)	0 (0/5)	27 (4/15)
13	40 (2/5)	60 (3/5)	60 (3/5)	53 (7/15)
14	40 (2/5)	20 (1/5)	40 (2/5)	33 (5/15)
15	20 (1/5)	100 (4/4)	40 (2/5)	50 (7/14)
16	25 (1/4)	75 (3/4)	33 (2/6)	43 (6/14)
17	100 (5/5)	75 (3/4)	100 (4/4)	92 (12/13)
18	100 (2/2)	100 (3/3)	50 (2/4)	78 (7/9)
19	40 (2/5)	50 (1/2)	80 (4/5)	58 (7/12)
20	60 (3/5)	60 (3/5)	40 (2/5)	53 (8/15)
21	80 (4/5)	60 (3/5)	75 (3/4)	71 (10/14)
22	60 (3/5)	100 (5/5)	100 (5/5)	87 (13/15)
23	80 (4/5)	100 (5/5)	60 (3/5)	80 (12/15)
24	20 (1/5)	80 (4/5)	100 (1/1)	55 (6/11)
25	60 (3/5)	100 (1/1)	67 (2/3)	67 (6/9)
TOTAL	47 (54/115)*	67 (58/86)*	47 (50/106)*	53 (162/307)

Table 3-1. Distribution of *E. coli* (n = 162) isolated from intrauterine swabs collected at 25 commercial dairy farms by clinical presentation group (CTL, MET, and PUS). **A**: swabs positive for *E. coli*; **B**: total number of swabs collected from cows in clinical group; **C**: total number of swabs collected at each farm.

1. Clinical presentation of cows when intrauterine samples were collected; 2. Percentage, (Swabs positive for *E. coli* / total number of swabs collected from cows in clinical presentation group); 3. Percentage, (Swabs positive for *E. coli* / total number of swabs collected at each farm). * Percentage, (Swabs positive for *E. coli* / total number of swabs from all farms for cows in clinical group)

Risk factors for *E. coli* isolation

Risk factors for *E. coli* isolation are presented in table 3-2 and supplemental table 3-1. The odds ratio for isolating intrauterine *E. coli* isolate from MET cows when compared to CTL cows was 2.0 (95% CI: 1.1 - 3.7, *P* value= 0.03). No significant difference between cows diagnosed as PUS and CTL, or a MET and PUS was observed for isolation of *E. coli* from intrauterine swabs. Days in milk of the cow sampled were significantly associated with lower odds of isolating intrauterine *E. coli* for each day increase in DIM for MET and PUS cows (table 3-2).

		OR (95% Confidence interval)		
Variable	**Odds Ratio**	**Lower**	**Upper**	***P* value**
Clinical Group[1]				**0.005**
MET vs PUS	1.67	0.87	3.2	0.11
MET vs CTL	2.00	1.07	3.7	**0.03**
PUS vs CTL	1.19	0.68	2.1	0.53
DIM[2]				**0.0008**
Clinical Group[1] * DIM[2]				**0.02**
MET	0.85	0.71	0.98	**0.01**
PUS	0.88	0.80	0.96	**0.004**
CTL	0.99	0.93	1.06	0.92

Table 3-2. Summary of the logistic regression model evaluating the effect of the clinical presentation groups (MET, PUS, or CTL) and the days in milk (DIM) on the odds ratio of isolation of *E. coli* from intrauterine swabs collected from cows at 25 commercial dairy farms.

1. Clinical presentation group (MET, PUS, or CTL) of cows when intrauterine samples were collected. (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; (PUS) purulent discharge defined as a non-fetid purulent or mucopurulent vaginal discharge; and (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia.
2. Days in Milk at sampling time.

E. coli antimicrobial resistance

The distribution of minimum inhibitory concentration (MIC) and resistance for intrauterine *E. coli* (n = 162) by individual drug for the BOPO6F panel are shown in table 3-3. The most common resistance profiles and the resistance profile for each isolate and are presented in supplemental tables 3-2 and 3-3, respectively. No significant association ($P > 0.05$) was observed at the animal level between *E. coli* AMR for the nine drugs with clinical breakpoints and the clinical presentation group.

The percent of all *E. coli* isolates classified as susceptible for the four antimicrobial drugs commonly used to treat cows with metritis in the U.S. [79], are presented in figure 3-3. Of the nine antimicrobials tested with available MIC breakpoints, AMP had the highest prevalence of AMR, with all isolates being classified as resistant (table 3-3). Although all isolates were resistant to ampicillin, nearly 60% of isolates (n = 97) were resistant to AMP alone (supplemental table 3-10). A total of 8.6% of isolates (n = 12) included ceftiofur resistance within their total AMR resistance profile (supplemental table 3-2).

The second most common resistance profile was ampicillin-chlortetracycline-oxytetracycline (18.5% of isolates). A total of 3.1% of isolates (n = 5) displayed resistance to ampicillin-ceftiofur-chlortetracycline-florfenicol-oxytetracycline and 2.5% of isolates (n = 4) displayed resistance to ampicillin-ceftiofur-chlortetracycline-oxytetracycline (supplemental table 3-2).

Heat map of MICs for antimicrobials for the 162 *E. coli* isolates grouped by clinical presentation group (CTL, MET, PUS) and farm, are shown in figures 3-1 and 3-2, respectively. When visually comparing CTL, MET, and PUS groups in figure 3-1, no clinical presentation group had a noticeable visual trend for percentile distribution toward higher MIC quantiles. When comparing farms in figure 3-2, there was a visual clustering of isolates in a higher MIC quantile

for specific farms, particularly for chlortetracycline and oxytetracycline for farms 18 and 21, which were the only farms that used tetracycline drugs as their primary treatment for metritis in dairy cows (table 3-4).

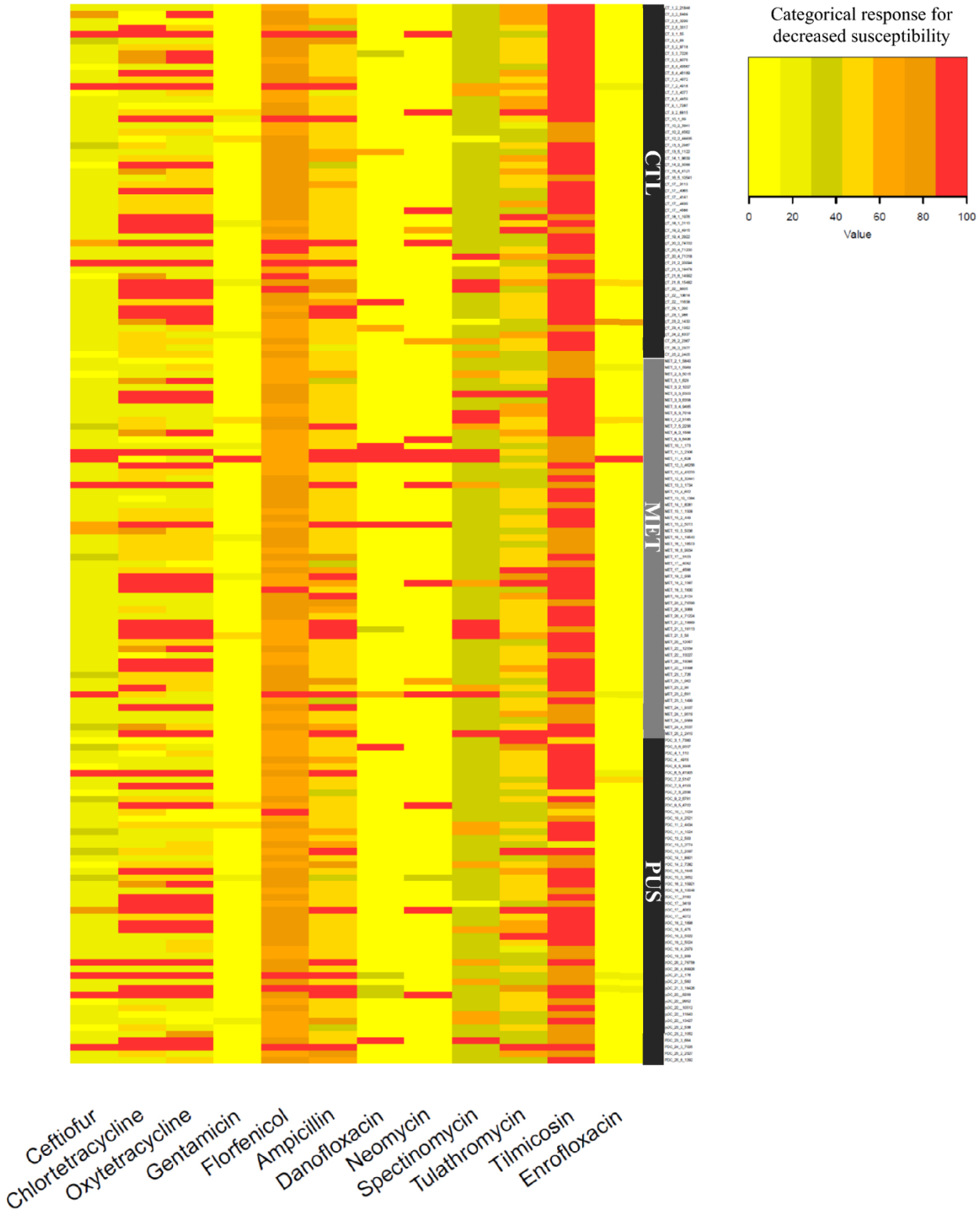

Figure 3-1. Heat map of minimum inhibitory concentrations for 12 antimicrobials compared against 162 *E. coli* isolates grouped by clinical presentation group (CTL, MET, PUS). Each row represents an isolate that was categorized by percent decrease in the susceptibility range.

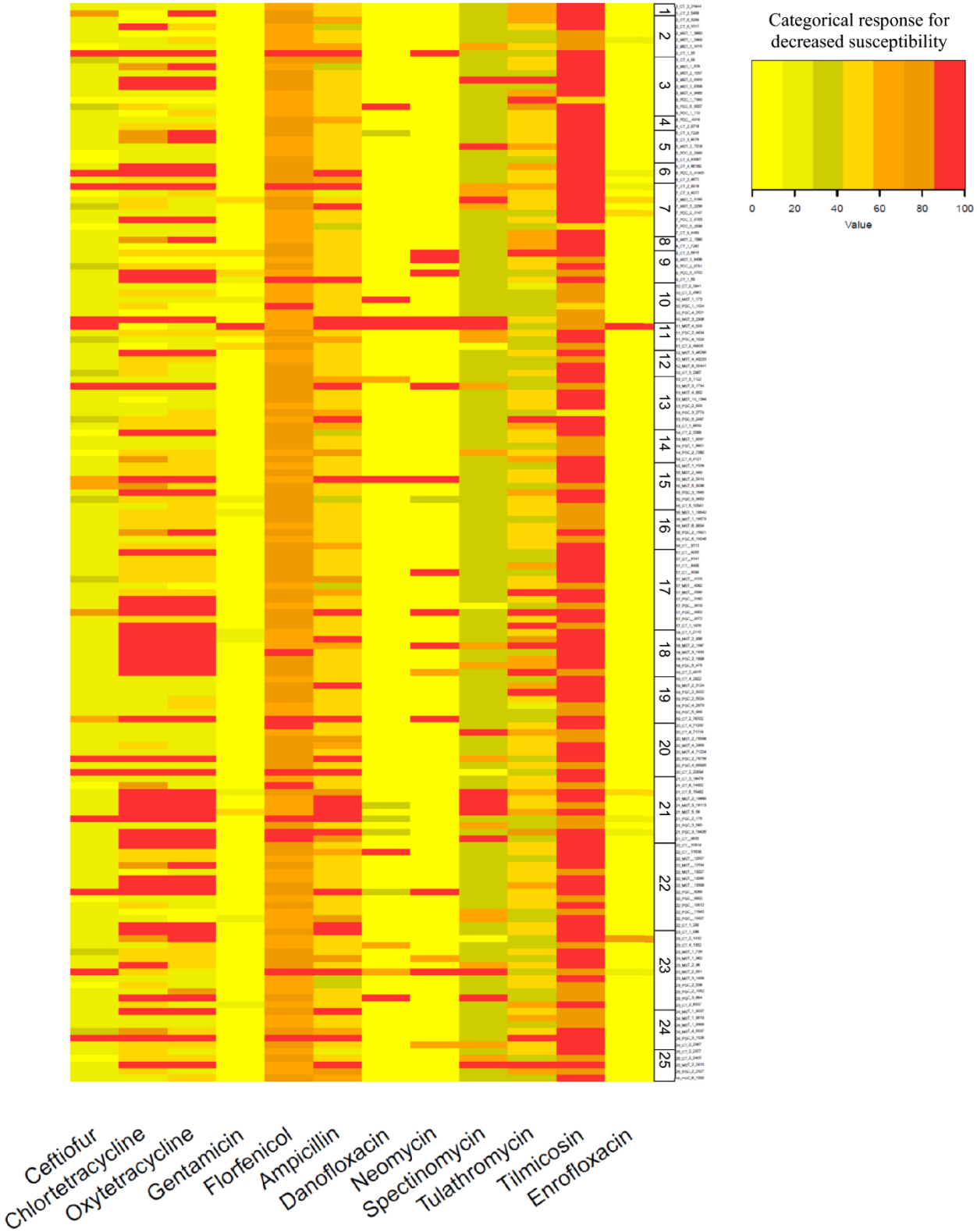

Figure 3-2. Heat map of MICs for 12 antimicrobials compared against 162 *E. coli* isolates grouped by the farm (n = 25). Each row represents an isolate that was categorized by percent decrease in susceptibility range. Boxed numbers indicate which of the 25 farms samples correspond

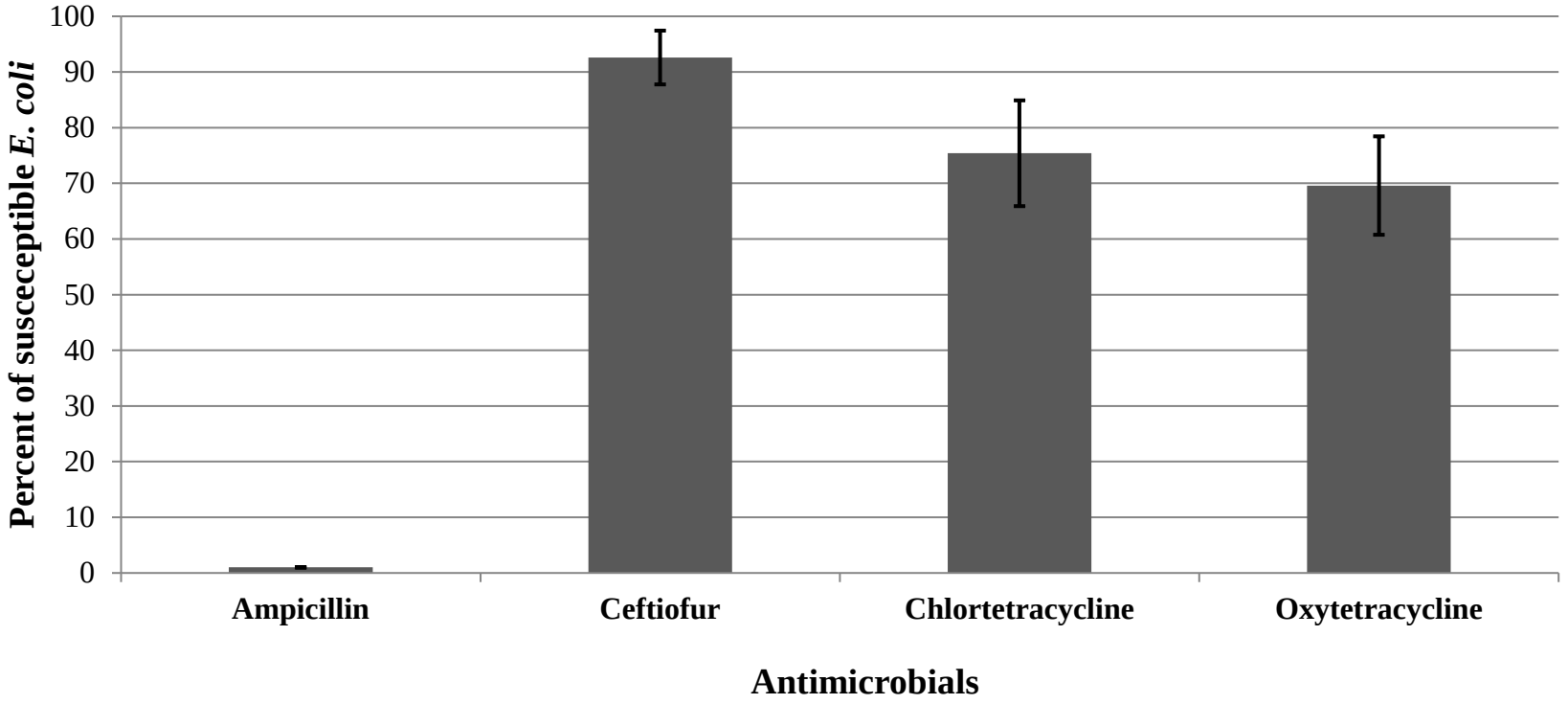

Figure 3-3. Percent of *E. coli* isolates susceptible to commonly used antimicrobial treatments for metritis. Current Clinical Laboratory Standard Institute veterinary breakpoints were used to define susceptibility [1]. A total of 162 *E. coli* isolates were obtained from the uterus of post-partum cows housed in 25 California dairies.

		% Distribution of MICs (µg/ml)[1]										
Antimicrobial	**%NS[2]**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**GAD[3]**
Ampicillin	**100%**			1.2	4.9	61.7	9.3	5.6	0.6			16.7
Ceftiofur	**8.6%**		7.4	75.3	6.8	1.9	1.2	0.6				6.8
Chlortetracycline	**37.1%**			8	29	26	6.8	1.9				28.4
Danofloxacin	**6.2%**	90.7	3.1	1.9	3.1							1.2
Enrofloxacin	**3.1%**	93.2	3.7	1.9	0.6							0.6
Florfenicol	**58.1%**					42	49.4	1.9				6.8
Gentamicin	**4.3%**				90.1	5.6	3.7					0.6
Neomycin[4]	-						88.9	0.6	1.9	1.9		6.8
Oxytetracycline	**34.5%**			6.2	32.7	26.5	0.6	0.6				33.3
Penicillin[4]	-							2.5				97.5
Spectinomycin	**8.6%**							2.5	78.4	10.5	4.3	4.3
Tilmicosin[4]	-							0.6	1.9	30.2	54.3	13
Tulathromycin[4]	-					1.2	30.9	43.8	14.8	1.9	3.1	4.3

Table 3-3. Distribution of minimum inhibitory concentration (MIC) and resistance for intrauterine *E. coli* (n = 162) by individual drug for the BOPO6F panel. Highlighted areas in blue corresponds to susceptible, green corresponds to intermediate, and orange corresponds to resistant classification. For antimicrobials without a MIC breakpoint, the dilution scale tested is highlighted in gray. For the lowest MIC value in the dilution range, results indicate lowest MIC detected, but should be interpreted as less than or equal to (≤) the lowest MIC detected.

1. Distribution of minimum inhibitory concentration (**MIC**); 2. Percent of isolates classified as non-susceptible (Intermediate and Resistant) to the referred antimicrobial drug (**%NS**); 3. Percent of bacterial growth in all antimicrobial dilutions tested (**GAD**), Read as MIC > highest drug concentration available; 4. *Enterobacterales* are highly susceptible to these drugs or no CLSI breakpoint available.

		Criteria used for metritis diagnosis (Q2)						Metritis Treatment		
Farm ID	Range for Number Cows in herd	Foul-Smell	Watery discharge	Pus discharge	Fever	Depressed attitude	Drop in milk	Antimicrobials for all cases	Preferred antimicrobial*	Anti-inflammatory drug use
1	1000 to 3000	■	■		■	■	■	■	Ceft	■
2	1000 to 3000	■	■		■	■		■	Ceft	
3	1000 to 3000	■	■		■	■	■	■	Ceft	■
4	1000 to 3000				■	■	■	■	Ceft	
5	1000 to 3000	■	■	■	■	■	■	■	Ceft	■
6	1000 to 3000	■		■	■	■	■	■	Ceft	■
7	1000 to 3000	■	■		■	■	■	■	Ceft	■
8	1000 to 3000	■			■			■	Ceft	■
9	500 to 999	■	■	■	■	■		■	Ceft	■
10	1000 to 3000	■		■	■	■		■	Ceft	■
11	3000 to 5000	■	■		■	■	■	■	Ceft	■
12	3000 to 5000	■		■				■	Ceft	■
13	3000 to 5000	■	■		■			■	Ceft	■
14	1000 to 3000	■	■						Amp	
15	500 to 999	■	■	■		■	■		Ceft	
16	3000 to 5000	■		■	■	■	■		Ceft	■
17	500 to 999	■		■	■	■	■		Ceft	■
18	500 to 999	■		■				■	Tet	■
19	1000 to 3000	■		■	■	■	■	■	Amp	■
20	>5000	■			■	■		■	Ceft	
21	1000 to 3000	■			■				Tet	■
22	1000 to 3000		■					■	Ceft	
23	1000 to 3000	■			■	■		■	Amp	
24	1000 to 3000	■		■	■	■	■	■	Amp	■
25	200 to 499	■			■	■	■		Ceft	

Table 3-4. Farm level factors related to the number of dairy cows and metritis diagnosis and treatment criteria. Grey filled cells indicate a yes to the questions. *Ceft = ceftiofur; Amp = ampicillin; Tet = tetracycline

Effect of antimicrobial treatment on *E. coli* AMR

A total of 11% (18/162) of animals sampled for which *E. coli* was isolated received antimicrobial treatment within the fourteen days prior to sampling. For cows diagnosed with metritis, 26% (12/46) had a prior treatment with antimicrobials, and 11% (5/45) and 2% (1/53) for PUS and CTL cows, respectively. Based on survey data collected for farms, we would expect a higher number of cows with metritis being treated; however, our research team selected cows for enrollment independent of prior diagnosis of metritis, resulting in a metritis diagnosis occurring prior to that of the farm. This is not surprising, given that most farms used systemic signs of disease to diagnose metritis (e.g., depressed attitude, drop in milk) (table 3-4). Indicating a more severe case; the approach used by our study was based on vaginal discharge, which allows an earlier diagnosis of metritis, and therefore explaining the reason for metritis cases identified in not having received a prior antimicrobial treatment by the farm.

Results for Fisher's Exact Test analysis evaluating the effect of individual animal treatment with antimicrobials is shown in supplemental table 3-4, and was not associated with a significant increase of AMR in *E. coli*. When this analysis was stratified by clinical presentation, again, no significant effect of individual animal treatment on AMR within *E. coli* was observed. Results for Fisher's Exact Test analysis evaluating the effect of individual animal treatment with ceftiofur alone is shown in supplemental table 3-5; Individual animal treatment with only ceftiofur 14 days prior to sampling did not result in any significantly increased odds ratios for antimicrobial resistance to the nine antimicrobials analyzed. When this analysis was conducted stratifying by clinical presentation group (CTL, MET, and PUS), there were also no significantly increased odds for antimicrobial resistance to the seven antimicrobials analyzed for (supplemental table 3-6).

Results for the Fisher's Exact Test evaluating the effect of individual animal treatment with tetracycline alone is shown in supplemental table 3-7; individual animal treatment with tetracycline 14 days prior to sampling did not result in any significantly increased odds ratios for antimicrobial resistance to the nine antimicrobials analyzed. When this analysis was conducted stratifying by clinical presentation group (CTL, MET, and PUS), there were also no significantly increased odds for antimicrobial resistance to the seven antimicrobials analyzed for (supplemental table 3-8).

The mixed-effect multinomial logistic regression model used to evaluate the association between intrauterine *E. coli* AMR and animal-level variables, did not identify treatment group or any other animal-level variable evaluated, including prior antimicrobial treatment, as being significantly associated with AMR in *E. coli*.

Farm-level antimicrobial treatment and management practices

Descriptive data by farm related to the number of dairy cows and metritis diagnosis and treatment criteria are presented in table 3-4. Farms in which cows were treated for metritis using intrauterine treatment with oxytetracycline had a significantly higher farm-level prevalence of intrauterine *E. coli* with AMR to oxytetracycline (LSM ± SEM: 0.82 ± 0.09) and chlortetracycline (LSM ± SEM: 0.70 ± 0.14) when compared to farms in which cows were not treated for metritis using intrauterine treatment with oxytetracycline (LSM ± SEM: 0.03 ± 0.03 and LSM ± SEM: 0.25 ± 0.04, respectively) (table 3-5).

Farm-Level Risk Factors	Chlortetracycline				Oxytetracycline			
	Estimate[1]	**LSM[2]**	***SEM*[3]**	***P* value[4]**	**Estimate[1]**	**LSM[2]**	***SEM*[3]**	***P* value[4]**
Intrauterine treatment with oxytetracycline[4]				0.0005				0.019
No	Ref	0.82	0.09		Ref	0.70	0.14	
Yes	2.68	0.03	0.03		1.94	0.25	0.04	

Table 3-5. Association of farm-level management of using oxytetracycline as an intrauterine infusion as the most common drug for the treatment of metritis and farm-level prevalence of AMR to tetracycline drugs in intrauterine *E. coli*.

1. Parameter estimate for the multivariate model evaluating resistance to the referred drug

2. Least-square means (LSM) of farm-level prevalence of intrauterine *E. coli* resistant to the referred drug

3. Standard error of the means (SEM) for the LSM

4. *P* value from analysis comparing farm-level prevalence of AMR between farms for the referred drug, adjusted using Bonferroni.

4. DISCUSSION

Recovery of *E. coli* from intrauterine swab samples

E. coli was recovered from 53% of intrauterine swab samples collected from all post-partum dairy cows in our study, which is within the range previously observed by other researchers. Bicalho et al. (2010), recovered 125 (33.4%) intrauterine *E. coli* from 374 total lactating Holstein cows sampled in upstate New York [a subset of 117 cows displayed clinical signs of metritis] [72]. In another study, De Boer et al. (2015), recovered 209 (76.8%) intrauterine *E. coli* from 272 pasture-raised cows in New Zealand [85]; and Kasse et al. (2016), recovered 156 (42%) intrauterine *E. coli* from 371 Holstein dairy cows in Canada [192]. Furthermore, recovery of *E. coli* was higher in cows with MET when compared to PUS and CTL.

MET cows had significantly higher odds ratio for isolation of intrauterine *E. coli* when compared to CTL cows (table 3-2), and is in agreement with previous studies [192, 193]. A study by Pohl et al. (2018) that isolated intrauterine *E. coli* from cows using two clinical signs to define metritis (reddish-brown fetid discharge and rectal temperature > 39.5℃) had a 90% recovery of intrauterine *E. coli*, and 70% recovery when using solely one clinical sign (reddish-brown fetid discharge or rectal temperature > 39.5℃). In contrast, cows not displaying clinical signs of metritis had an *E. coli* recovery of 54% [86]. The Pohl et al. (2018) study also observed that cows with two clinical signs of metritis had 7.16 times the odds of having intrauterine *E. coli* compared to cows without metritis, in agreement with our findings. The difference in the magnitude of *E. coli* recovery in cows with metritis between Pohl et al. (2018) and our study may be explained by differences in herd management practices and geographical factors from German dairy farms that resulted in a different magnitude of recovery of *E. coli* in cows with metritis.

A higher days in milk (DIM) at the time of MET or PUS diagnosis was found to be significantly associated with a lower odds of isolation of intrauterine *E. coli* (table 3-2). The relationship between DIM and odds to isolate *E. coli* agrees with previous findings [62], where the progression of the uterine microbiota from calving was evaluated, with an observed rapid decrease in the relative abundance of Proteobacteria, a major phylum of Gram-negative bacteria that includes *Escherichia coli*, from 0 to 6±2 DIM. Jeon et al. (2015) also observed a subsequent increase in the relative abundance of bacteria in the phylum Bacteroidetes from 0 to 6±2 DIM. While the dynamics of the uterine microbiome are complicated, particularly at the time of parturition, increases in relative abundance of other microbes likely drive the decrease in abundance of Proteobacteria; therefore decreasing the odds of isolating intrauterine *E. coli* [71]. A definitive explanation behind this phenomenon remains elusive and continues to be a topic of research.

E. coli AMR

When comparing CTL, PUS, and MET cows, no significant difference in the odds ratio for isolating AMR *E. coli* isolates to the AMDs tested was observed. This is in discordance with our hypothesis, that had an assumption that cows with MET may have been colonized by *E. coli* carrying both virulence and antimicrobial resistance genes. Previous studies have shown that specific virulence genes are associated with intrauterine *E coli* isolated from cows with metritis [72]. Furthermore, a study from cows from a single farm, using whole genome sequencing to characterize intrauterine *E. coli*, observed a correlation between intrauterine pathogenic *E. coli* (characterized based on presence of virulence genes) and extended spectrum β-lactamase (ESBL) genes, which confer resistance to expanded-spectrum cephalosporins [194]. Discordance between our results using phenotype methods for AMR diagnosis and those using genomic approaches could be due to disagreements that have been reported between these two methods.

Resistance to ceftiofur, the most common systemic antimicrobial treatment for metritis in California, was low in our study with 8.6% of isolates (n = 12) phenotypically resistant [79]. Similar studies conducted in New York State, New Zealand, and Germany also observed low AMR (as specified by CLSI breakpoints available at the time of publication) to CEFT within uterine *E. coli*; with 1.2%, 0%, and 5.9% of isolates resistant, respectively[84-86].

Extra-label use of ampicillin has previously been reported as the second most common treatment option for metritis in California dairy cows [79]. In the literature, intrauterine susceptibility of *E. coli* to AMP has varied, possibly due in part to the use of breakpoint values that have been periodically updated [85]. As an example, a study by [84] observed that approximately 34% of early post-partum cows harbored ampicillin-resistant *E. coli*. Due to the lack of specific MIC breakpoints for *Enterobacterales* from cattle for AMP at the time, this study used a resistance breakpoint of ≥ 16 µg/mL based on a CLSI breakpoint used for human isolates [195]. The use of human-based CLSI breakpoints has been a common standard in veterinary studies evaluating MIC, with specific breakpoints against AMP for *E. coli* from cattle only being available starting in 2018 with the release of the 4th edition of "Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals" allowing for SIR classification of *Enterobacterales* for ampicillin [196]. As an example, had we used the human clinical breakpoint for ampicillin for *Enterobacterales*, as per the CLSI M100 31st edition for which the breakpoint for resistance is MIC ≥32 µg/mL, only 16.7% of our isolates would have been classified as resistant. This large discrepancy is a reflection of how MIC breakpoints are defined for *Enterobacterales* in humans and animals. Traditionally, MIC breakpoints are set using a range of data, including *in vitro* microbiological data, animal and human pharmacokinetic/pharmacodynamic (PK/PD) data, and clinical and bacteriological outcome data

from prospective clinical studies [197]. Furthermore, the accuracy of the data used to determine MIC breakpoints for specific animal species and tissues will directly affect the validity of the results related to SIR classification.

Oxytetracycline is approved in the US for the systemic treatment of metritis caused by species of staphylococci and streptococci. A study by de Boer [85] reported that of 209 intrauterine *E. coli,* 83.2% of were susceptible and 4.8% were resistant to oxytetracycline. In Germany, a study reported that of 85 intrauterine *E. coli* isolates, 81.1% were susceptible and 9.5% were resistant to tetracycline [86]. In our study, 30.3% of *E. coli* tested against chlortetracycline and 33.9% of *E. coli* tested against oxytetracycline were classified as resistant. The observed higher prevalence of resistance to tetracyclines in our study when compared to previous studies may reflect specific practices for managing metritis in California. In figure 3-2, the visual clustering of isolates in a higher MIC quantile collected from the two farms that indicated using tetracycline drugs as their first choice for treatment of metritis in dairy cows suggests that treatment of cows with metritis using tetracyclines may increase the selection of AMR to drugs in that class. Future studies should be designed to allow for the determination of causation.

Our study revealed that using oxytetracycline intrauterine infusion as a treatment tended to increase the odds of recovering intrauterine *E. coli* displaying resistance to either chlortetracycline or oxytetracycline. In California, approximately 27% of farms used intrauterine infusion with oxytetracycline for treatment of metritis [79]. However, researchers have recommended against intrauterine infusions due to lack of evidence to support any added benefit in reproduction or cure [198, 199]. Given the extra-label nature of the use of intrauterine oxytetracycline in cattle, this practice can only occur though the prescription of a veterinarian, and appropriate milk withhold periods should be followed [200].

The fluoroquinolone enrofloxacin is approved for use in treating respiratory disease in non-lactating dairy cattle under 20 months of age, however, its extra-label use to treat bovine metritis is illegal [201]. Due to drug dilutions present on the plate, we could only classify isolates as susceptible and intermediate. As such, we can only report that enrofloxacin susceptibility was high with 96.9% of isolates classified as susceptible (figure 3-3). None of the farms in our study reported using enrofloxacin as a treatment option for metritis. Our findings suggest very low resistance of intrauterine *E. coli* to enrofloxacin, supporting the expected lack of use of this drug in lactating dairy cattle. Fluoroquinolone resistance within our isolates could potentially be originating from horizontal gene transfer or from co-selection due in part to the use of approved AMDs to treat metritis; however, further study to investigate this would be necessary [202, 203].

While resistance to each of the antimicrobials discussed above is concerning, multidrug resistance (MDR) is an especially pressing issue if an isolate is resistant to multiple common treatment options. The most common resistance profile was to AMP alone (59.9% of isolates), while resistance to ampicillin-chlortetracycline-oxytetracycline (18.5% of isolates) was the 2nd most common profile. Our study showed the presence of resistance to the common drugs used to treat metritis in California with 2.5% of isolates (n = 4) resistant to ampicillin-ceftiofur-chlortetracycline-oxytetracycline. The relatively low prevalence of MDR within intrauterine *E. coli* isolates contrasts with Santos et al. (2010) in which a total of 35% (n = 80) of *E. coli* isolates from cows with metritis were MDR, with the major MDR profile being ampicillin-chloramphenicol-florfenicol resistance, observed in 96.4% of MDR isolates [84]. However, our results do more closely resemble those of Abdelfattah et al. (2021) in which a total of 14.14% (n = 307) of 2,171 *E. coli* isolates, recovered from fecal samples from healthy, adult dairy cows from

10 farms in California, were MDR [204]. Their major MDR profile being florfenicol-sulphadimethoxine (16.2%), and tetracycline-florfenicol-sulphadimethoxine (6.82%).

For farm level analyses, an important consideration when extrapolating results, is that the results represent findings for a specific population within the herd. Specifically, fresh cows (within the first 21 days in milk) that were sampled and also had an *E. coli* culture-positive result (cows without a culture positive result were not included in this analysis). Within these constraints, our results aimed to have internal validity for this specific population.

5. CONCLUSION

An increase in DIM at the time of sampling was significantly associated with a decreased odds for *E. coli* recovery, while classification within the MET clinical presentation group at the time of sampling was significantly associated with increased odds of recovery of *E. coli* from intrauterine swabs. A low prevalence of AMR to CEFT, the most common metritis treatment in our study, within *E. coli* was observed. The extra-label use of intrauterine infusion with oxytetracycline on two farms was observed as a significant factor for increased farm-level prevalence of intrauterine *E. coli* with AMR to oxytetracycline and chlortetracycline, highlighting the potential impacts of this practice on antimicrobial stewardship. Our findings support the need for further research to support a better understanding of farm practices driving AMR in cows with metritis, as well as data to support breakpoints that will result in more accurate AMR identification within intrauterine *E. coli* from cattle.

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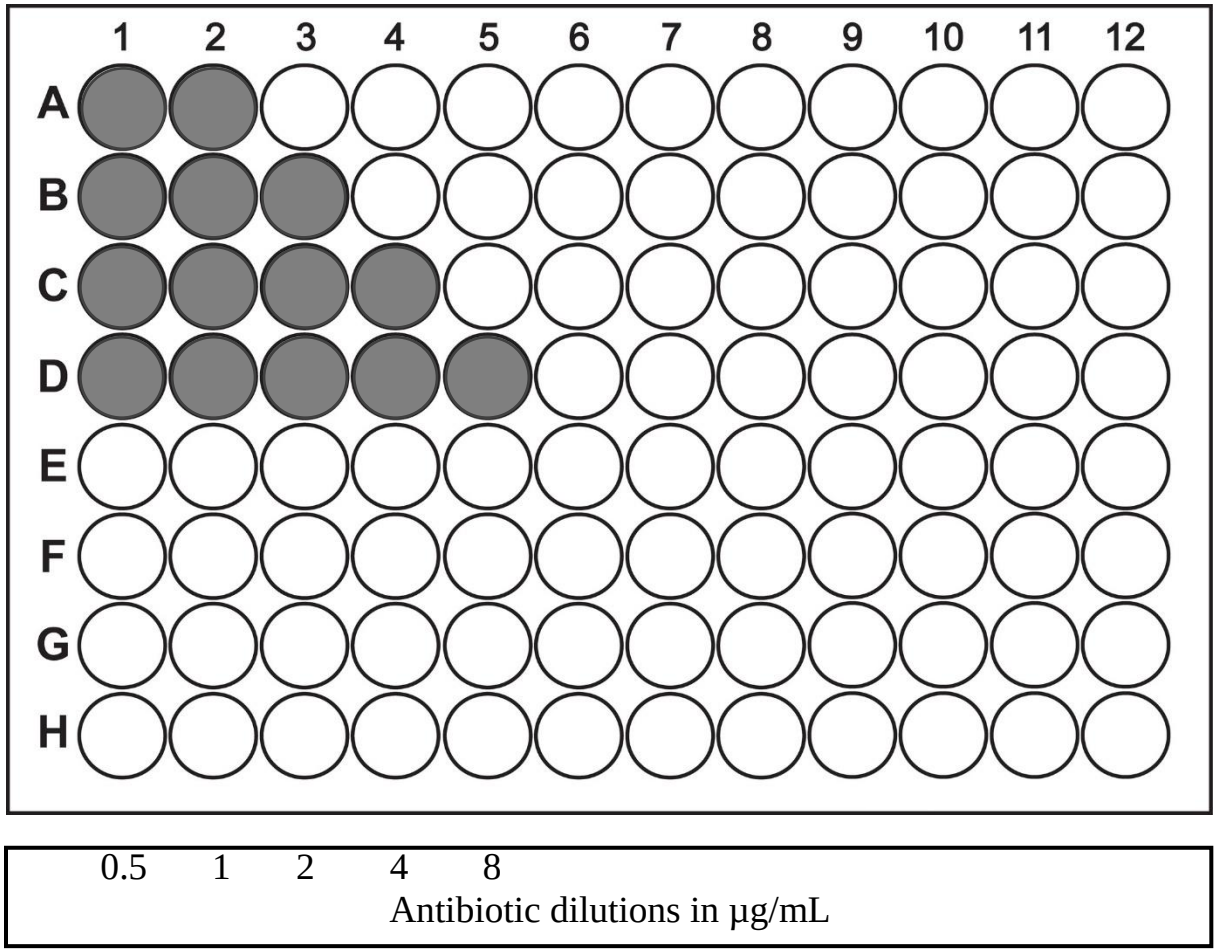
CHAPTER 3 SUPPLEMENTAL

Supplemental Figure 3-1. Visual examples of oxytetracycline susceptibility determination based on growth patterns in minimum inhibitory concentration (MIC) microtiter plates.



A. MIC = 2 µg/mL; Classified as susceptible
B. MIC = 4 µg/mL; Classified as intermediate or non-susceptible
C. MIC = 8 µg/mL; Classified as resistant
D. MIC > 8 µg/mL; Classified as "Growth in all dilutions" or GAD

Supplemental Table 3-1. Outcome for univariate analysis evaluating individual animal factors associated with the odds of isolating intrauterine *E. coli* from cows on commercial dairy farms in California.

Variable	χ^2	*P* value[1]
Clinical Group[2]	10.32	**0.005**
Farm[3]	55.98	**0.002**
DIM[4]	12.8	**0.003**
Rectal Temp[5]	1.72	0.18
Lactation[6]	2.62	0.27

1. *P*-value for univariate analysis evaluating the association between the referred variable and the odds of isolating intrauterine *E. coli*.
2. Clinical presentation group of cows when intrauterine samples were collected. (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; (PUS) purulent discharge defined as cows with a non-fetid purulent or mucopurulent vaginal discharge; and (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia.
3. Farm where cows were sampled (n = 25)
4. Days in milk
5. Rectal temperature of cows when intrauterine samples were collected.
6. Categorical variable with three levels: first, second, third, or greater lactations

Supplemental Table 3-2. Most frequently observed resistance profiles for intrauterine *E. coli* isolates

Drug Resistance Profile[1]	Count (n = 162)	Percent of Isolates[2]
Amp	97	59.9
AmpChtetOxtet	30	18.5
AmpOxtet	8	4.9
AmpChtetFlorOxtet	5	3.1
AmpXnlChtetFlorOxtet	5	3.1
AmpXnlChtetOxtet	4	2.5
AmpDano	3	1.9
AmpFlor	3	1.9
AmpChtet	2	1.2
AmpChtetOxtetDano	2	1.2
AmpXnlChtetOxtetDano	1	0.6
AmpXnlFlor	1	0.6
AmpXnlGenEnroDano	1	0.6
AmpXnlGenEnroDano	1	0.6

1. Drugs for which isolate displayed phenotypic resistance. Amp: ampicillin, Xnl: ceftiofur, Chtet: chlortetracycline, Flor: florfenicol, Genta: gentamicin, Oxytet: oxytetracycline, and Enro: enrofloxacin.

2. Proportion of isolates displaying resistance profile out of 162 *E. coli* isolates tested for antimicrobial resistance

Supplemental Table 3-3. Antimicrobial resistance profiles for intrauterine *E. coli* isolates selected for MIC determination

Isolate Number	Clinical Group[1]	Resistance Profile[2]
1	CTL	Amp
2	MET	Amp
3	MET	Amp
4	MET	Amp
5	CTL	Amp
6	CTL	AmpChtet
7	PUS	AmpDano
8	CTL	AmpXnlChtetFlorOxtet
9	MET	Amp
10	CTL	Amp
11	MET	AmpChtetOxtet
12	MET	AmpOxtet
13	MET	AmpChtetOxtet
14	MET	Amp
15	PUS	Amp
16	PUS	Amp
17	PUS	Amp
18	PUS	Amp
19	CTL	Amp
20	CTL	AmpOxtet
21	MET	Amp
22	CTL	AmpChtetOxtet
23	CTL	Amp
24	PUS	AmpXnlChtetOxtet
25	PUS	AmpChtetOxtet
26	MET	Amp
27	PUS	Amp
28	PUS	Amp
29	CTL	AmpXnlChtetFlorOxtet
30	CTL	Amp
31	CTL	Amp
32	MET	Amp
33	CTL	Amp
34	MET	AmpOxtet
35	PUS	Amp
36	CTL	Amp

37	MET	Amp
38	PUS	AmpChtetOxtet
39	CTL	Amp
40	CTL	AmpChtetFlorOxtet
41	CTL	Amp
42	PUS	AmpFlor
43	CTL	Amp
44	PUS	Amp
45	MET	AmpDano
46	PUS	Amp
47	MET	AmpXnlGenEnroDano
48	MET	AmpXnlChtetOxtetDano
49	PUS	Amp
50	MET	AmpChtetOxtet
51	MET	Amp
52	CTL	Amp
53	MET	Amp
54	PUS	Amp
55	PUS	Amp
56	PUS	Amp
57	CTL	Amp
58	CTL	Amp
59	MET	Amp
60	MET	Amp
61	MET	AmpXnlChtetOxtet
62	PUS	Amp
63	CTL	AmpChtetOxtet
64	MET	Amp
65	PUS	Amp
66	CTL	Amp
67	MET	Amp
68	MET	Amp
69	MET	Amp
70	PUS	Amp
71	CTL	Amp
72	PUS	AmpChtetOxtet
73	MET	AmpChtetOxtetDano
74	MET	Amp
75	CTL	Amp
76	MET	Amp
77	PUS	Amp

78	PUS	AmpOxtet
79	CTL	Amp
80	PUS	AmpChtetOxtet
81	CTL	Amp
82	MET	Amp
83	CTL	AmpChtetOxtet
84	CTL	Amp
85	PUS	AmpChtetOxtet
86	PUS	Amp
87	CTL	Amp
88	PUS	AmpChtetOxtet
89	MET	Amp
90	MET	Amp
91	MET	AmpChtetOxtet
92	CTL	AmpChtetOxtet
93	PUS	AmpChtetOxtet
94	PUS	AmpChtetOxtet
95	MET	AmpChtetOxtet
96	CTL	AmpChtetOxtet
97	MET	AmpChtetFlorOxtet
98	CTL	AmpChtetOxtet
99	PUS	Amp
100	MET	Amp
101	CTL	Amp
102	PUS	Amp
103	PUS	Amp
104	PUS	Amp
105	MET	Amp
106	MET	Amp
107	MET	Amp
108	PUS	Amp
109	PUS	AmpXnlChtetOxtet
110	CTL	Amp
111	CTL	AmpChtetFlorOxtet
112	CTL	AmpFlor
113	CTL	AmpChtetOxtet
114	CTL	AmpXnlChtetFlorOxtet
115	PUS	AmpChtetFlorOxtet
116	PUS	AmpXnlChtetFlorOxtet
117	MET	AmpChtetOxtet
118	MET	AmpChtetOxtet

119	CTL	AmpFlor
120	CTL	Amp
121	PUS	Amp
122	MET	AmpChtetOxtet
123	PUS	AmpXnlChtetOxtet
124	CTL	AmpChtetFlorOxtet
125	CTL	AmpChtetOxtet
126	CTL	AmpDano
127	PUS	Amp
128	PUS	Amp
129	PUS	Amp
130	PUS	Amp
131	MET	Amp
132	MET	AmpChtetOxtet
133	MET	AmpOxtet
134	MET	AmpChtetOxtet
135	MET	Amp
136	CTL	AmpChtetOxtet
137	CTL	AmpChtetOxtet
138	CTL	Amp
139	CTL	AmpOxtet
140	MET	AmpXnlFlor
141	PUS	Amp
142	MET	Amp
143	MET	AmpChtet
144	MET	Amp
145	PUS	AmpChtetOxtetDano
146	PUS	Amp
147	MET	Amp
148	MET	Amp
149	MET	AmpChtetOxtet
150	MET	Amp
151	MET	Amp
152	PUS	AmpXnlChtetFlorOxtet
153	CTL	Amp
154	PUS	Amp
155	CTL	Amp
156	MET	AmpChtetOxtet
157	CTL	Amp
158	PUS	Amp
159	CTL	Amp

160	CTL	AmpOxtet
161	CTL	AmpOxtet
162	MET	Amp

1. Clinical presentation group of cows when intrauterine samples were collected. (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; (PUS) purulent discharge defined as cows with a non-fetid purulent or mucopurulent vaginal discharge; and (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia.

2. Drugs for which isolate displayed phenotypic resistance. Amp: ampicillin, Xnl: ceftiofur, Chtet: chlortetracycline, Flor: florfenicol, Genta: gentamicin, Oxytet: oxytetracycline, and Enro: enrofloxacin

Supplemental Table 3-4. Outcome for Fisher's Exact Test analysis evaluating effect of individual animal treatment with any antimicrobial 14 days prior to sampling on AMR in *E. coli* to nine drugs tested for with MIC breakpoints.

Drug Resistance[1]	OR[2]	95% CI OR[3]	*P* value[4]
Ampicillin	-*	-	**
Ceftiofur	3.0	0.73-12.3	0.13
Chlortetracycline	0.87	0.29-2.6	1.0
Florfenicol	1.38	0.28-6.7	0.66
Gentamicin	-*	-	0.11
Oxytetracycline	0.72	0.24-2.14	0.79
Enrofloxacin	-*	-	0.11
Spectinomycin	-*	-	**
Danofloxacin	3.48	0.62-19.4	0.18

1. Resistance to antibiotic test against
2. Odds ratio for *E. coli* in the cows being treated with any antibiotic on antibiotic resistant to the referred drug when compared to cows not being treated with ceftiofur.
3. The 95% confidence interval of the odds ratio
4. *P*-value for univariate analysis evaluating the association between antimicrobial treatment of individual cows and increased resistance to drugs tested

*Odds ratio could not be calculated because one of more of the 2x2 cells were populated with a zero (no events in one of the treated vs antibiotic resistance combinations).
** All isolates susceptible or resistant; unable to evaluate effect of drug treatment on AMR

Supplemental Table 3-5. Outcome for Fisher's Exact Test analysis evaluating effect of individual animal treatment with ceftiofur 14 days prior to sampling on AMR in *E. coli* to nine drugs tested for with available MIC breakpoints. Thirteen of the 162 cows from which *E. coli* was isolated were treated with ceftiofur.

Drug[1]	**OR[2]**	**95% CI OR[3]**	***P* value[4]**
Ampicillin	-*	-	**
Ceftiofur	4.67	1.09-20.01	0.06
Chlortetracycline	0.67	0.18-2.56	0.76
Florfenicol	2.08	0.41-10.47	0.31
Gentamicin	-*	-	0.08
Oxytetracycline	0.55	0.15-2.12	0.55
Enrofloxacin	-*	-	0.08
Spectinomycin	-*	-	**
Danofloxacin	5.24	0.91-30.15	0.10

1. Drug in which prevalence of AMR was tested for
2. Odds ratio for *E. coli* in the cows being treated with ceftiofur on antibiotic resistant to the referred drug when compared to cows not being treated with ceftiofur.
3. The 95% confidence interval of the odds ratio
4. *P*-value for univariate analysis evaluating the association between ceftiofur treatment of individual cows and increased resistance to drugs tested

*Odds ratio could not be calculated because one of more of the 2x2 cells were populated with a zero (no events in one of the treated vs antibiotic resistance combinations).
** All isolates susceptible or resistant; unable to evaluate effect of drug treatment on AMR

Supplemental Table 3-6. Outcome for Fisher's Exact Test analysis evaluating effect of individual animal treatment with ceftiofur 14 days prior to sampling, stratified by clinical presentation groups (CTL, MET, or PUS), on AMR in *E. coli* to nine drugs tested for with available MIC breakpoints. Spectinomycin was omitted as all isolates were susceptible to the drug. Ampicillin was also omitted as all isolates were resistant to the drug. Thirteen of the 162 cows from which *E. coli* was isolated were treated with ceftiofur. One of 54 CTL cows, nine of 58 MET cows, and three of 50 PUS cows were treated with ceftiofur.

Drug[1]	**OR**[2]	**95% CI OR**[3]	***P* value**[4]
Ceftiofur			
CTL[5]	-*	-	1.0
MET	6.7	0.8-55.6	0.11
PUS	5.4	0.4-73.1	0.28
Chlortetracycline			
CTL	-*	-	0.31
MET	0.26	0.03-2.24	0.26
PUS	1.18	0.10-14.08	1.0
Florfenicol			
CTL	-*	-	0.15
MET	-*	-	1.0
PUS	7.33	0.51-105.91	0.23
Gentamicin			
CTL	-*	-	**
MET	-*	-	0.16
PUS	-*	-	**
Oxytetracycline			
CTL	-*	-	0.37
MET	0.22	0.02-1.86	0.25
PUS	1.07	0.09-12.71	1.0

Enrofloxacin				
	CTL	-*	-	**
	MET	-*	-	0.16
	PUS	-*	-	**
Danofloxacin				
	CTL	-*	-	1.0
	MET	6.71	0.81-55.64	0.11
	PUS	-*	-	1.0

1. Drug in which prevalence of AMR was tested for
2. Odds ratio for *E. coli* in the cows being treated with ceftiofur on antibiotic resistant to the referred drug when compared to cows not being treated with ceftiofur.
3. The 95% confidence interval of the odds ratio
4. *P*-value for Fisher's Exact test analysis evaluating the association between ceftiofur treatment of individual cows within specific clinical presentation and increased resistance to drug tested
5. Clinical presentation group (CTL, MET, or PUS) of cows when intrauterine samples were collected. (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as cows with a non-fetid purulent or mucopurulent vaginal discharge.

*Odds ratio could not be calculated because one of more of the 2x2 cells were populated with a zero (no events in one of the treated vs antibiotic resistance combinations).
** All isolates susceptible or resistant; unable to evaluate effect of drug treatment on AMR

Supplemental Table 3-7. Outcome for Fisher's Exact Test analysis evaluating effect of individual animal treatment with tetracyclines (chlortetracycline and oxytetracycline) 14 days prior to sampling on AMR in *E. coli* to nine drugs tested for with available MIC breakpoints. Four of the 162 cows from which *E. coli* was isolated were treated with tetracyclines.

Drug[1]	**OR[2]**	**95% CI OR[3]**	***P* value[2]**
Ampicillin	-*	-	**
Ceftiofur	-*	-	1.0
Chlortetracycline	2.36	0.32-17.27	0.59
Florfenicol	-*	-	1.0
Gentamicin	-*	-	1.0
Oxytetracycline	1.98	0.27-14.46	0.61
Enrofloxacin	-*	-	1.0
Spectinomycin	-*	-	**
Danofloxacin	-*	-	1.0

1. Drug in which prevalence of AMR was tested
2. Odds ratio for *E. coli* in the cows being treated with tetracycline on antibiotic resistant to the referred drug when compared to cows not being treated with ceftiofur.
3. The 95% confidence interval of the odds ratio
4. *P*-value for univariate analysis evaluating the association between tetracycline treatment of individual cows and increased resistance to drugs tested

*Odds ratio could not be calculated because one of more of the 2x2 cells were populated with a zero (no events in one of the treated vs antibiotic resistance combinations).
** All isolates susceptible or resistant; unable to evaluate effect of drug treatment on AMR

Supplemental Table 3-8. Outcome for univariate analysis evaluating effect of individual animal treatment with tetracyclines (chlortetracycline and oxytetracycline) 14 days prior to sampling, stratified by clinical presentation (CTL, MET, or PUS), on AMR in *E. coli* to nine drugs tested for with available MIC breakpoints. Spectinomycin omitted as all isolates were susceptible to the drug. Ampicillin also omitted as all isolates were resistant to the drug. Four of the 162 cows from which *E. coli* was isolated were treated with ceftiofur. None of 54 CTL cows, two of 58 MET cows, and two of 50 PUS cows were treated with tetracyclines. As no CTL cows received tetracycline, analysis could not be conducted for CTL cows.

Drug[1]	**OR**[2]	**95% CI OR**[3]	***P* value**[4]
Ceftiofur			
MET[5]	-*	-	1.0
PUS	-*	-	1.0
Chlortetracycline			
MET	-*	-	0.08
PUS	-*	-	1.0
Florfenicol			
MET	-*	-	1.0
PUS	-*	-	1.0
Gentamicin			
MET	-*	-	1.0
PUS	-*	-	**
Oxytetracycline			
MET	-*	-	0.10
PUS	-*	-	1.0
Enrofloxacin			
MET	-*	-	1.0
PUS	-*	-	**
Danofloxacin			
MET	-*	-	1.0
PUS	-*	-	1.0

1. Drug in which prevalence of AMR was tested for
2. Odds ratio for *E. coli* in the cows being treated with ceftiofur on antibiotic resistant to the referred drug when compared to cows not being treated with ceftiofur.
3. The 95% confidence interval of the odds ratio
4. *P*-value for Fisher's Exact test analysis evaluating the association between ceftiofur treatment of individual cows within specific clinical presentation and increased resistance to drug tested
5. Clinical presentation group (CTL, MET, or PUS) of cows when intrauterine samples were collected. (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as cows with a non-fetid purulent or mucopurulent vaginal discharge.

*Odds ratio could not be calculated because one of more of the 2x2 cells were populated with a zero (no events in one of the treated vs antibiotic resistance combinations).
** All isolates susceptible or resistant; unable to evaluate effect of drug treatment on AMR

Supplemental Table 3-9. Antimicrobials included in the BOPO6F Vet Antimicrobial Susceptibility Testing Plate, dilution ranges, and breakpoints for *E. coli* isolates (µg/mL).

			Breakpoints*				
Antimicrobial Class	**Antimicrobial Drug**	**Dilution Range**	**S**	**I**	**R**	**Organism**	**Source or Comment**
Cephalosporins	Ceftiofur	0.25-8	≤2	4	≥8	*E. coli*	CLSI VET01S ED5- Mastitis in cattle
Pleuromutilins	Tiamulin	0.5-32	-	-	-		**
Tetracyclines	Chlortetracycline	0.5-8	≤2	4	≥8	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Aminoglycosides	Gentamicin	1-16	≤2	4	≥8	*Enterobacterales*	CLSI VET01S ED5- Adult horse
Amphenicols	Florfenicol	0.25-8	≤2	4	≥8	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Tetracyclines	Oxytetracycline	0.5-8	≤2	4	≥8	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Penicillins	Penicillin	0.12-8					
Penicillins	Ampicillin	0.25-16	≤0.03	0.06-0.12	≥0.25	*E. coli*	CLSI VET01S ED5- Metritis in cattle
Fluoroquinolones	Danofloxacin	0.12-1	≤0.25	0.5	≥1	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Sulfonamides	Sulphadimethoxine	256					**
Aminoglycosides	Neomycin	4-32	-	-	-		**
Folate pathway antagonist	Trimethoprim / sulfamethoxazole	2 / 38	-	-	-		**
Aminocyclitols	Spectinomycin	8--64	≤32	64	≥128	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Macrolides	Tylosin	0.5-4	-	-	-		**
Macrolides	Tulathromycin	1-64	≤16	32	≥64	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle
Macrolides	Tilmicosin	4-64	-	-	-		**
Lincoamides	Clindamycin	0.25-16	-	-	-		**
Fluoroquinolones	Enrofloxacin	0.12-2	≤0.25	0.5-1	≥2	*Pasturella multocida*	CLSI VET01S ED5- Respiratory in cattle

* "S" susceptible, "I" intermediate, and "R" is resistant.
** Breakpoints not available for Gram negative bacteria from large animals

Supplemental Table 3-10. Treatment with any antimicrobials of the cows sampled according to clinical presentation group.

	Antimicrobial Treatments[2]			
Clinical Group[1]	**Ampicillin**	**Ceftiofur**	**Tetracyclines**	**N[3]**
CTL	0	1	0	**54**
MET	1	9	2	**58**
PUS	0	3	2	**50**

1. Clinical presentation group (CTL, MET, or PUS) of cows when intrauterine samples were collected. (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as cows with a non-fetid purulent or mucopurulent vaginal discharge.
2. Number of cows sampled given any antimicrobial treatments within 14 days prior to sampling
3. Total number of cows sampled belonging to corresponding clinical presentation group.

CHAPTER 4. Metagenomic Analysis of Intrauterine Microbiome of Cows with Metritis on California Commercial Dairy Farms

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ABSTRACT

The goal of this study was to assess the microbial ecology and diversity present in the uterus of post-partum dairy cows with and without metritis from commercial California dairy farms using shotgun metagenomics. A subset of 96 intrauterine swab samples, taken from a larger selection of 307 individual cow samples previously collected, were analyzed for α and β diversity and differential abundance at the genus level. Cows within 21 days post-partum were categorized into one of three clinical groups during sample collection: metritis (**MET**, n = 33), defined as a cow with watery, red or brown colored, and fetid vaginal discharge; cows with purulent discharge **(PUS**, n = 31), defined as a non-fetid purulent or mucopurulent vaginal discharge; and control cows, (**CT**, n = 32) defined as cows with either no vaginal discharge or a clear, non-purulent mucus vaginal discharge. In general, all three clinical groups (CT, MET, and PUS) were highly diverse with the top 12 most abundant genera only accounting for 10.3%, 8.8%, and 10.1% of mean relative abundance, respectively. The α diversity indices generally revealed a lower diversity from samples collected from MET and PUS when compared to CT cows. Nonmetric multidimensional scaling (NMDS) ordinations in conjunction with PERMANOVA and ANOSIM, revealed a significant difference in genus-level diversity between CT and MET samples (R^2 = 0.11, P = 0.003). When MET samples were stratified by antimicrobial treatment, significant differences were observed between CT and MET cases not treated with antibiotics (MET _No Treatment*;* P = 0.003) and between CT and MET cases treated with antibiotics (MET _Treatment*;* P = 0.003). Antimicrobial treatment resulted in a significant effect for distinguishing the uterine microbiome, when compared to CT, greater than metritis alone. To assess what bacteria might be associated with these differences, differential abundance testing was performed using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). Of the top 12 most abundant

genera, seven genera were significantly increased in the log-fold change of abundance in MET when compared to CT samples: *Bacteroides, Clostridium, Fusobacterium, Phocaeicola, Porphyromonas, Prevotella,* and *Streptococcus*. Two genera, *Dietzia* and *Microbacterium*, were significantly decreased in abundance in MET when comparing MET and CT, while no significant changes in abundance were observed for *Escherichia, Histophilus*, and *Trueperella*. Seven genera infrequently associated with metritis were increased in the log-fold change of abundance for MET_No Treatment, MET_Treatment, and PUS group when compared to CT. Three genera were decreased in the log-fold change of abundance for all clinical groups when compared to CT. *Bacteroides*, *Porphyromonas,* and *Fusobacterium,* genera which have previously been associated with metritis, were found to be significantly increased in abundance in metritic cows when compared to CT cows, while *Escherichia* and *Trueperella,* genera typically isolated from cows with metritis, were not significantly changed in abundance when comparing MET and CT samples. The results presented here, one of the deepest shotgun metagenomic analyses conducted on the bovine uterine microbiome to date, support the hypothesis that metritis is greatly associated with dysbiosis, and that specific aerobic and anaerobic pathogens are likely associated with metritis.

1. INTRODUCTION

As the fourth most common health issue in cows as identified by U.S. producers, metritis remains a major detriment to the American dairy industry [38]. Metritis is a uterine disease in cattle and typically occurs within 21 days post-partum, characterized by an enlarged uterus, fever, and fetid, watery red-brown uterine discharge [56]. Metritis negatively impacts milk production, reproductive performance, and increases the risk of culling [188]. The economic impacts of these production issues cost producers a mean of $511 per case of metritis [55]. In North America, metritis is estimated to affect 10 to 30% of post-partum dairy cows [53, 68].

Generally, bacteria have been most commonly implicated as the cause of bovine metritis. Traditional, culture-based methods have isolated certain bacteria from uterine swabs collected from cows with metritis, in particular, *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica* [59, 205]. However, such studies were limited to only those microbes that could be isolated and identified while growing on the media type and in atmospheric conditions provided. With the advent of culture-independent 16s rRNA gene sequencing a substantially larger array of microbes could be identified, leading to additional bacteria including those belonging to the genera *Bacteroides* and *Porphyromonas* being associated with metritis [62, 70, 206]. Such 16s rRNA-based studies also identified bacteria potentially associated with uterine health, albeit with occasionally conflicting findings. For example, the species belonging to the genus *Escherichia* are well-known uterine pathogens as evidenced by culture-based studies. Yet, various studies have demonstrated either an association between *E. coli* and uterine health or found little to no reads matching *E. coli* from uterine samples taken from cows with metritis [62, 207].

Despite the inability of 16s rRNA-based studies to reach a uniform agreement on a specific etiology for metritis, these analyses have allowed for the study of the overall community dynamics within the uterine microbiome in addition to increases or decreases of relative abundance for specific taxa. A common output of microbiome studies is the calculation of various metrics to estimate both α-diversity (diversity of microbes within a sample) and β-diversity (diversity of microbes between samples)[67]. 16s rRNA-based studies are again inconclusive on whether uterine samples taken from metritic cows have significant changes in α-diversity. Some studies have reported decreased α-diversity in samples taken from metritic cows when compared to healthy cows [62, 70, 208]; while other studies have reported no statistical difference in α-diversity metrics between samples collected from metritic and healthy cows [69, 206, 209]. One possible explanation for the disagreement between these studies is the wide range of sample size and sequencing depth, with one of the larger studies [62] (n = 60) resulting in nearly 5 million 16s reads, while one of the smaller studies [209] (n = 28) resulted in approximately 27,000 16s reads. As metrics for the estimation of α-diversity are heavily biased when taxa are unobserved, the ability of shotgun metagenomics to identify low abundance taxa more accurately and without the biases introduced by the necessary PCR amplification used in 16s rRNA methods may prove useful in discerning the community interactions within the uterine microbiome [210, 211].

The β-diversity (diversity of microbes between samples) of uterine microbiota from cows with and without metritis is also frequently measured in uterine microbiome studies. Typically, various metrics (e.g. Bray-Curtis and UniFrac) analyzing the similarity or dissimilarity of the microbiota sequenced are calculated; ordination plots based on these metrics (e.g. PCoA and NMDS) visually display how diverse the microbiota are and are generally paired with analyses (e.g. PERMANOVA and ANOSIM) to determine significance [67]. While the 16s rRNA-based studies examined

previously disagreed on the α-diversity of healthy and metritic uterine microbiota, those that did analyze β-diversity all concluded that the uterine microbiome from healthy and metric cows were significantly different [69, 70, 208, 209].

We previously conducted an evaluation of intrauterine *E. coli* isolated from 307 dairy cows with and without metritis throughout California for phenotypic antimicrobial resistance (AMR) and analyzed risk factors impacting the isolation of intrauterine *E. coli* [212]. In this study, we performed shotgun metagenomic analyses on a subset of intrauterine swabs (n = 96) collected from the same cows to assess the microbial ecology and diversity of microbes associated with both uterine health and metritis.

2. MATERIAL AND METHODS

Sample collection

Samples used for metagenomic analyses were collected as part of a study analyzing AMR to common antimicrobials used for the treatment of bovine metritis [212]. In short, a cross-sectional study was designed to collect uterine swabs from post-partum cows between 3 and 21 days in milk (DIM). A convenience sample of 25 commercial dairy farms from the Sacramento and San Joaquin Valleys in California was recruited with the help of local veterinarians and UC Davis faculty and UC ANR extension advisors. Cows sampled were classified into three clinical groups based on vaginal discharge (VD) characteristics: [190] normal discharge (**CT)**: clear lochia, clear mucus, or no vaginal discharge, metritis discharge (**MET)**: watery, reddish or brownish, and fetid), and purulent discharge (**PUS)**: non-fetid purulent or mucopurulent vaginal discharge).

Before uterine sample collection, the rectal temperature was measured with a GLA M900 thermometer (GLA Agricultural Electronics, San Luis Obispo, CA, USA). Researchers cleaned the vulva using dry paper towels and 70% isopropyl alcohol prior to uterine swab collection. A

30-inch double-guarded sterile culture swab (McCullough; Jorgensen Labs Inc., Loveland, CO, USA) was gently passed through the vulva and cervix until reaching the uterine body. The swab was exposed and rolled against the uterine wall, retracted within the double sheath, removed from the cow, and placed immediately in sterile cryogenic tubes (Thermo Scientific™ Nalgene™, Rochester, NY). These tubes were transported on ice until storage in the laboratory at -80°C for future DNA extraction.

Sample Selection

In order to select 96 out of the 307 swabs collected, certain criteria were created to prioritize which swabs would undergo DNA extraction and sequencing. We selected swabs collected from cows less than or equal to 14 DIM at sampling (n = 224) [214]. When possible, 1 swab from a metritic cow matching the DIM criteria was randomly selected from each of the 25 farms enrolled in the study. After the selection of a metritic cow, swabs from CT and PUS cows from the same farm were randomly selected for each of the 25 farms. Due to DNA quality issues, swabs chosen for sequencing were biased towards those acquired towards the end of the collection period. When possible, swabs from metritic cows from which multi-drug resistant *E. coli* were isolated were also chosen. After final selection (n = 96), 32, 33, and 31 samples were taken from cows belonging to CT, MET, and PUS clinical groups, respectively. Cows in this study belonged to 24 of the 25 farms sampled in the initial study.

DNA Extraction

DNA was extracted using the QIAamp DNA minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions for buccal swabs. The addition of the following steps to increase the recovery of high-quality DNA was based on methodology from previous publications [213]. Frozen swabs were placed in sterile 2 mL microcentrifuge tubes with 400 µL of buffer AL and left

to thaw at room temperature for 30 minutes. Once thawed, the swab was removed with sterile forceps and the micro centrifuge tube was centrifuged at 13,200 x g for 10 minutes before the supernatant was discarded and the pellet re-suspended in 245 µL of buffer AL. 5 µL of lysozyme (50 mg/mL; ThermoFisher Scientific, Waltham, MA) and 150 µL of mutanolysin diluted to 1000 Units/mL from *Streptomyces globisporus* ATCC 21553 (Sigma-Aldrich, Saint Louis, MO,) were added to each sample before incubating at 37℃ for one hour. DNA extraction was performed as specified by QIAamp DNA minikit protocol. To ensure adequate purity for DNA sequencing, eluted DNA was purified according to the Zymo Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). After subsequent DNA extraction and purification, DNA quantification was conducted for all samples using a NanoDrop One^{C} (Thermo Fisher Scientific, Wilmington, DE).

Library Prep and Metagenomic Sequencing

Illumina DNA libraries were prepared using the seqWell plexWell LP384 Library Preparation kit (seqWell, Beverly, MA) using 10 ng of genomic DNA. The prepared libraries were amplified with 8 PCR cycles, analyzed using Bioanalyzer 2100 (Agilent, Santa Clara, CA), quantified with Qubit (Life Technologies, ThermoFisher Scientific, USA), and combined into one pool at equimolar ratios. The library pool was quantified by qPCR with the Kapa Library-Quant kit (Kapa Biosystems/Roche, Basel, Switzerland) and sequenced on an Illumina NovaSeq system (Illumina, San Diego, CA) with paired-end 150-bp reads.

Bioinformatics

Raw sequence data were trimmed using trimmomatic and quality filtered using FastQC. Trimmed and quality filtered reads were sorted into bovine and non-bovine reads using the sort function in Kraken2 [215]. Microbial reads were identified from non-bovine reads using the identify function in Kraken2. Microbial reads were classified to the phyla, genus, and species level

and counted using Bracken [216]. Antimicrobial resistance and virulence factor genes were identified within non-bovine reads using the Comprehensive Antibiotic Resistance Database (CARD) and Virulence Factor Database (VFDB), respectively, using the Antimicrobial Resistance Identification by Assembly (ARIBA) program [217]. Identification of AMR and virulence factor genes were done to the SNP level. Classification of AMR and virulence factor gene hits into theoretical and validated catageories is ongoing.

Diversity Analysis

Cumulative Sum Scaling (CSS) was used to normalize reads via metagenomeSeq in R at the genus-level [218, 219]. To facilitate diversity analyses, prior to the creation of a *phyloseq* object, Taxallnomy was used to create a hierarchical taxa table [220]. *Phyloseq* objects were created using both CSS-normalized and non-normalized read counts [221]. Using non-normalized data, α diversity metrics (Shannon index and Chao1) were calculated at the genus level. After testing for normality via Shapiro-Wilk test, significance between clinical groups was tested using the Wilcoxon Sum Rank test for Chao1 and Simpson values and the Tukey-Kramer HSD test for Shannon values in JMP Pro 16. α diversity metrics were graphed using the "alpha_boxplot" function of R (Figure 4-1) [222].

β diversity was visualized at the genus-level using Bray-Curtis dissimilarity distances calculated from CSS normalized read count data using nonmetric multidimensional scaling (NMDS) using the "ordinate" function of *phyloseq* in R [221]. Ordination fit was assessed using stress values and when stress values ≥ 0.2 were obtained, NMDS was repeated with an increased *trymax* of up to 200 until stress values < 0.2 were obtained. Two NMDS ordinations were created, figure 4-2A analyzing β diversity between clinical groups of cows sampled (CT, MET, and PUS) and figure 4-2B analyzing β diversity between the same clinical groups, but with samples from

MET cows separated by whether cows sampled received antimicrobial treatment within fourteen days prior to intrauterine swab collection (CT, MET _No Treatment, MET _Treatment, and PUS). Colored ellipses were added to represent the 95% confidence interval for the various clinical groups. Three-dimensional scatterplots of both NMDS ordinations (Figure 4-3) were created using the "beta_diversity_3d" function of *plotly_microbiome.*

For both NMDS ordinations, significant differences were tested by permutational multivariate analysis of variance (PERMANOVA) via "adonis2" in *vegan* using 999 permutations and by analysis of similarities (ANOSIM) in R. For PERMANOVA, differences were considered significant when *p*-adjusted < 0.01. For ANOSIM, differences were considered significant when *p*-adjusted < 0.01. For PERMANOVA, post hoc pairwise comparisons between groups (Supplemental Table 4-1) was conducted using "pairwise.adonis" with *P* values adjusted using Benjamini-Hochberg [223]. Following PERMANOVA, the "betadisper" function was used to test for homogeneity of multivariate dispersion for NMDS of clinical groups for cows sampled and for NMDS of clinical groups, but with samples from MET cows separated by antimicrobial treatment. Pairwise ANOSIM between clinical groups of cows sampled and between clinical groups, but with samples from MET cows separated by antimicrobial treatment was also conducted with *P* values adjusted using Benjamini-Hochberg (Supplemental Table 4-2).

Stacked mean relative abundance boxplots of the top 12 genera vs all other genera were created using excel for each clinical group (CT, MET, and PUS) in figure 4-4A. Data for only the top 12 genera were visualized in figure 4-4B by including only relative abundance of the top 12 genera.

Differential Abundance Testing

Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was used to detect differences in microbial compositions between clinical groups [224]. CSS normalized

read count data was input into ANCOM-BC, which was selected as it provides *P* values and confidence intervals for each taxa, controls the false discovery rate, and is relatively computationally simple to implement.ANCOM-BC utilizes the Wilcoxon rank-sum test for identifying taxa that are differentially abundant and includes multiple hypothesis corrections by the Holm-Bonferroni method. It is a useful tool for comparing relative abundance between groups due to its capacity to control the false discovery rate at nominal levels while maintaining power. An ANCOM-BC detection q value <0.05 was considered significant (q values are the *P* values adjusted for the optimized false discovery rate).

ANCOM-BC computes log fold changes between groups and this data was used to create heatmaps to compare significant changes between clinical groups for various taxa either by using native ANCOM-BC code or by the "heatmap" function in R. Figure 4-5 presents a heatmap of log fold changes of phyla abundance in MET_No Treatment, MET_Treatment, and PUS clinical groups compared to CT. Figure 4-6 presents a heatmap of log fold changes of abundance of 10 genera for which all three clinical group pairwise comparisons (MET_No Treatment, MET _Treatment, and PUS when compared to CT) were significant (*p*-adjusted < 0.05). Figure 4-7 presents a heatmap of log fold changes in abundance of 31 selected genera for MET _No Treatment and MET_Treatment clinical group pairwise comparisons when compared to CT. Data used to create figure 4-7 is presented in Supplemental Table 4-3. Table 4-1 displays the log fold change in abundance, along with the mean percent relative abundance within MET samples, of the top 12 most abundant genera when MET (regardless of treatment) was compared to CT.

Data Availability

All sequence data are in the process of being submitted.

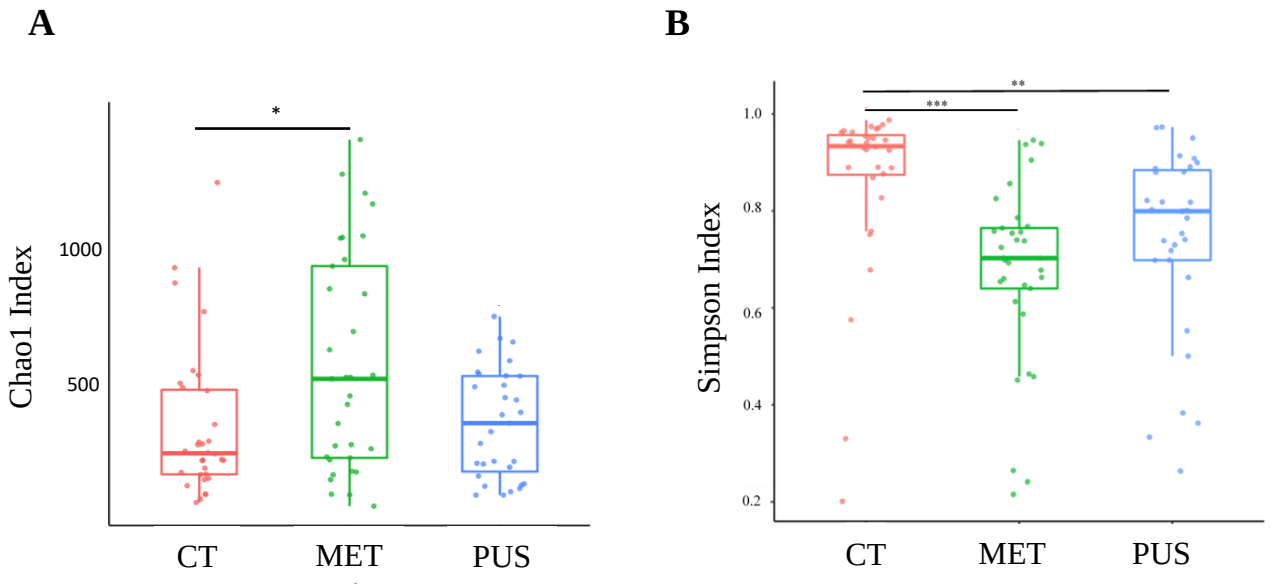
3. RESULTS

Shotgun metagenomics sequence descriptive data

After initial quality trimming and assignment of microbial reads to the genus level, sequencing of 96 samples resulted in a total of 24,616,858 reads prior to CSS normalization. The mean number of genus-level reads per sample was 256,425 (95% CI: 124,471-388,379). After CSS normalization, 96 samples resulted in a total of 135,190 reads. The mean number of genus-level reads per sample was 1,408 (95% CI: 1,265-1,551).

Intrauterine microbiome diversity analysis

α diversity was assessed at the genus-level using Chao1, Simpson, and Shannon index values (Figure 4-1). Chao1 index values were significantly different between CT and MET samples ($p = 0.021$) (Figure 4-1A). Simpson index values were significantly different between CT and PUS samples ($p = 0.0005$) and CT and MET samples ($p < 0.0001$) (Figure 4-1B). Shannon index values were significantly different between CT and PUS samples ($p = 0.0003$) and CT and MET samples ($p < 0.0001$) (Figure 4-1C).



C

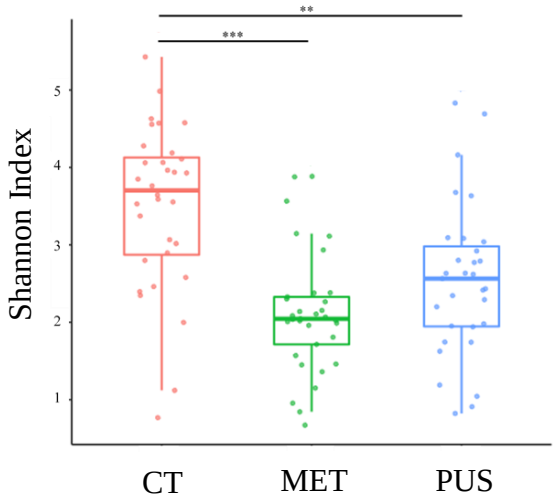
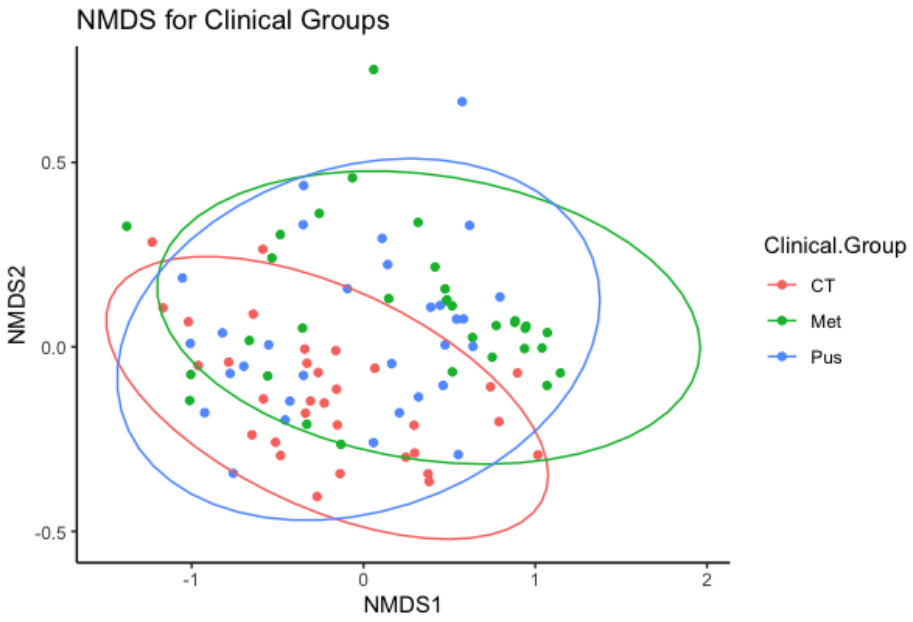


Figure 4-1. Bovine intrauterine swab microbiome α diversity. Genus-level (**A**) Chao1, (**B**) Simpson, (**C**) Shannon. For microbiome α diversity data, only Shannon data were normally distributed (Shapiro-Wilk *P* value = 0.073). Horizontal black lines indicate significant pairwise comparisons of clinical groups had significantly different mean Chao1 and Simpson values based on Wilcoxon Sum Rank Test or significantly different mean Shannon values based on Tukey-Kramer HSD $p < 0.05$ (*), $p < 0.001$ (**), and $p < 0.0001$ (***).

Bray-Curtis dissimilarity distances calculated from CSS normalized read count data were used to create nonmetric multidimensional scaling (NMDS) ordinations to visualize β diversity. Two NMDS ordinations were created to identify differences between clinical groups of cows sampled (Figure 4-2A) and between the same clinical groups, but with samples from MET cows separated by whether cows sampled received antimicrobial treatment (Figure 4-2B). Three-dimensional scatterplots of both NMDS ordinations were also created to help better visualize significant differences between clinical groups (Figure 4-3A) and clinical groups with MET separated by antimicrobial treatment (Figure 4-3B). PERMANOVA statistical testing revealed a significant difference in genus-level diversity between CT and MET samples ($R^2 = 0.112$, $P = 0.003$). When analyzing NMDS with MET separated by antimicrobial treatment, pairwise

PERMANOVA revealed significant differences between CT v. MET_NoTreat (R^2 = 0.075, P = 0.006), CT v. MET _Treat (R^2 = 0.242, P = 0.003), and PUS v. MET _Treat (R^2 = 0.155, P = 0.003) (Supplemental Table 4-1). In comparison, pairwise ANOSIM revealed significant differences between only CT v. MET_NoTreat (P = 0.003) and CT v. MET_Treat (P = 0.003) (Supplemental Table 4-2).

A



B

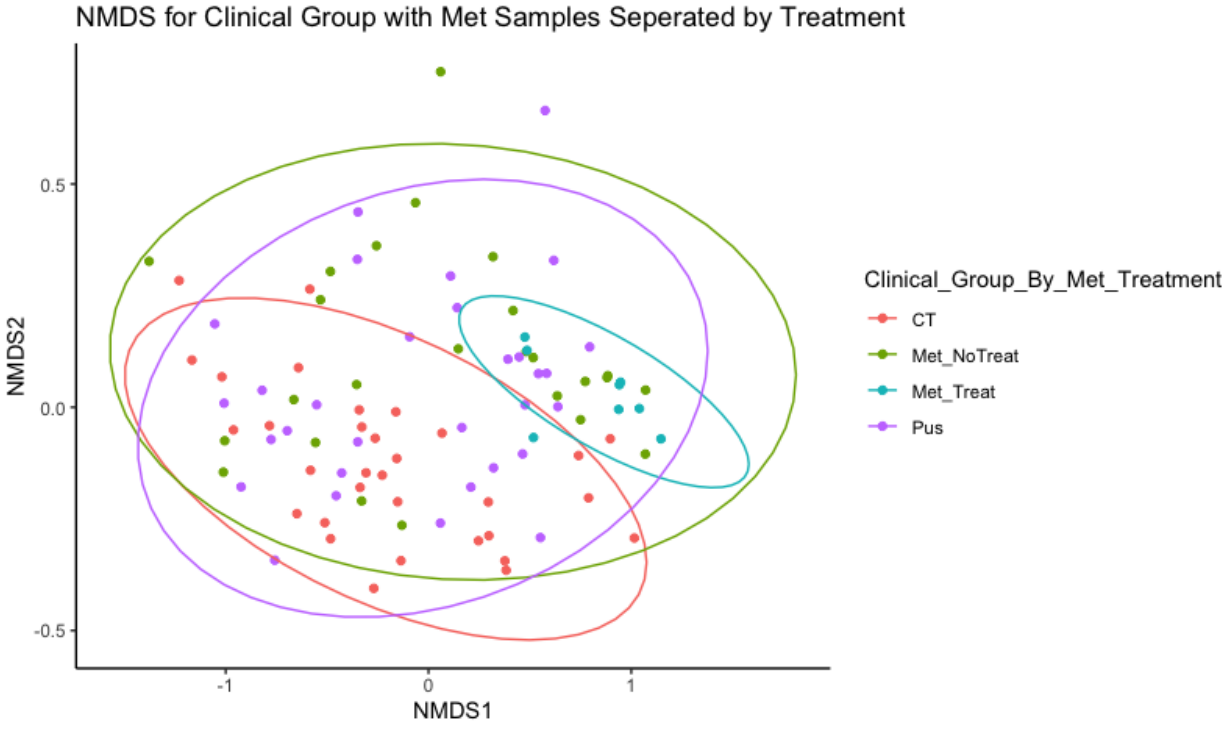
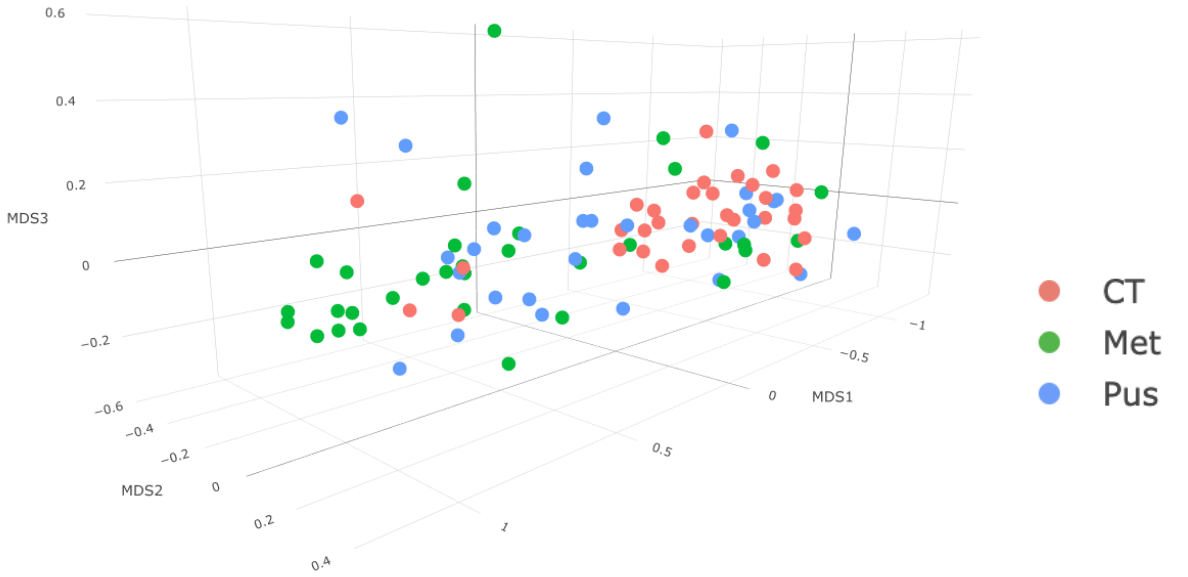


Figure 4-2. Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis (BC) dissimilarity of cumulative sum scaling normalized genus-level read counts of bovine intrauterine microbiome. Ellipses correspond to 95% confidence interval. (**A**) NMDS ordination by three clinical groups of cows sampled (ANOSIM, $P = 0.001$, $R = 0.128$; PERMANOVA, $R^2 = 0.085$, $P = 0.001$) (**B**) NMDS ordination by clinical groups of cows samples with MET cows (i.e., cows with clinical signs of metritis) stratified by treatment with antimicrobials or not (ANOSIM- analysis of similarities $P = 0.001$, $R = 0.129$; PERMANOVA- permutational multivariate analysis of variance $R^2 = 0.126$, $P = 0.001$).

A



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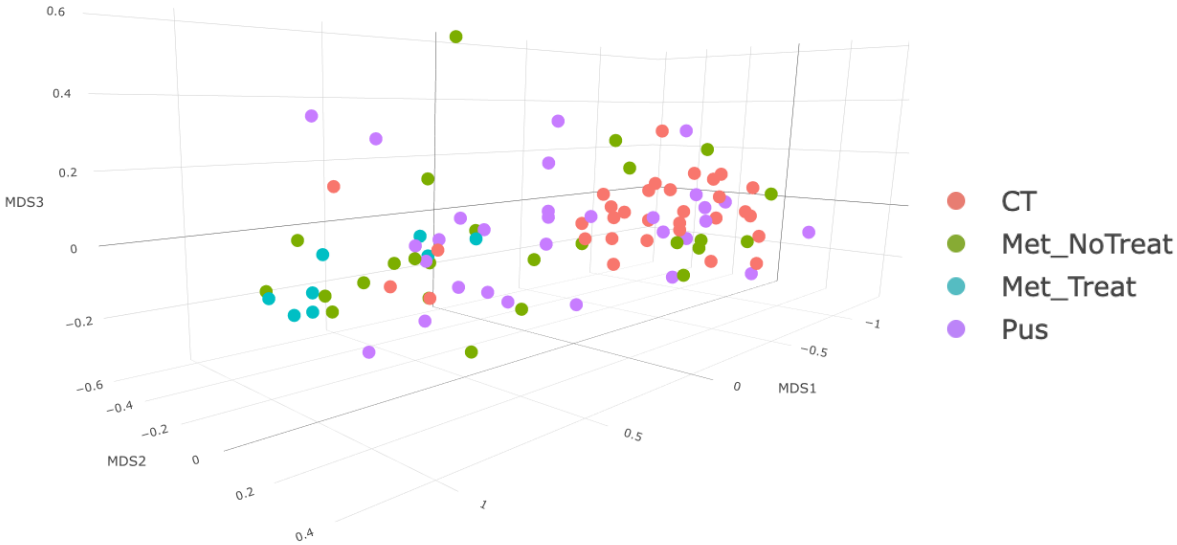
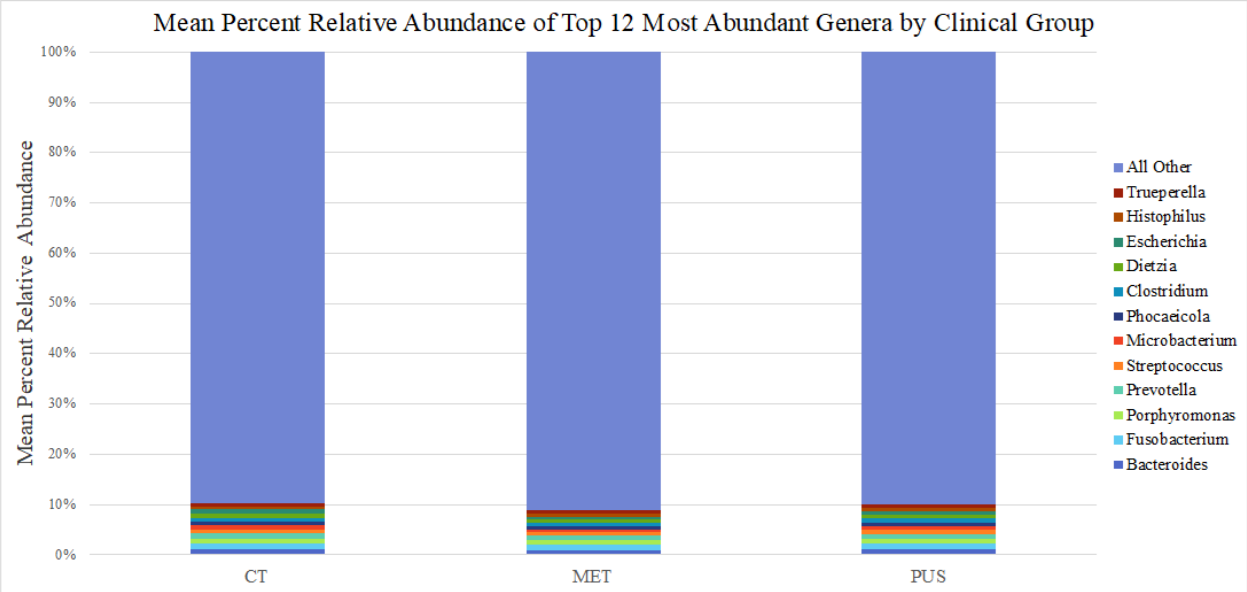


Figure 4-3. 3D scatterplot of nonmetric multidimensional scaling (NMDS) based on Bray-Curtis (BC) dissimilarity of cumulative sum scaling normalized genus-level read counts of bovine intrauterine microbiome. (**A**) 3D scatterplot of NMDS ordination of the three clinical groups of cows sampled (CT, MET, and PUS) (**B**) 3D scatterplot of NMDS ordination of the clinical groups of cows sampled, with MET cows (i.e., cows with clinical signs of metritis) stratified by treatment with antimicrobials or not (CT, MET_NoTreatment, MET_Treatment, and PUS).

Differentially abundant taxa

At the genus level, all three clinical groups (CT, MET, and PUS) were highly diverse with the top 12 most abundant genera only accounting for 10.3%, 8.8%, and 10.1% of mean relative abundance, respectively (Figure 4-4A). In other words, all other genera made up 89.7%, 91.2%, and 89.9% of CT, MET, and PUS sample microbiota. Within the top 12 most abundant genera, the most abundant genus was *Bacteroides* (1.2% of CT, 1% of MET, 1.1% of PUS) and the least abundant genus was *Trueperella* (0.64% of CT, 0.56% of MET, 0.64% of PUS) (Figure 4-4B).

A



B

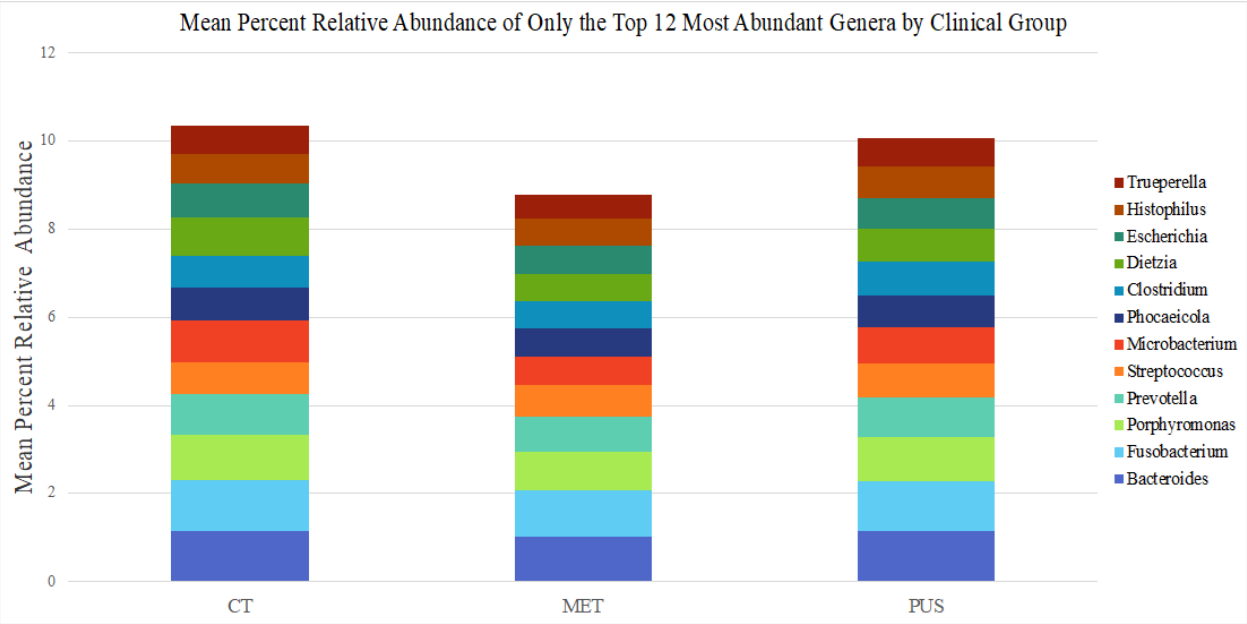


Figure 4-4. Mean relative abundance plots for top 12 most abundant genera. (**A**) Including all other genera and stratified by clinical group (CT, MET, and PUS). (**B**) Not including other genera and stratified by clinical group (CT, MET, and PUS). (CT) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as a non-fetid purulent or mucopurulent vaginal discharge.

Phyla level changes in log fold change in abundance

To detect if any taxa were differentially abundant between clinical groups, ANCOM-BC was used to generate log fold change in abundance data. Figure 4-5 presents this data at the phyla level for three clinical group comparisons: MET_No Treatment, MET_Treatment, and PUS clinical groups compared to CT. For MET_No Treatment samples, seven phyla had increased log fold changes in abundance (Ignavibacteriae, Gemmatimonadetes, Deferribacteres, Chlorobi, Chlamydiae, Bacteroidetes, and Aquificae) and the phylum Actinobacteria has a decreased log fold change in abundance when compared to CT. The MET_Treatment samples, when compared to CT, had the largest number of significantly increased (n = 14) and decreased (n = 12) phyla. Of

these phyla, three were increased by one log fold change or greater (Thermotogae, Planctomycetes, and Chlorobi) and three were decreased by one log fold change or greater (Evosea, Euglenozoa, and Apicomplexa) when compared to CT. Lastly, PUS samples had four phyla with increased log fold changes in abundance (Gemmatimonadetes, Chlorobi, Chlamydiae, and Bacteroidetes) and the phylum Actinobacteria has a decreased log fold change in abundance when compared to CT.

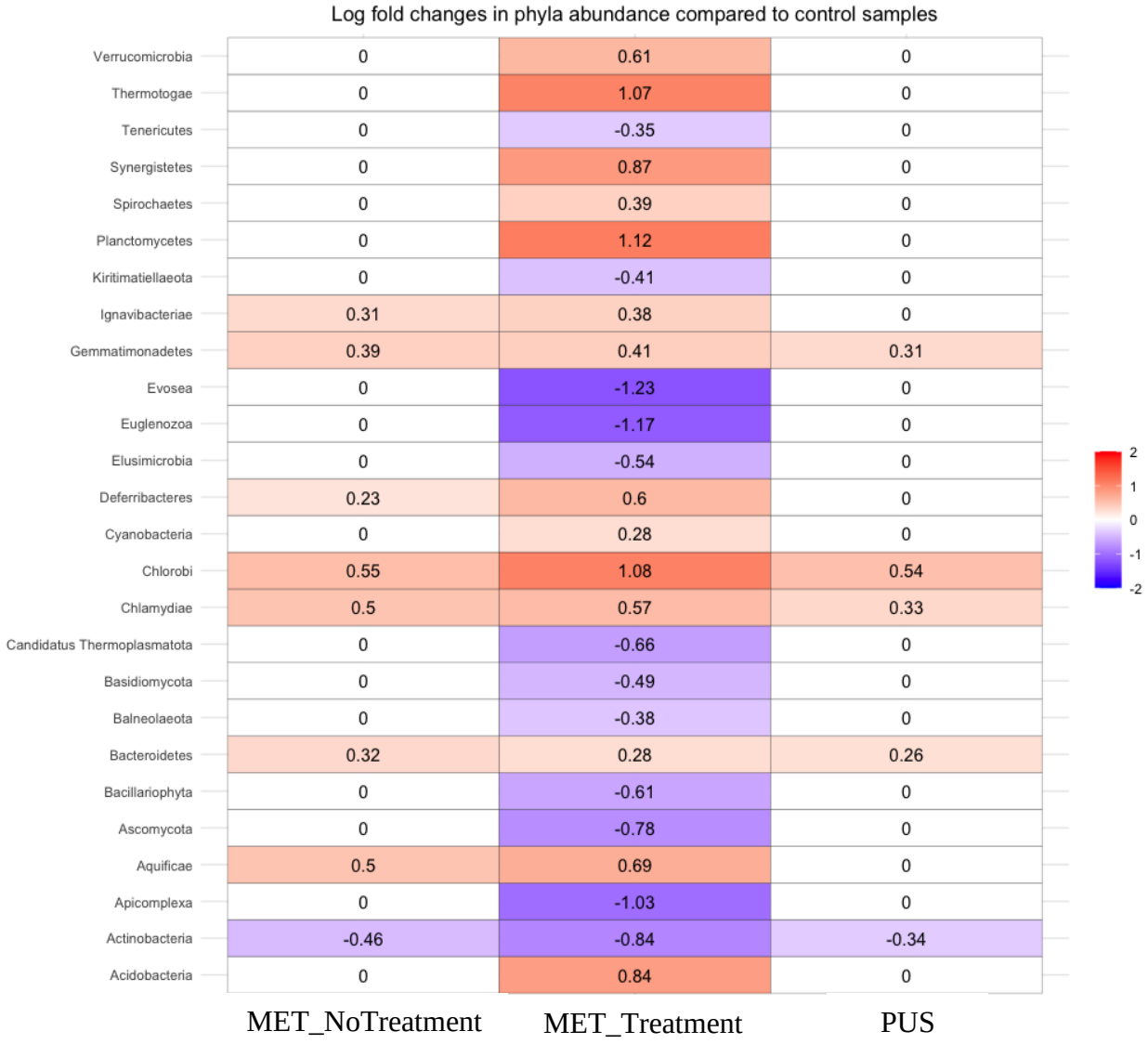


Figure 4-5. Heatmap of log fold changes in abundance of the 26 phyla with an adjusted $p < 0.05$ for MET_No Treatment, MET_Treatment, or PUS when compared to control. Red indicates increased abundance in the comparison group versus control; blue indicates decreased abundance in the comparison group versus control. Zero values in white cells indicate non-significant log fold change of abundance (adjusted $p > 0.05$).

Genus level changes in log fold change in abundance

Table 4-1 presents log fold changes in abundance of the top 12 most abundant genera, as also presented in figure 4, alongside the mean percent relative abundance of these genera in MET samples. Of the 12 genera, *Dietzia* and *Microbacterium* were significantly decreased in abundance in MET when compared to CT. Seven genera, namely *Bacteroides, Clostridium, Fusobacterium, Phocaeicola, Porphyromonas, Prevotella,* and *Streptococcus* were significantly increased in abundance in MET when compared to CT. The three genera found to be non-significantly changed in abundance in MET samples compared to CT were *Escherichia, Histophilus,* and *Trueperella.*

Genera	Log Fold Change of MET vs. CT[1]	MET (Combined Treatment) Mean Percent Relative Abundance[2]
Bacteroides	0.20	1.03
Clostridium	0.09	0.61
Dietzia	-0.19	0.61
Escherichia	-	0.63
Fusobacterium	0.2	1.03
Histophilus	-	0.63
Microbacterium	-0.16	0.67
Phocaeicola	0.16	0.64
Porphyromonas	0.19	0.89
Prevotella	0.17	0.79
Streptococcus	0.22	0.70
Trueperella	-	0.56

Table 4-1. Log fold change of abundance data collected from ANCOM-BC for MET vs. CT for top 12 most abundant genera.

(**1**) Log fold change of abundance for MET (regardless of antimicrobial treatment) vs. CT for top 12 most abundant genera.
(**2**) Mean of percent relative abundance for top 12 genera for all MET samples, regardless of antimicrobial treatment (n = 33).

-Indicates genera with non-significant log fold change values for MET vs CT (*p* adjusted > 0.05).

To compare significantly increased or decreased abundances for genera significant for all three clinical group comparisons, a heatmap was created in figure 4-6. Seven genera (*Liquorilactobacillus*, *Cyclobacterium*, *Owenweeksia*, *Anoxybacter*, *Flavihumibacter*, *Dyadobacter*, and *Oceanobacillus*) were increased in log fold change abundance for MET_No Treatment, MET_Treatment, and PUS clinical groups compared to CT. Conversely, three genera (*Pseudonocardia*, *Glutamicibacter*, and *Rathayibacter*) decreased in log fold change abundance for all three clinical groups when compared to CT.


0.42
0.66
0.36
Liquorilactobacillus
0.48
0.83
0.41
Cyclobacterium
0.40
0.79
0.35
Owenweeksia
-0.71
-0.77
-0.54
Pseudonocardia
0.36
0.66
0.40
Anoxybacter
0.37
0.64
0.39
Flavihumibacter
0.49
0.82
0.54
Dyadobacter
-0.54
-0.88
-0.60
Glutamicibacter
0.41
0.57
0.51
Oceanobacillus
-0.76
-0.69
-0.74
Rathayibacter
MET_No Treatment
MET_Treatment
PUS
Min (-0.88)
1st Quartile (-0.54)
Median (0.4)
3rd Quartile (0.53)
Max (0.83)

Figure 4-6. Heatmap of log fold changes in abundance of 10 genera for which pairwise comparisons between MET_No Treatment, MET_Treatment, and PUS when compared to CT had an adjusted $p < 0.05$. Colors correspond to 25% increments of log fold changes in abundance. Purple indicates decreased abundance in the comparison group versus control. Green indicates increased abundance in the comparison group versus control.

To determine whether genera often associated with metritis or uterine health by other studies were differentially abundant within our sample population, a heat map was created in figure 4-7 to analyze log fold changes in abundance of MET_No Treatment and MET_Treatment compared to CT for 31 selected genera. None of the selected genera were significantly changed in log fold abundance for Pus when compared to CT.

For MET_No Treatment, 21 genera (e.g. *Trueperella*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Escherichia*, and *Bacteoides*) were not significantly different when compared to CT. Four genera (*Streptobacillus*, *Filifactor*, *Citrobacter*, and *Chlamydia*) were significantly increased in log fold change of abundance in MET_No Treatment samples when compared to CT. Five genera (*Streptomyces*, *Micrococcus*, *Corynebacterium*, *Brucella*, *Brevibacterium*, and *Bifidobacterium*) were significantly decreased in log fold change of abundance in MET_No Treatment samples when compared to CT.

For MET_Treatment, four genera (e.g. *Porphyromonas*, *Fusobacterium*, *Enterococcus*, and *Chlamydia*) were not significantly different when compared to CT. Ten genera (e.g. *Streptobacillus*, *Salmonella*, *Filifactor*, *Cryptococcus*, *Citrobacter*, and *Arcanobacterium*, and *Anaerococcus*) were significantly increased in log fold change of abundance in MET_Treatment samples when compared to CT. Seventeen genera (e.g. *Trueperella*, *Streptomyces*, *Staphylococcus*, *Prevotella*, *Micrococcus*, *Klebsiella*, *Escherichia*, *Corynebacterium*, *Brucella*, *Brevibacterium*, *Bifidobacterium*, *Bacteoides*, and *Bacillus*) were significantly decreased in log fold change of abundance in MET_Treatment samples when compared to CT. Of these 17 genera,

only *Micrococcus* was decreased by one log fold change or greater in MET_Treatment when compared to CT. Ignoring stratification by antimicrobial treatment, three genera (*Streptobacillus, Filifactor,* and *Citrobacter*) were increased and six genera (*Streptomyces, Micrococcus, Corynebacterium, Brucella*, *Brevibacterium*, and *Bifidobacterium*) were decreased in log fold change of abundance in MET samples compared to CT.

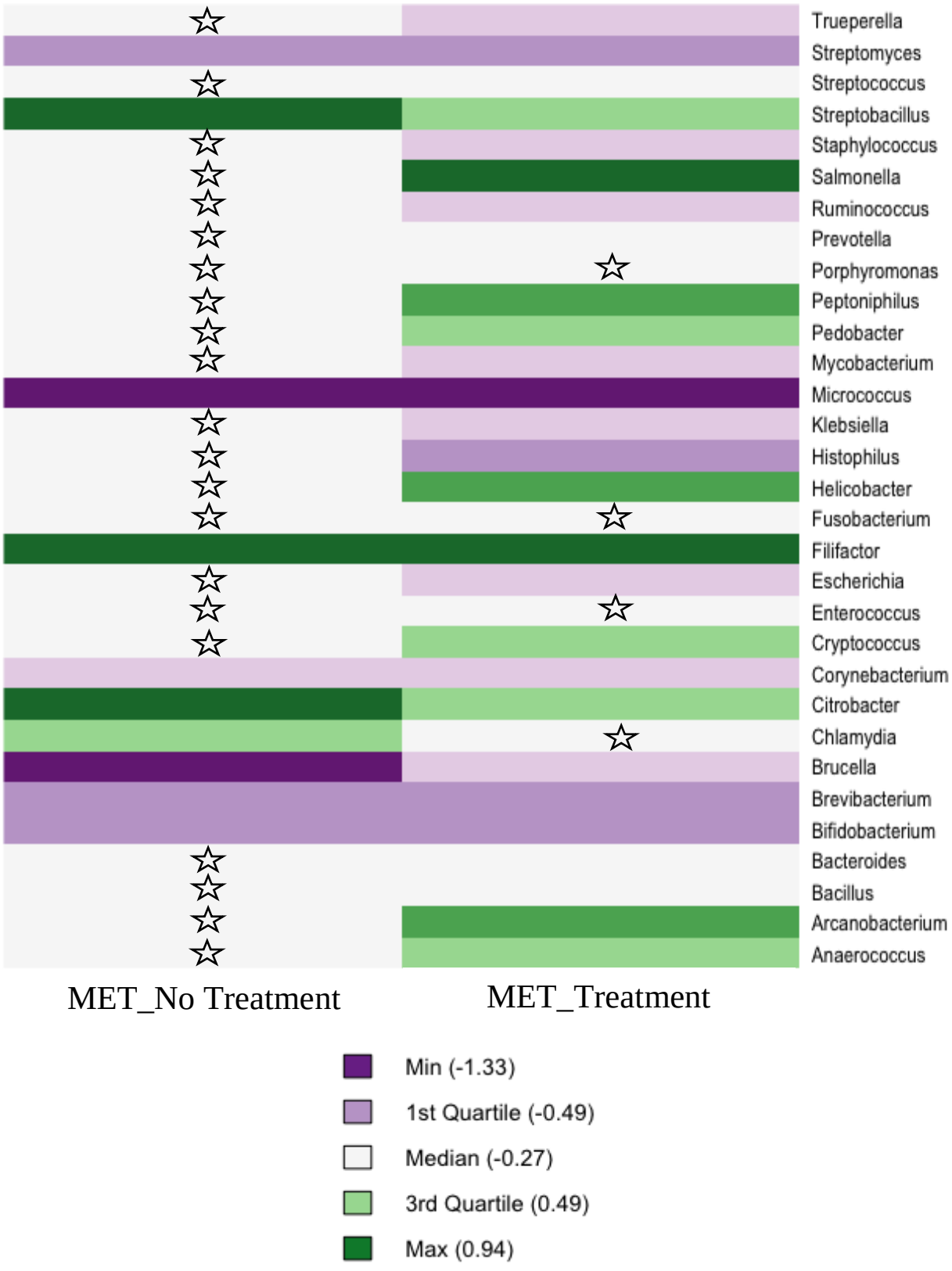

Trueperella
Streptomyces
Streptococcus
Streptobacillus
Staphylococcus
Salmonella
Ruminococcus
Prevotella
Porphyromonas
Peptoniphilus
Pedobacter
Mycobacterium
Micrococcus
Klebsiella
Histophilus
Helicobacter
Fusobacterium
Filifactor
Escherichia
Enterococcus
Cryptococcus
Corynebacterium
Citrobacter
Chlamydia
Brucella
Brevibacterium
Bifidobacterium
Bacteroides
Bacillus
Arcanobacterium
Anaerococcus
MET_No Treatment
MET_Treatment
Min (-1.33)
1st Quartile (-0.49)
Median (-0.27)
3rd Quartile (0.49)
Max (0.94)

Figure 4-7. Heatmap of log fold changes in abundance of 31 selected genera for which MET_No Treatment and/or MET_Treatment when compared to CT had an adjusted $p < 0.05$. PUS was not included as none of the selected genera were significant for this clinical group. Colors correspond to 25% increments of log fold changes in abundance. Purple indicates decreased abundance in the comparison group versus control. Green indicates increased abundance in the comparison group versus control.

☆ Non-significant (adjusted $p > 0.05$) for the clinical group comparison for that genus.

4. DISCUSSION

Comparison to previous shotgun metagenomics studies

The past twenty years of advancement in high-throughput, next-generation DNA sequencing has allowed major advancements in bovine metritis research. This study currently represents one of the largest cross-sectional metagenomic characterizations of the uterus of cows with and without metritis, with a total of 96 animals from 24 commercial dairy farms. Furthermore, the data generated represents the highest current sequencing coverage for the uterine microbiome of dairy cows, using high sequencing depth for individual samples. Altogether, the findings from our study represent a broad population of dairy farms in California, using next-generation sequencing approaches allowed for a genera-level characterization and comparison of the microbiota between cows with and without metritis. A unique aspect of our study compared to other information currently available in the literature for metagenomic characterization of the uterus, is the use of much higher sequencing depth, allowing for a more effective characterization of the uterine microbiota at lower taxa levels. Most research into bovine metritis and uterine microbiota has relied on amplicon 16s rRNA sequencing [57, 225]. As of early 2023, only one study, conducted by Bicalho et al. in 2017, has analyzed the bacterial microbiota present in the bovine uterus using shotgun metagenomics [68]. Their study performed shotgun metagenomics sequencing on uterine swabs samples collected from 20 cows (nine healthy and eleven metritis

cows) located on a single dairy farm near Ithaca, New York. The Bicalho et al. study resulted in 6.3 million quality-filtered reads that were then passed through MG-RAST [226] annotation, which relies on the alignment of 16s genes to classify bacterial reads, producing nearly 3 million bacterial reads. Of these nearly 3 million reads, 25,334 reads matched 16s sequences at a 97% similarity level. In contrast, our study was conducted using 96 uterine swab samples from 96 cows (CT = 32, MET = 33, and PUS = 31) located on 24 commercial dairy farms throughout California. Furthermore, our study of 96 samples resulted in 1.39 trillion raw reads and 1.34 trillion quality-filtered reads that were then classified through Kraken2 [215]. Genus-level abundance data was then created using Bracken [216] resulting in 24,616,858 reads assigned at the genus level. The high sequencing depth of the current study comparatively resulted in a near thousand-fold increase in the number of reads matching bacteria at the genus level between the Bicalho et al. and the present study. Additionally, the transition from bacterial identification by alignment of reads towards 16s sequences to the use of exact alignment of *k*-mers has allowed more accurate taxonomic identification of bacterial sequence data [227]. Furthermore, MG-RAST advises against classification past the genus level, while both Kraken2 and Bracken are able to provide species-level identification [228]. However, the majority of the analyses presented here were conducted at the genus level to allow for comparison to other metritis microbiome studies, with analysis at the species level pending.

α and β diversity of intrauterine microbiome

Analysis of α-diversity (diversity of microbes within a sample) and β-diversity (diversity of microbes between samples) was conducted to assess variation in the type or abundance of microbes between clinical groups. Current research suggests that cows with metritis or in the process of developing metritis have a dysbiosis of the uterine microbiota characterized by

homogenization of taxa and a decrease in bacterial richness [57, 71]. Figure 4-1A displays the Chao1 index values for the three clinical groups and the only significant difference was between CT and MET samples, with MET samples having higher mean Chao1 index values. Because Chao1 is an estimator of microbial richness that is heavily influenced by rare taxa, the higher Chao 1 index for cows with metritis could represent the invasion of the diseased uterus by less commonly observed microbes due to a disruption in the resistance to colonization that otherwise would be observed in a healthy uterus [229]. Conversely, figures 4-1B and 4-1C display Simpson and Shannon index values, two measures of diversity that account for not only the number of species present, but also the relative abundance of each species. Lower Simpson and Shannon indicies are observed for MET compared to CT, aligning with what previous studies have observed [208]. As both Simpson and Shannon α-diversity indices account for microbial richness and abundance, these indices provide a more representative estimation of the microbial community presence within samples. Notably, the lack of a significant difference for the α-diversity indices between MET and PUS samples, supports the hypothesis of a very low abundance level invasion of the uterus of cows with metritis by random opportunistic microbes, which results in the contrasting high richness and low diversity outcomes observed for MET.

To analyze β-diversity between clinical groups, NMDS ordinations were generated for CT, MET, and PUS clinical groups (Figure 4-2A) and the same clinical groups with an additional group, MET_Treatment, generated for samples taken from MET cows that received antimicrobial treatment (Figure 4-2B). Although the colored ellipses representing the 95% confidence intervals for the various clinical groups analyzed in figure 4-2 overlap, both ordinations in figures 4-2A and 4-2B were found to be significantly different by PERMANOVA and ANOSIM. While NMDS provides a visual approach to access β-diversity, PERMANOVA and ANOSIM statistically test

for significant differences between groups and are a mainstay of microbiome studies [230]. This discrepancy between ordination and statistical analysis of the β-diversity between metritic and healthy cows has also been observed by other studies [208, 209]. Interestingly, in figure 4-2B the 95% confidence intervals for CT and MET_Treatment are only slightly overlapping, with a clear separation of the two clinical groups in the 3D NMDS in figure 4-3B. This suggests that within the MET_Treatment group, antimicrobial treatment resulted in a significant effect for distinguishing the uterine microbiome, when compared to CT, greater than metritis alone. This supports the findings of Jeon et al. 2021 in which ceftiofur treatment of metritic dairy cows lead to a decrease in the relative abundance of *Fusobacterium*, and Jeon et al. 2018 in which *Porphyromonas* was significantly increased after ceftiofur treatment [231, 232].

Differential abundance of bacterial genera previously associated with metritis

Previous culture-independent studies such as PCR-type methods and 16S rRNA sequencing have observed a higher relative abundance of *Bacteroides*, *Porphyromonas,* and *Fusobacterium* in the uterine microbiota of cows with metritis, and a decrease in the relative abundance of these same genera in healthy cows [62, 71]. In addition to these three genera, bacteria in the genera *Escherichia* and *Trueperella* continue to be pathogens of interest in the etiology of bovine metritis [225]. Figure 4-4 confirmed *Bacteroides*, *Porphyromonas*, *Fusobacterium*, *Escherichia*, and *Trueperella* to be within the top 12 most abundant genera; however, the difference in mean relative abundances for these genera between CT, MET and PUS was minor (Table 4-1, Figure 4-4B). To further investigate if these five genera, and 26 additional genera of interest, were differentially abundant between clinical groups a heatmap of log fold changes in abundances was created using data from ANCOM-BC analysis (Figure 4-7). Notably, none of these five genera previously identified as present at a higher prevalence were found to be

significantly different in abundance (adjusted $p < 0.05$) when comparing MET_No Treatment to CT (Figure 4-7, Supplemental Table 4-3). A significant decrease in abundance of *Bacteroides, Escherichia,* and *Trueperella* in MET_Treatment compared to CT cows was observed, suggesting antimicrobial treatment with antibiotics (the most commonly used in the farms sampled was ceftiofur), was associated with a lower bacterial load of these genera.

The lower abundance of *Trueperella* is further supported by the lower log fold change in abundance of the phylum Actinobacteria (i.e., Actinomycetota), in which *Trueperella* is a member, for all clinical group comparisons versus CT as presented in figure 4-5. This is unsurprising as previous studies have demonstrated low minimum inhibitory concentration values and resistance to ceftiofur in *E. coli* and *T. pyogenes* isolates collected from uterine swabs [85, 212]. A recent 16s rRNA-based study analyzing the impact of ceftiofur treatment on the uterine microbiome of metritic cows revealed a significant decrease in the relative abundance of *Fusobacterium,* in contrast to our results [231]. The same study also observed that ceftiofur treatment had no significant effect on the bacterial load of *Porphyromonas* and *Bacteroides* within the uterine microbiota of metritic cows. Interestingly, these observations align with our result for *Porphyromonas,* but contrast our observed decrease in log fold change in abundance of *Bacteroides* in MET_Treatment compared to CT cows. Our data are from a cross-sectional study design and limit findings to potential associations. Further studies would need to be conducted to evaluate potential causation impacts in these pathogens of the uterus following treatment or not with ceftiofur.

However, when comparing these five genera from MET to CT, *Bacteroides, Porphyromonas,* and *Fusobacterium* were significantly higher by 0.2, 0.19, and 0.2 log fold, respectively, in metritis cows when compared to healthy cows (Table 4-1). *Escherichia* and

Trueperella were found to be not significantly different in log fold change of abundance (adjusted $p > 0.05$). The observed higher log fold change in abundance of *Bacteroides, Porphyromonas,* and *Fusobacterium* in metritic cows supports previous 16s rRNA and shotgun metagenomics-based studies that discovered similar findings [68, 71]. The lack of significance for *Escherichia* may be explained by the role different strains of *E. coli* play in the pathogenesis of metritis and by the decreasing likelihood of *E. coli* identification following parturition [194, 233]. Our observed lack of significance for *Trueperella* contrasts previous 16s rRNA -based studies that reported the genus as more abundant in metritic than healthy cows [207, 208].

Two additional genera, *Micrococcus* and *Filifactor* are also of interest due to their large log fold changes in abundance, as contrasted by other genera, when comparing MET to CT (Figure 4-7, Supplemental Table 4-3). Specifically, *Micrococcus* was decreased in log fold change in abundance (-0.66 and -1.33) and *Filifactor* was increased in log fold change in abundance (0.53 and 0.94) when comparing MET_No Treatment and MET_Treatment to CT. Unlike the five genera previously discussed, both *Micrococcus* and *Filifactor* have not commonly been associated with bovine metritis, respectively. A culture-based study of the uterine microbiome of cows with and without metritis at the time of insemination found *Micrococcus* to be the 5th most isolated genus of bacteria with 7.8% of cows being culture-positive [234]. *Micrococcus luteus* was also the 2nd most commonly isolated species in the study explaining why the authors found low species diversity within this genus. Another culture-based study of the uterine microbiome also isolated *Micrococcus luteus* from cows with and without metritis (n = 6 out of 279) [235]. The lower log fold change in abundance of *Micrococcus* in MET cows compared to CT may be due to a lower overall bacterial diversity of the uterus in metritic versus healthy cows. Bacteria in the genus *Filifactor* have been either associated with or found in high abundance in metritic cows [62, 70].

In particular, Jeon et al. 2015 observed a significant association between *Bacteroides* and *Filifactor* with metritic cows. Little is known about the role *Filifactor* may play in the development of metritis; however the species *Filifactor alocis* has been cited as an emerging pathogen in the development of human periodontal disease, a polymicrobial disease affecting the tissues around the teeth [236, 237]. As bacteria in the genus *Filifactor* have been previously associated with metritis and have been implicated in the development of another disease with a multifactorial etiology, further research into the role of *Filifactor* within the uterine microbiome may prove insightful.

Differential abundance of bacterial genera significant for all clinical group comparisons

Ten genera were found to be significantly (*p*-adjusted < 0.05) increased or decreased in log fold change of abundance for all three clinical group pairwise comparisons (MET_No Treatment, MET_Treatment, and PUS when compared to CT) and are presented in figure 4-6. Seven genera (*Liquorilactobacillus*, *Cyclobacterium*, *Owenweeksia*, *Anoxybacter*, *Flavihumibacter*, *Dyadobacter*, and *Oceanobacillus)* were found to be increased in log fold change of abundance for all three clinical group comparisons suggesting an association with uterine disease.

Members of the genus *Liquorilactobacillus* are lactic acid bacteria most often isolated from fermented plant materials (e.g. ciders, molasses, cocoa beans, and olives) [238]. Many strains of *Liquorilactobacillus* are capable of producing the exopolysaccharide dextran from sucrose [239]. Such exopolysaccharides are currently being explored as next-generation prebiotics. Interestingly, *Liquorilactobacillus satsumensis* isolated from water kefir (also known as tibicos), a fermented beverage produced from incubating water kefir grains in water with added sugar and fruits, produced an exopolysaccharide, that when hydrolyzed, was observed to promote *Bacteroides* growth within an *ex vivo* model of the large bowel [240]. This synergy between

Liquorilactobacillus and *Bacteroides*, a genus frequently associated with metritis [62, 71], may explain why *Liquorilactobacillus* was increased in log fold change of abundance within cows presenting with uterine disease.

Bacteria belonging to the genus *Cyclobacterium* are non-motile, strictly aerobic, ring-like or horseshoe-shaped, and are typically isolated from marine environments [241]. *Cyclobacterium* are also catalase-positive, oxidase-positive or negative, and slightly halophilic with preferred NaCl concentration ranging from 3–5% [242]. An explanation as to why an increased abundance of *Cyclobacterium* was associated with uterine disease has yet to be determined.

Owenweeksia are gram-negative, halophilic, non-flagellated, and motile short rods first isolated from seawater and are a member of the phylum Bacteroidetes [243]. *Owenweeksia* was previously identified in a 16s rRNA-based study of human endometrial fluid and vaginal aspirates [244]. The significance of identification of *Owenweeksia* within the bovine uterine microbiome remains unknown.

Little is known of bacteria belonging to the genus *Anoxybacter* as only one species (*Anoxybacter fermentans*) has been described. *Anoxybacter fermentans* was isolated from a deep-sea hydrothermal vent on July 2011 at a depth of 2891 meters and incubated anaerobically, without light, and at 60 °C [245]. An unclassified species of *Anoxybacter* was identified in a 16s rRNA-based study of human upper gastrointestinal tract samples studying dyspepsia (i.e. indigestion) [246]. More research is needed to elucidate the role of *Anoxybacter* in humans and cattle.

Flavihumibacter is a genus of aerobic, gram positive, non-motile, shot rods first isolated from a subtropical rainforest soil sample from Nepal in 2010 [247]. More recent detections of the genus have come from the bacterial culture of forest soil and freshwater stream samples [248, 249].

As isolation of *Flavihumibacter* has typically been from environmental sampling, it is likely that the genus was present in the environment of farms sampled in this study and was a potential opportunistic contaminant of the uterus.

The Gram negative bacterial genus *Dyadobacter* was first reportedly isolated from surface-sterilized *Zea mays* stems [250]. These bacteria are capable of fermenting glucose and sucrose and have been found in various other environments including biological soil crusts and gill and mucus microbiomes of farmed Atlantic salmon [251, 252]. The detection of *Dyadobacter* in gill and mucus samples of salmon immediately preceding the first signs of gill disease is interesting as both gills and the bovine uterus are protected by a layer of mucus [253, 254]. The significance of the identification of *Dyadobacter* within the bovine uterine microbiome is not fully understood.

The first reported isolation of the genus *Oceanobacillus* was from deep sea sediment at a depth of 1050 meters in 2001 [255]. Since then, the genus has been detected in various environmental samples, including Korean Kimchi, human gut, and pork at slaughter [256-258]. In general, *Oceanobacillus* are Gram positive, obligate aerobes or facultative anaerobes, and moderately halophilic rods. One isolate taken from pork samples was positive for the bla_{TEM} and $bla_{\text{CTX-M}}$ antimicrobial resistance genes, conferring resistance to β-lactam antibiotics [258]. *Oceanobacillus* was detected previously in uterine samples of metritic and healthy cows, but appears to be a rarely identified member of the uterine microbiome [69]. Further research is necessary to determine if *Oceanobacillus* is a contaminating bacteria of uterine samples or present in the uterine microbiome.

The genera *Pseudonocardia*, *Glutamicibacter*, and *Rathayibacter* decreased in log fold change abundance for all three clinical groups when compared to CT (figure 4-6). *Pseudonocardia* are aerobic, Gram positive, non-motile bacteria that may form hyphae and have primarily been

isolated from soil and other environmental samples [259]. The genus has been identified in 16s rRNA-based studies dealing with the microbiome of reproduction, including from vaginal and rectal swabs taken from human mothers and from amniotic fluid samples from healthy human pregnancies [260, 261]. *Pseudonocardia* has also been cultured from cattle manure and was detected in low abundance (<0.01% prevalence) in a 16s rRNA-based study of bovine uterine swabs [62, 262]. Given its low abundance within bovine uterine swabs and isolation from cattle manure, it is likely that *Pseudonocardia* is an environmental contaminant of the uterine microbiome.

Glutamicibacter are aerobic, gram-positive, rod-shaped bacteria often found in soil and often used in bioremediation [263, 264]. Interestingly, *Glutamicibacter nicotianae* was found to be resistant to tetracycline and has been observed degrading oxytetracycline and tetracycline, an antibiotic frequently used in veterinary medicine [265]. However, the significance of *Glutamicibacter* within the bovine uterine microbiome is unknown.

Members of the genus *Rathayibacter* are Gram positive, aerobic, non-motile, irregular shaped bacteria first isolated on annual grasses [266]. *Rathayibacter toxicus*, a Select Agent due to its production of a lethal toxin, is responsible for deaths of grazing animals and is able to survive in arid conditions when not found on ryegrass [267]. The genus was previously detected in high abundance in a 16s rRNA-based study of Nasopharyngeal swabs taken from healthy feedlot cattle [268]. As *Rathayibacter* have primarily isolated from annual grasses, it is likely the genus is a contaminant of the bovine uterine microbiome.

5. CONCLUSION

The analysis of shotgun metagenomics sequencing data of 96 intrauterine swab samples from commercial dairy cows with and without metritis revealed swabs from healthy (i.e., CT) cows had increased α-diversity compared to metritis or PUS cow swabs. NMDS ordinations, and subsequent statistical analyses, illustrated that the microbiome of cows from the CT and MET clinical groups were different at the genus level and that antimicrobial treatment further differentiated CT and MET. ANCOM-BC detected no significant difference in the abundance of *Bacteroides, Porphyromonas, Fusobacterium, Escherichia,* or *Trueperella* when comparing MET_No Treatment to CT. However, *Bacteroides*, *Escherichia,* and *Trueperella* were significantly decreased in abundance in MET_Treatment compared to CT. When comparing MET to CT (regardless of treatment) *Bacteroides*, *Porphyromonas,* and *Fusobacterium* were significantly higher in log fold change of abundance, while *Escherichia* and *Trueperella* were not significantly different in log fold change of abundance. *Liquorilactobacillus* and *Oceanobacillus*, two genera either infrequently or not previously associated with bovine uterine disease, were found to be associated with metritis and have biologically plausible explanations for this observation. Further research should be conducted to determine what role these two genera may play in the development of metritis. Our shotgun metagenomics analysis, in agreement with various 16s rRNA-based studies of the bovine uterine microbiome, highlights the decrease in α-diversity of the uterine microbiome within cows with metritis and reinforces the idea that metritis is strongly associated with dysbiosis.

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CHAPTER 4 SUPPLEMENTAL

Supplemental Table 4-1. PERMANOVA (permutational multivariate analysis of variance) to test the significance of genus-level Bray-Curtis dissimilarity distances of different clinical group comparisons (999 permutations).

Variable	Degrees of Freedom	Sum of Squares	R^2	F	*p*-adjusted[4]
Clinical Groups					
CT, MET, PUS [1]	2	0.93	0.08459	4.2972	**0.001**
CT, MET_Treat, MET_NoTreat, PUS [2]	3	1.391	0.12649	4.4401	**0.001**
Pairwise[3]					
CT v. MET	1	0.855	**0.112**	7.911	**0.003**
CT v. PUS	1	0.267	0.041	2.607	0.046
MET v. PUS	1	0.264	0.036	2.312	0.046
CT v. MET_NoTreat	1	0.491	**0.075**	4.485	**0.006**
CT v. PUS	1	0.267	0.041	2.607	0.034
CT v. MET_Treat	1	1.06	**0.242**	12.123	**0.003**
MET_NoTreat v. PUS	1	0.098	0.015	0.846	0.471
MET_NoTreat v. MET_Treat	1	0.46	0.12	4.241	0.011
PUS v. MET_Treat	1	0.658	**0.155**	6.785	**0.003**

(**1**) Comparison of clinical groups used at intrauterine swab sampling (CT, MET, and PUS). (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as a non-fetid purulent or mucopurulent vaginal discharge.
(**2**) Comparison of clinical groups stratified by whether MET cows received antimicrobial treatment.
(**3**) Pairwise comparisons of clinical groups
(**4**) *P* values adjusted using Benjamini-Hochberg. Significant values ($p < 0.01$) presented in bold.

Supplemental Table 4-2. ANOSIM (analysis of similarities) to test the significance of genus-level bray-curtis dissimilarity distances of different clinical group comparisons.

Variable	R	Adjusted *p*-value[4]
Clinical Groups		
CT, MET, PUS [1]	0.1277	**0.001**
CT, MET_Treat, MET_NoTreat, PUS[2]	0.1289	**0.001**
Pairwise[3]		
CT v. MET_NoTreat		**0.003**
CT v. PUS		0.018
CT v. MET_Treat		**0.003**
MET_NoTreat v. PUS		0.343
MET_NoTreat v. MET_Treat		0.648
PUS v. MET_Treat		0.168

(**1**) Comparison of clinical groups used at intrauterine swab sampling (CT, MET, PUS). (CTL) control, healthy discharge defined as cows with either no vaginal discharge, clear mucus, or clear lochia; (MET) metritis discharge defined as a watery, red or brown colored, and fetid vaginal discharge; and (PUS) purulent discharge defined as a non-fetid purulent or mucopurulent vaginal discharge.
(**2**) Comparison of clinical groups stratified by whether MET cows received antimicrobial treatment.
(**3**) Pairwise comparisons of clinical groups
(**4**) *P* values adjusted using Benjamini-Hochberg. Significant values ($p < 0.01$) presented in bold.

Supplemental Table 4-3. Table of log fold changes in abundance of 31 selected genera for which MET_No Treatment and/or MET_Treatment when compared to CT had an adjusted $p < 0.05$.

- Non-significant (p adjusted > 0.05) for the clinical group comparison for that genus.

GENERA	MET_NO TREATMENT	MET_TREATMENT
Trueperella	-	-0.43
Streptomyces	-0.27	-0.68
Streptococcus	-	-0.15
Streptobacillus	0.56	0.51
Staphylococcus	-	-0.44
Salmonella	-	0.91
Ruminococcus	-	-0.40
Prevotella	-	-0.20
Porphyromonas	-	-
Peptoniphilus	-	0.66
Pedobacter	-	0.35
Mycobacterium	-	-0.35
Micrococcus	-0.66	-1.33
Klebsiella	-	-0.51
Histophilus	-	-0.79
Helicobacter	-	0.65
Fusobacterium	-	-
Filifactor	0.53	0.94
Escherichia	-	-0.34
Enterococcus	-	-
Cryptococcus	-	0.59
Corynebacterium	-0.23	-0.56
Citrobacter	0.49	0.47
Chlamydia	0.23	-
Brucella	-0.62	-0.49
Brevibacterium	-0.34	-0.76
Bifidobacterium	-0.38	-0.74
Bacteroides	-	-0.20
Bacillus	-	-0.27
Arcanobacterium	-	0.67
Anaerococcus	-	0.40

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DISSERTATION CONCLUSION

The four projects presented in this dissertation have added important knowledge to the study of antimicrobial resistance within California cattle populations. These projects utilize a wide range of traditional and advanced diagnostic methods, including a combination of culture-based and sequencing-based techniques to analyze the bacteria present in bovine gastrointestinal, mammary, and intrauterine environments. In chapters one and three, bacteria from bovine fecal and intrauterine samples were further scrutinized, genotypically and phenotypically, for the presence of genetic elements or expression of antimicrobial resistance. In chapters two and four, 16s rRNA and shotgun metagenomic sequencing were used to examine the microbial community dynamics and diversity of the mammary and intrauterine microbiome prior to and following dry-off and for cows with and without metritis, respectively.

Chapter one details the prevalence of extended-spectrum β-lactamase (ESBL) genes, AmpC-type β-lactamase (ACBL) genes, and plasmid-mediated quinolone resistance (PMQR) genes in *Salmonella* isolated from bovine fecal samples at a Veterinary Medical Teaching Hospital microbiology laboratory. To examine trends in the identification of these resistance genes and to explore the correlation between phenotypic resistance and the presence of these genes a single, novel multiplex qPCR was developed. Only the genes $bla_{\text{CMY-2}}$ and bla_{TEM} were detected in the 110 *Salmonella* isolates tested. PMQR genes were not detected in the isolates screened. Of 94 third-generation cephalosporin resistant isolates, representing eight serotypes, 48% (n = 45) were positive for $bla_{\text{CMY-2}}$ only and 50% (n = 47) were simultaneously positive for $bla_{\text{CMY-2}}$ and bla_{TEM}. Overall, our results suggest that the prevalence of resistance to cephalosporins and fluoroquinolones due to ESBLs, ACBLs, and PMQR genes present in bovine nontyphoidal *Salmonella enterica* isolates has remained relatively constant in the isolates screened over a 14-

year period. Whole genome sequencing of these *Salmonella* isolates may reveal the presence of other antimicrobial resistance genes not assayed in our multiplex qPCR, likely explaining third-generation cephalosporin resistant isolates that were not positive for ESBL or ACBL genes.

Chapter two used 16s rRNA sequencing to evaluate the effects of dry cow antimicrobial therapy on the udder milk microbiota by comparing the microbial populations in milk at dry-off (DRY) (~60 days before calving) and post-partum (FRESH) (4-11 days after calving) from cows receiving intramammary antibiotic infusion prior to dry-off (IMT) and cows that did not receive treatment (CTL). The genus *Staphylococcus* was the most abundant taxa for DRY&CTL and FRESH&IMT. The genus *Delftia* was the most abundant taxa for DRY&IMT and FRESH&CTL. The only Shannon diversity values deemed significantly different (P value < 0.05) were those from DRY&CTL and FRESH&IMT. The lack of taxa differentiating FRESH samples between CTL and IMT treatment groups indicated that, within our study, intramammary antimicrobial dry cow therapy had no significant effect on the udder milk microbiota post-partum. Significant research has been conducted to examine the role of the mammary and milk microbiota in the development of mastitis, however, few studies have been conducted in commercial dairy cow populations like the study presented here. Lessons learned from 16s rRNA sequencing and subsequent analysis were used to inform the need for shotgun metagenomics sequencing and analysis specifically designed for compositional data used in the study presented in chapter four.

Chapter three presents a cross-sectional study designed to evaluate factors affecting recovery and antimicrobial resistance (AMR) in intrauterine *E. coli* in which a total of 307 cows with and without metritis were sampled from 25 farms throughout California. During sample collection, cows were segregated into three clinical groups: metritis (**MET**, n = 86), defined as a cow with watery, red or brown colored, and fetid vaginal discharge; cows with purulent discharge

(PUS, n = 106), defined as a non-fetid purulent or mucopurulent vaginal discharge; and control cows, (**CTL**, n = 115) defined as cows with either no vaginal discharge or a clear, non-purulent mucus vaginal discharge. From these intrauterine swab samples, a total of 162 intrauterine *E. coli* isolates were recovered and tested for phenotypic AMR. All intrauterine *E. coli* were resistant to ampicillin (AMP), with an AMR prevalence of 30.2% and 33.9% observed for chlortetracycline and oxytetracycline, respectively. Only 8.6% of isolates were resistant to ceftiofur (CEFT), one of the most common drugs used to treat cows on farms sampled. Interpretation of minimum inhibitory concentration (MIC) data into resistant or susceptible categories was complicated by a lack of specific MIC breakpoints in *Enterobacterales* from cattle, particularly when collected from the female reproductive tract, for antimicrobials commonly used to treat metritis. Nonetheless, our study was one of the largest studies of AMR prevalence within intrauterine *E. coli* recovered from California post-partum dairy cows with and without metritis.

Chapter four reveals the microbial ecology and diversity of the microbiota present within the uterus of post-partum dairy cows with and without metritis using shotgun metagenomics on a subset of the sample population analyzed in chapter three. In total, 96 samples from the same clinical groups (MET = 33, PUS = 31, and CT = 32) were selected, with MET being further stratified by whether the cow received antimicrobial treatment. In general, all three clinical groups (CT, MET, and PUS) were highly diverse with the top 12 most abundant genera only accounting for 10.3%, 8.8%, and 10.1% of mean relative abundance, respectively. α diversity indices generally revealed a decrease in diversity between samples collected from MET and PUS when compared to CT cows. Of the top 12 most abundant genera, seven genera: *Bacteroides, Clostridium, Fusobacterium, Phocaeicola, Porphyromonas, Prevotella,* and *Streptococcus* were significantly increased in log fold change of abundance in MET (regardless of treatment) v. CT samples. Two

genera, *Dietzia* and *Microbacterium*, were significantly decreased in abundance when comparing MET to CT; while *Escherichia, Histophilus,* and *Trueperella,* were non-significantly changed in abundance. Various microbiome studies have been conducted on intrauterine samples from healthy and metritic cows, however, our study is only the second to be conducted utilizing shotgun metagenomic sequencing, rather than 16s rRNA sequencing, enabling bacterial identification to the species level. Future analysis of our data will include species-level analyses and evaluation of AMR genes present within the uterine microbiota.

The overall goal of the dissertation research presented here was to analyze the impact of AMR within various microbiomes of California cattle and within pathogenic bacteria. This work has resulted in the creation of a novel multiplex qPCR for the identification of AMR genes in *Salmonella*, 16s rRNA sequencing data from milk, novel MIC data in *E. coli* for antimicrobials commonly used to treat metritis, and cutting-edge shotgun metagenomic sequence data of the healthy and metritic bovine uterus. In conclusion, the work presented here has added to the body of knowledge within veterinary medicine and, hopefully, improved the lives of producers, veterinarians, cattle, and the people of California.